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2453 Opposing Roles for Gab2 and Gab3 in M-CSF-mediated Chemotaxis
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Macrophage-colony stimulating factor (M-CSF)-induced chemotaxis involves Phosphatidylinositol 3-kinases (PI3-kinases); the Rho GTPase, Rac; and the Rac-GEF Vav. In this study, we investigated how the Gab (Grb2 associated binder) family of adaptor/scaffolding proteins, Gab2 and Gab3 coordinate with these proteins to regulate M-CSF-induced chemotaxis and proliferation. While Gab2-, Gab3-, and Vav1,2,3-deficient macrophages proliferated comparably in response to M-CSF the Vav1,2,3- and Gab2-deficient cells were defective in M-CSF cell-derived induced chemotaxis. In contrast, Gab3-deficient macrophages showed elevated M-CSF-induced chemotaxis, indicating Gab3 is inhibitory to M-CSF-driven migration. The changes in chemotaxis between these Gab2- and Gab3-deficient strains correlated with Rac and Vav activation, defined by plasma membrane recruitment, tyrosine phosphorylation, and Rac-GTP binding. The Gab2-deficient macrophages showed a partial decrease in PI3-K activation, suggesting that the block in Vav-Rac activation is due to limited activation of this upstream regulator. We therefore describe a novel role for Gab proteins in M-CSF mediated chemotaxis, such that while Gab2 and Gab3 are singly dispensable for proliferation, they have distinct and opposing roles in migration.

2454 Differentiation of AR42J Cells Is Not Dependent on ErbB1 Stimulation
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Betacellulin (BTC) is one of the members of epidermal growth factor (EGF) family. BTC has dual functions. One is the mitogenic activity stimulating EGF receptor as well as EGF and TGF-α. The other is the differentiating activity of pancreatic endocrine precursor cells into pancreatic β-cells secreting insulin. Especially, this differentiation activity is unique to BTC among the members in EGF family. BTC independently and preferentially binds to two different type-I tyrosine kinase receptors of ErbB1 and ErbB4, which belong to ErbB family. However, the factors, which lie in the structure of BTC, responsible for the preferential affinity for these two receptors have not been been inquired. In the study, to investigate the essential amino acid residues in BTC for the binding to the two receptors, we introduced spot mutations into the EGF domain of BTC randomly employing error-prone PCR. One hundred ninety of mutants were expressed in E. coli and assessed their receptor binding affinity by enzyme immunoassay (EIA). As the result, we found a mutant that showed 160-fold weaker affinity to ErbB1 than wild type BTC while the affinity to ErbB4 was remained. Simultaneously, this mutant showed less growth promoting activity on Balb/c 3T3 cells than that of the wild type BTC. Interestingly, this mutant still induced differentiation of AR42J cells with much more insulin production in individual cells when compared with the cells treated with the wild type BTC. The two functions of BTC were successfully separated implying the responsible receptors for each activity were independent. Especially the differentiation should not be depending on ErbB1 stimulation, which is essential for the mitogenic activity.

2455 TGF-β1-induced Plasminogen Activator Inhibitor-1 Expression in Vascular Smooth Muscle Cells Requires Cooperative pp60-src/EGFRY845 and Rho/ROCK Signaling
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TGF-β1 and its target gene encoding plasminogen activator inhibitor-1 (PAI-1) are major causative factors in the pathology of tissue fibrosis and vascular disease. The increasing appreciation of the complexity of TGF-β1 action in vascular cells requires analysis of specific TGF-β1-initiated signaling events that impact PAI-1 transcriptional regulation in a physiologically-relevant cell system. TGF-β1-induced PAI-1 expression in vascular smooth muscle cells (VSMC) was preceded by EGFR phosphorylation on Y845 (a src kinase target residue), required pp60-src activity and was completely blocked by inhibition of EGFR activity or adenoviral delivery of a kinase-dead EGFR(E521A) construct. Infection of VSMC with an adenovirus encoding the EGFRY845 mutant or transfection with a dominant negative pp60-src (DN-Src) expression vector effectively decreased TGF-β1-stimulated PAI-1 expression implicating EGFRY845 phosphorylation in the inductive response. Consistent with these findings, TGF-β1 failed to induce PAI-1 synthesis in src kinase-deficient (SYK−/−) fibroblasts and reexpression of a wild-type pp60-src construct in SYK−/− cells restored the PAI-1 response to TGF-β1. Genetic EGFR deficiency also virtually ablated TGF-β1-stimulated ERK1/2 activation as well as PAI-1 expression but not SMAD2 phosphorylation. Transient transfection of a dominant negative RhoA (DN-RhoA) expression construct or pretreatment of VSMC with C3 transferase and Y-27632 (inhibitors of Rho and ROCK respectively) also dramatically attenuated TGF-β1-initiated PAI-1 induction. In contrast to EGFR pathway blockade, interference with Rho/ROCK signaling effectively inhibited TGF-βR-mediated SMAD2 phosphorylation and nuclear accumulation. SMAD2 activation, moreover, was not sufficient to induce PAI-1 expression by TGF-β1 in the absence of EGFR signaling. Thus, two distinct but cooperative pathways involving EGFR/MEK-ERK signaling and Rho/ROCK-dependent SMAD2 activation are required for TGF-β1-induced PAI-1 expression in VSMC. The identification of such novel interactions between two TGF-β1-activated signaling networks that specifically impact PAI-1 transcription in VSMC may provide therapeutically-relevant targets to manage the pathophysiology of PAI-1-associated fibrotic diseases. (Supported by NIH grant GM57242)

2456 EGFR Is Involved in Clusterin-induced Astrocyte Proliferation via ERK Activation
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Astrocyte proliferation is one of pathophysiological features in response to brain injury and neurodegenerative diseases, and thus controlling the astrocyte growth is of great interest. Clusterin, a secretory glycoprotein, has been shown to be upregulated in the reactive astrocytes. In a previous report, we demonstrated that clusterin stimulates proliferation of rat primary astrocytes through ERK activation, suggesting an important role of clusterin in astrogliaosis. However, cell surface molecule(s) that transactivate ERK and proliferating signal was uncovered. To identify the receptor that mediates mitogenic effect of clusterin, cells were treated with various inhibitors against cell surface receptors existed in astrocytes. Clusterin-induced cell proliferation was prevented by Genistein (tyrosine kinase inhibitor) and AG1478 (EGFR inhibitor), indicating potential involvement of EGF receptor tyrosine kinase in the signal transduction by clusterin. In addition, ERK phosphorylation induced by clusterin was blocked by AG1478 in a dose-dependent manner. We further confirmed that EGF receptor is the signal transducing molecule underlying clusterin-induced astrocyte proliferation, using EGFR siRNA. Both cell growth and ERK activation by clusterin was abolished as EGFR siRNA was applied. Taken together, these results suggest that EGFR is involved in clusterin-induced ERK activation and subsequent growth in astrocytes. [This study was supported by the Korea Research Foundation grant (KRF-2006-312-E00031) and Medical Research Center of Korea University]

**Signal Transduction (2457-2469)**

**2457**

Effect of Tetrahydrobiopterin on Advanced Glycation End-Product-induced Hypertrophic Growth in Renal Tubular Epithelial Cells

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Renal tubulointerstitial fibrosis is believed to play a key role in the development of diabetic nephropathy, and advanced glycation end-product (AGE) may contribute importantly to this. In the present study, the mechanisms by which tetrahydrobiopterin (BH₄) modulates the AGE-induced hypertrophic growth in renal tubular epithelial (LLC-PK1) cells were examined. We found that AGE time- and dose-dependently decreased nitric oxide (NO) production and GTP cyclohydrolase I (GTPCH I)-inducible NO synthase (iNOS) activation. These effects were not observed when cells were treated with non-glycated BSA. NO and iNOS stimulated by BH₄ and the NO donor N-nitroso-N-acetylpenicillamine (SNAP) prevented AGE-induced JAK2/STAT1/STAT5, Raf-1/ERK, and JNK/p38 MAPK activation. Nevertheless, addition of 2,4-diamino-6-hydroxypyrimidine (DAHP) that inhibits GTPCH I activity may enhance AGE-induced these effects. The ability of iNOS/NO to inhibit AGE-induced hypertrophic growth was verified by the observation that BH₄ and SNAP inhibited both cyclin-dependent kinase inhibitors (CDKI) p21Waf1/Cip1 and p27Kip1 expression. Furthermore, BH₄ significantly decreased extracellular matrix (ECM) proteins fibronectin and collagen IV synthesis in AGE-treated cells. These findings suggest that BH₄ supplementation is renoprotective partly by attenuating AGE-induced renal tubular hypertrophy by increasing GTPCH I/iNOS activation and reducing CDKI/ECM expression.

**2458**

Rho Kinase 2 Is Present in Early Endosomes and Its C-terminal PH Domain Interacts with Vesicle Trafficking Proteins

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Rho Kinase 2 (ROCKII) is a Serine/Threonine kinase of ~160kD that is an effector of small GTPases of the Rho family (Rho GTPases). The kinase activity of ROCKII is believed to be activated following RhoA binding to the central coiled-coil domain, thereby inhibiting the autoinhibitory activity of the C-terminus. However, the role of the ROCKII C-terminal Pleckstrin Homology (PH) domain in ROCKII activity has not been fully investigated to date. In this study we investigated whether the ROCKII PH domain interacts with ROCKII substrates. Two separate pull-down experiments using the ROCKII-PH domain as bait were performed: separate nickel-sepharose bead columns, one with a His-tagged ROCK II PH-domain recombinant protein and another with a control sample lacking recombinant protein were used in a pull-down experiment. Eluted proteins from both experiments were subjected to 2D SDS-PAGE followed by mass-spectrometry analysis. The Mascot search algorithm was used to match peptides derived from interacting partners with archived sequences. Database interrogation revealed association between ROCK II PH domain and a number of previously known partners like CRM2 and profilin 2. Biochemical analyses we have performed confirm the association between ROCKII and these two proteins. In addition, we also identified the potential association between ROCKII and important vesicle trafficking proteins. These findings, led to the prediction that ROCK II would be localized to membranous structures. To test this hypothesis, we have performed subcellular fractionation experiments by way of flotation-gradient fractionation using a sucrose step-gradient which in our experiments enables the enrichment of early endosomes in a known and characterized interphase between two distinct sucrose densities. These experiments show that significant amounts of ROCK II are present in the early endosome fraction - an important trafficking organelle.

**2459**

Molecular Chaperone, Cyclophilin, in Activation of MAPK Pathways

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Cyclophilins (Cyps) were identified as cellular binding proteins for the Immunesuppressive drug cyclosporin A (CsA) and are constitutively expressed in most tissues. To date, more than 10 subtypes of Cyps including CypA, CypB, CypC, CypD, and Cyp40 have been identified. Cyps have peptidyl-prolyl cis-trans isomerase (PPIase) activity that catalyzes protein folding reactions in cells. Moreover, some of the Cyps are involved in apoptosis. CypB is found mainly in the ER lumen, whereas CypA is the most abundant CsA binding cytosolic protein. Although there is speculation that CypB plays a role in protein folding in the ER, the exact cellular function of CypB has yet to be discovered. Reactive oxygen species (ROS) are generated as by-products of the mitochondrial respiratory process. Transient fluctuations in ROS serve important regulatory functions, but when present at high and/or sustained levels, they can cause severe damage to DNA, proteins and lipids, which may finally lead to cell death. The aim of this study was to determine whether CypB can protect cell against ROS-mediated cell death. We performed immunoblotting and MTT Assay in Huh-
7 cell, Human Hepatoma cell, treated with H2O2 for 24 Hours. According to our results, overexpressed CypB increased cell viability though activation of ERK and JNK pathways.

2460
8-Chloro-cAMP Induces Growth Inhibition through the Activation of AMPK and p38 MAP KINASE
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8-Cl-cAMP (8-chloro-cyclic AMP), which induces differentiation, growth inhibition and apoptosis in various cancer cells, has been investigated as a putative anti-cancer drug. Although we reported that 8-Cl-cAMP induces growth inhibition via p38 MAP kinase (MAPK) and a metabolite of 8-Cl-cAMP, 8-Cl-adenosine mediates this process, the action mechanism of 8-Cl-cAMP is still uncertain. In this study, it was found that 8-Cl-cAMP-induced growth inhibition is mediated by AMP-activated protein kinase (AMPK). 8-Cl-cAMP activated AMPK, which was also dependent on the metabolic degradation of 8-Cl-cAMP. A potent agonist of AMPK, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) could also induce growth inhibition and apoptosis. To further delineate the role of AMPK in 8-Cl-cAMP-induced growth inhibition and apoptosis, we used two approaches: pharmacological inhibition of the enzyme with compound C and expression of a dominant negative mutant (a kinase-dead form of AMPKα2). AICAR was able to activate p38 MAPK and pre-treatment with AMPK inhibitor or expression of KD-AMPK blocked this p38 MAPK activation. Cell growth inhibition was also attenuated. Furthermore, p38 MAPK inhibitor attenuated 8-Cl-cAMP- or AICAR-induced growth inhibition but had no effect on AMPK activation. Also, since it was reported that 8-Cl-cAMP decreases the intracellular ATP pool, we tested if this ATP depletion could be related with the modulation of cellular growth. Intracellular ATP level decreased after the treatment with 8-Cl-cAMP time-dependently, and excess addition of ATP could mitigate 8-Cl-cAMP-induced growth inhibition. These results demonstrate that 8-Cl-cAMP induced growth inhibition through AMPK activation and p38 MAPK acts downstream of AMPK in this signaling pathway.

2461
The Transcription Factor Stp1 Is a Novel Regulator of TOR Signaling Pathway in Yeast
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TOR (target of rapamycin) signaling pathway plays crucial roles in regulation of eukaryotic cell growth. In Saccharomyces cerevisiae, nitrogen sources in extracellular environment activate TOR pathway. However, how nitrogen sources activate TOR pathway and what components participate in upstream signaling events of TOR pathway are poorly understood. Here we report that Stp1, a key transcription factor for amino acid uptake, is a novel upstream regulator of TOR pathway in S. cerevisiae. Through genome-wide protein localization study, we found that Stp1 disappeared from the nucleus upon inactivation of TOR kinases by rapamycin, suggesting involvement of Stp1 in TOR pathway. Supporting this notion, expression level of Stp1 affected rapamycin sensitivity of cells. Interestingly, we found that rapamycin-induced disappearance of Stp1 from the nucleus resulted from Stp1 degradation, which was triggered by action of a protein phosphatase 2A (PP2A)-like phosphatase Sis4, a well-known downstream effector of TOR kinases. Taken together, our findings suggest a model for regulation of TOR pathway in which inactivation of TOR kinases by rapamycin leads to degradation of Stp1 through a novel feedback loop.

2462
Differential Regulation of HSP70 Expression by the JNK Kinases SEK1 and MKK7 in Mouse Embryonic Stem Cells Treated with Cadmium
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JNK, a member of the mitogen-activated protein kinases (MAPKs), is activated by the MAPK kinases SEK1 and MKK7 in response to environmental stresses. In the present study, the effects of CdCl2 treatment on MAPK phosphorylation and HSP70 expression were examined in mouse embryonic stem (ES) cells lacking the sek1 gene, the mkk7 gene, or both. Following CdCl2 exposure, the phosphorylation of JNK, p38, and ERK was suppressed in sek1 Δ mkk7 Δ cells. When sek1 Δ mkk7 Δ cells were treated with CdCl2, JNK phosphorylation, but not the phosphorylation of either p38 or ERK, was markedly reduced, while a weak reduction in p38 phosphorylation was observed in sek1 Δ cells. Thus, both SEK1 and MKK7 are required for JNK phosphorylation, whereas their role in p38 and ERK phosphorylation could overlap with that of another kinase. We also observed that CdCl2-induced HSP70 expression was abolished in sek1 Δ mkk7 Δ cells, was reduced in sek1 Δ cells, and was enhanced in mkk7 Δ cells. Similarly, the phosphorylation of heat shock factor 1 (HSF1) was decreased in sek1 Δ mkk7 Δ and sek1 Δ cells, but was increased in mkk7 Δ cells. Treatment with either U0126 or SB203580 reduced HSP70 expression in wild-type ES cells treated with CdCl2. Transfection with siRNA specific for JNK1, JNK2, p38Δ, ERK1, or ERK2 suppressed CdCl2-induced HSP70 expression. In contrast, silencing of p38Δ or p38Δ resulted in further accumulation of HSP70 protein. These results suggest that HSP70 expression is up-regulated by SEK1 and down-regulated by MKK7 through distinct MAPK isoforms in mouse ES cells treated with CdCl2.

2463
Role of AMP-activated Protein Kinase in T Cell Receptor-induced FasL Expression and Activation-induced Cell Death in Human Leukemic Jurkat T Lymphocytes
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T cell activation results in enhanced expression of FasL, a critical regulator of activation-induced cell death (AICD) of T lymphocytes and an inflammatory protein. In this study, we examined the roles and signaling mechanisms of AMP-activated protein kinase (AMPK) in T cell receptor induced FasL expression and AICD. AMPK is activated at the downstream of Ca2+/Calmodulin-dependent protein kinase kinase (CaMKK) during T cell receptor (TCR) activation. Inhibition of AMPK by pharmacological agent or small interfering RNA suppressed FasL expression in T cells stimulated with PMA plus ionomycin (PMA/lo). Inhibition of AMPK blocked mRNA levels and prompter activation of FasL in PMA/lo-stimulated Jurkat T cells. Moreover, inhibition of AMPK reduced transcriptional activation of NF-AT and AP-1 but not of NF-kappaB. Moreover, we found that inhibition of AMPK suppressed phosphorylations of p38, c-Jun-NH2-terminal kinase (JNK), and Akt/GSK-3 but not of extracellular signal-regulated
kinase (ERK). Finally, we showed that inhibition of AMPK slightly enhanced T cell receptor-activation induced cell death and caspase-3 activation, suggesting AMPK activity might be critical for T cell survival. Taken together, the results show that AMPK pathway plays an essential role in T cell receptor-induced FasL expression and that AMPK may compromise the activation induced cell death of and homeostasis of T lymphocytes.

2464

Hydrogen Peroxide Mediate TGFβ1-induced EGF Receptor Activation

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TGFβs are multi-functional growth factors that have various effects on cell growth and differentiation. As one attempt to investigate the molecular basis for the TGFβs’ diverse cellular responses, we examined crosstalk between TGFβ1 and receptor-type tyrosine kinase (RTK) in SCC13 cells. EGF receptor (EGFR) was main phosphorylated RTK upon TGFβ1 treatment. TGFβ1 increased the phosphorylation of Erk1/2 as well as EGFR. To explore possible mediator for the crosstalk, N-acetyl cystein was treated before TGFβ1 treatment. Inhibition of EGFR phosphorylation by N-acetyl cystein suggests that reactive oxygen species are involved between TGFβ1 and EGFR activation. Peak induction of hydrogen peroxide was observed at 30 min treatment of TGFβ1 and catalase inhibitor prolonged TGFβ1-induced EGFR activation. This suggests the involvement of hydrogen peroxide in this crosstalk. Here, we show that TGFβ1 activates EGFR in hydrogen peroxide-dependent mechanism, which then activated genuine EGFR signaling pathway leading to the activation of Erk1/2.

2465

Identification of Arginine Methylated Proteins Undergoing Time Dependent Changes in the Cytosol of Regenerating Rat Liver

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Post-translational methylation of arginine residues of proteins is known to plays a crucial role in transcriptional regulation, signal transduction and protein-protein interactions. Previously, we showed that in vivo asymmetric dimethylarginine formation was most prominent in the proteins with molecular weights of about 18 or 32 kDa, which peaked at 1 Day following partial hepatectomy, and gradually declined to a basal level within the next 7 Day. Symmetric dimethylarginine formation detected by SYM 10 antibody was prominent in a 10-kDa protein and a 25-kDa protein. The 10-kDa methylation peaked at 3 day and the 25-kDa methylation peaked at 5 day. On the other hand, SYM11 bind to symmetric dimethylarginine in sequence different to that for SYM10, detected 25-kDa and 50-kDa arginine-methylated proteins which peaked at 3 day and 1 day, respectively. In this study, our interest was to identify the arginine-methylated proteins that showed changes in vivo methylation status during the early period of rat liver regeneration. Thus, we performed 2-D electrophoresis and immunoprecipitation. Thereafter, we conducted MS-analysis for arginine-methylated proteins. Asymmetric arginine-containing proteins identified were carbonic anhydrase3(29kda), guanidinoacetate methyltransferase(27kda), unnamed protein (35kda), Hsc70-ps1(75kda), and symmetric arginine-containing proteins were tubulin beta 2c(50kda) and guanidinoacetate methyltransferase(27kda) detected by SYM11 and glutathion-s-transferase(26kda), albumin(70kda) detected by SYM10. Intriguingly, carbonic anhydrase3 and glutathion-s-transferase are related to ROS pathway, acting as antioxidant. Therefore, it was suggested that arginine methylation is implicated with ROS pathway activation known to accompany liver regeneration. Since the in vivo arginine-methylation status of proteins changes preceding proliferation of hepatocytes upon partial hepatectomy, it was tempting to speculate that sequential methylation of several proteins are involved in an early signal critical for liver regeneration.

2466

The Hominoid-specific Gene TBC1D3 Regulates Growth Factor Signaling through Ras Activation

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TBC1D3 is a hominoid-specific family of genes that has recently appeared within the hominoid lineage. The TBC1D3 genes are encoded by a cluster of 8 paralogues arrayed along a region of human chromosome 17 that has undergone extensive intrachromosomal rearrangement and segmental duplication. TBC1D3 induces tumors in nude mice, growth in low serum and is significantly amplified in selected prostate and breast cancers (Pei et al, 2002, Cancer Res.). In this study we describe a potential mechanism by which TBC1D3 may act as an oncoprotein. Here we show that TBC1D3 increases the basal level of cellular growth and stimulates proliferation in response to epidermal growth factor (EGF). Moreover, Erk and PKB/Akt activation responses were increased after stimulation with EGF and other growth factors (i.e. PDGF and IGF-1) in cells expressing TBC1D3; meanwhile RNA silencing suppressed that activation. By measuring EGF receptor turnover, quantification of the receptor levels after EGF stimulation and light microscopy to follow receptor localization, we found that TBC1D3 expression extends the life span of EGFR. These observations suggest that increased signaling in response to EGF is coupled with significant delayed in EGFR trafficking and degradation. The measurement of Ras-GTP levels in cells expressing TBC1D3, using Raf1 Ras binding domain (RBD), revealed an augmented Ras activation in steady-state cells. This activation was further enhanced in response to serum and EGF. Immunoprecipitation in cells transfected with TBC1D3 and Grb2 showed that both proteins interact. In vitro transcription/translation and pull-down experiments using GST-coupled proteins confirmed a direct interaction. We hypothesize that TBC1D3 acts as a potent oncogenic trigger enhancing Ras activation in response to serum and growth factor receptors through its interaction with Grb2.
epidermal growth factor (EGF) receptor (EGFR) is involved in cancer development and progression. However, the role of EGFR in LPS-induced cancer behavior needs to be examined. Here, we examined the hypothesis that LPS promotes cancer cell growth and invasion through EGFR activation via a novel signaling pathway on the surfaces of the plasma membranes of carcinoma cells (biliary carcinoma Mz-ChA-1 cells and lung adenocarcinoma A549 cells). First, we show that LPS induced cancer cell growth and invasion in vitro dose- and time-dependently, effects that were prevented by pretreatment with selective EGFR inhibitors, implicating EGFR activation in LPS-induced responses. Second, we show that LPS induced EGFR phosphorylation, an effect that was inhibited by preincubation with an EGFR or a TGF-α-neutralizing antibody, which also prevented LPS-induced cancer cell growth and invasion, implicating TACE activation. Third, we examined the role of the mitogen-activated tumor necrosis factor (TNF)-α-converting enzyme (TACE), which cleaves pro-TGF-α to release mature soluble TGF-α. Small interfering (si)RNA for TACE inhibited LPS-induced TGF-α release, EGFR phosphorylation, cell growth and invasion, implicating TACE. Finally, we show that dual oxidase (Duox)1 siRNA inhibited LPS-induced generation of reactive oxygen species (ROS), activation of TACE and EGFR, cancer cell growth and invasion, implicating Duox1. These results show that LPS induces cancer cell growth and invasion via a signaling pathway on the surfaces of cancer cells and provide new insights into how bacterial products promote cancer progression.

Proteolytic Activation of T widow–Epsin Receptors during Mitochondrial Membrane Perforation

Bacterial products [e.g., lipopolysaccharide (LPS)] are implicated in the pathogenesis of chronic inflammation-associated cancer, notably in the digestive and respiratory tracts, in which the gram-negative bacteria (the major resources of LPS) are the predominant infectious agents. The epidermal growth factor (EGF) receptor (EGFR) is involved in cancer development and progression. However, the role of EGFR in LPS-induced cancer behavior needs to be examined. Here, we examined the hypothesis that LPS promotes cancer cell growth and invasion through EGFR activation via a novel signaling pathway on the surfaces of the plasma membranes of carcinoma cells (biliary carcinoma Mz-ChA-1 cells and lung adenocarcinoma A549 cells). First, we show that LPS induced cancer cell growth and invasion in vitro dose- and time-dependently, effects that were prevented by pretreatment with selective EGFR inhibitors, implicating EGFR activation in LPS-induced responses. Second, we show that LPS induced EGFR phosphorylation, an effect that was inhibited by preincubation with an EGFR or a TGF-α-neutralizing antibody, which also prevented LPS-induced cancer cell growth and invasion, implicating TACE activation. Third, we examined the role of the mitogen-activated tumor necrosis factor (TNF)-α-converting enzyme (TACE), which cleaves pro-TGF-α to release mature soluble TGF-α. Small interfering (si)RNA for TACE inhibited LPS-induced TGF-α release, EGFR phosphorylation, cell growth and invasion, implicating TACE. Finally, we show that dual oxidase (Duox)1 siRNA inhibited LPS-induced generation of reactive oxygen species (ROS), activation of TACE and EGFR, cancer cell growth and invasion, implicating Duox1. These results show that LPS induces cancer cell growth and invasion via a signaling pathway on the surfaces of cancer cells and provide new insights into how bacterial products promote cancer progression.

A Novel Mechanism of Bacterial Products-induced Cancer Growth and Invasion

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Bacterial products [e.g., lipopolysaccharide (LPS)] are implicated in the pathogenesis of chronic inflammation-associated cancer, notably in the digestive and respiratory tracts, in which the gram-negative bacteria (the major resources of LPS) are the predominant infectious agents. The epidermal growth factor (EGF) receptor (EGFR) is involved in cancer development and progression. However, the role of EGFR in LPS-induced cancer behavior needs to be examined. Here, we examined the hypothesis that LPS promotes cancer cell growth and invasion through EGFR activation via a novel signaling pathway on the surfaces of the plasma membranes of carcinoma cells (biliary carcinoma Mz-ChA-1 cells and lung adenocarcinoma A549 cells). First, we show that LPS induced cancer cell growth and invasion in vitro dose- and time-dependently, effects that were prevented by pretreatment with selective EGFR inhibitors, implicating EGFR activation in LPS-induced responses. Second, we show that LPS induced EGFR phosphorylation, an effect that was inhibited by preincubation with an EGFR or a TGF-α-neutralizing antibody, which also prevented LPS-induced cancer cell growth and invasion, implicating TACE activation. Third, we examined the role of the mitogen-activated tumor necrosis factor (TNF)-α-converting enzyme (TACE), which cleaves pro-TGF-α to release mature soluble TGF-α. Small interfering (si)RNA for TACE inhibited LPS-induced TGF-α release, EGFR phosphorylation, cell growth and invasion, implicating TACE. Finally, we show that dual oxidase (Duox)1 siRNA inhibited LPS-induced generation of reactive oxygen species (ROS), activation of TACE and EGFR, cancer cell growth and invasion, implicating Duox1. These results show that LPS induces cancer cell growth and invasion via a signaling pathway on the surfaces of cancer cells and provide new insights into how bacterial products promote cancer progression.

Profilig the Activity of 83 Protein Tyrosine Kinases with Five Substrates

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Protein tyrosine kinases (PTK) play key roles in signal transduction and normal cell growth. They are also involved in numerous proliferative diseases like cancer and atherosclerosis. Five PTK substrates were developed for highly sensitive time-resolved fluorescence energy transfer (TR-FRET) assays. The substrates are polyGT (4:1), poly GAT(1:1:1), and three peptides of 14-18 amino acids in length derived from known phosphorylation sites: CDK-1 (Tyr15), IRS-1 (Tyr983), and JAK-1 (Tyr1023). The substrates were validated using one of the cited PTKs. For example, the JAK-1 peptide was validated with its kinase JAK3. The objective was to further evaluate these substrates with a large number of PTKs. A panel of 83 purified human PTKs were evaluated. In the developed PTK assay, the enzyme was incubated with dye-labeled substrate, the enzyme reaction was stopped with EDTA, and the phosphorylated substrate was detected with a Europium chelate-labeled anti-phospho-antibody. The results demonstrated that polyGT and polyGAT are indeed universal substrates for PTKs. The CDK-1 peptide was a substrate for the SRC family of kinases including BLK, FYN, HCK, LYN/a/b, SRC, and YES. CDK-1 was also a substrate for some of the EPK family members including EphA5/B1/B2/B4. The IRS-1 peptide contains the YmxM motif and select kinases were active with this peptide including ALK, FGFR1/2, FLT4, IGFR1, JAK2, ROS, and TRKA/B. On the other hand, the JAK-1 peptide was quite generic in that kinases from 9 different families (EPK, FGFR, VEGFR, INSR, JAKA, MET, RET, SYK, TRK) phosphorylated the peptide. To our knowledge, this is the first time such extensive analysis of PTK activity versus different substrates has been undertaken. The information will benefit the investigation of PTK biology. Additionally, the data will serve as a guide for the implementation of a PTK assay in the lab.

Biphasic Activation of AMP-activated Protein Kinase during the Onset and Progression of Liver Regeneration after Partial Hepatectomy

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Liver regeneration after 70% partial hepatectomy (70PHx) involves priming of hepatocytes in the remnant liver (0.5-4 hrs) to activate the transition from the quiescent state (G0), progression through G1 and entry into S phase by 24 hrs, with restoration of liver mass by 1-2 weeks. The signals for the onset of regeneration remain poorly understood. We observed a marked (30%) and rapid (30sec) decrease in ATP and total adenine nucleotides (TAN) levels in the remnant liver following PHx, resulting in a sustained decrease in ATP/AMP ratio. These changes were accompanied by a transient activation of AMP-activated protein kinase (AMPK) peaking at 1 min after PHx, with a subsequent decline. A later, more prolonged activation of AMPK occurred peaking at 12 hrs after PHx, at the onset of the G1-S transition. The early changes in ATP and AMPK were inhibited by pretreatment with connexin-mimetic peptide inhibitors (GAP26), suggesting involvement of connexin/pannexin membrane channels. GAP26 treatment selectively suppressed a subset of gene expression changes during liver regeneration as detected by cDNA microarray analysis. 30% partial hepatectomy (30PHx) activates the priming phase of liver regeneration, but is not effective in promoting cell cycle progression and DNA synthesis in the remnant liver, as was evident in the lack of activation of markers of cell cycle progression (cyclin D1, cyclin E, PCNA). 30PHx caused a loss of ATP and early, transient activation of AMPK similar to that observed after 70PHx, with a subsequent decline. A later, more prolonged activation of AMPK was observed. These results demonstrate that polyGT and polyGAT are indeed universal substrates for PTKs. The CDK-1 peptide was a substrate for the SRC family of kinases including BLK, FYN, HCK, LYN/a/b, SRC, and YES. CDK-1 was also a substrate for some of the EPK family members including EphA5/B1/B2/B4. The IRS-1 peptide contains the YmxM motif and select kinases were active with this peptide including ALK, FGFR1/2, FLT4, IGFR1, JAK2, ROS, and TRKA/B. On the other hand, the JAK-1 peptide was quite generic in that kinases from 9 different families (EPK, FGFR, VEGFR, INSR, JAKA, MET, RET, SYK, TRK) phosphorylated the peptide. To our knowledge, this is the first time such extensive analysis of PTK activity versus different substrates has been undertaken. The information will benefit the investigation of PTK biology. Additionally, the data will serve as a guide for the implementation of a PTK assay in the lab.

Activation of AMPK by treatment with AICAR during the G1 phase promoted cell cycle progression and the onset of S phase, indicated by increased expression of markers of cell cycle progression. These data suggest that AMPK activation after partial hepatectomy is required for effective liver regeneration.
Oncogenes and Tumor Suppressors (2470-2475)

2470
Centrosome Amplification and Genomic Instability Contribute to Mammary Tumor Initiation
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Sporadic breast cancers develop through multiple genetic alterations including those in cyclin D1, Neu, EGFR, Ras, Myc, and p53. We hypothesized that K-Ras4bG12D and c-Myc initiate mammary cancers by rapidly altering the homeostasis of mammary epithelial cells. To test that hypothesis, we selectively expressed K-Ras4bG12D and c-Myc in mammary epithelial cells for five days, using the MMTV-rtTA; tetO inducible transgenic model. Acute induction of K-Ras4bG12D and/or c-Myc in the mammary epithelium led to various degrees of hyperplasia and dysplasia. Expression of c-Myc resulted in mild hyperplasia of mammary epithelial cells without significant alterations in lateral polarity, measured by E-cadherin and β-catenin immunostaining; c-Myc expressing mammary ducts maintained the epithelial lumen, and the stromal compartment was clearly visible. On the other hand, expression of K-Ras4bG12D led to severe hyperplasia, loss of epithelial lumen, and of lateral polarity; those epithelial cells occupied much of the stromal compartment. Co-expression of K-Ras4bG12D and c-Myc cooperated to induce more severe dysplasia and hyperplasia than expression of either oncogene alone; otherwise, those glands had the same structural characteristics of those expressing K-Ras4bG12D. Frequencies of proliferation and double-strand breaks foci were high amongst mammary glands expressing c-Myc and/or Ki-Ras4bG12D, while c-Myc led to elevated frequencies of apoptosis relative to Ki-Ras4bG12D. Importantly Ki-Ras4bG12D induced high frequencies of centrosome amplification, which may dictate the shorter times-to-onset of mammary tumors induced by H-Ras relative to Myc, as centrosome amplification can rapidly induce aneuploidy. Ki-Ras4bG12D induced gene expression profiles consistent to its ability to induce centrosome amplification; in addition, Ras and Myc induced molecules that may de-regulate the cell cycle, as well as those of CDKs. Our experiments demonstrate that K-Ras4bG12D and c-Myc initiate precursor lesions which morphological and cellular changes similar to those of end-stage mammary tumors, and that centrosome amplification and chromosome instability are involved in tumor initiation.

2471
Gefitinib (ZD1839, Iressa) Reduces Tumor Volume through BRAK/CXCL14 Expression
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(Objectives) Epidermal growth factor (EGF) receptor signaling plays a crucial role in the growth, metastasis, and angiogenesis of human head and neck squamous cell carcinoma (HNSCC). BRAK, also known as CXCL14, is a non-ELR motif chemokine. It plays a role not only in immune recognition processes but also acts as an anti-angiogenic factor. We recently found that mRNA expression of BRAK was specifically down-regulated by EGF treatment in HSC-3 tongue carcinoma cells and that over-expression of the gene in the cells showed anti-cancer activity (Ozawa et al., Biochem. Biophys. Res. Commun. 348: 406-412, 2006). Here, we investigated whether gefitinib (Iressa, ZD1839), a specific inhibitor of the EGF receptor, would restore BRAK mRNA expression of the cells and, if so, whether this restoration would have an anti-cancer activity. (Materials) HNSCC lines were cultured Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum. Nearly confluent cells were cultured overnight in serum-free DMEM. After starvation, they were incubated with or without EGF (10 ng/ml) and/or gefitinib (1 µM). HSC-3 cells were subcutaneously injected into athymic nude mice. HSC-3-xenografted mice were orally administered gefitinib (50 mg/kg/day). (Results) Gefitinib reduced the size of the tumors formed by HSC-3 cells in nude mice, concomitant with increases in BRAK mRNA expression in vivo. (Conclusion) Our results indicate that oral administration of gefitinib reduced tumor size, at least in part, through elevation of BRAK expression. Thus, the use of gefitinib for treatment of patients with HNSCC in whom there is an inducing effect of the drug on the BRAK expression of their cancer cells may be advantageous. Furthermore, BRAK may be a promising molecule for gene therapy of HNSCC.

2472
Effects of Txnip Ablation on Cellular Growth and Metabolism
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Cellular redox is an important regulator of proliferation, apoptosis and intracellular signaling. Thioredoxin (TRX) is a NADPH-dependent oxidoreductase that is important for the reduction of protein disulfide bonds as well as reactive oxygen species (ROS). Thioredoxin interacting protein (Txnip) inhibits TRX-mediated reduction by forming a disulfide bond with TRX’s redox active site. Deletion of Txnip in mice impaired mitochondrial oxidative phosphorylation of fuels including: glucose, fatty acids and ketones and increased the amount of phosphorylated Akt during prolonged fasting. Activation of Akt has been proposed to explain the survival advantage of cancer cells which exclusively rely on glycolysis for the production of energy (the Warburg effect). Our hypothesis was that deletion of Txnip would cause cultured cells to exhibit characteristics reminiscent of the “Warburg” cancer cell phenotype and thereby alter the metabolic and growth profile of cells in culture, as well as conferring a survival advantage to the Txnip knock-out (TKO) cells. To investigate this hypothesis embryonic fibroblasts were isolated from wild-type (WT) and TKO mice. When placed in an atmosphere of 95% O2/5% CO2 the 1st rate constant describing the growth of TKO fibroblasts was ~2-fold greater than that of WT cells. TKO fibroblasts exhibited a markedly (83%) reduced capacity to oxidize [14C]-glucose to 14CO2 and increased the concentration of lactate in culture medium by 35%. TKO fibroblasts exhibited increased expression (~3 fold) of phosphorylated Akt after 18 hour serum starvation relative to WT cells. We also observed that fibroblasts from TKO mice exhibited a marked resistance to the chemotherapeutic doxorubicin, which
induces the production of ROS as well DNA strand breaks. These combined data suggest that deletion of Txnip caused fibroblasts to exhibit a Warburg cancer cell phenotype. Thus, the thioredoxin-NADPH inhibitor Txnip links mitochondria respiration to Akt downstream signalling of energy metabolism, growth and survival.

**2473**

**Premature Dephosphorylation of γ-H2AX by Wip1 Delays Double Strand Breaks DNA Repair**

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DNA double-strand breaks (DSB) represent the most potentially serious damage to a genome, cause deadly chromosomal adjustments and, as a result, highly increase risk of cancer development. Well-organized rapid damage response and proficient DNA repair became critically important for maintaining genomic stability and organism survival. Within minutes of the induction of DNA double-strand breaks in somatic cells, histone H2AX becomes phosphorylated at serine 139 and forms γ-H2AX foci at the sites of damage. These foci then play a crucial role in recruiting DNA repair and damage-response factors and changing chromatin structure to accurately repair the damaged DNA. Dephosphorylation of γ-H2AX serves as a signal for completion of DNA repair. Here, we report that Wip1 protein, which is often over-expressed in various cancers, effectively dephosphorylates γ-H2AX in vitro and in vivo. Over-expression of Wip1 significantly reduces the level of γ-H2AX after ionizing as well as UV radiation. Forced premature dephosphorylation of γ-H2AX by Wip1 disrupts recruitment of important DNA repair factors to damaged sites, and, as a result, dramatically decreases DNA damage repair. Taken together our studies demonstrate that Wip1 is an important mammalian phosphatase of γ-H2AX and can propose additional mechanism of acting Wip1 in tumor surveillance network.

**2474**

**Fumarate Hydratase Defects and Myoma Formation: Identification of Relevant Pathways**

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Dominantly inherited fumarate hydratase (FH) defects predispose mostly to skin and uterine myomas but also to papillary renal cell cancer, Leydig cell tumors, ovary adenocarcinomas and cerebral cavernomas. The mechanisms underlying these phenotypes remain unclear. We used control and FH-deficient (FH-) primary fibroblasts to explore the role of energetic and redox metabolism in FH deficiency and to identify the mechanisms connecting FH-deficiency with myoma formation. We also used primary fibroblasts with respiratory chain defects (RC-), to distinguish the general consequences of malfunctioning mitochondrial energy metabolism from those leading to tumor formation. We chose to use primary FH- cell lines, which are not biased towards anti-apoptosis and growth promotion as tumor cells would be, but that similarly to the tumors have severely reduced FH activity. All FH- cells lacked cytoplasmic FH, but had normal levels of mitochondrial FH. Both FH- and RC- cells had glycoalysis induced. To study the redox metabolism, we focused on H2O2 and the major cell redox buffer, glutathione (GSH). FH- cells had a more reduced redox state, with increased GSH and lower H2O2, a situation which is known to repress the differentiation of stem cell-like populations. To determine if pathways associated with differentiation have a role in myoma formation in FH- patients, we performed expression microarrays of FH-, RC- and control primary fibroblasts. The fibroblast expression data was crossed with myoma expression data, to identify transcripts similarly changed in FH-deficiency and in myoma formation. A fundamental pathway regulating smooth muscle differentiation was found to be repressed both in FH- and in myomas. We confirmed the result at transcriptional and protein level. In conclusion, myomas in FH- patients seem to arise due to decreased differentiation of smooth muscle cell progenitors.

**2475**

**Tumor Suppressor p14ARF Promotes Non-classic Proteasomal Independent Ubiquitination of COMMD1**

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Although tumor suppressor cyclin-dependent kinase inhibitor 2A/alternate reading frame (p14ARF) is generally accepted for its essential role in activating p53 pathway, some of its p53 independent functions have also been proposed. Here we report a novel pathway that p14ARF regulates the nuclear factor κB (NF-κB) via the interaction with and monoubiquitination of a multifunctional factor named copper metabolism gene Murr1 domain containing 1 (COMMD1) in the nucleoplasm. We found that p14ARF interacts with COMMD1 in vivo. Deletion analysis of p14ARF suggested that there are two distinct domains of p14ARF (amino acids 15-29 and 30-45) that are important for its interaction with COMMD1. Interestingly, p14ARF promotes non-K48 but K63 mediated ubiquitination of COMMD1 in a p53 independent manner. However, p14ARF mutant lacking the interacting domains could not promote COMMD1 ubiquitination, indicating that physical association is a prerequisite condition for the ubiquitination process. We also found that p14ARF specifically induces monoubiquitination of COMMD1 and the monoubiquitinated form of COMMD1 can further serve as an acceptor for non-K48 mediated polyubiquitination. Hence, we believe that p14ARF was found defective in promoting polyubiquitination of monoubiquitinated COMMD1 chimera, which could readily induce polyubiquitination by itself. Most importantly, NF-κB transactivation activity was inhibited by the monoubiquitinated COMMD1. Compared with unmodified COMMD1, monoubiquitination resulted in stronger inhibition on transactivation of NF-κB at basal level or in response to TNF stimulation. Taken together, these data suggest that p14ARF specifically interacts with COMMD1 in the nucleoplasm and promotes monoubiquitination of COMMD1. The addition of single ubiquitin on COMMD1 can further induce non-K48 mediated polyubiquitination which result in stronger inhibition on the transactivation activity of NF-κB.
**Cell Cycle Controls (2476-2484)**

2476

**Cdk2 Is a Major Mediator of Centrosome Amplification Induced by Genetic Ablation of p53**

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The centrosome controls chromosome segregation by directing the formation of two spindle poles and equal segregation of sister chromatids during mitosis. Unregulated centrosome duplication or cytokinesis results in centrosome amplification (CA) and chromosome instability (CIN). As most human cancers harbor elevated frequencies of CA and CIN, they may contribute to the genesis and sustenance of most cancers. Cyclins E/A/Cdk2 may coordinate the centrosome duplication cycle with the cell cycle, ensuring that replication of the genome and its segregation during mitosis is tightly coordinated. However, Cyclins E1/E2’ or Cdk2’ mouse embryonic fibroblasts (MEFs) grow slowly and harbor normal centrosomes, suggesting that other kinases may support Cdk2 in regulating the cell and centrosome duplication cycles. Cdk2 impinges on the centrosome duplication cycle by phosphorylating targets such as nucleophosmin B (NPM) in G1; indeed, NPM is a major suppressor of CA, as its genetic ablation or hyperactive G1 Cdkks cause CA. P53 might maintain normal centrosome complements by regulating Cyclin E/Cdk2 activity, as genetic ablation of p53 leads to constitutive Cdk2 activity, CA, and CIN. As p53 is a potent transcription factors that induce plethora of genes, the relationship between p53, activation of Cdk2 and CA remains correlative; to test that relationship genetically, we generated wt, Cdk2’/’ or p53’/’ mouse embryonic fibroblasts (MEFs) grow slowly and harbor normal centrosomes, demonstrating that Cdk2 is a major mediator of CA and CIN induced by the p53 pathway.

2477

**Cold Adaptation and Growth of Yeast at Low Temperatures Involves Diverse Cellular Functions and Physiological Processes**

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During studies of membrane biogenesis in yeast, we discovered an unexpected link between cold adaptation and the endoplasmic reticulum (ER) quality control system known as ERAD (ER-associated degradation). Specifically, a subset of ERAD genes (UBC7, CUE1 and DOA10) are required for both karmellae assembly and growth of yeast at low temperatures (≤10°C). Mutations in these genes leads to alterations in sterol composition, including increased levels of 4,4-dimethylzymosterol and fecosterol. The abnormal sterol composition of these cold-sensitive mutants suggests that a primary defect produced by loss of UBC7, CUE1, or DOA10 is the inability to properly regulate sterol metabolism, which may, in turn, lead to cold sensitivity. To further explore the physiology and genetics of growth at low temperatures, we screened the complete Sacchromyces cerevisiae deletion collection to identify all genes that are required for growth of yeast in the cold. Our screen of 4746 homozygous diploid deletion mutants and 4773 haploid deletion mutants identified 360 diploid mutants and 350 haploid mutants that grow slowly or not at all at 10°C. Among these mutations, 201 were identified in both haploid and diploid deletion sets. We also screened a collection of heterozygous deletion mutants to identify genes that are haploinsufficient for growth at low temperature. Of the 1143 mutants screened, 29 were haploinsufficient. Thus, our screens identify 230 genes that are required for growth of yeast in the cold. The majority (~90%) of these genes fall into Gene Ontology categories related to cell communication, establishment and/or maintenance of cell polarity, cell development, apotrophy, cell cycle processes, cytoskeletal organization, mitochondrial organization and biogenesis, and membrane organization and biogenesis. Thus, as expected from studies of bacteria and plants, cold adaptation in yeast involves a variety of cellular functions, many of which may be subject to regulation by ERAD.

2478

**ATM and ATR Check Point Kinases Control Cyclin D1 Expression**

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We have shown that cyclin D1 expression must decline during S phase. This suppression, which requires phosphorylation of Thr286, is a critical regulator of overall cell cycle progression. Glycogen synthase kinase-3 was reported to be the Thr286 kinase, but our data show that another kinase, one regulated by DNA synthesis directly, is responsible. Here we identify if ATR, the check point kinase known to be activated even during normal S phase, can phosphorylate cyclin D1 on Thr286. (Methods) These studies rely upon techniques that allow quantitative, single cell analyses through the cell cycle of actively proliferating cultures; including microinjection, time-lapse, and quantitative image analysis. All results, however, were confirmed by western analysis. (Results) ATR is activated by UV irradiation exclusively during S phase as indicated by phosphorylation of histone H2AX. UV also induces phosphorylation of cyclin D1 Thr286 exclusively during S phase. Pre-treatment of cells with siRNA against ATR blocks phosphorylation of both proteins. The specific ATR inducer, TopBP1, promotes phosphorylation of both H2AX and cyclin D1 throughout the cell cycle; except following caffeine treatment. Critically, even during normal cell proliferation, siRNA against ATR induced a slight suppression of cyclin D1 phosphorylation. Finally, a second check point protein, ATM; was specifically induced by double stranded DNA breaks, and specifically inhibited by Ku55933. With these tools we found that ATM is also able to phosphorylate cyclin D1. (Conclusions) These data indicate that both ATM and ATR control cyclin D1 phosphorylation, either directly or indirectly. This phosphorylation plays a role in the check point response following DNA damage; and in the case of ATR, plays a role in regulating cyclin D1 expression even during normal cell cycle progression. The connection between the check point response, cyclin D1, and normal cell growth has important implications.
2479

Novel Role for the CDK-activating Kinase Cak1 in Actively Growing Cells

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Cdc28 is the main cyclin-dependent kinase (CDK) directing the yeast cell cycle. Besides cyclin binding, Cdc28 requires phosphorylation by the Cak1 kinase to achieve full activity. We have previously found that carboxy-terminal cdc28 mutants exhibit temperature sensitivity and high chromosome instability, phenotypes that are both suppressed by high copy Cak1. Interestingly, the suppression is independent of Cak1’s catalytic activity. Further, overexpression of the thirteen carboxy-terminal amino acids of Cdc28 is deleterious in both cdc28CST and cak1 mutants. On the other hand, mutations in the carboxy terminus of Cdc28 render cells highly sensitive to changes in Cak1 integrity and dosage: These mutants are unable to survive in the presence of cak1 temperature sensitive mutations, or even when Cak1 is slightly modified by the introduction of a 13-Myc or 3-HA epitope tag. This modification causes only mild growth defects in a wild type CDC28 background. Based on these results, we suggest that for the Cdc28 complexes to remain stable and active, an interaction with Cak1 is needed via Cdc28’s carboxy terminus. Supporting this model, we found that actively growing yeast cells require maintaining an optimum Cdc28:Cak1 ratio that favors the interaction. Nutrient depletion and entry into stationary phase result in alteration of this ratio via active proteolytic degradation of mainly Cak1, and of Cdc28 to a lesser extent. We suggest that Cak1’s novel role promoting stability of Cdc28 complexes represents a means to most effectively coordinate nutrient availability and cell growth in S. cerevisiae.

2480

Role of Adherens Junction Complexes in Regulating Cell Proliferation in the Vertebrate CNS

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Regulation of the cell cycle in the central nervous system (CNS) is necessary to generate the proper number of neurons and glia. Misregulation of the cell cycle may lead to uncontrolled division, resulting in cancer or a reduction in the number of specific cell types (due to decreased precursor populations). Several pieces of evidence suggest that junctional complexes regulate the cell cycle. In vitro studies indicate that confluent cells do not normally respond to mitogenic factors. However, disruption of E-cad results in proliferation of confluent cells in response to mitogenic factors. Moreover, overexpression of E-Cad in vitro reduces the ability of FGFs to activate the ERK pathway. These observations suggest that the expression of cadherins might negatively regulate cell cycle progression by inhibiting cells from responding to mitogenic factors. In KIF3 mouse knockouts, in which the subcellular localization of N-cad and AJ proteins such as β-catenin is defective, enhanced proliferation of neural progenitor cells is observed. This suggests that disruption of epithelial organization, by preventing AJ assembly, might result in increased proliferation. Preliminary results in the zebrafish indicate that loss of N-cadherin (a classical cadherin expressed specifically in the neural tube) results in an increase in neurogenesis and cell division at the 14-15 s stage (a stage at which AJs are formed in the wildtype). There appears to be a 2 fold increase in cell division which was quantified using antibodies against phosphorylated histone H3 and BrdU. Here, we analyze the role of N-cad and other AJ components in regulating cell proliferation.

2481

Structural Conformational Changes in p27Xic1 Mediate Cyclin/Cdk Binding and Inhibitor Stability

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Progression through the cell cycle depends on modulation of the activity of Cyclin/Cdkks through post-translational modifications, protein degradation, and binding of inhibitors. The p27Xic1 protein is the only known cyclin/Cdk inhibitor found in Xenopus laevis. It shares over 40% identity with its mammalian counterpart, p27Kip1, and over 50% with the N-terminus domain of p21Cip1/WAF1. Our results show that conformational changes in p27Xic1 are associated with post-translational modifications and result in the release of the inhibitor from Cyclin E/Cdk. To determine the contribution of secondary structure changes in p27Xic1 binding to Cyclin E/Cdk2, we expressed and purified various constructs of p27Xic1 and measure structural changes by circular dichroism spectroscopy. Our data indicate Thr204Asp (mimics phosphorylation) releases the inhibitor from Cyclin E/Cdk2 and this event is associated with differences in alpha structure content. Accordingly, deletion in either the N-terminus region involved in cyclin binding or the C-terminus domain resulted in reduced association to the complex and loss of secondary structure in agreement with the fold-in-binding model proposed for cyclin-dependent inhibitors. Further experiments were devoted to identify the role of the Cyclin E/Cdk2 target site Thr204 for inhibitor stability and its function during cell cycle progression. One-cell stage embryos were injected with p27Xic1Thr204Ala, Thr204Asp or wild type protein and monitored for inhibitor stability and cell division progression. Our data indicate that p27Xic1Thr204Ala is remarkably more stable and thus causes a greater impact on cell progression in Xenopus embryos. As expected, cell cycle arrest resulted in increased levels of tyrosine phosphorylation in Cdc2/Cdk2 and cyclin B1 and B2. Overall, our results establish that, unlike p27Kip1, binding of p27Xic1 to Cyclin E/Cdk2 complex is not required for inhibitor turnover and thus alternative mechanisms exist. It also demonstrates for the first time the relevance of Thr204 modification for structural stability and in complex association.

2482

As2O3 Attenuates Mitotic Arrest and Spindle Checkpoint Activation Induced by Paclitaxel

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To human health, arsenic exhibits the property of a double-edged sword. Arsenic compounds such as As2O3 is effective for the treatment of relapsed/refractory acute promyelocytic leukemia, whereas chronic exposure to environmental arsenic is associated with the development of a variety of common cancers. To date, the mechanism by which arsenic compounds promote carcinogenesis remains unclear. Substantial evidence suggests that genomic instability in cells exposed to arsenic may account for its carcinogenic effect. Because As2O3 is capable of inducing mitotic arrest which appears to be due to its ability to inhibit tubulin polymerization, we examined whether there existed any functional interaction between As2O3 and...
paclitaxel, a well-known microtubule poison. Flow cytometry and fluorescence microscopy revealed that that although As2O3 alone caused a moderate level of mitotic arrest in HeLa cells, it greatly attenuated mitotic arrest induced by paclitaxel. Western blot analysis showed a dose-dependent suppression of paclitaxel-induced phosphorylation of BubR1, Cdc20, and Cdc27, suggesting that As2O3 compromised the activation of the spindle checkpoint. Our further studies revealed that the attenuation of paclitaxel-induced mitotic arrest by As2O3 resulted primarily from sluggish cell cycle progression. It is rather intriguing to observe that As2O3 significantly slows down taxol-induced mitotic arrest. The clinical efficacy of taxol is associated with its ability to induce mitotic arrest and subsequent mitotic catastrophe. To a less extent, As2O3 is also capable of inducing mitotic arrest. Our observation that As2O3 has a negative impact on the cell cycle checkpoint activation by taxol would have significant clinical implications. For example, if it also occurs in vivo, it may be necessary to take into consideration the spindle checkpoint status/integrity of myeloid leukemia cells when the patients undergo chemotherapy with As2O3.

2483

Regulation of Cyclin D in Neuronal Differentiation and Death
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It has been observed that neurons make a futile attempt to re-enter the cell cycle during neuronal apoptosis which is accompanied by modulation of the levels and activity of cell cycle proteins like cyclin D1 and cdk4. It is not entirely clear how Cyclin D1 levels are regulated in response to signals that induce neuronal differentiation and “de-differentiation” which could cause apoptosis. We are addressing this issue by using PC12 cells, these cells differentiate to neuron-like phenotype in response to NGF treatment. In response to NGF treatment, Cyclin D1 protein and RNA levels increased. Using an inhibitor of MEK1/2, U0126, we could demonstrate that the MAP kinase pathway regulates Cyclin D1 differentially. When NGF was added for 6h in the presence of U0126 a significant increase in cyclin D1 levels was observed, suggesting that the MAPK pathway suppresses the expression of cyclin D1 in the early stage of differentiation. In contrast, when NGF was added for 48 hours (when most cells are differentiated or are in the process of differentiation) in the presence of U0126, elevation in Cyclin D1 levels was not seen. To identify the transcription factors (TFs) involved in Cyclin D1 expression, regulation of Cyclin D1 promoter was studied. Deletion and mutational analysis of the promoter suggested at least two different classes of TFs may regulate Cyclin D1 transcription.

2484

Regulation of CDKN1A Expression in Keratinocytes by Distal-less 3
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The balance between proliferation and differentiation has always been a central question in development. Among the homeobox genes, it has been shown that some of them can act directly on the regulation of cell cycle checkpoints, inducing or preventing cells from exiting the cell cycle. Thus, the role of transcription factors involved in embryonic development has become increasingly relevant. The homeodomain transcription factor Dlx3 is expressed in the suprabasal layer of stratified epidermis and in the basal matrix of the hair follicle during the later stages of embryonic development. Previous studies in our laboratory show that the ectopic expression of Dlx3 in the proliferative basal layer of the epidermis in transgenic mice, lead to premature differentiation of the keratinocytes. To determine the role of Dlx3 in the control of the cell cycle, we analyzed by microarray the modified gene expression profile of keratinocytes transduced with a retroviral vector expressing Dlx3. The overexpression of Dlx3 in Normal Human Keratinocytes caused an arrest of the cell cycle in G1 phase due to an upregulation of CDKN1A. Analysis of the CDKN1A promoter determined a putative Dlx3 binding site 2.5 Kb upstream of the transcription start site. Performing Luciferase Reporter Assays, Electrophoretic Mobility Shift Assays and Chromatin Immunoprecipitation, we demonstrated that CDKN1A is a direct target of Dlx3 in human and mouse keratinocytes. Recently we confirmed the upregulation of CDKN1A and, furthermore, the downregulation of Cyclin D in synchronized cells using a PAM212 Dlx3 Tet-on system. We are currently assessing the CDKN1A regulation by Dlx3 using shRNA in human keratinocytes. These data demonstrate an important role of Dlx3 in the control of the cell cycle in keratinocytes.

Steroid Hormones and Receptors (2485)

2485

Tripartite Motif-containing Protein 22 Nuclear Bodies Exhibit Dynamic Changes during Cell Cycle Progression
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Tripartite motif containing protein 22 (TRIM22) is also referred to as Stimulated Transacting Factor of 50kDa (Staf50). It was originally identified as an interferon-inducible gene in human lymphoblastoid Daudi cells. It is a member of the RING-finger family of proteins and it has been implicated in antiviral response, cell growth and differentiation. In this study, we found that TRIM22 up-regulation by progesterone in breast cancer cells was associated with progesterone-induced growth inhibition. Polyclonal antibody generated against the 498 aa protein detected progesterone-induced nuclear bodies that were primarily in the nucleolus. The speckled distribution was also observed in cells that had a distinct nuclear boundary in the G2/M population. However, during M phase, when DNA condenses and segregates, TRIM22-EGFP became completely dispersed. Regular multiple TRIM22 bodies were evident in cells undergoing cytokinesis and became abundant after the cells entered G0/G1 phase. In addition, MCF7 cells stably expressing TRIM22-EGFP, TRIM22 bodies aggregated when serum-starved or when treated with the DNA synthesis inhibitor methotrexate while serum treatment led to dispersion of these bodies into multiple smaller foci. These results suggest that TRIM22 is a dynamic protein which may have a function in cell cycle regulation.
Late Abstracts                         Wednesday

Apopotosis (2486-2496)

2486
The Effects of Radiation on Xenopus laevis and Wild Population Amphibian Embryos
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UV-B radiation is hypothesized to be one of the environmental stressors that cause declines in amphibian populations. Due to high intensities of UV-B, DNA damage and developmental abnormalities arise. In the model amphibian, *Xenopus laevis*, treatments with ionizing radiation before the midblastula transition (MBT) can lead to apoptosis. The MBT is a major developmental transition where cell divisions become asynchronous and the cell cycle acquires gap phases and checkpoints. Early apoptotic death might explain some of the mysterious cases of amphibian decline. In *X. laevis*, the effect of ionizing radiation on native amphibian species. We are also testing the effect of UV-B on *X. laevis* and wild species at a more environmentally relevant source of DNA damage. Current studies with embryos from spring peepers, gray tree frogs, toads, green frogs and wood frogs collected in southwest Virginia indicate a diverse response to DNA damage with some species activating checkpoints before the MBT. Studies with UV-B on *X. laevis* embryos have demonstrated an apoptotic response similar to that induced by ionizing radiation. As we continue these studies, we will develop a better understanding of the plasticity in DNA damage response at both the developmental and evolutionary scales.

2487
Baicalein Protects Neuronal Cells against Endoplasmic Reticulum Stress-induced Apoptosis
J. Choi, I. Kang; Biochemistry, Kyung Hee University Medical School, Seoul, Republic of Korea
Baicalein (BE) and baicalin (BI), the major flavonoid component of *Scutellaria baicalensis*, have been demonstrated to prevent oxidative stress-induced cell damage. Endoplasmic reticulum (ER) stress is caused by disturbances in structure and function of ER with the accumulation of misfolded proteins and alteration of calcium homeostasis. ER stress has been suggested to be involved in some neurodegenerative diseases such as Alzheimer’s disease and Parkinson’s disease. Here we examined the roles of BE and BI in ER stress-induced apoptosis. BE but not BI inhibited apoptosis induced by thapsigargin, tunicamycin, and brefeldin, which are well known ER stress-inducing agents. We showed that BE but not BI reduced ER stress-induced accumulation of reactive oxygen species (ROS) and maintained mitochondrial membrane potential in ER stress-induced cells. We also showed that BE reduced ER stress-induced induction of glucose-regulated protein (GRP78), C/EBP homologous protein (CHOP), phosphorylated eukaryotic initiation factor-2α (phospho-eIF2α), x-box-binding protein-1 (XBP-1), and phosphorylated p38 mitogen-activated protein kinase (p38MAPK). In conclusion, these observations suggest that BE inhibits the ER stress-induced apoptosis by inhibiting ROS and ER stress protein induction in neuronal cells.

2488
Blm-s Participates in the Regulation of Neuronal Apoptosis at Later Embryonic Stage
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We have previously identified a novel member of Bcl-2 superfamily named as Blm-s, functioning as a pro-apoptotic molecule in the developing nervous system. In neuronal and non-neuronal cell lines, overexpression of Blm-s induces apoptosis through a conventional mitochondria-dependent pathway and in a Bax-dependent way. Because Blm-s transcript is expressed in the developing brain at later embryonic stage when CNS morphogenesis and physiological neuronal death occur, we wonder that Blm-s may participate in the regulation of neuronal apoptosis at this stage. We thus exposed E15.5 mice to γ-irradiation to create DNA double strand breaks (DSBs) and to see whether Blm-s is transcriptionally induced under such DSBs-induced neuronal apoptosis. Immunohistochemistry of mouse brain show that Blm-s is transcriptionally induced in postmitotic migrating neurons, and some of which undergoes apoptosis. Specifically, Blm-s transcript is abundantly induced in lateral ventricle susceptible to γ-irradiation-induced DSBs and apoptosis.

2489
Analysis of Apoptosis Signaling Pathways Using Luminex xMAP Technology
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Normal cellular processes involve the continuous turnover of cells within a tissue or organ. Apoptosis is the process whereby individual or select groups of cells are removed during development, as well as throughout the lifespan of an organism. Apoptosis can be initiated as a protective mechanism for the removal of cells containing damaged DNA as well. The dysfunction of apoptotic pathways plays a central role in several pathological states, including cancer and ischemic injury. Several proteins play key roles in the transduction of apoptotic signals within a cell. The ability to monitor the abundance of these specific apoptotic pathway member proteins is necessary for a thorough understanding of the physiological as well as pathological processes these pathways affect. To facilitate the analysis of several apoptosis pathway members, we have constructed a set of Luminex based immunoassays. Assays were developed to analyze the apoptosis pathway member proteins: cleaved PARP, phosphorylated Histone H2A.X (S139), phosphorylated Histone H2B (S14), total and phosphorylated Bad (S112), total and active Caspase 8 and GAPDH. Assay conditions were further optimized for the simultaneous analysis of active Caspase 3, active Caspase 8, cleaved PARP, phosphorylated Histone H2A.X and GAPDH in a single assay. Presented here are the results of several experiments designed to fully characterize these immunoassays using the Jurkat human T cell line treated with anisomycin or the A431 human carcinoma cell line treated with EGF (phosphorylated Bad). Assay response to both treated and non-treated cellular lysates was determined to be both specific and analyte concentration dependant. Western blotting experiments corroborated the results observed for the developed assays. These results demonstrate that the apoptosis analysis tools we have designed are an effective and efficient method to monitor critical constituents of the apoptotic pathway.

RETURN TO MAIN MENU
Inhibitory Effects of Vam3 on Apoptosis Induced by a Mitochondrially Targeted Ceramide in MCF7 Breast Carcinoma Cells

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Vam3, a dimer of diphenel ethylene, is an extract from Vitis amurensis (Rupr.), which grows in the northeast and central parts of China. Its roots and stems have been used in Chinese folk medicines for generations. Our previous studies found Vam3 showing obvious inhibitory effects on inflammation both in vivo and in vitro by reducing production of pre-inflammatory cytokines and mediums such as IL-8 and LTβ. Oral administration of Vam3 significantly inhibited ovalbumin-induced increase in eosinophil counts and upregulation of TNF-α, IL-4, IL-5 and IL-13 in the BALF (Bronchoalveolar fluid). It also decreased the ovalbumin-induced lung tissue damage and mucus production and showed anti-asthmatic effects. To investigate the effects of Vam3 on apoptosis, we have established MCF7 cells stably expressing GFP-tagged Bax. This allows us to track the movement of this protein upon apoptosis induction. Using a mitochondrially targeted ceramide as the apoptotic inducer, we found Vam3 significantly inhibited Bax translocation from the cytoplasm to mitochondria. Vam3 was shown to be effective at concentrations ranging from 0.5 μM to 2 μM. Vam3 inhibited cytochrome c release from mitochondria and reduced nuclear fragmentation. Overall, our study has identified a novel anti-apoptotic property of the extract from Vitis amurensis.

Apoptosis Is the Primary Phenotype Evoked by p190RhoGAP Overexpression

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p190RhoGAP (p190) is a RhoGTPase activator protein (GAP) that has been shown to regulate actin cytoskeletal dynamics via Rho-dependent signaling pathways. By enhancing Rho-mediated hydrolysis of RhoGTP to RhoGDP, p190 negatively regulates association of RhoGTP with its downstream effectors, such as ROCK and mDia. The p190 gene is located on chromosome 19q13.3, which is mutated in several solid tumor types, suggesting its involvement in malignant transformation. Further support of its role in cancer is the finding that p190 reduces the incidence of PDGF-induced gliomas in mouse models. Previous studies in our lab and others revealed that overexpression of p190 in epithelial cells led to the formation of multi-nucleated cells, implicating its importance in cytokinesis, or to chromatin condensation, a marker of apoptosis. In contrast, overexpression of p190 in fibroblasts led to dendrite-like formation or chromatin condensation. To gain further insight into which cellular processes p190RhoGAP influenced most, and how p190 might function as a tumor suppressor, both epithelial cells and fibroblasts were examined for the frequency of multi-nucleation, dendrite-like formation, or apoptosis in cycling cell populations overexpressing p190. Anti-p190 and TUNEL immunofluorescence indicated that such damage was due to apoptotic rather than necrotic cell death. A decrease in the Bcl2/Bax ratio by 9-folds during effects. To investigate the movement of this protein upon apoptosis induction. Using a mitochondrially targeted ceramide as the apoptotic inducer, we found Vam3 significantly inhibited Bax translocation from the cytoplasm to mitochondria. Vam3 was shown to be effective at concentrations ranging from 0.5 μM to 2 μM. Vam3 inhibited cytochrome c release from mitochondria and reduced nuclear fragmentation. Overall, our study has identified a novel anti-apoptotic property of the extract from Vitis amurensis.

Trichostatin A Induces Endoplasmic Reticulum Stress Proteins and Apoptosis in Neuroblastoma Cells

H. Yeon, I. Kang; Biochemistry and Molecular Biology, Kyung Hee University Medical School, Seoul, Republic of Korea

Endoplasmic reticulum (ER) stress is caused by disturbances in structure and function of ER with the accumulation of misfolded proteins and alteration of calcium homeostasis. ER stress has been suggested to be involved in various common diseases such as diabetes, ischemia, and neurodegenerative diseases as well as in some anticancer agents-induced apoptosis. Trichostatin A (TSA), a potent inhibitor of histone deacetylase activity, has been suggested to induce growth arrest and apoptosis in various cancer cells, however their mechanisms are not completely understood. Thus, we examined the effect of TSA on apoptosis in neuroblastoma cells and the mechanism of TSA-induced apoptosis. We showed that TSA but not other histone deacetylase inhibitors such as sodium valproate and sodium butyrate induced apoptotic cell death in neuroblastoma. We also showed that TSA induced generation of reactive oxygen species as determined by DCFH staining. Interestingly, we found that TSA at the cytotoxic concentration induced ER stress responsive proteins including glucose-regulated protein (GRP78), C/EBP homologous protein/growth arrest and DNA damage-inducible gene 153 (CHOP/GADD153), x-box-binding protein-1 (XBP-1), and phosphorylated eukaryotic initiation factor-2α (phospho-eIF2α), to a similar potency as thapsigargin, a well known ER stress-inducing agent. We also showed that pretreatment with catalase or calcium ion chelators BAPTA-AM/EGTA partially reduced TSA-induced CHOP induction. In conclusion, these observations suggest that ER stress contributes TSA-induced apoptosis and calcium ion may be involved in TSA-induced ER stress in neuroblastoma cells.

Molecular Characterization of Apoptotic Effects of Torsion of Testis

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Testicular torsion is associated with damage to the testicular tissue as a result of ischemia-reperfusion (I-R) injury of the testis caused by the twisted spermatic cord and its release. Although necrotic cell death has been thought to be the predominant type of cell death after I-R of the testis, evidence has shown the involvement of apoptotic cell death. Thus, we aim to investigate the involvement of apoptotic cell death following acute IRI in the germ cells of the rabbit testis. The left testes of 6-12 months old NZW rabbits were subjected to 1 hour of ischemia-inducing torsion by cross-clamping the left spermatic cord followed by reperfusion. The right testes served as internal control. Both testes were excised after 6 months. Germ cell apoptosis was evaluated by TUNEL assay. Gene expression of the survival genes: survivin, Bcl-2 and Bax was assessed by quantitative reverse transcription-polymerase chain reaction (RT-PCR). Johnsen score was used to assess morphological damage for control and testes undergoing I-R injury. After 6 months of ischemia/reperfusion injury (IRI), the decline in Johnsen score by almost 50% was reflective of testicular tissue damage. TUNEL staining indicated that such damage was due to apoptotic rather than necrotic cell death. A decrease in the Bcl2/Bax ratio by 9-folds during
IRI is suggestive of an apoptotic event. Reduction in the gene expression of Survivin, an inhibitor of apoptosis, during IRI by half its initial amount in control tests coincides with the above findings in support of apoptotic cell death. We postulate that the decreased expression of Survivin and Bel-2/Bax ratio in the testes after IRI may accelerate germ cell death by apoptosis. In conclusion, we postulate that the decreased expression of Survivin and Bel-2/Bax ratio in the testes after IRI may accelerate germ cell death by apoptosis.

2494
Cytocidal Effect of TALP-32 on Human Cervical Cancer Cell HeLa
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TALP-32 is highly basic protein with a molecular weight of 32 kDa purified from human term placenta. Some basic proteins such as defensins and cecropins are known to induce cell death by increasing membrane permeability and some of them are under development as an anticancer drug especially targeting multi-drug resistant cancers. Therefore, we investigated cytotoxic effect and mechanism of TALP-32. When HeLa cell was incubated with TALP-32, cytotoxicity increased in time and dose dependent manner. As time goes by, HeLa cells became round and plasma membrane was ruptured. Increase of plasma membrane permeability was determined with Annexin V/PI staining and LDH release assay. Also in transmission electron microscopy, typical morphology of necrotic cell death, such as plasma membrane swelling and intracellular organelle disruption was observed, but DNA fragmentation and caspase activation was not. These data suggests that TALP-32 increases permeability of cell membrane and induces necrotic cell death. TALP-32 may be novel peptide cancer therapeutics because it induces necrotic cell death and not apoptosis.

2495
Angiotensin II-induced Apoptosis Requires the Type 2 Receptor and SHP 1/2 Phosphatase
Y. H. Lee, O. Mungunsukh, R. M. Day; Pharmacology, Uniformed Services University, Bethesda, MD
Angiotensin II (ANG II) has been identified as a pro-apoptotic and pro-fibrotic factor in Idiopathic Pulmonary Fibrosis (IPF). Using bovine pulmonary artery endothelial cells (BPAEC), we studied the signaling mechanism of ANG II-induced apoptosis. ANG II induced apoptosis in BPAEC within 24 h as measured by Neutral Comet Assay; we also observed release of cytochrome C and activation of Caspase 3 within 16 h. ANG II-induced apoptosis was reduced with the inhibitor of angiotensin II receptor type 2-selective receptor antagonist PD123319 but not by Telmisartan, a selective inhibitor of the type 1 ANG II receptor. Moreover, ANG II type 2-receptor agonist (CGP-42112) resulted in a greater cellular apoptosis. ANG II has been shown to signal through the activation of protein tyrosine phosphatases (PTPase) and protein kinase C (PKC). ANG II induced apoptosis was also blocked by the non-specific inhibitor of tyrosine phosphatase (sodium orthovanadate) and SHP1/2 PTPase (NSC-87877), while inhibitors of PKC (Chelerythrine Chloride and Calphostin C) did not inhibit apoptosis. Previous studies demonstrated that hepatocyte growth factor (HGF) inhibits epithelial and endothelial cell apoptosis in culture, and ameliorates lung fibrosis in animal models for IPF. 24 h pretreatment of cells with HGF was reduced ANGII-induced apoptosis. Our data suggests that ANG II-induced apoptosis requires the type 2 receptor and SHP 1/2 PTPase; the pro-apoptotic activity of ANG II can be blocked by the anti-fibrotic factor HGF.

2496
Cytotoxic Effects of Azurin from P. aeruginosa on Oral Squamous Carcinoma YD-9 Cells
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The anticancer effect of purified azurin from Pseudomonas aeruginosa (P. aeruginosa) was investigated in human oral squamous carcinoma (YD-9) cells. Azurin isolated from P. aeruginosa has been reported to shrink the malignancy in tumor-bearing mice with no apparent harmful side effects. Since oral cancer cells are known to exhibit strong resistance to available anticancer drugs, the targeted elimination of oral squamous carcinoma cells by apoptosis has emerged to be the valued strategy to combat oral cancer. Thus, we chose to test the effects of azurin on YD-9 cells in this research. First, to test the effects of azurin on the cytotoxicity of p53 positive YD-9 cell, both p53 positive YD-9 and p53 negative MG-63 (osteosarcoma) were treated with various doses of azurin. Only YD-9 cells exhibited dose dependent cytotoxicity to azurin, which indicated a link between the tumor suppressor p53 gene to azurin. In YD-9 cells, azurin treatment increased the expression level of p53 by forming complexes with p53. This result suggests that azurin stabilizes p53 leading to apoptosis in YD-9 cells, and thus azurin may be a potentially powerful anticancer agent to YD-9 cells.

Cytokines (2497-2498)

2497
Cationic Protein Derived from Human Eosinophil Basic Granule Enhances the Stress Fibers in BALB/c 3T3 Cells and Accelerates Neonatal Cardiac Differentiation
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The basic granule of activated eosinophil contains eosinophil cationic protein (ECP). ECP has been demonstrated to show growth inhibitory effects on some cancer-derived cells as well as the bactericidal and anti-parasitic activities. In spite of the 3-D structure similar to pancreatic ribonucleases, ECP shows very low ribonucleolytic activity, which appears difficult to explain the cytotoxic effects. Although the mechanism of the effect is not clear
yet, ECP has been considered to be involved in the inflammation of respiratory epithelial cells in asthma. However, we have recently found that ECP supports the growth of Balb/c 3T3 cells. In the presence of ECP these cells showed also the flat shape together with the enhanced stress fibers and focal adhesions. ECP cancelled the typical morphological change into spindle shape in Balb/c 3T3 cells, but not the growth induced by FGF-2. The flat cell shape was abrogated by ROCK inhibitor, so that this effect of ECP should depend on the Rho/ROCK signaling pathway, which regulated the stress fiber formation. Since the Rho/ROCK signaling pathway controls the differentiation and muscle contraction in cardiomyocytes, the effect of ECP was assessed on primary cultures of cardiac myocytes derived from rat neonate. As the result, ECP promoted the differentiation of the myocytes showing accelerated beating rate of the cells as well as the expression of atrial natriuretic factor known as the cardiac muscle-specific markers. The stimulatory mechanism of ECP is not clearly understood yet, ECP is a potential cytokine working as a differentiation factor affecting the cytoskeletons.

2498
Effect of Oleic Acid on Lipopolyscharide-induced Cyclooxygenase-2 Expression in Microglial Cells
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Microglia, brain-resident macrophages, are activated in response to cerebral ischemia, infection, and neurodegenerative diseases and are major responsive cells to endotoxin LPS. Oleic acid (C18:1), a monounsaturated fatty acid, is known to have both pro- and anit-inflammatory effects in different cell types including macrophages and endothelial cells. In this study we examined the effect of oleic acid on LPS-induced cyclooxygenase-2 (COX-2) expression and the mechanism involved in BV2 microglial cells. Oleic acid inhibited LPS-induced COX-2 mRNA and protein expressions and it also modestly increased microglial cell proliferation and survival. Mitogen activated protein kinases (MAPKs) and PI 3-kinase/Akt pathways are activated and act mediators of LPS-induced expression of inflammatory genes. We found that oleic acid inhibited LPS-induced activation of Akt and p70S6 kinase, a downstream of Akt, whereas it had no effect on the activation of extracellular signal-regulated kinase (ERK1/2) and p38 MAPK. We also showed that oleic acid inhibited phosphorylation of inhibitor IkappaB kinase (IKK) and activation of NF-kappaB in LPS-stimulated microglial cells. Taken together, our results showed that oleic acid showed the anti-inflammatory effect via inhibiting Akt and NF-kappaB pathways in microglial cells and it deserves further study on the therapeutic benefits for suppression of inflammatory-related neuronal injury in neurodegenerative diseases.

Mitosis and Meiosis (2499-2512)

2499
Toward a Network View of Nuclear Envelope Function and Chromatin Biology
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In order to achieve a global view of early embryogenesis in C. elegans, we have undertaken a systems approach. By integrating different functional genomic datasets, we have represented interrelationships between genes using network models. The higher-order structure of these models indicates that most genes inhabit highly interconnected sub-networks called modules. Such modules are often enriched for genes representing a specific functional category. The nuclear envelope module shares several connections to the module enriched for genes required for chromatin maintenance, including a shared connection with mel-28, a gene known to be required both for proper function of the nuclear envelope and for chromosome decondensation, congression, and segregation. These observations and others indicate that chromatin maintenance and nuclear envelope function may be closely coordinated. To understand better the relationship between these two processes, we are probing the protein-protein interaction universe among the gene products in these two modules. By discovering the protein complexes these gene products can occupy in the course of cell division, we hope to better understand how nuclear envelope function and chromatin maintenance are interconnected.

2500
Regulation of Chromosome Segregation in Mitosis by CAML
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CAML, (Calcium modulating cyclophilin binding protein) is a ubiquitously expressed protein that has been found to play a critical role in thymocyte development and EGF receptor recycling in ES derived epithelioid cells. The molecular mechanism of its actions is not yet understood. In order to discover the function of CAML in signaling, we generated a CAML deficient MEF cell line using an in vitro retroviral-mediated conditional knock-out system. CAML deficient MEF cells showed severely impaired proliferative responses to fetal bovine serum. By live cell imaging microscopy, we found that more than 30% of CAML deficient MEF cells undergo anaphase failure, exhibiting thick chromatin bridges or ‘cut’ phenotype, cytokinesis without karykinesis. The incidence of failed mitosis in control cells was only 6%. At the same time, only 13% CAML deficient mitotic MEF cells showed normal mitosis versus 61% in CAML competent control cells. Reconstitution of full length CAML protein can restore normal mitosis in CAML deficient MEF cells. By using a standard nocodazole-challenge assay, we found that the spindle assembly checkpoint was severely weakened when CAML is lacking. Subsequent immunoprecipitation studies on nocodazole-arrested Hela cells revealed that CAML forms a complex with the Anaphase Promoting Complex/cyclosome (APC/C) component Cdc27 and its co-activator cdc20. Based on these observations, we propose that in MEF cells, CAML is critical for normal mitosis exit and normal checkpoint function by regulating APC/C function.
Late Abstracts

2501
Oxidative Stress and Mitotic Chromosome Segregation
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The main objective of our study was to investigate a possible causal correlation between telomere shortening/loss and erroneous mitotic chromosome segregation. Because several sources of evidence indicate that oxidative stress accelerates telomere shortening, we used hydrogen peroxide \((H_2O_2)\) as a tool to induce oxidative stress and, possibly, telomere shortening/loss in human primary fibroblasts. Cells were treated with 10 \(\mu M\) \(H_2O_2\) for 1 - 6 days. At the end of the treatment, mitotic chromosome segregation was analyzed by time-lapse phase-contrast microscopy. We found a time-dependent increase in chromosome mis-segregation, with almost 30% of the cells exhibiting some kind of chromosome segregation defect after 6 days of treatment. In addition, up to >20% of the cells either did not divide or died during the period of observation (2 - 3 hours) at the long treatment time (6 days). A common mitotic defect was the segregation of all the chromosomes to one spindle pole and subsequent failure of cytokinesis. We hypothesize that this defect could occur if telomeres were shortening enough to cause the ends of the chromosome arms to become ‘sticky’. In that case, the ends of the sister chromatid arms could fuse and not be able to segregate to opposite poles during anaphase. To investigate this possibility, we are currently performing quantitative fluorescence in situ hybridization for telomere sequences on cells treated as described above.

2502
The Roles of a Novel Human Microtubule Severing Protein in Mitosis
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Microtubule severing proteins are part of a class of the AAA+ (ATPases associated with diverse cellular activities) family. Most if not all of the members of this family are known to form hexamer rings in order to perform their functions. In Drosophila, microtubule severing proteins were found to play important roles in mitosis, specifically in the mechanisms of anaphase A chromatid-to-pole motion: Paacman-Flux. Using a human osteosarcoma cell line (U2OS) we have cloned an uncharacterized human microtubule severing protein, termed Katanin Subunit A like-1 (KLI). Using siRNA to suppress the expression of KLI, we have found that KLI significantly impacts mitosis. In addition, overexpression has shown KLI to be a microtubule severing enzyme. Consistent with work done in Drosophila, real time imaging in U2OS cells indicates a significant impact on chromosome dynamics in anaphase.

2503
Activation of Oncogenic Aurora A Kinase Induces Ciliary Disassembly
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Recently we have identified HEF1 as the centrosomal protein and have shown its role in centrosome maturation and mitosis initiation. We found HEF1 as the direct binding partner and activator of mitotic kinase AurA (Pugacheva et al NCB 2005). We found that AurA is an important for disassembly of another centrosome based structure called cilia. Primary cilia could be detected on the apical surface of ultimately all human cells and is considered as environment sensory organ implicated in cell proliferation and differentiation. Number of human diseases associated with ciliary dysfunction like polycystic kidney disease (PKD) and Bardet-Biedl Syndrome (BBS). Using immortalized retinal pigment epithelium cell line (RPE1-hTERT) as the model we identified AurA kinase as necessary and sufficient factor inducing cilium disassembly (Pugacheva et al Cell 2007). Current research is dedicated to outline molecular mechanisms governing AurA activation and finding AurA substrates responsible for cilium disassembly. One of the potent candidates we have identified is HDAC6. HDAC6 is directly regulates microtubule dynamic by deacetylation of microtubule bundles. Phosphorylation by AurA causes substantial increase in HDAC6 deacetylase activity and promotes microtubule instability. As upstream regulators of AurA activity HEF1 focal adhesion adaptor protein and PAK1 kinase were identified. Thus HEF1 have proved himself again as an important scaffolding hub for coordination adhesion, polarity and division.

2504
Direct Screening for Genes Involved in Meiotic Paring in C. elegans
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During sexual reproduction, haploid gametes are generated by a specialized cell division program (meiosis) in which homologous chromosomes pair, undergo reciprocal recombination, then segregate to opposite spindle poles. Failure in homologous pairing or inappropriate associations between heterologous chromosomes can lead to aneuploidy, a major cause of miscarriages and birth defects. In order to identify the genes required for proper homologous chromosome recognition and association during C. elegans meiosis, we have been conducting a screen using a system to visualize homologous pairing in live animals, and a feeding RNAi library to knock-down gene function. We assumed that pairing genes are enriched in the germline and/or expressed in a way similar to known pairing genes. Based on this assumption we selected 1762 clones from a genome-wide RNAi library. The strain we use contains both a LacI-GFP gene expressed in the germline and its target lacO repeat integrated into the genome. This strain allows fast and direct examination of pairing without the use of additional experimental technique for visualization. Pairing can be detected by observing two homologous chromosomal loci as one (paired) or two (unpaired) fluorescent GFP signal(s) in each germline nucleus. We have examined knocked-down phenotypes of 1196 clones so far. In a blinded scoring, we were able to successfully identify all genes that were previously shown to function in homolog pairing. Furthermore, we have newly identified 37 RNAi clones showing a potential pairing defect with either a normal or an altered (arrest or delay) meiotic progression. Since most of the previous screens for pairing mutants could not cover the latter case, this approach is advantageous to comprehensively identify genes that function in homologous chromosome pairing in meiosis.
2505

**Kinesin-5 Maintains Spindle Length in Metaphase Mouse Eggs by Promoting Microtubule Incorporation during Poleward Flux**

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During metaphase simultaneous depolymerisation of microtubules at the spindle poles and polymerisation at the kinetochores causes kinetochore microtubules (k-fibres) to move towards the poles in a process termed poleward flux. The coordinated balance between polymerisation and depolymerisation is crucial for the maintenance of spindle length. What drives poleward flux and how this balance is achieved is poorly understood. One motor implicated in poleward flux is kinesin-5 (Eg5), which may drive anti-parallel sliding of spindle microtubules, and is necessary for flux in *Xenopus* egg homogenous spindles. Here we use photoactivation of photoactivatable-GFP::tubulin to show that microtubules flux polewards at a rate of ~0.5µm/min in live metaphaseII-arrested mouse eggs. Kinesin-5 immunolocalises to egg spindles, and inhibition of kinesin-5 by monastrol causes spindles to shorten at the same speed as poleward flux. By photo-marking microtubules during kinesin-5 inhibition, we show that shortening occurs by persistent depolymerisation of kinetochore MTs at the spindle poles, in the absence of MT addition at the kinetochore. In anaphase, kinesin-5 inhibition has no effect upon spindle length or upon the poleward movement of chromosomes. Thus, in mouse eggs, spindle poles have the capacity to shorten k-fibres both in metaphase and anaphase, but kinesin-5 maintains spindle length and poleward flux in metaphase by promoting the incorporation of MTs at the kinetochore. Kif2a, a microtubule depolymerising kinesin which has been shown to contribute to poleward flux in somatic cells, is an excellent candidate as a component of the depolymerising activity at the poles in mouse eggs, since Kif2a is concentrated at spindle poles throughout metaphase and anaphase, and monastrol-induced spindle shortening is significantly reduced by microinjection of anti-Kif2a antibodies.

2506

**A Novel mRNA 3' Untranslated Region Translational Control Sequence Regulates Xenopus Maternal mRNA Translation**

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Cell cycle progression during oocyte maturation requires the strict temporal regulation of maternal mRNA translation. The intrinsic basis of this temporal control has not been fully elucidated but appears to involve distinct mRNA 3’ UTR regulatory elements responding to distinct signaling pathways. In this study we identify a novel translational control sequence (TCS) that enforces repression of target mRNAs in immature oocytes of the frog, *Xenopus laevis*, and directs early cytoplasmic polyadenylation and translational activation during oocyte maturation. The TCS is functionally distinct from the previously characterized Musashi/polyadenylation response element (PRE) and the cytoplasmic polyadenylation element (CPE). We report that TCS function contributes to the translational regulation exerted by the pericentriolar material-1 (Pcm-1) mRNA 3’ UTR. We demonstrate that adjacent CPE sequences in the 3’ UTR of the late class Wee1 mRNA suppress the ability of Wee1 TCS elements to exert early translational activation. Our results reveal novel functional hierarchies between distinct 3’ UTR regulatory elements that control the timing of maternal mRNA translational activation during oocyte maturation.

2507

**Cytoplasmic Dynein Slides Microtubules of Opposite Polarity: Insights from Spindle Micromanipulation Studies**

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*Xenopus* egg extract is a cell-free system uniquely amenable to biophysical analysis of mitotic spindle assembly. Micromanipulation studies of bipolar spindles assembled in these extracts demonstrated that two parallel spindles can align and fuse together into one metaphase spindle. If spindles are positioned proximally, but with no overlap between their poles, the poles come together and the spindles pivot around the shared pole, “jackknifing” into a bipolar metaphase spindle. Interestingly, this fusion mode can be blocked by placing a third spindle between the jackknifing spindle pair, resulting in stable, tripolar structures. Perturbation of cytoplasmic dynein function, either by antibody addition or by immunodepletion with antibodies to the 74 kDa intermediate chain, completely inhibits spindle fusion, implicating dynein as the responsible motor. To better understand the mechanics of dynein dependent fusion, we created radial arrays of microtubules with their minus ends at the pole and plus ends oriented outwards (monopoles) by biochemically inhibiting the Eg5 kinesin motor. Our previous studies demonstrate that monopoles will fuse if their centers are initially separated by distances less than ~60µm, forming a central spindle of overlapping microtubules that shortens as the poles come together. High-resolution imaging of fluorescently labeled EB1 comets revealed stabilized microtubule interactions between fusing monopoles. Microtubules extending from opposing poles at oblique angles to the interpolar axis often exhibit movement towards the axis (orthogonal to the direction of fusion). These observations support a model in which minus end directed “pulling in” forces contribute to central spindle formation. Furthermore, they suggest the motor forces generated at distal sites of microtubule-microtubule interaction are transmitted to the poles. We hypothesize that this same antiparallel, sliding-filament mechanism is involved in normal bipolar spindle assembly and the regulation of steady state spindle length. Supported by GM24364 and GM60678 to EDS and F32GM080049 to JCG.

2508

**Meiotic Phosphorylation of the Sum1 Transcriptional Repressor by Ime2**

M. Moore, E. Winter, Thomas Jefferson University, Philadelphia, PA

Meiosis produces haploid cells through a specialized cell division program involving DNA replication, genetic recombination, and two rounds of nuclear segregation. Successful completion of this process requires the choreographed transcription of genes specific to meiosis. In the budding yeast *Saccharomyces cerevisiae*, these genes can be grouped, according to the timing of their transcription, into early, middle, and late genes. The middle
Chromosomal instability (CIN) is one of the most common characteristics of solid tumors. Up to 80% of all breast carcinomas show some degree of aneuploidy. However, it remains unclear whether CIN is a cause or consequence of cancer. The spindle checkpoint prevents aneuploidy by ensuring correct distribution of sister chromatids between daughter cells during mitosis. Mad2 and Mps1 activities are essential for a functional spindle checkpoint. We have generated conditional knockout mice for Mps1 and Mad2, allowing specific inactivation of these genes in a tissue specific manner. Recent results from our lab show that Mps1 is a very potent tumor suppressor in the T-cell compartment, in a p53-negative background (S Xie and PK Sorger, in preparation). Additionally, conditional inactivation of both Mad2 and p53 in the liver leads to aggressive hepatocarcinomas (Y Yue and PK Sorger, in preparation). We are currently monitoring tumor development in Mps1 and Mad2-deficient mammary glands in the presence or absence of functional p53. By comparing tumor latency and incidence in p53-proficient and p53-deficient mice with a defective spindle checkpoint, we expect to get better insight in the role of CIN in mammary tumorigenesis. In parallel, we are developing tools to visualize chromosome segregation, but had no impact on cell growth and survival. These data suggest that the most critical function of BubR1 is to inhibit APC/C through Cdc20 binding, and that kinetochore-dependent checkpoint signaling through BubR1 is dispensable.

2511

Chromosomal Instability as a Primary Cause of Breast Cancer?
F. Foijer, Y. Yue, P. Sorger; Systems Biology, Harvard Medical School, Boston, MA

Chromosomal instability (CIN) is one of the most common characteristics of solid tumors. Up to 80% of all breast carcinomas show some degree of aneuploidy. However, it remains unclear whether CIN is a cause or consequence of cancer. The spindle checkpoint prevents aneuploidy by ensuring correct distribution of sister chromatids between daughter cells during mitosis. Mad2 and Mps1 activities are essential for a functional spindle checkpoint. We have generated conditional knockout mice for Mps1 and Mad2, allowing specific inactivation of these genes in a tissue specific manner. Recent results from our lab show that Mps1 is a very potent tumor suppressor in the T-cell compartment, in a p53-negative background (S Xie and PK Sorger, in preparation). Additionally, conditional inactivation of both Mad2 and p53 in the liver leads to aggressive hepatocarcinomas (Y Yue and PK Sorger, in preparation). We are currently monitoring tumor development in Mps1 and Mad2-deficient mammary glands in the presence or absence of functional p53. By comparing tumor latency and incidence in p53-proficient and p53-deficient mice with a defective spindle checkpoint, we expect to get better insight in the role of CIN in mammary tumorigenesis. In parallel, we are developing tools to visualize chromosome segregation, but had no impact on cell growth and survival. These data suggest that the most critical function of BubR1 is to inhibit APC/C through Cdc20 binding, and that kinetochore-dependent checkpoint signaling through BubR1 is dispensable.
Securin Regulates Entry into M-phase by Modulating the Stability of Cyclin B

P. Marangos, C. John; Cell & Developmental Biology, University College London, London, United Kingdom

The timely progression into mitosis is necessary for normal cell division. This transition is sensitive to the levels of cyclin B, the regulatory subunit of the master mitotic kinase, Cdk1. During G2 and prophase cyclin B levels are dependent on continued translation in the face of a low rate of destruction driven by APC-dependent ubiquitination. Securin, which is known to be ubiquitinated and degraded simultaneously with cyclin B through the APC, is present during prophase but it is not known whether this cell cycle protein can act as a co-competing substrate that plays a physiological role in pacing the rate of cyclin B accumulation. In prophase-arrested mouse oocytes, a 2-fold excess of securin, leads to stabilization of cyclin B and precocious entry into M-phase. In contrast, no effect is seen with a non-destructable form of securin or with excess Aurora B, an APC substrate which is ubiquitinated and destroyed after cyclin B. Depletion of endogenous securin using morpholino (MO) oligonucleotides increases cyclin B instability, resulting in reduced accumulation of cyclin B and a delayed progression into M-phase. This effect requires APC activity since it is reversed by the addition of a Cdh1 MO or the expression of wild type securin, but not the non-destructible form of the protein. These data show a novel role for securin at the G2/M transition and suggest a more general mechanism whereby physiological levels of co-competing APC substrates play a role in modulating the timing of cell cycle transitions.

Interaction of Zds2p with PP2A Subunit Cdc55p: Possible Mechanism of Polarized Growth Regulation

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Much research has focused on how a cell establishes an axis of polarized growth. Less well understood is how a cell regulates polarized growth once cell polarity is established, especially in relation to the cell cycle. ZDS1 and ZDS2 (zillon different screens) are paralogous genes that are conserved among fungi. A regulatory role for the latter gene in its N-terminal domain was suggested by a hyper-elongated bud phenotype when the genes are deleted in combination (Bi and Pringle. 1996. MCB 16: 5264-5275). Thus, to determine how polarized growth is regulated, we have focused on deciphering the functions of ZDS1 and ZDS2. Expression of either gene rescues the double deletion phenotype, indicating the genes are functionally redundant with respect to polarized growth. We posited that a region(s) of homology between Zds1p and Zds2p is necessary for regulating polarized cell growth. We show that of four regions of high homology (>75%), only region 4 (aa 812-913) of Zds2p is necessary for regulating polarized growth in a zds1Δ background. Furthermore, to identify Zds2-interacting proteins that regulate polarized cell growth, we found by differential two-hybrid analysis that Cdc55p (a PP2A regulatory subunit) binds to wild-type Zds2p but not to Zds2Δ812-913p. In contrast, both these proteins bound equally well to Pck1, a known interactor. Thus, region 4 may represent a binding domain for Cdc55p. Similar to the previously determined localization patterns of Cdc55p and Zds1p, we show that Zds2 containing an internal epitope tag (Zds2-i-9x myc) and expressed from an endogenous promoter localized at the bud cortex. No localization was observed at the bud neck. Random mutagenesis of region 4 produced several temperature-sensitive alleles that are lethal or phenocopy the zds1Δ zds2Δ mutant. These data suggest that region 4 of Zds2p represents a functional domain that regulates polarized growth via a PP2A-dependent mechanism.

N-Terminal Domain of the Y-Box Binding Protein, YB-1, Plays a Major Role in Cell Proliferation and Apoptosis

P. Khandelwal, M. Padala, R. Guntaka; Molecular Sciences, University of Tennessee Health Science Center, Memphis, TN

Y-box binding protein, YB-1, is involved in a number of cellular functions, including cell proliferation and transcription and translation regulation. YB-1 knock out mice die in embryonic stage due to defects in neural tube closure and cell proliferation. Our previous study showed that a targeted disruption made in one allele of YB-1 gene at its N-terminal domain resulted in major defects in cell cycle. The abnormalities seen in heterozygous YB-1 knock out mice die in embryonic stage due to defects in neural tube closure and cell proliferation. Our previous study showed that a targeted disruption made in one allele of YB-1 gene at its N-terminal domain resulted in major defects in cell cycle. In prophase-arrested mouse oocytes, a 2-fold excess of securin, leads to stabilization of cyclin B and precocious entry into M-phase. This effect requires APC activity since it is reversed by the addition of a Cdh1 MO or the expression of wild type securin, but not the non-destructible form of the protein. These data show a novel role for securin at the G2/M transition and suggest a more general mechanism whereby physiological levels of co-competing APC substrates play a role in modulating the timing of cell cycle transitions.
**Late Abstracts**

### Cytokinesis (2515-2520)

#### 2515

**Traffickling and Exocytosis of Post-Golgi Vesicles from Both Daughter Cells Contributes Membrane to the Cytokinesis Cleavage Furrow**

J. W. Goss, D. Toomre; Cell Biology, Yale University, New Haven, CT

Recent studies have implicated membrane traffic in cytokinesis. To address if constitutive exocytic membrane is focially delivered to the cleavage furrow, and if asymmetric or symmetric mechanisms of delivery are involved, we monitored constitutive exocytosis at the single vesicle level using Spinning Disc Confocal Microscopy and Total Internal Reflection Fluorescence Microscopy (TIRFM). We observed that post-Golgi vesicles from both daughter cells rapidly traffic along curvi-linear paths into the cleavage furrow. Fluorescence Recovery After Photobleaching (FRAP) and immunofluorescence experiments indicated that individual vesicles accumulate near the furrow and are distinct from endosomal and lysosomal organelles in this region. Additionally, we observe that not only do vesicles from both daughter cells traffic to this spatial landmark, but also exocytic fusion is more frequent there and are consistent with a symmetrically polarized model of exocytosis.

#### 2516

**Detailed Examinations on Monopolar Cytokinesis Characterize Unique Features of Normal Bipolar Cytokinesis**

C. Hu, M. Coughlin, C. Field, T. Mitchison; Systems Biology, Harvard Medical School, Boston, MA

Cytokinesis can be triggered in monopolar cells proceeding as normal bipolar cytokinesis (Canman, 2003), however, the relative simple geometric conformation of monopolar cells makes it a better system to tackle difficult cytokinesis questions. We have established a convenient monopolar system which grants efficient synchronizing mitotic exit and successful furrow completion during cytokinesis. By using monopolar HeLa cells to eliminate the asymmetric distributions of chromosomes and spindles, we trigger the mitotic exit from a symmetric initial state and observe a novel polarization mechanism which is essential for furrow formation but probably masked in bipolar cytokinesis due to the asymmetric nature of half-spindles. New features of known cytokinesis proteins are characterized in our monopolar system. Although the fully stabilized microtubules resemble midzone in both structure and function, its plus ends appear non-dynamic and are exposed in cytoplasm, revealing possible mechanism for midzone stabilization. This microtubule organization also provides new information on how different classes of cytokinesis proteins are spatially localized. Chromosome passenger proteins behave differently to other cortex- or microtubule associated proteins, relocating from microtubule tips to the cortex through actin filaments perpendicular to the cortex. Cytokinesis is traditionally viewed as a linear sequence of events, with microtubules localizing furrow components. However, during monopolar cytokinesis, we notice protein localizations on microtubules are changed by interruption of cortical components. It suggests during cytokinesis, not only the cortex is regulated by signals from microtubules, but microtubules are also regulated by signals from the cortex. Our observations point to the existence of feedbacks from furrow components to microtubules that promote polarization, and perhaps symmetry breaking. Together, the monopolar cytokinesis system we established in HeLa cells can be a useful tool for cytokinesis studies with unique insights which is difficult to be obtained by normal bipolar cytokinesis.

#### 2517

**Cytokinesis in Cells Containing Monopolar Spindles: Differential Recruitment of Factors Required for Contractile Ring Assembly**

S. Shrestha, J. Eyer, L. J. Wilmeth; Biology, New Mexico State University, Las Cruces, NM

The cleavage plane is defined by microtubule arrays that recruit factors required for organizing the contractile ring. And while a bipolar spindle is always required for equatorial positioning of the contractile ring, it remains unclear how these factors arrive at the cell equator. We have developed a highly reproducible assay where cells arrested in mitosis with monopolar spindles are forced into mitotic exit and cytokinesis with a small molecule inhibitor of p34cdc2 kinase. HeLa cells manipulated in this process undergo a rapid exit from mitosis within ten minutes, and form ectopic furrows at the cell periphery within twenty minutes. Unlike more adherent cells, the placement of ectopic furrows in spherical HeLa cells appeared to be random, with cells often exhibiting multiple sites of furrowing or blebbing. We examined the localization dynamics of four factors required for cytokinesis: Aurora B kinase, INCENP, Polo-like kinase (Plk1), and MKLP-1. Normally sequestered at the inner centromeres prior to anaphase onset, Aurora B and INCENP were found along microtubules as early as five minutes post-induction, and could be found on the cortex at sites of ectopic furrowing. MKLP-1, a homotetrameric kinesin required to organize the spindle midzone and recruit MgcRacGAP and Ect2, could be found at the microtubule plus ends, but was not enriched at the cortical cytoskeleton like Aurora B and Incenp. Plk1, which is found at both spindle poles and kinetochores and is required for the initiation of cytokinesis, was also not recruited to microtubule plus ends or the cortex or ectopic furrows. Preliminary results suggest that Plk1 is dispensable for Aurora B and MKLP-1 localization, as well as ectopic furrowing, raising the possibility that unlike the chromosomal passenger- and centralspindlin complexes, Plk1 requires a bipolar spindle for equatorial localization during cytokinesis.

#### 2518

**Role of Sav1/WW45 in the Regulation of the Cell Cycle**

S. R. Scholz, B. R. Mardin, E. Schiebel; ZMBH, University Heidelberg, Heidelberg, Germany

In *Drosophila melanogaster* the Salvador (Sav)-Warts-Hippo (SWH)-pathway was shown to be essential for the control of tissue size, cytokinesis and apoptosis. The SWH-pathway consists of the two kinases, Warts and Hippo, and the potential scaffold protein Sav and the kinase subunit Mob1. Together, they regulate apoptosis and cell cycle progression via the transcription factor Yorkie, a homolog of the mammalian YAP. In mammalian cells, homologues of the fly proteins have been identified, but little is known about the molecular architecture and functions of this pathway. Mammalian Mst1 and Mst2 (homologues of Hippo) have been shown to interact with Sav1/WW45 via their C-terminal SARA-H-domains provoking the phosphorylation of both proteins and the stabilization of the complex. We demonstrated that Sav1/WW45 is localized at the centrosomes during the interphase of the cell cycle and at the midbody during the abscission of the daughter cells. At the midbody Sav1/WW45 colocalizes with its interactor Mst2. Furthermore, when overexpressed as a GFP-fusion protein, Mst2 can also be detected at centrosomes. With a doxycyclin inducible...
expression system we can follow the localization of the Sav1/WW45-GFP fusion protein during the cell cycle and examine the effects of its overexpression. We measured the turnover rate of Sav1/WW45-GFP and found it to exchange extremely fast on centrosomes. We performed a yeast-two-hybrid screen and identified unsuspected proteins of the mitotic machinery. Furthermore we have investigated the effect of the Sav1/WW45-downregulation by RNA-interference in different cell lines. Our data suggest that the Sav1/WW45 protein interlinks components of the SWH-pathway with other cell cycle regulators.

2519
Katanin Ubiquitination by the Cul3/KLHL5 E3 Ligase Regulates Microtubule Structure in Mammalian Cells
C. M. Mesrobian,1 C. A. Bentley,2 S. A. Perdue,3 P. W. Baas,4 J. D. Singer2, 1Department of Molecular Biology, Cell Biology, and Biochemistry, Pathobiology Graduate Program, Brown University, Providence, RI, 2Genomics Institute of the Novartis Research Foundation, San Diego, CA, 3Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY, 4Department of Neurobiology and Anatomy, Drexel University College of Medicine, Philadelphia, PA. We demonstrate a novel regulatory mechanism for p60/katanin that occurs at the level of targeted proteolysis to allow completion of cytokinesis in mammalian cells.

2520
The Regulation of Mitotic p190RhoGAP and Its Role in Cytokinesis
S. A. Sánchez, J. M. Agati, L. Su, S. J. Parsons; Department of Microbiology, University of Virginia Health System, Charlottesville, VA P190RhoGAP (p190) is a GTPase Activating Protein (GAP) specific for the small GTPase RhoA and has been shown to act as a tumor suppressor, preventing Ras-induced transformation in NIH3T3 cells and PDGF-induced gliomas in mice. Inactivation of Rho signaling, through p190’s GAP domain, results in disruption of stress fibers and formation/contraction of the acto-myosin ring in cytokinesis. Our previous work has shown that cellular levels of p190 decrease in mitosis and that this decrease is mediated by ubiquitin-dependent proteasomal degradation. Furthermore, p190 overexpression prevents successful completion of cytokinesis. We endeavored to understand the mechanism by which p190 expression is regulated and the requirement of its decrease for successful completion of cytokinesis. Structure-function analysis of p190 mutants revealed that regions within the N-terminus are necessary for both the ubiquitination and degradation of p190. Co-immunoprecipitation assays have revealed that APC activators, cdh1 and cdc20, associate with p190 and overexpression of these activators results in an earlier and more pronounced decrease in mitotic p190 levels, thus implicating the Anaphase Promoting Complex (APC/C) as a putative E3 ligase involved in this process. Mass spectrometry analysis has revealed putative Cdk and Aurora phosphorylation sites raising the possibility that p190 levels are regulated by these important players in cell cycle progression. Finally, using a p190 degradation-resistant mutant in siRNA reconstitution experiments, we determined that the degradation of p190 is required for the completion of cytokinesis. In summary, our data suggest that 1) the downregulation of p190 protein levels is required for completion of cytokinesis, 2) p190 degradation is mediated by the APC/C, and 3) mitotic phosphorylation of p190 may be an important mechanism to integrate cell cycle control over the expression/activity levels of p190RhoGAP. These data suggest that p190 downregulation in mitosis may serve as a mechanism to prevent cytokinesis failure and aneuploidy.

Kinetochores (2521-2523)

2521
Construction of an Artificial Kinetochore in Budding Yeast
S. Lacefield, D. Lau, A. W. Murray; Molecular and Cellular Biology, Harvard University, Cambridge, MA Chromosome segregation must be a tightly regulated event to ensure that genetic information is not lost or duplicated. Chromosomes should only segregate if all kinetochores are attached to a bipolar spindle and sister chromatids are under tension. If chromosomes separate before proper attachment of kinetochores to spindles, missegregation events can occur leading to aneuploidy. The connection between microtubules and kinetochores must be secure enough to allow proper segregation, but also easily reversible if improper attachment occurs to allow a correction of the error. We are analyzing kinetochore components in budding yeast to determine how this regulated connection occurs. We have developed an assay that tethers individual kinetochore proteins to regions of DNA to ask if a kinetochore can form. We have found that tethering the protein Ask1 to a plasmid without centromeric DNA can allow this plasmid to segregate like a centromere containing plasmid. Furthermore, addition of the tether to a natural chromosome causes an increase in chromosome loss and breakage, similar to creating a dicentric. If this tether completely replaces a natural centromere, 80% of the cells can form a colony. We are currently investigating how faithful the artificial kinetochore segregates chromosomes, which other kinetochore proteins are recruited, and whether the connection is reversible.
2522
Recognition of Centromeric Chromatin by Centromere Protein N
C. W. Carroll, K. M. Godek, A. F. Straight; Biochemistry, Stanford University School of Medicine, Stanford, CA
Centromeres are specialized chromosomal domains that direct kinetochore assembly during mitosis and therefore play an essential role in chromosome segregation. Centromeric chromatin is distinguished from other chromatin by the replacement of histone H3 with the histone H3-variant centromere protein A (CENP-A) within centromeric nucleosomes. CENP-A is required for the recruitment of most kinetochore proteins to the centromere, but how CENP-A-containing chromatin is recognized as the site of kinetochore assembly is poorly understood. To explore the molecular basis for centromere recognition we developed a simple and rapid in vitro assay designed to identify human kinetochore components that directly and specifically interact with reconstituted CENP-A-containing nucleosomes. This approach led to discovery that centromere protein N (CENP-N) is a DNA-sequence independent CENP-A-nucleosome binding protein. Nucleosomes reconstituted with a chimeric histone H3 that contains only the centromere-targeting domain of CENP-A were sufficient for CENP-N binding. Point mutations that disrupted binding of CENP-N to CENP-A nucleosomes in vitro also blocked localization of CENP-N to the centromere in vivo, indicating that nucleosome binding is required for the centromere-specific recognition of CENP-N. Nucleosome binding was not sufficient for centromere targeting of CENP-N, however, because a CENP-N mutant that bound nucleosomes with the same affinity as the wild type protein was unable to localize to centromeres in vivo. These data suggest that the direct recognition of centromeric chromatin by CENP-N is a critical early step in the kinetochore assembly process.

2523
SGI-1 Helps Target the Spindle Assembly Checkpoint Component, MDF-1/Mad1 to the Kinetochore by Forming a Complex in Caenorhabditis elegans
T. Yamamoto, S. Watanabe, R. Kitagawa; Molecular Pharmacology, St. Jude Children's Research Hospital, Memphis, TN
To ensure faithful chromosome segregation, the spindle assembly checkpoint (SAC) delays anaphase onset until all kinetochores of chromosomes are properly attached to the spindles. The SAC genes MAD1-3, BUB1, and BUB3 have been identified, and the molecular mechanism for delaying anaphase onset is relatively well understood. However, how the SAC recognizes and transduces signals generated by unattached kinetochores remains unclear. By performing a genome-wide RNAi screen for synthetic genetic interactors with san-1/MAD3 and a secondary screen for genes whose depletion caused a defect in the C. elegans SAC, we identified an uncharacterized gene, sgi-1 (san-1 genetic interactor). The sgi-1 encodes a novel coiled-coil protein that has no apparent homologs in other organisms. Partial depletion of SGI-1 significantly reduced the viability of the san-1/MAD3-deletion strain but not that of the wild-type strain, and complete depletion of SGI-1 caused chromosome missegregation in wild-type embryonic cells, leading to embryonic lethality at a high penetrance. Immunofluorescence microscope analysis using anti-SGI-1 antibody and live-imaging analysis using a transgenic strain expressing a GFP::SGI-1 fusion protein in embryonic cells revealed that SGI-1 transiently localized to the kinetochore from prometaphase to metaphase and delocalized from the kinetochore at anaphase, as do some outer kinetochore components and SAC components. SGI-1 also localized to centrosomes during mitosis. Kinetochore localization of SGI-1 did not depend on NDC-80/Hec1 or HCP-1/CENP-F but did depend on KNL-1 and CZW-1/Zw10, a component of the RZZ (Rod, Zw10, Zwilch) complex. SGI-1 was required for microtubule damage-induced kinetochore localization of MDF-1/Mad1. SGI-1 also coimmunoprecipitated with MDF-1/Mad1 in vivo, suggesting that SGI-1 physically associates with MDF-1/Mad1. Together, these results indicate that SGI-1 functions as a kinetochore receptor of MDF-1/Mad1, thereby targeting MDF-1/Mad1 to the unattached kinetochore to exert SAC function in the RZZ complex pathway.

Actin (2524-2525)

2524
Expression of Mutated Sequences of β-actin in Fibroblast Interferes in the Stress Fibers Formation and Adhesion and Cellular Spreading Process
M. Cruz-Aguilar, R. Manning-Cela; Biomedicina Molecular, CINVESTAV, IPN, México, D.F, Mexico
Actin is one of the most abundant and highly conserved proteins in the cytoskeleton, which participates in multiple cellular functions as maintaining cellular architecture, cell motility, endocytosis, cytokinesis, establishment of adhesions cell-cell and cell-substrate among others. In mammals, six different actin isoforms has been described, four specific for muscular cells (α-actins) and two ubiquitous or cytoplasmic (β and γ-actin). Because each isoform is encoded by a different gene, its expression is tissue specific, display differences in its polymerization dynamics and in their ability to interact with ABPs, it has been suggested a possible isoform specific function. In non-muscular cells the actin isoform specific function has not been established. Then, in this work we obtained and characterized different stable transfected fibroblasts 3T3-Swiss with mutated sequences of β-actin fused to EGF that result in the obtaining of dominant negative mutants of β-actin. We evaluated in mutant and control (fibroblast expressing β-actin-EGFP and mock cells) cells, the regulation of endogenous iso-actins expression, microfilaments organization, adhesion and cellular spreading processes and their ability to form actin-rich structures (stress fibers) after the stimulus of Rho-dependant pathways. The mutated protein copolymerized with the endogenous actin, producing a disorganization of microfilaments and a decrease in the adhesion (40%) and cellular spreading (50%) process. This result was consistent with the reduced capacity of mutant cells to form stress fibers (80%) compared with control cells. Finally, the over-expression of normal or mutated β-actin induced a down-regulation of endogenous β-actin and an up-regulation for γ-actin at mRNA and protein levels showing a transcriptional regulation. Together, these results provide evidence that β-actin participates in the stress fibers formation and in the adhesion and cellular spreading process.
Direct Observation of the Uncapping of Capped Actin Filaments by CARMIL

I. Fujisawa, K. Remmert, J. A. Hammer; NHLBI, NIH, Bethesda, MD

Recent solution studies have shown that the ~80-residue CAH3 domain of CARMIL binds free Capping Protein (CP) with low nanomolar affinity, strongly inhibits the capping activity of CP, and rapidly and potently restores actin polymerization from actin filaments previously capped with CP. To investigate the mechanism of this uncapping activity directly, we observed individual capped actin filaments before and after the addition of the CAH3 domain of mouse CARMIL-1 (mCAH3) using TIRF microscopy. Real time observations showed that mCAH3 rescues the polymerization of capped filaments by driving the rapid dissociation of CP from the barbed end. The number of uncapped actin filaments was proportional to the concentration of mCAH3 added and saturated at ~250 nM mCAH3. The time interval between mCAH3 addition and uncapping decreased with increasing concentration of mCAH3, with the average half-life of CP at the barbed end decreasing from ~40 minutes without mCAH3 to ~10 seconds with saturating amounts of mCAH3. The actin polymerization rate itself was not altered at either the barbed or pointed ends by mCAH3. As anticipated from solution studies, mCAH3 containing a single point mutation (R993E) that blocks its binding to free CP was totally devoid of uncapping activity as measured by TIRF microscopy. We conclude that the isolated CAH3 domain of CARMIL, and presumably the intact molecule as well, possesses the ability to uncap capped actin filaments. This activity may drive, along with de novo nucleation and filament severing, the generation of free barbed ends in vivo, and may be responsible in part for the short half-life of CP at the barbed end inside cells (JCB, 2006). Our results contrast with a recent report that PIP2, which was also thought from previous solution studies to uncap capped filaments, does not do so when examined by TIRF microscopy (JBC, 2007).

Actin-associated Proteins (2526-2538)

Ezrin Function in the Retinal Pigment Epithelium (RPE)-Ezrin Knockout Mouse

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Ezrin, a member of the ezrin/moesin/radixin (ERM) family, localizes to microvilli of epithelia in vivo, where it functions as a bridge between actin filaments and plasma membrane proteins. In the posterior eye, ezrin has been localized to Muller cell microvilli and to apical microvilli and basal infoldings of RPE. We have previously shown that ezrin is expressed and plays an important role in establishing and maintaining apical microvilli of RPE and Muller cells, and RPE basal infoldings. In ezrin-/- mice, we noted that photoreceptor development was delayed as compared to their development in wild type littermates. Moreover, in these mice ezrin loss led to substantial reductions in the apical microvilli and basal infoldings in RPE cells and in the Muller cell microvilli. To dissect the effects of ezrin absence in the RPE, we generated an RPE-specific ezrin knockout mouse model. While RPE microvilli and basal infoldings are formed in this model, they appear abnormal ultrastructurally and express low levels of RPE microvilli transporters at 5 months. The membranes of apical microvilli and cytoplasm were very electron dense, as were the membranes of the basal infolding membranes. These observations suggest an alteration in the lipid to protein ratio. RPE-ezrin-/- expressed low levels of microvilli transporters such as the glucose transporter Glut-1. Although the cone opsins labeling were significantly decreased all of the major components of the dc-ERG were present in RPE-ezrin-/- mice. These mice will prove useful to understand the role of ezrin in RPE development and function. Supported in part by NIH grants EY017153, EY06603, EY14240, EY15638, a Research Center Grant from The Foundation Fighting Blindness, a Challenge Grant from Research to Prevent Blindness and funds from the Cleveland Clinic Foundation.

Rapid Activation of mDia1 to Restore Cellular Actin Polymers Regulated by G-actin

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Wide variety of actin-based cellular structures such as yeast actin cables, cytokinetic cleavage furrows and actin bundles in mammalian cells are dependent on formin family. Formin homology 2 (FH2) domain nucleates actin filaments and binds associated to the barbed end of filaments, while formin homology 2 (FH2) domain promotes the formation of barbed end elongation through profilin. A mammalian formin, mDia1 is auto-inhibited by its intramolecular interaction. Binding of activated Rho A relieves the intramolecular interaction of mDia1. mDia1 then promotes actin assembly by its exposed FH1-FH2 domain. However, the physiological regulation of formin-mediated actin filament formation, both temporally and spatially within the cell, is still unknown. By using single-molecule live-cell imaging, we found a low-dose actin monomer sequestering drug, latrunculin B (LatB), rapidly activates mDia1, but not Arp2/3 complex, another major actin nucleator. mDia1 activation by LatB requires Rho activity, but the FH2 region alone can be activated by LatB. By building a simulation model, we found that LatB treatment paradoxically increase free G-actin concentration. Furthermore, we found frequent actin nucleation by mDia1 around sites of vigorous actin disassembly. We propose that transient accumulation of actin monomer works as a cue to activate mDia1 to execute rapid re-assembly of actin filaments.

Interaction of Proline-Rich Region of Adapter Protein RIL with α-Actinin-1 Inhibits Actin Depolymerization

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Furthermore, we found frequent actin nucleation by mDia1 around sites of vigorous actin disassembly. We propose that transient accumulation of actin monomer works as a cue to activate mDia1 to execute rapid re-assembly of actin filaments.
Late Abstracts

Actin-associated proteins are known to participate in the maintenance and remodeling of actin cytoskeleton. RIL is a PDZ and LIM-domain containing adapter protein that has previously been shown to localize along actin fibers and interact with α-actinin-1, a major actin cross-linking protein. Several alternatively spliced isoforms of RIL that result in its protein domain shuffling have been previously identified. Here we aimed to characterize in more detail the interaction of RIL with α-actinin and its functional significance. It was previously postulated that binding to α-actinin was mediated by PDZ-domain of RIL. However, in reciprocal co-immunoprecipitation experiments using RIL splice isoforms and various deletion mutants we identified an additional interaction site within proline-rich region of RIL, which is adjacent to the LIM-domain. By using immunofluorescence approach we show that this interaction is necessary to localize both proteins to actin cytoskeleton and induces formation of thick actin fibers and actin aggregates. Moreover, in cell fractionation experiments proline-rich region of RIL induced dramatic redistribution of α-actinin from the free cytosolic to the cytoskeleton-bound state. This was accompanied by a significant shift in F/G-actin ratio towards polymerized actin measured by specific quantitative staining of fibrillar/globular (monomeric) actin. By utilizing a panel of actin-targeting drugs we show that RIL inhibits actin disassembly. The cytoskeleton rearrangements induced by RIL lead to increased motility of RIL-overexpressing cells. PDZ domain of RIL appears to be dispensable for these processes. These results provide a valuable insight into distinct functions of RIL alternatively spliced isoforms that either contain or not the proline-rich region. Taken together our data suggest that RIL is involved in regulation of actin structure and dynamics thus influencing cell behavior.

2529 Synthetic Actin Binding Domains Reveal Compositional Constraints for Function
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Calponin homology domains (CHDs) are modular protein domains of around 100 amino acids. A large number of actin binding proteins interacts with the actin filament via a structurally conserved actin-binding domain (ABD) that follows the canonical type1/type2 CHD arrangement. Using the ABD of α-actinin as a scaffold we have generated a series of synthetic ABDs by altering position and composition of the CHDs within the ABD, and tested the significance of the loop connecting helices A and B in the CHD fold by homologous replacement with similar regions from divergent CHD subtypes by co-sedimentation and fluorescence microscopy. We show that the presence of two CHDs alone is not sufficient for a functional ABD, and actin binding is almost undetectable in a homotypic type2 CHD tandem. The absence of a type2 CHD in single or homotypic type1 CHD tandems results in defective turnover rates on thin filaments, and in aberrant filament bundling in the context of the α-actinin molecule. The inverted position (type2/type1) of the two CHD in the ABD, however, is of negligible importance for the function of the isolated ABD and for normal actin bundling in the parent molecule. Replacement of the type2 CHD in the ABD of α-actinin with the type2 CHD from filamin also reduces the turnover rate on actin filaments and results in structural defects of the actin cytoskeleton. Finally, introducing the actin binding sites (ABS) ABS1 and ABS2 from a type1 CHD into the N-terminal type2 CHD of the synthetic type2/type2 tandem fails to restore actin filament association. Taken together our data demonstrate that the dynamics and specificity of actin binding via ABDs requires i) both the filament binding properties of the type1 CHD and regulation by type2 CHDs, and ii) appears independent of the position of the CHDs within the ABD.

2530 dDia2 Induced Polymerization Kinetics of Actin in the Presence of Fascin
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Formins are multi-domain proteins with a highly conserved formin homology domain 2. It can nucleate actin polymerization and may even trigger filament growth by a processive capping mechanism. A formin of the amoeba Dictyostelium discoideum, dDia2, has been shown to be important for the formation, elongation and maintenance of filopodia. Fascin, an actin-bundling protein, is essential for filopodial protrusion, too. To characterize the role of these two proteins in filopodia formation their interaction has been examined in vitro. The assembly rates of dDia2-induced actin polymerization of single filaments have been studied using total internal reflection fluorescence microscopy (TIRFM) as well as the polymerization kinetics in a bulk assay. A kinetic model allows the determination of the nucleation and elongation kinetics - the nucleation rate is found to be 5.5 10^{-4} µM^{-3}s^{-1}, which is an order of magnitude faster than reported before for the yeast formin Bni1p. The elongation rate is slowed down by a factor of 4 by dDia2, which is 2 times slower than the literature value for Bni1p. Adding fascin to the model system shows that bundling is not affecting the observed polymerization kinetics. With TIRFM the bundle formation of actin filaments in the presence of fascin and forming via a zipperping mechanism can directly be observed. This data contributes to the understanding of the formation of filopodia.

2531 Functional Study and Subcellular Organization of the EhABP152 Protein from Entamoeba histolytica
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The actin cytoskeleton is a three-dimensional dynamics structure that maintains the intracellular architecture. In the pathogenic parasite Entamoeba histolytica the actin cytoskeleton has great importance in different cellular processes related to the invasion of different cell targets. The mechanisms and proteins involved in the movement of E. histolytica are poorly defined. However, it is known that in higher eukaryotic cells, actin and the actin binding proteins (ABPs) can form highly ordered structures that provide shape and support to the cells. Due to the importance of the ABPs in the reorganization of the actin cytoskeleton during the pathogenicity mechanism of E. histolytica, in this project we focused on the study of a new ABP: EhABP152. This protein has a MW of 152 kDa and has the conserved putative ABD domain. By RT-PCR and WB assays we demonstrated that the gene EhABP152 is transcribed and translated in the amoeba. The biochemical assays revealed that EhABP152 is capable of binding to actin and Rho GTPases in vitro. By fluorescence microscopy assays we detected that EhABP152 protein is located at the cytoplasm as well as inside the nucleus of E. histolytica. Finally the EhABP152 protein colocalises with actin in the phagocytic cups of trophozoites formed during the erythropagocytosis assays, as well as in the uroid of the cell. These results suggest that probably EhABP152 should be a bridge between Rho GTPases and actin.
cytoskeleton during erythrophagocytosis and capping. Although up to now we do not know what could the function of this protein in the nucleus of the parasite be.

2532

Cortactin Splice Variants in Tumor Cell Growth and Invasion

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Cortactin is a src kinase substrate and cytoskeletal protein that is gene-amplified via 11q13 amplification in 30-45% of head and neck squamous cell carcinomas (HNSCC), leading to poor patient prognosis. Whereas a key function of cortactin in invasion is promoting the formation and function of invadopodia, not all cortactin-amplified, aggressive cell lines form these invasive subcellular structures. We hypothesize that different cortactin splice variants may lead to different cellular phenotypes, e.g. invasive vs. noninvasive. To test this hypothesis, we are using the FaDu HNSCC cell line, in which cortactin is amplified 13-20 times, but does not make invadopodia. Multiple bands were detected on a Western blot and it is not yet known if these represent phosphorylated cortactin or different isoforms. Three splice isoforms of cortactin, differing in the number of actin binding repeats, have been identified in humans. We have developed a quantitative real-time PCR strategy to determine the relative amounts of each in the FaDu cells. The full length isoform accounts for ~30% of the total cortactin in FaDu cells, leaving the possibility that one or both of the splice variants plays a major role in inhibiting invadopodia formation and promoting tumor growth. We PCR-amplified and cloned individual cortactin isoforms and found that all three splice variants were expressed. No mutations were identified by sequencing. The individual roles of the three major cortactin isoforms in HNSCC invadopodia formation will be determined by expressing them in a cortactin knockdown cell line to determine their effects on invasion.

2533

Btk Regulates BCR-mediated Antigen Transport by Modulating the Dynamics of the Actin Cytoskeleton

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Engagement of the B cell antigen receptor (BCR) by multivalent antigens leads to the activation of signaling cascades as well as rapid antigen internalization for processing and presentation to T cells. The efficient internalization and trafficking of the BCR-antigen complex requires BCR-initiated signaling pathways and dynamic reorganization of the actin cytoskeleton. It has been suggested that the actin cytoskeleton acts as a linker between signaling and antigen transport pathways. Bruton’s tyrosine kinase (Btk) is a tyrosine kinase with multiple protein-protein interaction domains that can potentially serve as a linker molecule between actin-binding proteins and the BCR signaling machinery. We used both biochemical inhibition (with LFM A-13) and xid mice which have a point mutation in the PH domain of Btk to study the role of Btk in actin regulation and BCR-mediated antigen uptake and transport. In both systems, the rate of BCR internalization and its movement to late endosomes was significantly reduced. The reduced BCR internalization and intracellular movement were concurrent with reduced cellular F-actin levels and a blockage of de novo actin polymerization. Furthermore, Btk mutation and inhibition interfered with BCR-triggered redistribution of WASP, an actin modulating protein in B cells. Taken together, these results suggest that Btk, when activated by BCR-initiated signaling, facilitates BCR-mediated antigen transport by regulating actin cytoskeletal dynamics.

2534

Developmental Characterization of δ-Catenin, a Cadherin Binding Molecule of the P120-Catenin Sub-Family Required for Xenopus Early Development

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δ-catenin is a member of the p120-catenin sub-family (p120-catenin, δ-catenin, ARVCF, p0071 and plakophilins) that is implicated in neural adhesion, developmental morphogenesis and tumor progression. In mammals, δ-catenin exhibits a near brain-exclusive expression pattern. Its knock out in mice results in abnormal synaptic functions and cognitive defects, while chromosomal deletions in humans that encompass δ-catenin are associated with mental retardation. Here we report the isolation and characterization of δ-catenin in Xenopus laevis (Xδ-ctn hereafter). Xδ-ctn is present as full-length cDNA, or as three alternatively spliced isoforms designated A, B or C. RT-PCR analysis indicates that each isoform is expressed throughout Xenopus development, although expression of isoform C varies according to developmental stage. Unlike the neural restricted expression of δ-catenin reported in mammals, Xδ-ctn transcripts and proteins are detectable in most adult Xenopus tissues although enriched in neural structures when visualized via in situ RNA hybridization. To characterize Xδ-ctn’s developmental functions, we employed anti-sense morpholinos to knock-down the endogenous protein. The Xδ-ctn loss-of-function results in significant gastrulation defects, phenotypes what we indicated to be specific based upon self-rescue experiments. The phenotypic effects of Xδ-ctn depletion were further enhanced by co-depletion of p120-catenin and were significantly rescued via exogenous p120-catenin, both of which suggested shared signaling components downstream of these two proteins. Biochemical approaches revealed that Xδ-ctn depletion leads to reduced levels of C-cadherin protein as well as activation of RhoA, a small GTPase that regulates actin dynamics and morphogenetic cell movements. Significant rescues were achieved via titrated doses of C-cadherin and dominant-negative RhoA/dominant-active Rac1. Thus our initial experiments suggest that Xδ-ctn plays essential roles in Xenopus early development by affecting multiple events in developmental morphogenesis including cadherin-mediated adhesion and small GTPase functions.
Role of Mammalian Actin Binding Protein 1 in Coupling BCR Signaling and Antigen Processing Pathways

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The B cell antigen receptor (BCR) serves as both signal transducer and antigen transporter and induces signaling cascades and antigen processing and presentation, the two essential cellular events for B cell activation. BCR-initiated signaling increases BCR-mediated antigen processing and presentation efficiency by increasing the rate and specificity of antigen transport. Our lab and others have previously shown a critical role of the actin cytoskeleton in these two processes. Here we found that mammalian actin binding protein 1 (mAbp1, HIP55, or SH3P7) functioned as an actin-binding adaptor protein, coupling BCR signaling and antigen-processing pathways with the actin cytoskeleton. Perturbation of mAbp1 functions by dominant negative transfection and inhibition of mAbp1 expression by gene knockout inhibited BCR-mediated antigen internalization and transport to the processing compartment and abrogate BCR-mediated antigen processing and presentation. The binding of the BCR with antigen induced transient tyrosine phosphorylation of mAbp1 and translocation of mAbp1 from the cytoplasm to plasma membrane, where it colocalized with the BCR, cortical F-actin, and dynamin 2. Mutations of the two putative tyrosine phosphorylation sites of mAbp1 and depolymerization of the actin cytoskeleton interfered with BCR-induced mAbp1 recruitment to the plasma membrane. These results demonstrate an essential role for mAbp1 in BCR-mediated antigen processing and the functional control of mAbp1 by BCR signaling.

The Z-disc Proteins Myotilin and FATZ Interact with Each Other and Are Potentially Connected to the Sarcolemma via Muscle-specific Filamins

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Myotilin and the calssarcan family member FATZ-1 (also called calssarcan-2 or myozennin-1) are sarcoemeric proteins implicated in the assembly and stabilization of the Z-discs in skeletal muscle. The essential role of myotilin in skeletal muscle is attested by the observation that certain forms of myofibrillar myopathy and limb girdle muscular dystrophy are caused by mutations in the human myotilin gene. Here we show by transfection, biochemical and/or yeast two-hybrid assay that: 1) myotilin is able to interact with the C-terminal region of FATZ-1 and that the N- or C-terminal truncations of myotilin abrogate binding; 2) myotilin can also interact with another calssarcan member, FATZ-2 (calssarcan-1, myozennin-2); 3) myotilin and FATZ-1 bind not only to the C-terminal region of filamin-C but also to the other two filamins, filamin-A and filamin-B, as well as the newly identified filamin-Bvar-variant; 4) the binding of myotilin to filamin-C involves binding sites in its N-terminal region, whereas FATZ-1 associates with filamin-C via sequences within either its NH2 or COOH extremity. Our findings further dissect the molecular interactions within the Z-disc that are essential for its organization, and provide evidence for a connection between Z-disc proteins and the sarcolemma via filamins. These data shed new light on the complex organization of the Z-disc that is highly relevant to understanding muscular dystrophies.

A Novel Role for the DH and the Dimerization Domain of betaPIX in Its Localization

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PAK-betaPIX-GIT1 complex (PIX complex) plays a vital role in a wide range of biological processes including neurite outgrowth, synapase formation, and migration. The PIX complex is targeted to the actin-rich areas, for instance, the leading edges in migratory fibroblasts and the growth cones and the dendritic spines in neuronal cells, in which it regulates actin dynamics. However, the molecular mechanism by which this complex in these destinations localizes has remained elusive. We show here that betaPIX specifically interacts with non-muscle myosin II (NMM II) and this interaction is responsible for localization of the PIX complex. Truncation of the dimerization domain (leucine-zipper domain) at the C-terminus of betaPIX resulted in improper targeting to the specific subcellular sites. Interaction of betaPIX and NMM II has been demonstrated in rat brain, PC12 cells and cultured hippocampal neurons. Consistent with this, betaPIX co-localized with NMM II in these neuronal cells. In vitro binding study revealed that the DH domain is responsible for interaction with NMM II. These results suggest that the DH domain and the dimerization domain may play a novel role in targeting of betaPIX, but in a distinct mechanism.

Characterization of Coronin7 in Dictyostelium discoideum

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WD-repeat proteins play a role in a variety of cellular events, including signal transduction, transcriptional regulation, cytoskeleton remodelling and regulation of vesicular trafficking. One family of WD-repeat containing proteins are the coronins. All coronins have a conserved N-terminal core domain that includes seven WD-repeat motifs folding into a three dimensional propeller like structure. Our work focuses on the coronin7 homolog in Dictyostelium. In contrast to other coronins, coronin7 possesses two WD-repeat motifs that form a core region in the N-terminal and another one at the C-terminal region. The homologous C. elegans POD-1 and Dictyostelium coro are known to regulate the actin cytoskeleton, but also govern vesicular trafficking as indicated by mutant phenotypes. Furthermore, mutants have severe developmental defects. Analysis of Dictyostelium coronin7 GFP fusions and immunofluorescence studies with specific monoclonal antibodies show localization at actin-rich structures in the cells. Dynamic accumulation at phagocytes and pinocytic cups leads to the assumption that coronin7 may participate in the remodelling of the cortical actin cytoskeleton. The 105 kDa protein is expressed throughout Dictyostelium development and is especially prominent during early aggregation state. Furthermore we present data from a coronin7 knockout cell line where first experiments show premature development in respect to wild type.
Autophosphorylation of the Actin-regulating Kinase Prk1p Negatively Regulates Its Kinase Activity

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Prk1p is a serine/threonine kinase that regulates endocytosis and actin cytoskeleton organization in budding yeast. It phosphorylates multiple important endocytic proteins including Pan1p and Sla1p on [L/I/V/M]xx[Q/N/T/S]xT/G motifs. Prk1p has also been reported to undergo autophosphorylation in vivo. In this study, we were interested in determining the site(s) and possible regulatory roles of the autophosphorylation. Two putative autophosphorylation sites in the C-terminal domain of the Prk1p kinase that closely resemble the previously defined recognition site were examined and validated in vitro and in vivo. We showed that constitutively non-autophosphorylated mutant kinase displayed a three-fold increase in kinase activity towards Pan1p in vitro, suggesting a negative effect resulted from autophosphorylation. This mutant also caused a marked elevation in the phosphorylation level of Sla1p in vivo. We also demonstrated that Abp1p, responsible for the cortical recruitment of Prk1p, induced autophosphorylation of the kinase. These results indicate that inhibitory autophosphorylation plays an important role in regulating the kinase activity.

Imaging Analysis of the Apical Structure of Neuroepithelial Cells: Evidence for Hindbrain Cytonemes as a Novel Aspect during Vertebrate Brain Development

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Compartmentalization and boundary formation are general strategies to make body patterns during embryogenesis in invertebrates and vertebrates. Boundaries that are specialized between adjacent epithelial compartments function as signaling centers for growth and differentiation. In the fly imaginal disc epithelium, lateral cells in one compartment directly extend actin-based cellular extensions, called cytonemes, into signaling centers. In the vertebrate hindbrain, obvious compartments and specialized boundaries producing secreted molecules are formed during brain development, although a possibility that filopodial protrusions mediate long-range signal transduction in the vertebrate neuroepithelium is still unknown. To characterize features of hindbrain neuroepithelial cells, we labeled these cells with membrane-bounded EGFP, EGFP-actin and EGFP-tubulin, and visualized their apical structure by applying confocal laser-scanning microscopy to the open-book hindbrains. We found that cytoneme-like cellular extensions protruded from dorsal side of the apical membrane of neuroepithelial cells vertically to the hindbrain. These cellular extensions contained actin cytoskeleton and extended in differential orientation according to the anterior-posterior (A-P) positions. These results suggest a possibility that a conserved long-range signaling mechanism serves both in patterning of the fly imaginal disc and the vertebrate hindbrain.

Cordon-bleu Is a Novel Actin Nucleation Factor and Controls Neuronal Morphology

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Despite the wealth of different actin structures formed, only two actin nucleation factors are well established in vertebrates, the Arp2/3 complex and formins. Here we describe the discovery and characterization of a novel actin nucleator, Cordon-bleu (Cobl). Cobl is a brain-enriched protein that contains three Wiskott-Aldrich syndrome protein homology 2 (WH2) domains. Biochemical analyses and in vitro reconstitutions demonstrate that all three WH2 domains are used for actin binding albeit with different affinities. Kinetic studies show that Cobl-induced actin nucleation is as powerful as that triggered by fully activated Arp2/3 complex and gives rise to non-bundled and unbranched filaments. Cobl-mediated filament formation hereby relies on barbed end growth and does not lead to pointed end protection. In order to nucleate actin, Cobl requires all three Cobl WH2 domains and the extended linker L2 between the second and third WH2 domain. We therefore suggest that the nucleation power of Cobl is based on the assembly of three actin monomers in a cross-filament orientation. Our findings thereby provide direct experimental evidence for the idea that minimally the assembly of three G-actin molecules is required for effective nucleation and that formation of cross-filaments seeds are the major source for actin filaments. In line with Cobl being involved in actin dynamics also in vivo, Cobl localizes to sites of high actin dynamics and modulates the morphology of fibroblasts via the induction of intense, three-dimensional ruffling. In primary hippocampal cultures, Cobl has drastic effects on neurromorphogenesis, both the induction of neurites and neurite branching is dramatically increased. These effects critically depend on Cobl’s actin nucleation ability. Nucleation-competent Cobl mutants did not lead to any effects. Our data therefore reveal Cobl to be a novel actin nucleator controlling neuronal actin morphology and development and provide detailed mechanistic insights into Cobl’s function in actin dynamics.

Actin Dynamics and Assembly (2539-2543)
Transmission electron microscopy (TEM) were highly ordered and have regularly spaced transverse striations. Filament bundle organization is influenced by Dynamin2 bundles actin filaments nucleated by Arp2/3 complex and cortactin. Dynamin2-induced filament bundles observed by negative-stained transmission electron microscopy (TEM) were highly ordered and have regularly spaced transverse striations. Filament bundle organization is dependent on the nucleotide state. Tightly bundled actin filaments formed in the absence of GTP became loosely associated and lost the distinct striated pattern following GTP addition. Dynamin2-induced bundled filaments observed by total internal reflection fluorescence microscopy (TIRFM) were dramatically reorganized upon GTP addition, with filaments splaying out from bundles, appearing as if many had been fragmented or unraveled. Since actin depolymerization factors function, in part, by severing actin filaments, we tested the hypothesis that dynamin2 GTPase activity may be involved in breakage of actin filaments or by altering access of cofilin to filaments. Thus, our findings suggest that dynamin GTPase activity promotes actin filament turnover via a mechanism that may involve breakage of actin filaments or by altering access of cofilin to filaments.

Regulation of Actin Filament Organization and Dynamics by Dynamin2
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Dynamin2 (dyn2) GTPase activity regulates actin filament turnover in cells but the mechanisms by which dynamin2 influences actin filaments are unknown. Cortactin binds to dynamin2, which could provide a biochemical link between dyn2 and actin filaments. To determine how dyn2 activity alters actin filaments, we observed the effects of dyn2 on actin filament organization and stability using biochemical and microscopic assays. Dynamin2 bundles actin filaments nucleated by Arp2/3 complex and cortactin. Dynamin2-induced filament bundles observed by negative-stained transmission electron microscopy (TEM) were highly ordered and have regularly spaced transverse striations. Filament bundle organization is dependent on the nucleotide state. Tightly bundled actin filaments formed in the absence of GTP became loosely associated and lost the distinct striated pattern following GTP addition. Dynamin2-induced bundled filaments observed by total internal reflection fluorescence microscopy (TIRFM) were dramatically reorganized upon GTP addition, with filaments splaying out from bundles, appearing as if many had been fragmented or unraveled. Since actin depolymerization factors function, in part, by severing actin filaments, we tested the hypothesis that dynamin2 GTPase activity may promote filament disassembly by a similar mechanism. Pyrene-labeled actin filaments formed in the presence of Arp2/3 complex, cortactin, and dyn2 were subjected to disassembly in the absence and presence of an actin filament severing protein, cofilin. GTP, but not GTPγS, promoted disassembly of filaments in the presence of cofilin. Thus, our findings suggest that dynamin GTPase activity promotes actin filament turnover via a mechanism that may involve breakage of actin filaments or by altering access of cofilin to filaments.

Conventional Myosins (2544-2545)

Myosin Network Contraction Is Coupled to Adherens Junctions to Drive Apical Constriction
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The bending and folding of epithelial sheets during development generates an embryo with distinct germ layers that give rise to separate organs and tissues. A common cell shape change that accompanies epithelial bending is apical constriction, which converts columnar epithelial cells to wedge-shaped cells. Apical constriction is thought to result from the contraction of a belt-like actin filament bundle that underlies the adherens junction. Consistent with this model, non-muscle myosin II is apically localized in constricting cells. However, the mechanism by which myosin II functions during constriction and how constriction is coordinated between cells is poorly understood. We have performed live-cell imaging of fluorescein-labeled myosin light chain to visualize apical myosin II dynamics during gastrulation in Drosophila. Myosin II is first present prior to cell constriction as spots on the apical surface that exhibit random movements. These spots coalesce into larger spots and fibers that exhibit coordinated movement between cells, forming a web-like network that spans the apical surface of the entire constraining epithelium. Surprisingly, this myosin II network is mostly on the apical surface, separate from adherens junctions, arguing against a purse-string model for constriction. Consistent with the contraction of myosin II at the apical surface rather than underlying the adherens junction, acellular embryos that have no junctions still assemble a contractile cortical myosin network. However, in cellular embryos mutant for armadillo (β-catenin), myosin II contraction results in loss of cell-cell adhesion, which causes myosin II fiber contraction in single cells and loss of the trans-cellular myosin II network. We propose a new model for apical constriction whereby the apical actin network on the roof of an epithelial cell contracts to drive cell constriction, and adherens junctions integrate these contractile structures across the epithelium to form a supracellular tensile network that is essential for tissue invagination.

Characteristics of Myosin from Peritubular Myoid Cells
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In the mammalian testis, peritubular myoid cells (PMCs) surround seminiferous tubules. These cells are contractile and express the cytoskeletal markers of true smooth muscle, such as alpha-actin and F-actin, and participate in the contraction of seminiferous tubules during the transport of spermatozoa and testicular fluid to the rete testis. The goal of this work was to analyze the properties of myosin from PMCs. Myosin from PMCs (PMC-myosin) was isolated from adult rat testes and purified by cycles of aggregation-disaggregation and sucrose gradient centrifugation. PMC-myosin was recognized by a monoclonal anti-smooth muscle myosin antibody, and the peptide sequence shared partial homology with rat smooth muscle myosin-II (SMM-II). A fraction (95%) of PMC-myosin was found soluble in the PMC cytosol, and purified PMC-myosin did not assemble into filaments in the in vitro salt dialysis assay at 4 °C, but did at 20 °C. PMC-myosin filaments are stable to ionic strength to the same degree as gizzard SMM-II filaments, but PMC-myosin filaments were more unstable in the presence of ATP. When PMCs were induced to contract by endothelin-1, a fraction of the PMC-myosin assembled into filaments. From these results we infer that PMCs express an isoform of smooth muscle myosin II, that is characterized by solubility at physiological ionic strength, a requirement for high temperature to assemble into filaments in vitro, and instability at low ATP concentrations. PMC-myosin is part of the PMC contraction apparatus and assembles into filaments when PMCs are induced to contract by hormone.
Unconventional Myosins (2546-2547)

2546
**Rab10 Interacts with Myosin Vb and Myosin Vc in an Exon-specific Manner**
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The proper trafficking of many cellular cargoes is dependent on the recruitment and regulation of molecular motors such as the Class V myosins. These motors are regulated by their association with members of the rab family of small GTPases. We have previously noted that Rab11a and Rab8a can interact with the tail region of myosin Vb and regulate distinct trafficking pathways. We have now determined that a related rab protein, Rab10, is able to interact with Myosin Vb in vivo on a network of long tubules. Rab8a- and Rab10-positive tubules have partially overlapping localizations in HeLa cells and both proteins are mislocalized by the expression of a dominant-negative Myosin Vb fusion protein. In addition, we have determined that an exon in Myosin Vb that is subject to alternative splicing, Exon D, is required for Rab10 binding. Fluorescent Resonant Energy Transfer (FRET) data show that Rab10 does bind to the tail of Myosin Vb in vivo and that the alternatively spliced Exon D of Myosin Vb is an important structural component of this binding. Like Myosin Vb, Myosin Vc is also able to interact with both Rab8a and Rab10 and expression of a dominant-negative Myosin Vc tail construct results in the mislocalization of both Rab proteins. These results demonstrate that Myosin Vb and Myosin Vc are associated with, and potentially regulated by, multiple rab proteins.

2547
**Navigation through the Actin Cortex: How Do Myosins Do It?**
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Cell movement encompasses cellular locomotion, as well as molecular mechanisms, which achieve movement. To achieve movement, living organisms must utilize molecular motors, machines that consume energy and convert it into motion. Even though these motors are quite small, they generate amazing amounts of force to accomplish vital tasks such as intracellular trafficking, muscle contraction, and cell division. Due to their central role in biology, motor proteins have begun to be characterized biochemically and biophysically. However, these efforts have only scratched the surface in understanding how these motors truly function in vivo to aide cellular movement. Understanding how myosin motors navigate through the meshwork of cortical actin within living cells is essential to comprehending cell motility. We have developed a technique that allows the actin networks in cells to be preserved, while labeled myosin motors are added the cells and observed moving on the actin networks. Determining if labeled myosins move along filopodia and how these motors interact with the plasma membrane is the goal in actin-preserved mammalian cells, although the exploration of many combinations of motors on native tracks will also be feasible.

Tubulin (2548-2551)

2548
**A Gradient of Glutamylated Tubulin Marks the Cell Periphery**
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Microtubules give a cell its shape and ability to divide. These filaments frequently undergo modifications. One such modification is the addition of Paclitaxel (PTX) is a highly successful anticancer drug that acts by affecting microtubule dynamics. Understanding the important interactions between the drug and its receptor, assembled tubulin, will assist in design of the next generation of drugs that share PTX’s mechanism of action. The 2’-hydroxyl moiety of PTX is well known to be necessary for the drug’s cytotoxicity; however, its role in the association of PTX with microtubules has not been quantitatively assessed. The PTX analog 2’-deoxy-PTX was synthesized from baccatin III, which lacks the C-13 side chain of PTX. The affinity of the ligand for GMPCPP microtubules was measured by competition with a well-characterized fluorescent PTX derivative and compared to...
those measured for PTX and baccatin III. The association constant for microtubule binding of 2'-deoxy-PTX is 83-fold less than that of PTX and about 3-fold greater than that of baccatin III. Thus, the 2'-hydroxyl group is responsible for about 75% of the binding free energy of the side chain at 37 °C. The efficacy of each ligand was assessed by evaluating the effect of the ligand on the critical concentration of tubulin. The relative potency of each ligand in this assay is in concordance with the affinity constants. The trend in cytotoxicities in PC3 cells mirrors the microtubule results: 2’-deoxy-PTX is about 6-fold more cytotoxic than baccatin III but is more than 300-fold less cytotoxic than PTX, confirming that the 2’-hydroxyl group is the critical feature of the side chain. We propose that the role of the two aromatic rings in the side chain is primarily to orient the hydroxyl group in a proper position for a hydrogen bonding interaction with the protein. Otherwise these structural features are only minor contributors to the receptor site interactions and therefore to the microtubule assembly-promoting and cytotoxic activities of PTX.

2550

Arylcarbonylindoles: Antimitotic Agents and Potent Inhibitors of Tubulin Polymerization

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Having previously shown that arythioindoles are potent inhibitors of tubulin polymerization, we continued our structure-activity relationship studies in a search for still more potent compounds. We have now identified arylcarbonylindoles as a variant scaffold, with several members that are excellent inhibitors of tubulin polymerization and of the growth of MCF-7 human breast carcinoma cells. The best of the new compounds, referred to herein as compound 1, inhibited tubulin assembly with an IC_{50} of 0.7 µM, so that it was about 2-4 times more active than colchicine and combretastatin A-4 (CSA4). The compound inhibited MCF-7 cell growth with an IC_{50} of 17 nM, comparable to the activity of colchicine. As an inhibitor of the binding of[^{3}H]colchicine to tubulin, compound 1 was almost as potent as CSA4. With inhibitor and[^{3}H]colchicine equimolar and in excess over the tubulin concentration, compound 1 inhibited colchicine binding about 80% compared with about 90% inhibition by CSA4. Further characterization of the interaction of compound 1 with tubulin is underway, but, from a structure-activity viewpoint, maximum activity required a carbonyl bridge, a trimethoxyphenyl ring, and additional substituents on the indole moiety.

2551

Regulation of γ-tubulin Gene Expression during Cell Differentiation in Naegleria gruberi

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The amoeba-to-flagellate differentiation of N. gruberi is accompanied by the de novo formation of basal bodies, flagella, and cytoskeletal microtubules. γ-tubulin plays an essential role in the initial stage of this event, the formation of basal bodies. We have cloned Naegleria γ-tubulin (Ng-γ-tubulin) and examined the gene expression of γ-tubulin mRNA. Ng-γ-tubulin and γ-tubulin mRNA were detected in N. gruberi by northern blot and in situ hybridization. The amount of Ng-γ-tubulin mRNA increased transiently reaching a maximum between 20 min and 40 min after initiation of differentiation, and its mRNA continuously maintained low expression level throughout the differentiation. The transient accumulation of Ng-γ-tubulin mRNA was not inhibited by cycloheximide, which blocked the accumulation of α- and β-tubulin mRNAs completely. These data showed that the regulation of Ng-γ-tubulin gene expression is separated from that of α- and β-tubulin genes. Nevertheless gene expression of Ng-γ-tubulin is regulated by different mechanism with α- and β-tubulin, at 80 min after the initiation of differentiation, two mRNAs were colocalized to the base of the growing flagella, adjacent to the basal bodies. Result of immunostaining at the mitosis stage, Ng-γ-tubulin might have no relationship in assembly of the mitotic spindle while play a part in formation of basal bodies and cytoskeletal flagella. "This work was supported by the Korea Research Foundation Grant funded by Korea Government (KRF-2005-070-C00089)"

Dynemin (2552-2554)

2552

Analysis of Dynemin Cortical Patches in Yeast Mutants Lacking Microtubule Plus End Targeting Mechanism

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Mitotic spindle positioning plays an important role in determining where replicated chromosomes are deposited at the end of cell division. In the budding yeast S. cerevisiae, cytoplasmic dynein mediates movement and positioning of the mitotic spindle into the mother-bud neck. We have previously reported localization of dynemin HC/Dyn1 and IC/Pac11 at the plus end of microtubules and at the cell cortex. We proposed an off-loading model whereby dynemin targeted to the plus ends is delivered to specific cortical Num1 receptor sites. Here we carried out detailed analysis of dynein cortical patches in wild type and mutants lacking dynemin plus end targeting mechanism. We quantified the number and intensities of Dyn1-3GFP and Pac11-3YFP cortical patches in bik1Δ, bik1ΔACT39, pac1Δ, pac1Δ bik1ΔACT39, and pac1Δ bik1Δ mutants. Our results suggested differential roles for Bik1 (yeast Clp1 homologue) and Pac1 (yeast L151 homologue) in dynein localization at cortical patches, providing new insights into a potential role for these proteins in dynein off-loading or anchoring.

2553

Sub-domain Mapping within the Dynemin Motor Domain

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Cytoplasmic dynein is a molecular motor that uses ATP to drive most minus-end directed traffic along microtubules, as well as specialized roles in mitosis. The force-generating dynein heavy chain forms a ring-like head containing AAA+ modules that hydrolyse ATP, a stalk that cyclically binds microtubules, and a tail that binds cargo. A lack of high-resolution structural information has hampered insights into how these components are
arranged and generate force. Here, using negative stain electron microscopy and image processing, we map several functionally important sub-domains within recombinant cytoplasmic dynein motors. These include the stalk, the primary catalytic AAA+ module and the motor N-terminus, which has been implicated as the main transmitter of mechanical motion. The results suggest the heart of the motor is a hexameric ring of sequentially arranged AAA+ modules, contrasting with current orthodoxy which sees a heptameric ring containing a C-terminal domain. The N-terminal sequence is shown to form the linker that spans the AAA+ ring and swings across it in a nucleotide dependent manner. The structural data support a model where the linker acts as a lever, amplifying conformational changes within the AAA+ ring.

2554

Engineering of a Monomeric Photoactivatable GFP for Analysis of Dynein Pathway Components in Budding Yeast

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In budding yeast, the anchor protein Num1 has been shown to play an important role in nuclear migration. Localized at the cell cortex, Num1 is a protein found in the bud cortex as well as the mother cortex, serving as a receptor and anchor for cytoplasmic dynein. Dynein is a multi-subunit motor protein complex, which generates movement towards the minus ends of microtubules. Num1 contributes to spindle movement during anaphase in a dynein-dependent manner. It is hypothesized that dynein at the plus end interacts with Num1 at the cell cortex. This interaction mediates anchoring of the motor at Num1 sites. Once anchored, it is proposed that dynein becomes activated to pull on the cytoplasmic microtubules, thus contributing to nuclear migration. Here, we set out to test this model with photoactivatable-GFP (PA-GFP) to examine the dynamic localization of dynein anchoring sites. We created a yeast tagging vector containing a conventional photoactivatable GFP. We also used site-directed mutagenesis to generate a vector containing a monomeric variant of codon-optimized and photoactivatable GFP. Using the PA-GFP tag, we have demonstrated selective photolabeling of Erg6, an abundant sterol methyltransferase that localizes to both lipid particles and mitochondrial outer membrane. Fluorescence activation of these PA-GFP tags will be compared and will enable protein tracking studies of Num1 in growing yeast cells, which may reveal key components and functions associated with Num1.

Kinesin (2555-2559)

2555

Effect of Spastic Paraplegia Mutations in KIF5A Kinesin on Transport Activity

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Hereditary spastic paraplegia (HSP) is a neurodegenerative disease caused by motoneuron degeneration. It is linked to at least 30 loci, among them SPG10, which causes dominant forms and originates in point mutations in the neuronal Kinesin-1 gene (KIF5A). Here, we investigate the motility of heterozygous patients, whereas the others (K253N, R280C) do not. Attached to quantum dots as artificial cargo, mixtures involving N256S mutants produced slower cargo populations lagging behind in transport, whereas mixtures with the other mutants led to populations of quantum dots instead uniquely recognize MT-ends and depolymerizing MT protofilaments. We have used electron microscopy (EM) and single particle image processing methods to visualize and understand the molecular interactions by which kinesin 13 induces depolymerization at MT-ends. Previous studies have shown that Dolastatin-induced tubulin rings mimic the properties of MT-ends and that these rings can be decorated with the motor domain of kinesin 13 in various nucleotide states. We have visualized the interactions of different kinesin 13 domain constructs with these tubulin rings. Whereas a construct consisting of only the motor domain of kinesin 13 binds to every tubulin heterodimer in the ring, a construct that contains the 60-residues of the class-specific neck linker in addition to the motor domain occupies alternate binding sites. Similarly, a monomeric C-terminal deletion construct and a dimeric full length protein also show alternate site binding. Furthermore, EM maps of the dimeric full length protein interacting with tubulin rings reveal density for only one of two motor "heads." These results indicate that the second head of dimeric kinesin 13 does not have access to neighboring binding sites on the protofilament and suggest that the neck linker alone is sufficient to obstruct access to neighboring binding sites. Future studies will investigate possible conformational changes in the kinesin 13 and tubulin ring complex when incubated with different nucleotides.
Analysis of the Localizations and Functions of Kinesins in the Filamentous Fungus Aspergillus nidulans

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The functions of kinesin family members are of interest in all eukaryotes and this is particularly true in A. nidulans. Two kinesins, BIMC and KLPA, have been shown to play roles in mitosis. In addition, we have shown that microtubules are essential for rapid and steady tip growth of A. nidulans (MBC, 2005, 16, 918-). This is almost certainly because they are required for long-range transport of vesicles containing cell wall precursors. This transport must, in turn, involve motor proteins such as kinesins. It is thus of interest to determine the localization patterns and functions of the kinesins. There are 11 kinesin family genes in the genome of A. nidulans. Among them, only bimC has been shown to be essential for growth so far. To characterize the functions of the kinesins, we deleted each of the kinesin genes except for bimC, tagged all of them with fluorescent proteins and observed the fusion proteins in living cells. None of the 10 kinesins deleted were essential for growth, but some of the deletants exhibited minor growth defects. Some kinesins exhibited nuclear/spindle localization suggesting that they play some role in mitosis. Several kinesins were observed as scattered dots in the cytoplasm, in some cases on microtubules. Accumulation to the growing tip region was observed in a subset of these kinesins. Kinesins in this group could, thus, be good candidates for transporters of tip-growth materials. We failed to observe any particular signal for two kinesins although we verified that both kinesins are expressed at levels comparable to other kinesins. These results indicate that, although these 11 kinesins belong to 10 different subtypes, only bimC has a unique function. All the remaining kinesins share overlapping functions. Supported by JSPS and the NIGMS.

Disruption of KIF17/Mint1 Interaction by CaMKII Dependent Phosphorylation: A Molecular Model of Kinesin/Cargo Release

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Establishment and maintenance of cell structures and functions is highly dependent upon the tight and efficient regulation of intracellular transport in which kinesin superfamilies (KIFs) play major roles. In this regard how KIFs regulate the release of their cargoes is a critical process that remains to be elucidated. To shed light on this specific question, we have investigated the mechanism behind the regulation of KIF17/Mint1 interaction. Here we report that the molecular motor KIF17 tail region is under spatial and temporal regulation by phosphorylation. Using direct visualization of protein-protein interaction by FRET and various in vitro and in vivo approaches we have demonstrated that CaMKII dependent phosphorylation of KIF17 on ser1029 disrupts KIF17/Mint1 association and results in the release of the transported cargo from its microtubule-based transport.

Spatial Regulation of Eg5

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Eg5 is a homotetrameric, plus end-directed kinesin known to play an important role in the spindle assembly process, a fact most clearly demonstrated by the monopolar spindles that result from its inhibition. Although the Eg5 motor domain has repeatedly been localized to mitotic microtubules, often with an enrichment at spindle poles, what remains unknown is how this localization is controlled. To some degree, the mitotic distribution of Eg5 depends on the phosphorylation of a Thr residue within Eg5’s tail domain, a process mediated by Cdk1. However, while this event appears to be essential for the Eg5-spindle association, it is not sufficient. We hypothesize that phosphorylation of Eg5 by Aurora A kinase, is additionally required for its ability to associate with mammalian spindles for the following reasons: i) in Xenopus, the Aurora A homolog, pEg2, has been reported to phosphorylate an Eg5 stalk domain Ser residue; and ii) inhibition of Aurora A leads to a centrosome separation phenotype, consistent with an impairment of Eg5 activity. To determine how Eg5 normally distributes within LLC-Pk1 mitotic spindles, we have generated a construct containing Eg5 C-terminally fused to photoactivatable GFP, thereby enabling us to look not only at the protein’s location, but also at more subtle spindle association features, like its dynamics. Transient transfection of this construct into LLC-Pk1 cells reveals a mitotic distribution similar to prior observations. We will now introduce point mutations at the putative Aurora A phosphorylation site to hinder Aurora A phosphorylation of Eg5.

Microtubule-associated Proteins (2560-2564)

Drosophila mars Is Required for Organizing Kinetochore Microtubules in Mitosis

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During mitosis, chromosomes are required to attach to the kinetochore microtubules and separate equally to the daughter cells. The formation of kinetochore microtubule is a complex process, which its molecular mechanism remains unclear yet. Here, we report that Drosophila mars gene is critical for kinetochore microtubules formation. Immunocytocchemical staining revealed that Mars expression is dynamically changed during cell cycle progression. In prophase, the Mars protein appears diffusively in the cytoplasm. In metaphase and early anaphase, Mars is localized at the mitotic spindles. Mars is then gradually degraded and relocated in the nucleus as cells entering telophase. We also found that Mars localization is microtubule dependent since chemicals interfere microtubule organization disrupts Mars localization. Using kinetochore marker and cold-treatment, we further found that Mars is specifically localized to the kinetochore microtubules. Using RNAi to deplete Mars expression in Drosophila S2 cells, we found high percentage of chromosomes is uncongressed along with defected kinetochore microtubules. Together, this study suggests that Mars plays a critical role in mediating formation of kinetochore microtubule.
The First TOG Domain of MOR1 Plays a Key Role in Microtubule Dynamics
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Members of the Dis1/XMAP215 family of microtubule-associated proteins are essential players in the control of microtubule (MT) dynamics. These proteins have up to 5 N-terminal, 250 residue TOG domains, so-called for their identification in the human homologue, Tumor-Overexpressed Gene protein. Here we investigate the specific function of the N-terminal TOG domain (TOG1a) of the MOR1 homologue from the higher plant Arabidopsis thaliana. Three point mutants that substitute single amino acids in TOG1a all cause disruption of MT arrays when shifted from a permissive temperature of 21°C to a restrictive temperature of 31°C. Until now, it was not clear how MT dynamics were altered in these disrupted arrays. We used spinning disc confocal microscopy to quantify MT dynamics using GFP reporters of β-tubulin and the plus end tracker EB1. In wild-type cells, MT growth and shrinkage rates nearly doubled when the temperature was raised from 21°C to 31°C. In the mor1-1 mutant, we found that MT growth and shrinkage rates were reduced compared to wild-type even at permissive temperature, despite no obvious differences in the organization of MT arrays. When shifted to 31°C, the mor1-1 MT growth and shrinkage rates were reduced even further, demonstrating that the mutations in the first TOG domain prevent MTs from adapting to the higher temperature. The frequency of transitions between growth, shrinkage, and pause was greatly increased in mor1-1 mutants, and this was reflected in the greatly reduced association of EB1 with MT ends. Interestingly, the accumulation of EB1 at MT ends also appeared to diminish in wild-type cells at 31°C, suggesting that EB1’s association with MTs is not dependent on MT growth rates. Taken together, our findings suggest that the N-terminal TOG domain of MOR1 is a critical player in maintaining the assembly and removal of tubulin subunits from the plus end of MTs.

A Novel MACF1 Isoform 3 Displays Dual Function in the Cytoplasm and the Nucleus
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MACF1 (microtubule actin cross-linking factor 1) is a multidomain protein that associates with microfilaments and microtubules. The MACF1 gene encodes several alternatively spliced transcripts, which differ in length, domain composition and their expression pattern. In particular, MACF1 isoform-3 shows an unusual N-terminus, which suggests novel functions. It is composed of a unique and highly conserved 1kb N-terminus that is fused to the second half of the actin-binding domain. Using a specific antibody against isoform-3, an unusual localization pattern was observed in and around the nucleus in various cell lines, in addition to the well-documented microtubule-network pattern. Digitonin permeabilization assays showed MACF1 isoform-3 to be located at the outer nuclear envelope, where it co-localizes with Nesprins. Using GST-pulldown assays, we demonstrate the association of the MACF1 N-terminus with Nesprin-3. Furthermore our results suggest interactions between MACF1 and additional nuclear proteins. Using affinity chromatography we identify histones 1b and d to associate with the isoform-3 specific domain. Interestingly, in lamin knockout mouse fibroblasts we observed MACF1 isoform-3 to be mislocalized from the nuclear envelope. In addition transiently expressed dominant-negative lamins also affect the localization of MACF1. In summary our results suggest that MACF1 isoform-3 is a dual linker protein, which functions in both the cytoplasm and the nucleus.

Polymerization and Tubulin Binding Properties of Calpain-cleaved Tau Proteins In Vitro and in Alzheimer's Disease
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Alzheimer's disease (AD) is partly characterized by the intracellular deposition of neurofibrillary tangles (NFTs), which are comprised of hyperphosphorylated and truncated tau filaments. NFTs pose a malignancy to normal neuronal physiology and morphology for tau normally regulates and stabilizes microtubule assembly. Proteolytic cleavage of tau at specific sites by calpain I and II is implicated in the pathogenesis of AD. Calpains I and II are calcium-activated cytosolic cysteine proteases whose activities are increased in AD brain and colocalize with NFTs in AD. It is plausible that the impaired calcium levels observed in AD brain contribute to the overactivation of calpains I and II, ultimately causing the pathogenic organization of tau proteins in NFTs. This study confirms that in vitro digestion of tau with calpain II produces a 45kD tau fragment cleaved at Lysine 254, in addition to other fragments. To monitor the polymerization and tubulin binding effects of calpain cleaved tau, recombinant tau proteins truncated at Lysine 254 were constructed from the HT40 cDNA (longest tau isoform, 441 residues) using site-directed mutagenesis and PCR. Laser light scattering analysis (LLS) of polymerization reactions demonstrates that tau 255-441 polymerizes to a greater extent than both HT40 and tau 1-254. Conversely, LLS reveals that tau 1-254 polymerizes to a lesser degree than HT40. Transmission electron microscopy (TEM) analysis shows that tau 255-441 forms large protein aggregates measuring about 2 μm in diameter, while tau 1-254 forms smaller and less frequent filaments than HT40 measuring approximately 20 nm in length. Addition of HT40 to tubulin increases the turbidity of the reaction compared to tubulin-only control and TEM reveals that microtubules readily form in the presence of tau. The increased extent of polymerization displayed by calpain-cleaved tau fragment 255-441 suggests that calpain II is involved in the pathogenic arrangement of tau proteins observed in NFTs.

EMAP, a Microtubule-associated Protein Important for Mechanosensory Transduction in Drosophila
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The tubular body- the distal part of a ciliated dendrite is filled with regular arrangement of microtubules and is the structural basis for mechanotransduction in Drosophila. The echinoderm-microtubule associated protein EMAP has been identified in a screen searching for genes specific for mechanotransducers in Drosophila. EMAP localizes to sensory structures in the fly’s campaniform receptor as well as in the Johnston’s organ- the fly’s auditory organ. A P-element insertion into the EMAP gene causes uncoordination and hearing impairment in Drosophila. The EMAP
protein seems to be responsible for microtubule organization in the tubular body as determined by electron microscopy. Interestingly, huEMAP-1 has been implicated to be a cause for Usher-syndrome. In zebrafish EMAP seems to be expressed in the lateral line organ. Therefore, EMAP might be a protein involved in microtubule organization with a conserved role in sensory systems.

**Microtubule Dynamics and Assembly (2565-2567)**

2565

**Modeling and Analysis of Microtubule Bending**

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The microtubule cytoskeleton in living cells generates and resists mechanical forces to mediate fundamental cell processes. Direct observations of individual microtubules in living cells show that microtubules are often highly bent and they deform dynamically. The complex nature of the intracellular forces makes it difficult to characterize the contribution of specific loading mechanisms that drive microtubule bending. To systematically investigate the origins of microtubule bending, we present a simulation framework for the modeling of semiflexible biopolymers (microtubules) embedded in a solvent with built-in thermal fluctuations. In order to make realistic comparisons with the experiments, we convolve the microtubule contours obtained from simulations with the point spread function of light and Gaussian noise to mimic the optical blur and digital noise inherent in the microscope-camera system, an approach we call model convolution. A useful measure of the deformation of microtubules is the curvature distribution obtained by discretizing the contours of these microtubules obtained from images. We use our computational model to validate this measurement technique, identify the effects of measurement and digitization error and provide a procedure for using this tool effectively. We show that curvature distribution measurements can serve as a powerful tool to analyze microtubule deformation and provide complementary information to traditional methods such as bending mode analysis. This approach is not limited to microtubules and can be used also to characterize the deformation of other biopolymers such as actin filaments. In addition, the effects of molecular motors, elastic forces in the surrounding cytoplasm, polymerization forces, as well as hydrodynamic forces due to the presence of other microtubules can be investigated using this computational framework.

2566

**A Novel Acetylation on β-tubulin Negatively Regulates Tubulin Dimer Formation In Vitro and Destabilizes Microtubules In Vivo**

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Microtubule plays central roles in maintaining mechanical integrity of the cell, facilitating intracellular trafficking, and forming dynamic macromolecular structures such as cilia and mitotic spindle. Various post-translational modifications, including acetylation, are used to generate diversified microtubules to carry out distinct functions. Our previous study shows that an acetyltransferase San is required for the maintenance of centromeric cohesin. In an effort to identify San substrates, we found that β-tubulin, but not α-tubulin, can be acetylated by San in vitro. We located the acetylation site on β-tubulin by both mass spectrometry and site-directed mutagenesis. A β-tubulin mutant mimicking San-acetylated form was generated. Immunoprecipitation assay showed that this mutant barely interacts with α-tubulin in vitro. When expressed in HeLa cells, the mutant disassembled microtubule network in interphase and resulted in either shortened multi-polar mitotic spindle or undetectable spindle. The mutant-expressing cells also showed reduced level of α-tubulin K40 acetylation, which is consistent with the observation that depletion of San elevated the level of α-tubulin K40 acetylation. Taken together, our data suggest that San-mediated acetylation on β-tubulin serves as a negative regulation of microtubule dynamics in vivo.

2567

**Mechanism of Spastin-mediated Microtubule Severing**

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Spastin, an AAA+ ATPase mutated in the neurodegenerative disease Hereditary Spastic Paraplegia, severs microtubules. While regulation of the dynamic properties of microtubule plus ends by plus end binding proteins and kinesins is better understood, nothing is known about how spastin makes internal breaks in microtubules. Many other AAA+ proteins form ring shaped hexamers and contain loops that project into the ring’s central cavity. These loops can act as ratchets that pull on target proteins leading, in some cases, to conformational changes. We show that spastin assembles into an ATP-dependent hexamer and that loops within the central pore recognize C-terminal amino acids of both alpha and beta tubulin. Key pore loop amino acids are required for severing, including one altered by a disease-associated mutation. Surprisingly, we find that spastin only interacts weakly with tubulin heterodimer as compared to its interactions with microtubules or with the isolated C-terminus of tubulin. We also show that spastin contains a second microtubule binding domain which makes a distinct ATP-independent interaction with microtubules and is required for severing. Therefore, spastin engages the microtubule in two places and both interactions are required for severing. These interactions suggest at least two possible mechanisms for microtubule severing. First, spastin might sever microtubules by translocating and unfolding tubulin. Second, spastin’s ATPase activity may result in relative motions between the two binding sites, inducing a conformational change in a tubulin heterodimer such that it is released from the microtubule lattice.
Cilia and Flagella (2568-2574)

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Expression of Cilia-associated Kinesins in Fibroblasts
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Lack of normal functioning cilia causes various defects and diseases including polycystic kidney disease, infertility, respiratory diseases, blindness, developmental defects and cancer. A number of receptors and ion channels/transporters localize to primary cilia in mammalian cells, including Platelet Derived Growth Factor Receptor α (PDGF-Rα), polycystin-2, the taurine transporter TauT and components of the Hedgehog and Wnt signaling pathways. The ciliary localization of these signaling components is necessary for their activation/function. While intraflagellar transport (IFT) motors of the kinesin-2 family are known to participate in building of the ciliary axoneme little is known about which kinesins participate in transport of ciliary membrane components. In Caenorhabditis elegans the kinesin-3 family protein Klp6 is required for localization of polycystin-2 to the membrane of sensory cilia (Peden EM, Barr MM. 2005. Curr Biol. 15:394-404), but whether any mammalian kinesin-3 family proteins play a similar role in ciliary localization of membrane proteins is not known. By RT-PCR we have shown that the kinesin-3-encoding genes Kif13A, Kif13B, Kif14, Kif16A (272-05-0411) and The Novo Nordisk Foundation.

growth arrest, supporting that the corresponding proteins have cilia specific functions. Consistent with a role in cilia assembly/function is currently being investigated. Supported by grants from the Lundbeck Foundation, the Danish Natural Science Research Council (272-05-0411) and The Novo Nordisk Foundation.

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Characterization of Primary Cilia with Signaling Properties in Human Pancreatic Epithelial Cells from the Exocrine Duct
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The primary cilium coordinates signal transduction pathways in mammalian development and tissue homeostasis (Christensen et al., Traffic, 2007), and defects in ciliary assembly or loss of functional cilia leads to a plethora of human diseases and disorders now commonly referred as to ciliopathies (Badano et al., Ann. Rev. Gen. Hum. Genet. 2007). In the pancreas, disruption of primary cilium assembly in epithelial cells results in e.g. cyst formation and pancreatitis (Cano et al., Development 2004 and Gastroenterology 2006; Zhang et al., Lab Invest. 2005). However, little is known about the sensory function of the primary cilium in pancreatic cells. We investigated the presence of specific signal transduction components in primary cilia of the pancreatic exocrine duct epithelium in the developing human embryo and in cultures of human cells derived from the exocrine duct, PANC-1 and CFPAC-1. After serum starvation, both cell lines formed 5-20 μm long primary cilia, which contain essential components of Ca2+ and Hedgehog (Hh) signalling pathways, including polycystin-2 (PC2), Patched (Ptc), Smoothened (Smo) and glioma transcription factor, Gli2. The ciliary localization of these proteins was evidenced by using specific antibodies and/or by the expression of fluorescent-tagged proteins. Further, stimulation with Hh agonists increased the ciliary targeting of Smo in CFPAC-1 cells, indicating a functional role of the cilium in coordination of Hh signaling. Similarly, the kinesin motor protein KIF13A, which is homologous to C. elegans KLP-6 that transports polycystins into the sensory cilium, uniquely localized to primary cilia of exocrine duct cells. These findings indicate that pancreatic primary cilia are sensory organelles involved in Ca2+ and Hh signaling, which in turn is important for development, patterning and homeostasis of pancreatic tissue.

The Primary Cilium Coordinates Directional Cell Migration via PDGFRα and NHE1 in Fibroblasts
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Previous work in our group has shown that PDGF-AA-mediated PDGFRα signaling is coordinated by the primary cilium in fibroblasts. During growth arrest, PDGFRα expression is up-regulated and the receptor is targeted to the primary cilium where ligand-dependent activation of the receptor occurs [Schneider et al. (2005) Curr. Biol. 15: 1861-1866]. Since PDGF signaling is critical in development and tissue repair, we investigated the function of the primary cilium during cell migration. Using micro pipettes to generate a PDGF-AA gradient we found that growth-arrested wild-type (wt) mouse embryonic fibroblasts (MEFs) respond immediately to PDGF-AA injection, and migrate uniformly towards the pipette. Tg737orpk MEFs, lacking primary cilia, do not respond to PDGF-AA and move around randomly. In wound healing assays primary cilia with GFP-PDGFRα in wt MEFs orient parallel to one another, perpendicular to the wound. Moreover, PDGF-AA incubation during wound healing increases the migration speed and the directional movement of the cells. In contrast, in Tg737orpk cells the migration speed is unaffected by PDGF-AA incubation and cells have decreased directionality. PDGFRα signaling in the cilium is associated with activation of central signaling and transport systems in cell migration, including protein kinase B (Akt) at the cilary base as well as the ubiquitous plasma membrane Na+/H+ exchanger, NHE1, at the leading edge. Notably, inhibition of NHE1 by EIPA abolished PDGF-AA-mediated migration speed and directionality of wt MEFs, and this inhibition was markedly reduced in Tg737orpk MEFs. These results support the conclusion that the primary cilium is an important mechanism of sensing chemotactic gradients and is part of the positioning machinery that coordinates directed migration in wound healing and developmental processes. Consequently, defects in building up the primary cilium may have crucial consequences on cellular and physiological levels and be responsible for developing a series of human diseases and migration-related disorders.
Role of Microtubule Plus End-Tracking Proteins (+TIPs) in the Assembly of Primary Cilia

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Microtubule (MT) plus-end-tracking proteins (+TIPs) such as EB1 play important roles in regulating MT dynamics and in linking the MT plus end to the cell cortex, mitotic kinetochores, and other cellular structures. EB1 binds directly to p150Glued and in addition to localizing to the MT plus end, both proteins also localize to centrosomes and are required for MT minus end anchoring at this site. Recently we showed that centriole-associated EB1 is required for assembly of primary cilia in fibroblasts and that interaction between EB1 and p150Glued is essential for EB1 to promote ciliogenesis (Schröder et al. 2007. Curr. Biol. 17:1134-1139). However, the exact mechanism by which this occurs is unclear. To address the role of p150Glued in ciliogenesis we partially depleted this protein from NIH3T3 fibroblasts by siRNA and analyzed the effect on primary cilia formation in growth-arrested cells using immunofluorescence microscopy. Preliminary results indicate that reduction in the cellular amount of p150Glued inhibits the efficiency of cilia formation in fibroblasts by ca. 50%, consistent with the notion that both EB1 and p150Glued are required for ciliogenesis in fibroblasts. We are currently using dominant-negative constructs to confirm the role of p150Glued in ciliogenesis and the role of other +TIPs (e.g. EB2 and EB3) in ciliogenesis is also being addressed. Supported by grants from the Danish Natural Science Research Council (272-05-0411) and The Novo Nordisk Foundation. JMS is the recipient of a BioCampus scholarship from the University of Copenhagen.

The Primary Cilium Coordinates Hedgehog Signaling in the Mouse P19.CL6 Embryonal Carcinoma Stem Cells

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The Hedgehog (Hh) pathway controls differentiation and patterning of cells and tissues during embryonic development. In the adult, Hh signaling is involved in the maintenance of stem cell niches and tissue homeostasis [Dellovade et al. (2006) Annu Rev Neurosci. 29:539-563]. Essential elements of vertebrate Hh signaling include a family of secreted Hh proteins that upon binding to the transmembrane patched protein (Ptc) abolish the inhibitory effect of Ptc on the seven-transmembrane receptor Smoothened (Smo). This allows Smo to transduce a signal via Gli transcription factors to the nucleus for expression of Hh target genes. Essential Hh signal components localize to primary cilia, indicating that the Hh pathway is regulated though this organelle in development and tissue homeostasis [Christensen and Ott (2007) Science 317:330; Rohatgi and Scott (2007) Nat. Cell Biol. 9:1005]. Here we investigated the role of primary cilia in cardiac development using P19 mouse embryonal carcinoma stem cells that differentiate into cardiomyoblasts, which is controlled by Hh [Gianakopoulos and Skerjanc (2005) JBC:21022]. Initially we used immunofluorescence microscopy analysis (IF) to demonstrate that P19 cells form primary cilia throughout differentiation into beating cardiomyoblasts. At all differential stages Ptc, Smo and Gli2 localized to the primary cilia, although the ciliary level of Ptc markedly increased in fully differentiated cells, indicating a stop in Hh signaling by a negative feedback inhibition via the primary cilium. Using real-time quantitative RT-PCR and IF we are at present analyzing the ciliary localization of Hh components as well as the expression of Hh specific genes, i.e., Gli1-3, Sufu, Smo, Gata4, Ptc1-2, Arrb2, Grk2 and Rab23 during differentiation of P19 cells in relation to ciliary disassembly by RNAi. Our current hypothesis is that the primary cilium controls differentiation of cells into cardiomyoblasts and partly via ciliary Hh signaling.

Characterization and Functionality of Hedgehog Signalling through the Primary Cilia in Human Embryonic Stem Cells

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Primary cilia are sensory organelles that coordinate key signal transduction pathways during embryonic development and tissue homeostasis [Christensen et al (2007) Traffic 8:97]. These include the hedgehog (Hh) pathway, whose essential components specifically localize to the primary cilium [Rohatgi and Scott (2007) Nat. Cell Biol. 9:1005]. Because of these signalling properties, which are crucial to development and tissue differentiation, we examined whether human embryonic stem cells (hESC) form primary cilia, and if so, whether these cilia control Hh signalling. Using hESC line LRB003 [Laursen et al. (2007) Reprod. Biomed. Online], we were able for the first time to show the presence of a single primary cilium per cell in hESC. When grown under feeder-free conditions, the cells formed monolayer cultures, whereas cells on 25% matrigel formed colonies of embryoid bodies. Using immunofluorescence microscopy analysis we demonstrate that both cell types form primary cilia with lengths of 5-10 μm, which emerge from the centrosomal mother cilium at the cell surface. We then went on to demonstrate that essential components of the Hh signaling pathway are present in hESC cilia, including Patched (Ptc), Smoothened (Smo) and the Glioma transcription factor, Gli2. To show the functionality of the cilium in Hh signaling, we stimulated undifferentiated cells with Hh agonists and observed translocation of Smo into and Ptc out of the cilium, indicating that the cilium acts as a cellular switch in Hh signaling and differentiation. These results indicate that hESC primary cilia are sensory organelles that play a critical role during self-renewal and/or differentiation of hESCs and during embryogenesis, and our results may provide the foundation for the development of tools to create specific stem cell lines that may have therapeutic potential. Work is in progress in order to further analyze the function of the hESC primary cilium in differentiation.

Transcriptional Up-Regulation and Targeting of PDGFRAs to the Primary Cilium Is Controlled by Inversin during Growth Arrest in Fibroblasts

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We previously demonstrated that the growth-arrest specific protein, PDGFRα, is targeted to the primary cilium during growth arrest in fibroblasts to regulate PDGF-AA-mediated cycle control and directional cell migration [Schneider et al. (2005) Curr. Biol. 15:1861; Schneider et al., submitted]. Up-regulation of the receptor is highly reduced in Tg737inv-/- mutant mouse embryonic fibroblasts, which form no or very short cilia, indicating that the receptor becomes enriched in the cilia under normal conditions to ensure a strong signal from this organelle during growth arrest [Schneider et al. (2005) Curr. Biol. 15:1861-1866]. Here we investigated the role of inversin (Nephrocystin-2) as a regulator of PDGFRα promoter activity and targeting of the receptor to the primary cilium. Inversin was previously shown to localize to primary cilia [Morgan et al. (2002) Hum. Mol. Gen. 11:3345-3350] and to act as a molecular switch between the canonical and non-canonical (PCP) Wnt pathways [Simons et al. (2005) Nat. Genet. 37(5):537-543]. Initially, we have shown that PDGF-AA-mediated activation of PDGFRα, Erk1/2 and protein kinase B (Akt) is blocked in growth-arrested inv-/- mouse embryonic fibroblasts, which are mutated in the gene encoding inversin, but form primary cilia. Further analysis with RT-PCR and western blotting showed that PDGFRα up-regulation in both inv-/- and Tg737inv-/- fibroblasts is blocked at the transcriptional level, indicating that ciliary signaling via inversin controls PDGFRα promoter activity. In addition, targeting of basic levels of PDGFRα to the primary cilium was blocked in inv-/- cells. These results indicate that inversin, or up-stream regulators of inversin in the primary cilium, specifically control the activity of transcription factors for the PDGFRα promoter, and regulate targeting of PDGFRα to the primary cilium, linking the PCP pathway to PDGFRα-mediated signalling in growth control and cell migration.

**Cell Motility – Techniques (2575)**

**Dynamic Quantification of Force-dependent Changes in Paxillin Turnover in Focal Contacts Quantified by Fluorescence Uptake after Photoconversion-Microscopy (FUAP)**

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The molecular machinery of integrin adhesions consists of a network of scaffolding and signaling proteins that link the integrin receptors to the actin cytoskeleton. Force is a critical component in the regulation of adhesion site behavior. However, the contribution of force to the regulation of protein-turnover on a molecular level remains elusive. We developed “fluorescence uptake after photoconversion-microscopy” (FUAP) to quantify paxillin turnover and its response to changes in force exerted on a cell. By using an EosFP-tagged paxillin construct this new technique allowed an exact quantification of protein turnover using a normal epi-fluorescence microscope. EosFP is a novel photoconvertible green fluorescent protein which changes emission from green to red upon illumination with UV light. EosFP-paxillin localized to typical focal contacts and was phosphorylated in response to matrix contact. The turnover rates of paxillin determined by FUAP were comparable to turnover quantified by FRAP. Interestingly, the application of force by shear-stress (95dyn/cm²) drastically delayed the turnover in adhesion sites (61,1s vs.130,7s) suggesting that either paxillin or binding partners were altered by the application of force. It has been suggested that phosphorylation of paxillin is a key event in the regulation of paxillin behavior. Indeed, inhibition of phosphorylation at major-phosphorylation sites (Y31F, Y118F or Y31F/Y118F) delayed turnover to the level of force-application with wt-paxillin (123,6s vs 130,7s). Surprisingly, application of force doubled turnover rates of the phosphorylation defective mutants (130,7s vs 245,0s). Taken together these data suggest that quantification of protein dynamics using FUAP is easy and feasible. In addition, force has an pronounced effect on the turnover rates of paxillin and force may either induce other paxillin modifications that have a super-additive effect on turnover or interaction with binding sites is affected by inhibition of phosphorylation in the response to force.

**Cell Motility – Regulation (2576-2585)**

**Reengineering the Substrate Specificity of a Src Family Kinase**

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**Objective:** To redirect the substrate specificity of a Src family kinase by replacing the associated modular domains. Src family kinases (SFKs) are modular signaling proteins possessing SH3, SH2, and tyrosine kinase domains. The SH3 and SH2 domains of SFKs have dual roles: they regulate the activity of the kinases, and they also target SFKs to their cellular substrates. To test the importance of the associated domains in governing substrate recognition, we generated a novel SFK (PDZ-Hck) by replacing the SH2 domain of Hck with the syntrophin PDZ domain. The negative regulatory tyrosine in the C-terminal tail was replaced with a PDZ ligand sequence. When expressed in mammalian cells, the overall substrate specificity of PDZ-Hck was redirected towards PDZ specific substrates. In contrast to wild-type Hck, PDZ-Hck phosphorylates nNOS due to a specific interaction between the PDZ domains of nNOS and PDZ-Hck. We also introduced a PDZ ligand sequence into the Src substrate Cas, and observed phosphorylation of the engineered Cas by PDZ-Hck upon co-expression in Cas-deficient cells. PDZ-Hck shows auto-regulatory properties similar to a natural SFK. Addition of a PDZ ligand to PDZ-Hck increases tyrosine kinase activity to a similar degree as observed for activation of Hck by an SH2 ligand. Thus, the PDZ-ligand interaction is able to functionally replace the normal SH2-pY527 interaction that regulates SFKs. Our data highlight the modularity and evolvability of signaling proteins.

**The Rho GAP DLC-1 Is Essential for Cell Migration**

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DLC-1 encodes a multi-domain protein with GTPase activating activity (RhoGAP) for specific Rho family members (Rho A-C and Cdc42). DLC-1 expression is lost in many human malignancies, while ectopic reexpression of DLC-1 in DLC-1 deficient tumor cell lines cause apoptosis and reduced proliferation. However, the regulation and mechanism of DLC-1 function remains poorly understood. We evaluated truncation and missense mutant of DLC-1 and identified constitutively activated and dominant negative mutants of DLC-1. Using these and other mutant DLC-1 proteins, we determined that the N-terminal SAM (sterile alpha motif) domain functions as an inhibitor of DLC-1 intrinsic GAP activity and that additional N-terminal sequences are important for DLC-1 association with focal adhesions. We report that DLC-1 activation profoundly alters cell morphology, enhances protrusive activity, and increases the velocity of cell migration, and that these effects are dependent on DLC-1 localization to focal adhesions. Finally, DLC-1 effects involve inhibition of both RhoA and Cdc42, with RhoA function mediated by both mDia and Rho kinase effector signaling. Our observations provide further insight into the mechanisms by which DLC-1 may function as a tumor suppressor.

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CPEB-1 Regulation of β-catenin mRNA Translation and Cell Migration in Astrocytes
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Astrocytes supply nutrients, provide insulation for neurons, and respond to injuries in the brain. This injury response requires astrocyte migration. Motility is a complicated process requiring signal transduction cascade(s) localized to the leading edge of moving cells. Activation of the signal transduction pathway leads to significant increases in β-catenin, a multifunctional protein known to be important for cytoskeletal rearrangement, at the leading edge. This lead to the hypothesis that β-catenin is produced locally from mRNA found at the leading edge, however how the β-catenin mRNA is translationally regulated remains unknown. Recently it has been shown that the 3’ untranslated region of β-catenin’s mRNA contains regulatory sequences called Cytoplasmic Polyadenylation Elements (CPEs). The Cytoplasmic Polyadenylation Element Binding Protein 1 (CPEB-1) is a protein that binds CPEs and serves as a translational regulator. The CPEs found in β-catenin mRNA suggests that it could be regulated by CPEB-1. Here, using an in vitro migration assay, we show that there is a correlation between CPEB-1 and expression in astrocytes. We determined CPEB-1 and β-catenin protein colocalize at the leading edge of a migrating astrocyte. In order to assay CPEB-1 function in migrating astrocytes, we infected astrocytes with an adenoviral vector encoding a mutant form of CPEB-1. This construct will inhibit the function of endogenous CPEB-1, thus preventing the translation of its associated mRNAs. We intend to use the adenoviral-induced expression of CPEB-RBD to determine whether CPEB-1 regulates the production of β-catenin and cell motility. A-10

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PRL-3 Promotes Cell Migration through Regulation of Arf Activity
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The phosphatase of regenerating liver 3 (PRL-3) is strongly associated with tumorigenesis and metastasis in many tumors. It was shown that overexpression of PRL-3 promotes cell migration, invasion and formation of metastases. PRL-3 substrates and signaling pathways have not been clearly defined. Here we report on a possible mechanism for PRL-3 action. Our data show that GFP-tagged PRL-3 localizes predominantly to the Golgi complex, endosomal compartments and areas of active membrane protrusions. Using a bacterial two-hybrid assay, we discovered a previously unknown interaction between PRL-3 and members of β-catenin, a multifunctional protein known to be important for cytoskeletal rearrangement, at the leading edge. This led to the hypothesis that β-catenin is produced locally from mRNA found at the leading edge, however how the β-catenin mRNA is translationally regulated remains unknown. Recently it has been shown that the 3’ untranslated region of β-catenin’s mRNA contains regulatory sequences called Cytoplasmic Polyadenylation Elements (CPEs). The Cytoplasmic Polyadenylation Element Binding Protein 1 (CPEB-1) is a protein that binds CPEs and serves as a translational regulator. The CPEs found in β-catenin mRNA suggests that it could be regulated by CPEB-1. Here, using an in vitro migration assay, we show that there is a correlation between CPEB-1 and expression in astrocytes. We determined CPEB-1 and β-catenin protein colocalize at the leading edge of a migrating astrocyte. In order to assay CPEB-1 function in migrating astrocytes, we infected astrocytes with an adenoviral vector encoding a mutant form of CPEB-1. This construct will inhibit the function of endogenous CPEB-1, thus preventing the translation of its associated mRNAs. We intend to use the adenoviral-induced expression of CPEB-RBD to determine whether CPEB-1 regulates the production of β-catenin and cell motility. A-10

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CaMK-II Induces Tyrosine Dephosphorylation of FAK and Paxillin to Promote Focal Adhesion Turnover
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We have previously shown that both CaMK-II inhibition and expression of constitutively active CaMK-II inhibit cell motility by over-stabilizing or destabilizing focal adhesions, respectively. Here, we show that CaMK-II regulates focal adhesion turnover by promoting dephosphorylation of FAK and paxillin on tyrosine residues. Specifically, CaMK-II induces dephosphorylation of Y925 on FAK and Y31 on paxillin. These residues are important for focal adhesion assembly and are regulated by the SH2 domain-containing tyrosine phosphatase 2, SHP-2 (also known as PTP1D and PTPN11). CaMK-II has been implicated upstream of SHP-2 activity in T-cell signaling (McGargill et. al., 2005), but this link has not been investigated with respect to focal adhesion turnover. Like CaMK-II inhibition, the knockout or inhibition of SHP-2 increases focal adhesion size and blocks cell motility. Thus, we suggest that CaMK-II promotes focal adhesion turnover through SHP-2-mediated dephosphorylation of FAK and paxillin to enable cell motility.
p38 Kinase Is Required for X-cytokine-mediated Umbilical Cord Blood-derived Mesenchymal Stem Cell Migration to U87MG, Human Glioma Cells

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Although umbilical cord blood mesenchymal stem cells (UCB-MSC) are being widely used as cell therapy in clinical trial, the mechanism which regulates migration to damaged site is poorly understood. Our interesting observation is that UCB-MSC has intensive ability to migrate against human glioma cell line, U87MG. In co-culture system using Transwell, UCB-MSC labeled PKH26 showed prominent migration to U87MG compared to several cancer cell lines and bone marrow MSC (BM-MSC). Since UCB-MSC migration also observed in incubation with conditioned media of U87MG cells alone, we analyze cytokines in media by cytokine array assay. Three up-regulated cytokines in U87MG cultured media were identified. Among them, X-cytokine treatment enhanced UCB-MSC migration by dose-dependant manner. However, treatment of monococyte chemoattractant protein-1 (MCP-1) did not affect UCB-MSC migration. In addition, pretreated neutralizing X-cytokine antibody reduces UCB-MSC migration to U87MG. As a down-stream signaling, X-cytokine activates p38 kinase but not Erk-1,2 in time-dependant manner. Furthermore, pretreatment of p38 inhibitor blocks X-cytokine mediated UCB-MSC migration by dose-dependant manner. Since the ability of UCB-MSC migration to U87MG is much higher than that of BM-MSC, we compared X-cytokine mediated p38 signaling pathway between UCB-MSC and BM-MSC, another source of cell therapy. Based on our results, glioma tracking properties of UCB-MSC provide a promising possibility for suicide gene delivery into the site of tumor formation. Our results suggest not only possible strategy for glioma therapy but also molecular mechanism of UCB-MSC migration. Acknowledgement This research was supported by a grant(SC3190) from Stem Cell Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology, Republic of Korea

IGF-I Produced from Osteoblasts Plays a Pivotal Role in Osteoblast Migration

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Osteoblast recruitment to the site of future bone formation is essential for skeletal development, bone remodeling and fracture healing, but the mechanism of which remains to be clarified. Here, we hypothesized that osteoblasts secreted chemoattractants for osteoblast recruitment, and the serum-free conditioned medium of mouse osteoblast-like cell line MC3T3-E1 was examined for its ability to induce osteoblast migration. Using a modified Boyden chamber assay, we found that the medium induced MC3T3-E1 chemotaxis in a dose-dependent manner. Employing several chromatography procedures and liquid chromatography equipped with tandem mass spectrometry analysis, we identified insulin-like growth factor-I (IGF-I) as a potent chemoattractive factor from the conditioned medium. IGF-I induced cell migration of both MC3T3-E1 cells and primary murine osteoblasts and checkerboard analysis revealed that IGF-I induced chemotaxis and not simply chemokinesis. By neutralization of IGF-I activity with specific anti-murine IGF-I antibody, both osteoblast monolayer wound healing and cellular polarization were impaired, whereas human IGF-I replenishment reversed these inhibitory effects. IGF-I also promoted cell spreading on fibronectin in an integrin β1-dependent manner. IGF-I-induced Akt and Erk phosphorylation in MC3T3-E1 cells and a PI3K inhibitor, LY294002, but not a MEK inhibitor, PD98059, inhibited IGF-I-induced cell migration and wound healing. Together, these findings suggest that IGF-I produced from osteoblasts regulates osteoblast migration through the activation of PI3K signaling.

dnfA-mediated Spatiotemporal Regulation of Ras Activity Is Essential for Directional Sensing and Cell Polarity

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During chemotaxis, cells amplify and differentially localize specific signaling responses at the future anterior and posterior of the cell leading to outwardly directed F-actin polymerization and myoII-mediated contractility at the front and back, respectively. When a cell first perceives a chemoattractant gradient, it responds globally to reset any intrinsic cell polarity and then extends a pseudopod in the direction of its source. During this sensing period, cells must integrate spatio-temporal signals that require both the proper activation and inhibition of upstream signaling pathways. Ras is activated at the leading edge in Dictyostelium and mediates directional sensing, in part, through the regulation of PI3K and TORC2 and other downstream effectors. We hypothesized that mis-regulation of the timing of Ras signaling would lead to defects in the ability to properly localize leading edge responses. We demonstrate that the Dictyostelium RasGAP dnfA, a homologue of human NF1, is required for the proper spatio-temporal regulation of chemoattractant-stimulated RasG activity. Loss of dnfA leads to extended and non-spatially restricted Ras activity in vivo. Downstream PI3K activity and F-actin polymerization are upregulated and not temporally and spatially restricted to the side of the cell closest to the chemoattractant source leading to severe directionality defects. Biochemical studies and those using time-lapse video microscopy show that dnfA cells exhibit significantly delayed Ras as well as PI3K inactivation and reactivation so that cells are unable to properly localize Ras signaling on the upstream side of the cell. Our findings suggest that proper spatial and temporal control of Ras is an integral component of the regulatory circuit of the cell’s compass. Further, dnfA cells exhibit elevated Ras-GTP levels and display enhanced random mobility, consistent with our model that a G protein-independent Ras/PI3K/F-actin circuit regulates the random cellular movement.

Activation of Cdc42 by the DOCK 180 Family GEFs Zizimin1 and ACG

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The Rho family GTPases Cdc42, Rac, Rho and TC10, regulate the dynamic organization of the cytoskeleton and membrane trafficking in physiologic processes such as cell proliferation, motility and polarity and pathological processes such as tumorigenesis, metastasis, cardiovascular diseases, diabetes, and neuronal degenerative diseases. The Rho GTPase Cdc42 functions as a molecular switch regulating cell polarization, growth, migration, and membrane trafficking through a number of downstream effectors. The activation of Cdc42 requires its conversion from a GDP bound
inactivate form to the GTP bound active form, which is catalyzed by the Guanine nucleotide Exchange Factors (GEFs). The classical GEFs of Cdc42, characterized by their conserved DH/PH tandem domains, remain typically bound to the inactive form of Cdc42 and catalyze the release of GDP from Cdc42. In our previous studies we identified a Cdc42 GEF, ACG (Activated guanine nucleotide exchange factor)/DOCK 11/Zizimin2, which binds to activated Cdc42 and its GEF activity requires interaction with GTP-Cdc42. Here we show that Zizimin1 is another DOCK180 GEF for Cdc42 with protein domains similar to ACG. Our studies have led us to the unexpected finding that both Zizimin1 and ACG bind to the activated form of Cdc42 in protein-protein interaction assays, possibly to generate a robust feedback activation of Cdc42 in the cell. As expected, Zizimin1 and ACG induced microspike formation in Cos7 cells upon overexpression. This microspike induction requires the binding capacity of ACG and Zizimin1 to active Cdc42. We also detected high levels of endogenous Zizimin1 in some cancer cell lines. Since their protein structure is similar we expect that ACG and Zizimin1 play similar roles in activation of Cdc42 but are likely to have distinct spatiotemporal distribution. We are currently testing the hypothesis that they form specific complexes in response to external stimuli to regulate cellular processes.

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Convergence of G Protein- and Integrin-dependent Signaling Pathways in Ascidian Sperm Activation
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During activation, Ascidia ceratodes sperm undergo a defining process called mitochondrial translocation (MTL) and characterized by mitochondrial rounding and translocation off the head and along the tail. Sperm-egg interaction includes cell-cell recognition (mediated by sperm N-acetylg glucosaminidase), adhesion (mediated by sperm integrin), and signaling (mediated by integrin- and G protein coupled receptor [GPCR]-initiated pathways) to actin and myosin. This study focuses on the activation of myosin by members of the integrin and GPCR signaling pathways. Specifically, we studied roles for the Rho family GTPases, Rho-activated kinase (ROCK), myosin light chain kinase (MLCK), and myosin II. To assay sperm activation, sperm cells were isolated, diluted, treated, fixed, and MTL assayed by light microscopy. Activation was initiated by mAb12G10 (integrin agonist) or mas-7 (G-protein agonist). Previous work showed that myosin is required for sperm activation. Here, we used the myosin II-specific inhibitor, blebbistatin, to establish a role for myosin II (IC50=12 nM). Previous studies established that sperm activation was calmodulin-dependent suggesting that MLCK was a likely candidate to activate myosin II. We tested this hypothesis using the MLCK inhibitor ML-9, which at 25µM decreased integrin-dependent activation from 30.4±2.9% to 3.1±3.5%. Next we tested whether MLCK activation could be initiated by ROCK. Using the inhibitor Y-27632 (10 µM), integrin-dependent activation levels were reduced from 40.5±4.0% to 11.8±2.6%. The general RhoGTPase inhibitor Clostridium difficile toxin A (150 pM) reduced integrin-dependent activation from 52.4±3.6% to 0.4±1.7%. As the second step in testing the roles of RhoGTPases, we treated cells with the Rac1 inhibitor NSC23766 (125 nM). Interestingly, both integrin and G protein-induced activation were decreased from 26.5±3.5% to 2.8±5.02% and 43.5±3.0% to 4.5±4.05%, respectively. We conclude that there are two avenues of cross-talk between the GPCR- and integrin-initiated pathways: (1) integrin→ρ→ROCK→MLCK and GPCR→CaM→MLCK and (2) integrin→ρ and GPCR→unknown agents→ρ.

Cell Motility – Structural Basis (2586)

2586

Simultaneous Analysis of Actin Motion and Substrate Deformation during Cell Migration: Mapping the Viscosity of Substrate/Cytoskeleton Connection
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During cell migration, forces generated by actin cytoskeleton are transmitted through adhesion complexes to the substrate. Forces at the substrate level, adhesion components, and the dynamics of actin system have been investigated in numerous studies, but little is known about the efficiency of force transmission from the actin cytoskeleton to the substrate. Here we analyze simultaneously actin movement and substrate deformation in a simple model system of persistently migrating fish epidermal keratocytes. The dynamics of actin labeled with microinjected Alexa 568-Phalloidin, and the movement of fluorescent beads incorporated in flexible gelatin substrate [Doyle et. al Biotechniques 33, 358-364 (2002)] were followed using Matlab tracking routine [Schaub et al. Mol. Biol. Cell 18: 3723-3732 (2007)]. Actin velocity maps demonstrated motion patterns similar to the ones on the solid glass substrate. The comparison of actin and substrate dynamics showed mostly similar direction of movement at the front and sides of the cell, but the substrate velocity was always smaller than actin velocity, indicating a slipping connection between the cytoskeleton and the substrate. Forces at the substrate were directed mostly backward under the front of the cell, and forward and towards the cell center under the sides, indicating that the cell front generated active traction forces, while the sides were dragging passively during migration. We considered the ratio of the substrate force to the relative velocity of actin with respect to the substrate as an index of the effectiveness of force transmission from actin cytoskeleton to the substrate, or apparent viscosity of actin/substrate connection. This index was high under the front and the sides of the cell and low under the cell body. The map of actin/substrate viscosity is a part of the data set to test forthcoming biophysical models of cell motion. Supported by Swiss Science Foundation grant 3100A0-112413.

Centrosomes (2587-2589)

2587

Mutational Analysis of Dictostelium Centrin B and Its Role in Cell Division
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Centrins are a family of calcium-modulated proteins related to calmodulins. Structurally, these proteins have two globular calcium-binding domains and a characteristic N-terminus extension, which is not present in calmodulins and in human centrin 2 is required for oligomerization. Centrins typically localize to the MTOCs, fibrous structures related to basal bodies, and the nucleus. Centrins are involved in diverse cellular roles, including centriole/SBP duplication, flagellar excision and DNA repair. Dictyostelium discoideum has two centrin proteins named DdCenA and DdCenB. We have previously shown that an RFP-tagged form of DdCenB predominantly localizes to the periphery of the nucleus of interphase cells in a cell cycle-dependent fashion, although Dictyostelium cells undergo a closed mitosis in which the nuclear envelope does not breakdown. To address the functional significance of this unusual cell cycle-dependent localization, we have initiated mutagenesis analysis of DdCenB. Our findings suggest that the C-terminal sequence of DdCenB is required for the normal progression of cell division, since mutations in this section of the protein generate considerable delay in the cell culture duplication time. Additionally, large numbers of multinucleated cells were observed in these mutants, suggesting a role for DdCenB in the later stages of cell division. To explore the function of the N-terminus extension, we have generated a 14 amino acid N-terminus deletion mutant. ΔN-DdCenB protein runs faster than can be accounted for by the simple deletion, when compared to full length protein. This downward shift in electrophoretic mobility can be explained by the loss of interaction with other cell components or by the inability to oligomerize. The latter explanation is consistent with the known function of the N-terminus extension of human centrin 2.

2588

Developmental Regulation of Centriole Replication in C. elegans
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Centrosomes, which consist of a centriole pair and pericentriolar material (PCM), are the key microtubule organizing centers (MTOCs) in most cells. In Caenorhabditis elegans, the kinase ZYG-1 is essential for the replication of centrosomes. Embryos lacking maternal ZYG-1 activity fail to replicate the paternally contributed centriole pair, and are thus unable to form bipolar spindles following first division. In contrast, loss of paternal ZYG-1 activity results in duplication failure during male meiosis, and the production of sperm with a single centriole. These sperm can still fertilize eggs but the resulting embryos assemble a monopolar rather than bipolar spindle at first division. We have found that small truncations of the c-terminus of ZYG-1 cause a novel phenotype: the production of embryos with supernumerary replication-incompetent centrosomes. These embryos inherit up to eight centrioles which are unable to duplicate. We have found the extra centrioles arise from defects in the male germ line and that this phenotype behaves as a gain of function. In contrast, failure of mutant embryos to duplicate these centrosomes is due to a loss of function of maternal ZYG-1. Thus, a single mutation behaves as a loss-of-function allele in one developmental context and a gain-of-function allele in another, suggesting that ZYG-1 is subject to different forms of regulation in different tissues. To gain insight into these regulatory mechanisms we are trying to determine the origin of these centrioles by studying centrosome duplication in the male germ line. So far we find that abnormalities in centrosome number are limited to the meiotic portion of the male germ line and have obtained evidence that centriole pairs are separating inappropriately. By comparing centriole behavior in the paternal and maternal germ lines of these zyg-1 mutants, we hope to determine how centrosome duplication might be regulated in a tissue-specific manner.

2589

CDK5RAP2 Is Essential for the Accumulation of AKAP450 and Pericentrin in the Mitotic Centrosome
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The centrosome cycle is intimately linked to the cell cycle. The centrosome, together with the DNA, replicates in S-phase. The centrosome consists of a pair of centrioles, each of which serves as a template for the assembly of a new centriole. In preparation for their role as mitotic spindle poles, the duplicated centrosomes then mature and separate during G2. Coincidently with mitotic exit, the tight cohesion between the centriole pair is severed by separase (Tsou and Stearns, 2006), but a flexible linker is sustained between the pair until G2 of the following cell cycle. Several proteins have been implicated in maintaining this connection, including c-Nap1, Rootletin and Nek2. The human centrosomal protein, CDK5RAP2 (Cep215) shares significant sequence homology with Drosophila Centrosomin, a protein implicated in maintaining centrosome integrity. Importantly, mutations in CDK5RAP2 cause microcephaly in humans, yet the function of CDK5RAP2 is largely unknown. In addition to its centrosomal localisation (Bond et al, 2005), we can also detect CDK5RAP2 in the Golgi apparatus. When overexpressed, CDK5RAP2 colocalises with the microtubule network in interphase cells. Moreover, endogenous CDK5RAP2 also co-pellets with microtubules in microtubule spin-down assays. RNAi-mediated depletion of CDK5RAP2 in tissue culture cells causes premature centrosome separation (i.e. the flexible linker between the parental centrioles is lost before G2). Furthermore, AKAP450 and Pericentrin fail to accumulate at the mitotic spindle poles in these cells. Their localisation at the interphase centrosome, however, is not affected. Co-immunoprecipitation experiments suggest that CDK5RAP2 can interact with AKAP450, but not with Pericentrin. Together these data point to a role for CDK5RAP2 in centriole cohesion as well as in maintaining centrosomal architecture during mitosis.

Cytoskeletal Organization (2590-2597)
interaction between mDia and POPX2 regulates each other’s effect on the actin cytoskeleton and SRF transcription. mDia and POPX2 may well serve to link Cdc42/Rac with RhoA pathways.

2591
**A Cytoplasmic Role of Stat3 in Regulating Cytoskeletal Organization and Cell Migration**

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Stat3 was originally identified as a member of the signal transducer and activation of transcription family, which is important for cytokine signaling as well as a number of cellular processes including cell proliferation, anti-apoptosis and immune responses. In recent years, there is emerging evidence that Stat3 is also involved in cell invasion and motility. However, how Stat3 regulates these processes remains poorly understood. In our current study, we use wild type Stat3 and ΔStat3 mouse embryonic fibroblasts (MEF) as a model to investigate the role of Stat3 in directional cell migration. Using a wound-healing assay, we find that ΔStat3 cells migrate less efficiently and more randomly onto the wound surface. In single cell random migration analysis, the ΔStat3 cells also migrate randomly with less directional persistence in contrast to WT cells. In addition, the ΔStat3 cells exhibit multiple protrusions during cell spreading. We show that the ΔStat3 cells have impaired actin organization during cell spreading and migration and this is independent of the defective microtubule network previously reported in the ΔStat3 cells. To confirm the role of Stat3 in directional cell migration, we reintroduce a transcriptionally-inactive mutant of Stat3 into the ΔStat3 cells for clonal isolation and characterization. We find that the rescued cells migrate more efficiently with increase in directional persistence. Interestingly, the actin network was also restored in these cells. These results reveal a cytoplasmic role of Stat3 in regulating cytoskeletal organization and cell migration.

2592
**Organization of Mechanical Forces Generated by Cells and Tissues on Micropatterned Surfaces**

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Tissues produce effective force when cellular lamellipodia and stress fibers generate ordered protrusion or contractility, which is delivered to the external environment through focal adhesions. These tissue forces, which are significant to wound healing, organism development, and tumor metastasis, are complex and multi-dimensional. We developed an in vitro assay to investigate the organization of force generating structures—lamellipodia and stress fibers—in individual cells and in tissues that were given complex, micropatterned spatial cues. B16F10 mouse melanoma cells were allowed to spread on 1000 um² adhesive islands in the shape of circles or triangles. We found that single cells which adhered to circular islands developed lamellipodia, stress fibers, and focal adhesions along the entire cell perimeter. Single cells which spread onto triangular adhesive islands preferentially localized lamellipodia and focal adhesions to the corners and distributed stress fibers along the straight edges. When two cells were allowed to spread onto adhesive islands with similar shape and size, they not only formed a ‘tissue’ with cell-cell and focal adhesions, but also demonstrated similar shape-dependent distributions of force-generating structures to that seen in single cells. These findings were consistent across a population of tissues, indicating that tissues and single cells similarly recognize and respond to the same spatial cues. Our results provide a quantitative method of studying the coordination of force-generating cell structures across a simple tissue in response to spatial cues.

2593
**Roles for Two γ-Tubulin Associated Proteins in Regulating MTOC Localization**

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The assembly of microtubule arrays depends on the spatial and temporal regulation of microtubule (MT) organizing centers (MTOCs). In the fission yeast, *Schizosaccharomyces pombe*, distinct MTOCs containing gamma-tubulin complexes are created to assemble different MT arrays throughout the cell cycle. During interphase, these complexes are associated with motile satellites that nucleate new MTs along existing MT bundles. Gamma-tubulin complexes further associate with additional proteins that regulate their localization and activity, such as mto1p and rsp1p. The centrosomin related protein mto1p is necessary for the assembly of cytoplasmic MTOCs on interphase MT bundles and at the cell division plane. Rsp1p, a J-domain protein (a chaperone regulatory protein), mediates the disassociation of the eMTOC during cell division and is needed for robust satellite formation. We are interested in the molecular function of rsp1p at MTOCs and its role in regulating the assembly of MT arrays. By examining mutant cells that lack an eMTOC, we find that rsp1p mutants have defects in MT organization that are independent of its effects on the eMTOC. Thus, rsp1p may directly regulate the localization and behavior of gamma-tubulin complexes. Using two-hybrid assays and purified proteins, we found that rsp1p directly interacts with mto1p and this interaction affects the localization of both proteins. Rsp1p binds to the C-terminus of mto1p, which we show mediates the localization of the mto1 protein and is separate from mto1p's other roles. These studies begin to dissect the molecular interactions that regulate MTOC localization and subsequent MT organization.

2594
**Nuclear Actin Regulates the Localization and Activity of the SRF Cofactor MAL**

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Myocardin family proteins (MRTFs) are coactivators of the transcription factor SRF which regulates the expression of many immediate-early, cytoskeletal and muscle specific genes. The MRTF family members are differentially regulated. Myocardin is nuclear even in unstimulated cells, whereas MAL accumulates in the nucleus only upon activation of RhoA-signalling, which leads to depletion of the actin monomer pool. It has remained unclear how actin regulates the localization of MAL, and why myocardin is not subjected to this regulation. By using photoactivation and
bleaching combined with live-cell imaging techniques, we show that MAL constantly shuttles in and out of the nucleus. We also demonstrate that actin has a dual role in regulating MAL localization by both inhibiting nuclear import and promoting nuclear export of MAL. Moreover, we show that nuclear MAL is able to activate SRF mediated transcription only if actin-binding is prevented, indicating that actin inhibits the activity of MAL within the nucleus. To corroborate these results, we show by using a FRET/FLIM approach that MAL interacts with actin both in the cytoplasm and nucleus, and that this interaction responds to Rho-signalling. Therefore our studies have demonstrated a novel role for nuclear actin as a signal responsive regulator of SRF through cofactor MAL. We also show that myocardin does not shuttle between cytoplasm and nucleus. The contrasting shuttling properties of MAL and myocardin are confined by their RPEL domains. The RPEL domain of MAL binds actin more avidly than that of myocardin both in vitro and in vivo. We also illustrate that the RPEL motif itself is an actin-binding unit. The distinct properties of MAL and myocardin are specified by the unit of the first and second RPEL motifs, and hence we propose that differential actin occupancy of multiple RPEL motifs regulate the nuclear transport and activity of MRTFs.

2595
Visualization of Pathogen Invasion Dynamics and Mechanisms at a Single Cell Level Using Ligand-coated Microspheres and a FRET Reporter
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Ligand-induced receptor clustering is thought to be a critical step in host cell invasion by bacterial pathogens. While parts of signaling cascades leading to pathogen internalization are known for a number of species, the exact dynamics and nature of the molecular switch that might be responsible for activating the process of bacterial uptake is much less certain. Our goal is to study pathogen entry into a host cell with high spatial and temporal resolution at a single cell level. We herein report our ongoing efforts on studying pathogen originated ligand-induced receptor clustering and the application of a FRET reporter to visualize the initial host cell response. We first use beads coated with InIA from Listeria monocytogenes as a model system to study the dynamics of pathogen-host cell interactions. We then measure host cell response both with GFP-actin and a FRET reporter designed to respond to changes in phosphoinositides (PIP3) levels. By controlling ligand density on the beads and monitoring recruitment of various markers at the site of bead-cell contact and quantifying the uptake efficiency, we determine the threshold ligand density necessary to initiate signaling inside the cell. The host cell showed a high FRET signal upon addition of InIA within 10 min. The FRET reporter is highly sensitive and ligand-specific holding promises in further applications to detect pathogens in a high throughput format. Furthermore, biophysical characterization of internalization process at a single cell, single bead level using these techniques will reveal details of the invasion process that cannot be obtained from ensemble measurements using standard biochemical techniques.

2596
ARL4D Recruits Cytohesin-2/ARNO to Modulate Actin Remodeling
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ARL4D is a developmentally regulated member of the ADP-ribosylation factor (ARF)/ARL family of Ras-related GTPases. Although the primary structure of ARL4D is very similar to that of other ARF/ARL molecules, its function remains unclear. Cytohesin-2/ARNO is a guanine nucleotide-exchange factor (GEF) for ARF and, at the plasma membrane, it can activate ARF6 to regulate actin reorganization and membrane ruffling. We show here that ARL4D interacts with the C-terminal pleckstrin homology (PH) and polybasic c domains of cytohesin-2/ARNO in a GDP-dependent manner. Localization of ARL4D at the plasma membrane is GTP- and N-terminal myristoylation-dependent. ARL4D(Q80L), a putative active form of ARL4D, induced accumulation of cytohesin-2/ARNO at the plasma membrane. Consistent with a known action of cytohesin-2/ARNO, ARL4D(Q80L) increased GTP-bound ARF6 and induced disassembly of actin stress fibers. Expression of inactive cytohesin-2/ARNO(E156K) or siRNA knockdown of cytohesin-2/ARNO blocked ARL4D-mediated disassembly of actin stress fibers. Similar to the results with cytohesin-2/ARNO or ARF6, reduction of ARL4D suppressed cell migration activity. Furthermore, ARL4D-induced translocation of cytohesin-2/ARNO did not require phosphoinositide 3-kinase activation. Together, these data demonstrate that ARL4D acts as a novel upstream regulator of cytohesin-2/ARNO to promote ARF6 activation and modulate actin remodeling.

2597
Functional Analysis of CLASP in the Model Plant Arabidopsis thaliana
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CLASP is a highly conserved protein that in animal cells is known to associate with microtubule (MT) plus ends to control the association of MTs with kinetochores and the cell cortex. Here we show that the CLASP protein in the higher plant Arabidopsis thaliana localizes along the full length of mitotic and interphase MTs, and in contrast to animal orthologues, exhibits only a moderate enrichment at MT plus ends. Furthermore, unlike animal counterparts, GFP-CLASP in dividing plant cells shows no obvious kinetochore localization within mitotic spindles. Overexpression of GFP-CLASP results in stable drug-resistant MT bundles, while clasp-1 knock-out mutants have slightly aberrant interphase and mitotic arrays, and exhibit hypersensitivity to the drug oryzalin. The lack of kinetochore association and the mild mitotic defects observed in clasp-1 plants indicate partially divergent functions for CLASP in plants. Despite the relatively normal MT organization, clasp-1 plants are dwarf as a result of cell expansion defects. We show that clasp-1 exhibits characteristics indicative of defects in the transport of the hormone auxin, which requires an intact actin cytoskeleton. Consistent with this, actin filament organization and auxin distribution patterns are abnormal in clasp-1 cells. We also determined that the membrane distribution of an auxin efflux carrier is reduced in clasp-1, which may account for the compromised auxin transport. Taken together, our data support roles for Arabidopsis CLASP in organizing both MTs and actin filaments, and that these changes impair transport of auxin, the key hormone governing cell expansion and division in plants.
ERM Proteins and ROCK Are Necessary for PSGL-1 and F-actin Reorganization Following PSGL-1 Ligation
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P-selectin glycoprotein ligand-1 (PSGL-1) is a well-studied P-selectin ligand that is constitutively expressed on leukocytes. Its role as a rolling molecule has been well established, and interactions between PSGL-1 and P-selectin are known to be critical for a strong inflammatory response. Signal transduction through PSGL-1 following P-selectin binding is not well understood. The purpose of this study was to investigate potential downstream events following PSGL-1 ligation. Cross-linking of PSGL-1 on HL-60 cells with KPL-1, a monoclonal antibody that binds to the P-selectin binding site on PSGL-1, induced PSGL-1 association with the actin cytoskeleton. Conversely, cross-linking of PSGL-1 with KPL-2, an antibody that recognizes a sequence on PSGL-1 away from the P-selectin binding site, did not. In addition, KPL-1 and soluble recombinant P-selectin (PRIgG) engagement of PSGL-1 induced PSGL-1 and F-actin reorganization, co-localization and capping/polarization as determined by confocal microscopy. ERM proteins have previously been shown to interact with PSGL-1. We utilized siRNA to knockdown ERM proteins to see if they play a role in PSGL-1 and F-actin reorganization. ERM knockdown HL-60 cells had a significant decrease in number of cells that capped/polarized following PSGL-1 activation. In addition to ERM proteins, we hypothesized that the Rho pathway might play a role in mediating PSGL-1 and F-actin reorganization. Therefore, we incubated cells with ROCK inhibitor Y27632 prior to antibody activation. The ROCK inhibitor prevented PSGL-1 and F-actin reorganization following PSGL-1 activation, strongly suggesting involvement of the Rho-mediated signaling pathway in reorganization of PSGL-1 and F-actin.

Characterization of a Novel Protein, CLIPR76, That Links Membranes and Microtubules
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The microtubule cytoskeleton plays critical roles in cell structure and intracellular transport. Cytoplasmic linker proteins (CLIPs) help to link microtubules with membrane bound organelles. We have undertaken a study of genes related to CLIP-170 (the founding CLIP) in hopes of expanding our knowledge of microtubule-membrane interactions. This presentation will focus on one of these, CLIPR76. The CLIPR76 transcript contains three C-terminal CAP-Gly motifs (instead of the normal one or two) and has ankyrin repeats at the N-terminus. When transiently expressed in tissue culture cells, this form localizes to and bundles microtubules. However, the CLIPR-76 gene is also alternatively spliced, producing at least three additional transcripts in humans. The smallest of these, CLIPR76-4, encodes a 37kD protein containing only one CAP-Gly motif followed by a hydrophobic C-terminus. This protein localizes to ER and reorganizes the ER at high levels of expression; the hydrophobic C-terminus is necessary and sufficient to target the protein to the ER. To examine the tissue-specificity of CLIPR76-4 expression, quantitative PCR analysis was performed. Initial studies showed high levels of this protein in heart, muscle, and brain. Experiments with C2C12 cells, a murine myoblast cell line, have shown that CLIPR76-4 expression levels increase as the muscle cells differentiate. Myoblast fusion is a highly ordered process beginning with the reorganization of the microtubule cytoskeleton and organelles. We are interested in the possibility that CLIPR76-4 plays a role in these early stages of muscle differentiation. Currently, MALDI Mass Spectrometry analysis is being carried out in order to identify which CLIPR76 isoforms are present in differentiating muscle cells. Additionally, siRNA knockdown of CLIPR76 is being utilized to test the hypothesis that this protein plays a role in muscle development.

Protein Kinase A Mediated Phosphorylation of the NF2 Tumor Suppressor Protein Merlin at Serine 10 Affects the Actin Cytoskeleton
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Merlin localizes to the cell membrane where it links the actin cytoskeleton to membrane proteins. Cell proliferation is regulated by merlin in many cell types, but merlin's tumor suppressor function still remains unclear. Phosphorylation has been suggested to regulate merlin’s activity. The only characterized phosphorylation site is the C-terminal serine 518, which is phosphorylated both by p21-activated kinases (PAKs) and protein kinase A (PKA) in tissue culture cells, this form localizes to and bundles microtubules. However, the CLIPR-76 gene is also alternatively spliced, producing at least three additional transcripts in humans. The smallest of these, CLIPR76-4, encodes a 37kD protein containing only one CAP-Gly motif followed by a hydrophobic C-terminus. This protein localizes to ER and reorganizes the ER at high levels of expression; the hydrophobic C-terminus is necessary and sufficient to target the protein to the ER. To examine the tissue-specificity of CLIPR76-4 expression, quantitative PCR analysis was performed. Initial studies showed high levels of this protein in heart, muscle, and brain. Experiments with C2C12 cells, a murine myoblast cell line, have shown that CLIPR76-4 expression levels increase as the muscle cells differentiate. Myoblast fusion is a highly ordered process beginning with the reorganization of the microtubule cytoskeleton and organelles. We are interested in the possibility that CLIPR76-4 plays a role in these early stages of muscle differentiation. Currently, MALDI Mass Spectrometry analysis is being carried out in order to identify which CLIPR76 isoforms are present in differentiating muscle cells. Additionally, siRNA knockdown of CLIPR76 is being utilized to test the hypothesis that this protein plays a role in muscle development.

CDDO-Im Alters TGFβ-dependent Signaling and Cell Migration by Affecting the Cytoskeleton and the Polarity Complex
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The anti-tumor synthetic triterpenoid CDDO-Imidazolide (CDDO-Im) ectopically activates the TGFβ-Smad pathway and extends the duration of signaling by an undefined mechanism. Here we show that CDDO-Im-dependent persistence of Smad2 phosphorylation is independent of Smad2 phosphatase activity and correlates with delayed TGFβ receptor degradation and trafficking. Altered TGFβ trafficking parallels the dispersal of EEAA1-positive endosomes from the peri-nuclear region of CDDO-Im-treated cells. We also observed that CDDO-Im alters microtubule dynamics by disrupting the microtubule-capping protein,Clip-170, and biontinated triterpenoid was found to localize to the polarity complex at the leading edge of migrating cells. Furthermore, CDDO-Im disrupted the localization of IQGAP, PKCζ, Par6 and TGFβ receptors from the leading edge of migrating...
cells and inhibited TGFβ-dependent cell migration. Thus, the synthetic triterpenoid CDDO-Im interferes with TGFβ receptor trafficking and turnover, and disrupts cell migration by severing the link between members of the polarity complex and the microtubule network.

**Nerve Cell Cytoskeleton (2602-2604)**

2602

**The Analyses of Rapid Neurite Retraction Caused by Calyculin A**

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Calyculin A (CL-A), a toxin isolated from the marine sponge *Discodermia calyx*, is a strong inhibitor of protein phosphatase 1 (PP1) and PP2A. Previous studies reported CL-A induced rapid neurite retraction in developing neurons, and some of them attributed this phenomenon to microtubule (MT) depolymerization which accelerated by enhanced tau phosphorylation through inhibition of PP2A. In this study, to get insight into a part of the mechanism of axon guidance, we investigated the CL-A induced rapid retraction with LC-PolScope as well as a fluorescent microscope using cultured hippocampal neurons. The LC-PolScope employs polarized optics and liquid crystals to get high contrast time-lapse images of birefringent materials, such as cytoskeletons, without fixing or staining. Our analyses of time-lapse image series acquired by the LC-PolScope revealed that birefringent materials in axon were pulled backed rapidly into the soma by CL-A treatment, but the plasma membrane were left ad locum. Fluorescent microscopy with EGFP conjugated actin and Tubulin Tracker showed that the birefringent materials included actin filaments and MTs. We observed bending and winding MTs during the rapid retraction by fluorescent time-lapse imaging. These observations imply that there are additional mechanisms for the neurite retraction other than MT depolymerization. Indeed, the involvement of actin motors is becoming clear; CL-A enhanced myosin II phosphorylation level, and the rapid retraction by CL-A was inhibited by Blebbistatin and Cytochalasin D, an inhibitor of myosin II and actin polymerization, respectively. Our on-going pharmacological studies clarify that Staurosporin-sensitive kinases are responsible for the rapid retraction, but MLCK and Rho kinase are not. Thus, we here suggest actomyosin-activation is related with CL-A-induced rapid neurite retraction on rat hippocampal neurons.

2603

**Microtubule-stabilizing Protein APC Is Differentially Distributed in Axonal Growth Cones and Modulates Their Steering**

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During development of the nervous system, neurons send out axons to establish connections with their targets. At the tip of the axon is a motile structure, the growth cone, which guides elongating axons by processing environmental information and transforming them into internal, directed structures. This transformation is achieved by differential modulation of the growth cone cytoskeleton; key elements are the microtubules which are regulated in their dynamics by proteins (de-)stabilizing them. We investigated a potential role of a microtubule-stabilizing protein, adenomatous polyposis coli (APC), for growing axons by employing the embryonic chick visual system as a model system. APC is concentrated in the distal-most, i.e. growing region of retinal ganglion cell axons in vivo and in vitro. Within the growth cone, APC is enriched in the central domain; it only partially co-localizes with microtubules. When axons are induced to turn towards a cell or away from a substrate border, APC is present in the protruding and absent from the collapsing growth cone regions thus indicating the future growth direction of the axon. To assess the functional role of the differential distribution of APC in navigating growth cones, the protein was inactivated via micro-scale chromophore-assisted laser inactivation (micro-CALI) in one half of the growth cone. If the N-terminal APC region -crucial for its oligomerization- is locally inactivated, the treated growth cone side collapses and the axon turns away. In contrast, if the seven 20 amino acid repeats in the middle region of APC -which can negatively regulate its microtubule association- are inactivated, protrusions are formed at the treated side and the growth cone turns towards. Our data thus demonstrate a crucial role of APC for axon steering due to its multifunctional domain structure and differential distribution in the growth cone.

2604

**The Rho-Kinase Pathway Is Affected in a Cell Culture Model for Spinal Muscular Atrophy**

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Spinal muscular atrophy is a neurodegenerative disease accompanied by a loss of motoneurons. Either mutations or deletions in the survival of motoneuron (SMN) gene are responsible for this defect. SMN is an assembly protein for RNA-protein complexes in the nucleus and is also found in axons of neurons. However, it is unclear which dysfunctions of SMN are important for disease progression. We analyzed the effects of SMN on neuronal differentiation associated with outgrowth of neurites in PC12 cells as a model system for neurogenesis. Suppression of endogenous SMN protein levels by siRNA decreased significantly growth of neurites, whereas cells overexpressing SMN displayed increased lengths of neurites. Neurite outgrowth is associated with changes of the actin cytoskeleton. Remarkably, the knock-down of SMN led to a significant change of the G-/F-actin ratio indicating a role of SMN in actin dynamics. Rho-Kinase (ROCK) affects actin polymerization by phosphorylation of LIM-Kinase, which in turn regulates Cofilin. In our SMN knock-down model for spinal muscular atrophy we could show that the phosphorylation of these signaling molecules has been changed. The data suggest that actin-regulating proteins downstream of ROCK are involved in SMN-dependent neuritogenesis defects. Importantly, analyses of this pathway could help to elucidate new molecular targets for a therapy of spinal muscular atrophy.
The Active Form of the Serine/Threonine Kinase Akt/PKB Associates with Keratins in Human Hepatocellular Carcinoma Cells HepG2

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Intermediate filaments form with microtubules and actin microfilaments the cytoskeletal network of most mammalian cells. Keratin 8 and 18 (K8/18) are the only intermediate filament (IF) proteins present in the simple-type epithelial cells hepatocytes. One clearly defined function for keratins is to protect hepatocytes from mechanical and non-mechanical form of stress. For instance, K8 deficient hepatocytes are more sensitive to drug induced apoptosis than wild type hepatocytes. K8 phosphorylation is important in the regulation of the protective function and a mutation on K8 S73 phosphorylation site predisposes to liver injury and apoptosis. However, the molecular mechanisms underlying this protective function are not fully understood yet. The serine/threonine kinase Akt/PKB (pAkt) is known to play a fundamental role in protecting hepatocytes from apoptosis induced by toxic stress. The aim of the present study was to evaluate if keratins could interact with Akt signalling pathway in the response of hepatocytes to stress. Hepatocellular carcinoma cells HepG2, which contain K8/18 were used as a model system to investigate keratins and AKT potential interaction. HepG2 cells were treated with the hepatotoxic agent griseofulvin (GF) [(0,07 and 0,2μg/ml in 0.1% DMSO) for 8 to 72h] and, biochemical and microscopical analysis were performed. The results show that GF treatment induced phosphorylation of K8 on S73 and S431 and, K18 on S52. The level of pAkt was increased after 48h of GF treatment. The translocation of AKT to the plasma membrane, which is important for AKT activation, was also observed. Keratin IFs formed a cytoplasmic network that was denser in cell areas where pAkt was associated to the membrane. Immunoprecipitation analysis revealed that keratins associate with pAkt in HepG2 cells. Taken together, these results indicate that keratins could accomplish their protective role by directly interacting with pAkt, a central player in cell survival. (Supported by NSERC)

Absence of Axonal Neurofilaments Causes Multiple Fibre Abnormalities but the Architecture, Composition, and Spacing of Nodes of Ranvier Are Unaffected

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Mutual interactions between axons and glial cells are crucial for neuronal function. To determine the contribution of neurofilaments (NF) to the global architecture of myelinated fibers, we used a stable transgenic model (NFH-LacZ) in which neurofilaments aggregate in the neuronal cell body leaving axons without a neurofilament cytoskeleton. In the absence of neurofilaments, axon calibres in both the CNS and PNS are reduced by 50%. Around such calibre reduced axons, oligodendrocytes elaborate correspondingly thinner myelin sheaths. In contrast, relative to their absolute calibres, Schwann cells elaborate thicker than normal myelin sheaths. Western-Blot analyses of the CNS revealed no changes in myelin protein composition (PLP, MBP, CNPase, and CD9). Similarly, in the PNS, normal values for MBP, CNPase and CD9 were observed. However, P0, the major myelin protein in the PNS is reduced in transgenic samples by 20% and this reduction is associated with an increased inter-lamellar spacing (from 11.1 nm to 12.2 nm). In both the CNS and PNS conduction velocities are reduced to 40% of normal. Immunohistochemical, ultrastructural and morphometric analyses of both CNS and PNS fibres revealed no obvious modifications to either the molecular organization, structure or spacing of nodes of Ranvier. These combined observations confirm the essential role played by the axonal neurofilament network in the radial growth of axons. They also reveal that the mechanism used to locate and construct nodes of Ranvier operates independently of axonal NF, absolute axon calibres, rates of action potential conduction and the absolute (CNS) or relative (PNS) thickness of myelin sheaths. This work was supported by AFM and ARSEP to JE (joel.eyer@univ-angers.fr), and by the MS Society of Canada to ACP.

Prokaryotic Cytoskeletal Systems: Organization and Regulation (2605-2609)

2606
In Vivo Visualization of Type II Plasmid Segregation: Bacterial Actin Filaments Pushing Plasmids

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The R1 Par operon is a self-contained plasmid partitioning system composed of three parts: parC, ParR and ParM. parC is a stretch of DNA consisting of 10 sequential repeats, each of which binds ParR. The ParR/parC complex in turn binds the actin homolog ParM. Previous studies have shown that ParM forms filaments nearly identical to those of eukaryotic actin filaments and that ParM filament bundles appear to position plasmids at each end of a rod-shaped cell. We recently demonstrated that ParM filaments are dynamically unstable and can elongate bidirectionally in vitro. These observations led to a model in which ParM filaments continually search the cytoplasm and eventually capture a ParR bound parC region on a plasmid. Insertional polymerization at the ParM/ParR interface will then push the plasmids to opposite ends of the cell and hold them in place until cell division, ensuring that each daughter cell receives a copy. To test this model directly in live cells, we used RFP labeled lacI to visualize lacO sites present on a plasmid containing a functional R1 Par operon and GFP labeled ParM to visualize the filaments directly at the same time. We find that ParM filaments are dynamically unstable in vivo and that the majority of filaments undergo assembly and rapid disassembly in less than a minute. In addition, the filaments appear to orient themselves along the long axis of a bacterium by running into the sides of the cell and then following them to the poles. The most striking result, however, is that the plasmids undergo continual and very rapid pole-to-pole movements even when not actively dividing. Our results indicate that plasmid partitioning by ParM is a very dynamic process and suggest a new model for polymer-based plasmid segregation.
2608
**FtsZ-directed Protein Localization in Caulobacter Cytokinesis**
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The bacterial tubulin relative FtsZ is a GTPase that polymerizes at midcell and serves as a scaffold for recruitment of the cell division machinery, or divisome. FtsZ is essential for all stages of cytokinesis in bacteria, and is hypothesized to exert constrictive forces that drive cell division. However, the molecular mechanisms underlying its role in cytokinesis are unknown. As the structure, dynamics and, therefore, function of FtsZ are likely to be regulated by interacting partners, we sought to identify all FtsZ-binding proteins in the dimorphic bacterium *Caulobacter crescentus*. To do this, we developed a microscopy-based assay in which we overproduce a GTPase-defective mutant of FtsZ (FtsZ-G109S) in cells also expressing fluorescent proteins. From this screen, we identified six proteins that clearly localized to the constrictions, four of which are previously uncharacterized proteins that we named FzlA, FzlB, FzlC, and FzlD (for *FtsZ*-localized). Surprisingly, we discovered an additional set of proteins that were specifically excluded from the FtsZ-rich constrictions, indicating a second mode of FtsZ-directed protein localization in *Caulobacter*. We are now taking genetic, cytological, and biochemical approaches aimed at uncovering the mechanisms by which FtsZ controls the localization of these factors.

2609
**The Structure of FtsZ Filaments In Vivo Suggests a Force-generating Role in Cell Division**
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In this work, we are applying electron cryotomography (ECT) to directly visualize the tubulin homologue FtsZ in intact *Caulobacter crescentus* cells. In prokaryotes, FtsZ is a nearly ubiquitous GTPase that localizes in a ring at the leading edge of constricting plasma membranes during cell division. Here we report electron cryotomographic reconstructions of dividing *C. crescentus* cells wherein individual arc-like filaments were resolved just underneath the inner membrane at constriction sites. The filaments' position, orientation, time of appearance, and resistance to A22 all suggested that they were FtsZ. Predictable changes in the number, length, and distribution of filaments in cells where the expression levels and stability of FtsZ were altered supported that conclusion. In contrast to the thick, closed-ring-like structure suggested by fluorescence light microscopy, throughout the constriction process the Z-ring was seen here to consist of just a few short (~100 nm) filaments spaced erratically near the division site. Additional densities connecting filaments to the cell wall, occasional straight segments, and abrupt kinks were also seen. An "iterative pinching" model is proposed wherein FtsZ itself generates the force that constricts the membrane in a GTP-hydrolysis-driven cycle of polymerization, membrane attachment, conformational change, depolymerization, and nucleotide exchange.

**Focal Adhesions (2610)**

2610
**Analysis of Molecular Interactions between Focal Adhesion Proteins Talin and Vinculin in Living Cells by FRET Microscopy**
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The development of cell based sensors as well as new material concepts for medical applications will be greatly advanced by tools that allow online life monitoring of cellular processes. Cell adhesion and generation of force on the extracellular matrix (ECM) play an important role for cell viability, migration, and differentiation. Primary sites of adhesion are formed between integrin receptors and the underlying substratum. These initial adhesions consist of a large number of proteins such as talin which in turn binds to integrin, actin and vinculin. By recruitment of vinculin initial integrin-ECM adhesions become capable of exerting migration force. Conformational changes are important to vinculin function as in the autoinhibited state intramolecular interactions between its globular head and the C-terminal tail domain prevent binding to talin and actin and hence focal adhesion formation. FRET microscopy between CFP/YFP is a powerful technique that enables the visualization of protein interactions, protein conformations and biochemical status inside living cells. The goal of our study is to investigate the molecular interaction of talin and its binding partner vinculin in focal adhesions. For FRET measurements we generated several constructs were yellow fluorescent protein is inserted in close proximity to vinculin binding sites of talin rod. As the binding sites for talin are located in the N-terminal vinculin head domain our first attempt was to position CFP in front of vinculin. These FRET constructs were used for nucleofection of human fibroblasts followed by confocal laser scanning microscopy to monitor the fluorescence localisation of fusion proteins. Our first results showed that transfection of cells with the fluorescently-labelled vinculin or talin is efficient and results in the expected accumulation of fluorescence signal at focal adhesion sites. The correct localisation of both proteins was confirmed by immunohistochemical staining against talin or vinculin, suggesting that both tagged proteins are correctly synthesized.

**Organization and Regulation of the Extracellular Matrix (2611-2613)**

2611
**Remodelling of the Extracellular Matrix (ECM) in the Sinoatrial Node with Age**
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The ECM consists of structural proteins (elastin, collagen types I and III), adhesive proteins (laminin, fibronectin, collagen types IV and VI), anti-adhesive proteins, proteoglycans and enzymes (matrix metalloproteinases). In the heart, the ECM is responsible for connecting myocytes, aligning contractile elements, transmitting force and preventing myocardial rupture. Although it is well known that there is an age-dependent remodelling of the ECM in the heart, it is unclear whether there is a remodelling in the pacemaker of the heart (sinoatrial node, SAN). The aim of the present study was to investigate (using quantitative PCR) the effect of age on the expression of components of the ECM and factors that control the ECM in the SAN from Wistar-Hanover rats aged 3 (n=8) and 24 (n=8) months. With age, at the mRNA level, there is a statistically significant increase in transforming growth factor β1 and tumour necrosis factor α (factors controlling fibrosis) and a significant decrease in elastin and collagen types I and III. In addition, Picro Sirius red staining showed that in the SAN there is a significant decrease in the total content of collagen at the protein level with age. The expression of matrix metalloproteinase 2 mRNA, responsible for collagen degradation, undergoes a significant decrease in the SAN with age. On the other hand, the expression of fibronectin 1 and vimentin mRNA does not change in the SAN with age. Furthermore, the expression of integrins (α1, α5 and β1) at the mRNA level does not change in the SAN with age. Integrins are a family of membrane receptors that play a role in the mediation of cell interactions. We conclude that in the SAN with age there is remodelling of the ECM and this remodelling may partly be responsible for the deterioration in the function of the SAN in old rats.

2612 Control of Fibronectin Assembly by Cell Interactions with Module 1F3 and the C-terminal Region of Fibronectin

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Fibronectin is an extra-cellular protein that is presented to the cell as a soluble protein and assembled into a functional fibrillar matrix. Assembly of exogenous fibronectin by fibronectin-null cells is dependent on the nature of the adhesive substrates. Fibronectin-null cells assemble exogenous fibronectin when adherent to intact fibronectin, but not when adherent to the cell-binding region (7F3-10F3) of fibronectin. To identify regions of adsorbed fibronectin other than the 7F3-10F3 modules that are required to support fibronectin assembly, various recombinant constructs built outwards from 7F3-10F3 modules were generated. Fluorescence microscopy and western blots tested the assembly of exogenous fibronectin by fibronectin-null cells adherent to the recombinant fibronectin constructs. Cells plated on constructs as large as 2F3-14F3 poorly assembled exogenous fibronectin. Addition of module 1F3 or the C-terminal modules to 7F3-10F3, resulting in constructs 1F3-10F3 or 7F3-C, supported fibronectin assembly as adherent substrate. Addition of both 1F3 and the C-terminal modules to 7F3-10F3, resulting in 1F3-C, further improved supportive activity among the recombinant fibronectin constructs. A co-coating of 1F3-10F3 and 7F3-C, in which include module 1F3 and the C-terminus are in different materials, was less active than 7F3-C. When tested for ability to overcome the suppressive effect of co-coated vitronectin, 1F3-C was also the best. Similar results were obtained on binding of 70-kDa N-terminal fragment to fibronectin-null cells, which is an accurate indicator of the cell’s ability to assemble fibronectin. These results demonstrate that module 1F3 acts in cis with the C-terminal modules of adsorbed fibronectin construct to support fibronectin assembly by fibronectin-null cells.

2613 Control of Proliferation and Extracellular Matrix Production by Growth Factors in the Tissue Engineering of an Aortic Valve

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BACKGROUND: Tissue engineered aortic valves require an appropriate autologous cell source, which can be controlled to produce specific extracellular matrix (ECM) components and to proliferate in a biological scaffold. Rat aortic smooth muscle cells (RASMCs) are used here as an analog to peripheral vascular smooth muscle cells which may be harvested from patients for use in an engineered aortic valve. We hypothesized that control of proliferation and extracellular matrix production by growth factors in the tissue engineering of an aortic valve (2613)

Control of Fibronectin Assembly by Cell Interactions with Module 1F3 and the C-terminal Region of Fibronectin

J. Xu,1 E. Bae,2 D. F. Mosher1,2, 1Biomolecular Chemistry, University of Wisconsin–Madison, Madison, WI, 2Pathology and Laboratory Medicine, University of Wisconsin–Madison, Madison, WI
Fibronectin is an extra-cellular protein that is presented to the cell as a soluble protein and assembled into a functional fibrillar matrix. Assembly of exogenous fibronectin by fibronectin-null cells is dependent on the nature of the adhesive substrates. Fibronectin-null cells assemble exogenous fibronectin when adherent to intact fibronectin, but not when adherent to the cell-binding region (7F3-10F3) of fibronectin. To identify regions of adsorbed fibronectin other than the 7F3-10F3 modules that are required to support fibronectin assembly, various recombinant constructs built outwards from 7F3-10F3 modules were generated. Fluorescence microscopy and western blots tested the assembly of exogenous fibronectin by fibronectin-null cells adherent to the recombinant fibronectin constructs. Cells plated on constructs as large as 2F3-14F3 poorly assembled exogenous fibronectin. Addition of module 1F3 or the C-terminal modules to 7F3-10F3, resulting in constructs 1F3-10F3 or 7F3-C, supported fibronectin assembly as adherent substrate. Addition of both 1F3 and the C-terminal modules to 7F3-10F3, resulting in 1F3-C, further improved supportive activity among the recombinant fibronectin constructs. A co-coating of 1F3-10F3 and 7F3-C, in which include module 1F3 and the C-terminus are in different materials, was less active than 7F3-C. When tested for ability to overcome the suppressive effect of co-coated vitronectin, 1F3-C was also the best. Similar results were obtained on binding of 70-kDa N-terminal fragment to fibronectin-null cells, which is an accurate indicator of the cell’s ability to assemble fibronectin. These results demonstrate that module 1F3 acts in cis with the C-terminal modules of adsorbed fibronectin construct to support fibronectin assembly by fibronectin-null cells.

Cell Attachment to the Extracellular Matrix (2614)

2614

The Effect of Temperature on Integrin-mediated Adhesion in the Sea Anemone, Aiptasia pulchella

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Temperature-induced coral bleaching results from the loss of the symbiotic dinoflagellate algae from the coral host. One potential mechanism underlying temperature-induced coral bleaching is loss of the host cell containing the endosymbiotic algae. We have been investigating how temperature affects integrin mediated cell adhesion in the tropical symbiotic sea anemone, Aiptasia pulchella. A β1-integrin has been sequenced from Cnidarians, but the function has not been investigated in these animals. Using both commercially available anti-β1-integrin antibodies and an antibody made against a conserved extracellular domain of the Cnidarian integrin (CN1β1), we have identified an approximately 120 kDa protein from tissue extracts from the sea anemone, Aiptasia pulchella. In addition, we have identified by immunoprecipitation using an anti-focal adhesion kinase (FAK) antibody an approximately 125 kDa protein in tissue extract from A. pulchella. Using immunohistochemistry, we have investigated the tissue localization of integrins using the CN1β1 antibody. Integrin staining is the strongest where the cells of the endoderm attach to the acellular mesoglea. To investigate how temperature affects the tissue distribution of these integrins, anemones were heat shocked for 24 hours at 30°C and then processed for immunohistochemistry. Temperature shock disrupts the strong integrin staining at the base of the endodermal cells suggesting that integrin adhesion is disrupted by temperature shock. Further studies will investigate how temperature affects FAK as well as the timing of the temperature effect on integrin distribution.

Extracellular Matrix and Cell Behavior (2615-2618)

2615 Tissue Stiffness Enhances Malignant Transformation through Increased PI3 Kinase Signaling
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Physical palpation has long been a means to detect tumor formation; however, the role of tissue mechanics in tumorigenesis is poorly understood. We are using MMTV-Her2/neu transgenic mice and organotypic mammary epithelial cell (MEC) three-dimensional (3D) tissue models to study how alterations in the physical properties of the extracellular matrix (ECM) could modulate mammary tumorigenesis. Using compression and shear analysis, we found that malignant transformation is preceded by and associated with a progressive increase in mammary gland stiffness. Because we found that gland stiffness, altered tissue morphology, and tumor invasiveness are functionally linked to increased collagen deposition, bundling, and lysyl oxidase (LOX) expression, we examined the relevance and molecular mechanisms whereby ECM stiffness might influence normal breast epithelia and breast tumor behavior. Concomitantly, 3D organotypic MEC studies illustrated that elevated tissue stiffness, through increased collagen crosslinking, synergistically promoted tumor invasion in cooperation with expression of an oncogene such as ErbB2. Consistently, pharmacological inhibition of LOX-mediated collagen crosslinking repressed mammary gland transformation in MMTV-Her2/neu mice. Finally, we determined that matrix stiffness enhances epidermal growth factor (EGF) receptor-dependent PI3 kinase activity and are currently investigating if matrix stiffness promotes transformation by altering EGFR-dependent PI3 kinase signaling and if so how. (Supp: NIH T32HL00795404 to KJR; DOD W81XWH-05-1-330 and NIH CA078731 to VMW).

2616 Extracellular Matrix Signaling via β1 Integrin Regulates DNA Double-Strand Break Repair
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Genomic instability and aberrant cell-extracellular matrix (ECM) interactions are hallmarks of malignancy. One cause of genomic instability is altered DNA double-strand break (DSB) repair. DSBs are caused by endogenous factors such as blocked replication, oestrogen intermediates, reactive oxygen species, and by exogenous factors such as ionising radiation during cancer treatment. Breaks can be repaired by homologous recombination (HR) or non-homologous end joining (NHEJ) pathways, both of which can lead to mutations depending on genomic location, presence of repeat sequences, and the sub-pathways invoked. HR is accepted as the less mutagenic pathway and is up regulated during S and G2 phases of the cell cycle. Apart from cell cycle effects, it is not clear what determines pathway choice in DSB repair. Here we show that in primary mouse mammary epithelial cells, as well as in a human breast epithelial cell line, ECM signalling via β1 integrin regulates HR of a break within a direct repeat: ECM up regulates HR if normal cell-cell junctions are present but down regulates it in single cells. This regulation is direct and independent of cell cycle effects of ECM. Furthermore, formation of γ-H2AX, MRE11, and RAD51 foci in response to double-strand break formation via ionising radiation in single non-dividing human epithelial cells is attenuated by ECM signalling via β1 integrin, whereas γ-H2AX foci formation in primary mouse mammary epithelial cells with normal junctions is up regulated. Therefore, the effects of ECM on HR are genome-wide and not limited to the repair of the single break within a direct-repeat substrate. Our results suggest that the role of ECM in tissue homeostasis includes regulation of DNA repair. The observation that the effects of ECM are modulated by cell-cell junctions suggests that ECM may function to balance processes relevant to genome stability with maintenance of tissue integrity.

2617 Effects of the Extracellular Matrix on Embryonic Stem Cell Self-Renewal and Differentiation
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Embryonic stem (ES) cells are defined by their ability to either self-renew or differentiate in culture. While soluble factors that stimulate these processes have been identified, the contributions of both the extracellular matrix (ECM) and cell-adhesion receptors remain poorly characterized. Expression of the cell-cell adhesion receptor E-cadherin correlates with self-renewal. ES cells also express integrin receptors for various ECM proteins, and we have found that, under self-renewal conditions, ES cells assemble a fibrillar matrix of the adhesive ECM protein fibronectin. Additionally, treatments that increase ES cell-ECM interactions tend to promote differentiation. These results suggest that the appropriate balance...
between cell-ECM and cell-cell interactions is critical in order to control the switch from self-renewal to differentiation. To test the contributions of cell-cell and cell-ECM interactions, cells were grown on various ECM proteins in the presence of LIF, a soluble factor that promotes self-renewal. Flow cytometry of ES cells that express a Nanog promoter-GFP self-renewal reporter construct was used to quantify self-renewing cells. On gelatin, the standard substrate for ES cell propagation, the majority of cells expressed GFP and assembled a fibrillar fibronectin matrix. Inhibition of fibronectin production through treatment with siRNAs completely abolished cell-matrix adhesion on gelatin, resulting in formation of non-adherent, differentiating cellular aggregates. These results suggest that cell-ECM interactions are necessary for ES cell self-renewal. Culture on fibronectin induced cell spreading and caused a concomitant loss of GFP expression, suggesting that increased cell-ECM interactions antagonize self-renewal and promote differentiation. Thus, ES cells can be switched from a self-renewal to a differentiating phenotype by modulating the levels of cell-ECM interactions through changes in the makeup of the ECM substrate.

2618

**Nanofiber Extracellular Matrix Is Equivalent to Laminin in Maintaining Function and Gene Expression of Rat Hepatocytes In Vitro**

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**Objective** The goal of the current study was to examine the biochemical function of rat hepatocytes cultured on nanofiber ECM over 14 days in vitro. Hepatocytes cultured on nanofiber ECM were compared to standard culture on laminin. **Methods** Freshly isolated rat hepatocytes were inoculated on nanofiber ECM placed 6 well plates and cultured in Williams-E media. Control condition was tissue culture plastic pre-treated with 1.23 pM/cm² of laminin. Media was sampled for albumin production by ELISA, diazepam metabolism (CYP3A and CYP2C11) by HPLC, and urea production by GC-MS at days 2, 7, and 14. CYP1A1 enzymatic activity was measured by fluorometric analysis using 7-ethoxyresorufin substrate. RNA was extracted from hepatocytes at each time point and analyzed on a custom microarray with comparisons to freshly isolated hepatocytes. Inducibility of gene expression was also tested with β-naphthoflavone. **Results** Hepatocytes cultured on nanofiber ECM maintained cuboidal morphology and demonstrated higher albumin protein production, CYP1A activity, and urea production compared to hepatocytes cultured on laminin for 14 days. Levels of diazepam metabolism were equivalent between the two culture conditions. Production of albumin declined over time in both culture conditions and paralleled the decline in albumin gene expression as measured by microarray. Many cytochrome P450 enzymes (CYP1A1, CYP1A2), phase II (UDP-GT), and urea cycle (NAGS) enzymes and cell adhesion molecule (intracellular adhesion molecule 1, fibronectin and E-cadherin) genes remained intact. Gene expression of CYP1A1, ALDH 3A1, and GSTA2 was induced by β-naphthoflavone in hepatocytes cultured on both nanofiber ECM and laminin. **Conclusion** Hepatocytes cultured on nanofiber ECM maintained biochemical functions better than hepatocytes cultured on laminin. Membranes of nanofiber ECM were comparable to laminin-treated tissue culture plastic for maintaining differentiated function of primary rat hepatocytes in vitro.

**Extracellular Matrix and Cell Signaling (2619-2623)**

2619

**Epithelial-mesenchymal Signaling Interactions Induce Mutual Differentiation of Dental Pulp Cells and Dental Epithelial Cells**

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Objective: Similar to other epithelial appendages, teeth form through reciprocal signaling interactions between the ectodermally derived dental epithelium and mesenchyme, which program to enamel matrix secreting ameloblast and dentin forming odontoblasts. In this study, we reconstituted the reciprocal signaling interactions between human fetal dental epithelial cells and dental pulp cells to study the signaling network involved in odontogenesis. Methods: Dental epithelial cells, isolated from human fetal tooth organs, were mixed with Matrigel and seeded on the upper chamber of the transwell in 24-well plate. Human dental pulp cells, including approximately 2% of STRO1 expressing mesenchymal stem cells, were grown on the lower chamber of transwell plate. Dental epithelial cells or mesenchymal cells alone were cultured on the upper chamber or lower chamber of transwell plate as controls. After three weeks co-culture, the dental epithelial cells/Matrigel complex were fixed and sectioned for H&E and immunohistochemical staining. Dental mesenchymal cells were collected for RNA purification and assayed for the collagen type I expression. Superarray analysis was used to compare the expression of extracellular matrix and adhesion molecules between epithelial cells control and co-cultured epithelial cells. Result: PCR showed that when dental mesenchymal cells were co-cultured with epithelial cells/Matrigel, collagen type I mRNA expression was significantly increased. Superarray analysis and immunostaining showed higher expression of collagen matrix proteins by epithelial cells in co-culture. Meanwhile, Superarray analysis indicated that E-cadherin, integrin β7 and α2b were up-regulated in the dental mesenchyme induced epithelial cells. Conclusion: Human fetal dental epithelial cells and dental pulp cells grown in co-culture undergo reciprocal signaling to induce cellular differentiation. These teeth derived dental epithelial cells and pulp mesenchymal cells are valuable tools for studying epithelial-mesenchymal signaling interactions.

2620

**Thromboxane A2 Stimulates Fibronectin Assembly by Platelets: Inhibition of Rho Increases Fibronectin Assembly by Platelets, but Not Fibroblasts**

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Recent studies have suggested an important role for fibronectin (FN) assembly by platelets in thrombus growth and stability. The molecular mechanisms and platelet substrate interactions that influence platelet FN assembly are poorly understood. In fibroblasts, cell contractility mediated by Rho is well-recognized as being required for FN assembly. We used U46619, a stable analog of thromboxane A2 that activates G12/13 and Q signaling pathways at concentrations of 1 µM and only the G12/13 pathway at 30 nM, to investigate the signaling mechanisms that contribute to
platelet FN assembly. By both fluorescence microscopy and a quantitative 96-well assay, both concentrations of U46619 increased FN assembly by aspirin-treated platelets adherent to absorbed FN. Because the G12/13 pathway is known to activate Rho leading to subsequent activation of Rho kinase and phosphorylation of myosin light chain, we investigated the effects of the Rho kinase inhibitor (Y-27632) and the myosin light chain kinase inhibitor (ML-7) on FN assembly. Pre-treatment of platelets with either Y-27632 or ML-7 inhibited FN assembly by platelets stimulated with 30 nM U46619, whereas only ML-7 inhibited assembly by platelets stimulated with 1 μM U46619. To investigate the role of Rho, platelets were pre-treated with C3 transferase, which ADP ribosylates Rho. Surprisingly, platelets pre-treated with C3 transferase and stimulated with 30 nM U46619 showed an increase in FN assembly compared to platelets stimulated with 30 nM U46619 in the absence of C3 transferase. This increase was lost in the presence of Y-27632. Parallel studies demonstrated the expected decrease in FN assembly by fibroblasts treated with C3 transferase. Thus, FN assembly by adherent platelets is stimulated by Gq initiated pathways that do not require Rho kinase. Further, in platelets Rho kinase appears to be coupled to Rho by pathways that are influenced by C3 transferase differently than in fibroblasts.

**2621**
The Modulating Effects of Type II Collagen on Bone Progenitor Cell Differentiation through MAPK Signaling
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During endochondral bone development, mesenchymal stem cell undergoes condensation and forms cartilage in the shape of ensuing bone. This cartilage is rich in type II collagen matrix and served as a template for the subsequent bone formation. These observed processes during bone development lead to a hypothesis that “the cartilage formation is essential for and precedes bone formation, which is promoted by the matrix replacement events in the preformed cartilage”. To test this hypothesis, type II collagen (CII), which is the major extracellular matrix (ECM) component during the osteochondral stage of developmental bone, was used to study the ECM-guided osteogenic differentiation. STRO-1+/-, SH2+, SH3+, CD34+ human mesenchymal progenitor cells (hMPCs) were isolated and expanded. An enhanced calcium deposition was observed in both CII-hMPCs co-condensed micro-mass culture and monolayer cultured hMPCs on CII coated plates. Furthermore, ERK inhibition experiment with U0126 revealed that the CII-enhanced osteogenic effect may work through MAPK signaling. Subsequently, artificial bone tissues fabricated with either type I collagen as scaffold, or type I collagen scaffold coated with CII, were compared histologically. The data indicate that the remodeling of cartilaginous matrix may be crucial to osteogenesis processes; the implantation of cartilaginous matrix might be beneficial for repairing large bone defect as an alternative strategy. This work is supported by NSC 96-2314-B-038-015, Taiwan, ROC.

**2622**
Nuclear Translocation of Smad1 and CD44 Intracellular Domain via BMP Activation
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Interplay between the cell and its extracellular matrix serves to fine tune cellular responses to cytokines and growth factors. For instance, changes in hyaluronan-rich matrices affect cellular responsiveness to bone morphogenic protein-7 (BMP-7) mediated by the interaction of the intracellular domain (ICD) of its principal receptor CD44 and Smad1. Previous studies demonstrated that the interaction of CD44 ICD and Smad1 was necessary for a robust cellular response to BMP-7, and alterations in the hyaluronan matrix or CD44 structure reduced Smad1 phosphorylation and subsequent nuclear translocation. Interestingly, it has also been demonstrated that a sequential cleavage produced by MMP in the extracellular domain followed by γ-secretase transmembrane cleavage, releases the CD44 ICD which translocates to the nucleus. In order to study whether the cleaved ICD still interacts with Smad1 to translocate into the nucleus, three tagged protein domains were prepared and characterized. A human CD44 ICD was subcloned into pcDNA3 with and without a myc-tag. Similarly, Smad1 was subcloned into pEGFP-N2 vector to generate a fusion protein. These constructs were transfected into a variety of cells (OS-7 cells, rat chondrosarcoma, C-28/I2 and bovine articular chondrocytes) to study expression and function. Expression and cytoplasmic localization is evaluated by western blotting and immunocytochemistry respectively. Western blots of lysates of COS-7 transfecteds showed endogenous Smad1 at ~52 kD and expression of the fusion protein at ~90kD immunoreactive for both EGFP and Smad1 antibodies. The EGFP-Smad1 construct was well expressed in all four cell types. In C-28/I2 transfected chondrocytes with EGFP-Smad1 construct, EGFP fluorescence was detected mainly in the cytoplasm, but after BMP-7 stimulation nuclear translocation was observed. BMPs are being used to induce nuclear translocation of CD44 ICD and Smad1 to test cross-talk between the signaling pathways of CD44 and Smad1. Supported by NIH grants RO1-AR43384 and RO1-AR39507.

**2623**
Bit1 Regulation of Apoptosis in Cardiomyocytes
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Heart disease is a leading cause of death in newborns and in adults. Cardiomyocyte apoptosis plays a significant role in the transition from hypertrophy to heart failure. Integrin-mediated cell adhesion to the extracellular matrix (ECM) is necessary for the survival of many cell types including endothelial and epithelial cells. Loss of cell attachment to the ECM causes apoptosis (cell death) in these cells. Integrins suppress apoptosis in attached cells by activating signaling pathways that promote survival and inactivating the ones that promote apoptosis. Integrin expression is tightly coordinated with ECM expression in the normal adult heart, whereas in models of cardiac hypertrophy, dilated cardiomyopathy and myocardial infarction changes occur in both ECM and integrin expression. We have identified Bit1 as a regulator of apoptosis. We have previously shown that Bit1 is a pro-apoptotic protein and is not a transcription factor. Bit1 is regulated by integrins and may be a guardian of anchorage dependence. We show here that Bit1 is highly expressed at the mRNA and protein levels in the heart and particularly in cardiomyocytes. Bit1 expression is upregulated in cardiomyocytes after myocardial infarction. Moreover, the outcome of Bit1 regulated apoptosis is determined in part by its localization in the cell. Which key apoptosis pathways are activated in cardiomyocytes after myocardial infarction is currently unknown. Hence, understanding the mechanism of cardiomyocyte apoptosis after myocardial infarction may have direct clinical relevance.
Extracellular Matrix and Morphogenesis (2624)

2624
Barx2 Regulates Matrix Metalloproteinase (MMP) Expression and Is Important for Ocular Gland Branching Morphogenesis and Eyelid Fusion
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It is well known that cell-ECM interactions and ECM remodeling by MMPs are crucial for many developmental processes including lacrimal gland (LG) development. However, the factors that regulate MMPs during LG development are not extensively studied. Here we report that the homeodomain transcription factor Barx2 is expressed in the conjunctival epithelium and LG, regulates expression of MMPs, and is necessary for normal LG morphogenesis and function. LGs in Barx2 null mice were found to be poorly developed or entirely absent. In most cases the Barx2 null LG was vestigial, closely associated to the eye, and characterized by poor branching and differentiation. Using gelatin zymography assays we found that MMP secretion is significantly reduced in Barx2 null lacrimal gland epithelium. Consistent with the Barx2 null phenotype, down-regulation of Barx2 in LG explant cultures using antisense oligonucleotides also resulted in branching defects. In addition, gain of Barx2 function experiments in cell culture indicated that Barx2 promotes cell migration through extracellular matrix, and regulates the expression of MMPs and cell adhesion molecules. The Barx2 null mouse also showed dramatic defects in Harderian gland and eyelid development. The Harderian gland was completely absent in 99% of studied Barx2 null mouse embryos, while 85% of embryos showed defects in eyelid fusion. The defects in lacrimal and Harderian gland development and eyelid fusion lead to a range of ocular surface phenotypes in Barx2/-/- mice. They include ocular surface inflammation (blepharitis and conjunctivitis), abnormal eyelid and corneal keratinization and corneal epithelial defects. Together these data suggest that Barx2 and MMPs play an essential role in regulating branching morphogenesis of the ocular glands as well as normal eyelid development.

Integrins (2625-2628)

2625
Cleavage of the α6 Integrin in Xenopus laevis Occurs Early in Development and Persists through Adulthood
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The α6 integrin is essential for early nervous system development in Xenopus laevis. Although expression of the α6 integrin in the developing embryo has been studied, there is no information on its expression in the adult frog. In addition, human epithelial tissues express a novel proteolytically cleaved form (α6p) that has not been investigated in Xenopus. The objective of the present study was to investigate the expression of the α6 integrin in Xenopus and to determine whether the α6 integrin cleavage occurs during frog development. Our results show that the α6 integrin is expressed throughout the CNS and the notochord with an elevated expression in the neural tube, the olfactory placode, the interneurons, the pronephros and the pronephric duct. The α6p integrin is expressed as early as embryonic stage 17 and its expression increases until it reaches maximum levels at tadpole stage. Surprisingly, analysis of protein lysates from organs of an adult male frog indicated that the major form of the α6 integrin present in all organs was α6p. Mass spectrometry analysis following digestion, of two proteins of approximate sizes 70 and 75 kDa from an α6 immunoprecipitation from protein samples isolated from tadpoles confirmed that the 70kDa fragment is α6p and the 75kDa fragment is α6n (the amino-terminal segment of α6) as seen in the human tissues. Sequence comparison of the human and Xenopus α6 reveals that the uPA cleavage site in the human α6 is not conserved in Xenopus. Additionally, treatments of Xenopus primary cultures and cell lines with urokinase inhibitors did not reduce α6p levels. Collectively these data suggest that a unique protease cleaves the Xenopus α6 in a region similar but distinct from the human α6. Current work is to determine the protease involved in α6 cleavage and whether this cleavage is essential for normal development.

2626
Direct Effect of Maternal Anti-HPA-1a Antibodies on Endothelial Cells
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Introduction: Neonatal alloimmune thrombocytopenia (NAIT) is caused by maternal Human Platelet Antigen alloantibodies (HPA Abs). The HPA-1a Abs can also bind to endothelium, via the β3 integrin (CD61). This binding may thereby induce vascular damage which could explain part of the bleeding tendency seen in NAIT. The aim of the study was to investigate the influence of HPA1a-Abs on endothelial cell function. Methods: Firstly, a CD61 monoclonal antibody (MoAb) was used as a model for the HPA-1a Abs. Secondly, purified IgG with HPA-1a Abs from alloimmunized women were used. The effect of these antibodies was examined by monitoring real-time the transcellular resistance of primary human endothelial cells, as an indicator of endothelial permeability. The effects on the endothelial cells were further examined by analysis of adhesion and cell spreading, and by detecting activation of small GTPases. Results: We found that the MoAb CD61 and HPA-1a Abs caused a reduction in endothelial cell spreading and monolayer formation. This was confirmed by microscopy and can be explained by the reduced activation of the GTPase Rac1, as observed the MoAb CD61-treated cells. In addition, CD61 and HPA-1a Ab caused an increase in endothelial monolayer permeability. The reduction in endothelial monolayer permeability could be partly reversed by blocking signalling through the RhoA effector Rho Kinase. Furthermore, in MoAb CD61 or HPA-1a Ab treated cells, the endothelial monolayer showed less β-catenin at the plasma membrane, suggesting impaired formation of cell-cell junctions. Conclusion: Our data suggest that anti-HPA-1a alloantibodies, besides inducing thrombocytopenia, also have a direct effect on
endothelial cell adhesion, spreading and monolayer permeability, most likely acting through the GTPases RhoA and Rac1. These effects may contribute to the increased bleeding tendency in children with NAIT. Funding: Zon MW 40-40600-98-06728

2627

Cytoplasmic Regions of β5 Integrin Required for Adhesion and Phagocytosis
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Retinal pigment epithelial (RPE) cells maintain function and viability of photoreceptor neurons in the eye. Two distinct receptor-mediated interactions between the apical RPE surface and adjacent photoreceptor outer segments are phagocytosis of spent photoreceptor outer segment fragments (POS) every morning and retinal adhesion maintaining tissue integrity at all times. Both functions require apical αvβ5 integrin receptors of the RPE. Lack of αvβ5 in mice causes weakened retinal adhesion, asynchronous phagocytosis and age-related blindness. POS derivatives accumulating in αvβ5-deficient aging RPE resemble those in age-related macular degeneration, the leading cause of blindness among the elderly. Signaling by αvβ5 may depend on whether it mediates adhesion or phagocytosis. POS recognition activates αvβ5-associated FAK and MerTK tyrosine kinases but the diurnal peak of retinal adhesion does not. To study different αvβ5 functions we generated plasmids and adenovirus encoding full-length β5 integrin fused to C-terminal GFP. Additionally, we generated β5-GFP mutants harboring 10 amino acid deletions within the C-terminal 40 residues. Full-length β5-GFP dimerized with endogenous αv subunits at the surface, co-immunoprecipitated FAK, and redistributed to bound POS in transfected RPE cells. Increasing αvβ5 receptors moderately enhanced POS phagocytosis by wild-type mouse RPE. De novo formation of αvβ5-GFP receptors greatly increased POS binding and internalization by β5 knockout mouse RPE. Furthermore, expressing full-length β5-GFP in β5-deficient CS-1 cells promoted robust cell-substrate adhesion. Thus, β5-GFP forms fully functional αvβ5 integrin receptors. All our β5-GFP deletion mutants dimerized with αv and localized to the cell surface like full-length β5-GFP. Deleting the terminal 10 residues did not alter substrate adhesion. However, deleting regions located 40-30, 30-20, or 20-10 residues from the C-terminus significantly decreased adhesion. We are currently testing whether and which of these regions promote phagocytosis. We are also testing point mutations of β5-GFP to identify individual residues relevant for adhesion and phagocytosis.

2628

Differential Function of ILK in Activated and Quiescent Hepatic Stellate Cells: Implications for Fibrogenesis in Wound Healing
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Integrin-linked kinase (ILK) is a multidomain focal adhesion protein implicated in signal transduction between integrins and extracellular receptors. We have previously shown that ILK expression is increased in liver fibrosis and ILK appears to be a key regulator of fibrogenesis in rat hepatic stellate cells, effectors of the fibrogenesis phenotype. Here we hypothesized that the mechanism by which ILK mediates the fibrogenic phenotype is by engaging the small GTPases, Rac and/or Rho in a fibrogenic signal transduction. We used a culture-based model including normal (quiescent) and injured (activated) cells that mimics in vivo fibrogenesis. Inhibition of ILK activity with a pharmacologic ILK inhibitor, QLT-0267, reduced GSK3-activity in activated, but not in quiescent cells. Inhibition of either Rho kinase or ILK kinase activity altered cell shape and stress fiber formation in ILK in activated stellate cells increased Rho activity, but did not effect Rho activity in quiescent cells. Interestingly, endothelin-1 stimulated Rho activity in activated, but not in quiescent cells. Inhibition of either Rho kinase or ILK kinase activity altered cell shape and stress fiber formation in activated stellate cells. However, RhoA induced smooth muscle actin expression independent of ILK. Finally, ILK kinase mediated Rho dependent functional effects, including cell adhesion, type I collagen synthesis, and TGF-β production. These findings demonstrate cross talk between ILK and Rho in fibrogenic effects. Signaling to the cytoskeleton appears to proceed independently through ILK and Rho. Finally, ILK dependent effects on Rac and Rho occurred only in activated cells. Taken together, these data demonstrate an essential role of ILK and Rho in stellate cell actin cytoskeletal reorganization and fibrogenesis and suggest that cellular activation is required to engage ILK and small GTPase effector activation.

Metalloproteases (2629-2631)

2629

CD147 Regulation of Hepatocyte Derived Matrix Metalloproteinasises
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We have shown CD147 is upregulated in the human cirrhotic liver and expressed predominantly on hepatocytes. CD147 is an abundant glycoprotein and a potent inducer of matrix metalloproteinasises (MMPs-1, 2, 3 & 9). Liver fibrosis is characterised by the dysregulation of extracellular matrix turnover, primarily mediated by MMPs. We hypothesise that CD147 regulates hepatocyte-derived MMP expression which is capable of matrix remodelling. Cellular localisation of CD147 and MMPs was determined by immunohistochemistry and immunofluorescence in human cirrhotic liver. Temporal changes in CD147 protein were analysed in a rat bile duct ligated model of injury (0, 1, 2, 3 & 4 weeks). MMP and TIMP (endogenous MMP inhibitors) activities were analysed in primary rat hepatocytes and hepatic stellate cells (HSCs) by zymography. In vivo functional studies of CD147 were performed in mice treated with carbon tetrachloride for 4 weeks using an anti-CD147 blocking antibody (mAb RL73.2). Injury was assessed by histological analysis and severity of fibrosis quantitated by hydroxyproline estimation. CD147 mRNA and protein increased with injury and cirrhosis in humans and rat livers. CD147 and MMPs-1, 2 & 9 was predominantly localised to hepatocytes but not HSC. Similarly in vitro MMP-2 & 9 activity was significantly increased in hepatocytes compared to isolated quiescent or in vivo activated HSCs. In contrast, significant TIMP-1
Here, we report that ectopic expression of TRE17(long) can induce degradation of extracellular matrix components. To identify the enzyme(s) MMP9/MMP10. In sum, our studies suggest that TRE17 may promote bone degradation in ABCs by inducing production of matrix proteases.

In previous studies, we reported that TRE17 induces activation of the Arf6 GTPase, which has been implicated in cell motility and invasiveness. Here, we report that ectopic expression of TRE17(long) can induce degradation of extracellular matrix components. To identify the enzyme(s) response for matrix degradation, we analyzed the expression of various matrix proteases in TRE17-expressing cells. Using reverse transcription/real-time PCR and reporter assays, we found that TRE17(long), but not TRE17(onco), induced expression of matrix metalloproteinase 9 (MMP9) and stromelysin-2 (MMP10). Furthermore, western blot analysis showed that both MMP9 and MMP10 accumulated exclusively in the conditioned medium of cells expressing TRE17(long). TRE17(long) encodes a de-ubiquitinating enzyme (DUB), while TRE17(onco) is C-terminally truncated and inactive as a DUB.

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The TRE17/Ubiquitin-Specific Protease 6 (USP6) Oncogene Induces Expression of Matrix Proteases

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The TRE17/USP6 oncogene was originally identified based on its tumorigenic activity in mice. Recent studies reveal that the TRE17 locus, located on chromosome 17p13, is a target of chromosomal translocation in an osseous neoplasm termed aneurysmal bone cyst (ABC). ABCs are rapidly growing and locally invasive tumors that cause destruction of the surrounding bone. Translocation of the TRE17 locus occurs in over 60% of ABCs, and leads to high level expression of TRE17 in these lesions. Despite this strong evidence implicating TRE17 in the etiology of ABC, the mechanism by which TRE17 induces transformation and bone degradation remains unknown. TRE17 encodes two isoforms which we have termed TRE17(long) and TRE17(onco). TRE17(long) encodes a de-ubiquitinating enzyme (DUB), while TRE17(onco) is C-terminally truncated and inactive as a DUB.

Enhanced Strategy for Separating Human Plasma Fibronectin from MMPs by Affinity Chromatography

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Fibronectin (FN) has multiple activities and is involved in wound healing, metastasis, and other biological processes. FN binds gelatin and is typically purified from plasma by gelatin affinity chromatography. Commonly used elution with buffer containing 4M urea or low pH (5.5) co-elute gelatin-binding matrix metalloproteinases (MMP) -2 and -9 that can cleave FN and alter FN stability. Since reported protocols for separating MMPs from FN significantly increase time and expenditures, present experiments tested enhanced purification strategies based on differential elution with dimethylsulfoxide (DMSO). Assays used analytical mini-columns (Vt 25 µl) and column fractions were analyzed by SDS-PAGE and enzymography gels co-polymerized with type I gelatin. FN yields were quantified from digitized gel images. Elution strategies included low pH, a stepwise gradient of DMSO (0 - 10% v/v), and 4 M urea, alone or in combination. Low pH and 4 M urea efficiently eluted FN, but co-eluted MMPs. In comparison, DMSO differentially eluted MMPs over FN. Increasing concentrations of DMSO enhanced elution of MMPs, but also resulted in partial although not complete elution of FN. Whereas 1% DMSO was insufficient to elute MMPs from columns, 2% DMSO eluted all MMPs in 10 x Vt, and 3% DMSO or greater elimated all MMPs from the columns with 6 x Vt. Optimal MMP removal and FN yields (63%) were achieved with 6 x Vt of 3% DMSO pre-elution and final elution with 4M urea. Cell attachment assays verified biological activities of purified FN. Half-maximal attachment of HT1080 cells was achieved at ~3 nM coating for FN purified with either 3% DMSO and 4 M urea combined, or 4 M urea only. The enhanced FN purification by DMSO pre-elution and final urea elution using one gelatin affinity column efficiently generated MMP-free FN with intact biological activities (Supported by NIDCR grants DE017139 and DE 016312).

Cell–Cell Interactions (2632-2634)

A Microfluidic Platform for Improved Throughput and Reliability of Cellular Adhesion Assays

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A large number of processes underlying immune system biology are dependent on leukocyte adhesion and neutrophil rolling phenomena. Laminar flow chambers remain the conventional in vitro approach to studying cellular adhesion under shear stress conditions, but suffer from low throughput, poor response time, and long setup procedures. Here we report on the development of a microfluidic platform which improves upon throughput, experimental control, and setup time. An assay showing rolling adhesion dependence on the presence of divalent ions was conducted on this platform and compared to literature data. Cell adhesion and neutrophil rolling assays are presented comparing conventional flow chamber experiments to microfluidic experiments at multiple shear forces. Jurkat cells were grown in RPMI supplemented with 10% FCS. The cell suspension was centrifuged and resuspended in PBS/EDTA, then resuspended in PBS containing the indicated amounts of Ca and Mg. Adsorption of cellular adhesion factors, such as VCAM-1 and PNA, was carried out by backfilling the desired microfluidic channels with the adhesion factors diluted in appropriate buffers. Nonspecific binding was reduced with a BSA-based blocking buffer for two hours prior to use. As expected (Chen, et al, 2004), no adhesion or rolling was observed in the absence of divalent ions. Adding 1mM Mg resulted in cell adherence with no rolling. Adding 1mM Ca without Mg resulted in slow rolling while including both Ca and Mg resulted in a similar slow rolling. The BioFlux™ 200 System facilitates the study of cell adhesion by providing a simple, higher throughput approach to shear-based assays. Principal advantages demonstrated were parallel processing of assays, real time imaging, minimized reagent consumption, and precise control over shear stress application. Future modifications to
the system will include further expansion of the throughput to a 96-well plate format, integrated image acquisition, and automated cell tracking analysis.

2633
**Cardiac Fibroblast-Myocyte Interactions Regulate Extracellular Matrix Remodeling**
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Degenerative myocardial remodeling, including fibrosis, is thought to be mediated primarily by cardiac fibroblasts, but the mechanisms governing collagen deposition and organization in the heart remain largely uncharacterized. Collagen-binding adhesion molecules, including integrins and discoidin domain receptor 2 (DDR2), are abundant on the surface of cardiac fibroblasts and appear to regulate these processes. In addition, direct and paracrine interactions between the fibroblasts and neighboring cardiac myocytes may regulate matrix remodeling in the heart. The objective of this work is to characterize the roles of integrin and DDR2 receptors and fibroblast-myocyte interactions in matrix remodeling. To this end, we’ve implemented a 3D fibrin scaffold for the in vitro culture of cardiac fibroblasts and myocytes. Neonatal rat heart fibroblasts (200k cells/construct), myocytes (200k cells/construct), or both (200k fibroblasts + 200k myocytes/construct) were suspended in 3.33mg/mL fibrin in 24-well plates. The constructs were detached from the plates and cultured for up to 1 week (n=4/group/time point) in the presence of 2mg/mL aminocaproic acid and 50μg/mL ascorbic acid. Cultures were terminated and conditioned media were collected at 1, 2, 3, and 7 days. Cell-gel constructs were fixed, immunochemically costained for collagen type I and DDR2 or beta-1 integrin, and examined by confocal microscopy. DDR2 and beta-1 were consistently colocalized with newly deposited collagen in fibroblast-containing constructs. Conditioned media were assayed for collagen by the chloramine-T-p-DAB reaction and for cytokines by the Bioplex assay. Co-culture constructs appeared to release less collagen than fibroblast-only constructs, and released significantly more collagen than hyalin-only cultures (p<0.05 at 1wk). Release of interleukin (IL)-6 and IL-10 was higher in co-cultures than fibroblast-only constructs (p<0.05), whereas release of tumor necrosis factor α was similar between the two groups. These results suggest that cardiac fibroblast-myocyte interactions regulate collagen remodeling in part through paracrine and/or autocrine signaling.

2634
**Sea Urchin Hyalin from Lytechinus pictus May Mediate Archenteron-Blastocoel Roof Attachment**
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The sea urchin embryo has been designated as a National Institutes of Health model system because of its simplicity, accessibility and the discovery using it of many physiological developments important in human health and disease. Here we isolate the large glycoprotein hyalin from *Lytechinus pictus* embryos and suggest that it mediates archenteron-blastocoel roof attachment. Hyalin was isolated using 0.475 M NaCl-0.025 M KCl from 30-45 minute old embryos by methods of Gray et al. (J. Biol. Chem. 261: 9282 (1986); and Razinia et al. (Zygote 15: 1 (2007)). *L. pictus* hyalin was incubated with living 24 hour *L. pictus* embryos for 24 hours using a microplate assay (Razania et al., 2007) that allows quantitative evaluation of the effects of exogenously added molecules on the specific archenteron elongation-attachment to the blastocoel roof. This interaction was blocked in a hyalin concentration-dependent manner. A hyalin dose-response curve showed that at high hyalin concentrations (0.15 mg/mL) 6.1% +/- 2.3% of the embryos displayed attached archenterons, while controls in the absence of hyalin displayed 95.1% +/- 1.8% attached archenterons. The differences were highly significant at p less than 0.001. Since large molecules can enter the sea urchin blastocoel (Latham et al. (Acta Histochemica 100: 193 (1998); Itza and Mozingo (Zygote 10: 255 (2005))), we propose that exogeneous hyalin blocks the interaction of the archenteron-blastocoel roof by binding to hyalin receptors or hyalin-binding ligands by entry into the blastocoel. We have shown that Strongylocentrotus purpuratus sea urchin hyalin (Razina et al. (2007)); Alvarez et al. (Zygote, in press) acts in a similar way to hyalin described in this report. These studies suggest an additional function for hyalin, i.e., in mediating a specific cellular interaction in the sea urchin embryo model (Supported by NIH NIGMS SCORE (S0648680), RISE, MARC and the Joseph Drown Foundation).

**Gap Junctions (2635-2637)**

2635
**Lipid Raft Targeting and Endocytosis of Gap Junctions in the Failing Heart**
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VENTRICULAR ARRHYTHMIAS RESULTING IN SUDDEN CARDIAC DEATH CONTRIBUTE SIGNIFICANTLY TO THE HIGH MORTALITY ASSOCIATED WITH HEART FAILURE (HF). A LIKELY ARRHYTHMOGENIC TRIGGER IN HF IS REENTRANT ARRHYTHMIAS IN PART DUE TO SLOWED VENTRICULAR CONDUCTION. THE CELLULAR MECHANISMS WHICH CAUSE CONDUCTION SLOWING IN HF ARE NOT KNOWN, BUT LIKELY INVOLVE DECREASED GAP JUNCTIONAL COUPLING BETWEEN MYOCYTES. WE HAVE BEGUN TO CHARACTERIZE CELLULAR MECHANISMS CONTRIBUTING TO ALTERED LOCALIZATION AND DECREASED EXPRESSION OF CXX43 (CXX43), THE PRIMARY GAP JUNCTION FORMING PROTEIN OF THE WORKING MYOCARDIUM. WE DEMONSTRATE PARTITIONING OF CXX43 INTO LIPID RAFT (LR) AND NON-LR MEMBRANE FRACTIONS AND SHOW THAT LR TARGETING OF CXX43 IS INCREASED IN HF, CONCOMITANT WITH DECREASED TOTAL CXX43 EXPRESSION. LR TARGETED CXX43 IS COMPRISED OF A DISTINCT phosphorylated form of the protein, PREVIOUSLY DEMONSTRATED TO BE ASSOCIATED WITH INTERNALIZATION AND DEGRADATION OF CXX43 GAP JUNCTIONS. FURTHERMORE, WE DEMONSTRATE THAT A MISLOCIALIZED POPULATION OF CXX43 KNOWN TO BE INCREASED IN HF DOES NOT LOCALIZE WITH ZONULA OCCLUDENS-1, A KNOWN REGULATOR OF CXX43 FUNCTION, BUT DOES CO-LOCALIZE WITH THE ENDOPLASMIC/RIBOSOMAL MARKERS EEA1, RAB5, AND LAMP-2. ELECTRON MICROSCOPY REVEALS THAT ABERRANTLY LOCALIZED CXX43 RESIDES IN INTERNALIZED GAP JUNCTIONS. THESE DATA PROVIDE NOVEL INSIGHT INTO THE CELLULAR MECHANISMS CONTRIBUTING TO ALTERED LOCALIZATION AND DECREASED EXPRESSION OF CXX43 ASSOCIATED WITH HF.
A Novel Connexin43 Interacting Protein Involved in Connexin43 Proteasomal Degradation

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Control of connexin43 (Cx43) protein levels can have an important role in regulating cell communication. Cx43 is known to be rapidly degraded with a half-life of 1.5-5 hours. While it has been shown that Cx43 degradation occurs via the lysosomal and proteasomal pathways, how this happens and what controls the degradation is not clear. We have identified a novel Cx43-interacting protein that we have named CIP75. Sequence analysis found that CIP75 contains an ubiquitin-like domain (UbL) and an ubiquitin-associated domain (UBA). Other members of the UbL-UBA family have been demonstrated to interact with proteasomal subunits and thus implicated in proteasomal degradation. We investigated the possibility of CIP75 acting in the Cx43 proteasomal degradation process. Immunofluorescence studies found that CIP75 localizes to the endoplasmic reticulum (ER). In addition, GST pull-down and co-immunoprecipitation experiments demonstrate that CIP75 interacts with components of the proteasome. Further overexpression and knock-down studies suggest that CIP75 is involved in Cx43 turnover in a proteasomal dependent manner. We propose that CIP75 has a role in the ER-associated degradation of Cx43.

In Vitro Motility Assays Demonstrate Microtubule-Motor Dependent Trafficking of Connexin(Cx)32-containing Liver Vesicles

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Direct cytoplasmic communication between cells is largely performed by gap junction channels between adjacent cells. These channels are formed by half-channels (connexons) contributed by each cell. Vectorial trafficking of vesicles containing connexons has been hypothesized to explain the polarized delivery of connexons to appositional regions of cells. Cytoskeleton disruption experiments have suggested microtubular and microfilament involvement in this process. We have investigated whether delivery of connexin32 (Cx32), the major liver gap junction protein, is mediated through microtubule motors, in particular kinesins. Using an in vitro chamber motility assay (Murray et al., Mol. Bio. Cell 11, 419, 2000), we observed ATP-dependent displacement of 5-10% of liver-isolated vesicles marked with fluorescent anti-Cx32 antibodies along rhodamine-labeled microtubules at an average speed of 0.45 um/sec. This motility was inhibited by 5'-adenylylimido-diphosphate (AMP-PNP) but not by vanadate, suggesting a role for kinesin rather than dynein motors. Vesicle co-labeling indicated moderate (~15%) overlap of kinesin and Cx32 in vesicles. Our immunoprecipitation experiments demonstrated that Cx32 and kinesin interact in liver lysates and in vesicles isolated from rat and mouse liver. Using surface plasm resonance spectroscopy we observed a direct interaction between Cx32 cytoplasmic peptides and recombinant conventional kinesin and identified the binding site for interaction within the membrane proximal cytoplasmic carboxyl terminus of Cx32. We conclude from our results that in liver, vesicles containing Cx32 likely traffic within the cell upon microtubules driven by kinesin motors. Support: NIH DK41918

Tight Junctions (2638-2641)

Phenotype Analysis of Human Epidermal Keratinocytes Lacking Tight Junction-related Molecules

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Tight junction (TJ) is the most apical component of the junctional complex and creates primary paracellular barrier that regulates movement of water and solutes. In the skin, the evidence that the epidermal barrier should be consisted of not only the stratum corneum but also TJs in the granular layer are accumulated. At ASCB in 2006, we have demonstrated the importance of two TJ-related molecules, claudin-1 and occludin, on epidermal barrier in differentiated human epidermal keratinocytes (HEKs) using RNA interference technique. This study aimed at morphological and functional characterization of occludin or claudin-1 deficient HEKs. For morphological analysis, immune fluorescence staining, conventional ultra-thin section and freeze-fracture replica methods were performed on the differentiated HEKs, whose claudin-1 or occludin was knocked-down. For the functional analysis, paracellular barrier function was assessed by transepithelial electrical resistance (TER). When HEKs were induced to differentiation in high Ca++ (1.45 mM) culture medium, they showed cellular stratification and cornification, and developed TJs at the cell-cell border. In the claudin-1 or occludin knock-down cells, although expressions of TJ molecules were blocked and TER was suppressed, typical stratified epithelia with well-developed cell organelles and cell-cell contacts with desmosomes alone were maintained. However, “kissing” contacts characteristic of TJs between apoposed cell membranes were almost completely absent. Furthermore, freeze-fracture electron microscopy revealed that TJ strands were poorly constructed and their total lengths were reduced in those knock-down HEKs. These data provided us the new insight that expression of TJ molecules, claudin-1 and occludin, is strongly related to TJ constructive microstructure and its epidermal paracellular barrier function.

The Classic Claudin Family: Principles of Homophilic Interactions in Tight Junctions

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Claudins (CLD) are transmembrane proteins specifically tighten the tight junctions (TJ) between adjoining cells. CLD-5, for instance, prevents the paracellular permeation of small molecules. However, the molecular interaction mechanism is unknown. We therefore investigated the CLD-CLD interaction and TJ strand formation by means of systematic single amino (aa) acid exchanges. CLD-5 mutants transfected into TJ-free cells demonstrated that the second extracellular loop (ECL2) is involved in the strand formation via trans-interaction (between two cells), but not via polymerization along the plasma membrane of one cell (cis-interaction). Three phenotypes were obtained: the TJ type (wild type-like trans- and cis-
intercellular type (disturbed folding). Combination of the results of site-directed mutagenesis, live-cell imaging, electron microscopy and molecular modeling led to an anti-parallel homodimer homology model of the ECL2. These data explain how two CLD hold onto each other and constrict the paracellular space. The intermolecular interface includes aromatic aa, F147, Y148, Y158, and hydrophilic aa, Q156, E159 (mouse nomenclature). The aromatic residues form a strong binding core between two ECL2 from opposing cells. Since nearly all these residues are conserved in CLD 1-10, 14, 15, 17, 19, and since all other segments of them also show very high common sequence similarity, our findings are of general relevance for this CLD group we define as classic CLD. On the basis of our data, we establish a novel molecular concept for TJ formation.

2640
**Regulation of Tight Junctions and the Actin Cytoskeleton by AT-1002, a Small Peptide Derived from Zonula Occludens Toxin**

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Tight junctions, the apical junctional complex, control paracellular permeability and epithelial barrier polarity. Proteins of the tight junction are intimately associated with the underlying perijunctional actin ring. Tight junctions are highly dynamic structures that are regulated in response to exogenous stimuli such as external antigens and endogenous cytokines. We synthesized a series of peptides derived from zonula occludens toxin (Zot), a protein secreted by Vibrio cholerae that transiently and reversibly opens epithelial tight junctions. To gain more insight into the mechanism of tight junction disassembly, we examined the effect of AT-1002, our “pilot agonist”, on epithelial cells. AT-1002 caused a reduction in TEER, trans-epithelial electrical resistance, followed by an increase in permeability, as measured by lucifer yellow flux across Caco2 cell monolayers. This change in tight junction assembly was associated with the redistribution of ZO-1 and occludin away from cell junctions, as seen by florescence microscopy. AT-1002 also activated src and MAP kinase pathways and increased occludin tyrosine phosphorylation in Caco-2 cells. Effects of AT-1002 on the actin cytoskeleton were studied in IEC6, rat intestinal epithelial cells. AT-1002 caused bundling of actin filaments at the cell periphery. Reorganization of the actin cytoskeleton was accompanied by increase in cofilin and src phosphorylation. AT-1002 mediated actin reorganization was prevented by a classical Protein kinase C inhibitor. These results suggest that agonist peptides such as AT-1002 open tight junctions reversibly and transiently by modulating tight junction proteins and actin-cytoskeleton structures.

2641
**Protein p63 Regulates Expression of Distinct Components of Cell Adhesion Machinery of Human Oesophageal Epithelium**

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A member of the tumour-suppressor family protein p53 has a fundamental morphogenic role for squamous epithelia, which in turn is characterized by its prominent cell adhesion properties. In normal human oesophageal epithelia, expression of p63 is limited by non-differentiated, highly proliferating cells. With the help of the laser scanning confocal microscope, we found that the p63-positive cell population expresses a prominently different set of cell adhesion proteins compared to differentiated p63 negative oesophageal cells. In particular, in normal human oesophageal epithelium, proliferating p63-positive cells specifically express adherence junction protein P-cadherin and over express catenin p120 and E-CAM. On the other hand, they are selectively deficient in principal components of tight junctions (occludin, claudin and ZO-1) and certain desmosomal proteins (Desmoglein, Envioplakin and to a lesser extent - Plakoglobin and Desmoplakin) as well as protein Afinad. Furthermore, a number of also expressed by p63-positive normal oesophageal epithelial cells cell adhesion proteins, such as N-cadherin and gap junction protein connexins - Cx26, Cx32 and Cx43, are functionally impaired, since they were found abnormally translocated from lateral membranes of cells inside their cytoplasm. siRNA-mediated knock out of p63 protein in oesophageal cancer TE13 cell line resulted in prominent changes in expression of different cell adhesion proteins. p63 ablation initiated expression of tight junction protein Claudine and significantly enhanced expression of another tight junction protein ZO-1, which resulted in better organization of the tight junction itself. It also enhanced expression of desmosomal proteins Desmoglein and Plektin and essentially increased the expression level of adherence junction component E-cadherin. TE13 cells without p63 also displayed improved organization of connexin32 and -43 mediated gap junctions due to translocation of these proteins from cytoplasm into lateral membranes. The obtained results strongly indicate an ubiquitous regulatory role of p63 of cell adhesion machinery in oesophageal epithelium.

**Cell–Cell Adherens Junctions (2642-2646)**

2642
**Alpha-catenin as a Tension Transducer at Adherens Junctions**

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α-catenin is an essential protein for cadherin-based cell-cell adhesion. It binds to cadherin through β-catenin, forming a cadherin/catenin complex. α-catenin can bind also to vinculin, a major component of adherens junctions and vinculin is known to be recruited to adherens junctions through this binding. Recently we have shown that force generation by myosin II is required for vinculin recruitment to adherens junctions. In the present study, we examined the molecular mechanism underlying this force-dependent recruitment. In α-catenin-deficient R2/7 cells, vinculin is never recruited to cadherin molecules. We introduced a number of α-catenin mutants into these cells to identify regions responsible for the force-dependent vinculin recruitment. An α-catenin mutant lacking the C-terminal half recruited vinculin always without force dependency. In an α-catenin mutant lacking only the C-terminal ~60 amino acids, vinculin-recruitment was always inhibited. In vitro binding experiments showed that no other cellular proteins are required for this inhibition. This inhibition was released force-dependently within cells by adding the C-terminal region known to bind to actin filaments. To test the importance of actin-binding, the actin-binding domain of α-catenin was replaced by actin-binding domains of other proteins. An α-catenin mutant with the actin-binding domain of vinculin were able to release the inhibition force-dependently and functioned as α-catenin. We concluded that actin filament-binding and myosin II contractile force are required for α-catenin/vinculin binding within cells and imagined conformational change of α-catenin according to the tension applied on the molecule through actomyosin contraction. Fluorescence recovery after
photobleaching analysis showed that α-catenin in adherens junctions is more stable than that in other regions of lateral membranes. Inhibition of force generation by myosin II also caused instability of α-catenin at adherens junction region. These results indicate that α-catenin dynamics is also influenced by actomyosin force.

2643
Ezrin/Fes Interaction Regulates the Cross-Talk between Cell-Cell and Cell-Matrix Adhesion
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The membrane-cytoskeleton linker ezrin participates in different cellular functions that require the remodeling of cytoskeleton such as cell adhesion, migration, and morphogenesis (Bretscher, et al. 2002, Nat Rev Mol Cell Biol 3-586-99 ; Fiévet, et al. 2007, Biochim Biophys Acta 1773-653-60). Upon stimulation by growth factor or extracellular matrix components, ezrin is phosphorylated and thus can transmit signals from the outside of the cells to downstream effectors (Crepaldi, et al. 1997, J Cell Biol 138-423-34 ; Gautreau, et al. 2000, J Cell Biol 150-193-203). In particular, ezrin phosphorylation by Src family kinases was shown to be important for the control of cell adhesion (Srivastava, et al. 2005, Mol Biol Cell 16-1481-90). In order to understand the molecular connection between ezrin and Src kinases in epithelial cell adhesion, we performed a modified two-hybrid screen with ezrin phosphorylated by a member of the Src family kinases as bait. We report that the Fes kinase interacts directly, through its SH2 domain, with ezrin phosphorylated at tyrosine 477 by Src. In epithelial cells, we show that Fes displays a dual localization depending on cell confluency: in non confluent cells Fes is present in focal contacts whereas in confluent cells Fes localizes to cell-cell contacts. We demonstrate that, in confluent epithelial cells, ezrin recruits Fes at the cell-cell contacts. Moreover Fes recruitment by ezrin at the membrane leads to its activation. Either depletion of ezrin or inhibition of the ezrin/Fes interaction causes the relocation of Fes from cell-cell contacts to focal adhesions. As a consequence, cells in which the ezrin/Fes interaction is impaired show delayed cell spreading and defective HGF-induced scattering. Altogether, these results provide a novel mechanism whereby the ezrin/Fes interaction at cell-cell contacts plays an essential role in cell adhesion and implicates Fes in the cross-talk between cell-cell and cell-matrix adhesion.

2644
E-cadherin Engagement Reorients the Interphase Centrosome Away from Cell-Cell Contacts
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Cell polarity is orchestrated by a variety of extracellular cues and guides events such as chemotaxis, mitosis and wound healing. In scratch wound assays of cell monolayers, it has been demonstrated that the formation of new cell-extracellular matrix (ECM) adhesions as cells spread into the wound orients centrosomes toward the open wound. This polarizing event appears to depend on serum factors and cdc42 signaling. Here, we sought to investigate whether the asymmetry of cell-cell adhesions resulting from monolayer disruption might also contribute to centrosome polarity. By using microengineered substrates to pattern groups of cells while preventing dynamic events such as spreading, we examined whether asymmetries in cell-cell contact were sufficient to drive polarity in kidney epithelial cells. Indeed, cell-cell contact induced centrosomal reorientation to the distal side of the nucleus with respect to the cell-cell contact by displacement of the nucleus toward the contactImportantly, neither siRNA-mediated Cdc42 knockdown nor serum starvation prevented cell polarization, suggesting that this contact-mediated polarity is regulated by a mechanism distinct from cell-ECM mediated polarity. Expression of a dominant-negative E-cadherin randomized nuclear position. Furthermore, blocking E-cadherin, but not serum starvation, inhibited scrape-wound induced cell reorientation. These findings point toward E-cadherin-mediated cell-cell adhesion as a key mediator for defining an axis of cell polarization during processes such as wound healing and developmental patterning.

2645
Crosstalk between Cell-Matrix and Cell-Cell Adhesion on Bi-Functionalized Surfaces
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We study the signaling and mechanical crosstalk between cell-matrix adhesion and cell-cell adhesion in epithelial cells. This crosstalk is of foremost importance since its fine tuning affects important biological functions such as embryo development or wound healing. We focus on its role during early cell-cell contacts. To control the adhesion cues, we functionalize surfaces with distinct patterns of collagen or E-cadherin by micro-contact printing. Collagen and E-cadherin patterns aim at triggering cell-matrix or cell-cell adhesion signaling, respectively. They shape as lines and squares and range over cellular length scales. We visualize on fixed and live cells the recruitment of adhesion proteins at the patterned surface, the organization of the actin cytoskeleton and associated proteins, cell morphology and cell motility. When a cell is spread over a plain E-cadherin surface, we observe the disappearance of focal adhesions, the loss of actin stress fibers and increased cell spreading. When a cell is simultaneously spread over collagen and E-cadherin patterns, it exhibits focal adhesions and stress fibers on collagen patterns only. In addition, it elongates along the direction of patterns interface. These results show that cell-matrix and cell-cell adhesion cues lead to different and localized cytoskeletal rearrangements and cell morphologies that coexist within the cell. Moreover, pattern geometry and spatial distribution affect overall cell shape. Our current efforts focus on designing patterns that could help understand how the balance between localized cytoskeletal arrangements within the cell drives the early steps of cell-adhesion.

2646
Characterization and Functional Importance of the PDZ Binding Domain of WTIP
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Podocyte differentiation is critical for glomerular filtration barrier function and regulated by WT1, a zinc finger transcription factor. We have identified a novel, WT1 interacting protein (WTIP) that maps to human chromosome 19q13.1, a region with familial focal segmental glomerulosclerosis genes. Here we show targeting of endogenous WTIP both in vivo in glomeruli and in vitro in podocyte junctions. WTIP is
expressed in glomeruli in a podocyte specific pattern and co-localizes with synaptopodin. After density gradient ultra centrifugation fractionation using rat glomerular lysates, WTIP co-fractionates with nephrin and caveolin, suggesting association with slit diaphragm proteins in podocyte lipid rafts. In differentiated podocytes in culture, endogenous WTIP localizes at the cell-cell junction. In the intermediate stages of junction formation endogenous WTIP goes to filopodia-like structures which form interdigitated, zipper-like cell-cell contacts. Interestingly other junctional proteins such as ZO-1 and -catenin, are localized as puncta at the base of these specialized structures, which finally seal to form linear cell junctions. We also characterized the C-terminal putative Class-1 PDZ binding domain (PDZBBD) of WTIP and its importance in junction assembly. We were able to immunoprecipitate candidate PDZ domain-containing proteins, Rhophilin-1, MAGI-1, MAGI-2 and MAGI-3 with the full length GFP-WTIP, but not with GFP-WTIP lacking the PDZBBD. When GFP-WTIP lacking the PDZBBD is stably expressed differentiated podocytes, cell junctions were disordered; -catenin and ZO-1 failed to target to the plasma membrane properly and filamentous actin cables were markedly reduced compared to the podocytes expressing GFP-WTIP. In summary WTIP targets to the cell junctions and the PDZ binding domain of WTIP is required for formation of adherens junctions in podocytes. Because WTIP coexists with Nephrin and colocalizes with synaptopodin, it may also play a role in slit diaphragm maintenance in vivo.

**Membrane Receptors (2647-2652)**

2647

**Oligomerization and Intracellular Trafficking of the V2 Vasopressin Receptors**

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Oligomerization of G-protein-couple receptors modulates their functional and pharmacological properties, and their surface expression. In the rat kidney, the V2 vasopressin receptor gene generates, by alternative splicing, two variants, V2a and V2b. While the V2a is transported to the cell surface the V2b is retained inside the cell. Co-expression of V2a and V2b precluded the surface expression of V2a via heterooligomerization between V2a/V2b, which resulted in trapping V2a inside the cell. The aim of this study was to monitor the oligomerization along the trafficking pathway of V2 receptors from the site of synthesis to the cell surface. Using confocal imaging of cells expressing CFP-tagged V2a and YFP-tagged V2b isoforms we showed that the V2b is transported from the endoplasmic reticulum (ER) to ER-Golgi intermediate compartment (ERGIC) and accumulated in the Golgi complex without being transported to the cell surface. Fluorescence Resonance Energy Transfer (FRET) detected V2a/V2b heterooligomers in the ER, ERGIC and Golgi, but not in the cell surface. These results suggest that oligomerization is an early event in the biosynthesis of the V2 receptors, and that the V2b binding to V2a prevent the trafficking of V2a to the cell surface, as the heteroligomer is retained in the Golgi compartment. Supported by grant from FONDECYT 1060158 and DIDUACH

2648

**The Roles of Lipid Rafts in CB1 Cannabinoid Receptor Signaling Is Cell Type Dependent**

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Lipid raft microdomains have been reported to play critical roles in G protein-coupled receptor (GPCR)-mediated signal transduction. CB1 cannabinoid receptor belongs to the rhodopsin family of G-protein-coupled receptor (GPCR) family. Previously, in glioma cells, CB1 receptor has been suggested to exist in lipid rafts, where the CB1 ligand binding and signaling is negatively regulated. However, many other studies have found general phenomena or cell type dependent, N1E-115 neuroblastoma cells expressing endogenous CB1 and HEK293 cells stably expressing recombinant CB1 were used in this study. With both cell lines, we have found that CB1 receptors are associated with lipid rafts. Cholesterol depletion by methyl-β-cyclodextrin (MCD) treatment strongly reduced the flotation of the CB1 protein on the raft-fractions of sucrose density gradients, which was restored by cholesterol repletion, suggesting that CB1 raft-association is cholesterol dependent. Interestingly, cholesterol depletion by MCD inhibited the cannabinoid agonist HU-210-induced phosphorylation of p42/p44 mitogen-activated protein kinase in N1E-115 neuroblastoma cells, but not in HEK293 cells stably transfected with CB1 receptor. In conclusion, our data demonstrate that the roles of lipid rafts in CB1 receptor signaling is cell type dependent.

2649

**Phosphatidylinositides Regulate Ciliary Targeting of PKD-2 and Sperm Activation in C. elegans**

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Ciliary localization of sensory receptors is essential for normal cellular function. In C. elegans, cilia are located at the distal ends of dendrites on sensory neurons, where they perceive environmental cues and modulate worm behavior. The polycystin-1 (PC1) receptor LOV-1 and transient receptor potential polycystin-2 (TRPP2) channel PKD-2 mediate mating behaviors (Barr and Sternberg, 1999). In humans, defects in PC1 or TRPP2 result in autosomal dominant polycystic kidney disease (ADPKD) (Ong and Harris, 2005). Cilia are the site of action for both the human and worm polycystins, but how the polycystins and membrane proteins in general are targeted to cilia is largely unknown. To identify new genes involved in localizing ciliary receptors, we performed a genetic screen looking for PKD-2::GFP ciliary localization defects. We focus here on the my15 mutant. In wild type, PKD-2::GFP and LOV-1::GFP localize to cilia and neuronal cell bodies. In the my15 mutant, both PKD-2::GFP and LOV-1::GFP are evenly distributed throughout the entire neuron including the cillum, dendrite, cell body, and axon. Interestingly, my15 mutants also exhibit a sperm defect in both male and hermaphrodite. my15 males produce normal spermatozoa that fail to activate, resulting in immotile and nonfunctional spermatozoa. Sperm activation in C. elegans involves membrane fusion. Our data suggest that the gene mutated in my15 animals may regulate ciliary targeting of PKD-2 containing vesicles in sensory neurons and membrane fusion during sperm activation. my15 is a nonsense mutation in a phosphatidylinositol (PI) 5-phosphatase gene. PI metabolism is important for many cellular processes including membrane trafficking. We are
testing the hypothesis that a common PI-mediated pathway regulates membrane trafficking in both male-specific sensory neurons and sperm. Our study will provide and an understanding of how a C. elegans male successfully navigates the complexities of mating, from copulatory behavior to sperm activation.

2650

Dynamic Imaging of Receptor-G Protein Interactions: Single-Molecule Microscopy Reveals Dynamic Interactions between Chemotactrant GPCR and Heterotrimeric G-proteins in Membrane of Living Cells

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Ligand binding to GPCR triggers signaling events through heterotrimeric G-proteins. But it is not clear whether receptors and G-proteins form receptor/G-protein complexes in the membrane prior to receptor activation. Here, we have used single-molecule with TIRF microscopy and FRAP imaging to characterize the behavior of YFP-tagged cAR1 GPCR and heterotrimeric G proteins in Dictyostelium discoideum. In the absence of cAR1 receptor activation, cAR1 proteins diffuse significantly more slowly than G-proteins in the membrane, suggesting that receptors and G-proteins are not associated in a complex. Upon activation of cAR1, the receptors increase their mobility while G-proteins slow down their movement. When the receptors are fully activated, a large population of receptors and G-proteins display similar diffusion co-efficiency. Our work suggests that ligand binding to cAR1 receptors promotes dynamic interactions between the activated receptors and heterotrimers G-proteins and thus facilitates activation of heterotrimeric G-proteins in living cells.

2651

A Novel Mechanism for STAT3 Activation by Estrogen

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Estrogen is the anorexigenic steroid hormone which can regulate synaptic plasticity in POMC neurons of hypothalamus, affecting energy balance. Estrogen-induced activation of STAT3 in the brain is essential for its anorexigenic effect in mice. But, molecular mechanisms how estrogen activates STAT3 signaling has not been investigated. Generally estrogen produces various cellular effects, many of which are through the activation of estrogen receptors α and β. GRP30 is a G protein-coupled receptor and recently identified as an estrogen-binding receptor which can mediate rapid intracellular signaling evoked by estrogen. Here we describe a new mechanism of STAT3 activation that involves GPR30. Estrogen didn’t activate STAT3 in HeLa cells which doesn’t express estrogen receptor α. Transfection of GPR30 in HeLa cells demonstrates that GPR30 is the receptor mediating the effect of estrogen on STAT3 activity. Treatment of HeLa cells expressing GPR30 with estrogen also induced calcium mobilization showing that GPR30 can provoke rapid signaling cascade involving second messengers in the GPR30-expressing cell system. We speculate that estrogen activates STAT3, at least in part, through the activation of GPR30. [This work was supported by Brain Korea 21 Project for Medical Sciences.]

2652

Changes in Cell Surface DCC at the Promotion of Neurite Branching by Its Ligand Netrin-1 in Hamster Primary Cortical Neurons

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DCC (deleted in colorectal cancer), a membrane receptor for an axon guidance cue netrin-1, is known to play an important role in the correct wiring of the central nervous system. In rat spinal commissural axons where DCC-mediated chemotaxis is observed, it is reported that intracellular vesicular pool of DCC exists and plasma membrane insertion of DCC occurs in response to netrin-1 / protein kinase A. On the other hand, netrin-1 is known to promote axon branching in hamster cortical neurons. We are interested in the events that would happen at the plasma membrane of the cortical neurons in response to netrin-1, in the course of intracellular trafficking of DCC in the neuronal cells. To address this issue, we analyzed the changes in the level and distribution of cell surface DCC in hamster primary cortical neurons treated by netrin-1, using cell-surface labeling technique as well as total internal reflection fluorescence microscopy. Elevation of the DCC level and formation of cluster-like structure of DCC were observed at the cell surface of the neurons in which neurite branching was promoted by netrin-1. We hypothesize the involvement of exocytosis in these surface DCC changes. (Supported by Grant-in-aid for Young Scientists (B) No. 19790157 from the Ministry of Education, Culture, Sports, Science and Technology, Japan.)

Membrane Channels (2653-2657)

2653

FSH-induced Gα/Phospholipase C-δ1 Signaling Mediating a Non-capacitative Ca\(^{2+}\)-Influx through T-type Ca\(^{2+}\)-Channels in Rat Sertoli Cells

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Our previous study demonstrated that follicle-stimulating hormone (FSH)-induced immediate Ca\(^{2+}\)-influx in rat Sertoli cells (SCs) is mediated by Gα/phospholipase C-δ1 (PLC-δ1) signaling pathway. As to which Ca\(^{2+}\)-channel is responsible for such Ca\(^{2+}\)-influx is not understood. In this study, thapsigargin, which triggers an in-stored calcium release, evoked a 1.5-fold elevation of intracellular Ca\(^{2+}\) in Ca\(^{2+}\)-free media. The re-addition of CaCl\(_2\) (2.5 mM) to FSH-pretreated and thapsigargin-sensitized SCs in Ca\(^{2+}\)-free media immediately elicited a 2-fold of second intracellular Ca\(^{2+}\) elevation. The addition of Ca\(^{2+}\) chelator, EGTA (0.2 mM), reduced the FSH-induced elevation of intracellular Ca\(^{2+}\) in SCs cultured without CaCl\(_2\).
However, the pretreatment with dantrolene (25 μM), which inhibits in-stored calcium release, did not affect the FSH-induced elevation of intracellular Ca²⁺. The Confocal Microscopic observation revealed that FSH-induced intracellular Ca²⁺-elevation is caused by an extracellular Ca²⁺-mobilization. NiCl₂ (10 μM), a T-type calcium channel blocker, abolished the FSH-induced SC Ca²⁺-influx. Furthermore, mibefradil (10 and 100 μM), another specific blocker for T-type Ca²⁺-channels, dose-dependently suppressed the FSH-induced Ca²⁺-influx. Whereas, nifedipine (10 and 50 μM) or o-conotoxin GVI (100 and 500 nM), blocker of L- or N-type Ca²⁺-channels, respectively, did not affect the FSH-induced SC Ca²⁺-influx. On the other hand, FSH-induced Ca²⁺-influx was significantly reduced by the pretreatment of SCs with myristoylated PLC-δ1 synthetic peptide (0.1 and 1 μM), but not interfered by 2',5'-dideoxyadenosine (3 and 15 μM), a selective inhibitor of adenylate cyclase. In conclusion, the FSH-induced Gou/HPLC-61 pathway-dependent Ca²⁺-influx of rat SCs is mediated by T-type Ca²⁺-channels and independent of in-stored-calcium release. This study was supported by 94-CGH-TMU-06 and NSC93-2314-B-038-027, Taiwan, Republic of China.

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Novel Kv3 Glycforms Differentially Expressed in Adult Mammalian Brain Contain Sialylated N-Glycans

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The N-glycan pool of mammalian brain contains remarkably high levels of sialylated N-glycans. This study provides the first evidence that voltage-gated K⁺ channels, Kv3.1, 3.3, and 3.4, possess distinct sialylated N-glycan structures throughout the central nervous system of the adult rat. Electrophoretic migration patterns of Kv3.1, 3.3, and 3.4 glycoproteins from: spinal cord, hypothalamus, thalamus, cerebral cortex, hippocampus, and cerebellum membranes digested with glycosidases were used to identify the various glycoforms. Differences in the migration of Kv3 proteins were due to the desialylated N-glycans. Protein expression patterns of Kv3.1 and 3.3 revealed similarities with the highest protein levels observed in cerebellum, and the lowest levels in thalamus and hypothalamus. However, Kv3.3 protein levels were higher in spinal cord than both cerebral cortex and hippocampus while those for Kv3.1 were quite similar to each other. The difference between the Kv3.4 protein and both Kv3.1 and 3.3 proteins was that both cerebellum and hippocampus expressed the highest levels while the other four regions expressed lower levels. We suggest that novel Kv3 glycoforms differentially expressed throughout the central nervous system may be an important component of these channels in: cell recognition events, targeting to specific subdomains of neurons, and in modulating channel activity.

2655

Basic 1D NMR: ²³Na Transport through Vesicles Mediated by Ionophores

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Ionophore antibiotics are substances commonly incorporated in animal feeds to promote growth and prevent diseases. They are produced by bacteria and function as ion transporters across cell membranes. Ionophores are classified into three groups: neutral, carboxylic and quasi ionophores. The overall goal of our research is the characterization of cation ionophoric transport by 1D and 2D ²³Na-NMR. The specific goal of this project was the development of basic 1D ²³Na-NMR protocols needed to study cation transport mediated by all three ionophore groups. We chose for this study Nonactin (neutral), Monensin (carboxylic) and Amphoterin B (quasi ionophore), all known cation transporting ionophores. Our data showed that Nonactin does have a strong preference for cations other than Na⁺. After the addition of 0.5 μL · 10⁻² M of 20mM stock solution of ionophore to LUV solutions the ²³Na-NMR line width did not change more than 2Hz. Monensin studies did not show any significant NMR line broadening either after the addition of 0.25 μL or 0.50 μL of 20mM ionophore. However, after the addition of 0.75 μL and 1μL the line width exhibited clear broadening (25 and 35 Hz respectively) after just 30 minutes. From these results we confirmed that Monensin had the ability to transport sodium ion. We are currently testing the ability of the quasi channel Amphoterin B to transport sodium in a similar manner. Literature shows that it should be able to do so, but that it will require the presence of sterols in the LUV preparation. We are in the process of carrying out these experiments.

2656

Molecular and Cellular Mechanisms of Fish Calcium Uptake: Zebrasfish as a Model

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Freshwater fish absorb calcium predominantly from ambient water, and more than 80% of calcium uptake is achieved by active transporting through gill mitochondria-rich cells (MR cells). In the current model for calcium uptake in gill MR cells, calcium enters passively the cytosol via the epithelium calcium channel (ECaC), and then, is extruded into the plasma through the basolateral sodium-calcium exchanger (NCX) and plasma membrane calcium-ATPase (PMCA). However, no convincing molecular and cellular evidence about the calcium transporters was available to support this model. Zebrasfish (Danio rerio) is a good model for analyzing isoforms of a gene because of the complete genome database and abundant ESTs (expression sequence tag) data. By a strategy of BLAST from the zebrasfish genome database (Sanger Institute), 6 isoforms of PMCAς (PMCA1a, PMCA1b, PMCA2, PMCA3a and PMCA4) and 7 isoforms of NCXς (NCX1a, NCX1b, NCX2a, NCX2b, NCX3, NCX4a and NCXb) were identified. In reverse transcriptase-PCR analysis, 5 PMCAς and 2 NCXς were expressed ubiquitously in various tissues including gill. From the results of triple fluorescence labeling, the mRNAs of PMCA2 and NCX1b were co-localized with ECaC in MR cells. The gene expressions in gill of high- or low-calcium acclimated zebrasfish by quantitative real-time PCR analysis showed that ECaC was the only one gene regulated by the environmental calcium levels. The present study provided the molecular evidence to support the current calcium uptake model, and ECaC was concluded to play the major regulatory target for this mechanism during environmental challenges.

2657

The Computational Prediction of the Cold and Menthol Activated Calcium Ion Gated Channel Transient Receptor Potential Melastatin 8

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The calcium gated ion channel Transient Receptor Potential Malestatin 8 (TRPM8) is a member of the superfamily of cation gated channels. Like many transmembrane proteins, crystallization has not yet been accomplished leaving little data about tertiary structure and stimuli response mechanisms such as voltage gating. This study offers a 3D prediction based on homology modeling using the program Molecular Operating Environment (MOE) 2006.08. We searched the Protein Data Bank (PDB) to select template structures and built ten separate homology models for each segment with the final product being the best intermediate. Homology modeling for the N-terminal end (residues 1-691) suggests a repeat domain similar to both those of “death domains” associated with apoptosis, and ankyrin repeats like those found in the TRPV class. Models of subsequent residues predicted an overall helix-turn-helix motif throughout the transmembrane domain (692-979) that may culminate in a coiled-coil at the C-terminus (1055-1104). The overall coiling/helix structure and pattern of hydrophobicity of both the transmembrane region and the C-terminal end suggests a channel assembly and voltage response mechanism analogous to KvAp and Kv7 class channels.

Structure and Function of Membrane Proteins (2658-2661)

2658

Protein Interactions of the Growth-related, Type IIc Renal Na/Phosphate Co-Transporter
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The sodium/phosphate (Pi) cotransporter NaPiIIc is responsible for at least 30% of the renal Pi reabsorption. It is also differentially regulated compared to NaPiIIa, the main Pi re-absorber, despite a similar molecular structure. These differences could be explained by the type of proteins the two transporters interact with. In this work, we have identified proteins that interact with NaPiIIc using bacterial and mammalian two-hybrid systems as well as coimmunoprecipitation. We have found that NaPiIIc interacts with the PDZ domain-containing proteins NHERF1 and NHERF3 via novel binding motifs in its C-terminus. Immunoprecipitation of endogenous NaPiIIc from rat proximal tubular brush border membranes co-precipitates both NHERF1 and NHERF3, with the coprecipitated NHERF3 more abundant in rats chronically fed a low Pi-containing diet. In addition, NaPiIIc colocalizes with both NHERF1 and NHERF3 at the proximal tubule brush border membrane of rats fed either a low or high Pi diet. In opossum kidney cells mouse NaPiIIc is expressed mainly in the apical microvilli and in the trans-Golgi. Both confocal microscopy and total internal reflection microscopy show that NaPiIIc colocalizes with NHERF1 and NHERF3 in the apical microvilli, and this is not altered by truncation of the last three amino acids of NaPiIIc, which abolishes the interaction with NHERF3 but not with NHERF1. Finally, the interactions of NaPiIIc with NHERF1 and NHERF3 are modulated by MAP17 similarly to those with NaPiIIa, i.e. only the MAP17-NaPiIIc-NHERF3 complexes are internalized to the trans-Golgi. In conclusion, NaPiIIc interacts with a limited number of PDZ proteins, and the mechanisms and consequences of such interactions differ from those of NaPiIIa.

2659

Electron Crystallography Studies of Human Multi-Drug Resistance Protein (MDR1)
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ATP-binding cassette (ABC) transporters are integral membrane proteins that utilize energy derived from ATP hydrolysis to transport molecules across cell membranes. A subgroup of ABC transporters are responsible for the efflux of hydrophobic drugs and lipids, and play an important role in multi drug resistance. The most widely studied of this subgroup of ABC transporters is the human multi drug resistance protein, P-glycoprotein, or human MDR1/ABCB1. This protein is the major factor responsible for endowing chemotherapeutic resistance to tumor cells. The objective of our work is to better understand the mechanism by which MDR1 utilizes ATP hydrolysis to achieve transport. To this end, we have grown 2D crystals of lipid-reconstituted MDR1 in various nucleotide states and will utilize electron crystallography to solve these structures. Crystals of MDR1 in the presence and absence of the non-hydrolyzable ATP analog AMP*PNP were successfully grown. Future studies will use diffraction data from these 2D crystals to solve the MDR1 structure with and without AMP*PNP, and with focus on obtaining crystals for other nucleotide states.

2660

A Functional Role for the Putative Cholesterol Binding Motif in the Influenza A Virus M2 Protein
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Influenza A virus assembly occurs at glycolipid rafts and at least two viral proteins, hemagglutinin (HA) and neuraminidase (NA), have been shown to localize to lipid rafts and play key roles in particle assembly. The influenza A virus M2 protein, an integral membrane protein whose ion channel activity is required during virus entry, is also incorporated into virus particles but does not associate with lipid rafts. The M2 protein is incorporated into virus particles at low levels when compared to either HA or NA, but its incorporation is critical for the production of infectious virus particles. Several cellular and viral proteins are known to bind cholesterol via a cholesterol recognition/interaction amino acid consensus (CRAC) motif, L/V-Y-X1,5-Y-X1,5-Y/R/K. Because cellular cholesterol is enriched in lipid rafts, integral membrane proteins containing this motif are believed to localize in or immediately adjacent to lipid rafts. The cytoplasmic tail of some influenza A virus M2 proteins contains this putative CRAC motif. Because this CRAC motif is not universally conserved in influenza A virus isolates, we hypothesized that it may be important but not essential for M2 function. Alteration of the CRAC motif in virus strains that encode this sequence resulted in decreased virus production from infected MDCK cells. Conversely, reconstitution of this motif in strains lacking it had a less significant effect on virus production. This data establishes a functional role for the putative cholesterol binding motif in the influenza A virus M2 protein.
Functions of the Membrane Phosphoinositide-binding and Palmitoylation of EW12/PGRL
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Immunoglobulin superfamily (IgSF) member EW1-2/PGLR associates with tetraspanins and regulates cell motility. The short cytoplasmic domain of EW1-2 is positively charged: five out of ten residues are basic. We demonstrated that EW1-2 cytoplasmic tail specifically interacts with phosphatidylinositol phosphates (PIPs) but not with most of other membrane lipids. Mutation of basic residue-clusters in EW1-2 cytoplasmic tail abolishes the PIP binding, and the PIP bindings are also determined by the positions of basic residues in EW1-2 cytoplasmic tail. In addition, EW1-2 is constitutively palmitoylated and the palmitoylation occurs at the cytoplasmic cysteine residues located at the N-terminal of those basic residues. The PIP interaction and palmitoylation appear to be independent events since the mutations of these basic residues did not significantly alter EW1-2 palmitoylation and the palmitoylation was not required for the PIP bindings. Functionally, EW1-2 PIP interaction and EW1-2 palmitoylation regulates cell migration, cell proliferation. Together, our study elucidated a novel mechanism of transmembrane protein-PIP interaction and the functional consequences of the membrane PIP binding and palmitoylation of EW1-2. *These authors made equal contributions to this work **To whom correspondence should be addressed, at Vascular Biology Center, Cancer Research Building Room 220, 19 S Manassas, Memphis, TN 38163. Tel: 901-448-3448; Fax: 901-448-7181; E-mail: xazhang@utmem.edu

Membrane Fusion (2662-2664)

The Requirement for Cholesterol in Membrane Repair Indicates a Possible Mechanism by Which Statins Cause Muscle Injury
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Plasma membrane disruptions occur in skeletal muscle, and many other cell types, under physiological conditions. Wounded cells repair these disruptions using a patch mechanism. Calcium entering through a disruption initiates homotypic fusion of intracellular membranes. This erects a 'patch' that is then annealed to the plasma membrane by exocytotic fusion. We report that depletion of cholesterol from BSC-1 cells using methyl-beta-cyclodextrin (MBCD) dose dependently inhibits membrane repair. Given recovery time (60 min) in the absence of MBCD these cells can partially restore repair capacity, presumably by replacing depleted membrane cholesterol from endogenous sources. Incubation of cells with a statin (Lovastatin) did not, by itself, inhibit membrane repair. However, pre-incubation with statin impaired the ability of cells to recover repair capacity after MBCD treatment. Full recovery was observed after the addition of exogenous cholesterol. We conclude that plasma membrane cholesterol is required for membrane repair, and that statins, by limiting the availability of cells to replenish cholesterol by an endogenous pathway, can interfere with repair recovery after cholesterol depletion. Since a major side-effect of statin therapy in humans is muscle injury, and since muscle injury can result from defective repair, we propose that one mechanism of statin toxicity might be inference in muscle with the fusion-mediated repair mechanism.

Identification of a Polymerization-dependent Association between Actin and SNAREs
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Traditionally, the actin cytoskeleton has been viewed as a barrier to exocytosis, requiring depolymerization prior to vesicle docking and membrane fusion at the plasma membrane. However, we have shown that drugs that stimulate either F-actin depolymerization or F-actin formation inhibit membrane fusion. This suggests that actin remodeling (i.e. de- and re-polymerization) is required membrane fusion. Using a sensitive in vitro pyrene-actin polymerization assay, we found that purified vacuole membranes stimulate actin polymerization, and this activity increases when vacuoles are incubated in conditions that support membrane fusion. To further confirm the need for actin polymerization during membrane fusion, we have examined an actin polymerization-deficient mutant strain. This strain shows in vivo vacuole fusion defects, and actin purified from this strain also inhibits vacuole fusion in vitro. Affinity isolation of vacuole-associated actin revealed a polymerization-dependent interaction between actin and the SNARE Ykt6p. In solution binding experiments we found two SNAREs, Nyv1p and Ykt6p, interact with F-actin. SNAREs are key components of the fusion machinery; they catalyze membrane fusion by forming a tightly bound complex of four-helical bundles via coiled-coil domains. Interestingly, Nyv1p and Ykt6p, both have additional N-terminal extensions called longin-domains. Taken together, our results suggest that actin polymerization is a subreaction of vacuole membrane fusion, interacting with the core fusion machinery via longin-domain containing SNAREs. (We acknowledge the CIHR for grant support)

Characterization of the Functional Role of VSM-1 in C. elegans
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VSM-1 is a highly conserved protein that was identified as a synaptobrevin binding partner capable of inhibiting SNARE complex formation. Analysis of VSM-1 mutant yeast as well as overexpression data suggest that VSM-1 negatively regulates constitutive exocytosis by limiting priming [1]. To better understand VSM-1 function in vivo and to test its role in synaptic vesicle exocytosis we began characterizing the C. elegans VSM-1 homolog (CO1G5.6). First, we established that C. elegans has a VSM-1 homologue by performing a BLAST search of the C. elegans protein sequences database (Wormpep) using the yeast VSM-1 protein (accession # AAC18522). The search results revealed a predicted VSM-1 protein with
strong homology to yeast VSM-1 (40% identity and 60% similarity). Moreover, expression of the predicted VSM-1 gene fused to GFP and driven by upstream regulatory sequences demonstrate that this protein is ubiquitously expressed at low levels in adults but is up-regulated in the intestine during larval development. Furthermore, integrated transgenic lines overexpressing the fusion protein VSM-1::GFP displayed abnormal gonad development, deficiencies in reproduction and significantly reduced longevity. Taken together these data suggest that intestinal overexpression of VSM-1 may inhibit exocytosis of important signaling molecules that regulate longevity and reproduction pathways.

Golgi to Cell Surface Transport (2665-2666)

2665

Genetic and Biochemical Analysis of Avl9 Function in Yeast and Mammalian Cells
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Multiple exocytic transport routes has made it difficult to identify components of the machinery involved in forming exocytic transport vesicles at the Golgi and endosomes. Mutations that primarily affect one transport route usually result in cargo missorting and transport by an alternative route, making such defects difficult to detect in mutant screens for transport blocks. A triple-mutant synthetic lethality screen in yeast for mutants that are defective in forming exocytic vesicles led to the identification of a previously uncharacterized protein, Avl9p (Harsay and Schekman, MBoC 2007). Phylogenetic sequence analysis showed that Avl9p is a member of a novel superfamily of ancient eukaryotic paralogs, most of which are still uncharacterized. Localization of myc-tagged human Avl9 in 293-T cultured cells is consistent with a role for Avl9 in exocytic vesicle formation and transport. Avl9p overexpression in yeast causes vesicle accumulation and an exocytic transport defect. This phenotype, which is toxic, can be efficiently suppressed by overexpression of an N-terminal actin fragment, suggesting that Avl9p may function in an actin-mediated process. A cytoplasmic deacetylase, Hst1p, is a weaker suppressor of Avl9p toxicity. Although this suppression may be indirect, bioinformatic analysis suggests that Avl9 superfamily proteins may interact with a deacetylase, so the suppression could indicate that Avl9p function is directly regulated by acetylation. For example, acetylation could regulate membrane association of Avl9p. A lipid-protein overlay experiment suggests that Avl9p may interact directly with phospholipids.

Activation of ADP-ribosylation Factor (Arf) Regulates Biogenesis of the ATP7A-containing trans-Golgi Network Compartment and Its Cu-induced Trafficking
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Copper is a vital co-factor for many enzymes, consequently the body is faced with maintaining a delicate balance, supplying sufficient amounts but conversely effluxing excess from the cells to avoid copper toxicity. The copper transporter ATP7A plays a key role in copper homeostasis by translocating cytoplasmic copper across membranes. This is underscored by the genetically inherited disorder Menkes disease in which this copper ATPase is mutated or deleted. Under basal copper levels ATP7A resides predominantly within a compartment of the trans-Golgi network (TGN). In response to increased copper load ATP7A traffics en masse to the plasma membrane (PM) or vesicles close to the PM. The mechanisms that regulate the biogenesis of the ATP7A compartment and the trafficking of ATP7A are unclear. Here, we show the biogenesis of the ATP7A compartment requires activation of Arf1 GTPase, shown previously to facilitate the biogenesis of the Golgi ribbon. Preventing activation of cellular Arf1 by (1) expressing an inactive "empty" form of Arf (Arf1/N126I), (2) expressing an inactive form of GBF1 (GBF1/E794K), guanine nucleotide exchange factor for Arf1, or (3) treating cells with Brefeldin A (BFA), an inhibitor of GBF1, disrupts ATP7A into a diffuse pattern. Importantly, preventing Arf activation inhibits copper-responsive trafficking of ATP7A to the PM. Our findings support a model in which active Arf is essential for the generation of the ATP7A compartment and for copper-responsive trafficking of ATP7A from here to the PM. Our findings provide an exciting foundation for identifying Arf1 effectors that facilitate the biogenesis of the ATP7A compartment and ATP7A traffic.

Membrane Domains and Polarity (2667-2669)

2667

Cellular Nonlinear Creep Response Depends on the Type of Membrane Anchor
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The physical properties of the plasma membrane have been probed by a number of methods, from high speed video to experiments with the laser trap. Many interesting phenomenon have been observed, from subdiffusive to superdiffusive behavior, caused by structures (somewhat hypothetical) such as corrals and lipid rafts [1,2,3]. We chose to look at the behavior of beads anchored to either the outer leaflet of the plasma membrane or anchored through a transmembrane link to the cytoskeleton. We used antibodies to glycosyolphosphatidylinositol (GPI) anchored or B1 integrin transmembrane receptor to specify the type of linkage expected. We report a new analysis approach, i.e. looking at the spectral characteristics of the thermal fluctuations of the bead over time while a force pulse is applied. We show how this approach reveals subtle differences in the diffusion and the creep response depending upon the nature of bead anchoring. We observed a nonlinear suppression in the thermal fluctuations upon application of force for GPI-anchored beads but not that of B1 anchored beads, which may be explained by the nature of the anchoring and the role of membrane skeleton.
2668

Oxysterol Binding Protein Homologs Localize to Organellar Junctions and Mediate Contacts with Lipid Droplets

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The specific mechanisms responsible for transporting lipid into and out of lipid particles remain unclear. Oxysterol binding proteins, a family distinguished by a unique conserved sterol-binding domain, have recently been identified as sterol transporters in yeast. Here we identify two yeast oxysterol binding protein homologs (OSH), Osh6p and Osh7p, as potential lipid droplet-associated sterol transporters. Purified Osh6p and Osh7p strongly promote an energy-dependent association between purified lipid droplets and purified microsomes, and as a result the normally buoyant droplets sediment with the dense microsomal fraction during high-speed centrifugation. Osh6p and Osh7p also induce the tight apposition of negatively-charged liposomes. GFP-tagged Osh6p and Osh7p localize primarily to the cell periphery and co-localize with an RFP-tagged endoplasmic reticulum marker (Sec63p), suggesting a preference for cortical ER tubules. Based on the collected evidence, we speculate that Osh6p and Osh7p localize to membrane contact sites and promote contacts between ER and lipid droplets, facilitating sterol transfer between these organelles.

2669

Detection of Intracellular Phosphatidylserine in Living Cells

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Phosphatidylserine (PS) is the major anionic phospholipid in mammalian cell membranes and participates in important cellular processes by interacting with signaling proteins. The intracellular distribution of PS has been studied 20-30 years ago by chemical and enzymatic methods but generated ambiguous results. Here, we investigate the intracellular PS distribution in neuronal cells, neuroblastoma cells and hippocampal neurons by visualization of GFP-AnnexinV in transfected cells. Cells were stimulated with a calcium ionophore and the localization of GFP-AnnexinV was monitored by fluorescence microscopy. Initially, GFP-AnnexinV distributed evenly in the cytosol and nucleus. Raising the intracellular calcium level with ionomycin induced translocation of cytoplasmic GFP-AnnexinV to the plasma membrane but not to the nuclear membrane, indicating that PS distributes in the cytoplasmic side of the plasma membrane. Nuclear GFP-AnnexinV subsequently translocated to the nuclear membrane, indicating PS localization in the nuclear envelope. GFP-AnnexinV also localized in a juxtanuclear organelle that was identified as the recycling endosome. However, minimal fluorescence was detected in any other subcellular organelles including mitochondria, endoplasmic reticulum, Golgi complex and lysosomes, strongly suggesting that PS distribution in the cytoplasmic face of these organelles is negligible. Similarly in hippocampal primary neurons, PS distributed in the inner leaflet of plasma membranes of cell body and dendrites, and in the nuclear envelope. To our knowledge, this is the first demonstration of intracellular PS localization in living cells, providing an insight for specific sites of PS interaction with soluble proteins involved in signaling processes.

Exocytosis: Plasma Membrane Events (2670)

2670

Role of Tetraspanin-enriched Microdomains in HIV-1 Budding

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Human immunodeficiency virus type 1 (HIV-1) is a complex retrovirus that is highly dependent on cellular mechanisms for successful replication, particularly with regard to virus assembly and budding. These processes are driven by the viral protein Gag, which alone is sufficient for the formation of virus-like particles (VLPs). The nonreplicating, self-assembled VLPs can be released from eukaryotic cells by budding, and are morphologically and antigenically similar to immature virions. An essential prerequisite to budding is aggregation of Gag into membrane microdomains. It has been shown that Gag accumulates specifically at sites on the plasma membrane enriched in a network of cellular proteins known as tetraspanins, a large family of transmembrane proteins that maintain a role in laterally organizing cellular membranes. We sought to determine the functional requirement for tetraspanins in Gag-mediated budding of VLPs. Using epifluorescence microscopy and flow cytometry, HeLa cells were shown to express the tetraspanins, CD9, CD63, CD81, and CD82, on their surface to varying degrees. The tetraspanins appear as discrete, punctate structures on the cell surface. HeLa cells transfected with Gag-GFP results in the production of VLPs that appear as domains of varying size as determined by epifluorescence microscopy. Examination of these cells using scanning electron microscopy reveals the presence of 165 nm particles on the plasma membrane. VLP release from the Gag-GFP-transfected cells was confirmed using a standard ELISA assay. To determine the functional requirement for tetraspanins in Gag-mediated VLP budding, siRNA technology was used to knockdown tetraspanin expression. In Gag-GFP-transfected HeLa cells wherein either CD63 or CD81 expression was decreased by at least 90%, Gag-mediated budding of VLPs was reduced by 75% and 80%, respectively. These findings suggest that Gag interactions at tetraspanin-enriched microdomains are necessary to promote optimal budding. Supported by NIH GM 41402, UNC CFAR P30 AI50410, and NIH T32 CA 09156.

Exocytosis: Regulated Secretion (2671-2674)

2671

Mast Cells Possess Distinct Secretory Granule Subsets Whose Exocytosis Is Regulated by Different SNARE Isoforms

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Mast cells degranulate and release the contents of intracellular secretory granules in response to the crosslinking of FceRI by multivalent antigens. These granules contain a variety of biologically active inflammatory mediators, however it is not clear whether these granules are homogenous or
whether there is heterogeneity within the secretory granule population in mast cells. Using genetically-altered mice lacking specific vesicle-associated SNARE membrane fusion proteins, we found that VAMP-8-deficient mast cells exhibited defects in FceRI-regulated exocytosis while synaptobrevin-2- or VAMP-3-deficient mast cells did not. Surprisingly, the defect in secretion in VAMP-8-deficient mice was limited to the subpopulation of mast cell secretory granules containing serotonin and cathepsin D, whereas regulated exocytosis of secretory granules containing histamine and TNF-α was normal. Confocal microscopy confirmed that serotonin and histamine were present in distinct intracellular granules and that most serotonin-containing granules were VAMP-8-negative. Thus this study demonstrates that mast cells do indeed possess distinct subsets of secretory granules and that these subsets use different SNARE isoforms for exocytosis.

2672
Myosin Va Mediates Docking of Secretory Granules at the Plasma Membrane
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Myosin Va (MyoVa) is a prime candidate for controlling actin-based organelle motion in neurons and neuroendocrine cells. Its function in secretory granule (SG) trafficking was investigated in enterochromaffin cells by wide field and total internal reflection fluorescence microscopy (TIRFM). The distribution of endogenous MyoVa partially overlapped with SG and microtubules. Impairing MyoVa function by means of a truncated construct (MyoVa tail) or RNA interference prevented the formation of SG-rich regions at the cell periphery and reduced SG density in the subplasmalemmal region. Individual SG trajectories were tracked to analyze SG mobility. A wide distribution of their diffusion coefficient, Dxy, was observed. Almost immobile SG (Dxy < 5 x 10^-9 µm^2.s^-1) were considered as docked at the plasma membrane based on two properties: (i) SG that undergo exocytosis have a Dxy below this threshold value for at least 2 s before fusion; (ii) a negative autocorrelation of the vertical motion was found in subtrajectories with a Dxy below the threshold. Using this criterion of docking, we found that the main effect of MyoVa inhibition was to reduce the number of docked granules, leading to reduced secretory responses. Surprisingly, this reduction was not due to a decreased transport of SG toward release sites. In contrast, MyoVa silencing reduced the occurrence of long-lasting, but not short-lasting, docking periods. We thus propose that, despite its known motor activity, MyoVa directly mediates stable attachment of SG at the plasma membrane.

2673
Involvement of Rac1 in Neuroendocrine Cell Secretion: Regulator and Effector Pathways
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Neuroendocrine cells release hormones and neuropeptides by exocytosis, a regulated process in which secretory granules fuse with the plasma membrane to release their content in response to a calcium trigger. In chromaffin cells, we previously reported that regulated exocytosis is regulated by RhoA and Cdc42, two RhoGTPases. Rac1, another RhoGTPase member at the cell periphery in chromaffin cells, is specifically activated during exocytosis suggesting a potential role of this GTPase during exocytosis. The aim of the present study is to dissect Rac1 effector and regulator pathway during secretory response. Using neuroendocrine PC12 cell line as a secretory model and RNA interference (RNAi) strategy, we demonstrated that Rac1 is an essential component of the exocytotic machinery. RhoGTPases act as molecular switches that cycle between inactive (GDP-bound state) and active (GTP-bound state) form. Rac1-GTP recognizes its effectors that lead to generate a specific response. To investigate one possible effector pathway, we focused our research on Phospholipase D1, an enzyme which produces phosphatidic acid during the late stages of exocytosis in neuroendocrine cells. Interestingly, silencing of Rac1 by RNAi prevents the activation of PLD1 required for secretion in PC12 cells. Then, these findings raised the question of how Rac1 is activated during exocytosis. β-Pix, a guanine nucleotide exchange factor for Rac1 and Cdc42, has been recently shown to be involved during secretion in PC12 cells and thus, appears as a likely candidate to fulfill this function. Silencing of β-Pix by RNAi was found to inhibit exocytosis and prevent secretagogue-induced activation of Rac1 indicating β-Pix is a factor which can activate Rac during exocytosis. Altogether, our results demonstrate for the first time that secretagogue-evoked stimulation induces the sequential ordering of β-Pix, Rac1 and phospholipase D1 at the plasma membrane, thereby providing lipid modification that makes the exocytotic machinery more efficient.

2674
Two Distinct Functional Domains in the Golgi-associated Vps74p for the Processing of Gas1p and the Apical Growth
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In Saccharomyces cerevisiae, apical bud growth occurs for a brief period in G1 when the deposition of membrane and cell wall is restricted to the tip of the growing bud. Vps74p was reported to alter the elongated bud morphology of cdc34-2 cells arrested in the apical growth phase. Here, we show that Vps74p is a Golgi-associated phosphorylated protein and the localization at the Golgi is dependent on its C-terminal domain, but not the phosphorylation or the N-terminal domain. Vps74p is not involved in known exocytic transport to the vacuole and endocytic pathways. Deletion of C-terminus, but not N-terminus, of Vps74p affects cell wall integrity, although both domains are involved in the glycosylation of a GPI-anchored protein Gas1p. Although deletion of Gas1 affected the elongated bud morphology of different cell-cycle mutants (cdc34, cdc4 and cdc28), only Vps74p and Arf1p, but not Arf1p, can specifically alter morphology of cdc34-2 cells. Together, we infer that Vps74p contains two distinct functional domains, one for processing the glycosylation of Gas1p and the other for modulating a specific vesicular transport involved in apical growth.
Endocytosis (2675-2681)

2675

**Dynamin-mediated Endocytosis Is Required for Sea Urchin Embryo Cytokinesis**
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The GTPase dynamin plays an important role in clathrin-mediated endocytosis (Slepnev and De Camilli, Nat Rev Neurosci. 2000 1(3):161-72). Recent studies suggest that dynamin and dynamin-related proteins are involved in cytokinesis (Konopka, et al., Traffic 2006. 7: 239-247). We have previously shown that phenylarsine oxide (PAO), a tyrosine phosphatase inhibitor, blocks both FM 1-43 uptake and cell division in developing sea urchin embryos. Over expression of Src kinase had the same effect, suggesting either that the mechanisms of endocytosis and cytokinesis are regulated in parallel by tyrosine kinase/phosphatase cascade, or that cytokinesis requires a form of endocytosis. To test this second hypothesis we inhibited endocytosis by three additional mechanisms using: 1. Anti-sense morpholinos against Dynamin, 2. An inhibitory peptide that interferes with Dynamin’s ability to interact with it’s down-stream binding partner Amphiphysin, and 3. Using anti-sense morpholinos against Cortactin, a protein that couples Dynamin to the actin network. All three approaches blocked endocytosis, and all three also blocked cytokinesis. Because reagents targeting five different proteins, a Tyrosine phosphatase, Src kinase, Dynamin, Amphiphysin and Cortactin all inhibited both FM 1-43 uptake and cytokinesis strongly suggest that a form of endocytosis is required for cytokinesis. Two-photon microscopy was used to image a form of endocytosis that specifically occurred at cleavage furrows and whose endosomes dissipated after cell division and then re-concentrate at cleavage furrows before the next round of cell division.

2676

**Positive and Negative Factors Regulate Membrane Trafficking during Axon Growth and Retraction**
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Axon retraction is a typical negative response of axons to the environment or guidance molecules, critical for axon pathfinding in vivo and small scale pruning of axonal connections. Cellular mechanisms, particularly membrane trafficking events, mediating the positive and negative axonal responses remain poorly understood. Here, by time-lapse microscopy, we show that axon retraction is associated with the formation of clathrin-independent, dextran-positive pinocytic vesicles in the growth cone. Negative factors induce axon membrane retraction and increase dextran uptake. Conversely, positive factors decrease dextran uptake and additionally increase the motility and turnover of these vesicles. These vesicles appear to be coated by F-actin and an F-actin-binding protein, cortactin. Disruption of F-actin or cortactin knockdown decreases vesicle formation and membrane retraction, whereas inhibition of clathrin-mediated endocytosis did not affect these processes. These results suggest that this type of pinocytic vesicle constitutes a critical component of membrane trafficking during axon growth and retraction.

2677

**Ras Interference 1 (Rin1) Regulates Heterotypic Endosome Fusion between Early Endosomes**
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Fluid phase and receptor-mediated endocytosis involve a series of intracellular membrane fission and fusion reactions that play an essential role in the regulation of the efficiency of both processes. We describe an endosome-endosome in vitro fusion assay using two different ligands, biotin-epidermal growth factor (b:EGF) and avidin-beta-galactosidase (av:GAL). The first ligand (b:EGF) is rapidly and efficiently internalized to the endosomal compartment via receptor-mediated endocytosis, and the second one (av:GAL) is internalized via fluid phase endocytosis. Both ligands were localized in endosomes as determined by fractionation on sucrose gradients. Incubation of endosomes prepared from the two sets of cells resulted in endosome-endosome fusion as indicated by the formation of EGF:b-av:GAL complexes. Under our experimental conditions, fusion was time-, ATP-, KC1, and temperature-dependent. Furthermore, Rab5 was required in both sets of vesicles as well as from the cytosolic fraction. Interestingly, Rin1 and Rabex-5, two Rab5-guanine exchange factors, also were required for optimal endosome-endosome fusion driven by EGF. However, these Rab5-guanine exchange factors were required only in one set of vesicles. We conclude that endosome-associated Rab5-guanine exchange factors were required for the heterotypic fusion between early endosomes.

2678

**Platelet Activating Factor Acetylhydrolase Ib Catalytic Activity Generates Endosome Membrane Tubules and Mediates Endocytic Trafficking**
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Membranes in the endocytic and secretory pathways are dynamic, requiring continuous curvature change and regulated sorting of both lipid and protein cargo. Both membrane vesicles and tubules accomplish this regulated trafficking of cargo. Although many proteins necessary for vesicle-mediated traffic have been identified, little is known about the formation of membrane tubules. Previous studies in our lab have shown that tubule-mediated trafficking from endosomes requires the activity of Ca\(^{2+}\)-independent, cytoplasmic phospholipase A\(_2\) (PLA\(_2\)) enzymes. These results prompted the hypothesis that phospholipid shape-changing activities of PLA\(_2\) enzymes alter local lipid composition to induce membrane curvature, leading to the formation of membrane tubules. We have identified platelet activating factor acetylhydrolase Ib (PAFAH Ib) as one such PLA\(_2\) tubulation factor. In mammalian cells, mild overexpression of the PAFAH Ib catalytic subunits, α1 and α2, showed partial colocalization with endosomal markers. Overexpression of these subunits resulted in an increase in tubulated endosomes and altered trafficking of endocytic cargo. The catalytically dead mutant counterparts had no such effects. Knockdown of both catalytic subunits slows BFA-stimulated endosome tubulation and affects the distribution of endosomal markers and cargo. These results demonstrate that PAFAH Ib catalytic activity is involved in generating membrane tubules and mediating endocytic trafficking. Moreover, the results support the hypothesis that membrane tubule formation is mediated by altering local membrane curvature through changes in phospholipid composition.
Role of Vps1p during Endocytosis in *Saccharomyces cerevisiae*

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Receptor mediated endocytosis (RME) is a process by which cells take up extracellular materials, recycle the plasma membrane, and downregulate expression of membrane receptor molecules. In mammals, Dynamin, a GTPase protein, has been shown to be a major player in the process of scission of endocytic vesicles from the plasma membrane. Dynamin and dynamin-related proteins are engaged in diverse cellular processes such as intracellular protein trafficking, and organelle partitioning (Hinshaw; 2000; Danino and Hinshaw; 2001). Budding yeast *Saccharomyces cerevisiae* has three Dynamin-like proteins: Dnm1p, Mgm1p and Vps1p. Of these Vps1p is important for normal actin cytoskeleton organization during the early step of RME in *S. cerevisiae* (Yu X and Cai M 2004). To understand the physiological role of Vps1p, we defined yeast RME into early, intermediate and late phases. Early phase is marked by the formation of endocytic vesicles in the plasma membrane and ends with the scission of newly formed vesicles into the cytoplasm. The intermediate phase involves the movement of vesicles in the cytoplasm until they come in contact with the endocytic vacuoles. The sequence of events resulting in the fusion of the endocytic vesicle to the vacuole is referred to as the late phase. To test the effect of loss of Vps1p in yeast endocytosis, we performed FM4-64 pulse chase labeling experiments. We fixed the yeast cells at different time points using formaldehyde and observed the FM4-64 labelled vesicles arrested at various phases of endocytosis. Our results showed that most of the endocytic vesicles labeled with FM4-64 were accumulated in the cytoplasm rather than fused to the endosomal compartments in Vps1p cells compared to wild type cells, suggesting an endocytic delay either in the intermediate or the late phase of endocytic pathway of *S. cerevisiae*.

Caveolin-1 Is a Component of the Apical Recycling System in Polarized Epithelial Cells

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Biochemical studies have identified caveolin-1 as a component of recycling endosomes in polarized epithelia cells, but little is known about the organization or function of caveolin-1 in these compartments. We have characterized a novel pool of caveolin-1 in polarized epithelial cell, which is enriched in a subapical compartment, utilizing two N-terminally directed antibodies that recognize structures in the most apical regions of MDCK cells and rat hepatocytes. Previous proteomic studies of the H/K-ATPase tubulovesicles of human stomach, known apical recycling vesicles, indicated that caveolin-1 might be associated with the apical recycling system. In MDCK cells, subapical caveolin-1 labeling was concentrated around the microtubule organizing center and partially overlapped with Rab11-positive structures and endocytosed plgA. Caveolin-1 in this compartment could be detected independently of the cell surface and Golgi pools, indicating it displays unique epitopes within this environment. This compartment was microtubule dependent, partially dispersing with nocodazole treatment and re-localizing with taxol treatment. In rat liver, caveolin-1 was enriched in the transcytotic subapical compartment hepatocytes, which is functionally closely related to apical recycling endosomes of MDCK cells. This suggests that caveolin-1 defines a subcompartment of the apical recycling system in MDCK cells and rat hepatocytes.

Yta6 Is a AAA-ATPase Involved in Organization of the Cell Cortex and Endocytosis

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AAA-ATPases constitute a structurally well studied and functionally well conserved class of proteins that has been associated with proteasomal degradation, disassembly of SNARE complexes and microtubule severing. Yeast (*Saccharomyces cerevisiae*) genome has 21 AAA-ATPases with characteristic Walker A and B motifs. While some have well characterized functions, e.g. NSF in the SNARE cycle, the function of other members of this family is still completely unknown. One of these proteins is Yta6 (Schnall R et al, Yeast 1994). Overexpressed Yta6 is localized in patches preferentially on the mother cell cortex (Beach DL et al, Mol Biol Cell 2001). Together with a bioinformatic analysis that detected a phylogenetic link of Yta6 with the cytoskeleton this suggests a function of Yta6 in plasma membrane and cell cortex organization (Swaffield JC et al, J Mol Evol 1997). Consistent with this notion, we found that endogenous Yta6 is also localized on the plasma membrane. To understand how this is mechanistically achieved we pursue a structure function analysis of Yta6 and a crystal structure of the protein. This is likely to also reveal the mechanism of Yta6 function. Null mutants of YTA6 results show decreased efficiency of FM4-64 uptake by endocytosis. To gain first insights how it mechanistically acts during endocytosis, we characterized its interactions with other proteins. We found that it interacts with the major eisosome component Pil1 (Walther et al, Nature 2006). Consistently, a pool of Yta6 is localized at eisosomes and overexpression of Yta6 has an effect on eisosome organization and assembly. In addition, we found that Yta6 deletion results in an abnormal actin cytoskeleton, particularly actin patches. Together our results suggest that Yta6 is involved in remodelling of the actin cytoskeleton to effect the architecture of the cell cortex and thereby endocytosis.

Endocytic Machinery: Structure, Function, and Regulation (2682-2686)

Alsin Mediates IGF-1 Receptor Signaling and Cell Survival

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Amyotrophic lateral sclerosis (ALS) is a heterogeneous group of neurological disorders characterized by motor neuron degeneration. Although ALS is predominantly sporadic, familial ALS accounts for approximately 10% of patients with this illness. Eight independent chromosomal loci have been
linked to ALS, and six specific genes have been identified that are mutated in familial forms: SOD1, SETX, DCTN1, ANG, ALS8 and ALS2. ALS2 is mutated in a recessive juvenile-onset form of ALS and has also been linked to juvenile primary lateral sclerosis (JPLS) and infantile-onset ascending hereditary spastic paraplegia (IAHSP). We have previously shown that the ALS2 gene product (Alsin) is a Rab5 and Rac1 guanine nucleotide exchange factor (GEF). In an effort to understand the molecular basis of juvenile ALS, we have chosen to examine the functional role of Alsin in neuron maintenance and survival using a cell culture model system. Insulin-like growth factor 1 (IGF-1) signaling was significantly impaired in cells expressing mutant Alsin lacking an intact Rab5 GEF domain. Though IGF-1 receptor activation and internalization were unaffected, trafficking of activated receptors to early endosomes was compromised. Since IGF-1 plays an important role in mediating neurotrophic support, we examined IGF-1 mediated survival in cells expressing the mutant form of Alsin. Cells expressing Rab5 GEF domain truncated Alsin showed reduced IGF1 protection from serum withdrawal-induced apoptosis. Furthermore, to understand the contribution of Alsin’s Rac1 GEF activity, we mutated the key catalytic residue in its Rac1 GEF domain. Overexpression of this catalytically inactive mutant reduced downstream signaling of IGF-1. We conclude that Alsin Rac1 and Rab5 GEF activities are required for efficient IGF-1 signaling, identifying a potential mechanism for motor neuron degeneration observed in juvenile ALS.

2683
VR: In Silico Identification of a Novel Protein Family Implicated in Clathrin-coated Vesicle Formation
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Formation of clathrin-coated vesicles (CCV) is an important process underlying nutrients uptake, signal transduction, receptor internalization and synaptic vesicle recycling in neurons. ANTH, ENTH and VHS protein domain families, which belong to the same super-family, were shown to be implicated in CCV formation. Despite the structural similarity among proteins from these three families, their roles in CCV formation are different. ENTH and ANTH bind membrane-embedded phosphoinositides (PIPs), thereby recruiting components of CCV-formation machinery to the membrane. PIPs’ headgroups are highly negatively charged, and PIP-binding sites are represented by patches or clefts enriched in basic amino acid residues and corresponding to the areas of strong and condensed positive potential. In contrast, none of the members of VHS family has been shown to be able to bind the lipid components of the membrane. Our objective was to search database for non-annotated VHS domains that would possess electrostatic properties compatible with binding PIPs. We used comparative modeling tool SKYLINE that creates structural models for all homologs of a given protein structure and assesses the reliability of these models. We identified a group of proteins with low sequence similarity (up to 20%) to VHS templates. We call them VR (VHS-related). Modeling of N-terminal sequences of VR proteins using VHS structures as templates resulted in very highly reliable models. Electrostatic analysis revealed that most of the models feature a strong positive patch similar to one displayed by ANTH proteins. Three-dimensional cluster analysis revealed that this region is evolutionarily conserved. Sequence analysis detected clathrin-binding boxes in most of VR proteins. We propose that VR is a novel family of protein domains pertaining to ANTH-ENTH-VHS superfamily. Our results indicate that VR protein domains are involved in the process of CCV formation using binding to PIPs or other acidic components of the membrane.

2684
SNX9 Activities Are Regulated by Multiple Phosphoinositides through Both PX and BAR Domains
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The sorting nexin SNX9 functions at the interface between membrane remodeling and the actin cytoskeleton. In particular, SNX9 links membrane binding to potentiation of N-WASP and Arp2/3 complex-mediated actin polymerization and also to enhancement of dynamin GTPase activity. SNX9 is one of a growing number of proteins that contain two lipid binding domains, a PX and a BAR domain, and localizes to diverse membranes that are enriched in different phosphoinositides. Here, we investigate the mechanism by which SNX9 functions at these varied membrane environments. We show that SNX9 is a low affinity lipid binding protein that harnesses a broad range of phosphoinositides to synergistically enhance both dynamin and N-WASP activities. Using point mutations that block phosphoinositide binding by the PX and/or BAR domains, we demonstrate that these domains function separately and in concert for SNX9’s membrane active properties. We also demonstrate that the two lipid binding domains are differentially required for N-WASP and dynamin regulation and localization of SNX9 to clathrin-coated pits and dorsal ruffles. In total, our results demonstrate that SNX9 integrates signals from varied lipids through two lipid binding domains to direct membrane remodeling events.

2685
ESCRT-III Family Members Did2 and Vps60 Stimulate Vps4 ATPase Activity via Vta1
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Multivesicular bodies (MVBs) are endocytic intermediates formed when the limiting membrane of the endosome invaginates and buds into its lumen, actively selecting transmembrane protein cargoes in the process. Fusion of a MVB with the lysosome represents a mechanism by which eukaryotic cells degrade endocytosed transmembrane proteins. MVB function is critical for maintaining cellular homeostasis, highlighted by its role in modulation of growth factor receptor signaling. Exvagination of the endosomal membrane from the cytosol is topologically similar to the budding of retroviral particles and cytokinesis, wherein membranes bud away from the cytoplasm, and the machinery responsible for MVB sorting has been implicated in these phenomena. For transport (ESCRTs) from endosomal membranes during the course of MVB sorting, but it is unclear how Vps4 ATPase activity is synchronized with ESCRT release. Vta1 potentiates Vps4 activity and interacts with the ESCRT-III family members Vps60 and Did2. We have investigated the impact of ESCRT-III family members on the stimulation of the Vta1-Vps4 complex ATPase activity. Vps60 and Did2 both stimulate Vta1-Vps4 in a manner dependent on the Vta1 amino-terminus and independent of the Vps4 MIT domain. These data support a model wherein interaction of the Vta1 amino-terminus with ESCRT-III subunits contributes to the coordination of Vps4 activity during MVB sorting.
Identification of New Rin1 Interaction Partners

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Rin1 is a guanine nucleotide exchange factor (GEF) for Rab5 that regulates endocytic protein traffic. It has been demonstrated that Rin1 directly interacts with the epidermal growth factor receptor (EGFR) and modulates its trafficking and signaling. However, the precise role and regulation of Rin1 in this pathway are unknown. To further explore the mechanisms of Rin1 function, we generated stable Tet-on PC12 cell lines capable of the inducible expression of C-terminally TAP (tandem affinity purification) tagged Rin1 (Rin1TAP). By utilizing the Tet-on inducible expression of Rin1TAP and tandem affinity purification methods, we were able to copurify seven putative novel interactors of Rin1, including Calmodulin Kinase II (CaMKII) and Grb10 GYF Containing Protein2 (GIGYF2). In addition, we analyzed post-translation modifications of Rin1TAP by mass spectrometry, and identified three novel phosphoserine sites (S258, S333, S337) in addition to two other known phosphorylation sites (Y36 and S351). Currently, we are in the process of verifying the CaMKII- and GIGYF2-interactions with Rin1, and we are exploring the roles of these interactions as well as the novel phosphorylation sites in Rin1 function in EGFR signaling and trafficking.

Protein Targeting (2687-2691)

Subcellular Localization and Translocation of Fatty Acid Transport Protein 5 in the Liver

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The liver plays a central role in fatty acid metabolism including energy utilization, incorporation into lipoproteins and membrane lipids, and storage. Uptake and activation of fatty acids into hepatocytes are the key steps in the metabolism and mediated by several transport proteins and enzymes. However, the physiological role, mechanism of the uptake, control of expression, and functional regulation of each molecule have not been elucidated. In this study, we examined these issues by focusing mostly on one of the fatty acid transport proteins (FATPs), FATP5 that is uniquely expressed in the rodent liver and also known as bile acid-CoA synthetase. Northern blot analysis revealed that FATP5 mRNA levels were not altered after feeding a diet containing a peroxisome proliferator-activated receptor (PPAR) agonist, in contrast to up-regulation of other FATPs in the liver. These results suggest that expression of FATP5 is under post-translational regulation and PPARa is indirectly involved in it. Unique characteristics of FATP5 were further examined by immunocytochemical and biochemical studies. FATP5 was localized around the vein and/or bile duct in the liver and in endoplasmic reticulum in primary hepatocytes. In the further localization studies, FATP5 protein was dispersed into tiny structures in bile acid-treated primary hepatocytes, and flowed up in the sucrose gradient by centrifugation of homogenate. These results indicate that the FATP5 translocates from endoplasmic reticulum to lipid droplet-like low density compartments, at least in part, after bile acid-treatment. Furthermore, the mode of oleic acid-induced lipid accumulation in culture cells was changed by expression of FATP5. Thus FATP5 may play a role in formation and remodeling of lipid droplets in the liver. These results suggest that expression, localization and activities of FATP5 in the liver are regulated at multiple steps depending on the physiological conditions.

Identification of Tail-anchored (TA) Membrane Proteins in the Arabidopsis, S. cerevisiae and Human Proteomes: Characterization of the Targeting Mechanisms of Arabidopsis Chloroplast and SNARE TA Proteins

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Although the biochemical functions and intracellular trafficking of some tail-anchored (TA) integral membrane proteins have been well characterized, our overall understanding of TA protein biogenesis is limited because only a relatively few members of this novel group of proteins have been identified in evolutionarily diverse organisms. Here, we employed a bioinformatics strategy based on the unique topology (Ncytosol-Cgenome) of TA proteins to identify 508, 100 and 378 candidate TA proteins in the Arabidopsis, Saccharomyces cerevisiae and human proteomes, respectively. While many of these TA proteins have been functionally annotated using PersonNameGivenNameGene SnOntology, ~45% of the proteins identified in each proteome are of unknown function. Furthermore, subcellular localization experiments revealed that many of these TA proteins localize to a surprisingly wide variety of compartments, suggesting that TA proteins participate in far more cellular processes than originally proposed. We also discovered that for Arabidopsis chloroplast TA proteins with both known and unknown functions their localization to different sites within the organelle (e.g., outer and inner envelope and thylakoid membrane) appears to be mediated by distinct targeting signals. While some chloroplast TA proteins employ the traditional N-terminal transit peptide utilized by most chloroplast-destined proteins, other TA proteins rely on novel internal or C-terminal targeting signals. Similarly, the targeting of the largest and most diverse group of TA proteins to lipid droplets are also mediated by several distinct targeting signals that are situated, depending on the SNARE, either in the protein’s internal SNARE-domain or at its C terminus. Taken together, these results suggest that the targeting mechanisms underlying TA protein biogenesis in plant cells are more complex than proposed previously.

Redistribution of 17β-Hydroxysteroid Dehydrogenase Type 11 from the Endoplasmic Reticulum to Lipid Droplets

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17β-hydroxysteroid dehydrogenase type 11 (17β-HSD11) is a member of the short chain dehydrogenase/reductase family involved in activation and inactivation steps of sex steroid hormones. We recently identified 17β-HSD11 as a gene most efficiently regulated by peroxisome proliferator-activated receptor (PPAR) α in the intestine followed by the liver (K. Motojima (2004) Eur. J. Biochem. 271:4141-4146). In this study, we studied its subcellular localization and redistribution under physiological conditions to obtain a clue about its new physiological role. In Chinese hamster ovary
cells, the green fluorescence protein-tagged 17β-HSD11 was mostly localized in the endoplasmic reticulum (ER) under normal conditions whereas it was concentrated on lipid droplets (LDs) when they were induced. A pulse-chase experiment suggested that 17β-HSD11 was redistributed to the LDs via the ER. In mice, immunohistochemical and biochemical studies showed that 17β-HSD11 was induced mostly in intestinal epithelia and hepatocytes with heterogeneous localization. 17β-HSD11 was localized mostly in the ER when mice were fed a normal diet but it was distributed in both the ER and LDs of which formation was induced by feeding a diet containing a PPARα agonist. These data indicate that 17β-HSD11 localizes both in the ER and LDs depending on physiological conditions, and that LD 17β-HSD11 is not a mere ER-contaminant or non-physiologically associated protein only found in the cultured cells, but a bona fide protein comprising the membrane component of both intracellular compartments of mouse cells. We next examined the structural determinant for the redistribution of 17β-HSD11 from the ER to LDs. Mutational studies indicated that the N-terminal short sequence is necessary and sufficient for the redistribution in contrast to multiple structural requirements for targeting to LDs in previously characterized LD-associated proteins. These unique properties of 17β-HSD11 may be related to its new physiological role.

2690
Genome-wide Analysis of the Pleckstrin Homology Domains of *Dictyostelium discoideum*
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Pleckstrin Homology (PH) domains are small protein modules, present in many proteins involved in cellular processes in which membrane association plays an integral role e.g. signal transduction, vesicular trafficking and cytoskeletal rearrangements. Many PH have been implicated in binding phosphoinositides, but the physiological role of many others has not been established. The present study focuses on the detailed analysis of all the PH domains in the organism *Dictyostelium discoideum*. Genomic scale modeling of the PH domains from various organisms can provide invaluable clues regarding their function as well as how the subtleties and complexities of the membrane targeting functions of the PH domains evolved over time; the current work is part of ongoing effort in the lab to accomplish the same. We have used computational databases and tools to predict the secondary structure of all forty-two *Dictyostelium discoideum* PH domains identified by the SMART database of protein domains, build and evaluate their three-dimensional models and study their electrostatic profiles. Our computational strategy of integrating the information available on sequence, structure and function and combining this with modeling and biophysical characterization allows us to make new biological predictions based on the models we have generated which can then be experimentally validated.

2691
Characterization of the Outer Mitochondrial Membrane Targeting Signal within the 36-kDa Carnation Italian Ringspot Virus Replicase Protein
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Carnation Italian Ringspot Virus (CIRV) is a positive-strand RNA virus that in plant cells causes extensive inward sphericulation/vesiculation of the mitochondrial outer membrane leading to the formation of a multivesicular body (MVB). While the role of a MVB is likely to provide an unique cellular environment for the efficient functioning of the membrane-bound viral RNA replication protein complex, the molecular mechanisms of how these viral proteins target to mitochondria and participate in MVB biogenesis are not well understood. Here we present the results of a comprehensive mutational analysis of the CIRV 36-kDa RNA-binding protein (p36) that indicate the protein’s outer mitochondrial membrane targeting information resides within its two transmembrane domains (TMD1 and TMD2) and intervening hydrophilic loop region. We show that replacement of the p36 TMDs, either individually or together, with an artificial TMD(s) led to all of the resulting mutant p36 proteins being mislocalized to the cytosol. However, TMD2, but not TMD1, of p36 was sufficient in preserving the sorting of the tail-anchored outer mitochondrial membrane protein cytochrome b₅₆₇, suggesting that TMD1 is involved in membrane integration and TMD2 has a more direct role in mitochondrial targeting. We show also that a positively-charged amphipathic face within the intervening loop region of p36 is critical for its mitochondrial targeting. Overall, these results, as well as bimolecular fluorescence complementation experiments with p36 and various components of the translocase of the outer mitochondrial membrane, indicate that p36 utilizes an internal targeting signal and sorting pathway similar to that employed by host-cell outer mitochondrial membrane proteins.

Protein Targeting to the Cell Surface (2692)

2692
A Regulated Interaction between NHE7 and CD44 Occurs in Specialized Membrane Domains
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Organellar and cytosolic pH homeostasis is central to most cellular processes, including vesicular trafficking, post-translational modification/processing of proteins, and receptor-ligand interactions. NHE7 was identified as a unique (Na⁺, K⁺)/H⁺ exchanger that dynamically cycles between the trans-Golgi network (TGN), endosomes and the plasma membrane. In this study, we show that endogenously expressed CD44, a cell surface glycoprotein weakly binds to NHE7 in human breast cancer MDA-MB-231 cells, and this interaction is enhanced by phorbol ester treatment. While NHE7 and CD44 are associated with both lipid raft and non-raft fractions, phorbol ester treatment of the cells caused a significant shift of CD44 to lipid raft fractions. The NHE7-CD44 interaction was also predominantly detected in the lipid rafts, and disruption of lipid rafts by saponin caused a significant reduction in NHE7-CD44 binding. Methyl-beta-cyclodextrin (MbCD) treatment partially dissociated CD44 and NHE7 from lipid rafts, and a small but significant population of CD44 and NHE7 retained in the raft fractions even after MbCD treatment. Interestingly, the CD44-NHE7 association appeared to take place predominantly in raft fractions. These results suggest that CD44 and NHE7 bind in a regulated
manner and that this interaction occurs in specialized membrane domains. We propose a model in which NHE7 regulates surface targeting and/or internalization of CD44, which might influence cell adhesion and migration.

**Protein Targeting to the Endocytic Pathway (2693-2694)**

**2693**

**Proteomic Analysis of the Supramolecular Organization of the AP-3 Adaptor Pathway**

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Transport of membrane-bound proteins (cargoes) is mediated in part by adaptor complexes, which bind and recruit cargoes into vesicles. In particular, the adaptor complex 3 (AP-3) functions in the transport of membrane proteins from endosomes to lysosome/lysosome-related organelles and synaptic vesicles. Presently few factors regulating the AP-3 route have been identified and some of them, like clathrin, remain controversial. In order to define the AP-3 protein interaction network, we implemented an in vivo cross-linking strategy. In vivo cross-linked AP-3 complexes were purified by sucrose gradient sedimentation and immunoaffinity chromatography with antibodies against AP-3. The molecular composition of isolated complexes was analyzed by nano-LC MS/MS. This allowed us to identify 54 proteins. Among them, we found neuronal and non-neuronal AP-3 subunits, clathrin chains, BLOC complex subunits, AP-3 cargoes, signaling molecules, and molecular chaperones. Using siRNA and pharmacological tools to perturb clathrin, we provide evidence for a role of clathrin in AP-3 vesicle formation from endosomes. In addition, we uncovered a multipronged mechanism that binds clathrin to AP-3 supracomplexes.

**2694**

**SNX27 Recruits the Cytohesin Associated Scaffolding Protein (CASP) to Lymphocyte Early Endosomes via PDZ Domain Interaction**

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CASP is a small cytokine inducible human protein, primarily expressed in hematopoietic cells, which associates with members of the Cytohesin/ARNO family of guanine nucleotide-exchange factors. Cytohesins are known activators of ARFs, a group of GTPases involved in vesicular initiation. Functionally, CASP is an adaptor protein containing a PDZ domain, a coiled-coil motif, and a potential carboxy terminal PDZ-binding motif (PDZbm) that we sought to characterize here. Using GST pulldowns and mass spectrometry, we identified the novel interaction of CASP with Sorting Nexin 27 (SNX27) in lymphocytes. CASP’s PDZbm was found to interact with the PDZ domain of SNX27. This N-terminal PDZ domain makes SNX27 a unique member of the sorting nexin family of proteins, a group generally involved in the endocytic and intracellular sorting machinery. Immunoprecipitation studies demonstrated that CASP might link members of the Cytohesin/ARNO family to SNX27 in a complex with potentially dynamic phosphoinositol affinity. While CASP expression is limited to hematopoietic cells, SNX27 is expressed in many tissues, indicating a cell-type restricted role for this interaction. Endogenous SNX27 and CASP co-localize at the early endosomal compartment in lymphocytes. In transfection studies, endogenous SNX27 is localized to the early endosomes regardless of CASP expression, while ectopically expressed CASP co-localizes at this compartment. Knockout of the CASP PDZbm (ΔSRF) abolishes its endosomal localization and redistributes CASP to the cytoplasm. These results demonstrate that SNX27 can recruit CASP to early endosomal structures through a classical PDZ-PDZbm interaction where they may orchestrate novel Cytohesin/ARNO-related intracellular trafficking and/or signaling complexes in lymphocytes.

**Caveolae (2695)**

**2695**

**Effects of Insulin and CL316243 (β3-Receptor Agonist) on Activation of Adipocyte PDE3B Associated with Caveolae and Internal Membranes**

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PDE3B is activated by insulin and CL (CL316243, a β3-receptor agonist) in adipocytes via PKB- and PKA- signaling, respectively. Activation of PDE3B by insulin seems to be important in its ability to reduce cAMP, and thereby inhibit lipolysis in adipocytes. Using sucrose gradient centrifugation and confocal microscopy techniques, PDE3B was found to be localized in internal membranes (fractions enriched in endoplasmic reticulum and Golgi markers) and in caveolin-enriched plasma membranes (PM) in 3T3-L1 adipocytes. Stimulation of adipocytes with insulin and CL indicated that insulin preferentially phosphorylated/activated PDE3B associated with internal membranes, whereas CL-phosphorylated/activated PDE3B was preferentially associated with caveolin-enriched PM. Superox 6 gel filtration chromatography of membrane proteins from adipocytes stimulated with insulin or CL demonstrated the reversible assembly of distinct macromolecular complexes that contain 32P-phosphorylated PDE3B and signaling proteins thought to be involved in its activation. Insulin- and CL-induced macromolecular complexes contain certain common signaling proteins (14-3-3, PP2A, and caveolin). Analysis of insulin- and CL-stimulated fractions also suggest that the complexes present in insulin-stimulated cells contain tyrosine-phosphorylated IRS and its downstream signaling proteins, whereas CL-activated complexes contain adenylate cyclase and PKA-regulatory subunit (PKA-R), which may be involved in the differential regulation of phosphorylation/activation of PDE3B by PKB- or PKA-mediated pathways. This research was supported in part by the intramural program of NHLBI.
Protein Folding and Assembly (2696)

Identification of the Motifs Regulating Uromodulin Secretion and Polymatisation
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Uromodulin (or Tamm-Horsfall protein) is the most abundant protein present in urine under physiological conditions where it is found as a high molecular weight polymer. It is a GPI-anchored glycoprotein exclusively expressed by kidney epithelial tubular cells of the thick ascending limb of Henle's loop (TAL) and distal convoluted tubule (DCT). Single uromodulin filaments can assemble in a helical fashion to form 10-15 nm wide bundles forming a mat-like structure that coats the lumen of TAL and DCT tubules. Uromodulin polymerisation depends on the presence of the Zona Pellucida (ZP) domain. ZP domain comprises about 260 amino acids and it is found in a wide range of extracellularly exposed or secreted proteins with different functions but all arranged into filaments and/or matrices. Two conserved motifs, mapping inside the ZP domain (Internal Hydrophobic Patch/IHP) and in the C-terminal tail (External Hydrophobic Patch/EHP) have been recently identified in mouse ZP3 protein as regulating protein polymerisation. By sequence homology and hydrophobicity profile analyses we identified putative IHP/EHP motifs in uromodulin sequence and generated site-specific or deletion mutant constructs to study the role of these sequence in the regulation of protein secretion and polymerisation. We assessed protein trafficking to the plasma membrane, polymerisation and release in the medium by biochemical and immunofluorescence analyses in transfected HeLa and stably transfected MDCK cells. These experiments allowed us to identify conserved motifs in the C-terminus of uromodulin that are important for ZP domain-mediated protein assembly and secretion, in accordance with previous studies on ZP3 protein. Understanding the molecular mechanisms regulating uromodulin assembly could gain new insights into the biology of this protein and into the molecular mechanisms underlying pathological conditions associated to uromodulin aberrant polymerisation.

Trafficking in Polarized Cells (2697-2699)

Palmitoylation Targets GAD65 from ER to the Trans-Golgi Network and Mediates Cycling between ER/Golgi and Post-Golgi Membranes
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GAD65, the smaller isoform of the enzyme glutamic acid decarboxylase, synthesizes GABA for fine-tuning of inhibitory neurotransmission. GAD65 is synthesized as a soluble hydrophilic protein but undergoes a hydrophobic post-translational modification and becomes anchored to the cytosolic face of Golgi membranes. A second hydrophobic modification, palmitoylation of cysteines 30 and 45 in GAD65, is not required for the initial membrane anchoring but is critical for post-Golgi trafficking of the protein to presynaptic clusters. The mechanism by which palmitoylation directs targeting of GAD65 through and out of the Golgi complex is unknown. In this study we show that prior to palmitoylation, GAD65 anchors to both ER and Golgi membranes. Palmitoylation, however, serves to clear GAD65 from the ER/Golgi, target it to the trans-Golgi network, and then to a post-Golgi vesicular pathway. FRAP analyses of trafficking of GAD65-GFP reveal a rapid and slow pool of protein replenishing the Golgi complex. The rapid pool represents non-palmitoylated hydrophobic GAD65-GFP that exchanges rapidly between the cytosol and ER/Golgi membranes. A second hydrophobic modification, palmitoylation of cysteines 30 and 45 in GAD65, is not required for the initial membrane anchoring but is critical for post-Golgi trafficking of the protein to presynaptic clusters. The mechanism by which palmitoylation directs targeting of GAD65 through and out of the Golgi complex is unknown. In this study we show that prior to palmitoylation, GAD65 anchors to both ER and Golgi membranes. Palmitoylation, however, serves to clear GAD65 from the ER/Golgi, target it to the trans-Golgi network, and then to a post-Golgi vesicular pathway. FRAP analyses of trafficking of GAD65-GFP reveal a rapid and slow pool of protein replenishing the Golgi complex. The rapid pool represents non-palmitoylated hydrophobic GAD65-GFP that exchanges rapidly between the cytosol and ER/Golgi membranes. The slow pool represents palmitoylation competent GAD65-GFP, that replenishes the Golgi complex via a non-vesicular pathway and at a rate consistent with a depalmitoylation step. We propose that a depalmitoylation/repalmitoylation cycle serves to cycle GAD65 between Golgi and post-Golgi membranes and dynamically control levels of enzyme directed to the synapse.

FAPP2 Regulates Exit of Basolateral and Raft-associated Apical Cargo Proteins from the TGN
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FAPP2 is a TGN-localized protein controlling the traffic of neosynthesized proteins to the plasma membrane (PM). FAPP2 is targeted to the TGN via its PH domain that binds phosphatidylinositol 4-phosphate and the small GTPase ARF1. It also possesses a glycolipid-transfer-protein (GLTP) homology domain, and it has been recently shown to control glycosphingolipid synthesis thanks to its ability to transfer glucosylceramide from its site of synthesis at the cis-Golgi to the distal Golgi compartments. A functional GLTP homology domain in FAPP2 is required for its activity both in glycosphingolipid synthesis and in TGN-to-PM trafficking. The question is now whether the role of FAPP2 in membrane trafficking is secondary to its activity in glycosphingolipid metabolism or whether it can exert a more direct control on TGN-to-PM trafficking. We addressed the question by “acutely” inhibiting FAPP2 through antibody injection thus not allowing the time required for a significant impact on glycosphingolipid levels. Using this approach we found that acutely blocking FAPP2 inhibits the TGN-to-PM transport of basolateral (the reporter protein VSVG and LDL-R) and of the apical raft-associated GPI-anchored proteins due to the impaired formation of their transport carriers at the TGN. In contrast apically directed non-raft associated proteins (P75-GFP) were efficiently transported to the PM in anti-FAPP2 injected cells. In conclusion our results indicate that FAPP2 can directly and specifically control at the TGN the formation of PM-directed pleiomorphic carriers containing VSVG and GPI-anchored proteins.
Dynamic Microtubules Are Required for Apical Membrane Recycling in Polarized WIF-B9 Cells and Provide the "Missing Link" to the Canaliculus Actin Network

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Microtubules (MTs) play a fundamental role in establishing and maintaining plasma membrane polarity in hepatocyte. However, little is known about which MTs (dynamic and/or stable MTs) are involved in directional cargo delivery to the canalicular (hepatocyte apical) membrane. Using polarized WIF-B9 hepatic cells, we investigated the role of dynamic MTs on apical membrane targeting and their linkage to the canalicular actin network. Immunostaining was performed with α-tubulin antibody and γ-tubulin antibody, a marker of the microtubular organizing center (MTOC) to visualize MT distribution around canaliculus. Immunostaining of 3D reconstituted images of α-tubulin revealed that longitudinal MT structures surround the canaliculus and form apical microtubulate web structures. The MTOC was proximate to the bile canaliculus, and EB1, a dynamic MT plus-end marker, was homogeneously distributed around the canaliculus. To determine the role of dynamic MTs in apical membrane trafficking, we performed live cell imaging of BSEP-YFP trafficking combined with fluorescence recovery after photobleaching. In control cells, BSEP fluorescence in the canalicular domain recovered within 20 minutes after photobleaching. Fluorescence recovery was abolished by nocodazole treatment that disrupt all microtubule structures. After treated with 201F, a marine sponge product, which specifically dissembled dynamic MTs and not stable MTs, fluorescence recovery after photobleaching at canalicular domain was abolished as is in nocodazole treated cells. These results reveal that apical membrane targeting to the canaliculus requires dynamic MTs. To explore the relation between dynamic MT plus-end and the actin network, we performed immunostaining of IQGAP, Rac, APC and EB, which link dynamic MTs and actin. These proteins all surrounded the bile canaliculus in association with actin. Conclusion: Dynamic MTs are required for canalicular targeting of apical membrane protein. Dynamic MT plus-end may provide the long sought link between the MT and actin-based endosome trafficking systems which surround the bile canaliculus.

Gene Structure and Expression (2700-2707)

Effect of DNA Contaminants on Transfection Efficiency and Mutation Rate in CHO Cells

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Plasmid DNA is widely used to deliver genes into mammalian cells for the construction of new cell lines, gene therapy and gene expression studies. There are different methods to isolate and purify plasmid DNA, which include: cesium chloride banding, ethanol precipitation and the use of commercial kits. Various contaminants are introduced together with the isolated DNA and cause the reduction of the efficiency of delivery and create mutations that decrease the expression level of the delivered genes. We evaluated the effect of different plasmid DNA contaminants on transfection efficiency in Chinese Hamster Ovary (CHO) cells. pCMVβ was prepared using an endotoxin-free commercial kit. This plasmid was transfected into CHO cells after spiking with different contaminants. Five conditions of varying concentrations were carried out, with the clean plasmid being used as a control. These conditions are: ethanol, endotoxin, cesium chloride, ethidium bromide and the latter two contaminants together in different ratios. The transfection efficiency as well as mutation rate were determined through X-Gal staining and counting of the transfected cells and quantitative PCR (qPCR). The reversibility of the interaction of the contaminants with DNA was examined through cleaning of the spiked DNA followed by transfection into CHO cells. In general, most of the examined contaminants appear to have a negative effect on the transfection efficiency. Furthermore, these contaminants cause a significant increase in transfected plasmid mutations.

A Versatile System for Stringent Regulation of Gene Expression and Knockdown in Mammalian Cells

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The generation of stable cell lines with inducible expression of transgenes has become a powerful routine method for the analysis of gene function. Random integration of the transgene into the genome is, however, very problematic, because variable chromosomal position effects typically result in unpredictable expression characteristics. We therefore developed a strategy for the pre-characterization of genomic integration sites. A bidirectional tetracycline inducible transcription unit, simultaneously expressing a fluorescent and an enzymatic reporter gene (e.gfp and luciferase), was genomically integrated into a large population of HeLa cells that constitutively express the reverse transcription-transactivator (Tet-On System). Transduced cells were then screened to identify those with a high regulatory potential. We were thus able to identify a gene locus that reached a reporter expression more than 104-fold above background after only 16 hours of induction. Expression levels could be fine-tuned in a dose-dependent manner. By applying Flip recombinase-mediated cassette exchange we replaced the reporter unit for a constitutively expressed selection marker. This site could then be used to insert a gene of interest by recombinase-mediated target and exchange. Negative selection is used to identify faithfully-recombined clones. The excellent expression characteristics of this cell system in respect to regulation and stability were maintained after recombination. As a proof of principle, endogenous Lamin A/C was efficiently down regulated using a single copy insertion of a Tet-inducible transcription unit producing microRNA-based short hairpin RNAs. Both, knockdown and subsequent recovery to original levels of this abundant protein occurred within only 96 hours of induction and depletion, respectively. The potential of stringent expression regulation of any gene of interest will make this cell system a versatile genetic tool. The strategy described here should allow the establishment of this regulatory system also in other cell lines.
2702
Analysis of Histone Modifications Surrounding a Suppressor of Hairy Wing Chromatin Insulator
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Chromatin insulators are DNA/protein complexes proposed to affect gene activity through formation of independent chromatin domains. Two properties of insulator complexes are enhancer-promoter blocking and protection of transgenes from chromosomal position effects. The gypsy insulator sequence of Drosophila melanogaster contains binding sites for the Suppressor of Hairy wing (Su(Hw)) protein. Two additional proteins of the complex are Centrosomal protein 190 (CP190) and Mod(mdg4)2.2. Previous studies identified a Su(Hw) binding site, 1A-2, immediately downstream of the yellow gene and 8.5 kilobases upstream from the achaete gene. Transgenic studies suggest that this insulator possesses enhancer-blocking activity. The yellow gene is highly transcribed during early larval and mid-pupal stages, whereas achaete is expressed strongly during the late larval stage. Four distinct enhancers regulate yellow while achaete is regulated in coordination with the downstream scute locus through several enhancers. To test whether 1A-2 demarcates independent chromatin domains, histone modifications were examined across the yellow-achaete locus in third instar larvae and pupae by chromatin immunoprecipitation. At both stages of development, Su(Hw), CP190, and Mod(mdg4)2.2 associate with 1A-2. In larvae, active Histone H3 Lysine 4 di/tri-methylation (H3K4me2/3) marks are enriched equally throughout the yellow-achaete locus. This same enrichment pattern is found for repressive Lysine 27 (H3K27Me2) and Lysine 9 di/tri-methylation (H3K9Me2/3) marks. In pupae, locus wide enrichment of H3K4me2 is observed; however, H3K27me2 and H3K9me2 are enriched within yellow. Therefore, we did not detect distinct regions of histone modifications in the yellow-achaete locus at either stage. We conclude that the 1A-2 insulator may exert enhancer-blocking activity independent of effects on histone modifications. Alternately, use of whole organisms may preclude the ability to detect changes in histone modifications due to the presence of cells lacking yellow and/or achaete expression. Future experiments using cell lines derived from tissues expressing either gene will be examined.

2703
Isolation of the U6 snRNA Gene in the Orb Web Spider Nephila clavipes
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We have developed the large ampullate glands of the orb web spider Nephila clavipes as a model system in which to study the elicited production of a tissue-specific secretory protein, a fibroin. The studies have revealed a finely orchestrated series of time and tissue specific synthesis of components of the protein synthesis system. These studies have been possible due to rather simple manipulations that allow us to completely abolish the process and subsequently elicit it to high levels of production. The studies reveal a series of transient events, the production of the full size fibroin peaking at ninety minutes after stimulation, the production of the template precedes the latter by sixty minutes. Two other peaks of activity are seen; one following the appearance of the template, which adaptively enriches the system with the tRNAs cognate to the fibroin predominant amino acids, and a very prominent peak of activity enriches the glands with two isoforms of SS RNA and all the members of the U subset of snRNAs. Since the responses are executed at the level of gene expression and regulatory controls, we isolated the corresponding genes and characterized many of them via cell-free transcriptions. Currently we are in the process of the isolation and the transcriptional characterization of the U6 snRNA gene. We have chosen this gene due to the novel regulatory controls it has exhibited in other systems. In so doing we are making use of a heterologous cell free system derived from the silk worm Bombyx mori, as done previously. This will allow us to pinpoint the transcriptional regulatory controls of these unusual set of genes.

2704
A BAC-based Integrated Linkage Map of the Silkworm, Bombyx mori
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We have constructed an integrated map between a high resolution SNP genetic linkage map and a physical map composed of BAC contigs. Using a male informative backcross population between strains p50T and C108T, 1755 SNP makers found in BAC end sequences were mapped on 28 linkage groups, of which 26 groups were assigned to standard silkworm chromosomes using morphological markers. We constructed 6221 BAC contigs by fingerprinting three BAC libraries prepared by different restriction enzymes. Among them, 782 major BAC contigs comprising more than 8 BAC clones spanned 376 Mb of the silkworm genome, which corresponded to 79% genome coverage. We mapped 523 ESTs onto the 28 linkage groups by BAC High-Density-Replica (HDR) filter hybridization using EST probes, and an additional 357 ESTs by BLAST search of 1,755 SNP-containing BAC end sequences against a B. mori cDNA/EST database. In addition, we assigned 1082 ESTs to the 28 linkage groups as RFLPs using DNA from a female informative backcross population. Among a total of 1689 silkworm genes we found 769 orthologs in Apis mellifera and 790 orthologs in Tribolium castaneum which we used to test for synteny relative to silkworm chromosomes. Whereas we found a high degree of synteny between most silkworm and beetle chromosomes, only 2 honeybee chromosomes showed significant synteny with silkworm. The present data will be a resource to construct and evaluate the accuracy of scaffolds for a forthcoming new assembly of integrated whole genome shotgun data. It will also be a powerful tool for investigating silkworm genome properties, mutation mapping and map-based cloning, as well as providing a valuable resource for testing synteny leading to gene discovery in other Lepidoptera.

2705
Globin Intergenic Regulatory Regions Are Highly Variable in the Antarctic Dragonfishes, the Sister Group of the Hemoglobinless Icefishes
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Late Abstracts

Wednesday

2706 Identification of a Highly Conserved Meis-linked Gene
A. S. Carpenter, B. Graham, A. S. Wellington, T. Zenucha; Biology, Appalachian State University, Boone, NC
We have identified two previously unstudied zebrafish (Danio rerio) genes, at least one of which is linked to the meis2 homeobox genes, using a comparative genomics approach. We have identified homologs of this gene in all animals examined and have named the genes m2lg1 and m2lg2 (for Meis2 linked gene). While zebrafish contain two paralogs of this gene, tetrapods and invertebrates appear to contain only one. This is consistent with what would be predicted based on the genome duplication which occurred in the teleost lineage following its phylogenetic divergence from the Antarctic icefishes, are a paraphyletic group composed of 16 species in 11 genera and 3 subclades (Bathydraconinae, Cygnodraconinae, Gymnodraconinae). To gain further insight into the evolution of globin gene loss and to delineate the minimal regulatory sequences necessary to support globin gene transcription, we have sequenced the intergenic regions of the adult αβ-globin gene loci of seven species from the three dragonfish subclades. The intergene of Gymnodraco acuticeps (Gymnodraconinae) is virtually identical to that of the nototheniids Dissostichus mawsoni and Notothenia angustata, and those of Akatoraxis nudiceps and Prionodraco evansi (Bathydraconinae) are quite similar, lacking ~1 kb adjacent to the nototheniid α-globin promoter. By contrast, four species of the Cygnodraconinae (Cygnodraco mawsoni, Gerlachea australis, Parachaenichthys charcoti, and P. georgianus) have lost most of the intergenic region. Furthermore, as intergene length decreases, its major enhancer element, a GATA- and CAC/Sp1-site-rich originally described in N. coriiceps [Lau et al. (2000) Am. Zool. 41, 113-132], loses the CAC/Sp1 sites. Thus, the globin intergenic regions of the dragonfishes have undergone rapid evolutionary change that is consistent with the trend to reduced Hb expression. Supported by NSF grants OPP-0336932 and ANT-0635470 to H.W.D.

2707 Transcriptional Regulation of δ-Catenin/NPRAP/Neurojungin (CTNND2) Gene Expression in Human Prostate Cancer
T. Wang,1 K. Kim,1 Y. Chen,1 H. Hong,1 Q. Lu1; 1Department of Anatomy and Cell Biology, East Carolina University Brody School of Medicine, Greenville, NC, 2Department of Pathology and Laboratory Medicine, East Carolina University Brody School of Medicine, Greenville, NC δ-Catenin (NPRAP/Neurojungin) is a unique β-catenin/ armadillo domain-containing p120<sup>th</sup> subfamily protein in that it is primarily expressed in the central nervous system. However, δ-catenin is upregulated in over 80% of human prostatic adenocarcinomas although how its expression is regulated is currently unclear. Analyses of δ-catenin CpG islands in the promoter region in benign and prostate cancer specimens revealed no significant hyper- or hypomethylation. Real time PCR analyses found no evidence of gene amplification in δ-catenin. However, we have observed an increased incidence of mutations in the promoter and 5'-untranslated region of δ-catenin gene when compared to that of benign prostatic tissue specimens as well as to that of peripheral blood samples of normal control subjects. δ-Catenin promoter region contains strong consensus binding domains for transcription factors such as E2F and LEF-1. Ectopic expression of E2F, but not LEF-1 or Notch intracellular domain (NICD), significantly increased δ-catenin expression. These studies indicate that δ-catenin promoter could be one target for its upregulation in human prostate cancer. This study was supported in part by NIH CA111891 and Department of Defense PC040569.

Mechanisms of Nuclear Transcription (2708-2709)

2708 SUMOylation of KAP1 Determines Its Interaction with Chromatin Repression Machinery and Repression Activity
A. V. Ivanov,1,2 H. Peng,3 V. Yurchenko,3 D. G. Negorev,2 K. L. Yap,2 M. Sadofsky,2 G. G. Maul,2 M. Zhou,4 F. J. Rauscher2; 1Department of Biochemistry, West Virginia University, Morgantown, WV, 2Department of Structural and Chemical Biology, Mount Sinai School of Medicine, New York, NY Posttranslational modifications of proteins play a pivotal role in regulation of euukaryotic gene transcription and chromatin organization. SUMO (Small Ubiquitin-like MOdifier) conjugation to many transcription factors and histones has been associated with decreased transcriptional potential and/or repression. However, the mechanism of SUMO-mediated repression is poorly understood. Here we report that repression function of most abundant family of transcriptional repressors in the human genome, KRAB domain containing zinc-finger proteins, is strictly dependent on SUMOylation of their universal co-repressor KAP1. We show that PHD domain of KAP1 directly interacts with SUMO E2 protein Ubc9 and mediates SUMO auto-ligase activity towards its Bromodomain. Structural mutation in the PHD domain compromises KAP1 SUMOylation while mutation of all six SUMOylation sites in KAP1 abolishes KRAB-mediated repression. Furthermore, we show direct interaction between SUMOylated KAP1 and the H3-K9 methyltransferase SETDB1 or the component of the NuRD histone deacetylase complex Mi-2a mediated through
their specific SUMO iteration motifs (SIMs). Thus, we propose that SUMO serves as universal protein tag employed for recruitment of repressive chromatin modifying complexes.

2709
Involvement of ER Stress Response in the Adipogenic Differentiation
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Induction of endoplasmic reticulum (ER) stress response has been observed in cell differentiation. We examined the expression of ER stress genes during the adipogenic differentiation. When we treated cells with the induction medium for 8 days, based on the expression of adipogenic marker PPARγ and Oil-red-O staining, mouse preadipocyte 3T3-L1 cells were differentiated into adipocytes. The expression of ER stress genes such as BiP and CHOP increased from 8 day, showing the occurrence of ER stress response in adipogenic differentiation. Expression of spliced form XBP1, a bZIP family of transcription factor, was also increased after 7 days after the induction, similar temporal pattern to that of PPARγ, indicating the close relationship between them. In the cells in which XBP1 is knock-downed by the infection with xbp1-shRNA retroviral vector, induction of xbp1 and PPARγ expressions during the differentiation were completely blocked. No lipid droplet containing cells were also found Oil-red-O staining results. Treatment of xbp1-shRNA treated cells with rosiglitazone, a PPARγ activator, adipogenic differentiation potential was partially recovered. These results strongly suggest the critical role of XBP1 during adipogenic differentiation.

Tissue–specific Gene Expression (2710-2713)

2710
Gene Expression Profiling of Bone Marrow Cells on the Enhanced Erythropoiesis Mouse Model
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Our previous result suggested that the Peroxiredoxin II knockout (Prdx II−/−) mice have a hemolytic anemia. In this study, we found that Ter-119+ cells were increased in Prdx II−/− mice bone marrow (BM) after postpartum 5 week till death, and Lin−c-Kit+Sca-1+ cells, hematopoietic stem cells (HSCs), were reduced at 8 wk old mice, but they recovered at 6 months old mice. We examined the differential expression profiles to bone marrow cells (BMcs) between Prdx II−/− and Prdx II+/+ mice using a cDNA microarray. We identified the differentially expressed 136 genes were expressed differentially a greater 2-fold increase or decrease than the erythropoietin (EPO) receptor. We also examined whether the selected nucleotide binding proteins (NBPs) are involved in in vitro erythropoiesis. Six genes of NBPs were up-regulated during erythropoiesis in Prdx II−/− mice. Among the six genes, elf3-p44, hnRNPH1, G3bp, and Zfpm-1 were initially up-regulated at day 7 of the erythropoietic differentiation process. These genes were dramatically down-regulated in glycohorin A (GPA)− cells at day 9. However, 2 genes, DJ-1 and Rbm3 increased only on day 12. Our results suggest that up-regulated NBPs might perform important functions in erythropoietic differentiation. This work was supported by Korea Health 21 R&D (01-P110-PG6-01GM02-002) by the Ministry of Health and Welfare, Republic of Korea. These authors are supported by Research Center for controlling Microbial Virulence.

2711
MicroRNA Profiling of Human Breast Cancer Cell Line MDA-MB-231 (sub line 4175) and MCF10A
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MicroRNAs are noncoding, endogenous small RNAs, which can regulate their target genes by cleavage of the targeted mRNA or translational repression. We have investigated the microRNAs in breast cancer cells, using highly invasive breast cancer cell line MDA-MB-231 (subline 4175) and normal human breast epithelial cell line MCF10A. A genome-wide microRNA microarray consisting of 471 human mature miRNAs was used to identify the differentially expressed miRNAs between these two cell lines. A simple T-test analysis showed that 13 miRNAs were up-regulated and 9 down-regulated significantly in MDA-MB-231 sub line 4175 cells (p<0.05, fold change>2), compared to MCF10A cells. The miRNA genes selected for further analysis include miR-99a and miR-27b (up-regulated miRNAs) and miR-205 (down-regulated miRNA). TaqMan Q-PCR analysis confirmed the expression levels of all three miRNAs from the microarray data which suggesting that our gene chip data are highly reliable and accurate. We also examined these miRNA expression levels in six other breast cancer cell lines (MCF7, MDA-MB-231, SKBR3, BT474, T47D, ZR75) by Q-PCR. The miR-99a expression was higher in MDA-MB-231 and its subline 4175 compared to other cell lines, while the miR-27b had lower expression levels in ZR75 and MCF7. Interestingly, the miR-205 is undetectable in MDA-MB-231 cell and its subline 4175, while it is highly expressed in MCF10A cells. Furthermore, we confirmed the expression levels of these three miRNAs in human breast normal and cancer tissues. The target genes of miR-99a, miR-27b and miR-205 were predicted by Target Scan, Pictar and miRanda softwares and their function analysis is currently being elucidated.

2712
MRF4 Gene Expression in the Xenopus laevis Frog
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Myogenic Regulatory Factors (MRFs) Myf5, MyoD, Myogenin and MRF4 are basic/helix-loop-helix transcription factors that help to specify skeletal muscle cells during embryogenesis and activate muscle structural genes. This family of transcription factors is considered muscle-specific in mammals, with its expression essentially restricted to somites and muscle fibers. However, Myf5 and MRF4 transcripts have been detected in limited regions of mouse brains. In our studies of MRF4 gene regulation in the frog *Xenopus laevis*, we have consistently obtained in situ hybridization results showing MRF4 mRNA not only in the somites but also in anterior regions of neurula-stage embryos, apparently in the neural ectoderm. If verified, this expression raises the novel possibility that MRF4 plays a role in neural differentiation in *Xenopus*. To confirm our in situ results, we conducted RT-PCR analysis of RNA prepared from embryos (st. 17-19) that were first microdissected into anterior, dorsal, and ventral regions. Electrophoretic analysis of the dorsal, ventral, and anterior RT-PCR reactions confirmed the presence of MRF4 gene expression in the anterior as well as the dorsal regions and revealed low-level expression in the ventral region. Comparison of signal intensities indicated that the ratio of MRF4 mRNA to EF1α mRNA (a ubiquitously expressed control) is similar in the dorsal and anterior regions. Although the presence of MRF4 transcripts in the anterior neural ectoderm demonstrates neither the presence of MRF4 protein nor a role for MRF4 in neural development, these results suggest that further analysis of such a possibility should be considered.

2713
**Gli1 Overexpression as a Result of Altered Splicing of Quaking Transcripts May Contribute to Infertility in Male Quaking Viable Mice**
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Quaking viable (qk') mice are homozygous for a spontaneous deletion of about 1 Mb from the proximal end of chromosome 17 and show a phenotype that is characterized by tremors and infertility in males, the latter due to oligospermia, postmeiotic spermatogenic arrest, and a flagellar defect. The deletion has been shown to affect the regulation of 3 nearby genes: Quaking, Parkin and Parkin-coregulated gene (Pacrg). Transgenic expression of Pacrg in the testis has been shown to partially restore spermatogenesis in qk' mice. We previously demonstrated that QKI-6 down-regulates Gli1 translation and that transgenic expression of Gli1 in the testis affects spermatogenesis, raising the possibility that altered Quaking might also contribute to infertility in qk' males through misregulation of Gli1. We demonstrate Quaking isoform QKI-7 transcripts by RT-PCR in qk' testes instead of QKI-5, which are normally expressed in WT testes. Although it remains unclear if QKI-5 or QKI-7 regulates Gli1, histologic sections of qk' mutant testes show overexpression and abnormal distribution of the Gli1 protein. It has been previously suggested that Leydig cells represent important targets of Hedgehog-Gli1 signaling in the testis. Indeed, histologic sections of qk' testes show Leydig cell hyperplasia with tight apposition of Leydig cell clusters to the basal lamina of the seminiferous tubules. Our findings suggest that alterations in Gli1, together with loss of otx2 transcripts, and alteration in Notch signaling as a result of altered splicing of Quaking transcripts may contribute to qk' male infertility.

**Developmental Control of Gene Expression (2714-2716)**

2714
**Identification of Gene Regulatory Elements Associated with the Meis Family of Homeobox Genes**
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Homologues of the Meis homeobox-containing gene family (originally named for myeloid ecotropic leukemia virus integration site because a disruption of the first member of this gene family discovered was found to lead to Leukemia) have been identified in all animals studied. The products of the Meis genes appear to function as cofactors, directly interacting with other transcription factors as well as DNA to facilitate transcriptional regulation. Most notably, they appear to act as co-factors of the evolutionarily well-conserved Hox proteins and have also been described as acting with members of other homeobox genes including Pbx and potentially the Distalless (Dlx) family of homeobox genes. The vertebrate Meis homeobox-containing gene family consists of at least three members and while little to nothing is known about their regulation, they are expressed in conserved patterns throughout the embryonic development of those vertebrates that have been examined. We are currently using comparative genomics / phylogenetic footprinting to search for regulatory elements associated with the Meis family of homeobox-containing genes. We have, to date, identified four elements that are all located downstream of the Meis2 gene. These elements are very well-conserved in sequence and relative position amongst the genomes of all land vertebrates that we have been able to examine, including human, mouse and chicken, and we have recently identified one of these elements in both zebrafish and the pufferfish *Takifugu rubripes*.

2715
**Developmental Expression of Eph Receptors and Ephrin Ligands in Small Intestine**
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Eph receptor tyrosine kinases EphB2 and EphB3, and ephrin B1 ligands, play a critical role in regulating small intestinal epithelial cell migration (Batlle, Cell, 2002). Although well studied in tissues such as the developing brain, the expression pattern of Ephs and ephrins has not been delineated in the developing small intestine. Therefore, we undertook to define the expression of all 21 known A and B family members in mouse small intestine. mRNA expression profiles of all known Eph/ephrins were established by RT-PCR. In addition, differential expression for selected Ephs/ephrins was measured by real-time RT-PCR. Localization of EphB2, EphB4, and ephrin B3 was determined by immunohistochemistry. mRNAs for ephrins A1 - A5, B1 - B3; EphA1 - EphA4, EphA6, EphB1 - EphB4, and EphB6 are expressed constitutively from E13 through maturity. EphA5 and EphA7 showed very weak or no detectable expression at any age. In contrast, EphA8 was strongly expressed in fetal and newborn intestine, but low or undetectable in the adult. Quantitative analysis for EphA4, EphA8, EphB4, and ephrin B2 indicated that expression was strong at E13, then declined with age. Adult crypt cells expressed EphB2 and EphB3, but not ephrin B1, B2, or B3, indicating a shift in expression as cells cease proliferating and differentiate. EphB2 and EphB4 were localized to cell membranes of stratified epithelium at E15, then restricted to intervillus...
regions at E17, and later to cells at the bottom of adult crypts, marking proliferating cells. Most of the family members are expressed at similar levels throughout development, suggesting a continuing role in cell migration. EphA5 and EphA7 are unlikely to play a role in the normal intestine. Restricted expression of EphA8 during the fetal period suggests that it may play a regulatory role in the morphogenesis of the small intestine which occurs at this time.

The Effect of Peroxiredoxin III on the Late Erythroid Differentiation of K562 Cells
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Peroxiredoxin III (Prdx III), the mitochondrial peroxidase, was preferentially expressed in murine erythroleukemia (MEL) cells. However, the mechanisms by which Prdx III regulates erythroid differentiation are unknown. In this study, K562 cells were differentiated by Ara-C treatment, and Prdx III was dramatically increased until day 5. We also investigated Prdx III expression pattern on in vitro erythropoiesis of human CD34 cells. When human CD34 cells became proerythrocyte on day 7, Prdx III was diminished, and then augmented on day 12. We established the stable sublines of Prdx III overexpression (O/E), and dominant-negative (D/N). The intracellular ROS level of Prdx III O/E cell line was lower than D/N stable cell lines. Moreover, Prdx III O/E cell line was placed in G1 arrest, but not D/N cell lines. Finally, the expression level of b-globin and GATA-1 was dramatically increased in Prdx III O/E cell line. This work was supported by Korea Research Foundation Grant (KRF-2007-070-C00086) from MOEHRD.

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Chromatin and Chromosomes (2717-2724)

Role of Bacterial Histone-like Protein HIU in Caulobacter Chromosome Organization
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It has been demonstrated that the single circular chromosome in Caulobacter crescentus is highly organized. In a non-replicating cell, the position of chromosomal loci within the cell correlates with the distance of their map position from the single origin of replication, which is always located at one pole. This order is maintained during replication, as newly replicated loci move rapidly and directly to the same relative position in the incipient daughter cell. Many proteins are known to be involved in chromosome replication and segregation, but the mechanisms underlying their function are unknown. One of these factors, HIU, is a small, two-subunit histone-like protein that binds DNA non-specifically and is thought to condense the chromosome by bending the DNA. We have shown that deletion of either subunit alone or both together yields slow growing, but viable Caulobacter strains. To examine chromosome organization in cells lacking HIU, we assessed the localization of individual chromosomal loci using the fluorescent repressor-operator system (FROS). A tetO array was inserted at the origin of replication and a lacO array at a second locus of interest. Induced expression of fluorescently tagged TetR and LacI allowed visualization of the arrays in vivo and the distance of the fluorescent foci from the cell pole was determined. Deletion of either subunit alone had no effect on the organization of the chromosome. However, deletion of both subunits caused a significant variation in the cellular position of individual chromosomal loci while maintaining their same mean distance from the pole. The essential nature of chromosome organization is illustrated by the maintenance of the overall positioning of the studied loci despite the loss of HIU. However the greater variation in locus position suggests that loss of both HIU subunits results in some decondensation of the chromosome allowing more movement of individual DNA loci.

Distribution of Gene Silencing Proteins in Relation to Transcriptionally Active Sites
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Polycomb (PCG) and Trithorax (TrxG) proteins are considered to be regulators of gene expression. They act at chromatin level and can represent the example of a tight relationship between large-scale chromatin folding and gene activity. In order to study this connection, we have analyzed the localization of PCG and TrxG proteins in relation to functional domains of cell nucleus. As markers of transcriptionally active sites we used acetyl-histone H4 and nascent RNA labeled after incubation with halogenated uridine. Experiments were performed on high-pressure frozen and cryosubstituted mammalian cells. This approach allowed us to reduce the artifacts of conventional electron microscopy specimen preparation together with excellent preservation of antigenicity for immunogold labeling. Moreover, condensed areas can be easily differentiated. We found out that the surface of condensed chromatin areas is the most important sites of both the nucleoplasmic RNA transcription and the PCG and TrxG localization. Furthermore, we show that PCG and TrxG proteins are in a rather diffuse pattern, without notable local accumulation of colloidal gold grains. Interestingly, gene silencing proteins are also distributed in sites where nucleolar transcription takes place, i.e. in the dense fibrillar component. It suggests that PCG and TrxG can have a potential contribution in ribosomal gene regulation. Taken together, our fine structural results indicate that active and epigenetically silenced genes are closely associated in space. This work was supported by grants MSM0021620806, LC535, 304/06/1662, AVOZ50110509, and WT075834/04/Z.

Centromere Architecture Breakdown Induced by the Viral Protein ICP0
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We have recently described a novel cellular defence response that we named interphase centromere damage response or iCDR (Morency et al., J. Cell. Biol., 2007). The iCDR consists in the centromeric accumulation of the proteins col, fibrillarin, and SMN, components of the Cajal bodies and Gems, following the destabilisation of interphase centromeres. To provoke this destabilisation we mainly took advantage of the peculiar properties of the ICP0 protein of herpes simplex virus type 1. ICP0 is an E3-ubiquitin ligase nuclear protein that possesses the singular property to temporarily localize at centromeres. Subsequently, ICP0 induces the proteasomal degradation of constitutive centromeric proteins CENP-A and -C, provoking considerable damages to interphase centromeres, resulting in failure in microtubules binding during mitosis. To better understand the iCDR it is thus important to know if the stability of other constitutive CENPs is similarly affected by ICP0. Here, we show that major constitutive proteins such as CENP-B, -H, -I, and hMsi1 are removed from centromeres in an ICP0- and proteasome-dependent manner. Recent reports described the presence at interphase centromeres of multi-subunit centromeric protein domains that are associated with the CENP-H-I complex and/or with the centromere-specific CENP-A-containing nucleosomes. Using cells expressing GFP-fusion versions of proteins present in these complexes, i.e., CENP-M, -N, -O, -P, and -Q, we show that those proteins also disappear from centromeres as a result of ICP0 activity. Our results highly suggest that ICP0 is thus responsible for the complete collapse of the proteinaceous scaffold of interphase centromeres. In addition, the degradation of CENP-A, the histone H3 centromeric variant, is likely to result in major modifications of centromeric chromatin-associated nucleosomes. Therefore, the iCDR is probably set off as a consequence of a complete breakdown of the centromere architecture.

2720
Genomic Instability Caused by Chromium (VI) and/or γ-Radiation In Vitro
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Humans being may be exposed to radiation and/or heavy metals in orthopaedic surgery procedures, when radiation is performed to provide a diagnosis and metal ions may then be released by a prosthesis, for example after a hip replacement. Genomic instability (GI), which is considered to be an important component in carcinogenesis, can be caused by exposure to agents, which appear to act through induction of stress-response pathways related to oxidative stress. These agents have been studied mostly in the radiation field but evidence is accumulating that heavy metals, can also act in the same manner. Therefore, either Cr (VI) or γ-Radiation can initiate long-term GI in generations of daughter cells from parent human primary fibroblasts. This phenomenon is regulated by telomerase. The aim of this study was to examine the difference in clonogenic survival of normal human fibroblasts (NHFs) and engineered human fibroblasts (EHFs), infected with a retrovirus carrying a cDNA encoding hTERT, which rendered these cells telomerase positive and replicatively immortal. Cr (VI) induced GI in NHFs but not in EHF, whereas γ-Radiation induced GI in EHF and in less extent in NHF. Combined exposure caused GI in both types of fibroblasts. This GI was more pronounced in NHFs when γ-Radiation was followed by Cr (VI), and more pronounced in EHF when Cr (VI) was followed by γ-Radiation. Moreover, the biological effects provoked by combined exposure of Cr (VI) and γ-Radiation gave evidence of a synergistic action in both types of cells, compared to Cr (VI) treatment only or γ-Radiation exposure only. This study suggests that telomerase does prevent GI caused by Cr (V), but not γ-Radiation. Furthermore, GI induced by combined exposure appears to be prevented by telomerase only when γ-Radiation was followed by Cr (VI), and not vice versa.

2721
Nucleolus and Positioning of NORs and NOR-bearing Chromosomes
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It is known that chromosomes occupy non-random positions in the cell nucleus. But it is not clear to what extent their nuclear positions, together with their neighbourhood, are conserved in daughter cells. To address specific aspects of this problem, we used the model of the chromosomes carrying ribosomal genes, organized in clusters termed Nucleolar Organizer Regions (NORs). Using FISH and immunocytochemistry, we found a regular and stable pattern of distribution of the transcriptional competence for the different NOR-bearing chromosomes (NOR-chromosomes) in two human-derived cell lines: transformed HeLa and primary LEP cells. Next, we analyzed this pattern of transcriptional competence in relation to nucleolar association of the corresponding chromosomes in HeLa and LEP cells. Our data show that the tendency of rDNA bearing chromosomes to contact nucleoli correlates with the number of transcriptionally competent NORs on these chromosomes. Additionally, we found that not only competent, but also most of the non-competent, NORs are included in the nucleoli. Finally, we compared the association of chosen NOR-chromosomes with nucleoli in the pairs of daughter cells, and established how frequently the daughter cells had equal numbers of the homologs of certain NOR-chromosomes associated with individual nucleoli. The cell pairs with identical combinations appeared significantly more frequently than predicted by the random model. Although the daughter cells typically have different numbers of nucleoli, and the total number of chromosomes associated with nucleoli is variable, our data indicate that the position of the NOR-chromosomes in relation to nucleoli is partly inherited through mitosis. This work was supported by grants 075834/04/Z, MSM0021620806, LC535, AVOZ50110509, 304/06/1662 and 304/06/1691.

2722
Do the Mammalian Chromosomes "Remember" Their Nuclear Position in the Daughter Cells?
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Chromosomes in interphase nucleus occupy mutually exclusive chromosome territories. Number of experiments have shown that in vertebrate cells these chromosome territories are non-randomly arranged within the nuclear space. Important point is to what extent the chromosome positioning, together with the chromosome neighbourhood, is transmitted through mitosis to daughter cells. Several recent studies that dealt with this problem provided divergent conclusions. To expand the knowledge in this respect, we addressed a specific aspect of this problem and studied nucleolus associated chromatin. During early G1-phase chromosomes that carry ribosomal genes associate and give rise to the new nucleoli. To label nucleolus associated chromatin, we photoconverted the closest vicinity of nucleoli in HepG2 cells stably expressing histone H4-Dendra. By time-lapse live cell imaging we followed the fate of labeled interphase chromatin through mitosis to consequent generation. We observed that the labeling pattern remains stable from the moment of photoconversion in late S- or G2-phase as long as mitosis onset. After dynamic reestablishment of chromatin during mitosis and early G1-phase of the next cycle, nucleolus associated chromatin to a large extent did not re-entered to the vicinity of nucleoli, nevertheless the statistical analysis of labeled areas of chromatin showed that there was a certain extent of its conservation within nuclear space. The results obtained are thus not entirely in harmony with studies claiming that the nuclear positioning of chromosomes is basically conserved in the daughter cells. This work was supported by grants MSM0021620806, LC535, WT075834/04/Z and AV0Z50110509.

2723
**Characterization of the Novel Telomere Associated Protein hSnm1B**

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The *c. elegans* protein mrt1 shares homology with the single-strand telomere-binding protein POT1, and the DNA-damage response protein Snm1, prompting us to test whether human Smn1 proteins function at telomeres. The Smn family in humans is composed of three proteins: Artemis, which has already been shown to be important in maintaining telomere length and stability, hSnm1A, and hSnm1B, neither of which had been shown to interact with the telomere. We found that while hSnm1A did not localize to telomeres, hSnm1B localized to telomeres, as assessed by immunofluorescence, and co-immunoprecipitated specifically with telomeric DNA. However, hSnm1B did not associate with telomeric DNA directly, rather it bound to telomeres via an association with the double-strand telomere-binding protein TRF2. Moreover, we recently discovered that this association with TRF2 reduced poly-ubiquitination of Smn1B, and stabilized the protein. Future studies will be aimed at determining the importance of the interaction between hSnm1B and TRF2 as well as the consequence of hSnm1B stabilization.

2724
**Novel Function of Human Nucleolar Protein, Fibrillarin in Nuclear Morphology and Cellular Growth**

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Fibrillarin is a key small nucleolar protein in eukaryotes, which has an important role in pre-rRNA processing during ribosomal biogenesis. Though several functions of fibrillarin are known, its novel function during the cell cycle is still unknown. In this study, we revealed the dynamic localization of fibrillarin during the cell cycle of HeLa cells. During mitosis, fibrillarin is localized in the chromosome periphery and nuclear organizing regions and eventually accumulated in pre-nuclear bodies. Using a combination of immunofluorescence microscopy and RNAi technique, we also studied novel functions of fibrillarin. The depletion of fibrillarin revealed almost no effect on the nucleolar structure. However, fibrillarin-depleted cells showed significant abnormal nuclear morphology observed in live cell images. Moreover, fibrillarin depletion resulted in the reduction of the cellular growth, which was further supported by prolonged mitosis and modest accumulation of cells with 4n DNA content obtained by FCM analysis. Our data currently obtained suggest that fibrillarin would play a critical role for the maintenance of nuclear shape and cellular growth.

**Chromatin Remodeling (2725-2726)**

2725
**TIPS SANT Domain Is Required for TIP-p300-mediated Histone H3 and Histone H4 Acetylation and TIP-6-induced Adipogenesis**

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We previously identified a set of transcription regulators, referred to as TIPS (tension-induced/inhibited proteins), with a role in myogenic versus adipogenic differentiation. Here we report that the TIP family comprises eight isoforms, all bearing a SANT domain and some of them presenting SAM and NRB motifs. These functional sites are characteristic of histone modifying enzymatic complexes. HAT, HDAC and HMT assays with immunoprecipitated and recombinant TIPS plus siRNA interference studies showed that TIPS had SANT-dependent, p300-mediated HAT activity. TIP-6 was the only SANT(+)/SAM(-)/NRB(-) isofrom. Transfections and promoter-reporter assays showed that TIP-6 but not TIP-6ΔSANT induced a novo PPARγ2-mediated adipogenic gene expression in NIH-3T3 cells and promoted preadipocyte differentiation into fat cells. Furthermore TIP-6 was identified in adipose tissue in vivo. TIP-6 and TIP-6ΔSANT commomunoprecipitated p300 and histone H4. GST-pull downs showed that TIP-6 directly interacted with p300 and histone H4 and deletion of the SANT domain eliminated TIP6 binding to p300 but not to histone H4. ChiP assays showed that TIP-6 and TIP-6ΔSANT were recruited to the PPARγ2 promoter along with p300, but histone acetylation occurred only when p300 was associated with TIP-6. These studies demonstrated the importance of TIPS as regulators of p300 catalytic activity and identified a new member in the adipogenic cascade.

2726
**Dynamic Regulation of p53-Target Gene Expression by Peptidylarginine Deiminase 4**

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Histone Arg methylation has been correlated with transcriptional activation of p53 target genes. However, whether this modification is reversed to repress p53 target genes remains unknown. Here, we report that peptidylarginine deiminase 4 (PAD4), a histone citrullination enzyme, is involved in the repression of p53 target genes. Interference of PAD4 functions elevated the expression of a subset of p53-target genes, including p21/CIP1/WAF1, GADD45, and PUMA, leading to slow cell growth and apoptosis. Moreover, protein-protein interaction studies showed an interaction between p53 and PAD4, suggesting that p53 may recruit PAD4 as a corepressor for transcriptional regulation. Further, permanganate genomic footprinting and chromatin immunoprecipitation (ChiP) detected dynamic RNA Pol II activities at the p21 promoter during UV irradiation. Before UV treatment, the p21 promoter is associated with paused RNA Pol II and high levels of histone citrullination. After UV irradiation, an increase of histone Arg methylation and a decrease of citrullination were correlated with the activation of p21. Finally, at later time points, a loss of RNA Pol II and an increase of histone citrullination were detected at the p21 promoter. Together, these results implicated PAD4 in dynamic regulation of p53-target genes.

Ribonucleoproteins (2727-2728)

2727

Proteomic Analysis of Yeast Ribosomal Protein L35 Mutant

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Ribosomes are large ribonucleoprotein particles, consisting of two unequal subunits (40S and 60S), that carry out protein synthesis. Eukaryotic ribosomes contain one copy of each of four ribosomal RNAs (5S, 5.8S, 18S, and 25–28S) and about 75 different ribosomal proteins. Recently, it has been suggested that many ribosomal protein genes may act as haploinsufficient tumor suppressors in zebrafish (Danio rerio). Furthermore, some ribosomal protein mutations are known to be associated with human diseases. To obtain an insight into the novel roles of ribosomal proteins related to tumorigenesis or other pathological events, we sought to analyze changes in proteome expression in ribosomal protein mutants using Saccharomyces cerevisiae as a model system. We introduced rpl35aΔ or rpl35bΔ mutation into the 4,159 GFP-tagged yeast strains by synthetic genetic array method, and carried out genome-wide protein expression analysis in those ribosomal protein mutants using multilabel microplate reader and flow cytometer. We identified several overexpressed or underexpressed proteins in each ribosomal protein mutant. These proteins were functionally classified into the Gene Ontology categories of biosynthetic process, translation, metabolic process, spindle checkpoint, ribosome biogenesis and assembly, dephosphorylation, and mitotic checkpoint. Our results show that ribosomal protein mutation causes perturbation in expression of several proteins, which may form the basis for tumorigenesis or other pathological events in higher eukaryotes.

2728

SENP3 and SENP5 SUMO Proteases Regulate Ribosome Biogenesis

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SUMO proteins are ubiquitin-related proteins that have been implicated in a wide variety of cellular processes. Fission and budding yeast each express a single SUMO, while there are three commonly expressed mammalian SUMO paralogues. SUMO-2 and -3 are 96% identical in their processed forms. SUMO-1 is roughly 45% identical to either SUMO-2 or -3. Newly synthesized SUMO proteins are processed, to expose a C-terminal di-glycine motif that is essential for their conjugation. After conjugation, the linkage between SUMO proteins and the substrates can be hydrolyzed by SUMO proteases. SUMO processing and deconjugation are mediated by a family of proteases, called Sentrin specific proteases/Ubiquitin like proteases (SENP/Ulps). In mammals, there are six members of this family that act on SUMO proteins, although little is known about the individual biological roles of these enzymes. We have analyzed the sequences of human SENP/Ulps, and find that human SENP3 and SENP5 are most similar to each other and share a common localization to the nucleolus. Both of these enzymes show a strong SUMO parologue preference for SUMO2 and 3, but do not act efficiently on SUMO1. After their co-depletion by RNA interference (RNAi), normal exclusion of SUMO2 and 3 from nucleoli is lost, and SUMO2 and 3 become abundant within nucleoli. At the same time, we observe substantial defects in ribosomal RNA synthesis and processing, suggesting that deconjugation of SUMO2/3 is essential for normal ribosome biogenesis. These findings are interesting in light of earlier observations that Ulp1, the S. cerevisiae SUMO protease most closely related to SENP3 and SENP5, plays an essential role in pre-ribosomal particle export in yeast. Finally, we have found novel interactions of human SENP3 and SENP5 with nucleolar proteins, which are essential for their nucleolar localization and stability.

Nuclear Import and Export Signals (2729)

2729

The C-terminal Fragment of RXRα Inhibits Nuclear Import, Dimerization, and Transcriptional Activity of RARγ

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Retinoic acid receptors (RARs) mediate retinoic acid-induced transcriptional activation as homodimers or heterodimers with retinoid X receptor (RXR). Transfected RARγ, unlike endogenous one, was localized exclusively in cytoplasm, but imported into nucleus by ligand-binding or cotransfection with RXRα, suggesting that quantitative balance between RARγ and RXRα is an additional determinant for the distribution of RARγ. C-terminal 30 kDa fragment of RXRα, which has been reported to result from cleavage by cathepsin L type protease, completely blocked nuclear import of RARγ. This RXRα fragment interrupted RARγ homodimerization and heterodimerization with RXRα, and subsequently inhibited
transcriptional activity of RARγ. The RXRα fragment did not affect the localization and transcriptional activity of RXRα or other RAR subtypes, indicating that the action of RXRα fragment is specific to RARγ. Ectopic expression of RXRα fragment in SiHa cells abolished the p53 induction by retinoic acid treatment, while p53 induction by ionizing radiation was not affected. Taken together, we hypothesize that RXRα cleavage fragment acts as a physiological modulator for biological response to retinoic acid by restricting the activity of RARγ.

**Structure of Nuclear Envelope (2730-2737)**

2730

**Characterizing Nup153 as a Target of Sumoylation**

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SUMO (Small Ubiquitin-like Modifier) is a small peptide that can be covalently conjugated to a variety of protein substrates through regulated activities of SUMO E1 (SAE1, SAE2), E2 (Ubc9), and E3 (PC3, PIAS, Nup358) enzymes. Since SUMO modification on protein substrates can also be reversed by regulated activities of SUMO proteases, SUMO modification represents a powerful post-translational modification that dynamically modulates a variety of cellular processes. Interestingly, certain enzymatic components of the SUMO modification pathway localize at the NPC (Nuclear Pore Complex), a macromolecular structure that bridges the inner and outer nuclear membranes to form a gateway for nucleocytoplasmic trafficking, as well as to coordinate other events at the nuclear envelope. In a quest to understand physiological roles for the SUMO modification machinery at the NPC, experiments were undertaken to probe for SUMO-modification of the nuclear pore component, Nup153. Our observations suggest that the SUMO pathway may modulate the role of this nucleoporin. Specifically, in in vitro assays, the recombinant zinc finger domain of Nup153 can be SUMO modified and endogenous full-length Nup153 in *Xenopus* egg extract can also be sumoylated. The zinc finger domain of Nup153 is a distinctive secondary structure at the NPC that is not found in any other nucleoporins except Nup358. With the recent findings that the zinc finger domains of Nup153 and Nup358 are involved in mediating mitotic nuclear envelope disassembly by recruiting the coatomer complex COP1 to NPC, the SUMO modification of these zinc finger domains is hypothesized to have a regulatory function on mitotic nuclear envelope disassembly. Current work is focused on mapping sumoylation sites in the Nup zinc finger domains in order to determine whether these proteins are targets of sumoylation regulation during nuclear disassembly at the interphase to mitotic transition.

2731

**Nesprin-2 Giant Safeguards Nuclear Envelope Architecture in LMNA S143F Progeria Cells**

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The S143F lamin A/C point mutation causes a phenotype combining features of myopathy and progeria. We demonstrate here that patient dermal fibroblast cells have dysmorphic nuclei containing numerous blebs and lobulations, which accumulate as cells age in culture. The lamin A/C organisation is altered, showing intranuclear and nuclear envelope aggregates and presenting often a honeycomb appearance. Immunofluorescence microscopy showed that nesprin-2 C-terminal isoforms and LAP2α were recovered in the cytoplasm, whereas LAP2β and emerin were unevenly localised along the nuclear envelope. The intranuclear organisation of acetylated histones, histone H1 and the active form of RNA polymerase II were markedly different in patient cells. A subpopulation of mutant cells, however, expressing the 800 kDa nesprin-2 giant isoform did not show an overt nuclear phenotype. Ectopic expression of p.S143F lamin A in fibroblasts recapitulates the patient cell phenotype, whereas no effects were observed in p.S143F LMNA keratinocytes, which highly express nesprin-2 giant. Overexpression of the mutant lamin A protein had a more severe impact on the nuclear envelope of nesprin-2 giant deficient fibroblasts when compared to wild type. To explore whether nesprin-2 giant exhibits a general protective function on the NE architecture, fibroblasts from the classical LMNA G608G mutation and Zmpste24/-/- mutants were examined. The results show unequivocally that nesprin-2 giant presence at the NE is indicative of a normal nuclear shape. Inhibition of farnesylation by using FTI-277 in LMNA S143F and Zmpste24/-/- mutants not only ameliorates the nuclear morphology defects of the mutants but also coincidences with the restoration of the nesprin-2 localisation at the NE. Our results suggest that the p.S143F lamin A mutation affects nuclear envelope architecture and composition, chromatin organisation, gene expression and transcription. Furthermore, our findings implicate a direct involvement of the nesprins in laminopathies and propose nesprin-2 giant as a structural reinforcer at the nuclear envelope.

2732

**Kinesin Light Chain 1 - a New Binding Partner of Nesprin-2**

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Nesprin-2 is a giant nuclear envelope actin-binding protein, which belongs to the spectrin superfamily. The largest Nesprin-2 isoforms exhibit an N-terminal actin-binding domain followed by a long spectrin repeat containing rod and a C-terminal type II transmembrane domain, sufficient to anchor these proteins specifically to the nuclear membrane. A prerequisite and important aspect to understand the function of a protein is to specify and to characterize its interactions. In order to identify additional Nesprin-2 binding partners we performed a yeast-two-hybrid screening, of a human brain cDNA library using a C-terminal Nesprin-2 segment (Nesprin-2-SR) as bait. The screening yielded the microtubule motor associated protein KLC1 (kinesin light chain 1) as a new binding partner for Nesprin-2. Preliminary data show that endogenous KLC1 colocalizes with the microtubule cytoskeleton and with Nesprin-2 along the nuclear envelope in human keratinocytes and fibroblasts. Ectopically expressed GFP/Myc-tagged KLC1 confirmed the localization of KLC1 at the nuclear membrane, demonstrating unequivocally the ability of this protein to associate with the nucleus. Using GST-pulldown assays we provide biochemical evidence, corroborating the interaction of the Nesprin-2 spectrin...
repeat 20 and the sequence between spectrin repeat 20 and 21 with KLC1 in vitro. Our data suggest that Nesprin-2 may act as a linker, which may physically connect the nucleus to the microtubule cytoskeleton through KLC1 mediated interactions.

2733

α-N-Catenin: A Novel Binding Partner of Nesprin-2
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Nesprin-2 belongs to the family of α-actinin type actin binding proteins. It is ubiquitously expressed and primarily located at the nuclear envelope (NE). The largest Nesprin-2 isoform is composed of an N-terminal calponin homology domain that mediates binding to actin, followed by a massive spectrin repeat containing rod domain. At the C-terminus resides the KASH domain (Klarsicht/ANC-1/Syne-1 homology), which is sufficient for the NE localization. The ability to bind to membranes and the actin cytoskeleton simultaneously suggests a role as a scaffold, linking the nucleus to the actin cytoskeleton. In search for new binding partners for Nesprin-2, a yeast-two-hybrid screen was performed using a C-terminal part of the central rod domain (SR) as bait in a human pretransformed brain cDNA library. This study revealed a C-terminal fragment of an unknown α-N-Catenin isoform as a novel binding partner. α-Catenins are essential components of the classical Cadherin-β-Catenin-complex responsible for cell-cell adhesion. At present there is increasing evidence for additional regulatory functions, such as coordination of actin dynamics, cell proliferation and migration. Beside the interaction of Nesprin-2 with α-catenin in vivo in yeast cells we further verified the interaction biochemically by GST-pulldown assays. Using different GFP- and Myc-tagged α-N- and α-E-Catenin constructs including the full length protein we could show that all localize to the NE in Cos-7 and MDCK cells. These data were further verified by the localization of endogenous α-E-Catenin at the NE and Nesprin-2 at the plasma membrane in primary human keratinocytes. Taken together these data collectively suggest new roles for α-N-Catenin at the NE and for Nesprin-2 at the plasma membrane.

2734

Characterization of the Vertebrate Nup93 Nuclear Pore Sub-complex
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Vertebrate Nuclear Pore Complexes (NPCs) are large complexes, with a mass of about 125 MD. NPCs are conduits for active transport of large substrates between the cytoplasm and nucleus, and they allow the passive diffusion of small molecules into and out of the nucleus. NPCs contain around 30 distinct polypeptides, which have been assigned to a number of sub-complexes based on their biochemical interactions. Nup93 is the vertebrate homologue of Nie96, an essential yeast nucleoporin that localizes near the cytoplasmic and the nuclear periphery of the central NPC channel. Along with Nup155 and Nup205, Nup93 has previously been found as an interaction partner for Nup35, the mammalian homologue of budding yeast Nup53. It has therefore been proposed that Nup205, Nup155 and Nup93 stably interact within mammalian cells. We have examined the interaction partners of Nup93 by gel filtration and co-precipitation in mammalian cell extracts and Xenopus egg extracts. Under both interphase and mitotic conditions, we observe stable association of Nup93 with Nup205, but not with Nup155. Nup93 also interacts tightly with Nup188, and we observe association to a number of other nucleoporins, including Nup35. Based upon our findings, we propose that Nup205, Nup188 and Nup93 are constituents of a NPC sub-complex within vertebrate cells that is stable throughout the cell cycle. We will discuss interactions of this complex with other nucleoporins.

2735

Nuclear Envelope Assembly by Fusion between Nuclear Membrane Vesicles and Protein-free Liposomes
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We used an in vitro reconstitution system to study the assembly of nuclei and endoplasmic reticulum (ER). When membrane vesicles and cytosol isolated from Xenopus laevis egg extracts are mixed in the presence of sperm chromatin, nuclei with active DNA replication, functional nuclear pores and expanded nuclear envelopes (NE) are formed. Simultaneously branched membrane network of ER is observed. We previously showed that protein-free dioleoylphosphatidylcholine liposomes fused with each other at the surface of chromatin in the presence of cytosolic proteins but failed to form functional nuclei in the absence of membrane vesicles (C. Ramos at al, 2006). In this study we report that a 10-fold decrease in the concentration of membrane vesicles resulted in the formation of nuclei that were 3 times smaller in the surface area than control ones. These undeveloped nuclei failed to activate DNA replication and import substrates. Thus, neither liposomes nor membrane vesicles at lowered concentrations can form expanded, functional nuclei. However adding liposomes to diluted membrane vesicles compensated for the membrane shortage. rescued nuclei demonstrated “normal” size, active DNA replication and nuclear transport. Formation of rescued nuclei was active-transport dependent and involved fusion between liposomes and membrane vesicles. Liposomes also participate in ER formation if incubated with membrane vesicles in the presence of cytosol. Similarly membrane vesicles inactivated by treatment with trypsin were able to participate in the NE and the ER formation in the presence of cytosol. Therefore liposomes can participate in NE formation by fusing with membrane vesicles in GTP-dependent manner, compensate for shortage of membrane vesicles and restore nuclear activity. As for control nuclei, the growth of rescued ones requires active nuclear transport. Thus, in this in vitro system, NE assembly is limited not by the amount of transmembrane proteins but by the amount of available lipid material.
2736

A Mitotic Role for Nup153

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The mammalian nuclear pore complex is a large macromolecular structure that spans the two lipid bilayers of the nuclear envelope, creating a channel through which all nucleocytoplasmic transport is facilitated. Recent studies have implicated additional roles for certain components of the nuclear pore during mitosis, specifically in nuclear envelope remodeling, chromosome segregation, and mitotic progression. For instance, in an in vitro nuclear disassembly assay, Nup153 has been shown to be involved in nuclear envelope breakdown; the zinc-finger motifs of Nup153 bind COPI and are thought to recruit membrane remodeling complex to help disperse the nuclear envelope. The current study focuses on further understanding the molecular role of Nup153 during mitosis in mammalian somatic cells. Knockdown of Nup153 using RNAi resulted in a significant increase in the number of unresolved cytokinetic midbodies, multilobed nuclei, and multinucleated cells, suggesting that Nup153 is required for proper nuclear division and mitotic exit. Time-lapse imaging of cells expressing histone H2B-CFP confirmed these observations as depletion levels of Nup153 resulted in nuclear division defects, delayed mitotic progression, and, in some cases, complete mitotic failure. Consistent with its role in nucleocytoplasmic transport, knockdown of Nup153 also resulted in delayed transport kinetics. To determine whether the transport defect can be uncoupled from the mitotic defects and to address the role of the zinc-finger domain of Nup153 in these processes, a depletion/rescue strategy is underway using several different recombinant Nup153 constructs.

2737

Nup98 Uses Distinct Mechanisms to Target to Nuclear Bodies and the Nuclear Pore Complex

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Nup98, the only GLFG nucleoporin in vertebrates, is a dynamic protein that shuttles between the NPC and nuclear bodies. The GLFG repeat domain in the N-terminal half of Nup98 is required for association with nuclear bodies. The C-terminal domain contains a pore-targeting motif that binds to Nup96 and Nup88. However, in vivo this domain is inefficient and requires a synergistic contribution of the GLFG domain for efficient targeting. We previously hypothesized that this occurs through many weak but cumulative interactions between the GLFG repeats and FG repeats of other nucleoporins. In myelogenous leukemias, the Nup98 gene can be translocated with one of several homeobox genes to form Nup98/Homeobox fusions. Fusion proteins contain the GLFG domain but form novel nuclear bodies distinct from those of wild type Nup98. We hypothesized that these bodies assemble through interactions between FG repeats, as proposed to occur at the NPC. Through FRAP of Nup98/HoxA9 truncations containing varying numbers of FG repeats, we found that the dynamic association of Nup98/HoxA9 is indeed directly proportional to the number of FG repeats with a threshold of 21 FGs. At the NPC, the efficiency of Nup98 targeting was also proportional to the number of repeats. Unexpectedly however, FG domains from other nucleoporins were unable to substitute for the GLFG domain in NPC targeting or, in most cases, formation of nuclear bodies. In contrast, a GLFG domain from the yeast Nup116p was fully functional. Thus, enhanced NPC targeting is a specific characteristic of GLFG repeats. Both Nup98 and Nup98/HoxA9 nuclear bodies are dispersed by hexanediol; Nup98 at the NPC is not sensitive to this treatment. We conclude that although the GLFG repeats do self-assemble, the NPC-targeting function of the GLFG repeats is likely to occur through a specific binding partner. Potential partners and functional implications will be discussed.

Germ Cells and Fertilization (2738-2742)

2738

Cleavage Status of ZP2 Determines Initial Sperm-Egg Recognition in Mice

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The zona pellucida is an extracellular matrix surrounding mammalian eggs that mediates fertilization and ensures passage of the pre-implantation embryo through the oviduct. Sperm recognize the mouse zona (ZP1, ZP2, ZP3) prior to, but not after, fertilization. Several biochemical changes have been inferred to account for this dichotomy, but only proteolytic cleavage of ZP2 has been experimentally demonstrated. Although widely accepted that initial sperm-egg recognition is mediated by zona pellucida ligands binding to sperm surface receptors, definitive identification of putative zona ligand(s) and sperm receptor(s) has been elusive. We have proposed an alternative model in which cleavage status of ZP2 determines whether the zona pellucida is permissive (intact ZP2) or non-permissive (cleaved ZP2) for sperm-egg recognition. This model is based on the observation that replacement of endogenous mouse ZP2 with the human homologue results in a chimeric zona which preserves sperm-egg recognition in 2-cell embryos. The persistence of gamete interactions correlates with human ZP2 remaining uncleaved despite cortical granule exocytosis. To confirm the central role of ZP2 in sperm-egg recognition, the cleavage site was determined by microscale Edman degradation and transgenic mouse lines with moZP2 mutated to prevent cleavage (mutZP2) were established. Each of six founder lines expressed both normal and mutated ZP2 proteins as determined by immunoblot of eggs and 2-cell embryos. Two lines have been crossed into the Zp2 null background and eggs/embryos will be analyzed for sperm-binding assays and in vitro fertilization. The mutZP2 rescue female mice are fertile in vivo and we anticipate that sperm will bind and fertilize their eggs in vitro; and that sperm-egg recognition will persist in pre-implantation embryos. The molecular basis by which ZP2 cleavage blocks sperm-egg recognition remains under investigation.

2739

Compartmentalization and Processing of Acrosomal Matrix Proteins SP-10 and AM50 in Guinea Pig Sperm Support a Sequential Mechanism for Zona Pellucida Adhesion and Acrosomal Matrix Disassembly during Fertilization

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The zona pellucida is an extracellular matrix surrounding mammalian eggs that mediates fertilization and ensures passage of the pre-implantation embryo through the oviduct. Sperm recognize the mouse zona (ZP1, ZP2, ZP3) prior to, but not after, fertilization. Several biochemical changes have been inferred to account for this dichotomy, but only proteolytic cleavage of ZP2 has been experimentally demonstrated. Although widely accepted that initial sperm-egg recognition is mediated by zona pellucida ligands binding to sperm surface receptors, definitive identification of putative zona ligand(s) and sperm receptor(s) has been elusive. We have proposed an alternative model in which cleavage status of ZP2 determines whether the zona pellucida is permissive (intact ZP2) or non-permissive (cleaved ZP2) for sperm-egg recognition. This model is based on the observation that replacement of endogenous mouse ZP2 with the human homologue results in a chimeric zona which preserves sperm-egg recognition in 2-cell embryos. The persistence of gamete interactions correlates with human ZP2 remaining uncleaved despite cortical granule exocytosis. To confirm the central role of ZP2 in sperm-egg recognition, the cleavage site was determined by microscale Edman degradation and transgenic mouse lines with moZP2 mutated to prevent cleavage (mutZP2) were established. Each of six founder lines expressed both normal and mutated ZP2 proteins as determined by immunoblot of eggs and 2-cell embryos. Two lines have been crossed into the Zp2 null background and eggs/embryos will be analyzed for sperm-binding assays and in vitro fertilization. The mutZP2 rescue female mice are fertile in vivo and we anticipate that sperm will bind and fertilize their eggs in vitro; and that sperm-egg recognition will persist in pre-implantation embryos. The molecular basis by which ZP2 cleavage blocks sperm-egg recognition remains under investigation.
The acrosome is a modified secretory vesicle in sperm and the acrosomal matrix (AM) is a large complex analogous to the dense core of a secretory vesicle. When sperm bind to the egg’s extracellular matrix, the zona pellucida (ZP), at the onset of fertilization, the acrosomal contents are released; but the role of the AM in this process is not fully understood. Previous studies have shown that the guinea pig sperm AM is comprised of only a few proteins including AM67, a homologue of the ZP-binding mouse protein sp56, and proacrosin, a serine protease. In this study we show by immunofluorescence microscopy and Westerns that the acrosomal protein SP-10 is a component of the AM in guinea pig and mouse sperm. Like the other AM proteins in guinea pig sperm, SP-10 has a discrete distribution but several different patterns of distribution were observed in different cells. During exocytosis a significant proportion of SP-10 remains associated with sperm for a longer duration than other AM proteins. Westerns of isolated AM showed that SP-10 is modified during AM disassembly, but whether this modification is due to proteolysis is unclear. These Westerns confirmed the N-terminus of another AM protein, AM50, is cleaved, but AM67, an sp56 orthologue, did not undergo any major processing during AM disassembly. In experiments to evaluate the mouse sperm AM, the distribution of SP-10 overlapped with sp56 but was more extensive in the posterior region. These results support a model of sperm-ZP interactions that involve, in part, a sequential series of adhesions with several AM proteins accompanied by processing of AM50 (and possibly SP-10) and AM disassembly that would serve to detach a sperm embedded in the ZP from restrictive adhesions and allow the traversal of the ZP. Supported in part by NIH HD41552.

2740

Phthalates are infused with polymers to increase flexibility of plastics. Exposure to phthalates is ubiquitous, e.g., food containers, toys, baby bottles, IV tubing, blood storage bags, PVC flooring, and household dust. Di-(2-ethylhexyl) phthalate (DEHP), one of the most commonly used phthalates, has adverse effects on the male reproductive system. Moreover, DEHP may shorten the lifespan of the fruit fly, Drosophila melanogaster, as well as increase lipid peroxidation, an indicator of oxidative stress. Therefore, this study determined whether DEHP affects Drosophila development. Adult wild-type flies were grown in glass vials containing insect medium (control) or insect medium with 500 μM DEHP. After 7 days this parental generation (P) was transferred to new vials and exposed to DEHP (500 μM) for an additional 14 days. The resulting F1 generations from both long and short-term exposure were counted on day 21. Both exposures to 500 μM DEHP were found to have no adverse effects on the P generation as compared to control. No differences in the number of flies of the F1 generation were observed after short-term exposure (7 days) to DEHP when compared to the matched control. Interestingly, longer exposure (21 days) to 500 μM DEHP reduced the number of flies in the F1 generation when compared to the matched control. Phase contrast imaging of testicular squashes revealed abnormal association of nuclei with the nebkern (mitochondrial structure) in round spermatids, which is indicative of a meiotic cytokinesis defect. Our preliminary findings suggest that acute exposure to 500 μM DEHP, although not lethal to adult flies (i.e. P generation), appears to affect the development of the fly; possibly through meiotic defects in spermatogenesis. Further studies will be designed to elucidate whether these effects observed after acute exposure to 500 μM DEHP exposure result from increased oxidative stress.

2741
Effects of In Utero Exposure to Tributyltin on Epididymal Sperm Motility and Epididymal Junctional Proteins in the Rat

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Tributyltin (TBT) is an environmental contaminant used in anti-fouling agents for boats and is a by-product of industrial processes. TBT is known to have adverse effects on the male reproductive system. Moreover, DEHP may shorten the lifespan of the fruit fly, Drosophila melanogaster, as well as increase lipid peroxidation, an indicator of oxidative stress. Therefore, this study determined whether DEHP affects Drosophila development. Adult wild-type flies were grown in glass vials containing insect medium (control) or insect medium with 500 μM DEHP. After 7 days this parental generation (P) was transferred to new vials and exposed to DEHP (500 μM) for an additional 14 days. The resulting F1 generations from both long and short-term exposure were counted on day 21. Both exposures to 500 μM DEHP were found to have no adverse effects on the P generation as compared to control. No differences in the number of flies of the F1 generation were observed after short-term exposure (7 days) to DEHP when compared to the matched control. Interestingly, longer exposure (21 days) to 500 μM DEHP reduced the number of flies in the F1 generation when compared to the matched control. Phase contrast imaging of testicular squashes revealed abnormal association of nuclei with the nebkern (mitochondrial structure) in round spermatids, which is indicative of a meiotic cytokinesis defect. Our preliminary findings suggest that acute exposure to 500 μM DEHP, although not lethal to adult flies (i.e. P generation), appears to affect the development of the fly; possibly through meiotic defects in spermatogenesis. Further studies will be designed to elucidate whether these effects observed after acute exposure to 500 μM DEHP exposure result from increased oxidative stress.

2742
The Investigation of meg-1, an X Linked Gene Required for Germline Development, in C. elegans

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In C. elegans, germ granules called P granules are directly inherited from mother to daughter and segregate with the germ lineage as it separates from the soma during the initial embryonic cell divisions. The gene meg-1 (maternal-effect germ cell defective) is an X-linked gene expressed in the maternal germ line and the encoded protein localizes to P granules during embryonic germ line segregation. It has been shown that meg-1 is required for proper germ cell function and the progeny of meg-1 mutants are sterile with a dramatic lack of germ cells in a shortened germ line. Here we continue the investigation of meg-1 during embryonic germ line segregation. To determine whether the decrease in germ cell proliferation is caused by an increase in apoptosis, we crossed meg-1 mutants to a ced-4 mutant in which apoptosis is blocked. We show that ced-4 does not rescue ger
cell proliferation in the meg-1 background suggesting that apoptotic cell death is not responsible for the lack of germ cells in the meg-1 mutant. It has previously been demonstrated that MEG-1 localizes to P granules with the assistance of MES-1 however it was unclear if this is a specific relationship between MES1 and MEG1 or a generalized relationship for other P granule proteins. To determine whether the role of mes-1 is a specific to MEG-1, we assessed the localization of other P granule components in a mes-1 mutant background. Our preliminary data suggests that GLH-1 also requires MES-1 for localization, but PGL-1 does not. We speculate that MES-1 is a general recruitment factor for P-granule component proteins, and maybe GLH-1 and MEG-1 share similar signals for recruitment while PGL-1 is localized in a MES-independent manner by an undefined mechanism.

A-17

Organogenesis (2743-2745)

2743

Alpha 2-Macroglobulin Regulation of Axial and Gut Morphogenesis in Xenopus laevis
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Alpha 2-macroglobulin (A2M) is a major serum protein that inhibits protease activity and binds growth factors. In Xenopus laevis, two A2M genes, Endodermin (Edd) and Panza, have been isolated. Edd is expressed in endoderm and dorsal mesoderm cells from gastrula to neurula stage and with the onset of gut coiling expression is restricted to the liver. In contrast, Panza is expressed in the dorsal domain of the gut endoderm from neurulation through the late tadpole stage. During gut coiling Panza expression is initiated in the liver and is maintained in the liver as Panza expression is lost from other gut regions. The distinct and overlapping expression patterns of Edd and Panza in gut endoderm suggests that A2M function may be important for differentiation and morphogenesis of the gut. To examine the role of Edd and Panza in gut development, loss-of-function experiments using morpholino oligos (MO) were performed to knockdown Edd and Panza individually and together. Panza knockdown resulted in a ventral bending of the posterior axis, lack of gut coiling, kinking of the notochord and somites defects. Edd knockdown resulted in a shortened body axis, a failure of tail outgrowth, lack of gut coiling, notochord shortening and somites defects. Knockdown of Edd and Panza resulted in a dramatically shortened body axis, an absence of tail structures, head and eyes defects, lack of gut coiling, notochord degeneration and disorganized somites. Mesodermal and endodermal patterning was examined from the gastrula stage through the coiled gut stage in single and double knockdown embryos. Formation and patterning of the mesoderm and endoderm was unaffected by Edd or Panza knockdown. These results suggest that A2M function is essential for axial and gut morphogenesis by influencing the shape and movement of cells and tissues, rather than by perturbing developmental patterning of mesodermal and endodermal tissues.

2744

MicroRNAs in Pancreas Development
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Objective: The long-term goal of this study is to elucidate the role of microRNAs (miRNAs) in vertebrate pancreas development and disease. Recently a number of studies have implicated miRNAs as critical regulators of diverse biological processes including cell proliferation, apoptosis, lineage specification, embryonic development and cancer. However, the role that miRNAs might play in regulating pancreatic development has not been fully explored. Methods: To assess the role of miRNAs in mouse pancreas development we selectively deleted (a key enzyme required for miRNA processing) in mouse embryonic pancreas by crossing Pdx1-Cre mice with floxed Dicer mice. We have also performed extensive miRNA expression profiling in four different developmental stages of mouse pancreas (E11.5, 13.5, 15.5 and adult), using miRNA microarrays (Invitrogen). Validation of microarray results was performed using quantitative PCR and in situ hybridization (ISH). Results: Pancreatic tissue in Pdx-Cre; Dicerloxp/wt mice displayed profound defects in normal development compared to Pdx-Cre; Dicerloxp/wt littermate controls. Pancreatic tissue lacking Dicer failed to undergo normal growth and morphogenesis and showed a severe reduction in both endocrine and exocrine cell types. In our microarray analysis, 48 different miRNAs showed statistically significant changes across developmental time points. Among these, miR-7, miR-99a, miR-125b, miR-200c and miR-217 showed distinct dynamic expression patterns confirmed by quantitative PCR. ISH using a locked nucleic acid (LNA) probe for miR-125b suggested expression throughout the pancreatic epithelium at E11.5, with progressive restriction to the islet and ductal compartments in adult pancreas. Conclusion: miRNA processing is absolutely required for mouse pancreas development and the observed dynamic expression of a discrete set of miRNAs as a function of developmental stages that they might act as novel regulators of pancreatic development and differentiation.

2745

Involvement of TIP-1 in Mouse Embryonic Kidney Development
Y. Zhou, K. Badri, X. Zhe, S. Lucia; Pathology, Wayne State University, Detroit, MI

TIPs are a family of transcriptional regulators originated by alternative splicing from a single gene in chromosome 2q22-23 in mouse and chromosome 2q31.1 in human. TIPs were originally isolated from mesenchymal cells of E11 mouse embryonic lungs and have been shown to promote myogenesis or adipogenesis in an isoform-specific manner through their abilities to interact with proteins that are functional on myogenic or adipogenic promoters. The current study shows that TIP-1 is found in the ureteric buds of developing mouse embryonic kidneys and in the distal convoluted tubules in the adult organ. Treatment of E12 mouse kidney explants with TIP-specific siRNAs inhibited endogenous TIP-1 mRNA expression by 84.92 ± 18.70%. Decrease in TIP-1 significantly inhibited ureteric branching morphogenesis and suppressed subsequent glomeruli formation. An initial study of some of the proteins with a known role in kidney morphogenesis showed down-regulated expression of factors involved in ureteric bud outgrowth, such as GDNF and Sal1, and factors that contribute to mesenchyme-epithelium transition, such as Wnt4b and Wnt11. These data explicit that TIP-1 plays a role in mouse embryonic kidney development.
Mammalian Development (2746-2752)

2746 Balance between Bmp4 and Activin Signalling Regulates Fgf3 Expression and Epithelial Stem Cells in Mouse Incisors
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In a continuously growing mouse incisor epithelial stem cells are located in cervical loops. Stem cells proliferate within stellate reticulum and progeny invade the basal epithelial cell layer. These cells differentiate into enamel forming ameloblasts on the labial side whereas on the lingual side differentiation does not occur and cervical loop also remain small. Bone morphogenetic protein4 (BMP4) is known to induce the differentiation of ameloblasts while the proliferation of the epithelium is stimulated by Fibroblast growth factor10 (FGF10). Follistatin inhibits signalling of several Transforming growth factor β superfamily members such as BMP4. Fgf3 have partially overlapping expression pattern with Fgf10 in the mesenchyme. We analysed mice overexpressing Follistatin in the epithelium (Keratin-14 Follistatin) and noticed that they had small incisors with a hypoplastic cervical loop on the labial side. In contrast, Follistatin-/- mice had enlarged stellate reticum on the lingual side which contained active proliferation. The expression of Fgf3 was completely down regulated in the Keratin-14 Follistatin mice but surprisingly in the Follistatin-/- incisors there were ectopic expression on the lingual side. In tissue culture experiment BMP4 was able to inhibit Fgf3 expression while ActivinA induced ectopic expression of Fgf3 on the lingual side and stimulated epithelial cell proliferation in the cervical loops. This effect was reversed by inhibition of Activin receptor-like kinase receptors which caused reduction in proliferation. The enamel formation and growth of incisors were more affected when there was less FGF signaling. Our results indicate that the epithelial stem cell niche in the incisor is redundantly regulated by FGF3 and FGF10, and BMP4 negatively controls the expression of Fgf3 and epithelial cell proliferation. Activin counteracts the inhibition on the labial side and thus stimulates stem cell proliferation. Follistatin contributes to the formation of normal asymmetric incisor by inhibiting the function of Activin on the lingual side.

2747 Investigation of the Developmental Expression of Yeast vps33 Mammalian Homologues Suggests Their Relevance to the Development of Human Kidneys
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Background VPS33A and B are homologues of yeast Vps33p. They are thought to be involved in tethering of endosomes to the target membranes prior to SNARE-complex formation. The naturally occurring buff mouse is a phenocopy of Hermansky-Pudlak (HPS) syndrome causing hypopigmentation and a platelet aggregation defect which is caused by a missense substitution in Vps33a. In mouse embryos, VPS33A and VPS33B in human tissues was studied using quantitative real-time PCR and immunoblotting. Expression of Vps33a and Vps33b in developing mouse embryos was studied by in-situ hybridisation. Results 1) The samples of mRNA and protein were obtained from 7-20 week gestation human embryos (liver, kidneys and brain), neonatal liver and adult liver, kidneys and brain. Quantitative real-time PCR and immunoblotting showed that the expression of VPS33A and VPS33B was upregulated (at least 4-fold) in the kidneys from 11-16 week gestation embryos. Expression of VPS33B was upregulated in neonatal compared to embryo liver. 2) In mouse embryonic tissues there was a generally higher level of expression of Vps33a. Low expression of VPS33B was seen in the fetal liver but otherwise the pattern of expression was similar between Vps33a and Vps33b. The expression in the renal cortex was identifiable at e14.5 stage. Conclusions Fetal expression of vps33 homologues suggests its importance in renal development. The surprisingly mild renal phenotype in the buff mouse may be due to the relatively weak effect of the genetic alteration on the Vps33a protein product.

2748 A Homeotic Gene, Hoxc8, Regulates the Expression of Proliferating Cell Nuclear Antigen in NIH3T3 Cell
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Hoxc8 is one of the homeotic developmental control genes regulating the expression of many downstream target genes, through which animal body pattern is established during embryonic development. In previous proteomics analysis, proliferating cell nuclear antigen (PCNA) which is also known as cyclin, has been implied to be regulated by Hoxc8 in F9 murine embryonic teratocarcinoma. The sequence analysis of the 5’ upstream region of PCNA revealed that about 1.17 kbp (kilo base pairs) region (-520 to -1690) contained twenty Hox core binding sites (ATTA). In order to test whether this region is responsible for Hoxc8 regulation, the upstream 2.3 kbp fragment of PCNA was amplified using PCR and then cloned into the pG3 basic vector harboring a luciferase gene as a reporter. When the luciferase activity was measured in the presence of effector plasmid (pcDNA.c8) expressing murine Hoxc8, the PCNA promoter driven reporter activity was reduced. To confirm whether this reduction is due to the Hoxc8 protein, the siRNA against Hoxc8 (5’-GUA UCA GAC CUA GGA ACU A-3’ and 5’-UAG UUC CAA GGU CUG AUA C-3’) was prepared. Interestingly enough, siRNA treatment up regulated the luciferase activity which was down regulated by Hoxc8, indicating that Hoxc8 indeed regulates the
Interestingly, we identified two genes with an obvious connection to the Wnt pathway: these observations indicate that Barx2 is an important regulator of muscle growth and repair. Downregulation of differentiation markers such as MyoD and Myogenin, suggesting that Barx2 could control activation of satellite cells. Together, parental strain alone. In satellite cell cultures, Barx2 regulates differentiation and directly controls the expression of muscle-specific genes in injury. In addition, loss of Barx2 in dystrophic mdx mice (Barx2/mdx double null) leads to a much more severe muscle phenotype than either cell function. Consistent with this notion, mice lacking the Barx2 gene show reduced body and muscle mass and defective repair after acute muscle postnatal muscle growth and repair. Barx2 is strongly upregulated in Pax7-expressing cells after muscle injury suggesting a role for Barx2 in satellite muscle development that interacts with other muscle-expressed transcription factors. Barx2 is expressed in a subset of cells in embryonic, juvenile, and adult muscle and overlaps strongly with Pax7 expression. Pax7 defines a muscle progenitor population called satellite cells that are essential for postnatal muscle growth and repair. Barx2 is expressed in a subset of cells in embryonic, juvenile, and adult muscle and overlaps strongly with Pax7 expression. Pax7 defines a muscle progenitor population called satellite cells that are essential for postnatal muscle growth and repair. Barx2 is strongly upregulated in Pax7-expressing cells after muscle injury suggesting a role for Barx2 in satellite cell function. Consistent with this notion, mice lacking the Barx2 gene show reduced body and muscle mass and defective repair after acute muscle injury. In addition, loss of Barx2 in dystrophic mdx mice (Barx2/mdx double null) leads to a much more severe muscle phenotype than either parental strain alone. In satellite cell cultures, Barx2 regulates differentiation and directly controls the expression of muscle-specific genes in cooperation with MyoD and SRF. Moreover, satellite cell cultures prepared from Barx2−/− muscle show a decreased proliferation rate and downregulation of differentiation markers such as MyoD and Myogenin, suggesting that Barx2 could control activation of satellite cells. Together, these observations indicate that Barx2 is an important regulator of muscle growth and repair.

2749

Mapping the Expression Pattern of FOXP2 in the Cerebral Cortex

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Much attention has been directed towards not only the molecular and environmental basis of speech and language development, but also its genetic and developmental determinants. FOXP2, a forkhead transcription factor, is considered a speech gene because chromosomal breakage leads to a mutation in its binding domain which in turn is linked with impairments in orofacial movements and verbal dyspraxia or other speech related problems. FOXP2 is expressed many areas of the developing brain further supporting a role in speech and language. Interestingly FOXP2 mRNA is expressed in homologous brain areas of postnatal mice as compared to fetal humans. In recent studies FOXP2 has been found to be expressed in the deep layers of the cerebral cortex, mainly in layers V and VI of mice and humans suggesting that cortical FOXP2 expression pattern is evolutionarily conserved. However, immunohistochemical staining of fetal human brain and mouse brain revealed that FOXP2 was expressed only in layers V and VI in mice, but in humans expression was expanded to include layer IV, the principal target of projections from the thalamus. Because of these differences we mapped the expression pattern of FOXP2 in the cortical layers of a wide variety of species. The results of our study of over 30 species of primates and non primates show that in almost every primate, as well as carnivores, FOXP2 is expressed in layers IV, V, and VI while other non primates only express FOXP2 in layers V and VI. We speculate that FOXP2 in layer IV of the cortices of carnivores and primates could be linked with the ability for higher cognitive tasks, and may serve as an indication of the higher intelligence in comparison to non-primates that do not express FOXP2 in layer IV.

2750

Identification of Dkk4 as a Target of Eda-A1/Edar Pathway Reveals an Unexpected Role of Ectodysplasin as Inhibitor of Wnt Signalling in Ectodermal Placodes

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Hypohidrotic ectodermal dysplasia (HED) is characterized by defect in number and shape of teeth, lack of certain glands (like sweat and lachrymal glands), and sparse hair. HED is caused by mutations in genes of the ectodysplasin (Eda) pathway including Eda, Eda receptor (Edar) or adapter protein Edaradd leading to defective signaling during ectodermal appendages development. The ectodysplasin A1 isoform (Eda-A1) and its receptor Edar activate NF-KappaB pathway. Eda-A1/Edar signaling pathway is one of the earliest ectodermal messages promoting the formation of the epithelial placodes that are the first signs of developing skin appendages. Formation of placodes is regulated by the interplay of both stimulatory and inhibitory signals. Both Eda-A1 and Wnts are critical activators of placode formation. To identify direct targets of ectodysplasin pathway, we performed microarray profiling of genes differentially expressed upon a short exposure to recombinant Eda-A1 in cultured embryonic skin. Interestingly, we identified two genes with an obvious connection to the Wnt pathway: dkk4 (most highly induced gene in the screen), and lrp4 were upregulated upon Edar activation. Both genes colocalized with Eda-A1 receptor Edar in placodes of ectodermal organs. However, low dkk4 and lrp4 expression was retained in the absence of NF-kappaB signalling in eda−/− hair placodes. Here, we provide evidence that their expression was dependent on Wnt activity present prior to Eda-A1/Edar signalling. Dkk4 was recently suggested as a key Wnt antagonist regulating lateral inhibition essential for correct patterning of hair follicles. Several pieces of evidence suggest Lrp4 as a Wnt inhibitor, as well. The finding that Eda-A1 induces placode inhibitors was unexpected, and underlines the importance of delicate fine-tuning of signalling during placode formation.

2751

The Homeodomain Transcription Factor Barx2 Regulates Satellite Cell Activation and Differentiation during Muscle Development and Regeneration

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Skeletal muscle development is regulated by several families of transcription factors including bHLH proteins (MRFs), MADS-box proteins (MEFs and SRF) and homeobox/paired-box proteins (such as Pax7 and Pax3). Here we report that the Barx2 homeobox protein is a novel regulator of muscle development that interacts with other muscle-expressed transcription factors. Barx2 is expressed in a subset of cells in embryonic, juvenile, and adult muscle and overlaps strongly with Pax7 expression. Pax7 defines a muscle progenitor population called satellite cells that are essential for postnatal muscle growth and repair. Barx2 is strongly upregulated in Pax7-expressing cells after muscle injury suggesting a role for Barx2 in satellite cell function. Consistent with this notion, mice lacking the Barx2 gene show reduced body and muscle mass and defective repair after acute muscle injury. In addition, loss of Barx2 in dystrophic mdx mice (Barx2/mdx double null) leads to a much more severe muscle phenotype than either parental strain alone. In satellite cell cultures, Barx2 regulates differentiation and directly controls the expression of muscle-specific genes in cooperation with MyoD and SRF. Moreover, satellite cell cultures prepared from Barx2−/− muscle show a decreased proliferation rate and downregulation of differentiation markers such as MyoD and Myogenin, suggesting that Barx2 could control activation of satellite cells. Together, these observations indicate that Barx2 is an important regulator of muscle growth and repair.
Effect of Retinoic Acid on Palate Formation during Rat Embryogenesis

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All-trans Retinoic Acid (RA), a Vitamin A derivative, is required for normal pattern formation during embryogenesis. High concentration of RA in embryos results in fetal malformations, such as cleft palate, cleft lip, microphthalmia, and unascended kidneys, etc. Since craniofacial abnormalities induced by the ectopic expression of HoxA7 has been reported to be similar to that induced by RA, we have studied the expression pattern of Hox genes in RA-treated embryos. Since the development of craniofacial structure started at day 11 p.c (E11), RA (100 mg/kg) was injected intraperitoneally into the pregnant (E11) female rat, and then embryos (E13 to E17) were isolated. The overall developmental process was shown to be delayed in the RA-treated embryos and the cleft palate was clearly detected after E16. After isolating the total RNA from the head region deleting BioGreen21 program, RDA, and KRF-2005-204-C00074, Republic of Korea.

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Invertebrate Development (2753-2754)

The Notch Signaling Pathway Is Required for Ommatidial Rotation in the Drosophila Compound Eye

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Many epithelial tissues are characterized by not only an apical-basolateral polarity but also polarization within the plane of the epithelium, a phenomenon known as Planar Cell Polarity (PCP). PCP establishment in the Drosophila compound eye requires correct cell-fate establishment, followed by the precise rotation of clusters of photoreceptor cells. The conserved Fz/PCP signaling pathway is required during these processes to establish the identity of R3 and R4 photoreceptors and to determine the direction of ommatidial rotation. However, recent studies suggest that input from additional pathways, such as EGFR, is required to coordinate ommatidial movement by signaling to molecules that regulate the cytoskeleton as well as cell adhesion. While genes such as the serine/threonine kinase Nemo have been implicated in rotation, very few additional genes and pathways have been identified to control this complex cell motility process. The overall objective of our study was to identify and characterize genes required in ommatidial rotation. Here we report several lines of genetic evidence that indicate the Notch signaling pathway may provide control of ommatidial rotation. Initial attempts in our lab to isolate modifiers of a Nemo overexpression phenotype identified Notch as a dominant suppressor. Modulation of signaling through Notch alleles or by overexpression of the Notch ligand Delta and the Delta-associated gene Neuralized in photoreceptors results in ommatidial rotation defects. In addition, loss-of-function mutations in other Notch pathway components can dominantly suppress the effects of Nemo overexpression. Our observations suggest a role for the Notch pathway in ommatidial rotation and that Notch may interact with Nemo during epithelial morphogenesis.

Ultrastructure in Testis of Mutant Mip40 of Drosophila melanogaster

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In Drosophila spermatogenesis, a single gonialblast cell undergoes four mitotic division and 16 primary spermatocytes go through each subsequent event. In the steps of this process, loss of function mutation lead to the production of abnormal spermatids arrest in progression or abnormal cellular differentiation, leading to male sterility. Mip40 (Myb -interacting protein 40) has been shown previsously to be involved in spermatid differentiation and loss of function in mip40 female have severely reduced fertility and mip40 mutant male were sterile. Using electron microscopy analysis, we examined the ultrastructure in testis of mip40 mutant of Drosophila melanogaster. And we found the altered structure of testis in mip40 mutant. Our data demonstrate Mip40 is a subunit in a testis-specific complex required for morphogenesis during spermatid differentiation.

Cell Polarity (2755-2761)

Robust Neuronal Symmetry Breaking by Ras-triggered Local Positive Feedback

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Neuronal polarity is initiated by a symmetry breaking event whereby one out of multiple minor neurites undergoes rapid outgrowth and becomes the axon. Axon formation is regulated by phosphatidylinositol 3-kinase (PI3K) related signaling elements that drive local actin and microtubule reorganization, but the upstream signaling circuit that causes symmetry breaking and guarantees formation of only a single axon is not known. Here
we use live FRET imaging in hippocampal neurons and show that the activity of the small GTPase HRas, an upstream regulator of PI3K, markedly increases in the nascent axonal growth cone upon symmetry breaking. This local increase in HRas activity results from a positive feedback loop between HRas and PI3K, locally reinforced by vesicular transport of HRas to the axonal growth cone. Recruitment of HRas to the axonal growth cone is paralleled by a decrease in HRas concentration in the remaining neurites suggesting that competition for a limited pool of HRas guarantees that only a single axon forms. Mathematical modeling demonstrates that local positive feedback between HRas and PI3K, coupled to recruitment of a limited pool of HRas, generates robust symmetry breaking and formation of a single axon in absence of extrinsic spatial cues.

2756

**lkbl Is Required for Adherens Junction Integrity and Apical Membrane Domain Morphogenesis in the Developing Drosophila Eye**

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Epithelial cells are characterized by distinct apical and basal membrane domains that are separated by sites of close cell-cell contact, including adherens junctions (AJs). Our understanding of the mechanism by which polarity-determining factors influence one another’s localization, and how this complex interplay results in the proper positioning of the AJs remains superficial. To address these questions we are using the photoreceptor of the genetically amenable Drosophila compound eye as an in vivo model cell. Photoreceptors are highly polarized along the apico-basal axis with a specialized light-gathering organelle, or rhabdomere, at the apical pole. Early during photoreceptor development the AJs are remodelled as individual cells of a columnar epithelia are recruited into discrete clusters of specialized epithelial cells. Later on during development, these cells elongate dramatically along the proximo-distal axis of the retina and this requires proper AJ specification. We show that in photoreceptors, the conserved polarity gene *lkbl* (*par4*) is required to properly define the AJ domain in the developing photoreceptor during the elongation phase. Loss of LKB1 function leads to the formation of ectopic AJs, and to the expansion of the rhabdomere and the sub-apical stalk membrane domains. Our results suggest that in the developing *Drosophila* photoreceptor, *lkbl* functions upstream of the conserved polarity gene *par1* and antagonises *bazooka* (*Dpar3*) function in promoting AJ assembly and apical membrane morphogenesis.

2757

**Addressing Mechanisms of Asymmetric Spindle Positioning in the One-Cell C. elegans Embryo**

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Asymmetric cell divisions are essential for creating cell diversity in many developing organisms. Cells divide asymmetrically by generating daughter cells of different sizes and/or by unequally partitioning cell-fate determinants. The first division in the *C. elegans* embryo shows both of these characteristics. During this division, forces exerted on the microtubules aid in positioning the mitotic spindle toward the posterior of the embryo, leading to an asymmetric division plane. Some key modulators that have been implicated in the initiation and regulation of these asymmetrically distributed forces include cortically localized PAR proteins, receptor independent G-protein signaling, and the microtubule motor protein dynein. While many of the players involved in asymmetric spindle positioning have been identified, the mechanisms behind the forces required for the spindle to shift are not completely understood. Labbé et al. 2003 and Kozlowski et al. 2007 have previously shown that microtubule plus-end residence times at the cortex are longer on the anterior half of the one-cell embryo when compared to the posterior half. These residence times become symmetric in both halves of the embryo in polarity-defective and spindle positioning-defective backgrounds. From this evidence, it has been hypothesized that differential regulation of microtubule stability at the cortex might play a role in asymmetric spindle positioning. Currently, I am developing reagents and imaging methods that will aid in testing this hypothesis and help elucidate the mechanism behind force generation during the time of the spindle shift.

2758

**Ectopic Pole Plasm Assembly in the Drosophila Oocyte**

A. N. Becalska, E. R. Gavis; Molecular Biology, Princeton University, Princeton, NJ

Patterning of the anterior-posterior (A-P) axis in *Drosophila* is governed by the localization of maternally derived RNAs and proteins to the anterior and posterior poles of the developing oocyte. Posteriorly localized determinants comprise the pole plasm, and are ultimately responsible for the formation of the germ line as well as the patterning of the A-P axis. While some basic mechanisms underlying localization of pole plasm components have been elucidated, the process by which these molecules are specifically targeted to the posterior and anchored at the posterior cortex is poorly understood. To further understand this process, we are characterizing two mutations that cause ectopic assembly of the pole plasm. One of these is a novel mutation in *bazooka*, the *Drosophila* homolog of *par-3*. Preliminary evidence suggests that microtubule polarity and organization within the oocyte may be disrupted in this mutant. We are investigating the mechanism by which these disruptions might lead to ectopic pole plasm assembly and whether our second mutation disrupts assembly via the same mechanism.

2759

**Clonal Analysis in the Mouse**

W. H. Sowby, J. R. Barrow; Physiology and Developmental Biology, Brigham Young University, Provo, UT

Historically, clonal analysis has been a powerful tool in *C. elegans* to analyze the molecular and behavioral characteristics of cells with altered gene activity in a wildtype milieu. We have previously reported a clonal analysis model in mouse that relies on making chimeras from an embryonic stem cell line (YFP3.1) that harbors a Cre inducible construct and tamoxifen inducible Cre (*CreER*). Injection of low levels of tamoxifen into surrogate mothers bearing the chimeric embryos induces widespread nuclear transport of *CreER*, resulting in the expression of a dominant acting gene product and a fluorescent reporter. We have used this technique with much success however, injection of surrogate mothers with tamoxifen quite frequently yields chimeras that exhibit a wide range of induction making it difficult to identify isolated clones. Here we report the generation of *CreER/Cre*-inducible transgene-GFP reporter and an exo-utero 4-OH tamoxifen injection approach as a means of inducing expression of a cDNA and GFP.
Using this method, we can closely control the timing and location of the induced clone. Further, the injected embryos can develop several days or to term to enable the analysis of the long term consequences of mutant clones. This approach will be especially useful in understanding the role of signaling pathways such as Wnt/Planar Cell Polarity that play critical roles in cell behavior during morphogenetic processes.

2760
**PAR Proteins May Act as a Bistable Switch in the Early *C. elegans* Embryo**
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PAR proteins establish distinct intracellular spatial domains in the one cell *C. elegans* embryo, polarizing the cell along the anterior-posterior axis. This polarization is persistent, lasting approximately 10-20 minutes until first cleavage. It has been established experimentally that PAR proteins interact through mutual phosphorylation, and recent genetic studies indicate that PAR-3 is capable of oligomerizing. Using mathematical modelling, I show that known interactions between the PAR proteins give rise to a bistable switch that may account for the stable segregation of the PAR proteins. The presence of a bistable switch in the *C. elegans* embryo is investigated experimentally by depleting PAR-6 using RNA interference, providing a direct test of the mathematical model.

2761
**Identifying the Wnt5a Signaling Pathway**
J. J. Barrott, J. J. Kendall, A. P. McMahon, J. R. Barrow; 1Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA

Wnt5a has been previously demonstrated to play critical roles in many important processes in embryonic development as well tumor suppression. Despite many studies, most of which lend credence to a non-canonical Wnt signaling pathway, it remains unclear through which signaling pathway(s) Wnt5a signals. We are focusing our efforts on three potential pathways for Wnt5a: the Wnt/β-catenin or canonical pathway, the Wnt/Planar Cell Polarity (PCP) pathway, and a signal transduction pathway involving the Ror family receptors. As an attempt to determine the downstream signaling pathway for Wnt5a, we are crossing Wnt5a heterozygous mice with mice heterozygous for components of each of these other pathways to uncover genetic interactions. Hence, we have performed Wnt5a X Wnt5a (Wnt/β-catenin); Wnt5a X Ror2 and Wnt5a X Looptail (Lp) (Wnt/PCP) heterozygous intercrosses. Double heterozygotes for each of the intercrosses exhibit phenotypes not present in each of the single heterozygotes (including embryonic lethality) demonstrating that Wnt5a interacts genetically with each of these pathways. The finding that Wnt5a genetically interacts with the canonical Wnt pathway is surprising as biochemical, in vitro and in vivo studies seem to suggest that Wnt5a signals in β-catenin independent fashion. We present evidence that the interaction between Wnt5a and Wnt5a is indirect. We also demonstrate here that Wnt5a mutants possess phenotypes indicative of convergent and extension defects and neural tube closure defects-phenotypes associated with defects in the Wnt/PCP pathway. Hence our studies indicate that Wnt5a is a complex ligand that elicits its effects through multiple pathways.

**Growth Factors in Development (2762)**

2762
**HB-EGF Dependent Stimulation of Adipogenesis by ADAM 12S**
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Obesity is a major healthcare crisis in today’s society. Objective: To begin to understand the molecular underpinning leading to obesity, we investigated the role of a disintegrin and metalloprotease (ADAM) 12S and its enzymatic action on heparin-binding EGF-like growth factor (HB-EGF). ADAMs are a large family of both secreted and membrane-bound proteins involved in a number of biological activities including cell matrix modification, regulation of growth factor availability by ectodomain “shedding”, and mediating cell-cell interactions during normal development as well as a number of pathologies. HB-EGF is a membrane bound protein that is proteolytically processed by a soluble form of ADAM (ADAM 12S). Methods: This study investigated the biological properties of mouse fibroblasts upon expression of cDNAs encoding ADAM 12S and/or HB-EGF proteins. Stable cell lines were examined for expression of recombinant ADAM 12S proteins (68kDa and 92kDa) and HB-EGF proteins (6.5, 20 and 22kDa) by western blot analyses using an ADAM 12S specific antibody and HB-EGF antisera that specifically recognizes the intracellular domain of HB-EGF. Results: Stable expression of either ADAM 12S or HB-EGF cDNA did not affect the fibroblast morphology; however, stable co-expression of both ADAM 12S and HB-EGF cDNAs in fibroblasts stimulated significant fat deposition (adipogenesis). Furthermore, co-expression of ADAM 12S and HB-EGF significantly slowed cell growth as compared to ADAM 12S or HB-EGF expressing cells. Conclusions: These data suggest that adipogenesis is mediated by processing of HB-EGF, providing insight into potential molecular targets to disrupt adipogenesis.

**Signal Transduction in Development (2763-2777)**

2763
**The Regulation of Neurogenin2 Function in the Specification of Pyramidal Neurons by Post-Translation Modifications**
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The proper production and differentiation of glutamatergic pyramidal neurons during development is critical for normal cognitive function. The basic Helix-Loop-Helix transcription factor Neurogenin2 (Ngn2) is expressed in the developing cortex where it plays a critical role in the specification of the phenotype of glutamatergic pyramidal neurons. We found that phosphorylation of a single tyrosine residue (in position 241) within Ngn2...
regulates the acquisition of two important traits of glutamatergic pyramidal neurons: their migration properties and dendritic morphology. Interestingly, the phosphorylation of this tyrosine residue does not regulate the ability of Ngn2 to promote the acquisition of pan-neuronal properties. We hypothesized that Ngn2 differentially regulates the expression of genes involved in neuronal differentiation versus genes involved in subtype specification. Furthermore, the mechanisms underlying the function of phosphorylated Ngn2 are likely mediated by protein-protein interactions with transcriptional co-regulators. This is supported by recent results from our laboratory as well as others demonstrating that interaction between Ngn2 and the histone acetyltransferase(s) (HATs) CBP/p300 requires phosphorylation on tyrosine 241. HATs acetylate non-histone proteins including transcription factors. The acetylation of transcription factors regulates a wide variety of both transcriptional and cellular events. We have demonstrated that Ngn2 is acetylated using both in vivo and in nucleo acetylation assays. We have mapped the acetylation of Ngn2 and begun testing identify the molecular mechanisms regulating Ngn2 function during cortical development.

2764
Cdk8 is Essential for Preimplantation Mouse Development
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The Cdk8 kinase and associated proteins form a non-essential transcriptional repressor module of the Mediator in budding yeast. Genetic analysis of this module have demonstrated functions ranging from environmental responses in budding yeast to organogenesis and development in worms, flies, and zebrafish. Here we have investigated the function of mammalian Cdk8 using mice harboring a gene trap insertion at the Cdk8 locus inactivating this kinase. No phenotypes were noted in heterozygote Cdk8 +/- mice, but intercrossing these did not produce homozygous Cdk8 +/- offspring. Developmental analysis demonstrated a requirement for Cdk8 prior to implantation at E2.5-3.0. Cdk8 +/- preimplantation embryos had fragmented blastomeres and did not proceed to compaction. As Cdk8 deficiency in cultured metaozoan cells did not affect cell viability the results suggest that transcriptional repression of genes critical for early cell fate determination underlies the requirement of Cdk8 in embryogenesis.

2765
The Cerebral Cavernous Malformations Proteins CCM1, CCM2, and CCM3 Form a Ternary Complex and Are Required for Cardiovascular Function in Zebrafish
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People with loss-of-function mutations at any of the three loci for cerebral cavernous malformations are susceptible to hemorrhagic stroke caused by dilated and weakened capillaries in their central nervous system. Here we demonstrate that ccm1, ccm2, and ccm3, are not only genetically linked but also linked physiologically and biochemically. Morpholino knockdown of expression for each of the three ccm genes results in reduced circulation and pericardial edema in the developing cardiovascular system of zebrafish embryos. In addition loss of ccm1, 2 or 3 expression decreases pigmentation and craniofacial cartilage formation, demonstrating a shared role for each ccm gene in the development of these neural crest-derived cells. At the biochemical level, pulldown assays using CCM3-Sepharose show that CCM3 interacts directly with CCM2 and that CCM2-CCM1 complexes efficiently interact with CCM3, demonstrating that the three CCM proteins form a ternary complex. Threading analysis suggests CCM3 is a helix bundle protein with five lysines forming a highly conserved amphipathic carboxy-terminal helix. Mutation of these lysine residues showed they are necessary for CCM3 binding to PI-3,4,5P, suggesting a role for CCM3 in regulating phosphatidylinositol-dependent signaling. Together these studies indicate that the three CCM proteins form a biochemical complex, that each is similarly required for normal cardiovascular, cartilage and melanocyte development, and that CCM3 binds PI-3,4,5P. Thus, CCM1, 2 and 3 are predicted to function in a common biochemical signaling network required for postnatal vascular maintenance in the CNS.

2766
PLD Regulates Myoblast Differentiation through the mTOR-IGF-II Pathway
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A mammalian target of rapamycin (mTOR) pathway is essential for the differentiation of cultured skeletal myoblasts in response to growth factor withdrawal. Previously phospholipase D (PLD) has been found to play a role in cell growth regulation and mitogenic activation of mTOR signaling. However, a role for PLD in the autocrine regulation of myoblast differentiation—a process seemingly counteracts cell growth—is not known. Here we show that upon induction of differentiation in the mouse C2C12 satellite cells, the expression of both PLD1 and PLD2 is upregulated. C2C12 differentiation is markedly inhibited by 1-butanol, an inhibitor of the transphosphatidylation reaction catalyzed by PLD. Moreover, RNAi knockdown of PLD1, but not PLD2, significantly diminished the myogenic capacity of C2C12 cells, suggesting that PLD1 plays a critical role in the regulation of myogenesis. Previously, PLD1 was proposed to regulate arginine-vasopressin stimulated L6 myoblast differentiation through modulation of the actin cytoskeleton. However, we have found that the actin cytoskeleton is unlikely to be the target of PLD1 function in the autocrine regulation of myoblast differentiation. Instead, PLD1 positively regulates mTOR signaling and the cellular PLD activity is sensitive to amino acids availability during differentiation. As a consequence, PLD1 is required for the production of IGF-II—an autocrine factor instrumental for the initiation of satellite cell differentiation, and exogenous IGF-II fully rescues the differentiation defect resulting from PLD1 knockdown. Hence, PLD1 is critically involved in skeletal myogenesis by regulating a nutrient-sensing mTOR-IGF-II pathway.
**Late Abstracts**

**Wednesday**

**2767**

**Lysophosphatidic Acid-induced Survival of Immortalized Hippocampal Progenitor Cells by Inactivation of Glycogen Synthase Kinase 3 in PTX-sensitive Pathways**

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Lysophosphatidic acid (LPA) is a lipid growth factor known to regulate cell proliferation, death, and survival through a family of cognate G protein-coupled receptors. Tight regulation cell survival and death in correct times and places would be essential in ensuring correct size and morphogenesis during brain development. Recently, increasing data have showed that many neurotrophic factors including LPA played roles in controlling cell survival and apoptosis in developing neurons, however, underlying detailed mechanisms remained to be elucidated. To explore how LPA regulates cell survival and apoptosis in developing neuron, cell viability and signaling mechanism by LPA were determined in H19-7 cells, an immortalized hippocampal progenitor cell line. In the present study, we showed that LPA promotes cell survival in H19-7 cells, which was attributed to protection of the cells from apoptosis instead of cell proliferation. LPA stimulated phosphorylation of glycogen synthase 3 (GSK-3) α and β at ser21 and ser9, respectively, but little change in phosphorylation of GSK-3 at tyrosine. Both cell survival and the GSK-3 phosphorylation by LPA were attenuated by pertussis toxin (PTX) and inhibitors of Gαlo downstream signaling effectors of LPA receptor, such as p38, Erk, and PKC. In addition, inhibition of GSK-3 activity by GSK-3 specific inhibitor, BIO or PMA known to inhibit GSK-3 activity through PKC activation also promoted cell survival. Knock down of LPA receptors by specific siRNAs demonstrated that LPA1 and LPA2 are responsible for LPA-induced cell survival in H19-7 cells. Taken together, these results suggested that LPA induced-cell survival in H19-7 cells occurs via Gαlo coupling of the LPA receptors following inactivation of GSK-3.

**2768**

**Src Kinase-PLC gamma Signaling Complex in Eggs at Fertilization**

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The fertilization induced pathway of Ca²⁺ release in the eggs of deuterostomes is regulated in a timely, spatially and specific fashion, ensuring successful initiation of development. Ca²⁺ is released from the egg’s endoplasmic reticulum (ER) by inositol1,4,5-trisphosphate (IP₃), produced when phospholipase C gamma (PLCγ) hydrolyzes phosphatidylinositol bisphosphate (PIP2). PLCγ is activated by tyrosine phosphorylation requiring Src family kinases (SKFs). Previous work demonstrated that one such SKF, SpSFK1, was required for normal Ca²⁺ release during fertilization in sea urchin (Strongylocentrotus purpuratus) eggs (Giusti et al. 2003 Dev Biol. 256: 367-78). We have identified 4 additional SKFs expressed in the sea urchin egg and early embryos. SpSFK5 and SpSFK7 are novel SKF forms and Frk and SKF3 are similar to sea star SKFs previously identified (O’Neill et al. 2004 J Cell Sci. 117: 6227-38). Dominant interference experiments were conducted to test the necessity of each SKF for Ca²⁺ release at fertilization. The function of SpFrk, SpSFK5 and SpSFK7 are under investigation. Finally, using antibodies and GFP fusion constructs, we have observed the dynamic co-localization of PLCγ, SKF1, and SKF3 at the point of sperm/egg interaction during fertilization. These data suggest that active signaling complexes are formed at fertilization, serving to regulate the spatio-temporal release of Ca²⁺.

**2769**

**The PDZ Protein Syntenin Interacts with Frizzled 7 and Supports Non-Canonical Wnt Signaling**

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Wnt signaling pathways are essential for embryonic patterning and are disturbed in a wide spectrum of diseases, including cancer. An unresolved question is how the different molecular pathways are regulated. We previously established that the PDZ protein syntenin binds to syndecans Wnt co-receptors, and regulates cell-shape. Here we show that syntenin interacts with the C-terminal PDZ binding motif of several Frizzled Wnt receptors, without compromising the recruitment of Dishevelled, a key downstream signaling component. Syntenin is co-expressed with cognate Frizzled receptors, and regulates cell-shape. This study was initiated to identify changes in proteins following ectopic Snail2 expression by using comparative proteomic analysis of 3 control and 4 experimental gels, we chose 15 differentially-regulated protein spots (4 downregulated, 11 upregulated) for analysis and identification using nano LC- ESI-MSMS mass spectrometry. Twelve proteins were identified by this process including alpha-actinin-4, HSC70, and EBP50/NHERF-1.

**2770**

**Proteomic Analysis of Changes in Protein Expression Following Ectopic Snail2 Expression in the Chicken Neural Tube**

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Snail2 (Slug) is a member of the Snail family of zinc-finger transcriptional regulators which play an important role in epithelial-mesenchymal transitions (EMTs). Snail family members are expressed in embryonic tissues that undergo an EMT, including the neural crest, and are upregulated in many cancers and during fibrosis. We found that overexpression of Snail2 in the trunk region of the chicken neural tube resulted in loss of the epithelial phenotype in the neuroepithelial cells. This study was initiated to identify changes in proteins following ectopic Snail2 expression by using a 2-dimensional gel electrophoresis (2DE) -based comparative proteomics approach. Soluble protein samples extracted from neural crest explants cultures ectopically expressing Snail2 or a control vector were subjected to 2-DE and the gels analyzed with PDQuest 8.0. From the comparative analysis of 3 control and 4 experimental gels, we chose 15 differentially-regulated protein spots (4 downregulated, 11 upregulated) for analysis and identification using nano LC- ESI-MSMS mass spectrometry. Twelve proteins were identified by this process including alpha-actinin-4, HSC70, and EBP50/NHERF-1.
Drosophila Nucleostemin 3 Is a Conserved Regulator of Cell Growth and Body Size

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Genetic regulation of cell, organ, and body size is central to processes of normal development, disease, and evolution. Drosophila Nucleostemin 3 (NS3) is one of a family of proteins implicated in stem and cancer cell proliferation. We find that Drosophila NS3 controls cell growth and overall body size. The majority of ns3 mutant animals die prior to reaching adulthood. Those that reach adulthood require approximately twice as long to develop as controls, but appear healthy and properly proportioned. However, ns3 mutants are less than 60% of the size of controls and have fewer cells. The cells are also reduced in size. Activation of the insulin signaling pathway is altered in ns3 homozygotes. Expression of NS3 in neurons, but not fat body or ring gland, of otherwise ns3 mutant flies mostly rescues their growth defects, indicating that NS3 may act in a neuronal "growth signaling center" to control organisinal size.

Examining the Role of C. elegans Uterine-vulval 1 (uv1) Cells in Egg-laying Function

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Wild type C. elegans have four cells from the ventral uterine lineage that express the EGF Receptor LET-23 and are specified to adopt the uterine-vulval 1 (uv1) fate by a LIN-3 EGF signal from the underlying vulval cell vulF. Although the role of uv1 cells in C. elegans is unknown, we hypothesize a positive or negative role in the function or development of the egg-laying apparatus. Known mutants that lack uv1 cells exhibit other defects in the development of the egg laying apparatus, such as vulval defects, which causes difficulty in determining the role of uv1 cells in egg laying. Thus, we have generated a strain lacking uv1 cells with no other obvious defects in the vulva, uterus, or associated muscles and neurons. To generate animals without uv1 cells, we expressed let-23 RNA hairpin in the ventral uterine pi lineage using the egl-13 promoter, causing reduced uv1 fate specification. The resulting strain shows 42.5% penetrance of the uv1 cell defect, defined as three or fewer uv1 cells per animal. 31.9% of animals have zero uv1 cells. To determine the role of the uv1 cells in egg-laying, we synchronized animals at the L1 stage, scored them for uv1 cell defects at the L4 stage, and assayed for egg-laying 24 hours later by counting the number of eggs retained in the uterus. We did not find a significant difference between the number of eggs retained in uv1-cell defective worms compared to control worms from the same strain with 4 uv1 cells. We conclude that uv1 cell elimination has no effect on the rate of egg-laying, but may still affect other aspects of egg-laying, such as the stage at which eggs are laid or egg-laying regulation in response to stimuli.

A Requirement for β-catenin-mediated Wnt Signaling in Vertebrate Neurogenesis

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Wnt signaling regulates proliferation and differentiation of different cell types in vertebrate embryos, however its specific roles during neuronal differentiation have been elusive due to the reutilization of this pathway at different developmental stages. Here we examine a role for the Wnt pathway in the specification of primary neurons in Xenopus embryos. Altered Wnt/β-catenin signaling in whole embryos and ectodermal explants resulted in corresponding changes in the number of cells expressing the neuron-specific genes N-tubulin and X-nga-R1, but did not have significant effects on the mesodermal marker MyoD and the pan-neural genes Sox2 and Nrp1. Similar results have been obtained using hormone-inducible TCF fusion proteins activated at late blastula stages, indicating that the observed changes in neurogenesis do not depend on early axis specification. To examine whole embryos we performed in situ hybridization and for the ectodermal explants RT-PCR analysis. We conclude that Wnt/β-catenin signaling is required for primary neuron specification independently of its functions in earlier developmental processes.

FoxO1 Negatively Regulates Skeletal Myocyte Differentiation through Degradation of mTOR Pathway Components

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The forkhead transcription factor FoxO, a downstream target of PI3K/Akt signaling, has been reported to suppress skeletal myocyte differentiation, but the mechanism by which FoxO regulates myogenesis is not fully understood. We have previously demonstrated that a nutrient-sensing mTOR pathway controls the autocrine production of IGF-II and the subsequent PI3K/Akt signaling downstream of IGF-II in myogenesis. Here we report a regulatory loop connecting FoxO1 to the mTOR pathway, in which activation of FoxO1 induces proteasome-dependent degradation of a specific subset of components in the mTOR signaling network, including mTOR, raptor, TSC2, and S6K1. This function of FoxO1 requires new protein synthesis, consistent with the idea that a transcriptional target of FoxO1 may be responsible for the degradation of mTOR; candidates for such a target include the E3 ligase Atrogin-1 and the polyubiquitin binding protein Znf216. We further show that active FoxO1 inhibits IGF-II expression at the transcriptional activation level, presumably through the modulation of mTOR protein levels. Moreover, the addition of exogenous IGF-II fully rescues myocyte differentiation from FoxO inhibition. Taken together, we propose that the mTOR-IGF-II pathway is a major mediator of FoxO’s inhibitory function in skeletal myogenesis.

Roles of β-arrestin-like Proteins in GPCR Signaling in Dictyostelium discoideum

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β-arrestins are well known for their role as mediators of G-protein-coupled receptors (GPCR) desensitization. Recently, they have been implicated as key regulators in cell motility and directional cell migration by serving as multifunctional adaptor and scaffold proteins in mammalian cells. However, the detailed functions and the molecular mechanisms remain to be determined. We have identified three β-arrestin-like proteins, named arrestin1-3, in D. discoideum using a genomic approach. By using real-time PCR, we measured the mRNA abundance of the three arrestins. We found that all of them showed similar decrease during the early development. To visualize the localization of arrestins in live cells, we tagged the proteins with YFP. We observed that arrestin1 has a uniform localization through the cells, while arrestin2 localizes on membrane and vesicles in cytosol. To study the functions of arrestins, we have generated arrestin null cells (arr1−/−, arr2−/− and arr3−/−) through homologous recombination. To investigate the potential roles of arrestins in GPCR signaling, we analyzed the development of wild-type, arr1−/−, arr2−/− and arr3−/− cells. The arr2−/− cells showed a developmental delay on bacteria lawn and non-nutrient agar, while arr1−/− and arr3−/− cells displayed a wild-type like phenotype. Since arrestin 1 and arrestin 3 have a similar protein domain structure, they may have a redundant function. To test this possibility, we constructed arr1−/− and arr3−/− double null cells by using Cre-loxP system. The resulting arr1−/−arr3−/− cells showed a clear aberrant developmental delay, while over-expressing YFP-tagged arrestin 1 in this cell line can rescue this phenotype. The phenotypes of arr1−/−arr3−/− and arr2−/− cells suggest that arrestins may play roles in the regulation of cAMP signaling and chemotaxis.

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Neddylation-induced CRL Degradation and Its Possible Implications
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Cullins are scaffold proteins that assemble substrate binding receptors/adaptors and Roc1 into cullin-RING ligase (CRL) complexes transferring ubiquitin moiety onto many proteins. Nedd8 is an ubiquitin-like polypeptide that can be transferred onto the conserved lysines of cullins in a process called neddylation. Deneddylation, the reverse reaction that removes the Nedd8 moiety from cullins, is carried out by COP9 signalosome (CSN). In genetic analyses, both neddylation and CSN deneddylating activities are required for the ubiquitylation and degradation of some CRL substrates. While it has been shown that neddylation activates individual CRL activity, we found that constitutive neddylation of CRL in the absence of CSN deneddylating activities leads to the degradation of cullins, substrate receptors like F-box protein Slimb and Nedd8. Because of the neddylation induced CRL degradation that reduces CRL concentration in cells, the total ligase activities of CRLs within a cell reduce in the absence of CSN deneddylating activities. In examining the degradation of two SCFSlimb substrates, Cubitus interruptus (Ci) and Armadillo (Arm) in CSN defective cells, we found that Ci protein levels decrease in contrast to the protein levels of Arm that increase in response to the neddylation induced CRL degradation. Nevertheless, Ci accumulates in CSN mutant cells if a dominant negative Slimb is expressed simultaneously. Interestingly, overexpressing Cul1 in CSN mutant cells also leads to the accumulation of Ci by further decreasing the protein levels of Slimb. This result implies that Cul1 can either promote substrate degradation in wild type cells as part of CRL holoenzyme or compromise substrate degradation in CSN mutant cells by promoting the degradation of substrate receptors. In this sense, we speculate that the significance of Cul1 degradation in CSN mutant cells is to prevent overwhelming degradations of substrate receptors. This may explain why the CRL substrate phenotype in CSN mutants tends to be subtle.

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Dermal Fibroblasts from Long-lived Ames Dwarf Mice Exhibit Resistance to H2O2 and Reactive Oxygen Species (ROS) Generated by Mitochondrial Dysfunction
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The Free Radical theory of Aging proposes that oxidative stress caused by mitochondrial dysfunction plays a major role in determining longevity. This theory is supported by studies in C. elegans, Drosophila, and rodents that shown resistance to reactive oxygen species (ROS) is a characteristic of longevity. Previously we demonstrated that activation of p38 MAPK stress pathway by mitochondria-generated ROS is linked to the level of ASK1 dissociated from an inhibitory thioredoxin (Trx)-ASK1 complex. Thus, the level of free ASK1 regulates the activity of p38 MAPK. Furthermore, the inhibitory Trx-ASK1 pool level is significantly higher in both young and aged long-lived Snell dwarf mice which explains their decreased levels of endogenous ASK1 and downstream p38 MAPK activities. We propose that these are characteristics indicative of resistance to oxidative stress and decreased levels of endogenous ROS production. In this study we isolated dermal fibroblasts from young, middle-age, and aged long-lived Ames dwarf mice and treated them with inhibitors of mitochondrial electron transport chain (ETC) and H2O2 to investigate whether they exhibit more resistance to ROS generated by mitochondrial dysfunction. Our results indicate that the dwarf fibroblasts exhibit resistance to rotenone (Rot)-, 3-nitropropionic acid (3-NPA)-, antimycin A (AA)-, and H2O2-mediated activation of p38 MAPK activity. The reduced p38 activity correlates with a higher level of Trx-ASK1 complex in the dwarf fibroblasts. Higher doses of Rot or H2O2 were required to activate the dwarf fibroblasts p38 MAPK activity to the level that occurs in the wild-type cells. Our results suggest that Ames dwarf fibroblasts are resistant to the ROS generated by dysfunction of mitochondrial ETC. Based on these results we conclude that the ROS-stimulated dissociation of the inhibitory Trx-ASK1 complex is the mechanism that links mitochondrial dysfunction (ROS) to the increased endogenous activity of the p38 MAPK stress pathway in aged tissues.

Plant Development (2778-2779)

2778

The Calcium Sensor CBL10 Mediates Salt Tolerance by Regulating Ion Homeostasis in Arabidopsis
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Calcium serves as a critical messenger in many adaptation and developmental processes. Cellulur calcium signals are detected and transmitted by sensor molecules such as calcium-binding proteins. In plants, the calcineurin B-like protein (CBL) family represents a unique group of calcium sensors and plays a key role in decoding calcium transients by specifically interacting with and regulating a family of protein kinases (CIPKs). We report here, that the calcineurin B-like protein CBL10 functions as a crucial regulator of salt tolerance in Arabidopsis. Cbl10 mutant plants exhibited significant growth defects and showed hypersensitive cell death in leaf tissues under high salt conditions. Interestingly, the Na⁺ content of the cbl10 mutant, unlike other salt-sensitive mutants identified thus far, was significantly lower than in the wild type under either normal or high salt conditions, suggesting that CBL10 mediates a novel Ca²⁺-signaling pathway for salt tolerance. Indeed, the CBL10 protein physically interacts with the salt-tolerance factor CIPK24/SOS2 and the CBL10-CIPK24/SOS2 complex is associated with the vacuolar compartments that are responsible for salt storage and detoxification in plant cells. These findings imply that CBL10 and CIPK24/SOS2 constitute a novel salt tolerance pathway that regulates the “sequestration/compartamentalization” of Na⁺ in plant cells. Because CIPK24/SOS2 also interacts with CBL4/SOS3 and regulates salt export across the plasma membrane, our study identifies CIPK24/SOS2 as a multi-functional protein kinase that regulates different aspects of salt tolerance by interacting with distinct CBL calcium sensors.

2779
Antisense Transcripts in Light Regulated Gene Expression in Nipponbare Rice
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Natural antisense transcripts (NATs) are RNA molecules that are complementary to other “sense” RNAs. NATs are known to play regulatory roles within the cell, but their specific functions have not been widely explored. This research examined eighteen genes with antisense partners which are involved in light regulated pathways in Nipponbare rice (Oryza sativa japonica). The goal of the research was to discover which of the identified antisense transcripts exist inside the cell using RT-PCR, Northern blotting, and RNAse protection. With the exception of one gene (PS II reaction center W protein), all genes were tested using at least two different primer sets. Of the 18 genes with identified antisense partners, 14 were found by RT-PCR to be expressed inside Nipponbare shoot and root cells. Light regulation was observed in 5 of the antisense transcripts. Northern blot and RNAse protection analyses, which are less sensitive than RT-PCR, only detected NAT expression in overlapping gene pairs, indicating that NATs from other genes are rare. Low molecular weight northerns of the ribulose-3-P epimerase/axi protein gene pair revealed a root-specific small RNA (~40 nucleotides). Similarly, low molecular weight northerns of the TGA/phosphatase gene pair showed small RNAs derived from exon 13 of TGA that were not present in phosphatase. RT-PCR of the rbcS gene on chromosome 12 suggests that the gene contains multiple small antisense fragments rather than one large, continuous one. The results of this research will be used to determine whether NATs play regulatory functions in Nipponbare light responses.

Development and Aging (2780)

2780
Identification and Characterization of Selenoprotein MsrA from Clostridium sp. OhILAs
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Methionine sulfoxide reductases are important repair enzymes that catalyze the reduction of free- and protein-bound methionine sulfoxide to methionine. There are two distinct enzyme families that reduce stereospecifically oxidized methionine residues: MsrA (methionine-S-sulfoxide reductase) and MsrB (methionine-R-sulfoxide reductase). These enzymes have been implicated in defending cells from oxidative stress, controlling protein function and regulating aging process. In this study, we identified and characterized a bacterial selenoprotein MsrA from Clostridium sp. OhILAs. A SECIS element required for selenocysteine incorporation was located immediately downstream of UGA selenocysteine codon. This SECIS element was recognized by an E. coli selenocysteine insertion machinery. The selenoprotein MsrA expressed in E. coli had a 20-fold increased activity than the cysteine mutant form, indicating a critical role of selenocysteine residue in the catalytic function. Also, our data revealed that the Clostridium MsrA was inefficiently reduced by thioredoxin, which is a known in vivo reducing agent, suggesting that there may be other in vivo reductants for the reduction of this enzyme. Supported by KOSEF grant R13-2005-005-01004-0.

Stem Cells (2781-2791)

2781
Differentiation of Human Adult Adipose-derived Mesenchymal Stem Cells (MSC’s) into Adipocytes and Osteocytes, but Not Neurons In Vitro
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Adult mesenchymal stem cells (MSC) are found in the supportive matrix of bone marrow and adipose tissues. Procedures for differentiation of human MSC have been established for several cell types including osteogenic, chondrogenic, myogenic, and adipogenic tissue. We have confirmed the differentiation of human adipose-derived stem cells into adipocytes and osteocytes. Adipose derived stem cells (ASC) were isolated from human lipoplasty (liposuction) obtained from Loma Linda University Medical Center with IRB approval at both Loma Linda University and California State University, San Bernardino. Liposaprate cells were harvested by washing with phosphate buffered saline followed by treatment with collagenase. Cells were initially grown in a control medium of RPMI, 10% fetal calf serum and 1% antibiotic/antimycotic. For osteocyte differentiation, RPMI, 10% FCS, 1% antibiotic/antimycotic, 0.1 µM dexamethasone, 50 µg/ml ascorbic acid, and 10mM β-glycerophosphate was used. For adipocyte
differentiation, adipogenic differentiation supplement in MesenCult medium (Stem Cell Technologies) was used. Protocols have been suggested for differentiation of MSC’s into neurons. For neural differentiation, we tested in serum free RPMI medium: N2 supplement, N2 + B27 supplement, 1 mM dibutyryl cyclic AMP (dbcAMP), and 20 ng/ml basic fibroblast growth factor (bFGF) for 24 hours followed by 100 mM BHA (butylated hydroxy anisole). These protocols for neural differentiation create changes in cell morphology resulting in a neural-like appearance within hours. The agents appear to cause retraction of cytoplasm towards the center of the cell resulting in a rounded appearance with moderate to thin processes extending outward. However, these are not neurites. De novo neurite growth and extension does not occur. In addition, retraction of the cytoplasm appears to concentrate low levels of endogenous neural markers to a detectable level. It is unlikely that expression is due to induction of neural genes. MSC’s appear to be unable to converted into fully differentiated neural cells.

2782
**Enrichment and Transcriptome Analysis of Human Embryonic Stem Cell-derived Cardiomyocytes**

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Human embryonic stem cells (hESCs) are a source for generating a virtually unlimited supply of cardiomyocytes for cell replacement therapies or drug screening. However, differentiation of hESCs invariably leads to a heterogeneous cell population with only a fraction of the desired cell type. To achieve enrichment of functional cardiomyocytes, we have employed a genetic lineage selection strategy to generate transgenic hES cells lines which express an antibiotic resistance under the control of the cardiac-specific alpha myosin heavy chain (αMHC) promoter. Directed differentiation of the transgenic cell lines resulted in essentially pure cardiomyocyte cultures. To characterize the gene expression signature of these cardiomyocytes, whole genome technology we employed. By comparing pure cardiomyocytes with a control population of differentiated hESC (21-day old embryoid bodies) and undifferentiated, pluripotent hESCs, we have identified a set of 1201 differentially expressed probe sets (p<0.05). Gene Ontology analysis showed that a subset of upregulated genes encoded for striated muscle structural proteins, ion transport proteins, and proteins involved metabolism. In contrast, genes involved in mitosis, cell proliferation and protein synthesis were downregulated in selected cardiomyocytes. A further comparison of the dataset from hESC-derived cardiomyocytes with samples from fetal and adult human heart tissue showed a clear similarity, and a series of unique cardiac markers was identified. Our results provide the basis for a comprehensive understanding of gene expression patterns of cardiomyocytes derived from hESCs and will help define biological process and signaling pathways for hESC cardiogenesis.

2783
**Cardiomyocyte Progenitors Isolated from Populations of Differentiated Human Embryonic Stem Cells**

W. L. Rust, T. Balakrishnan, R. Zweigerdt; Institute of Medical Biology, Singapore

Current protocols designed to direct the differentiation of cardiomyocytes from human embryonic stem cells (hESCs) can generate a population containing approximately 15% of cells which express characteristics of the cardiomyocyte lineage. These are found amongst a heterogeneous population of cells of various identity and developmental stage. For the purpose of producing cell therapies or diagnostic cell products, pure cardiomyocyte populations are desired. We have developed a system for selecting wild-type cardiomyocyte progenitors from differentiated hESC cultures using antibody-mediated Magnet Assisted Cell Sorting. The selected population is enriched for cells which express the cardiac transcription factor Nkx2.5 and cardiac contractile proteins. Although only a small proportion of cells survive the selection procedure, these cells proliferate rapidly when plated in tissue culture dishes and form monolayers of spontaneously contracting cell clusters. Molecular analysis suggests that the selected cells represent a subpopulation of the cardiomyocytes which is equivalent to a fetal-stage ventricular cardiomyocyte.

2784
**Using Hybrid Spheroids to Assay Cancer Stem Cell Sensitivity to Ionizing Radiation and Chemotherapeutics**

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Background: The cancer stem cell (CSC) hypothesis postulates that only a small fraction of a tumor is responsible for its growth, and cure requires sterilization of these CSCs. Studying the use of hybrid spheroids (HS) in predicting an assay of tumor radio- and chemo-sensitivity. The HS system includes but is not limited to, 1) Maintaining a healthy cell line of human fibroblast (AG 1522) to make hybrid spheroids; 2) chemical digestion of cervical carcinoma tumors to model in situ cell interactions with the spheroids; 3) scoring growth of the hybrid spheroids; 4) transferring spheroids to attachment dishes to observe mitotic cellular morphology; 5) data entry and analysis. The HS measurements shrank form their original size while incubated for several days, and then either stayed at that size or grew as the AG 1522s died off. Therefore when HS grew past original size, it was due to proliferation of the cancer cells, not the fibroblast. Results: We found it is possible to discriminate between normal and neoplastic cells by virtue of whether or not they grow in HS. This could lead to a system that should be suitable for testing cancer treatment strategies for individual patient, in an in vivo-like environment.

2785
**Functional Endothelial Progenitor Cells Derived from Human ES Cells**

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Endothelial progenitor cells (EPC) differentiated from human embryonic stem cells (hESC) were efficiently obtained by hypoxia culture condition and cell sorting with EPC markers (CD133, KDR). Hypoxic condition is considered as a microenvironment that affects the early embryo development and angiogenesis, when we compared to the normoxic condition, the hypoxic condition had 3 times higher population of cells with ECP markers. The isolated hESC-EPC maintained specific endothelial characteristics such as endothelial-specific marker expression, capillary formation, and ac-LDL uptake. For in vivo test, a hindlimb ischemia was surgically generated in athymic mice, and hESC-EPC were injected intramuscularly into near ischemic limbs. Human ESC-EPC treatment significantly increased limb salvage (4 of 5), compared to treatment with medium (0 of 5). In addition,
laser Doppler image showed that hESC-ECs treatment enhanced blood perfusion. After 5 months of treatment, the relative ratios of blood flow (ischemic to normal limb) were significantly increased by hESC-EPC treatment group (about 80%) compared to medium injection group (about 0%). Immuno-staining analysis using human-specific antibody showed that transplanted hESC-EPC induced the expression of human angiogenic growth factors including VEGF, bFGF, and angiopoietin-1 in the ischemic region. The presence of human cells in the ischemic region was also confirmed with the human specific nuclei antibody. Also, capillary and arteriole formation was confirmed with the human specific SMA, PECAM, vWF, VECadherin in the ischemic region. However, the expressions of these human angiogenic factors and various human specific antibodies were not observed in the ischemic limbs receiving medium injection. This study demonstrated that hypoxic culture condition was effective for hESC-derived EPC differentiation, and transplantation of hESC-EPC into a mouse model of hindlimb ischemia improved postnatal neovascularization, and consequently enhanced blood perfusion and limb salvage. Thus, this result suggests that hESC-EPC would be useful as an alternative cell source for angiogenesis-mediated therapy.

2786
**The Role of Bone Marrow Derived Stem Cells in Liver Injury**

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Bone marrow stem cell contribute to liver regeneration, the cells involved are poorly characterized and it remains unclear what relevance this response has to the development and/or resolution of intrahepatic injury. We hypothesized that there is specific bone marrow stem cell mobilization in response to liver injury. A model of chronic liver injury was investigated in B10.BR mice which were lethally irradiated and reconstituted with 178.3 mouse bone marrow. Mice received 300mg/L thioacetamide ad. lib. in drinking water for 20 weeks. Bone marrow and peripheral blood mononuclear cells were depleted of lin- cells using magnetic bead separation. Further purification of the stem cell fraction was performed using flow cytometry separation of Sca-1+ cells. Cells were subsequently sorted based on CD45 and CXCR4 expression. Analysis of bone-marrow cell engraftment into the injured liver was analysed by immunohistochemistry and RT-PCR. Treated mice had reduced body mass, enlarged livers and cirrhotic. With chronic liver injury there was a 12-fold increase in the number of lin-Sca-1+ stem cells in the bone marrow and a 13-fold increase in the number of lin-Sca-1+ stem cells in the peripheral blood suggesting that resident stem cell populations in the bone marrow are proliferating and migrating into the periphery. Analysis of the relative proportions within the lin-Sca-1+ stem cells, revealed that hematopoietic stem cells (CD45+, CXCR4+) were seen to decrease in the bone marrow and increase in the blood, while mesenchymal stem cells (CD45-, CXCR4-) and other non-hematopoietic stem cell populations decreased in proportion in the bone marrow. These non-hematopoietic fractions (CD45+) had exclusive expression of sonic hedgehog and c-fos, α-fetoprotein and insulin-like growth factor 2 in thioacetamide treated mice. The results support the hypothesis that a population of liver-specific stem cells reside within the bone marrow and are recruited during injury to the liver.

2787
**JAK-STAT Signal Inhibition Regulates Competition within the *Drosophila* Testis Stem Cell Niche**

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Stem cells are essential for generating, sustaining, and repairing tissues. Stem cells are maintained in microenvironments, or niches, that control their behavior, but little is known about the extrinsic cues and intrinsic signaling that regulate stem cell niches. In the *Drosophila* testis, germline stem cells (GSCs) and somatic stem cells (SSCs) both adhere to a cluster of non-mitotic cells called the hub, which prevents stem cell differentiation by local secretion of the JAK-STAT pathway ligand Unpaired. The JAK-STAT signaling pathway is required for GSC and SSC maintenance. Although factors required for stem cell identity have been characterized for many different niches, little is known about signal attenuation. Proteins of the mammalian Suppressor of Cytokine Signaling (SOCS) family are the best-characterized negative regulators of the JAK/STAT pathway. Several studies have indicated that *Drosophila* *socs36E* like its mammalian orthologues, negatively regulates JAK/STAT signaling. However, loss-of-function studies of *socs36E* have not been reported. We have found that *socs36E* is a STAT target in the testis and is expressed strongly in the hub as well as in SSCs. An enhancer trap line with a P-element insertion in the coding region of the *socs36E* gene, which is a loss-of-function allele designated as *socs36E*<sub>21967</sub>, causes testes to have a significantly lower number of GSCs. Mosaic analysis indicates that GCSs do not directly require *socs36E* in order to prevent GSC loss, but rather SSCs need *socs36E* to suppress SSC accumulation in the niche and subsequent GSC loss. This work demonstrates how a single niche coordinate regulates two stem cell populations. By carefully regulating the level of signaling within each cell lineage, a proper balance of each stem cell population is maintained in the tissue.

2788
**Differentiation of Umbilical Cord Matrix Stem Cells into Hepatocytes**

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Stem cells have the capability to differentiate into many different cell types. This characteristic gives them the potential for therapeutic use in many human diseases, particularly where there is damaged tissue. Stem cells from the human umbilical cord matrix (HUCM) express many of the proteins important for regulation of embryonic and adult stem cells. HUCM cells can be differentiated into multiple tissue types that could be used for cell-based therapies including cells from mesodermal and ectodermal lineages. In the current study, the potential of HUCM cells to differentiate into hepatocytes, an endodermal lineage cell is explored. The ability to direct HUCM stem cells to differentiate into hepatocytes would extend the utility of this abundant source of stem cells for use in drug research. Our preliminary studies show that HUCM stem cells are able to differentiate into presumptive hepatocytes by treatment with a differentiation protocol that exposes the HUCM cells to specific growth factors in a sequential fashion. The HUCM cells undergo a change from a spindle-like fibroblast morphology into flattened polygonal shaped cells typical of hepatocytes. After differentiation, the HUCM-derived hepatocytes store glycogen, take up and indocyanin green and low density protein while the undifferentiated HUCM cells do not. The differentiated HUCM cells also expressed the hepatocyte markers: albumin, HNF3-beta, HNF4-alpha, glutamine synthetase, and cytokeratin 18 which are not expressed by undifferentiated HUCM cells. In addition, smooth muscle actin, and nanog, proteins normally
expressed in undifferentiated HUCM cells, were no longer expressed in the HUCM-derived hepatocytes. Importantly, a number of transcription factors and Cyp3A4 were expressed in the HUCM-derived hepatocytes and induced by treatment with phenobarbital and/or rifampicin. These results are the first step toward developing a powerful new model for drug development and toxicity studies in man and animals.

2789 
**Isolation of Multipotent Stem Cells from Adult Rat Periodontal Ligament by Neurosphere-forming Culture System**  
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Adult multipotent stem cells have been successfully isolated from various non-neural tissues. We isolated multipotent stem cells from 6-week-old rat periodontal ligament (PDL) using neurosphere-forming culture system. Enzymatically dissociated PDL cells were cultured in serum-free basal medium containing EGF, bFGF and LIF. By seven days into culture, free-floating spheres with various sizes were distinctly recognized in the supernatant. Intermediate-sized spheres (50-100 μm in diameter) expressed Nestin, GFAP and Vimentin as determined by immunocytochemistry. Thus, there were cells of neural and mesenchymal precursor status, at least in part, within free-floating spheres derived from PDL. In addition, spheres expressed mRNA of neural crest-associated transcription factors Twist, Slug, Sox2 and Sox9. Methylcellulose colony-forming (CFC) assay revealed that spheres were formed by a single dissociated PDL cell and the frequency of sphere-forming cell was approximately 0.01%. Upon differentiation, PDL-derived spheres were able to generate multi-nucleated MyoD-positive myotubes, neurofilament M (NFM)-positive neuron-like cells, GFAP-positive astrocyte-like cells as well as CNPase-positive oligodendrocyte-like cells in the appropriate conditions. All these neural and mesodermal progeny could be grown in a single sphere. Our study suggests that teeth with healthy PDL extracted in the course of standard dental practice could be a novel source of adult multipotent stem cells. These cells might be used in the treatment of various diseases such as neuronal degenerative diseases and muscle dystrophy.

2790 
**Identifying Populations of Human Muscle Derived Stem Cells to Participate in Skeletal Muscle Regeneration Based on Phenotypic Differences**  
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**Introduction.** Muscle-derived stem cells (MDSC) isolated from murine models have displayed the capability to commit to the myogenic lineage and efficiently regenerate skeletal and cardiac muscle in murine Duchenne Muscular Dystrophy models (mdx). We characterized potential human MDSC (hMDSC) counterparts, testing if these populations, isolated by the preplate technique, exhibited similar myogenic and regenerative characteristics similar to their murine counterparts. **Methods.** Candidate populations obtained from CookMyosite™ were grown in EGM™-2 under standard conditions and passaged every 72-96 hours. hMDSCs were analyzed for the cell surface differentiation markers CD34, CD56, and CD146 and were stained for desmin, myosin heavy chain, and dystrophin. We compared hMDSC phenotypic profiles among different preplate, or isolation, fractions (pp2, pp4, pp6) to identify molecular and behavioral characteristics that might predict in vivo regeneration efficiency. These fractions were also transplanted to gastrocnemius muscles of mdx/SCID mice and harvested 2 weeks post-transplantation and frozen sections analyzed using dystrophin and human nuclear staining. **Results.** All 3 preplate fractions were negative for CD34 and CD144 with positive, decreasing expression of CD56 and CD146. Myogenic potential was evidenced by desmin, myosin, and dystrophin staining, showing the fusion of cells into multi-nucleated myotubes. Time-lapsed image analysis has not shown any differences in parameters such as cellular division time, population doubling time, and cellular motility behavior across preplate fractions. We injected several preplate populations; pp2 (n=11 muscles), pp4 (n=19), pp6 (n=20). The number of regenerating dystrophin positive fibers was significantly higher in transplantations using pp6 fraction as compared to pp2 fraction (P = 0.037, 2-tailed 2-test). **Discussion.** This study suggests that hMDSCs may be obtained from the pp6 which appears to be distinct from pp2, obtained from human muscle biopsy. Current studies are underway to further identify phenotypic and behavioral differences across preplate fractions corresponding to differing regeneration levels.

2791 
**miRNA Inhibitor Screen Identifies Several miRNAs That Are Critical for Human Adult Mesenchymal Stem Cell Differentiation**  
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Adult Mesenchymal Stem Cells (MSCs) are pluripotent progenitor cells that can differentiate into a variety of cell types, including osteoblasts and adipocytes. MicroRNAs (miRNAs) are a class of 17-25 nt non-coding RNAs with more than 400 known miRNAs in the human genome (miRdatabase v 9.0). miRNAs are regulators of gene expression and implicated in a wide variety of biological processes, including stem cells differentiation regulation. The activity of miRNAs can be modulated by unique molecular tools, miRNA inhibitors and mimics. The objective of our study was to identify miRNAs that are critical for human adult mesenchymal stem cell differentiation. Here we describe the development and synthesis of a complete collection of potent miRNA inhibitors and mimics. We then perform a screen of 396 miRNA inhibitors to evaluate miRNA involvement in human MSCs (hMSCs) osteogenic and adipogenic differentiation. We have identified at least 3 miRNAs, whose expression is both necessary and sufficient for either induction or repression of hMSCs osteogenic differentiation. In conclusion, our data suggests that the miRNAs have critical roles in regulating adult hMSCs differentiation. Furthermore, miRNA modulation may be used in hMSC based tissue engineering and development of novel therapeutics.
Stem Cell Biology (2792-2793)

2792
**Comparison of Potential 3D Organic Gels for Cartilage Tissue Engineering to Support Growth of Novel Human Umbilical Cord Cells**

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Objective: Cells from the Wharton’s Jelly of the umbilical cord are reported to have mesenchymal characteristics. Since cartilage repair remains an existing clinical challenge, novel reconstruction techniques are needed. This project tested the viability of UC-derived cells, as an alternative to BM-MSCs, in alginate and type I collagen 3D scaffolds. Methods: Male human umbilical cords (IRB #0305079) were digested in Collagenase. Cells were cultured in EGM-2. Alginate gels were prepared by combining a 1.5% alginate solution and 40 w/v% CaSO4 with a NaCl-cell suspension. Gels were cut into 5 x 5 x 2.5 mm3 sections, with final cell densities of 1x106 cells/cm3. Collagen gels were made from10X PBS, supplemented EGM-2 media, and PureCol (Inamed) and were formed in a 24-well tissue culture plate for a final cell density of 1x106 cells/cm3. Results: We found no difference in mesenchymal marker expression for cells grown on collagen vs. alginate substrate (CD44/CD73/CD90/CD105), but a trend toward faster monolayer growth rate on collagen substrate (14.2 doublings) as compared to alginate substrate (9.5 doublings) after fourteen days. Next, we assessed the feasibility of gel formation and cell viability after embedding into the 3D matrix. Alginate gels polymerized within 2 minutes of mixing the cells and reagents at room temperature. Although the final gel was solid and supported cell viability for days, the rapid polymerization time would make it difficult to inject the cells into a cartilage defect before gelation. Collagen gels polymerized within 45-60 minutes, occurring more quickly at 37°C. Cells were also viable up to at least 10 days. Conclusion: Although reports show that alginate gels also support chondrogenic cell growth, our results suggest that they would pose practical clinical challenges. Collagen type I is a compatible substrate for UC-derived cells; collagen gels offer a practical cell therapy approach for cartilage repair.

2793
**IxB Plays a Role in STO Cell as a Feeder Cell for Human Embryonic Stem Cell Culture**

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Embryonic stem cell (ESC) can be propagated in definitely in vitro on feeder layers of mouse STO fibroblast cells. The STO cells secrete several cytokines that are essential for ESCs to maintain their undifferentiated state. BMP4 (Bone Morphogenetic Protein 4) plays key roles in a variety of processes in embryonic development. Recent study has shown that putative consensus NF-κB binding sites are located in human BMP4 promoter region and activation of NF-κB brings about transcriptional repression of Bmp4 gene. In this study, we investigated whether the STO cells, which were infected with adenovirus containing dominant negative mutant of IκB, might lose functional activity as feeder cell to mouse ESCs. Higher MOI increased infection rate but decreased proliferation and growth of mESC, suggesting that blockage of NF-κB signal pathway can destroy the capability of STO cells to secrete essential cytokines for ESCs culture.

Bacterial Developmental Programs: Quorum Sensing, Sporulation, and Beyond (2794)

2794
**Confirmation of Akinete Cell-Type-specific Gene Expression**

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*Nostoc punctiforme* is a filamentous cyanobacterium capable of cellular differentiation into nitrogen-fixing heterocysts, motile hormogonia, or akinetes. Akinetes are spore-like resting cells induced by exposure to environmental stress and capable of surviving cold and desiccation. A *zwf* mutant strain that synchronously differentiates into akinetes upon dark incubation in the presence of fructose was used to study cellular differentiation in this one-dimensional model system of development. RNA harvested from *zwf* cultures before and during akinete development was used in DNA microarray analysis to identify genes induced in akinetes of *N. punctiforme*. Two of the genes exhibiting significantly higher expression in akinete cells versus vegetative cells were chosen for study. These genes encode an RNA Polymerase Sigma Factor and a Helix-turn-Helix DNA binding protein. Rapid amplification of cDNA ends (RACE) was used to determine the +1 transcriptional start sites for each of these genes. The promoter region of each gene aligned with the intergenic region of orthologous genes from other akinete-forming cyanobacteria, identifying putative regulatory elements involved in cellular differentiation. Polymerase chain reaction (PCR) was used to amplify the promoter regions from wild-type genomic DNA, and these fragments were cloned into a promoterless GFP transcriptional reporter plasmid. Following electrottransformation, of the *N. punctiforme* wild-type strain, akinetes were induced in each reporter strain using phosphate starvation and low light. Epiflourescence microscopy confirmed the array results indicating cell-type-specific gene expression unique to akinete formation.
Neurotransmitters, Peptides and Receptors (2795-2801)

2795
Netrin-1/UNC5 Signaling
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Netrins comprise a conserved family of guidance cues that direct the growth of neurons and their axons in a wide range of organisms. Four secreted netrins have been identified in vertebrates, netrin-1-4, and two families of netrin-1 receptors have been identified. In vertebrates, Deleted in Colorectal Cancer (DCC) family has two members, DCC and Neogenin. The UNC5 family has four members, (UNC5a-d). Functionally, DCC family mediates attraction towards netrin-1 while the UNC5 family mediates repulsion. Netrins have also been implicated in cell migration, tissue morphogenesis and tumor growth. Although the importance of netrin and its receptors in development of neuronal and non-neuronal network systems has been well characterized, the mechanisms by which signals are transduced downstream of the receptors are only partially understood. Previously it has been shown that Netrin-1 blocks axon outgrowth from DRG explants in vitro, and Unc5e/RCM mutant mice show abnormal invasion of DRG axons into spinal cord. To analyze Netrin-1/UNC5 signaling, we have employed mouse DRG explant cultures on PDL/Laminin-coated glass coverslips, a well established method to study the response of DRG axons to guidance cues. DRG neuron axons growing out of explants were stimulated with recombinant Netrin-1 or control protein for various amount of time. Netrin-1 stimulation leads to increased collapsing of DRG neuron growth cones and decrease in growth cone area. Netrin-1 stimulation also decreased axon growth of DRG neurons. It was shown that Src kinase mediates UNC5 signaling in C.elegans and can directly interact and phosphorylate UNC5. I next analyzed src kinase involvement in mediating the response of DRG axons to Netrin-1. The Src-family kinase inhibitor, PP2, blocks both Netrin-1 induced changes of DRG growth cone morphology. Thus I can conclude that Netrin-1 can control DRG axon growth and that src kinases are involved downstream of Netrin-1.

2796
Early Upregulation of C5a Receptor in a Mouse Model of ALS: Evidence for C5a and AMPA Receptor Synergy in Cell Death
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Background: Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder, characterized by the degeneration of motor neurons. While the cause of this degeneration is largely unknown, it is increasingly evident that neuro-inflammatory processes also contribute to the death of the motor neurons. In this study, we have examined whether the expression of the complement receptor, C5aR, is altered in degenerating motor neurons and its potential role in mediating neuronal degeneration. Objective: We have examined the role of C5aR in protecting against glutamatergic and kainate toxicity in cell culture, and also C5aR expression by immunohistochemistry in NFL-/- mice and ALS tissue. Methods: We examined the expression of C5aR in mediating neuronal injury in Neuro2a and NGF-differentiated PC12 cells by using MTT assay. The expression of C5aR, ubiquitin and TDP-43 was assayed in human spinal cord tissue from neurologically intact control (n=3) and ALS patients (n=3) by immunohistoch emistry. Results: We observed prominent C5aR immunoreactivity in motor neurons in the form of diffuse homogeneous cytosolic and cell membrane staining in NFL-/- mice as compared to C57BL/6 mice. In tissue from ALS patients a significant increase in C5aR staining was also found in motor neurons and the receptor appeared to be localized to the cell membrane and cytoplasm as vesicles. C5aR immunoreactivity co-localized with ubiquitin-positive intraneuronal aggregates. Interestingly there was no evidence of nuclear C5aR immunoreactivity when abnormal TDP-43 skeins were present. Finally, we observed that exogenous recombinant human C5a is neurotoxic with a similar potency to that observed with glutamate, and that the C5a and AMPA receptors appear to work synergistically in inducing this neurotoxicity. Conclusions: C5aR activation with AMPA receptor signalling appears to induce neurotoxicity; however, the upregulation of C5aR and concomitant mislocalization of C5aR in ALS affected motor neurons may be a neuroprotective response against synergistic AMPA-C5aR neurotoxicity.

2797
Ethanol-induced Behavioral Sensitization Is Associated with Dopamine Receptor Changes in the Mouse Olfactory Tubercle
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Objective: We have examined the role of C5aR in protecting against glutamatergic and kainate toxicity in cell culture, and also C5aR expression by immunohistochemistry in NFL-/- mice and ALS tissue. Results: We observed prominent C5aR immunoreactivity in motor neurons in the form of diffuse homogeneous cytosolic and cell membrane staining in NFL-/- mice as compared to C57BL/6 mice. In tissue from ALS patients a significant increase in C5aR staining was also found in motor neurons and the receptor appeared to be localized to the cell membrane and cytoplasm as vesicles. C5aR immunoreactivity co-localized with ubiquitin-positive intraneuronal aggregates. Interestingly there was no evidence of nuclear C5aR immunoreactivity when abnormal TDP-43 skeins were present. Finally, we observed that exogenous recombinant human C5a is neurotoxic with a similar potency to that observed with glutamate, and that the C5a and AMPA receptors appear to work synergistically in inducing this neurotoxicity. Conclusions: C5aR activation with AMPA receptor signalling appears to induce neurotoxicity; however, the upregulation of C5aR and concomitant mislocalization of C5aR in ALS affected motor neurons may be a neuroprotective response against synergistic AMPA-C5aR neurotoxicity.
Extracellular Calcium Dependent Coupling of P2X7 Receptor and Hemichannel in Neuro 2A Cell

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In mammalian cells extracellular Ca\(^{2+}\) plays a pivotal role in several signaling processes by modulating ion channels and receptors. We have shown that Neuro 2a cells, loaded with small fluorescent molecule calcein, release the dye if Ca\(^{2+}\) is omitted in the extracellular medium. In nominally Ca\(^{2+}\)-free solution, the fluorescence intensity of calcein loaded cells decreases 46±3 % (mean ± S.E. of 90 cells from 3 independent experiments) in 30 minutes. The leakage of calcein is blocked by 10 \(\mu\)M Carbeneoxolone (Cbx), a blocker of pannexin hemichannels. However LaCl\(_3\), a general blocker of gap junctions failed to block the dye-release. Fluorescence intensity does not decrease significantly if cells are incubated in Mg\(^{2+}\)-free solution. Interestingly, P2X7 receptor blocker, Brilliant blue (1\(\mu\)M) also blocks the dye-release completely. Externally added ATP caused the dye-release in a dose dependent manner which can be blocked by Cbx as well as Brilliant blue. Potency of ATP seems to be dependent on extracellular Ca\(^{2+}\). ATP (10 \(\mu\)M)-induced dye leakage increased 6 fold when extracellular Ca\(^{2+}\) was decreased from 2mM to 1 mM. Based on our results, we propose a coupling between P2X7 receptor and pannexin in neuro 2a cells. This work is supported by the Department of Science and Technology, Govt. of India.

Dopamine Inhibits Mitochondrial Motility in Hippocampal Neurons

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Axonal transport of mitochondria is critical for proper neuronal function. However, little is known about the extracellular signals that regulate this process. Previously, we demonstrated that serotonin (5-HT) stimulates axonal transport of mitochondria in hippocampal neurons by increasing the activity of Akt/Protein kinase B and decreasing the activity of glycogen synthase kinase 3\(\beta\) (GSK3\(\beta\)), a direct target of Akt. In the present study, we show that another neurotransmitter, dopamine, exerts the opposite effect on mitochondrial movement. Again, the Akt-GSK3\(\beta\) signaling cascade is involved. These results suggest that opposing dopamine and 5-HT signals may regulate mitochondrial movement in hippocampal neurons, and perhaps determine the local energy allocation of ATP among synapses within these neurons. The actions of psychiatric drugs such as fluoxetine (Prozac) and haloperidol, which affect the serotonin and dopamine systems respectively, are known to involve Akt-GSK3\(\beta\) signaling. The results reported here may therefore have important implications for understanding the effects of these drugs on the brain. Research was supported by the Neurosciences Research Foundation.

Kainic Acid Induces Brain-derived Neurotrophic Factor Expression by Activation of AMP-activated Protein Kinase

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Glutamate is a major excitatory neurotransmitter in the mammalian central nervous system (CNS). Kainic acid is a potent agonist of the AMPA/Kainate class of glutamate receptors. It is known that kainic acid induces expression of brain-derived neurotrophic factor (BDNF) in brain. Here we examined the role of and signaling mechanism of AMP-activated protein kinase (AMPK) in kainic acid-induced BDNF expression. We showed that kainic acid induced activation of AMPK via kainate and/or AMPA receptor in neuroblastoma and glioma cells. We then showed that inhibition of AMPK by compound C, a specific inhibitor of AMPK or small interfering RNA of AMPK mRNA and protein expressions. We also showed that inhibition of Ca\(^{2+}\)/calmodulin-dependent protein kinase kinase (CaMKK) by pharmacological inhibitor (STO609) or by CaMKK siRNA transfection significantly blocked kainic acid-induced AMPK activation and BDNF expression. Finally, we showed that inhibition of AMPK reduced kainic acid-induced CaMKII activation and calcium responsive transcription factor CaRF DNA binding activity. These data suggest that AMPK regulates kainic acid-induced BDNF expression through regulation of CaMKII and CaRF pathway.
Synapse Formation and Function (2802-2805)

2802
CASK: A Neurexin Kinase
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CASK belongs to MAGUK family of protein bearing a unique N-terminal CaMKinase domain. Mammalian CASK was discovered due to its ability to interact with Neurexins, a cell adhesion molecule at the neuronal synapse. Its interaction with cell surface and adhesion molecule through its PDZ domain has made it a likely candidate as an organizer of focal adhesion including synapse. CaM kinase domain of CASK has long been deemed as a pseudokinase due to critical substitutions in invariant residues responsible for Magnesium coordination. In our current study we demonstrate that unlike any known kinase CASK can transfer phosphate to Neurexin cytoplasmic tail independent of Magnesium. Counter intuitively Magnesium has a negative effect on this reaction. Moreover we demonstrate that this phosphotransfer activity is possible in vivo. We use a combined structural and biochemical approach to explain the rationale behind this anomalous reaction. Using high resolution crystal structure we demonstrate that unlike most CaM kinases CASK CaMK domain maintains an intrinsically active conformation readily accessible to nucleotide binding. We also demonstrate that CASK CaMK domain binds to ATP avidly in a canonical orientation and this binding is inhibited in presence of Magnesium. In line with ATP binding we demonstrate that CASK CaMK can autophosphorylate catalytically albeit with poor kinetics, a reaction inhibited by excess free magnesium. CASK could also phosphorylate neurexin C-tail, however the kinetics of this reaction strongly depended on its interaction with CASK PDZ domain. Thus CASK combines adaptor activity of MAGUK with a slow kinase activity to generate a very specific phosphorylating module.

2803
Structure of the MARVEL Domain Protein Synaptophysin: Implications for the Synaptic Vesicle Cycle
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Synaptophysin 1 (Sypl) is an archetypal member of the MARVEL domain family of integral membrane proteins and one of the first synaptic vesicle (SV) proteins to be identified and cloned. MARVEL domain proteins are known to be involved in membrane apposition and vesicle trafficking events but the precise role is unclear. Here we have purified mammalian Sypl from calf brain and determined its three-dimensional structure using electron microscopy and single-particle 3-D reconstruction. The hexameric structure resembles an open basket with a large pore and tenuous interactions within the cytosolic domain. The structure suggests a model for Sypl’s role in SV fusion and recycling that is regulated by known interactions with the SNARE machinery. This is the first three dimensional structure a MARVEL domain protein and provides a foundation for understanding the specific role of Sypl in the synaptic vesicle cycle and the general role of MARVEL domain proteins in other biological processes.

2804
Liquid Facets (Lqf) Is Required for Ubiquitin-dependent Synaptic Growth and Function, but Not for Synaptic Vesicle Recycling in Drosophila
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The ubiquitin-proteasome system (UPS) plays an important role in synaptic development and function. However, many components of this system and how they act to affect synapses are still not well understood. Here, we use the Drosophila neuromuscular junction to study the in vivo function of Liquid facets (Lqf), a homolog of the mammalian epsin 1. Contrary to prior expectations, we report that Lqf is not required for clathrin-mediated endocytosis of synaptic vesicles. Our data show that Lqf plays a novel role in synaptic development and function. Further, Lqf acts as a specific substrate of the deubiquitinating enzyme Fat facets (Faf), but not of the Highwire (Hiw) E3 ubiquitin ligase, to stimulate synapse overgrowth. This demonstrates that Lqf co-immunoprecipitates with tubulin in adult head extracts. Our data show that Lqf plays a novel role in synaptic development and function. Further, Lqf acts as a specific substrate of the deubiquitinating enzyme Fat facets (Faf), but not of the Highwire (Hiw) E3 ubiquitin ligase, to stimulate synapse overgrowth. However, Lqf converges to the Hiw pathway by negatively regulating transmitter release in the hiv background. Finally, Lqf co-immunoprecipitates with tubulin in adult head extracts. The microtubule cytoskeleton within synaptic boutons is altered by loss or gain of function of Lqf. These observations reveal a unique feature of structural and functional plasticities of the synapse regulated by two distinct but interactive ubiquitin pathways.

2805
Dysregulated NCAM Shedding Impairs Interneuron Development in the Prefrontal Cortex
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The extracellular domain of NCAM (NCAM-EC) is overproduced in brain and cerebrospinal fluid by proteolytic cleavage in schizophrenia. Transmembrane isoforms (NCAM140, NCAM180) regulate normal axonal and dendritic arborization and synaptic plasticity. NCAM is subject to cleavage (ectodomain shedding) in normal neurons, and requires tyrosine kinase activation of ERK1/2 MAP kinase and metalloprotease activity (Hinkle, et. al., J. Neurobiol. 2006). Transgenic mice that overexpress the soluble NCAM extracellular domain (NCAM-EC) exhibit reduced GABAergic inhibitory synapses in adult cortex and altered behavioral phenotypes (Pillai-Nair, J. Neurosci., 2005). Here, we examined the role of dysregulated NCAM shedding during development of interneurons in the prefrontal cortex, a region associated with cognition and working memory in the NCAM-EC mouse model. The early postnatal development of basket cells was impaired in these mice during the approach to adolescence (P10-P20), resulting in decreased arborization and perisomatic innervation of target neurons in adult prefrontal cortex. GAD65- and synaptophysin-positive synaptic terminals followed a similar developmental pattern. In cortical neuron cultures, NCAM-EC acted as a dominant inhibitor of NCAM-dependent interactions to inhibit neurite branching and outgrowth. Conversely, inhibiting normal NCAM shedding with the metalloprotease inhibitor GM6001 or expression of dominant negative ADAM10 (a disintegrin and metalloprotease) increased NCAM-dependent outgrowth and branching of cortical neurons. Coimmunoprecipitation of ADAM10 and NCAM further implicated ADAM10 as the NCAM sheddase. Taken
together, the results suggest that ADAM10-mediated NCAM shedding normally serves to regulate interneuronal arborization and refine the number of synapses during development. Excessive shedding, modeled in the NCAM-EC mouse, results in underdeveloped interneuronal arbors and fewer synapses, which may reflect GABAergic interneuron defects observed in schizophrenia.

Chloroplasts and Mitochondria (2806-2810)

2806 Identification of a Drosophila Gene Required for Proper Timing and Configuration of Mitochondrial Aggregation during Spermatogenesis
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Mitochondria normally aggregate in early post-meiotic spermatids of the Drosophila testis, enabling subsequent mitochondrial fusion and elongation along the growing flagellar axoneme. Mutations that affect mitochondrial aggregation lead to male sterility. To understand the molecular mechanisms underlying mitochondrial aggregation, we have characterized the mitoshell strain in which homozygous mutant male flies are viable but sterile and show dramatic defects in the timing and nature of mitochondrial aggregation. Homozygous mitoshell females are viable and fertile. Mitochondria in mitoshell mutants prematurely aggregate prior to meiotic entry and surround the nucleus instead of gathering beside it. Spermatocyte nuclei undergo meiotic nuclear divisions in the mutant, but meiotic cytokinesis is never initiated. We mapped the mitoshell mutation by deficiency to a narrow chromosomal region containing twenty-three genes, of which five show enriched expression in the testis. We performed sequence analysis of two of those five candidate genes in two independent EMS-induced mitoshell alleles. One of the candidate genes is mutated in both mitoshell strains, with a nonsense mutation early in the gene in one allele and a different nonsense mutation later in the gene in the second allele. This candidate gene is conserved within insect lineages and encodes a novel protein. Preliminary localization in mitoshell mutant testes of selected GFP-tagged proteins involved in the microtubule cytoskeleton and in cytokinesis demonstrated no obvious structural difference. We will present further subcellular localization of relevant gene products in mitoshell spermatids to dissect the defect more fully. Characterization of the mitoshell gene will allow better understanding of regulatory and/structural mechanisms underlying directed mitochondrial movement in cells.

2807 Subpopulations of Myocardial Mitochondria with Distinct Susceptibility to Mitochondrial Permeability Transition
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Mitochondrial permeability transition (MPT) describes the opening of nonselective mitochondrial pores, which causes the loss of membrane potential, mitochondrial swelling, and ultimately, membrane rupture. MPT was found to be involved in diseases, such as ischemia, where the susceptibility to MPT correlated with infarct size in an animal model. Previously, we have shown that mitochondria purified by zone electrophoresis in a laminar flow (ZE-FFE) separate in two major fractions (Mol Cell Proteomics. 2006; 5: S21). Mitochondria in cardiomyocytes are localized in the intermyofibrillar and subsarcolemmal space possibly explaining the two populations in our purification. Indeed, proteomics analysis showed that myosin heavy and light chain co-purified at low stoichiometric amounts with one subpopulation. In an attempt to further characterize the mitochondrial subpopulations in a physiological context, we tested their susceptibility to MPT. MPT of isolated mitochondria was assayed by Ca2+-induced swelling. Both subpopulations responded to the treatment with swelling, which could be intervened by the MPT inhibitor Cyclosporin A. Remarkably, the magnitude of mitochondrial swelling was significantly different between the subpopulations. In this context, it should be noticed that the approach enables parallel mitochondrial isolation in the same physiological buffer used for the MPT assay. Thus, a different magnitude of swelling was not induced by different treatment as in sequential isolation procedures. In conclusion, our study demonstrates that there exist mitochondrial subpopulations in the myocardium, which possess different susceptibility to MPT. Analyses of the modulation or a conceivable conversion of the subpopulations under pathophysiological conditions will improve the understanding of mitochondrial physiology in diseases.

2808 Lipin 1b Is Recruited to Mitochondria by MitoPLD-generated PA
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We recently identified a lipid-modifying enzyme, MitoPLD that is required for the progression of mitochondrial fusion. The overexpression of MitoPLD generates elevated levels of Phosphatidic Acid (PA) on the mitochondrial surface, resulting in mitochondrial aggregation. In the current study, we demonstrate that a newly-identified PA phosphatase, Lipin 1b, is recruited to mitochondria when the level of PA is increased via MitoPLD overexpression. This recruitment is specific to Lipin 1; the other two members of the Lipin family in mammals, Lipin 2 and Lipin 3, are not similarly recruited. There are two potential PA-binding sites in Lipin 1: the C-terminal DIDGIT catalytic motif, and the N-terminal highly basic region (KKRRKRRRRK). The N-terminal basic region suffices for PA binding, although the C-terminal one may also exhibit significant affinity. The basic region also functions as a nuclear localization signal, potentially in competition for lipid binding. As a PA phosphatase, Lipin 1b presumably converts PA into Diacylglycerol (DAG) when recruited to the exofacial mitochondria, which may have effects on the fusion reaction or mitochondrial metabolism. In Summary, our findings reveal that Lipin 1b is recruited to mitochondria by MitoPLD-produced PA. Moreover, our findings suggest that a lipid signaling pathway involving PA and DAG regulates mitochondrial fusion.
2809

Mitochondrial Biogenesis during Liver Regeneration: A Role for AMPK Signaling
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Liver regeneration after 70% partial hepatectomy (PHx) in rats involves priming of hepatocytes in the remnant liver (0.5-4 hrs) to activate the transition from the quiescent state (G0), progression through G1 and entry into S phase by 24 hrs, ultimately resulting in the restoration of liver mass by 1-2 weeks. We studied the effects of PHx on hepatic energy management in order to understand how the increased energy demand is met under conditions where the remnant liver meets the metabolic needs of the organism while supplying energy required for regeneration. A marked 20-30% loss of total adenine nucleotides and a drop in ATP/AMP occurs in the remnant liver within a minute of PHx. Adenine nucleotide levels and ATP/AMP remain low through the progression through G1 and do not fully recover to sham levels until 24-48 hrs. The early decline in ATP/AMP is associated with a sharp increase in the activity of AMP kinase (AMPK), a signaling protein that functions as an energy sensor coordinating cellular energy demand and supply and may be involved in mitochondrial biogenesis during liver regeneration. We investigated mediators of mitochondrial biogenesis that may act downstream of the increased energy demand following PHx. A significant increase of PGC1α, TFAM, and polymerase γ mRNA was observed by quantitative PCR within 1-4 hours post-hepatectomy. Concurrently, there is an increase in mRNA of other mitochondrial-related markers including ALAS-1, Cox4I1, mt 12S rRNA, and VDAC1. Preliminary experiments on rats expressing a dominant negative form of AMPK-α1 suggest that expression of TFAM and polymerase γ, but not PGC1α was suppressed. These findings suggest that the priming events following PHx may include activation of signals that lead to mitochondrial biogenesis, well ahead of the onset of the nuclear DNA synthesis.

2810

Perinatal and Maintained Calorie Restriction in Rats: Effects to Mitochondrial Level of Oxidative and Nitrosative Stress

Calorie restriction (CR) without malnutrition is an experimental manipulation that is known to extend the lifespan of a number of organisms including yeast, worms, flies, rodents and perhaps non-human primates. In addition, CR has been shown to reduce the incidence of age-related disorders (for example, diabetes, cancer and cardiovascular disorders) in mammals. Several studies have demonstrated that CR decreases the age associated increases in cellular ROS production and damage to cellular macromolecules. The aim of this project is to determine the effect in oxidative and nitrosative stress in liver mitochondria of a perinatal and maintained calorie restriction in rats. Calorie restriction during gestation led to growth retardation at birth. If CR was prolonged throughout lactation, adult body weight was permanently reduced. Offsprings of one month of CR regimen showed a decreased in body weight of 48.36% in female and 55.77% in male rats, levels of NO• in CR rats increased 179.47% in females and 281.97% in males rats compared with controls, the determination of lipoperoxidation showed an increased of 211.16% in female and 116.42% in males rats under CR regimen against controls. Rats of five months of calorie restricted regimen weighted 35.42% in both in female and 50.23% in male rats less than controls. The experimental determination of NO• shows a diminution on the production of NO•, 56.28% in female and 35.46% in male rats under calorie restriction versus controls. The same can be seen in the rats under calorie restriction regimen versus control in the determination of lipoperoxidation with a diminution of a 53.54% in male rats but, in contrast, an increased in lipoperoxidation levels in female rats of 96.5% were observed versus controls. **Acknowledgements:** The authors appreciate the partial economic support of: CIC-UMSNH (2.16), COECYT, Fondos Mixtos CONACYT-Gob. Estado de Michoacán (64277 and 64308).

Peroxisomes (2811-2813)

2811

A Role for ESCRT in the Formation of Multivesicular Peroxisomes in Tombusvirus-infected Plant Cells
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Peroxisomes are highly dynamic organelles in terms of their shapes, mobility and metabolic functions, and also serve as the sites for the development of some viral pathogens. For instance, in plants, certain tombusviruses recruit peroxisomes for viral RNA replication and in doing so cause extensive rearrangements of the organelle’s boundary membrane leading to the formation of a peroxisomal multivesicular body (pMVB). While it is unclear how a pMVB is formed, results from previous studies suggest that components of the tombusvirus replication complex, namely the 33 kDa RNA-binding protein (p33) and 92 kDa RNA polymerase (p92), are targeted specifically to peroxisomes and are essential for pMVB biogenesis (McCartney et al. 2005. Plant Cell 17:3513). Here we provide a role for p33 and p92 in pMVB formation by presenting evidence that, analogous to the ability of certain retroviral Gag proteins to recruit constituents of the ESCRT (Endosomal Sorting Complex Required for Transport) machinery to execute virus budding from infected mammalian cells, p33 and p92 appropriate ESCRT to facilitate internal vesicle formation at the peroxisomal membrane. For instance, we show as part of our comprehensive yeast two-hybrid analysis of the Arabidopsis ESCRT interactome that at least two components of ESCRT-I, Vps23 and Vps28, interact with the cytosolic-exposed C-terminal portions of p33 and p92. Notably, these regions of p33 and p92 posses sequences that resemble the -YXXL-type late domain found in some Gag proteins. We show also that transient expression of Vps23 or Vps28 in tombusvirus-(co)transformed tobacco cells resulted in both ESCRT components being relocalized from late endosomes to pMVBs. These data support a role for ESCRT in pMVB biogenesis and suggest that parallels exist between the molecular mechanisms underlying host-cell membrane rearrangements associated with positive-strand RNA virus and retrovirus infections.
Distinct Subsets of Resident ER Proteins Are Localized in Separate Sub-Regions to Facilitate Protein Synthesis versus Protein Destruction

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The endoplasmic reticulum is a major site of protein synthesis in eukaryotic cells. Resident ER chaperones and folding enzymes assist and monitor the folding and assembly of newly synthesized proteins in this organelle. Those that succeed are transported to exit sites for trafficking to the Golgi, while those that fail must be identified, unfolded in some cases, and transported to the cytosol for degradation by the 26S proteasome. Recent studies have found that different subsets of resident ER proteins partition in physically distinct regions of the ER, which suggests that these two opposing functions of the ER might be enabled by a physical separation of the proteins involved. We have further examined this possibility by determining the sub-organelar localization of a large number of resident ER proteins involved in either protein folding or protein degradation in an LPS-stimulated B cell line, which synthesizes large quantities of antibody molecules. Our study included chaperones and folding enzymes (i.e., BiP, GRP94, calnexin, and PDI), ER localized DnaJ homologues that regulate BiP’s activity (i.e., ERdj3, ERdj4, and ERdj5), nuclear exchange factors for BiP (i.e., BAP and GRP170), and proteins involved in the recognition and extraction of unfolded/unassembled Ig subunits (i.e., Herp, Derlin-1, Hrd1, and p97). We found that molecular chaperones involved in the folding of Ig molecules partitioned between the rough and smooth ER at ratios that were very similar to those observed for the bulk of Ig heavy and light chains. However, proteins involved in the degradation of Ig proteins that fail to mature properly were more preferentially localized to the smooth ER fraction compared to the resident ER proteins involved in folding. These data are compatible with the two earlier studies on partitioning of resident ER proteins and suggest that contrasting ER functions may occur in physically distinct regions of the ER.
Receptor Chaperone at ER-Mitochondrion Interface: Sigma-1 Receptor Regulates Ca\(^{2+}\) Signaling and Cell Survival
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Communication between endoplasmic reticulum (ER) and mitochondrion is important for bioenergetics and cell survival. The ER supplies Ca\(^{2+}\) directly to mitochondrion via inositol 1,4,5-trisphosphate receptors (IP3Rs) at close contacts between ER and mitochondrion called mitochondrion-associated ER membrane (MAM). Mitochondrial Ca\(^{2+}\), thus supplied by IP3Rs, subsequently activates key enzymes in the TCA cycle. IP3Rs are known to be, upon IP3 stimulation, rapidly ubiquitinated and degraded by proteasomes via the ER-associated degradation pathway. Although ER luminal Ca\(^{2+}\) is suggested to play a role in the degradation of IP3Rs, exactly how the ER luminal Ca\(^{2+}\) regulates the stability of IP3Rs is unknown. Further, whether ER chaperones play a role in this regard has never been explored. We discovered here that an ER protein, the sigma-1 receptor (Sig-1R), which is implicated in cellular differentiation, neuroprotection, carcinogenesis, and neuroplasticity, is a novel Ca\(^{2+}\)-sensitive and ligand-operated receptor chaperone at MAM. Normally, Sig-1Rs form a complex at MAM with another chaperone BiP. Upon ER Ca\(^{2+}\) depletion or via ligand stimulation, Sig-1Rs dissociate from BiP and begin chaperoning IP3Rs at MAM, leading to a prolonged Ca\(^{2+}\) signaling into mitochondrion. Knockdown of Sig-1Rs, on the other hand, potentiates ubiquitination, degradation and aggregate formation of IP3Rs. Importantly, synthetic compounds can regulate the dissociation of Sig-1Rs from BiP, a key step for activation of the Sig-1R chaperone, in an agonist-antagonist fashion. Increasing Sig-1Rs in cells counteracts ER stress response whereas decreasing them enhances apoptosis. These results reveal that the novel receptor chaperone at MAM, by sensing ER Ca\(^{2+}\) concentrations, regulates ER-mitochondrial interorganellar Ca\(^{2+}\) signaling and cell survival. Sig-1R agonists have been shown to prevent neurodegeneration induced by ischemia and beta-amyloid. Thus, this unique property of the ligand-operated chaperone machinery may represent a new avenue for therapeutic opportunities. (Supported by IRP/NIDA/NIH/DHHS).

Effect of Bax Inhibitor-1 on the Production of Reactive Oxygen Species by Cytochrome P450 2E1 and the Resultant Cell Death against ER Stress
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This study showed the effect of Bax inhibitor-1 (BI-1) on the production of ROS and membrane lipid peroxidation on the endoplasmic reticulum (ER) and the resultant cell death. As a mechanism, cytochrome P450 2E1 (P450 2E1), a major ROS-associated mono-oxygenase on ER membrane was examined in this study. The P450 2E1 was highly increased in Neo cells but relatively slower and lower in the BI-1 overexpressed cells. In a model system containing purified BI-1, NADPH-P450 reductase (CPR), and P450 2E1 incorporated into liposomes, the production of hydroxyl radicals and hydroxyl radicals (and/or superoxide) was decreased with an increasing amount of BI-1. Using fluorescence resonance energy transfer, it was found that CPR and BI-1 show a physical association with each other. This interaction was also confirmed by co-immunoprecipitation of BI-1 with CPR in Neo cell extract. As a possible mechanism, it is thought that the flow of electrons from CPR to P450 2E1 is modulated by BI-1, considering that the catalytic activity of P450 2E1 decreased at a limited concentration of CPR without any significant change of NADPH oxidation level in the presence of BI-1. Taken together, these results suggest that BI-1 reduces the production of P450-induced ROS in vivo and in a reconstitution system.

Golgi Complex (2817-2819)

Molecular Mechanism of Mitotic Golgi Disassembly and Reassembly Revealed by a Defined Reconstitution Assay
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In mammalian cells, flat Golgi cisternae closely arrange together to form stacks. During mitosis, the stacked structure undergoes a continuous fragmentation process. The generated mitotic Golgi fragments are distributed into the daughter cells, where they are reassembled into new Golgi stacks. In this study, an in vitro assay has been developed using purified proteins and Golgi membranes to reconstitute the Golgi disassembly and reassembly process. This technique provides a useful tool to delineate the mechanisms underlying the morphological change. There are two processes during Golgi disassembly: unstacking and vesiculation. Unstacking is mediated by two mitotic kinases, cdc2 and polo-like kinase (plk), which phosphorylate the Golgi stacking protein GRASP65 and thus disrupt the oligomer of this protein. Vesiculation is mediated by the COPI budding machinery ARF1 and the coatomer complex. When treated with a combination of purified kinases, ARF1 and coatomer, the Golgi membranes were completely fragmented into vesicles. After mitosis, there are also two processes in Golgi reassembly: formation of single cisternae by membrane fusion, and restacking. Cisternal membrane fusion requires two AAA ATPases, p97 and NSF (N-ethylmaleimide-sensitive fusion protein), each of which functions together with specific adaptor proteins. Restacking of the newly formed Golgi cisternae requires dephosphorylation of Golgi stacking proteins. This systematic study revealed the minimal machinery that controls the mitotic Golgi disassembly and reassembly process.

Nucleo-cytoplasmic Shuttling of the Golgi Phosphatidyl 4-kinase Pik1 Is Regulated by 14-3-3 Proteins and Coordinates Golgi Function with Cell Growth
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In mammalian cells, flat Golgi cisternae closely arrange together to form stacks. During mitosis, the stacked structure undergoes a continuous fragmentation process. The generated mitotic Golgi fragments are distributed into the daughter cells, where they are reassembled into new Golgi stacks. In this study, an in vitro assay has been developed using purified proteins and Golgi membranes to reconstitute the Golgi disassembly and reassembly process. This technique provides a useful tool to delineate the mechanisms underlying the morphological change. There are two processes during Golgi disassembly: unstacking and vesiculation. Unstacking is mediated by two mitotic kinases, cdc2 and polo-like kinase (plk), which phosphorylate the Golgi stacking protein GRASP65 and thus disrupt the oligomer of this protein. Vesiculation is mediated by the COPI budding machinery ARF1 and the coatomer complex. When treated with a combination of purified kinases, ARF1 and coatomer, the Golgi membranes were completely fragmented into vesicles. After mitosis, there are also two processes in Golgi reassembly: formation of single cisternae by membrane fusion, and restacking. Cisternal membrane fusion requires two AAA ATPases, p97 and NSF (N-ethylmaleimide-sensitive fusion protein), each of which functions together with specific adaptor proteins. Restacking of the newly formed Golgi cisternae requires dephosphorylation of Golgi stacking proteins. This systematic study revealed the minimal machinery that controls the mitotic Golgi disassembly and reassembly process.
The yeast phosphatidylinositol 4-kinase Pik1p is essential for growth and normal Golgi morphology and regulates transport of newly synthesized proteins from this compartment. Phosphatidylinositol 4-phosphate, which is generated by Pik1p recruits cytosolic effectors involved in formation of post-Golgi transport vesicles. A second pool of catalytically active Pik1p resides in the nucleus. The physiological significance and regulation of this dual localization of the lipid kinase is not understood. We report here that Pik1p binds to the redundant 14-3-3 proteins Bmh1p and Bmh2p through a phosphorylated consensus motif found in other 14-3-3 targets. We demonstrate that nucleo-cytoplasmic shuttling of Pik1p involves phosphorylation and that 14-3-3 proteins bind Pik1p in the cytoplasm. A dramatic relocation of Pik1p from the Golgi to the nucleus is observed upon nutrient deprivation, a process rapidly reversed upon restoration of the nutrient supply. Pik1p relocation is accompanied by a transient increase of the Pik1p -14-3-3 complex. The data presented suggest a role of Pik1p nucleo-cytoplasmic shuttling in coordination of biosynthetic transport from the Golgi with nutrient signaling in a pathway that involves 14-3-3 proteins.

2819 Soluble Proteins Traverse the Golgi Complex by a Novel Diffusion-based Transport Mechanism
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While in mammalian cells supramolecular cargo complexes are secreted by compartment maturation-progression through the Golgi complex, less is known about the transport of other protein classes. Here, we have examined the secretion of the soluble cargo proteins, using albumin and α1-antitrypsin as prototypes. We find that these serum proteins traverse the Golgi by a novel mechanism that involves diffusion via intercisternal tubular continuities and pH-dependent concentration in Golgi export domains. This results in maximization of their transport efficiency. In the same cells, the large transmembrane protein VSVG traverses the Golgi by the maturation-progression process. Thus, different transport mechanisms operate in the same cells.

Endosomes and Lysosomes (2820-2821)

2820 Actin Dynamics Plays a Role in the Endocytic Pathway beyond the Internalization Step
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The role of actin in the first step of endocytosis has been well characterized (reviewed in Lanzetti, 2007). Less understood is how actin regulates the trafficking through the endocytic pathway. We have observed that the depolymerization of actin, by treatment with Latrunculin A after internalization of transferrin or EGF, inhibits recycling and degradation, respectively. Colocalization experiments of endosomal markers, such as EGF, LDL, pepstatin A, EEA1 and Lamp1, with actin or actin binding proteins, revealed that actin is often found to be in close proximity or to colocalize with endosomes. The actin filaments associated with endosomes were organized in two main shapes. Typically, the actin structures observed either are round resembling actin “clouds” or elongated with a “snake”-like shape. These structures have different dynamic behaviors as observed by live imaging of actin-GFP. The actin clouds accompanied the movement of endosomes where the snakes waved but did not exhibit directional movement. Endosomes associated to these structures appeared trapped by the head of the actin snakes. Importantly, we have been able to reconstitute in vitro the assembly of actin clouds on purified endosomes, even in the absence of cytosol. We conclude that, after internalization, endosomes can also interact with the actin cytoskeleton; this interaction occurs in two different ways and may be necessary for efficient cargo recycling and degradation. We hypothesize that actin dynamics can be involved in the regulation of endocytic sorting events.

2821 Microscopic Evidence for HGSNAT-containing Microdomains in Lysosomal Membrane
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Human Acetyl-CoA: glucosaminide N-acetyltransferase (HGSNAT) is a lysosomal membrane protein that catalyses a key step in the degradation of glycosaminoglycan heparan sulfate (HS). Biochemical data showed that HGSNAT is localized in microdomains of lysosomal membrane (Taute et al., 2003). Its deficiency in humans results in mucopolysaccharidosis type III C (MPS IIIC, Sanfilippo IIIC syndrome). We have obtained antibodies against peptide epitopes derived from sequence of recently discovered human HGSNAT gene (Hrebicek et al., 2006). Immunofluorescence confocal microscopy showed that in control cultured human fibroblasts the anti-HGSNAT antibody stained a subpopulation (approximately 25-50% of LAMP2- positive vesicles) of lysosomes. The signal did not systematically co-localize with any of the following other organelar markers: COX1 (mitochondria), PDI (ER), Golgi58K (Golgi), EEA1 (early endosomal compartment) or mannoso-6-phosphate receptor (M6PR). The anti-HGSNAT antibody stained discrete regions of the lysosomal membrane - corresponding well with the microdomain localization of HGSNAT previously proposed by biochemical methods. In spite of apparent microdomain localization, HGSNAT did not co-localize with tested protein and lipid microdomain markers such as flotillin 1, GM1 ganglioside or globotriaosylceramide. Filipin staining for cholesterol was also not prominent in HGSNAT-positive regions, which makes HGSNAT presence in cholesterol microdomains unlikely. HGSNAT, however, partially co-localized with sortilin, a receptor in the alternative pathway for transport of soluble or membrane-associated lysosomal proteins into lysosome. The transport of HGSNAT apparently does not depend on M6PR as it reached lysosomes in N-Acetylg glucosamine-1-phosphotransferase deficient cells. It is not clear whether HGSNAT transport is sortilin-only dependent. As HGSNAT catalyses essential step in the lysosomal degradation of HS, its absence in a number of lysosomes may suggest existence of subpopulations of lysosomes with specialised metabolic functions.
Targeting to Lysosomes (2822)

2822
Palmitoylation Is Required for the Retrograde Transport of the Lysosomal Sorting Receptors
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The addition of palmitic acid to proteins is a reversible process that occurs on cysteine residues via a thioester bond. There have been many membrane proteins reported to be palmitoylated and theories for the role of palmitate on membrane protein function abound but there are only a few precise examples of changes in protein function or localization caused by palmitoylation. Studying the family of lysosomal sorting receptors, we have identified palmitoylation as a critical regulator for the efficient recycling of these receptors from the endosome to the Golgi apparatus. The lysosomal sorting receptors (the cationic dependent and independent mannose 6-phosphate receptors (CD-MPR and CI-MPR) and sortilin) bind cargo in the Golgi apparatus, are packaged into clathrin coated trafficking vesicles and traffic to the endosomes. Once in the endosomes, the receptors release their cargo into the endosomal lumen and recycle back to the Golgi for another round of trafficking, a process that requires the retromer complex. It has previously been proposed that palmitoylation may play a role in the efficient recycling of the CD-MPR. However, the mechanistic details of this process have not been elucidated nor have the enzymes that add this important post-translational modification been identified. Here, we demonstrate that in addition to CD-MPR, the other family members of the lysosomal sorting receptors, CI-MPR and sortilin are also palmitoylated and that this modification is crucial for its endosome to Golgi transport. In addition, we characterize the mechanism of this modification and provide evidence that it is required for the recycling of the lysosomal sorting receptors back to the Golgi. This mechanism is reminiscent of the on/off cycle of myristoylate for the proper function of the ras protein, and could be the first such example of an on/off cycle of palmitic acid required for recycling of a membrane receptor.

Epithelia (2823-2828)

2823
Spatiotemporal Localization of the Melanocortin 1 Receptor and Its Ligand Alpha Melanocyte Stimulating Hormone during Murine Cutaneous Wound Healing
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The melanocortin 1 receptor (MC1R) and its ligand alpha melanocyte stimulating hormone (αMSH) are best known for their role in melanogenesis/pigment regulation. However recent papers suggest that αMSH and MC1R also participate in the immune response and inflammation. We hypothesized that healing murine wounds would have increased MC1R and MSH expression. Methods: Using an excisional murine wound model in C57BL6 mice, we evaluated the expression and immunolocalization of MC1R and αMSH @ 6hr, 24hr, 48hr, 72hr, 7d, 14d and 21d after injury. Results: We found that αMSH is highly upregulated in the epidermis at 6hrs and remains relatively high until the 21st day mark when αMSH levels return to a baseline expression. αMSH is also seen in a subset of infiltrating immune cells at 6 and 24hrs and in hair follicles and sebaceous glands adjacent to the wound bed. MC1R expression undergoes dramatic temporal and spatial changes during wound healing. The receptor appears first in a subset of infiltrating immune cells at 24hrs and by 48hrs begins to localize to epidermal keratinocytes at the leading edge of the wound. By day 14 when the wound has epithelialized, there is no expression of MC1R in the epidermal keratinocytes. MC1R also appears in a small percentage of hair follicles adjacent to the wound at 48hrs and gradually increases expression by 21days. RT-PCR validates elevated MC1R expression during the course of murine wound healing, peaking at d21 when remodeling and re-pigmentation occurs. Conclusion: alpha MSH and its receptor MC1R contribute to the highly coordinated cutaneous responses to injury.

2824
Probing the Mechanical Properties of Epithelial Cells In Vivo by Laser Nanodissection: Relaxation of Cell Contacts during Intercalation in Drosophila Embryos
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During tissue morphogenesis, cells can exchange neighbours (a process called intercalation) by the remodelling of cell contacts. Junction remodelling involves a spatial regulation of actomyosin contractility and cadherin-based adhesion. In Drosophila embryos, junction remodelling during epithelial intercalation is irreversible and drives tissue elongation. This process requires the polarized recruitment of the actin-bound motor Myosin-II [1]. How force generation and transmission at the cellular level impact on tissue morphogenesis? We propose to use laser ablation to directly assess this question. In cell cultures, laser ablation has been successfully used to perturb intercellular mechanical components, e.g. by cutting actin stress fibres [2]. In this context, we devised a microscope set-up which allows us to ablate locally the cortical network in cells of Drosophila embryos, thereby destabilizing cell interfaces. A tightly focused near-infrared femtosecond laser delivers nJ pulses which disrupt cortical elements and modify the force balance at cell interfaces. We monitored topological and geometrical changes during relaxation after ablation. The rupture of the actomyosin cortex drowse a redistribution of the actin and cadherin patches revealing the interdependence between these two protein structures. We also show that cell response (geometry and dynamics) to mechanical perturbation depends on the intercalation stage at which nanosurgery is performed. [1] C. Bertet, L. Sulak and T. Lecuit, “Myosin-dependent junction remodelling controls planar cell intercalation and axis elongation”, Nature, vol. 429, pp. 667-671, 2004. [2] S. Kumar, I. Z. Maxwell, A. Heisterkamp, T. R. Polte, T. P. Lele, M. Salanga, E. Mazur, and D. E. Ingber, “Viscoelastic retraction of single living stress fibers and its impact on cell shape, cytoskeletal organization and extracellular matrix mechanics”, Biophysical Journal, vol. 90, no. 10, pp. 3762-3773, 2006.
Possible Roles of Uroplakins in Frog Bladder Epithelium in Regulating Water Flux

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Unlike mammalian bladder urothelium, which mainly functions as a water barrier, frog bladder epithelium is involved in water absorption which plays a key role in body fluid and salt homeostasis. We have previously shown that the apical surface of mammalian bladder urothelium is almost entirely covered by 2D crystals of 16 nm uroplakin particles (“urothelial plaques”) consisting of four major uroplakins (UPs; the 27kd UPla, 28kd UPb, 15kd UPII and 47kd UPIIIa) and a minor 35kd UPIIIb, that the formation of UPIa/II and UPb/III heterodimers is a prerequisite for ER exit, and that genetic ablation of uroplakin genes leads to a loss of urothelial plaques and a compromised permeability barrier function. These results established that uroplakins are the structural components of urothelial plaques that contribute to urothelial barrier function. In this study, we demonstrate that Xenopus bladder epithelium expresses homologues of all five mammalian uroplakins, that xUPla, Ib, II and IIIa are also expressed in kidney, oocytes and fat body, and that only xUPIIIb is bladder-specific. Using rabbit antibodies monospecific for each of the Xenopus uroplakins, we detected xUPIa (27kDa), UPb (21kDa), UPII (15kDa), UPIIIa (30kDa), and UPIIIb (35kDa) in the crude membrane fraction of Xenopus bladder epithelium. Similar to their mammalian homologues, these frog uroplakins are mainly associated with the apical surface of Xenopus bladder epithelium indicating that their expression is differentiation-dependent and that they primarily function at the apical surface. The apical location of uroplakins at the apical membrane suggests that uroplakins may be involved in regulating the passage of small molecules, including water, across the frog bladder epithelium. These results indicate that the structure and function of uroplakins are much more diversified than previously appreciated.

Characterization of a Novel Bves-VAMP3 Interaction

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Bves (Blood vessel/epicardial substance) is an integral membrane protein that is highly expressed in a wide variety of cell types, including epithelia, heart, skeletal muscle, and brain. Bves is necessary in preserving the integrity of epithelial tight junctions and for localization of junctional proteins to the cell membrane in a polarized monolayer. Bves-depleted Xenopus embryos exhibit disrupted migration of epithelia during gastrulation, while epithelial sheets devoid of Bves are inhibited in repair and movement. Due to the lack of predicted protein domains within Bves, a split ubiquitin screen was utilized to reveal potential binding partners, and subsequently, Vesicle-Associated Membrane Protein-3 (VAMP3) was identified. VAMP3 is a ubiquitously expressed member of the SNARE protein complex that binds syntaxin and SNAP to facilitate the docking and fusion of vesicles to the plasma membrane in the recycling endosome. Recently, VAMP3 has been implicated in the movement and adhesion of polarized epithelial sheets. Thus, VAMP3 and Bves potentially have related functions. Previous work has implicated Bves in several functions also ascribed to VAMP3. Preliminary immunochemical studies show that endogenous and transfected VAMP3 co-localize with Bves in several different cell types and tissues, while biochemical analyses demonstrate interaction between Bves and VAMP3. Thus, there is strong support for an interactive role for these two proteins in shared cell functions. Further analysis will confirm and characterize the interaction of Bves and VAMP3 proteins and determine how this association regulates basic epithelial cell functions dependent upon protein trafficking. Based upon previous studies indicating a role for VAMP3 in epithelial adhesion and movement, characterization of this interaction may reveal the molecular mechanism underlying the role Bves plays in these processes, and establish a novel, more global function for Bves within the cell.

Development and Mesenchymal Transformation of the Epicardium: A Role for Notch Signaling

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Epithelial-to-mesenchymal transformation (EMT) is essential for heart development. The outer covering of the heart, the epicardium, arises from an extracardiac source of mesothelial cells called the proepicardium (PE). After the PE attaches to the embryonic heart, an epicardium forms by migrating PE cells which cover the naked myocardium. Using a mechanism of EMT, outer epicardial cells invade into the underlying myocardium. Information is lacking on the precise mechanisms for epicardium formation, epicardial EMT, and how these processes are linked. To further characterize epicardial formation and EMT, we employed confocal imaging of developing quail hearts. We also examined whether Notch signaling plays a role in maintaining an epicardial phenotype by treating proepicardial and whole heart cultures with DAPT (a gamma-secretase inhibitor) and PDGF-BB (an inducer of EMT and coronary smooth muscle cell differentiation). We found that cell divisions within the epicardial layer nearly always orient so that the daughter cells form within the plane of the epicardium. This planar cell polarity is accompanied by formation of a primary cilium, and a highly organized epithelial-type cytoskeleton. EMT begins with a break in planar polarity as seen by loss of primary cilia, random orientation of cell divisions, and remodeling of actin cytoskeleton to a mesenchymal organization. Inhibition of Notch signaling with DAPT in proepicardial cultures leads to disorganized cell-cell contacts within the epicardial monolayer, while a combined treatment with DAPT and PDGF-BB synergistically triggers an early onset of EMT. DAPT treatment in whole hearts produces large increases in the number of epicardial cells undergoing EMT, suggesting that Notch signaling normally prevents EMT in the epicardium. These results suggest that planar polarity and Notch signaling pathways play important roles in maintenance of the epicardial phenotype, and that EMT requires suppression of Notch signaling which increases the responsiveness of the epicardium to EMT-activating signals.
Late Abstracts

2828
Effects of Scaling and Root Planing Therapy on Laminin-5 Gamma 2 Amount in Gingival Crevicular Fluid
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Periodontal disease is defined as inflammation of the periodontium, the tooth supporting structures in the oral cavity. The periodontium includes a connective tissue attachment from alveolar bone to the tooth with an overlying soft tissue connection, the junctional epithelium. Periodontal disease process is initiated by a host immune response to bacterial plaque producing an inflammatory response which may lead to alveolar bone loss and eventual tooth loss. There have been attempts to recover markers in the oral cavity which may help identify periodontal disease, including various cytokines, cellular fragments and proteins. Some of these efforts have focused on the sampling of the gingival crevicular fluid (GCF), an exude migrating from the gingival crevice surrounding teeth, in an attempt to correlate markers of inflammation with disease. Ten patients will be randomly selected from the periodontics clinic at NNMC Bethesda. Periodontal parameters and GCF samples will be collected from six teeth, three demonstrating bleeding on probing and clinical attachment loss and three healthy control teeth. Data will be collected before non-surgical scaling and root planing therapy and then monthly thereafter for six months. The purpose of this study is to determine the effect scaling and root planing has on the amount of laminin-5 gamma2 chain protein recovered in the gingival crevicular fluid. The hypothesis is patients with active periodontal inflammation will have lowered laminin-5 gamma2 amounts recovered from the gingival crevicular fluid following scaling and root planing therapy.

2829
Estradiol Regulates ERα Expression in Human Macrophages
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Objective: Monocytes and macrophages (Mφs) are exposed to fluctuations in estradiol (E2) throughout the menstrual cycle. E2 has been shown to regulate expression of its own receptor in some cell types. Since E2 levels peak pre-ovulation and during pregnancy, we hypothesized that E2 up-regulates estrogen receptors (ER) to increase sensitivity to E2, and subsequently influences E2-regulated inflammatory responses. As human monocytes and Mφs express ERs, we determined whether E2 regulates ER mRNA and protein expression in these cells. Methods: Monocytes were isolated from male and pre-menopausal female donors. Mφs were differentiated for 7 days with 10 ng/ml GM-CSF. Cells were treated in the presence or absence of 17β-estradiol or vehicle control, and ER mRNA and protein were measured by qRT-PCR and western blot. Transcriptional regulation was determined by luciferase assay. Results: We confirmed expression of ERα in monocytes and Mφs from both male and female donors. There was no gender difference in ER α mRNA and protein expression in either cell type. E2 treatment augmented ERα mRNA and protein expression in Mφs derived from females, and this was at least in part mediated transcriptionally. Intriguingly, E2 had no effect on monocyte ERα mRNA and protein levels; however ERα mRNA levels increased in monocytes with time in culture. Conclusions: These data indicate that ERα is differentially expressed and regulated in human monocytes and Mφs. Our previous studies have shown that E2 attenuates LPS-induced pro-inflammatory cytokine secretion, and we now show that E2 acts as a positive regulator of ERα expression. This implies that up-regulation of ERα by E2 may serve as a mechanism for the control of inflammation by human monocytes and Mφs. This may be of critical importance during times of the menstrual cycle when E2 levels are high and tolerance of sperm and an allogeneic fetus are imperative.

2830
Morphological and Functional Evaluation of Peritoneal Macrophage of Intoxicated Mouse with Peroxisomicine A1
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Peroxisomicine A1 (PA-1) one of the toxic substances present in the fruit of Karwinskia, causes lung damage characterized by polymorphonuclear (PMN) infiltration and breakage of the alveolo-capillary membrane, and the presence of tumor necrosis factor alfa (TNFα). To evaluate the activation of resident peritoneal macrophages and the presence of TNFα in circulating blood and supernatant fluid, mice groups were injected intraperitoneally with the following treatments:1) Peroxisomicine A1 dissolved in carthamus oil, 2) peroxisomicine A1 in salt solution, 3) carthamus oil, 4) salt solution, 5) lipopolysacharide (LPS, positive control), 6) negative control without any treatment. After treatments mice were sacrificed at 1, 1.5 and 2 hours and resident peritoneal macrophages were harvested by peritoneal lavage, cell suspensions were centrifugated and the pellet containing peritoneal macrophages was separated in two fractions: one for quimiotaxis studies and the other fraction was embedded in epoxy resin for light and electron microscopy evaluation. Serum and supernatant fluid were analyzed for TNFα quantification by ELISA. The present study demonstrates that PA1 induces the activation of peritoneal macrophages and mitochondrial damage characterized by swelling and rupture of the inner membrane in these cells. Secretion of TNFα was increasing with time. PA1 in salt solution causes an increment in quimiotaxis. Also we established an in vivo model for current studies to evaluate PA1 effects in other peritoneal cells.

Cell Culture (2831-2833)

2831
Heat Shock Protein of 70kDa and Ecological Potencies of Unicellular Organisms
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In protists of different macrotaxa heat shock protein of 70 kDa family (HSP70) was revealed, and its content being rather high in unstrained, intact cells. We presume this protein to play some role in adaptation of unicellular organisms to environmental conditions, p.e. temperature and salinity. Ecological potencies of organisms might be reflected by the initial constitutive level of HSP70 in their cells, as well as by behaviour mode of their chaperone system during long-term acclimation and/or after the stresses. In this connection we studied eurialine ciliate *Paramecium nephridiatum* and freshwater *Tetrahymena pyriformis* (amicronuclear strain GL); both of them were acclimated to 0 ‰ and 10 ‰, and treated by salinity stresses. In the cells of *P. nephridiatum*, acclimated to 0 ‰, constitutive HSP70 level was much higher, than in 10 ‰. In the cells of *T. pyriformis* from 0 ‰ and 10 ‰ the contents of HSP70 did not differ much from each other, but the sets of constitutive HSPs included different polypeptides. In *P. nephridiatum* salinity shock (transfer from 0 to 10 ‰ for 1 h) did not cause the induction of HSP70 synthesis, *a contrario*, some decrease of the protein content was observed, whereas the reciprocal transfer (from 10 to 0 ‰ for 1 h) resulted in the increase of HSP70 level. In *T. pyriformis* we could not cause the induction of HSP70 synthesis after shocks in both directions of salinity changes. It looks like eurialine ciliates are preadapted to abrupt environmental changes by the highest extent of constitutive HSP70 level and reactivity of their chaperone system, to compare with that of stenohaline freshwater *T. pyriformis*.

2832

**Characterization of PICM-19 Porcine Liver Stem Cell Line for Use in In Vitro Toxicological Assays**


In vitro models of the liver are needed to replace animal models for the rapid assessment of drug biotransformation and toxicity. One hepatocellular model, the PICM-19 pig liver stem cell line, may fulfill this need since these cells have many activities associated with xenobiotic phase I and phase II metabolism lacking in other liver cell lines. The objective of this study was to characterize phase I and phase II metabolic functions of a PICM-19 derivative cell line, PICM-19H. The PICM-19H cell line was compared to the tumor-derived human HepG2 C3A cell line and to primary cultures of adult porcine hepatocytes (APH). PICM-19H, HepG2 C3A, and APH were induced for 1 h with a variety of agents. Cells were assayed with fluorescent substrates to quantify cytochrome P450 1A2 and 3A4 activities. Relative to APH, PICM-19H cells exhibited 80% and 45%, respectively, of P450 activities, while HepG2 C3A cells exhibited 7% and 0% of those activities. Fluorescent metabolites were extensively conjugated; 52% and 96% of P450 1A2 and 3A4 metabolites were released from medium samples following treatment with aryl-sulfatase and β-glucuronidase. Rifampicin induction of P450 3A4 activity was also confirmed by the conversion of testosterone to 6β-HOH testosterone as determined by mass spectrometry. A toxicity experiment was performed by treating PICM-19H with either 5 μM 3-MC or vehicle control and then exposing them to various concentrations of aflatoxin B1. PICM-19H cells treated with the CYP1A2 inducer 3-MC had a TC50 of 0.7 μM compared to controls with a TC50 of 20 μM. Therefore, metabolic biotransformation of the aflatoxin B1 toxin by CYP 1A2 greatly enhanced its toxicity. The results demonstrate the positive potential for the use of PICM-19H cells in drug biotransformation and toxicity testing.

2833

**Characterization of Transformed T-lymphocytes Using Herpesvirus saimiri**

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Transformation of human B-lymphocytes with Epstein Barr virus (EBV) is an effective means of producing lymphoblast cell lines in order to secure a renewable source of DNA. However, the 98% efficiency of EBV transformation means that up to 2% of critical samples in individual and small population studies could be lost. Therefore, it is of interest to the scientific community to procure other means of transformation. *Herpesvirus saimiri* (*H. saimiri*), a γ-herpesvirus that is capable of stable transformation of human T-lymphocytes, may offer an alternative method to obtain a transformed cell line for donors not easily transformed by EBV. Therefore, the in vitro characteristics of *H. saimiri*-transformed lymphocytes versus EBV-transformed lymphocytes from the same individual were investigated. Freshly isolated lymphocytes from three individuals were transformed with either EBV or *H. saimiri* and assessed for time to freeze, surface antigen expression and chromosomal stability. The time to freeze (10³ viable cells) for EBV transformed lymphocytes was approximately 5 to 7 weeks post-infection, while *H. saimiri*-transformed lymphocytes required approximately 6-8 weeks incubation time before significant lymphocyte outgrowth was observed. As expected, EBV transformed cell lines were mostly comprised of CD19+ B lymphocytes with a small (< 3%) population of CD3+ T-lymphocytes while *H. saimiri*-transformed cell lines consisted largely of CD8+ T-lymphocytes with a minor subpopulation of CD8+CD4+ T lymphocytes. Cell lines were also analyzed for chromosomal abnormalities by g-banding. It was determined that in two of the three *H. saimiri* cultures there were multiple chromosomal abnormalities detected suggesting that the lines may be somewhat unstable. Only one of the three EBV cell lines from the same individuals contained significant chromosomal abnormalities. Finally, Affymetrix expression arrays are underway to assess expression in EBV and *H. saimiri* cell lines from the same individual.

**Endothelial Cells (2834-2835)**

2834

**Determining the Apoptotic Mechanism in Homocysteine Exposed Human Umbilical Vein Cells**

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Homocysteine (Hcy) is a biologically important amino acid occupying a pivotal position in the metabolism of the essential amino acid, methionine. Elevation of plasma homocysteine (hyperhomocysteinemia; HH) has been implicated as an independent risk factor for cardiovascular disease and is associated with various other diseases and/or clinical conditions including Alzheimer's disease and noninsulin-dependent diabetes. The HH condition
can lead to atherosclerosis (blood vessel hardening and blockage) by damaging to the endothelial cells that line the blood vessels, followed by production of collagen and smooth cell growth. Human umbilical vein cells (HUVECs) serve as an endothelial model cell to study the mechanism of apoptosis caused by Hcy exposure. Apoptosis was analyzed by DNA laddering and western blot techniques, with staurosporine being the positive control. Results indicate that Hcy exposure up to 1mM for up to 72 hours is not sufficient to cause DNA laddering. However, HUVECs exposed to increasing Hcy levels (0.01 mM up to 1 mM) are entering apoptosis as indicated by an increase in cleavage of caspase-3, caspase-9 and PARP proteins. Therefore, the mechanism of Hcy-induced apoptosis appears to be through a mitochondrial-mediated pathway. Future experiments include knocking down genes in the Hcy metabolism pathway in order to investigate the role of these enzymes in apoptosis of endothelial cells. Supported by a FSU West Virginia NASA Space Grant (stipend for Alyssa Childers) and NIH Grant SP20RR016477 to the West Virginia IDEa Network for Biomedical Research Excellence.

2835
C-terminal S-shaped Motif, Structural Determinant of Positive Cooperativity of Human Arginase I
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Arginase catalyzes the hydrolysis of L-arginine to L-ornithine and urea. Mammalian tissues contain the isoenzyme I, highly expressed in the liver and traditionally associated with ureagenesis, and the extrahepatic isoenzyme II, commonly associated to proline and polyamine biosynthesis. The interest in arginase has been renewed by the most recent evidence indicating that, by competing with nitric oxide synthase for L-arginine, both isoforms can effectively regulate NO-dependent processes. As a part of studies addressed to define the structural determinants of substrate specificity, kinetic and regulatory properties, we have examined a C-terminal S-shaped motif, which begins at Phe-304 and it is located at the subunit-subunit interface of arginase I. In contrast with positive cooperativity of wild-type species for L-arginine at pH 7.5, hyperbolic kinetics was exhibited by the monomeric R308A mutant. No significant differences in $K_a$ and $k_{cat}$ values were detected at the optimal pH of 9.5. Both variants were almost equally and competitively inhibited by high concentrations of guanidinium chloride (Gdn$^+$); $K_i$ ~ 60 mM. However, only R308A was activated by low Gdn$^+$ concentrations (< 5 mM). Activation was accompanied by reassociation to the trimeric state and recovery of cooperative properties. No activation was produced by other guanidino compounds, including agmatine (decarboxylated arginine). Deletion of the S-shaped motif (from residue 309 and maintaining Arg-308), yielded a trimeric variant totally insensitive to activation by Gdn$^+$ and exhibiting hyperbolic kinetics. Gdn$^+$ is suggested to mimic the guanidino group of Arg-308 as an intersubunit linkage. The entire S-shaped motif is concluded to represent an structural determinant of the cooperative properties of arginase I. Supported by Grant FONDECYT 1070467

Parasitology (2836-2841)

2836
Participation of Macrophage Membrane Rafts in Trypanosoma cruzi Invasion Process
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The establishment of the infection by Trypanosoma cruzi, the ethiologic agent of Chagas' disease, depends on a series of events involving adhesion of the parasite to surface receptors of the cell, recruitment of additional receptors to the infection-site, a re-organization of the membrane and, in particular, the formation of a parasitophorous vacuole. Distinct microdomains in the plasma membrane are responsible for the invasion of some virus, bacteria and protozoan. Membrane rafts are small and dynamic regions enriched in sphingolipids, cholesterol, ganglioside GM1 and protein markers like flotillins (1 or 2), forming flatter domains, or caveolins (1, 2 or 3), which are characterized as stable flask-shape invaginations. We explored whether membrane rafts participate on the entry of T. cruzi`s trypomastigote into marine macrophages. Transient depletion of macrophage membrane cholesterol with methyl-beta-cyclohexetrin and treatment with filipin caused a reduction of trypomastigote adhesion and internalization. Treatment with crescent concentration of cholera toxin B that binds GM1, demonstrated the ability to inhibit the adhesion and invasion of trypomastigote and amastigote forms. The same wasn’t viewed with epimastigote forms. By immunofluorescence we observed a colocalization of GM1, flotillin 1 and caveolin 1 in T. cruzi parasitophorous vacuole. Together our results suggest that membrane rafts are involved on the process of T. cruzi invasion of macrophages. Supported by CNPq, FAPERJ and CAPES.

2837
Application of Novel Proteomics Techniques for the Identification of Toxoplasma gondii Parasitophorous Vacuole Membrane Proteins
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The obligate intracellular protozoan parasite Toxoplasma gondii resides in the host cell within its replicative niche, the parasitophorous vacuole (PV). This vacuole is delimited from the host cytosol by the PV membrane (PVM) which is modified during infection by parasite proteins. It is across this membrane that nutrients are acquired, signaling between parasite and host cell occurs, and interactions with host cytoskeletal elements take place to support the growing vacuole. Additionally, the PVM is likely the foundation for the cyst wall of the bradyzoite form of the parasite responsible for chronic infection. Despite its importance, the protein complement of the PVM has been very poorly defined. The PVM is formed only in infected cells, comprises a low percentage of total cellular membrane, and cannot be biochemically purified due to intimate host organelle association. This precludes its analysis by modern proteomics technology. We have generated two rabbit polyclonal antibodies against PVM enriched fractions purified from infected rabbit fibroblasts. Using these antibodies as affinity reagents we developed new proteomics sample preparation techniques in order to generate starting material for 2DLC/MS/MS identification of extreme low abundance proteins in the presence of unfavorable detergents, salts, and contaminating proteins. We further used these antisera for the screening of a T. gondii cDNA library to complement our proteomics approach. In addition to 14 known PVM proteins, a total of 63 novel proteins have been identified. These include proteins with enzymatic activities
(including kinases and phosphatases), chaperones, and, surprisingly, sugar modifying enzymes and proteophosphoglycans. The localization of several novel proteins is being corroborated using immunofluorescence assays of tagged proteins. In addition the contribution of these factors to \textit{T. gondii} biology is being assessed by the generation of targeted knockouts of PVM-localized proteins. With these studies we open the door to our understanding of this enigmatic organelle.

2838

\textbf{Spinning Disk Confocal Microscopic Studies of Live, Intraerythrocytic Malaria Parasites}

B. Gligorijevic, 1, 2 R. McAllister, 1 J. Urbach, 1 P. D. Roepe 2; 1Anatomy & Structural Biology, Albert Einstein College of Medicine, Bronx, NY; 2Chemistry, Georgetown University, Washington, DC. We have recently customized a Nipkow Spinning Disk Confocal Microscope (SDCM) to acquire data in 3D vs time for live malarial parasites under perfusion. Using SDCM, we can acquire datasets at a high spatio-temporal resolution (20 fps) and analyze the key aspects of parasite subcellular biochemistry on a physiologically meaningful timescale. In the previous work, we reported that chloroquine (CQ) affected the stage progress only post-schizogony, ie in the cell cycle following the administration [Gligorijevic, B., et al. (2006) \textit{Biochemistry} 45, pp 12400-12410]. Further, we demonstrated that digestive vacuoles in CQ-resistant parasites were two-fold larger compared to CQ-sensitive parasites while the rates of hemozoin production were equal. [Gligorijevic, B., et al. (2006) \textit{Biochemistry} 45, pp 12411-12423]. This data suggested that the CQ mechanism and CQ resistance cannot be fully explained by the existing models. Now we probe the stage specificity of CQ action and CQ resistance in details. In order to do this, we have developed a new technique for fast nuclei counting within post-segmented schizonts. Using distribution of nuclei numbers in schizonts, in combination with previously developed hemozoin quantification and standard Giemsa counts, we have analyzed an array of different CQ effects depending on the dose, time of exposure, length of exposure and the resistance status of the parasite strain. [Gligorijevic, B., et al. submitted]. We demonstrate that CQ is more toxic to rings and schizonts than previously expected. Surprisingly, we also find that in mutant \textit{pfcr} transfectant lines, level of resistance is unchanged throughout the cell cycle. Our data suggests that CQ action involves more than one mechanism and/or more than one target.

2839

\textbf{Molecular Characterization, Expression, and Functional Analyses of LdNuc: A Novel Class-I Secretory Nuclease from the Human Pathogen \textit{Leishmania donovani}}

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\textit{Leishmania} are protozoan pathogens of humans that reside/multiply as extracellular promastigotes in the gut of their sandfly vectors and as intracellular amastigotes within the phago-lysosomal compartments of infected macrophages. These parasites are obligate purine auxotrophs and therefore must salvage such essential nutrients from their hosts. In that regard we have identified, characterized and epismally-expressed a gene (\textit{LdNuc}) encoding a unique 35 kDa, Class-I “secretory nuclease” from \textit{L. donovani}. Features of the \textit{LdNuc} deduced protein are consistent with those of secretory proteins. Both promastigotes and amastigotes forms of this parasite constitutively expressed \textit{LdNuc} mRNA and secretory nuclease activity. Results of Western blots and immunoprecipitation (IP) assays showed that an anti- \textit{LdNuc} peptide antibody recognized both the native 35 kDa secretory enzyme and the \textit{LdNuc} expressed enzyme. Further, results of coupled IP-enzyme activity assays demonstrated that this parasite secretory enzyme could hydrolyze a variety of synthetic polynucleotides as well as, both ss- and ds-DNAs and RNA. Results of PCR-, Southern-, Western- and zymogram-analyses all indicated that homologs of \textit{LdNuc} are conserved amongst all pathogenic species of \textit{Leishmania}. These observations suggest that this “secretory” nuclease could function away from the parasite, to hydrolyze host-derived nucleic acids to satisfy their essential purine requirements. Thus, the \textit{LdNuc} might play critical role(s) in facilitating the survival, growth and development of these important human pathogens. This study was supported by the Intramural Research Program of the DIR, NIAID, NIH.

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\textbf{Regulation of a Phosphatidylinositol-3 Kinase (PIPK3) and a FYVE Domain Containing Protein (FCP) in Plasmodium falciparum}

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Phosphoinositides (PIs) are potent second messengers, which regulate signaling and trafficking events in most eukaryotic cells. There is little information about the role and metabolism of PIs in the malaria parasite. PIPK3 is the only P-3 kinase homologue present in \textit{P. falciparum}. PIPK3 was found to be a versatile kinase as it phosphorylates PI and PI4P to yield PI3P as well as PI(3,4)P2 respectively. We have also identified a FYVE domain containing protein (FCP) as a target of PIPK3 as it specifically binds PI3P. FCP localizes to endosome-like compartments of mammalian cells in a P-3 kinase dependent manner. Interestingly, it is located exclusively inside the food vacuole of \textit{P. falciparum} trophozoites. The targeting of FCP to the food vacuole is independent of PIPK3 activity as well as brefeldin A (BFA). In contrast, PIPK3 was present inside the food vacuole as well as at compartmentalized locations in the parasite and erythrocyte cytoplasm. Treatment of parasites with BFA resulted in blockade of PIPK3 transport to the erythrocyte and parasite cytoplasm without affecting its transport to the food vacuole. Our results indicate that PIPK3 generates PI3P inside the food vacuole to regulate the function of proteins like FCP whereas the presence in the host erythrocyte may suggest that it may be involved host signaling or trafficking.

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\textbf{Evaluation of the Role of PfRab7 in Autophagy in Asexual Plasmodium falciparum Malaria Parasites}

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The intraerythrocytic (asexual) stage of \textit{Plasmodium falciparum} causes devastating morbidity and mortality worldwide. During their development, malaria parasites undergo massive intracellular remodeling. In other organisms, this remodeling is partly accomplished by autophagy processes,
which is induced in nutrient-limiting conditions and delivers unnecessary proteins and damaged organelles to lysosomes via double membrane autophagosomes for degradation and recycling of amino acids. In the last decade, 27 autophagy-specific genes and more than 40 genes functioning in other pathways, such as vesicle traffic, were identified as being required for autophagy in yeast and the other eukaryotes. Recently, the small GTPase Rab7, known to be involved in late endosome traffic, was identified as a key player in autophagosome maturation and fusion with lysosomes. We searched the Plasmodium falciparum genome for autophagy-related genes and found mainly autophagosome-expansion genes (Atg3, 7, 8) and fusion machinery genes (Rab7, Mon1, and Vps11, 16, 18, 33). To determine if P. falciparum parasites have autophagy pathways, infected erythrocytes were incubated in glucose-free medium for 2 h at 37°C and analyzed by electron microscopy. Double membrane structures were observed in cytosol, which are likely to be autophagosomes. Infected erythrocytes were also incubated with aluminum tetrafluoride (AlF₄⁻), a small GTPase activator, for 1 h at 37°C and double membrane structures (~ 500 nm) containing fibrous material were observed directly adjacent to the parasite food vacuole, which is a lysosome equivalent. By immunofluorescence using an anti-PfRab7 antibody, we observed 33.3% of AlF₄-treated parasites (n = 57) had round, punctuated structures associated with PfRab7 in parasite cytosol, whereas 14.3% of non-treated cells (n = 14) showed the same structure. Microarray analysis of AlF₄-treated and non-treated cells was performed and no autophagy related genes were upregulated. Our results suggest AlF₄ keeps Rab7 in the active GTP-bound state, which interferes with autophagosome fusion. Rab7 appears to be essential for autophagosome fusion with the parasite food vacuole.

## Cancer (2842-2863)

### 2842

**Caspase-dependent Cleavage Disrupts the ERK Scaffolding Function of KSR1**

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Kinase suppressor of Ras 1 (KSR1) is a protein scaffold that facilitates ERK cascade activation at the plasma membrane, a critical step in the signal transduction process that allows cells to respond to survival, proliferative, and differentiative cues. Here, we report that KSR1 undergoes caspase-dependent cleavage in apoptotic cells and that cleavage destroys the ERK scaffolding activity of the full-length KSR1 protein and generates a stable C-terminal fragment (CTF) that can function as a dominant inhibitor of ERK signaling. KSR1 is cleaved in response to multiple apoptotic stimuli and cleavage of endogenous KSR1 occurs during the involution of mouse mammary tissues, an in vivo apoptotic system. In addition, we find that in comparison to KSR1-/- MEFs expressing WT-KSR1, cells expressing a cleavage-resistant KSR1 protein (DEVA-KSR1) exhibit reduced apoptotic signaling in response to TNFα/CHX treatment. The effect of DEVA-KSR1 expression was found to correlate with increased levels of activated phosphoERK and could be significantly reversed by treating cells with the MEK inhibitor U0126. In contrast, reduced phosphoERK levels and enhanced apoptotic signaling were observed in cells constitutively expressing the C-terminal KSR1 fragment (CTF-KSR1). Moreover, we find that cleavage of WT-KSR1 correlates with a dramatic reduction in activated phosphoERK levels. These findings identify KSR1 as pro-survival/anti-apoptotic signaling molecule targeted by caspases during apoptosis and indicate that KSR1 cleavage is another mechanism whereby caspases down-regulate ERK survival signaling to promote cellular apoptosis.

### 2843

**Genome-wide Drug-induced Haploinsufficiency Screening of Fission Yeast for Identification of Hydrazinocurcumin Targets**

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Curcumin and its derivatives possess diverse anti-inflammatory and anticancer properties. Its mechanisms of action include inhibition of several cell signaling pathways effects on angiogenesis and cell to cell adhesion. Hydrazinocurcumin (HC), a synthetic derivative of curcumin, has been reported to inhibit angiogenesis via unknown mechanisms. Understanding the molecular mechanisms of action of HC is important for the development of improved compounds with better pharmacological properties. The genome-wide drug-induced haploinsufficiency screening of heterozygous deletion mutants of Schizosaccharomyces pombe has been applied to identify the mode of action of HC. Treatment of wild-type cells with HC showed growth inhibition in a dose-dependent manner with the inhibitory concentration 50 (IC₅₀) at 2.2 µM. A set of 4,158 heterozygous deletion strains covering 83% genome were screened at concentrations of 2, 3, and 4 µM of HC. The resulting sensitive strains were screened again on solid plates and 4 candidate strains were finally identified. Analysis of the targets of HC has shown that they are categorized two groups: the general transcription, taf4 and mcs2 and septin formation, spin3 and bar adaptor protein. The following experiment identified that the former is essential for growth and showed about 65% growth inhibition in response to HC while the latter which is dispensable in vegetative growth showed about 85% growth inhibition. Moreover, the treatment of HC showed in vivo abnormal septin formation. In conclusion, our results provide the critical clues to understand the basic mechanism of the diverse biological action of curcinoids.

### 2844

**MicroRNAs in TGF-β Signaling**

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Transforming growth factor-β (TGF-β) signaling induces a diversity of cellular responses depending on cell context. It is known that cells of different tissue origins exhibit unique gene expression profiles in response to TGF-β, yet little is understood about the regulatory mechanisms contributing to differential TGF-β-mediated gene expression. MiRNAs are a novel class of small RNAs that post-transcriptionally regulate gene expression. Numerous reports indicate that miRNAs function in various biological processes, including development, cell growth and apoptosis, and cancer. Interestingly, miRNAs also act in signaling pathways. For example, miR-15 and miR-16 mediate body patterning in Xenopus by regulating Nodal protein expression, and miR-7 regulates TGF-β signaling in mouse embryonic stem cells.
signaling. Thus, it is plausible that miRNAs play a role in TGF-β signaling. To investigate this idea, experiments were conducted in LIM1863 cells (human colon carcinoma), which respond to TGF-β with epithelial-to-mesenchymal transition (EMT) in about 6 days. Total RNA was isolated from LIM1863 cells pre- and post-TGF-β stimulation and profiled for miRNA expression via microarray. Microarray profiling revealed that TGF-β differentially modulated miRNA expression between non-EMT (1 day TGF-β) and EMT (6 days TGF-β) phenotypes. We performed additional studies for 1 miRNA that both microarray and northerns showed to be upregulated ~2-fold after 6 days of TGF-β stimulation. TGF-β did not alter candidate miRNA expression in other TGF-β-responsive cell lines. This suggests that TGF-β modulates miRNA expression in a cell-type-specific manner. Next, we explored if TGF-β transcriptionally regulated the candidate miRNA. Indeed, quantitative RT-PCR (qRT-PCR) demonstrated that within ~2 hours, TGF-β upregulated the primary transcript of the candidate miRNA ~2-fold, illustrating that miRNA genes can be TGF-β transcriptional targets. Together, these findings suggest a role for miRNAs in TGF-β-mediated EMT. Additional investigation to elucidate the functions of the candidate miRNA in TGF-β-mediated EMT may provide valuable information about how miRNAs regulate TGF-β signaling in cancer.

2845

Stable Association between a Ubiquitin Ligase and a Multi-functional Nuclear Protein
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Msp58 (microspherule protein 58 kDa) is evolutionarily conserved from fly to human and has been shown to play roles in various nuclear processes, including transcriptional regulation and miRNA export. Over-expression of Msp58 has been shown to induce cell transformation. The precise mechanism by which Msp58 carries out its normal nuclear functions is not known, nor is it understood why aberrant expression of Msp58 leads to cellular transformation. To elucidate the molecular mechanism of Msp58’s functions, we employed co-immunoprecipitation assays to isolate Msp58 associated proteins. One of the proteins identified by tandem mass-spectrometry is E3 ubiquitin ligase EDD (E3 identified by differential display). EDD, a member of the HECT (homologous to E6-AP carboxy-terminus) domain containing E3 family, has been shown to play a critical role in cell proliferation and differentiation. Our in vitro binding results demonstrated that Msp58 directly interacts with EDD. Furthermore, using deletion analysis, we have mapped the region of EDD that interacts with Msp58. Currently we are investigating the biological function of Msp58/EDD complex.

2846

Effects of Chitosan Treatment on Cell Proliferation, Morphology, and the Expression of TGF-β1 in CaOV-3 Cells
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Notwithstanding new technologies in treatment, there has been little change in the survival rate of ovarian cancer over the past 15 years. Therefore, novel treatments of this fatal disease are currently being investigated. Previous studies have shown that chitosan significantly lower the proliferation of cells. Chitosan is a derivative of chitin and is a polymer consisting of repeating units with a molecular weight of 348. Chitosan is known to be biodegradable and thus non-toxic and is therefore tissue compatible. A number of other factors, such as Transforming Growth Factor-beta (TGF-β1), has been implicated in influencing the incidence of ovarian cancer. TGF-β1 is a secreted protein that performs many cellular functions, including the control of cell proliferation, cell differentiation and apoptosis. The objective of this study was to evaluate the effect of chitosan on 1) proliferation, 2) the morphology of the CaOV-3 cell, and 3) the expression of TGF-β1 in CaOV-3, an ovarian cancer cell line. We hypothesized that chitosan will decrease the expression of TGF-β1, decrease CaOV-3 cell proliferation and change the morphology of the cells in a dose dependent manner. CaOV-3 cells were cultured and treated over a period of four days with 100, 250, 500 and 1000 µg/ml of chitosan in duplicate. A drastic decrease in cell proliferation was observed in all chitosan treated wells in a dose dependent manner. Chitosan-treated cells exhibited major differences in morphology compared to control. The expression of TGF-β1 in chitosan-treated CaOV-3 cells increased in a dose-dependent manner, except for the 100 µg dose, which induced the highest TGF-β1 expression. These data suggest that treatment with chitosan may alter the expression pattern of TGF-β1 in CaOV-3 cells and may thus promote the activities of TGF-β1 in ovarian cancer. Furthermore, chitosan may be considered as an alternative ovarian cancer treatment.

2847

Increase of ROK Activity and IRS-1 Serine Phosphorylation in Uterine Leiomyoma
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Uterine leiomyomas are common, benign, smooth muscle tumors of the uterus. IRSs (insulin receptor substrates) have been suggested to be involved in development of uterine leiomyoma. Rho/rho-kinase is a mediator of insulin signaling via interaction with IRS-1. And growth factors such as insulin and IGF (insulin like growth factor)-1 are thought to be involved in the leiomyoma. On myogenic process, IRS-1 serine phosphorylation drives to muscle proliferation and IRS-1 tyrosine phosphorylation proceed to muscle differentiation. Therefore, we investigated the level of phosphorylation of IRS-1 and ROK (rho-associated kinase) activity in leiomyoma and myometrium. Serine and tyrosine phosphorylation of IRS-1 were also examined. Normal myometrium and leiomyoma tissues were collected in paired 55 patients underwent hysterectomy with leiomyoma from Kyunghee university hospital. The ROK activity was assessed by kinase assay and western blotting by phospho-MYPT1 (effector of ROK), MYPT1, and ROK2 antibodies. IRS-1 antibody was used for immunoprecipitation before phospho-tyrosine (PY20), phospho-serine antibodies were used for western blotting. The activity of ROK was more increased in leiomyoma than normal myometrium in more than 70% of 55 paired samples. The increased serine phosphorylation and decreased tyrosine phosphorylation of IRS-1 was shown in leiomyoma. But, the increased tyrosine phosphorylation of IRS-1 was observed in the myometrium. In conclusion, the increase patterns of the ROK activity and serine phosphorylation of IRS-1 were demonstrated in leiomyoma. The present findings suggest that the increased serine phosphorylation by activation of Rho-ROK mediated...
signaling pathway may be involved in the leiomyoma development and inhibition of this pathway will be a novel therapeutic strategy for the treatment of leiomyoma.

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Depletion of ARMS Enhances Hydrogen Peroxidase-induced Apoptotic and Autophagic Cell Death in Malignant Melanoma Cells
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Alteration in programmed cell death pathway, apoptotic or autophagic cell death, is tumorigenic. ARMS (ankyrin repeat-rich membrane spanning), also known as Kidins220 (kinase D-interacting substrate of 220kD), is a transmembrane protein functioning in neurotrophin-mediated MEK/ERK pathway. Previously, we have shown that overexpressed ARMS confers growth advantage onto malignant melanoma. To investigate the role of ARMS in the tumorigenesis of melanoma under oxidative stress, we established siRNA-mediated ARMS-knockdown/B16-F0 stable cells and compare its growth characteristics with the control. We found that depletion of ARMS significantly increased H2O2-induced apoptotic cell death, as revealed by DNA fragmentation assay and flow cytometry. In addition, knockdown of ARMS in B16-F0 melanoma cell lines enhanced melanoma cell autophagy, as evidenced by autophagic vacuolization examined by electron microscopy, punctate translocation of GFP-LC3 (microtubule-associated protein light chain 3) by immunofluorescence, and increased LC3-II/LC3-I proportion by Western blotting. Moreover, hydrogen peroxide-induced extracellular signal-regulated kinase activation is compromised in ARMS-knockdown/B16-F0 melanoma cells, suggesting the involvement of MAPK pathway in ARMS-mediated melanoma cell death. We conclude that, through negatively regulating oxidative stress-induced apoptotic and non-apoptotic autophagic cell death, overexpression of ARMS may promote melanoma formation.

2849
Potential Cathepsin B Binding Partners
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Invasive properties of tumor cells depend on the activity of proteases in the tumor microenvironment. One of these proteases may be the cysteine protease, cathepsin B, a lysosomal protease that is secreted and becomes associated with the tumor cell membrane. Potential mechanisms for secretion and membrane association include binding partners that traffic cathepsin B to, or localize it on, the cell surface such as S100A10 (p11), the light chain of the annexin II tetramer, and DP1, the mammalian homologue of yeast Yop1p, a Rab interacting protein localized to Golgi in yeast. Cancer cells secrete cathepsin B, yet the distribution of cathepsin B pericellularly differs among cancer cells. We have shown that procathepsin B interacts with S100A10 in vitro and in situ on the tumor cell surface. Cathepsin B and S100A10 and annexin II are present in Versene wash fractions, but cathepsin B is also present in control wash fractions. Our data suggest that S100A10 serves as one mechanism for association of cathepsin B with the tumor cell membrane. We hypothesize that DP1 and perhaps other REEP family members are involved in the transport of cathepsin B to the tumor cell surface. REEPs, Receptor Expression Enhancing Proteins, are an evolutionary conserved family of proteins that are essential components of the rough ER. Our results show that increases in invasive tumor cells in the expression of REEP5/DP1 correlate with increases in cathepsin B expression. Furthermore, in double transfection studies utilizing REEP5/DP1-mRFP and cathepsin B-mGFP fusion proteins, we have observed colocalization of cathepsin B and REEP5/DP1 in Golgi and early and late endosomal compartments in the tumor cells. On this basis, we propose that the interaction of members of the REEP/DP1 family with cathepsin B leads to its altered localization in cancer.

2850
Disruption of Peripheral Circadian Rhythm Genes Initiates Breast Tumorigenesis
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Exposure to artificial light correlates with higher incidence of breast cancer. Shift workers, whose day/night rhythms are altered by their odd hours, appear more prone to develop breast cancer. In response to natural light, a master clock in our brain regulates molecular clocks in cells of the peripheral tissues, triggering clock-regulated genes that govern fundamental cellular functions. Critical clock genes - the period genes PER1, PER2, and PER3 - were found to be deregulated in breast cancer. It is currently unknown whether disruption of the peripheral clock in human breast epithelial cells leads to transformation. By using a modified serum shock protocol, we entrained human untransformed breast epithelial cells in vitro and found that a few key clock genes, including the PER genes, are indeed transcribed in a rhythmic fashion in untransformed but not in transformed breast epithelial cells. For this reason, we tested whether disruption of one of the key clock genes, PER2, can induce breast epithelial transformation in vitro. Stable knock down of PER2 in human untransformed breast epithelial cells by RNA interference leads to three-dimensional (3D) morphological phenotypes that recapitulate the changes observed in early breast tumorigenesis. Thus, disruption of peripheral circadian rhythm genes initiates breast tumorigenesis. This work has been supported by US Army DOD Award W81XWH0610657

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Altered Dynamic Behavior of the JAK2-STAT5 Signaling Pathway by STAT5 Overexpression
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In a wide array of human malignancies the JAK-STAT signaling cascade is constitutively activated. Core components of this signaling pathway are a tyrosine kinase of the Janus kinase family (JAK) and a latent transcription factor of the signal transducers and activators of transcription (STAT) family. Although the components of JAK-STAT signaling have been studied in detail very little is known regarding their dynamic interplay regulating biological functions. By data-based mathematical modeling of the core module of JAK2-STAT5 signaling (Swameye et al., 2003, PNAS), we could previously show that STAT5 undergoes rapid nucleocytoplasmic cycling with a nuclear residence time of approximately seven minutes and identified that steps from nuclear import to nuclear export of STAT5 are most sensitive to perturbation. However, the details of kinetic parameters
characteristic for nuclear import, export and retention remained unresolved. Therefore, we combined biochemical studies with live cell imaging to extend the model of JAK2-STAT5 signaling. Quantitative time lapse microscopy and photobleaching assays were employed to generate data on spatial dynamics of STAT5 labeled with green fluorescent protein (GFP) over time. For the expression of STAT5-GFP a tightly regulatable Tet-on expression system was developed. We show that at low expression levels, this allows to avoid artefacts caused by changes in the stoichiometry of signaling molecules whereas upon overexpression the dynamic behavior of STAT5 activation is critically altered. By time-lapse microscopy and fluorescence recovery after photobleaching (FRAP), import and export kinetics could be determined under various conditions. These studies provide a more detailed understanding of the consequences of constitutive activation of the JAK-STAT signaling cascade and thereby could facilitate the identification of novel therapeutic targets.

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Capsaicin Induces Synergistic Apoptosis of Cisplatin-resistant Stomach Cancer Cell with Cisplatin by Blocking Aurora-A-mediated Signal Pathway
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Combined cancer therapy, such as trimodality treatment, has been extensively investigated to enhance the antitumor activity of cisplatin. In this study, we investigated that capsaicin induces apoptotic death of cisplatin-resistant stomach cancer cell by MTT assay and apoptotic cell staining. Capsaicin or cisplatin alone in nontoxic doses did not give damage on growth of these cells. However, the combination treatment resulted in a synergistic effect. Recent study showed that down-regulation of NF-kB via Aurora-A depletion enhanced cisplatin-dependent apoptosis of cancer cells. In our study, western blot analysis and qPCR analysis indicated that Aurora-A was down-regulated in tumor cells treated with the combination of capsaicin with cisplatin. Our findings suggest a novel anticancer strategy that capsaicin increases cytotoxicity of cisplatin on human stomach cancer cell by inhibiting Aurora-A mediated signal pathway.

2853

Induction of Nuclear Autophagy by a New Anti-Inflammatory Compound Is Coupled to HIF-1α Degradation
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Many tumors possess an increased inducible nitric oxide synthase (iNOS) synthesis and activity. In breast cancers, such increase can be associated to poor prognosis and metastatic capacity. Interestingly, hypoxic environment stimulates iNOS through recognition of hypoxia responsive elements (HRE) on iNOS promoter by the hypoxia inducible factor 1α (HIF-1α). Therefore, a new and specific inhibitor of iNOS, named CR-3294, was designed and used to study the possibility to induce cell death in cancer cells under hypoxia. In the breast carcinoma cell line MDA-MB-231 hypoxia rapidly increased mRNA levels of iNOS starting at 1 hour. However, 300μM of CR 3294 in 1% O2 drastically reduced the amount of iNOS mRNA. Interestingly, treatment for 1 day or more with CR-3294 in 1% O2 resulted in the accumulation of autophagosomes and eventually in the death of the cells. Induction of autophagy was assessed by the processing of microtubule-associated protein 1 light chain 3 (LC3) from a cytosolic to a membrane bound protein. Interestingly, autophagosome-like vacuoles were present also in the nucleus of treated cells. Pretreatment of the cells with 3-Methyladenine (3MA) and Wortmannin, reduced the number of cells with autophagosomes. We next studied the distribution of HIF-1α. By immunofluorescence, we observed that there was a co-localization between LC3 and HIF-1α in the nucleus of the cells treated with CR-3294 in hypoxia. Moreover, immunoprecipitation studies revealed an interaction between LC3 and HIF-1α in the nucleus of the cells treated with CR-3294 in hypoxia. In conclusion, we showed that inhibition of iNOS resulted in the activation of autophagy with HIF-1α degradation and inhibition of cell invasion.

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GIF52, Regulated by PAD4 and p53, Is Critical for Cellular Apoptosis
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PAD4 can convert monomethyl-Arg residues in histones to Cit and release methylamine via a previously uncharacterized reaction termed demethylaminlation. Using a PAD4 inhibitor CI-amidine, we identified a novel p53 target gene, called GIF52 here. Expression of GIF52 is induced by inhibiting the PAD4 activity. Chromatin immunoprecipitation assays (ChIP) showed that p53 recruits to the promoter of GIF52. Elevated expression of p53 or DNA damage can induce the expression of GIF52. In addition, ChIP assays indicated that the increase of histone Arg methylation and the decrease of citrullination are associated with transcriptional activation of GIF52. Further, ectopically expressed GIF52 inhibits cell growth and is targeted to the mitochondria, results in cytochrome c release from mitochondria and promotes apoptosis in cancer cells.

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The isolation and structural characterization of new chemical entities from natural sources has been invaluable for the development of innovative therapeutic approaches. For centuries, the heartwood of Guaiacum officinale L. (Zygophylaceae) has been utilized by natives of South America and the Caribbean Islands to treat a variety of ailments including cancer. These applications in traditional medicine led us to believe that Guaiacum may
possess pharmacological properties worthy of investigation. This study evaluates the structure-activity relationship of the diaryltetrahydrofuran-type lignans (Nectandrin B, Isonectandrin B and Machilin I) and the dibenzylbutane lignans (meso-dihydroguaiaretic acid), which we isolated from the heartwood of Guaiacum officinale L. and showed to induce apoptosis in human breast cancer lines (MD-MBA-231). Our studies indicate that partial methylation of the aromatic hydroxyl groups and the presence of the tetrahydrofuran ring, which increases rigidity of the structure, enhance activity. Accordingly, it appears that the open chained dibenzylbutane lignans were less apoptotic. The increased antioxidant properties associated with meso-dihydroguaiaretic acid did not appear to enhance apoptotic activity. Furthermore, the positioning of the hydroxyl and methoxy groups on the aromatic rings seems to be important; however, their activity structure relationship is somewhat ambiguous at this point. In this study we also considered the relevance of stereochemistry for apoptotic activity. Our results show that the (R,S,R,S) and (S,S,R,R) stereoisomers are significantly more active than the (R,S,R,R) stereoisomer. Moreover, it appears that the (R,S,S,R) and (S,S,R,R) stereoisomers inhibit the cell cycle at the S (prereplication) phase whereas the (S,S,R,S) stereoisomer affects the G2/M (premitosis) phase in human breast cancer cell line MD-MBA-231. Suggesting that these compounds may pose effective treatment for a variety of types of human breast cancers. (The study was supported by NIH T37TW00076).

Decursin Protects Kidney Cells from Cisplatin Toxicity by Decreasing Oxidative Stresses

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Major side effects of chemotherapy are frequently shown in the kidneys, because they are the site for filtration, concentration, and excretion of the drugs. Cisplatin is a widely used anticancer drug, which has a major side effect of kidney injury. Our previous studies showed decursin mediated protection of cisplatin-induced nephrotoxicity in rats, which wouldn’t obstruct cisplatin’s anticancer activity. Decursin isolated from the root of Angelica gigas is known to be a PKC activator. This study was conducted to examine whether decursin ameliorates cisplatin-induced cell death and oxidative stress, as characterized by reactive oxygen species (ROS) production, lipid peroxidation, and inhibition of antioxidant activity in renal mesangial cells (RMCs) and primary kidney cells, including proximal tubular cells. We also compared the effects of decursin with a strong antioxidant, N-acetylcysteine (NAC). Cells were maintained as monolayer cultures in DMEM supplemented with FBS. After pretreatment with decursin or NAC, cisplatin was added. Whole cell lysate was collected for immunoblot analysis. For analysis of intracellular ROS levels, the oxidation-sensitive probe, dechlorofluorescin-diacetate (DCF-DA) was used. Malondialdehyde (MDA) level was measured using the thiobarbituric acid reactive substances (TBARS) method. SOD activity was estimated from the ability to remove superoxide anion generated from a mixture of xanthine and xanthine oxidase. Aquaporin-1 antibody was used for characterization of proximal tubular cells. Cisplatin-treated cells showed significant increase in cell death, cellular ROS levels, malondialdehyde (MDA) production, and the activity of antioxidant enzymes, such as SODs. In contrast, pretreatment of cells with decursin prevented cisplatin-induced decrease in cell viability. Moreover, decursin recovered the rise of ROS levels and the induction of SOD-1 protein expression. All together, our data indicate that decursin has a protective effect against cisplatin-induced nephrotoxicity in renal mesangial cells and proximal tubular cells.

Cell Surface Marker Candidates in Brain Tumor

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Cell surface proteins of tumor tissues are believed to have different expression patterns from those of their counterparts of normal tissues. Thus, cell surface proteins are attractive markers for tumor targeting. To identify differentially expressed cell surface markers in brain tumor, we analyzed gene expression profiles of nine human brain tumor cell lines and normal brain tissues by utilizing an oligonucleotide microarray. The array chips were originally fabricated in our laboratory so that the target oligonucleotides are restricted only to cell surface proteins avoiding the effect of alternative splicing. Arrays contained 1809 probes. 1795 human genes coding cell surface proteins, 9 housekeeping genes and 5 exogeneous artificially designed controls, which are sorted in duplicate. Fluorescent intensity from each spot of the array was captured by a scanner with image analysis software. The expression level of each gene was taken as the mean value derived from the two spots. Then, the raw fluorescent intensity of each gene referred to as “relative fluorescent intensity (RFI)”, which represented the expression level of each gene relative to that of the control spot hybridized to the known amount of RNAs with artificially designed sequence and exogeneously supplemented to the total RNA. Gene expression levels were compared to one another by RFI value to identify differentially expressed genes. To evaluate high-throughput screening procedure and analyze the data from microarray, we applied a spherical self-organizing map (sSOM). Based on the sSOM patterns, several genes that were highly expressed in the tumor cell lines were selected as candidates of tumor-specific cell surface markers. As the result, we nominated nine genes whose expression appeared significantly upregulated in all the tumor cell lines to the level higher than those in adult normal brain tissue. These genes were validated by real-time quantitative PCR to be consistent with those from microarray analysis.

Phenotypic Profiling and Analysis of Live Cells Treated with Anti-Mitotic Kinesin-5 Inhibitors

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Kinesin-5 is an attractive drug target for anti-mitotic cancer therapy. Prolonged mitotic arrest due to Kinesin-5 inhibition results in cell death, but the phenotypes that occur, and the precise timing of phenotypic progression and death in different cell lines are unclear. This study profiles and analyzes the phenotypes resulting from Kinesin-5 inhibition in different cancer and normal cell lines. At saturating drug concentration, all cell types arrest equally, but there are significant differences regarding the duration of arrest, the progression to and timing of death and the total extent of death. Importantly, normal diploid cells show less death than most cancer cells, despite having a strong mitotic arrest. For cells that die, they either die during mitotic arrest, or after mitotic slippage and the commitment to death appears to occur during mitotic arrest for most cell types. We also tested...
for relationships between 1) the length of arrest and probability of death and 2) the arrest time and timing of death after mitotic slippage. MCF7 breast cancer and diploid N/TERT1 cells show greater death with longer arrest, but this is not the case for other cell lines. Survival analyses indicate that multiple rates of cell loss occur simultaneously within a total population. Drug reversibility and long-term response after drug removal are also evaluated. Nearly 50% of arrested MCF7 cells at 24 hours of drug treatment recover and divide after drug removal, but 60% of these divisions display chromosome segregation defects that directly contribute to poorer long-term recovery compared to cells treated with drug for 48 hours; normal RPE1 cells do not show the chromosome segregation defects. This study provides important phenotypic and kinetic information of the Kinesin-5 drug response and insights into how different cancer and normal cells respond to these drugs.

2859

Twist Mediates Actin Cytoskeleton Reorganization & Amplifies TGF-β, EGF & Axl Signaling

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Metastasis formation is the leading cause of advanced cancer related deaths and the mechanism of this process is largely unknown. Understanding the molecular regulation of metastasis formation in breast cancer is especially critical, because the survival rates of breast cancer patients dropped from 98% to 26% when they developed distal metastases. A molecule that receives growing attention for its ability to facilitate the invasive properties of breast epithelial cells is Twist. Twist is often over-expressed in advanced cancers and found correlated with cancer metastasis formation. To examine the role of Twist in breast cancer metastasis we developed two Twist over-expressing human breast cell lines, MCF-7 Tw and MCF-10A Tw. Distinct morphological changes are observed in Twist over-expressing cells, featuring mobilization of actin cytoskeleton and formation of filapodia, suggesting a role of Twist in cytoskeletal regulation. Small Rho GTPases studies revealed that Twist over-expressing cells displayed enhanced activation of Cdc42, which indicates a novel mechanism of Twist in cell migration and cancer metastasis. Additionally, we found that the expression levels of TGF-β receptors (TβRI, II & III), EGFR and Axl are up-regulated in Twist over-expressing cells, corresponding to signaling pathways that were previously shown to participate in breast cancer cells invasion and metastasis formation. Further experiments demonstrated that these signaling pathways were hyper-activated when treated with their cognate ligand, suggesting that Twist sensitizes breast cancer cells in response to the extracellular signals that favor cell invasion. We postulate that Twist activates actin cytoskeleton reorganization through a novel mechanism to assist cellular dissemination concomitant with modulation of cell surface receptors expressions, making a cell permissive to extracellular cues in the microenvironment that facilitates movement out from the original tumor bed.

2860

Cross-Reactivity Studies of Human and Mouse Tissues Using Affinity Purified Polyclonal Anti-Human Antibodies

S. L. Djerbi, 1 A. Monazzami, 2 M. Hansson, 1 D. Cerjan, 2 K. Wester, 1 Atlas Antibodies AB, AlbaNova University Center, Stockholm, Sweden, 2Department of Twist of aktin cytoskeleton regulation. Small Rho GTPases studies revealed that Twist over-expressing cells displayed enhanced activation of Cdc42, which indicates a novel mechanism of Twist in cell migration and cancer metastasis. Additionally, we found that the expression levels of TGF-β receptors (TβRI, II & III), EGFR and Axl are up-regulated in Twist over-expressing cells, corresponding to signaling pathways that were previously shown to participate in breast cancer cells invasion and metastasis formation. Further experiments demonstrated that these signaling pathways were hyper-activated when treated with their cognate ligand, suggesting that Twist sensitizes breast cancer cells in response to the extracellular signals that favor cell invasion. We postulate that Twist activates actin cytoskeleton reorganization through a novel mechanism to assist cellular dissemination concomitant with modulation of cell surface receptors expressions, making a cell permissive to extracellular cues in the microenvironment that facilitates movement out from the original tumor bed.

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2861

Gonadotropin Effects on the Secretion and Expression of KLK 5 in SKOV-3 Cells

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Epidemiological evidence strongly suggests that gonadotropin hormones; Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) are important players in ovarian carcinogenesis. However, the possible mechanisms whereby these hormones affect the risk of ovarian cancer are not elucidated. Ovarian cancer is linked to kallikreins (KLK), a family of 15 serine proteases clustered on chromosome 19q13.4. These molecules are hormonally regulated and are implicated as players in many types of cancer. One member of this family, KLK 5, has been implicated in the aggressive progression of ovarian carcinogenesis. Therefore, the objective of this project was to investigate the effect of gonadotropin treatment on the secretion and expression of KLK 5 in an ovarian cancer cell line, SKOV-3. We hypothesized that LH and FSH will upregulate KLK 5 expression and secretion in these cells. To test this hypothesis, SKOV-3 cells were treated with (1) 10µg/mL LH, (2) 100µg/mL LH, (3) 500µg/mL LH, (4) 10µg/mL FSH, (5) 100µg/mL FSH and (6) 500µg/mL FSH for 24 and 48 hours. Culture supernatants were collected daily and the concentration of
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Mitomycin C and the In Vitro Effect on Cell Division

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Mitomycin C (C11H15N2O2) or MMC is known as an antitumor antibiotic agent isolated from Streptomyces lavendulae. Gastrointestinal and breast cancer is usually treated intravenously by this compound. Side effects on lung, kidney and bone marrow are always detected by long-term use. There have been unclear reports on the effect of this compound on cellular level. However, MMC was usually indicated to inhibit DNA synthesis, nuclear division and cell division. This research aimed to investigate the in vitro effect of MMC on viability, cells death, mitotic index and morphology of the cells. The 3T3 cells were exposed to MMC 10 µl/ml on different duration: 15, 30 minute, 1½, 2, 3 and 4 hour. The percentage viability of the 4-hour group, evaluated by trypan blue staining method, was significantly less than the unexposed control cells. The other exposed cell groups non-significantly different from the control. There was non-significant different of necrotic death among the cells of all exposed duration evaluated by the hoechst dyes No.33342 with propidium iodide counter stain, and no apoptotic cells observed. The mitotic index was found significantly greater in the 4-hour group, compared to the untreated control. In agreement with the enhanced GLUT4 translocation activity, the purified compound exhibited an increase in the GLUT4 translocation by this compound are in progress.

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Studies to Determine the Role of Allografts on Devil Facial Tumor Disease (DFTD) Spread

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Sarcophilus harrisii, better known as the Tasmanian devil, is the largest marsupial carnivore. Its geographical distribution is restricted to the island of Tasmania. Since 1996 this species has been affected by a disease known as the Devil Facial Tumor Disease (DFTD), which is characterized by the apparition of necrotic tumors that interfere with their feeding ability. The devil facial tumor disease has decreased populations dramatically, threatening this species with extinction. Studies have shown that Tasmanian devils show low genetic diversity. Studies of tumor cells do not detect the presence of any viruses. The hypothesis that tumor is spread by an allograft states that the devil facial tumor disease is caused by a seed cell line of unknown primary origin. This study focused on the use of microsatellites as a mechanism to test the allograft theory and in sequencing mitochondrial DNA of Tasmanian Devils in order to compare host and tumor tissues. Samples of DNA of affected and non affected Tasmanian devils were collected from tumor cells and non tumor cells (liver and kidney). The samples came from individuals from different sites in Tasmania. Primers which recognize the sequence of microsatellites from devils and quolls, a related marsupial species, were generated. The microsatellites were analyzed with the ABI genotyper. Our results provide evidence supporting the allograft as a transmission mechanism for the DFTD. We were also able to sequence mitochondrial DNA of Tasmanian devils for future studies.

Metabolic Diseases (2864-2872)

2864

An Aliphatic Compound from Bauhinia acuminata Leaves Exhibits GLUT4 Translocation and Glucose Uptake Activity

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GLUT4, a facilitative glucose transporter found in the membrane of muscle and adipose tissue, catalyzes the transport of glucose from the blood to the cells. GLUT4, generally resides in the specific intracellular vesicles and it can be translocated to the cell membrane in an insulin-dependent as well as independent manner. The purpose of the present study was to identify and characterize the modulators of GLUT4 translocation from Bauhinia acuminata, a plant belonging to Leguminosae family. B. acuminata leaves were sequentially extracted with solvents of increasing polarity and the active fraction that stimulated the GLUT4 translocation was purified to homogeneity. Structural elucidation of the purified compound using a combination of spectroscopic techniques such as 1H Nuclear magnetic resonance (NMR), 13C NMR, mass spectrometry and infrared spectroscopy revealed that it is a small aliphatic compound. This compound showed a 3-fold increase in GLUT4 translocation activity in 3T3 L1 adipocytes when compared to the untreated control. In agreement with the enhanced GLUT4 translocation activity, the purified compound exhibited an increase in glucose uptake activity in cell lines of adipocytes and muscle origin. Studies on the molecular mechanisms that mediate increased GLUT4 translocation by this compound are in progress.

2865

Fibronectin Fragmentation in Diabetic Wounds Alters Cell Behavior

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Fibronectin (FN) undergoes fragmentation in periodontal disease and diabetic wounds. This study characterized patterns of FN fragmentation in these diseases and effects of FN fragments on cellular behavior. Polyclonal antibodies were raised against proteolytically cleaved FN and three recombinant FN segments for Western blot detection of fragmentation in exudates from periodontal disease and longstanding leg and foot wounds in healthy and diabetic patients. Fragments were mapped by masses and frequencies from digitized blots. Experiments quantified effects of intact FN or three recombinant FN fragments on cell attachment, migration, and chemotaxis for human gingival fibroblasts (hGF) and HT1080 cells. FN fragmentation was detected in periodontal fluids from 18 patients with 135 and 155 kDa fragments at increased frequencies in both diabetic and healthy patients. Furthermore, 125 and 174 kDa fragments occurred only in diabetic and a 148 kDa fragment only in healthy patients. In 22 diabetic leg and foot wounds, samples included frequently 112, 144, and 182 kDa fragments. Although multiple fragments were present in both periodontal disease and leg wounds, no fragmentation pattern distinguished healthy and diabetic patients. Recombinant FN fragments supported cell attachment ~100-fold less efficient than intact FN. Moreover, hGF had reduced spreading with extension of delicate filopodia on all FN fragments compared to full-length FN, and cells were 10-24% longer (P<0.01) and had 28-39% larger areas (P<0.01) on intact FN than on FN fragments. Migration of hGF on two coated FN fragments decreased by 20-30% (P<0.01) whereas HT1080 migration increased by 15-25%. Treatment with soluble FN fragments differentially inhibited or stimulated on chemotactic migration of HT1080 by up to 50% (P<0.01) compared with intact FN. The experiments demonstrated that FN fragmentation occurs both in periodontal and chronic leg and foot wounds in diabetes and that FN fragments alter cell behavior (Supported by NIH grants DE017139 and DE016312).

2866 Characterization of Inorganic Phosphate Transport in Rat Vascular Smooth Muscle Cells under Calcifying Conditions

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Hyperphosphatemia and inorganic phosphate (Pi) transport by vascular smooth muscle cells (VSMC) have been implicated in the pathogenesis of vascular calcification (VC). The aim of this work has been to characterize Pi transport in VSMC. Primary cultures of VSMC express both high affinity Na-dependent and Na-independent components of Pi transport. Under physiological conditions both transport systems are saturated, show similar activity, and are inhibited by increasing pH. The Na-dependent transport is also weakly inhibited by phosphonoformic acid (PFA) (3.9 mM IC50 at 0.05 mM Pi). Real-time PCR shows that Pit1 and Pit2 are expressed to the same degree, and no other Pi transporters are significantly expressed. When expressed in Xenopus oocytes they are strictly Na-dependent, with high affinities for Pi, and are inhibited by increasing pH, but only weakly inhibited by PFA. We have used RNA interference to demonstrate that Pit1 and Pit2 are the transporters responsible for Na-dependent Pi transport in VSMC. 1 mM PFA prevented VC in VSMC induced with 2 mM Pi through a mechanism that was independent of Pi transport, cytotoxicity or adenylyl-/guanylyl-cyclases inhibition. Instead, PFA also prevented VC in calcifying VSMC lysates. We also studied Pi transport in VSMC under different calcifying conditions: 2 mM Pi and several concentrations of platelet-derived growth factor (PDGF-BB), and tumor necrosis factor (TNF-α). All three agents increased the expression of osteogenic genes (Msx2, Cbfα and Bmp2), but Pi transport was not modified, even if PDGF-BB increased the expression of Pit-1 transporter. PDGF also increased the protein content in VSMC, but DNA and number of cells was unchanged. Taken together, these novel findings suggest new roles of Pi transport in the pathogenesis of VC and have implications as potential future clinical targets.

2867 Pleiotrophic Effects of Diabetic Plasma Autoantibodies on Rat Differentiated Pheochromocytoma PC-12 Cells, Endothelial Cells and Cardiomyocytes: Associations with Rho Kinase, Stress Fiber Activation, and Increased Intracellular Calcium

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Neuropathy and retinopathy are common in diabetes. Previous studies suggested a relationship between endothelial cell binding autoantibodies and retinopathy in type 1, but not in type 2 diabetes. Since endothelial cell inhibitory autoantibodies in cancer sera were described were anti-neurotrophic, we analyzed the mechanism of diabetic plasma protein-A-eluted fractions effects on neurites in PC12 cells, on endothelial cells and cardiomyocytes. Subjects were 14 adults with type 2 diabetes, mean duration 11 yrs, from the Veterans Affairs Diabetes Trial. We now report that autoantibodies from type 2 diabetes blocked neurite outgrowth in bFGF-stimulated PC12 cells. The process is Rho kinase dependent, as evidenced by the finding that a specific Rho kinase inhibitor, Y27632 (1uM) completely restored neurite outgrowth by bFGF (10 ng/mL) in the presence of ‘blocking’ autoantibodies from three different diabetic patients. Inhibition of bFGF-induced neurite outgrowth was dose-dependent (1-20 ug/mL, n=8) and was not exhibited in autoantibodies (n=3) lacking significant inhibitory activity on endothelial cells. The autoantibodies from diabetic retinopathy subjects (maculopathy) caused significant inhibition of endothelial cell growth (72± 20%, n=7) compared to autoantibodies from diabetic subjects without retinopathy (101 ± 8%, n=7, p = 0.004 for the difference). Inhibitory autoantibodies caused endothelial cell retraction from attachment points in extracellular matrix, strong expression of F-actin immunoreactive stress fibers, and dose-dependent increases in intracellular calcium. Effects were substantially reduced upon denaturation by boiling antibodies. Purified inhibitory endothelial autoantibodies (2 ug/mL) interrupted spontaneous calcium oscillation and caused large increases in cytosolic intracellular calcium in HL-1 cardiomyocytes. Control diabetic autoantibodies had much less if any effect on intracellular calcium. These results suggest that potent inhibitory endothelial autoantibodies in plasma from type 2 diabetes with macular edema and/or painful neuropathy may have inhibitory effects in cardiac and neuronal cells. More study can correlate antibodies with severity and specific kinds of diabetic complications.
**Altered Bone Metabolism in Prostacyclin Synthase Deficient Female Mice**

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Bone metabolism is regulated by some hormones, autacoids and cytokines. Among them, prostaclin (PGI2) has critical roles in bone formation and resorption. It has been reported that cyclooxygenase (COX)-2 gene deletion produced negative effects on bone formation by reducing osteoblastogenesis, but no bone abnormality was found on mPGE synthase gene deletion. Therefore, we focused on the effects of PGI2, a product of arachidonic acid by COX and prostacyclin synthase (PGIS) found in osteoblasts, on bone metabolism. We examined bone morphology in multiple trabecular and cortical regions within the distal metaphyseal tibia from 5-week-old- to 8-month-old-PIGISA and PIGISA mice using DEXA, pQCT and micro-CT analysis. Bone marrow cells were obtained from mice for measuring alkaline phosphatase activity, mineralized nodule formation and osteoclast formation. The pQCT time-course analysis shows that PGI2 female mice displayed a decrease in trabecular and cortical density since early age (5 weeks), but increase in trabecular density in adult age (6 and 8 months). Micro-CT analysis in adult age-PIGISA mice also showed the increase in bone volume/tissue volume (BV/TV) of trabecular bone in the metaphysis (mean BV/TV in PGI2−/− = 3.29% and PGI2+/- = 7.18%) and net effect of decrease in total bone mass density. In cell culture experiments, it was observed that PGI2 deficiency led to suppressing osteoblast differentiation, while unaltered TRAP-positive osteoclast formation. Conversely, this phenotype was not observed in PGI2-/- male mice. In conclusion, PGI2-/- female mice displayed overall reduction in the levels of parameters in bone mass via suppression of osteoblast differentiation. However, they were associated with regional trabecular bone increase at the adult age. These data indicate that PGI2 contributes a role in maintaining normal bone mass and micro-architecture in mice. This is a first report that PGI2 is involved in bone metabolism in vivo.

**The Role of P2Y1 and P2Y6 Receptors in Ca2+ Mobilization and Insulin Secretion in Mouse Pancreatic Islets**

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Subtypes of purinergic receptors involved in modulation of cytoplasmic calcium ion concentration ([Ca2+]i) and insulin release in mouse pancreatic β-cells were examined in two systems: pancreatic islets in primary culture and Beta-TC6 insulinoma cells. Some physiological responses occurred in both, such as acetylcholine-stimulated [Ca2+]i rise via cytoplasmic Ca2+ mobilization. Addition of ATP, ADP and 2-MeSADP (each 100 µM) transiently increased [Ca2+]i, in single islets in culture at 5.5 mM (normal) glucose. The potent P2Y1 receptor agonist 2-MeSADP reduced insulin secretion significantly in cultured islets at high glucose (16.7 mM), with a slight stimulation at normal glucose. The selective P2Y6 receptor agonist UDP (200 µM) transiently increased [Ca2+]i and reduced insulin secretion at high glucose, whereas the P2Y2/4 receptor agonist UDP (200 µM) transiently increased [Ca2+]i and reduced insulin secretion at high glucose, whereas the P2Y2/4 receptor agonist UDP was inactive. [Ca2+]i transients induced by 2-MeSADP and UDP were antagonized by suramin (100 µM), U73122 (2 µM, PLC inhibitor), and 2-APB (100 µM, IP3 receptor antagonist), but not by staurosporine (1 µM, PKC inhibitor) or Ca2+ chelation by EGTA (100 µM). The effect of 2-MeSADP on [Ca2+]i was also significantly inhibited by MRS2500, a P2Y1 receptor antagonist. Thus, P2Y1 and P2Y6 receptor subtypes are involved in insulin release in mouse islets and Ca2+ is mobilized from intracellular stores. In Beta-TC6 cells, ATP, ADP, 2-MeSADP and UDP transiently elevated [Ca2+]i, and slightly decreased insulin secretion at normal glucose, while UDP and NECA were inactive. RT-PCR analysis detected mRNAs of P2Y1 and P2Y6 receptors in mouse pancreatic β-cells.

**Chemokine Gene Expression Profile of Peritoneal Adipose Tissue in KKAy Diabetic/Obese Mouse**

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Increasing evidences suggest that chemokines involve in adipose tissue inflammation and systemic insulin resistance. Chemokines are important mediators characterized by the ability to induce migration of different cell types to sites of inflammation through the action on specific receptors. In this study, we examined the chemokines and their receptors gene expression profile from in KKAy mice, which is a diabetic/obese animal model, treated with high fat diet (HFD). Microarray and qPCR analysis showed that HFD increased significantly CC119, CCL 25, CCR6, and CCR7 mRNAs in the KKAy. When four different adipose tissues, such as mesenteric (ME), subcutaneous(SE), epididymal(E), and renal(RE), were examined from HFD-induced diabetic/obesity mice, HFD upregulated CCR6 and CCR7 expression in ME tissue. Since enhanced expression of such molecules likely have been known to contributes to the maintenance of inflammation in chronic inflammatory disease, our data suggest that the up-regulated CCR6 and CCR7 may have specific functions for gut adipocyte inflammation in the obese/diabetic mice.

**Effect of Neuronatin on Insulin Secretion and High Glucose-induced Apoptosis in Pancreatic β-cells**

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Neuronatin (NNAT) was initially identified as a selectively-expressed gene in neonatal brains, and may be involved in neuronal cell differentiation. Expression of NNAT has been also identified in pancreatic β-cells, but its functions are largely unknown. To investigated the possible functions that NNAT may serve in pancreatic β-cells, two NNAT isoforms (α and β) were expressed using adenoviruses in murine MIN6N8 pancreatic β-cells, and the cellular fates and the effects of NNAT on insulin secretion, high glucose-induced apoptosis, and functional impairment were examined. NNATα and NNATβ were primarily localized in the endoplasmic reticulum (ER), and their expression increased insulin secretion by increasing intracellular
calcium levels. However, under diabetes-like conditions, whereby glucose levels are chronically elevated, the NNATβ to NNATα ratio gradually increased in proportion to the length of exposure to high glucose levels. Moreover, adenovirally-expressed NNATβ were inclined to form aggresome-like structures, and we found that NNATβ aggregation inhibited the function of the proteasome. Therefore, when glucose is elevated, the expression of NNATβ sensitizes MIN6N8 cells to high glucose stress, which in turn, causes ER stress. As a result, expression of NNATβ increased hyperglycemia-induced apoptosis via transcriptional induction of CHOP/GADD153, activation of c-JUN NH2-terminal kinase (JNK), and activation of caspase-12. In addition, the expression of NNATβ under high glucose conditions decreased the expression of genes important for β-cell function, such as glucokinase (GCK), pancreas duodenum homeobox-1 (PDX-1), and insulin. Collectively, NNAT may play a critical factor in normal β-cell function, as well as in the pathogenesis of type 2 diabetes.

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Effects of BMP-7 on Oxidative Stress and the Expression of TGFβ in Glucose-treated Mesangial Cells

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Glucose is believed to induce oxidative stress, increase transforming growth factor (TGF-β) expression, and promote apoptosis in renal cells for leading to diabetic nephropathy. Bone morphogenetic protein-7 (BMP-7) is mentioned to counteract TGF-β in kidney and reverse chronic renal injury. But the relation of TGFβ in the actions of BMP-7 including the prevention of glucose-induced ROS and apoptosis is not clear. Thus, we employed mesangial cells to investigate the influence of BMP-7 in glucose-added medium. We observed that mesangial cells became apoptosis by high glucose (25 mM) but to be the pro-apoptotic stage at medium-high glucose (15 mM). Both glucose treatments at 25 mM and 15 mM increased the expressions of TGFβ and extracellular matrix (ECM), collagen IV but lowered BMP-7 expression in mesangial cells. Tiron, one of the ROS scavengers, decreased the expressions of TGFβ and collagen IV but increased BMP-7 expression in 15 mM glucose-treated mesangial cells. However, tiron produced less effect in 25 mM glucose-treated mesangial cells. Either TGFβ or H2O2 inhibited BMP-7 expression in mesangial cells. Also, BMP-7 showed the direct decrease for the formation of ROS, and/or the expressions of TGFβ and collagen IV in 15 mM glucose-treated mesangial cells. Thus, we suggest that mesangial cell receiving glucose-treatment at 15 mM is under pro-apoptotic stage which seems suitable to be an in vitro model mimicking the early stage of diabetic nephropathy. BMP-7 showed a protective role in 15 mM glucose-treated mesangial cells due to the lower of oxidative stress and the decrease of TGFβ expression and ECM expansion. BMP-7 will be helpful as a therapeutic strategy to handle the early diabetic nephropathy.

Neuronal Diseases (2873-2886)

2873

cDNAs Which Protect PC12 Cells against Mutant Huntington

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Huntington’s disease is a neurodegenerative disorder caused by the expression of polyglutamine-expanded forms of huntingtin in CNS neurons. To identify genes whose expression can provide protection against the mutant huntingtin protein, mouse cDNA’s were introduced in a PC12-Q103 cell line which can be induced to express the toxic exon I of huntingtin with a Q103 repeat (Aiken et al.). This was achieved by infecting the cells with a retroviral cDNA library (derived from mouse testis) at low multiplicity before induction. Expression is under control of an edcsyne-regulated promoter and can therefore be induced by tebufenozide. The PC12-Q103 cells were taken through repeated cycles of selection by addition of tebufenozide until a majority of the cells died, followed by return to growth medium. Cells were harvested after 1-7 iterations of this cycle +/- induction. Both naïve non-transduced cells and cells which had been cycled without induction were highly sensitive to induction. By contrast, clones of cells which had been repeatedly challenged tolerated continuous culture with tebufenozide and continued to synthesize huntingtin, as judged by induction. Both naïve non-transduced cells and cells which had been induced to express the toxic exon I of huntingtin with a Q103 repeat (Aiken et al.). This was achieved by infecting the cells with a retroviral cDNA library (derived from mouse testis) at low multiplicity before induction. Expression is under control of an edcsyne-regulated promoter and can therefore be induced by tebufenozide. The PC12-Q103 cells were taken through repeated cycles of selection by addition of tebufenozide until a majority of the cells died, followed by return to growth medium. Cells were harvested after 1-7 iterations of this cycle +/- induction. Both naïve non-transduced cells and cells which had been cycled without induction were highly sensitive to induction. By contrast, clones of cells which had been repeatedly challenged tolerated continuous culture with tebufenozide and continued to synthesize huntingtin, as judged by the presence of GFP-positive inclusions. Since this strategy selects resistant cells it should allow identification of protective cDNAs Supported by the HiQ foundation.

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Podosome Loss and Impaired Phagocytosis in Primary Macrophages from an Alzheimer's Disease Patient

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Accumulation of fibrillar, amyloid beta (Aβ) plaques in the brain of patients with Alzheimer’s disease (AD) may be due in part to an inability of the innate immune system to clear the toxic protein deposits. Consistent with this, primary macrophages from AD patients have a reduced ability to internalize (Aβ)1-42 peptide compared with macrophages derived from age-matched control subjects. To investigate the basis of this internalization defect, we used live cell imaging approaches to examine the phagocytic pathway and the actin cytoskeleton of primary human macrophages from an AD patient and from an age-matched control subject. The ability of the macrophages to undergo phagocytosis was analyzed by testing whether they could internalize 2 μm beads coated either with Aβ1-42 peptide or rabbit IgG. Control macrophages effectively bound and internalized both types of beads, whereas AD macrophages showed lower affinity for the beads and an inability to internalize them. Fluorescently-labeled phalloidin was next used to examine actin organization within these cells. Whereas total F-actin staining was similar in the control and AD macrophages, a significant difference in the shape and distribution of F-actin was observed. AD macrophages displayed an overall rounded shape with less lamellipodia compared to the control cells. Most notably, AD macrophages lacked podosomes. These actin-rich, adhesive structures have been implicated in cell migration and invasion, and are a characteristic feature of macrophages. We discuss how podosome loss and impaired phagocytosis within AD macrophages could prevent these cells from removing toxic, fibrillar Aβ deposits in the brains of AD patients.
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Protective Effect of Decursin on Chronic Restraint Stress-induced Behavioral Changes and Dopaminergic Neurotransmission Alterations in the Nigrostriatal System of C57BL/6J Mouse

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The regulation of dopamine levels has been suggested as a potential mechanism of anxiety-like behavior of chronically stressed animals. In this study, we investigated whether the administration of a chemical agent inhibits stress-mediated impairments in nigrostriatal systems associated with dopaminergic neurotransmissions of stressed mice. Specifically, we analyzed sympathetic markers by western blot and immunochemistry, neuromelanin by Schmorl’s reaction and Masson-Fontana staining, neurodegeneration by Fluoro-Jade C staining, and oxidative damage by determination of superoxide dismutase activity and lipid peroxidation. Here, for the first time, it was observed that daily administration of decursin strongly suppressed the behavioral change in chronically stressed mouse and significantly alleviated the decrease of dopamine transporter in the substantia nigra region. At the same time, the levels of neuromelanin, neurodegeneration and oxidative damage were significantly decreased by decursin. Our experimental results indicate that decursin be can used as a protecting agent against the induction of anxiety and depression caused by chronic stress.

2876

Functional Characterization of Chorein, the Protein Altered in Chorea-Acanthocytosis, and Its Homologous Proteins

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Chorein, encoded by the VPS13A gene, is altered in Chorea-acanthocytosis (ChAc), a rare neurodegenerative disorder whose neurological findings resemble those found in Huntington’s disease. Chorein belongs to the VPS13 protein family, which includes also VPS13B, C and D proteins. VPS13B gene, also known as COH1, is mutated in Cohen syndrome. Their yeast homologous, Vps13p has been shown to be involved in the trafficking of several proteins between the trans-Golgi network and the prevacuolar compartment. We have studied the localisation of these proteins in transfected cell lines. They seem to be soluble cytoplasmic proteins that interact with membranes. A characteristic vesicular-like pattern of sub-cellular distribution is easily detected in cells expressing chorein; similar structures can also be detected for VPS13B, C or D but at a much more lower rate. Most of the mutations detected in VPS13A and VPS13B genes lead to frameshift or stop codons that produce shorter proteins or no protein at all. Interestingly, several disease missense mutations, important from the functional point, have also been reported. We have introduced these missense mutations in expression plasmids and compared the sub-cellular distribution of mutant and wild-type proteins. The characteristic vesicular-like pattern of chorein is altered in several of the mutant proteins suggesting that this pattern reflects a functionally important localisation. We have used an immunoprecipitation approach to check if the VPS13 proteins form homo- or heterodimers (or multimers) as it was reported for their yeast homologue, Vps13p. No interactions were detected, suggesting that these proteins do not form such protein complexes.

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Manganese Disruption of Mitochondrial Respiration in the Bivalve Crassostrea virginica and Its Protection by p-Aminosalicylic Acid

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Manganese is an essential metal that at excessive levels in the brain produces extrapyramidal symptoms called Manganism which is similar to Parkinsons disease. The mechanism of action of manganese is not completely understood but is thought to be due to factors including decreased brain dopamine levels, altered dopamine receptor activity and/or oxidative stress in mitochondria. p-Aminosalicylic acid (PAS) is a drug which recently is being shown to alleviate symptoms of Manganism. We studied the effects of manganese and PAS on mitochondrial respiration in gill of the bivalve mollusc, Crassostrea virginica. C. virginica gill is a tissue which is innervated by dopaminergic neurons. Mitochondrial respiration was measured using a YSI Micro-Biological Oxygen Monitor with a micro-batch chamber. Additions of manganese (0.1 - 10 mM) caused dose dependent decreases in mitochondrial O2 consumption. Adding PAS (0.1, 1 mM) prior to manganese additions protected the mitochondria. The study demonstrates that manganese does adversely affect mitochondrial respiration and that the protective actions of PAS may in part be due to its ability to shield mitochondria from manganese induced oxidative stress. This work was supported in part by grants 2R25GM06003-05 of the Bridge Program of NIGMS, 0516041071 of NYSDOE, 0622197 of the DUE Program of NSF and 67876-0036 of PSC-CUNY.

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Protein Misfolding and Neuronal Toxicity: The Case of Familial Amyotrophic Lateral Sclerosis

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Protein misfolding has been linked to neurodegenerative diseases as a consequence of mutations. A common feature of these disorders is the intracellular accumulation of these proteins, segregated into aggregates, probably exerting toxic effect on neuronal cells. For example, the aggregates may block axonal transport and mitochondria trafficking, or may sequester proteins essential for cell viability and impair the Ubiquitin-Proteasome-Pathway function. Amyotrophic lateral sclerosis is a motor neuronal disorder characterized by selective loss of upper and lower motor neurons. Some familial forms are associated to point mutations in Cu/Zn-Superoxide dismutase (SOD1), one of the major antioxidant enzymes. In this study we have analyzed the mechanisms involved in SOD1 misfolding and neurotoxicity. We have obtained a cellular model of fALS transfecting immortalized motor neurons (NSC34) with plasmids coding for wild type or mutants human SOD1. We found that wt SOD1 is diffused in cytoplasm and in nuclei, mutant SOD1 is mainly present in the cytoplasm and excluded from the nuclear compartment, except when present in aggregated forms. We have then produced chimeric proteins of wt or mutant SOD1, fused with YFP, carrying a nuclear exporting signal (NES) or a nuclear localization signal (NLS), and confirmed nuclear exclusion for mutant SOD1. Using YFPu-reporter plasmid for proteasome activity, we found impaired proteasome functions in the cytoplasmic compartment for cells expressing mutant SOD1. The effects of reduced levels of mutant SOD1 in
the nuclear compartment was investigated, by evaluating oxidative damage on DNA using COMET assay. We found increased DNA fragmentation, under oxidative stress induced with H2O2 treatment, in cells expressing mutant SOD1, compared to wt SOD1. The data suggest that nucleus may be a target of SOD1 neurotoxicity in fALS, arising from initial misfolding (gain-of-function), generating nuclear deprivation of the enzyme (loss-of-function in the nuclei), a process that may be involved in ALS pathogenesis.

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**SMN 1, SMN 2, and NAIP Deletions and Severity Spinal Muscular Atrophy (SMA) in the Puerto Rican Population**
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Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disease characterized by degeneration and death of cells in the spinal cord. SMA is classified into four groups based in age of onset. Type I (Werdnig-Hoffman disease), Type II (Dubowitz disease), Type III (Kugelberg-Welander disease), Type IV is the adult-onset variant. PCR amplification was performed on stored DNA of 18 Puerto Rican SMA patients. PCR targeted exon 7 and exon 8 of both Survival of Motor Neuron (SMN1 and SMN2) genes. To distinguish between the two genes, digestion was performed by DdeI and DraI enzymes in exon 8 and exon 7 respectively. In addition, PCR for exon 5 of the neuronal apoptosis Inhibitory Protein (NAIP) was performed to observe absence or presence of the gene. Sixty-six (66%) percent of the patients with all types of SMA were deleted for exon 7. For exon 8 thirty-three (33%) percent were deleted. NAIP shows the particularity of being present in the experimental group and deleted in control group. This result implies that NAIP is not completely related to SMA severity and is not the main cause for SMA expression in Puerto Rican population.

2880
**Neuroprotective Mechanisms of Shikonins**
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Microglia are the prime effector cells involved in immune and inflammatory responses in central nervous system (CNS). They are activated during pathological conditions to restore CNS homeostasis. However, chronic microglial activation endangers neuronal survival through the release of various pro-inflammatory molecules which are toxic to neurons. Thus, negative regulators of microglial activation have been considered as potential therapeutic candidates targeting neurodegenerations such as Alzheimer’s and Parkinson’s diseases. Shikonin, napthoquinone pigment, is a phytocompound isolated from the root of *Lithospermum erythrorhizon* which has long been used as oriental traditional medicine ointment for wound healing. Shikonin has been reported to confer anti-bacterial, anti-tumor, and anti-inflammatory effects. The aim of this study was to examine whether shikonin possibly represses the microglial activation. Among shikonin and six different derivatives of shikonin, we observed that isobutyrylshikonin (IBS) and isovalerylshikonin (IVS) were the most effective in inhibiting LPS-induced nitric oxide (NO) release from microglial cells. Reverse transcriptase real-time PCR analysis revealed that pretreatment of rat brain microglia with IBS and IVS attenuated LPS-induced mRNA expression for inducible NO synthase, tumor necrosis factor (TNF)-α, interleukin-1β, and cyclooxygenase-2. In primary cultured microglia, IBS and IVS reduced LPS-stimulated production of TNF-α and prostaglandin E2. In addition, IBS and IVS significantly decreased LPS-induced iKB phosphorylation and NF-kB DNA binding activity. In organotypic hippocampal slice cultures, propidium iodide staining exhibited prominent cell death in the hippocampal layer after 96 h of LPS treatment. Both IBS and IVS almost completely blocked the effect of LPS on neuronal death concomitant with inhibition of LPS-induced NO production in culture medium. These results suggest that IBS and IVS may play provide neuroprotective means by reducing various pro-inflammatory molecules from activated microglia.

2881
**Functional Study of Drosophila melanogaster Omega-Class Glutathion S-Transferases (GSTs)**
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Glutathione S-transferases are a family of multifunctional enzymes, which play important roles in cellular detoxification. Biological function of omega-class GSTs (GSTOs), however, have not yet been well elucidated. In our previous study, five DmGSTOs including DmGSTO1, DmGSTO2, DmGSTO3, DmGSTO4 and DmGSTO5 were cloned and characterized from *Drosophila melanogaster*. All cloned and expressed five DmGSTOs exhibited high thiol transferase and dehydroascorbate reductase (DHAR) activities and low activity towards 1-chloro 2,4-dinitro benzene, characteristics of GSTO. DmGSTO1 is found to be the structural gene for *seqio* which encodes PCA synthase. Especially, recombinant proteins of DmGSTO2 and DmGSTO3 had much higher DHAR activity compared to other DmGSTOs. Km values for glutathione (GSH) of the DmGSTO2 and 3 were 0.40 mM and 4.33 ± 0.23 mM, respectively. DHAR which converts DHA to ascorbate (AsA), is a key enzyme for recycling AsA. To investigate this function in the nuclei), a process that may be involved in ALS pathogenesis.

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**GPI-anchored Protein Localizes with 14-3-3 and HSP60 on the Mitochondria in Human Neural Progenitors**
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Most of neurodegenerative diseases have been characterized by the accumulation of the protein's self aggregates, or the formation of short amyloid filaments. The molecular architecture of their diseases proteins inside cells plays an indispensable part to understand the mechanism regulating their disease pathogenesis. The 14-3-3 protein, which is abundantly expressed in the CNS, recently detected in the cerebrospinal fluid of Creutzfeldt-Jacob
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disease (CJD) and used as a biochemical maker in the diagnosis of CJD. Currently, we have examined the formation of the molecular complex of the cellular prion protein (PrPC), a glycosylphosphatidylinositol (GPI)-anchored protein, in the human neuronal progenitors, by using electron microscopy. Though it is well known that the PrPC localizes on the outer surface of the plasma membrane, caveolae and clathrin-coated pits in fibroblasts, the function of PrPC and the structural change from PrPC to the pathogenic prion (PrPSc) remain to be unclear. In this research, we showed that the PrPC could localize with 14-3-3 and HSP60 on the mitochondria in human neural progenitors. These proteins might form a molecular complex in the human CNS under physiological conditions. It is suggested that the conformation change of the PrPC might act on the mitochondria membrane. These findings are consistent well with the previous biochemical assay.

2883

Dicarbonyl Modification Inhibits the Fibrillization of Alpha-synuclein In Vitro

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Alpha-synuclein is a major fibrillar component of intraneuronal inclusions called Lewy bodies, a pathological signature of Parkinson’s disease. Various post-translational modifications of alpha-synuclein have been reported to have significant effects on its amyloidogenic properties. Two dicarbonyl compounds, methylglyoxal and glyoxal, metabolites accumulated in intracellular environment, were investigated for their effects on the structural and fibril-forming properties of alpha-synuclein. Both compounds were found to induce the oligomerization of alpha-synuclein. By adding the substoichiometric amounts of the modified protein mixture, the protein fibrillation was remarkably inhibited. The heterogeneous modified mixtures were separated into three fractions of monomers, dimers, and high molecular mass oligomers. Interestingly, the fibril-forming property of alpha-synuclein completely disappeared in all of modified species of alpha-synuclein and the protein fibrillation was also significantly suppressed by the seeding of modified alpha-synuclein species. Temperature scanning and the interaction with SDS micelles mimicking the vesicle surface revealed that both methylglyoxal- and glyoxal-modified monomers were not as susceptible as the intact protein in the temperature- and vesicle-induced conformational changes to partially folded intermediates and alpha-helix, respectively. Therefore, our observations suggested that the dicarbonyl modification of alpha-synuclein should reduce the conformational flexibility of the protein, thereby contributing to the loss of the fibril-forming property of alpha-synuclein itself and the inhibitory activity of the modified protein against the fibrillization of the unmodified alpha-synuclein.

2884

Increased AICD Generation Does Not Result in Increased Nuclear Translocation or Activation of Target Gene Transcription

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A sequence of amyloid precursor protein (APP) cleavages culminate in the sequential release of the APP intracellular domain (AICD) and the amyloid β peptide (Aβ) and/or p3 fragment. One of the environmental factors identified favouring the accumulation of AICD appears to be a rise in intracellular pH. AICD can activate the transcription of artificially expressed constructs and many downstream gene targets have been documented. Here we further identified the metabolism and subcellular localization of the constructs used in this gene reporter assay. We also examined possible functions for its increased expression, including influence in transcriptional activity over two postulated target genes, neprilysin and KAI1. We found that most of the AICD generated under pH neutralized conditions is likely cleaved from C63. Furthermore, the AICD surplus does not further activate transcription of neprilysin or KAI1 but rather remains membrane tethered and free in the cytosol where it interacts with Fe65. We also demonstrated that AICD mediated activation of neprilysin and subsequent degradation of Aβ is likely not the result of a feed back mechanism, since AICD levels were insensitive to Aβ. However, Fe65 is still essential in AICD mediated transcriptional transactivation although its exact role in this set of events is unclear.

2885

Alterations in the Lysosomal Proteases (Cathepsins B and D) and NFKB during Oxidative Stress-induced Apoptotic Neuronal Death

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Various studies have demonstrated that lysosomes are vulnerable organelles following oxidative stress-induced injury and their proteases (cathepsin B and cathepsin D) may have an initiating role in apoptotic cell death. Activation of NF-kB has also been demonstrated in oxidative stress-induced injury such as transient forebrain and global ischemia in the rat. However the influence of NF-kB on the lysosomal system following injury has not been investigated. HYPOTHESIS: We hypothesize that NF-kB, will influence activation of cathepsin B and cathepsin D resulting in SHSY-5Y cell-death following oxidative stress induced by Hydrogen Peroxide (H2O2). METHODS: In this study we examined cell viability and the protein levels of NF-kB, cathepsin B and cathepsin D following exposure to H2O2 in SHSY-5Y cells. Co-localization and expression of NF-kB, Cathepsins B and D were assessed by Immunocytochemical analysis. RESULTS: Utilizing a MTS assay, our results indicate that when SHSY-5Y cells are exposed to H2O2, there is a dose and time dependent decrease in cell viability at 1, 4, 6, 12 and 24hrs. Immunocytochemical analysis demonstrate enhanced expression and co-localization of NF-kB, cathepsins B and D in SHSY-5Y cells following oxidative stress induced at 1, 4, 6, 12 and 24Hr time points. Additionally, Western Blot analysis confirm temporal alterations in Cathepsin B, Cathepsin D and NF-kB protein expression respectively following oxidative stress induced by H2O2 . CONCLUSION: We therefore conclude that H2O2 causes alterations in NF-kB, cathepsins B and D protein levels and may contribute to cell death in SHSY-5Y cells following oxidative stress.

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Cell Death Regulation and Recovery in Neuronal NG108-15 Differentiated Cells

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The study’s objective was to explore cell death regulation and recovery in neuronal differentiated cells in vitro. The NG108-15 cells differentiated in neurons by 1mM sodium butyrate (NaB) treatment. NaB differentiated cells showed a slower apoptotic kinetic. After 24h of 0,5 μm staurosporine (STS) treatment 96% of proliferating cells died, while only 54,1% of differentiated cells were dead. After STS treatment the anti-apoptotic Bcl-2 and Bcl-xL mitochondrial protein levels decreased in proliferating cells, but not in differentiated cells. The pro-apoptotic Bad was phosphorylated, in differentiated cells and not in proliferating cells. Staining mitochondria by 5,5’,6,6’-tetrachloro-1’,3’,3’-tetraethylbenzimidazole carbocyanide iodide (JC-1) showed that mitochondrial membrane potential was lost in proliferating cells after 30 min of STS treatment, while the mitochondria of differentiated cells were energized even after 100 min. Moreover, in proliferating cells Endo G and AIF translocated to the nucleus after 2 h of STS treatment, NaB differentiated cells did not show such translocation. When differentiated cells were co-treated with Insulin Growth Factor (IGF) and STS, cell viability increased by 17,1%. The effect on neuronal functionality of both STS and/or IGF treatments was also studied by electrophysiology. In STS-treated cells (with or without IGF), membrane potential oscillations were reduced in amplitude and never gave rise to spontaneous oscillations of the membrane potential (APs). However STS effect was reversible. After removing STS from the cultures, the percentage of cells yielding overshooting APs increased to 80% -100%. In conclusion, NaB differentiated NG108-15 were more resistant to STS-induced apoptosis. Such resistance was achieved at two levels, mitochondrial and nuclear. Moreover, IGF co-treatment increased cell viability and STS removal allowed the neuron cells to recover functionality. These results suggest that the neuronal resistance to cell death could be pharmacologically exploited to recover a structural and/or functional damage induced by neurodegenerative diseases.

Other Diseases (2887-2902)

2887

Cytogenetic Analysis of a Patient Carrying a Balanced Chromosomal Translocation
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A balanced chromosomal translocation is a common genetic abnormality that involves the exchange of genetic material between two chromosomes. This chromosomal abnormality generally has no affect on an individual’s phenotype however in rare cases balanced translocations have the ability to disrupt genes causing unfavorable phenotypes. When this occurs, molecular cytogenetic techniques must be employed to fine map the affected chromosomes. Although routine, karyotyping has a limited microscopic resolution and is unable to detect abnormalities smaller than 3-5Mb in size. This limitation prevents the identity of genes that may be involved in causing the unfavorable phenotypes Fluorescence in situ Hybridization (FISH), however, is a molecular cytogenetic technique that greatly increases this resolution, enabling abnormalities as small as 20Kb in size to be detected. In this study, patient BG071.04 and several members of her family have a translocation between chromosomes 6 and 18, (6;18)(p12;q12.3). Even more interesting than the familial history of this translocation is the similarity of phenotype, seizure and Attention Deficit Hyperactivity Disorder (ADHD), between both the patient and her carrier brother. We used FISH analysis to fine map the patient’s chromosomes and locate the two chromosomal breakpoints. We successfully identified the exact breakpoint on chromosome 18 while the breakpoint region on chromosome 6 has been fined tuned to a small. Only one gene lies within the breakpoint region of chromosome 18, gene BC045816 whose function is unknown. Within the breakpoint region of chromosome 6 there are several interesting candidate genes including MUT, a mitochondrial enzyme Methylmalonyl Coenzyme A mutase. It has previously been speculated that a mutation or disruption of MUT may lead to Methylmalonic Aciduria, a condition whose phenotype can include seizure. Ongoing research will be devoted towards finding the function of human gene BC045816, locating the exact breakpoint on chromosome 6, and further characterization of MUT.

2888

Antioxidant-dependent HIF-1α Expression during Acute Hypoxia in Mouse Kidney
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Hypoxia-inducible factor (HIF-1) is an oxygen-dependent transcriptional activator. HIF-1 is consists of α and β subunits. HIF-1α subunit is remarkably high during hypoxia and is degraded at low levels in most cells under normoxic conditions. Numerous studies have shown that reactive oxygen species (ROS) was generated from NADPH oxidase in hypoxic condition. However, the precise mechanism mediated by the ROS remains poorly understood. In this study, to investigate the relationship between the ROS and HIF-1α mediated protein expression, we used EC-SOD over expressed transgenic mouse. Extracellular (EC)- superoxide dismutase (SOD) is one of the major tissue defense enzymes that is highly expressed in the kidney. In hypoxic condition, NADPH oxidase activity and H2O2 were more increased in EC-SOD transgenic mouse kidney than normal mouse. Activation of antioxidant enzymes, peroxiredoxin(Prx) 2, 5, also more increased in EC-SOD transgenic mouse following the hypoxic condition. Interestingly, Prdx5 translocated to the nucleus during hypoxia in immunohistochemistry analysis. To determine the role of prdx2, 5, we examined the hypoxia related proteins level in knock down expression of Prx5 and knock out expression of Prx2 mouse. Taken together, these data show that specific antioxidant enzymes are regulate ROS levels and HIF-1α stabilization during hypoxic condition. This work was supported by the Korea Research Foundation Grant funded by Korea Government (MOEHRD, Basic Research Promotion Fund), (KRF-2006-070-E00238). *These authors are supported by Research Center for controlling Microbial Virulence.

2889

Characterization of the Role of the RECQ1 Helicase in DNA Damage Repair
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DNA helicases are enzymes that unwind DNA duplexes in a specific direction. RecQ helicases, named after the recQ gene of E. coli, are a family of DNA helicases essential for maintaining genomic stability in both eukaryotes and prokaryotes. There are five different RecQ helicases found in humans: RECQ1, BLM, WRN, RECQ4 and RECQ5. Of these five human RecQ helicases, three of them, namely BLM, WRN, RECQ4 have been
associated with distinct genetic disorders. Although human RECQ1 has not been linked to any disorders thus far, it has been shown that its deficiency causes DNA strand breaks and mild cellular sensitivity to ionizing radiation in a mouse model, suggesting that RECQ1 plays a role in genomic maintenance. To gain insight into the role of RECQ1 in the repair of DNA damage, we investigated possible phosphorylation on the helicase, and the effect of post-translational changes on its activity. We also examined possible interactions of RECQ1 with proteins known to have a role in DNA repair, and whether the helicase localizes to chromosomes upon DNA damage. Western blotting and helicase assay results showed that RECQ1 exhibits more unwinding activity when phosphorylated, suggesting that it could be activated through phosphorylation triggered by DNA damage. We did not find any interaction between RECQ1 and the limited number of DNA repair proteins studied. In addition, we found that RECQ1 does not localize to chromosomes upon DNA damage. These results suggest that RECQ1 participates in DNA repair by carrying out other functions in the nucleus.

2890
The Effect of UV Rays on Corneal Light Absorption Properties and Corneal Damage Is Dependent on UV Wavelength
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Purpose of this study was to examine the differences between the effect of UVA and UVB rays on light absorption properties of the rabbit cornea and corneal damage. In the first group of rabbits the corneas were irradiated with UVB rays (312 nm, once a day during 5 days, a dose per day 1.01 J/cm²). In the second group the corneas were irradiated with UVA rays (365 nm, once a day during 5 days, a dose per day 1.01 J/cm²) and in the third group the corneas were irradiated with UVA rays (365 nm, once a day during 5 days, a dose per day 2.02 J/cm²). Absorbances of corneal centers of normal corneas (as controls) and irradiated corneas were measured over a range of 190-650 nm using a scanning spectrophotometer (HELIOSb 84021). To investigate the damage of the cornea, rabbit corneae were examined histologically and immunohistochemically for peroxynitrite formation (demonstrated by nitrotyrosine residues) and lipid peroxidation (evaluated by malondialdehyde staining). Comparing the effect of the same doses of UVA or UVB rays with the normal cornea, UVA rays did not evoke corneal damage and did not significantly change corneal light absorption properties. Similar results were obtained with two times higher dose of UVA rays. In contrast, repeated irradiation of the cornea with UVB rays evoked the increase in corneal light absorption throughout the whole measurable UV-VIS spectral range than the normal cornea. Nitrotyrosine, a cytotoxic byproduct of nitric oxide and superoxide and malondialdehyde were highly expressed in the cornea after UVB rays. In conclusion, UVB rays (not UVA rays) were dangerous to the cornea.

2891
The Role of Recurrently Elevated Macro Creatine Kinase Type 1
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Background: The typical creatine kinase (CK) isoenzymes include CK-BB (brain), CK-MB (myocardium) and CK-MM (skeletal muscle). Macro CK type 1, one of the atypical CK enzymes with a higher than normal molecular mass, has been identified in human serum but the clinical significance still remains uncertain. Methods: From March 2004 to May 2007, 105 patients who expressed serum macro CK isoenzyme type 1 were identified. The relevance of macro CK isoenzyme type 1 was 0.73 in our study. Moreover, there were 16 patients (15.2%) with an 81 set analysis demonstrating repeated presences of macro CK type 1. Twelve patients were male and four were female. Patient ages at the initial presentation ranged from 5 months to 70 years (mean: 20.3 years). Results: The mean of macro CK type 1 and the CK-MM fraction was 0.18% and 0.08% respectively. Almost all CK-MB fractions were more than 4%. The linear regression analysis between macro CK and total CK revealed a good correlation (y = 3.5742x + 2105.6, R2 = 0.8579); and between macro CK type 1 and CK-MM (y = 0.3923x - 7.2234, R2 = 0.771) and CK-MM (y = 2.182x + 2112.9, R2 = 0.7019). Clinical diagnoses were myopathy in 15 patients, but only one had acute coronary syndrome. Conclusion: In conclusion, currently elevated macro CK type 1 is a benign course that is not a predictive factor of prognosis or severe illness, and further elucidation is needed.

2892
Cell Proliferation and Apoptosis in Cyst Formation
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Autosomal dominant polycystic kidney disease (ADPKD) is a common genetic disorder caused by mutation in PKD1 or PKD2, encoding polycystin-1 (PC1) and -2 (PC2). The disease is characterized by progressive development of epithelial lined, fluid-filled cysts from various kidney tubular segments, eventually leading to end-stage renal disease. Increased cell proliferation and apoptosis in cystic kidneys are two key features of the disease. In order to understand how inactivation of polycystins disturbs these cellular processes, we investigated the role of the tumor suppressor protein Rb which came out of a screening of the transcriptome in Pkd1 mutant kidneys. Western blot analyses revealed that there is a reduction or loss of Rb expression in both cystic kidneys and Pkd1 null cells. E2Fs are downstream targets of Rb. To determine a potential role of E2Fs in cyst formation in junction with a reduction of Rb, we went on to determine the expression of E2Fs in PKD kidneys. As expected, the expression of activating E2Fs is increased in both human and mouse cystic kidneys by western blot as well as by immunofluorescence staining, accompanied by increased apoptosis. These data indicate that mutation in polycystins results in an inactivation of Rb and subsequent activation of E2Fs, which subsequently lead to increased cell proliferation and apoptosis, eventually to cystogenesis in PKD. Further studies are underway to investigate the significance of the alteration in the Rb/E2F pathway in the loss of tube lumen control in PKD.
Cytokine Evaluation of Seven Different Respiratory Infections
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Positive samples from Influenza A, B, Parainfluenza (Para) 1, 2, and 3, Adenovirus and Respiratory Syncytial Virus (RSV) were tested for 16 different cytokines to evaluate the presence or absence of cytokines during acute infection. Two nasal lavage samples from each infection were taken and tested in duplicate using the Quansys Biosciences Multiplexed ELISA (MÉ™) Human Cytokine Array - Screen Plus. This panel contains 20 spots of capture antibodies to the following cytokines: IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-15, IL-17, IL-23, IFNγ, TNFα and TNFβ. The samples were loaded into a 96 well plate and incubated at room temperature for 1 hour. Following the sample incubation the plate was washed and incubated with detection antibody mix containing antibodies to each of the 16 analytes. Post incubation, the plate was washed again and incubated with streptavidin-HRP and incubated for 15 minutes. Following an additional wash step chemiluminescent substrate was added and the plate was imaged for 90 seconds using a Quansys Imaging system. The Flu A, Flu B and RSV samples showed a typical inflammatory response with a high expression of IL-6 and IL-8. The Para 1 samples showed a similar response yet one sample showed elevated values of IL-1α, IL-1β, IL-5 and TNFα. Para 3 samples showed similar elevate levels of IL-1α, IL-6 and IL-8. Yet, one sample showed elevated levels of IL-5, IL-12p70 and IL-13. The Adenovirus samples showed similar cytokine expression levels of IL-1β, IL-6, IL-8, IL-10, IFNγ and TNFα. Many of the cytokine responses were expected such as IL-1β, IL-6 and IL-8 as they are seen in typical inflammation conditions. However, a disparity was observed between samples of the same disease conditions showing different cytokine profiles such as that observed in the Para 1, 2 and 3 samples.

A New Immobilization Method for Skeletal Muscle Atrophy Induction in Mice
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Skeletal muscle atrophy occurs in various disease states and normal aging. It is also a serious concern for the rehabilitation of patients afflicted with nerve injury or immobilized after surgery. Although it is well-established that muscle atrophy results from an imbalance in protein degradation and synthesis, the molecular events leading to this debilitating condition is poorly understood. Moreover, a mouse model based on a convenient procedure for promoting muscle atrophy in mice is lacking. In this work, we describe a new hind-limb immobilization procedure that induces skeletal muscle atrophy by using a surgical staple to impede one hind leg. This method is simple, cost effective and most importantly less invasive than other methods currently used. The tibialis anterior (TA) muscle of mice in which one hind-limb was immobilized with a surgical staple for 2, 6 and 16 weeks, were compared to contra-lateral un-stapled limbs as well as sciatic nerve crushed muscle atrophy inducing mice. Results indicated that TA weight diminished drastically (21%) 2 weeks after staple immobilization as compared to the muscle from contra-lateral non-stapled hind-limbs (p<0.05). In addition, TA weight loss at 6 and 16 weeks remained similar to the 2 week time point (p=0.001) which indicated that muscle wasting by this procedure is a rapid process. Furthermore, myofiber cross-sectional area analysis revealed significant reductions of almost 50% after 2 weeks (p<0.05) for staple hind-limbs. Moreover, known molecular markers for skeletal muscle atrophy were significantly up-regulated in the TAs of stapled hind-limbs compared to their contra-lateral TAs after 6 weeks. In conclusion, we validated a new and convenient method for studying skeletal muscle atrophy through immobilization. This model will be useful for determining molecular and cellular processes involved in this debilitating state and will be beneficial for the development of effective therapies.

Pro-Inflammatory Study of Gastric Fluid Acid in Raw 264.7 Cells
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Asthma is now characterized as a chronic inflammation disease. Many investigators indicated that chronic gastric fluid acid aspiration is considered as the inflammatory factor in asthma. Connections between GERD (Gastro Esophageal Reflux Disease) and upper and lower respiratory disease had been suggested for decades. However the mechanisms involved in the inflammatory effect caused by gastric fluid acid is not clear at present time. Macrophage had been suggested as major cells in many chronically inflammatory diseases. To investigate the potential role of gastric fluid acid in chronic aspiration, we had demonstrated that gastric fluid acid exert pro inflammatory effect in murine macrophage Raw 264.7 cell line. Pro-inflammatory cytokine TNF-α production was enhanced by the gastric fluid acid stimulation. Recently, Rhee etal reported that MMP and NF-κB signal pathway was considered as an important factor in the progression of inflammation. Therefore we had also investigated the potential role of gastric fluid acid in macrophage MMP-9 expression and the activation of NF-κB signal pathways. The activity of MMP-9 expression was observed after gastric fluid acid stimulation in macrophage cells; however, no difference was seen in MMP-2 expression. The NF-κB pathway elements p65 and p50 were activated in a time dependent manner in gastric fluid acid stimulated cells. Taken together, these results suggest that, gastric fluid acid may play an important role in the inflammation progress in GERD associated asthma, and may be involved in the airway remodeling.

Mitomycin C Induces Apoptosis in Cultured Corneal Fibroblasts Derived from Type II Granular Corneal Dystrophy Corneas
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Objective: The present study investigated the effect of MMC on cell viability, apoptosis and transforming growth factor beta-induced protein (TGFβp) expression in cultured fibroblasts. Methods: Keratocytes were obtained from normal cornea or heterozygote or homozygote GCD II patients after lamellar or penetrating keratoplasty. To measure cell viability, corneal fibroblasts were incubated with 0, 0.01, 0.02, or 0.04% MMC for 3, 6, or 24 h, and then tested using LDH and MTT assays. To measure apoptosis, cells were analyzed using FACS analysis and annexin V staining. Bcl-XL, Bax and TGFβp mRNA expression was measured using reverse transcription polymerase chain reaction (RT-PCR) assays. Cellular and media levels of TGFβp protein were measured using immunoblotting. Results: MTT and LDH assays showed that MMC reduced cell viability in all 3 cell types in a dose- and time-dependent manner. FACS analysis and annexin V staining showed MMC caused apoptosis, with GCD II homozygote

Skeletal muscle atrophy by using a surgical staple to impede one hind leg. This method is simple, cost effective and most importantly less invasive than other methods currently used. The tibialis anterior (TA) muscle of mice in which one hind-limb was immobilized with a surgical staple for 2, 6 and 16 weeks, were compared to contra-lateral un-stapled limbs as well as sciatic nerve crushed muscle atrophy inducing mice. Results indicated that TA weight diminished drastically (21%) 2 weeks after staple immobilization as compared to the muscle from contra-lateral non-stapled hind-limbs (p<0.05). In addition, TA weight loss at 6 and 16 weeks remained similar to the 2 week time point (p=0.001) which indicated that muscle wasting by this procedure is a rapid process. Furthermore, myofiber cross-sectional area analysis revealed significant reductions of almost 50% after 2 weeks (p<0.05) for staple hind-limbs. Moreover, known molecular markers for skeletal muscle atrophy were significantly up-regulated in the TAs of stapled hind-limbs compared to their contra-lateral TAs after 6 weeks. In conclusion, we validated a new and convenient method for studying skeletal muscle atrophy through immobilization. This model will be useful for determining molecular and cellular processes involved in this debilitating state and will be beneficial for the development of effective therapies.
cells being most affected. RT-PCR analysis showed MMC caused decreased Bcl-xl mRNA expression and increased Bax mRNA expression in all cell types, with GCD II homozygote cells being the most affected. RT-PCR and immunoblotting analysis showed that MMC reduced TGFβp mRNA levels and cellular and media TGFβp protein levels in all cell types. Conclusion: MMC induced apoptosis and reduced production of TGFβp in all 3 types of corneal fibroblast. The effects of MMC were greatest in GCD II homozygote cells. These findings may explain the additional therapeutic effect of MMC in GCD II patients undergoing PTK treatment.

2897

The Danger from Oxidative Injuries to the Ocular Surface in Dry Eye Disease (Sjögren’s Syndrome)

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Dry eye syndrome is a chronic condition in which some components of the precorneal tear film are dysfunctional. The factors leading to abnormalities of the tear film are complex and may involve autoimmune disease (i.e. Sjögren’s syndrome), loss of hormonal support, and glandular inflammation. The resulting disorders may lead to reduced antiinflammatory activity and oxidative damage. The purpose of this study was to detect antioxidant enzymes in conjunctival epithelial cells of the human dry eye (Sjögren’s syndrome). Antioxidant enzymes cleave reactive oxygen species and are important in the maintenance of pro-oxidant and anti-oxidant balance on the corneal surface. Conjunctival epithelial cells were obtained using the method of impression cytology. Normal eyes served as controls. In conjunctival epithelium superoxide dismutase, catalase and glutathione peroxidase were examined immunohistochemically. In contrast to normal eyes where antioxidant enzymes are highly expressed in the conjunctival epithelium, in dry eye the expression of these enzymes is much less pronounced, particularly that of catalase, an enzyme cleaving hydrogen peroxide. Results show that the oxidative injuries of the ocular surface in Sjögren’s syndrome might be associated with the decreased expression of antioxidant enzymes.

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Transforming Growth Factor Alpha Signaling Mechanisms in Articular Cartilage Degeneration in Osteoarthritis

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BACKGROUND: Articular cartilage degeneration is a hallmark of osteoarthritis (OA). Previously, we identified increased expression of transforming growth factor alpha (TGF-α) in early stage degenerating cartilage in experimental knee OA (1,2). We determined that increased TGF-α signaling modified chondrocyte cytoskeletal organization, increased catabolic and decreased anabolic gene expression and suppressed Sox9 expression. We hypothesized that these effects are mediated by Rho/ROCK and MEK/ERK signaling pathways. METHODS: Primary cultures of chondrocytes and articular cartilage explants were used to determine which intracellular signaling pathways mediate TGF-α signals in chondrocytes. Pharmacological inhibitors of MEK1/2 (U0126), ROCK (Y27632), Rho (C3), p38 MAPK (SB202190) and PI3K (LY294002) were used to specify pathways are involved. RESULTS: Using a RhoA G-LISA assay, we determined that stimulation of primary chondrocytes with TGF-α activates RhoA. Reciprocally, inhibition of RhoA/ROCK signaling prevents modification of the actin cytoskeleton in response to TGF-α. Inhibition of other pathways did not prevent cytoskeletal re-organization. Conversely, inhibition of MEK/ERK signaling rescued the TGF-α-induced abrogation of anabolic gene expression in addition to rescuing the mRNA and protein expression of Sox9, as well as Sox9 activity. Inhibition of additional pathways did not confer this protective effect in the presence of TGF-α. Finally, inhibition of MEK/ERK, Rho/ROCK, p38 MAPK and P3K signaling pathways had variable effects on the induction of MMP13 and TNFα gene expression in chondrocytes. CONCLUSIONS: Rho/ROCK signaling mediates TGF-α-induced re-organization of the actin cytoskeleton, while MEK/ERK signaling mediates signals resulting in the suppression of Sox9 and anabolic genes activated by Sox9. However, multiple pathways exert influence over the activation of MMP13 and TNFα gene expression in chondrocytes. These pathways may represent strategic targets for intervention approaches to treating cartilage degeneration in osteoarthritis.


2899

Fibrocytes Home Lungs from Patients with Idiopathic Pulmonary Fibrosis through the CXCR4/CXCL12 Axis

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Fibrocytes are a unique population of circulating progenitors of fibroblasts implicated in wound healing and tissue fibrosis. However, their role in idiopathic pulmonary fibrosis (IPF) remains unresolved. In this study we aimed to identify fibrocytes in IPF lungs through fluoroscences confocal microscopy using different fibrocyte-specific combinations of antibodies. Stromal cell-derived factor-1 (CXCL12) was determined in plasma and bronchoalveolar lavage (BAL) by ELISA and its cellular source in lungs was examined by immunohistochemistry. Fibrocytes were identified in 8 out of 9 IPF lungs. Staining including CXCR4 and a mesenchymal marker identified significant (p<0.003) more fibrocytes/mm² compared with the other combinations: CXCR4/procollagen-1 (10.3±2.9), CXCR4/prolyl-4-hydroxylase (4.1±3.1), CD34/procollagen-1 (2.8±3.0), CD34/αSMA (2.2±1.6) and CD45/α4-hydroxylase (1.3±1.6). There was a positive correlation between the abundance of fibroblastic foci and the amount of lung fibrocytes (r=0.79; p<0.02). No fibrocytes were identified in normal lungs. Immunoactive CXCL12, the specific ligand of CXCR4, was strongly expressed by reactive alveolar epithelial cells of IPF patients. Plasma levels of CXCL12 were significantly increased in untreated IPF patients (n=42) compared to healthy controls (n=23) [median: 2707.5 pg/ml (648.1-4884.7) versus 1751.5 pg/ml (192.9-2686.0); p<0.003]. Likewise, CXCL12 was detectable in the BAL fluid of 40% (8/20) of patients with IPF and none (0/5) of the normal controls. A negative correlation between plasma SDF1/CXCL12 levels with lung DLCO (r=-0.56; p<0.03) and oxygen saturation on exercise (r=-0.41; p<0.04) was found. These
findings suggest that circulating fibroblasts recruited through the CXCR4/CXCL12 axis may contribute to the expansion of the fibroblast population in IPF and that CXCL12 plasma levels may be a biomarker of disease severity.

2900  
**Mechanical Strain and Prostaglandin E₂ Induce Reactive Oxygen Species Formation by Podocytes**  
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Elevated intraglomerular pressure, an underlying factor in the development of diabetic nephropathy, results in podocyte loss and compromised glomerular filtration barrier. The mechanism(s) underlying podocyte damage remain incompletely defined. Reactive oxygen species (ROS) play a major role in diabetic vascular complications; moreover, they are known mediators of podocyte injury, death and detachment. Podocytes are capable of generating ROS in response to a diverse range of stimuli and in this cell type, the primary source of superoxides are the NADPH-dependent oxidases. We investigated ROS production by conditionally immortalized mouse podocytes that were subjected to mechanical strain, and a well-established lucigenin assay was used to measure NADPH oxidase-dependent ROS generation. Mechanical strain stimulated ROS production by podocytes (2.3-fold over non-stretched controls). Our laboratory has previously shown that mechanical stretch of podocytes induces the expression of both COX-2 and the prostaglandin E₂ (PGE₂) EP4 receptor, but not the EP1 receptor (Martineau et al., 2004). We therefore evaluated the effects of PGE₂ on podocyte ROS production. PGE₂ (1 uM) similarly increased the generation of superoxides, and both EP1 and EP4 selective agonists induced ROS formation, implicating the involvement of both receptors. ROS generation stimulated by PGE₂ and mechanical strain were reversed by pre-treatment with the NADPH oxidase inhibitor, apocynin. Podocytes express the requisite molecular components of the phagocytic NADPH oxidase, including p22phox, p47phox, and NOX-2. More recently, we and others have demonstrated the presence, in podocytes, of NOX-4, the major renal NADPH oxidase. We are currently identifying the NOX(s) responsible for mechanical stretch- and PGE₂-mediated ROS generation. Overall, these data suggest that, within the diabetic milieu, PGE₂ and glomerular capillary hypertension may contribute to podocyte injury via increased NADPH oxidase-generated ROS.

2901  
**Gliadin Alters the Actin Cytoskeleton Via a Tyrosine Kinase Signaling Pathway in Intestinal Epithelial Cells**  
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Celiac disease, a T-cell mediated autoimmune disease, is triggered by the ingestion of glutenin/gliadin-containing grains (wheat, barley, rye) in genetically susceptible individuals. The disease is characterized by chronic small bowel inflammation, malabsorption, still birth, neurological disorders and gastro-intestinal lymphomas and co-morbidity with other autoimmune diseases. Gliadin proteins are known to trigger immune response to self tissue transglutaminase therefore the biological effects of gliadin are of particular interest. Early in the disease, epithelial tight junctions are opened followed by an innate immune response on the mucosal surface of the intestine that is not very well characterized. Peptides generated by a complete digestion of gliadin with trypsin and pepsin are used for in vitro studies and frequently referred to as PF-gliadin or PTG. We studied the effects of PTG on actin cytoskeletal organization and tight junction assembly in IEC6 (rat intestinal epithelial) cells. PTG caused redistribution of tight junction proteins ZO-1 and occludin from cell-cell junctions and dissolution of actin stress fibers and stress fiber associated phosphorylated myosin light chain (pMLC). PTG also caused increased phosphorylation of src kinase. Effects of PTG on the actin cytoskeleton could be prevented by inhibitors of src kinase and PI-3-kinase. These results provide novel insights into the signaling pathways modulated by gliadin to cause changes in epithelial cells during the pathogenesis of Celiac disease.

2902  
**The Mechanism of Transfer of Cholesterol from Aggregated Low Density Lipoprotein to Macrophages**  
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An early step in atherogenesis is the interaction of macrophages with aggregated lipoproteins that are associated with the extracellular matrix. Cellular processes involved in the interaction of macrophages with aggregated low density lipoprotein (agLDL) differ substantially from processes involved in their interaction with monomeric LDL. In particular, uptake of agLDL does not involve receptor-mediated endocytosis but rather the aggregate is sequestered in deep invaginations at the cell surface, termed surface connected compartments (SCCs). Further, initial hydrolysis of the cholesteryl ester (CE) moiety of agLDL is 5 fold greater than that of the protein moiety. However, the location of CE hydrolysis remains unclear. One possibility is that CE is transferred to lysosomes, either via a selective CE transport mechanism or by endocytosis of the CE-containing core of the aggregates. An alternative possibility is that lysosomal acid lipase (LAL), responsible for intracellular degradation of CE, is able to digest the CE extracellularly during the prolonged contact of the macrophage with the aggregate. Data will be presented that demonstrate delivery of lysosomal contents, including LAL, to SCCs via lysosome fusion and acidification of SCCs, thereby enabling LAL functioning. We used time-lapse confocal fluorescence imaging to demonstrate the active acidification of SCCs. Further, an increase in free cholesterol is witnessed in cell-engaged but extracellular aggregates during macrophase uptake of agLDL. Together, these results indicate that during macrophage sequestration and internalization of agLDL CE hydrolysis occurs extracellularly in SCCs. This novel endocytic pathway may more accurately reflect events that occur when macrophages encounter subendothelial lipoproteins in vivo, since there is increasing evidence that LDL is aggregated in atherosclerotic plaques.
Microtubule Motors Couple Adenovirus Transport to Disassembly

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Cells are frequently exposed to foreign DNA and RNA and regulate the subcellular transport of their own RNAs, but the underlying transport mechanisms are incompletely understood. Microtubule-mediated transport shuttles viruses to and from the nucleus. Incoming adenovirus capsids with their linear DNA genomes are transported on microtubules by the minus end-directed dynein/dynactin motor complex to the nucleus, bind and disassemble at the nuclear pore complex (NPC), and import their genome into the nucleoplasm. Here, we report that the heterotetrameric kinesin-1 is recruited to the viral capsid protein IX through the tetratricopeptide repeat (TPR) of kinesin light chain (KLC) 1/2. Protein IX-deficient virions, knock down of KLC1, Kif5C heavy chain or the Kif5C binding protein Nup358/RanBP2 of the nuclear pore complex reduced viral disassembly, recruited to the viral capsid protein IX through the tetratricopeptide repeat (TPR) of kinesin light chain (KLC) 1/2. Protein IX-deficient virions, knock down of KLC1, Kif5C heavy chain or the Kif5C binding protein Nup358/RanBP2 of the nuclear pore complex reduced viral disassembly, capsid accumulation in the periphery and infection. In unperturbed cells, disassembled capsids were retro-transported to the cell periphery with TPR and KLC2, and dynamically colocalized with the nucleoporins Nup358/RanBP2, Nup214/CAN, Nup62, and the export factors CRM1 and RanGAP but not Nup153. Our data reveal an unexpected role of microtubules and conventional kinesin in infectious entry of a large DNA virus.

EGFR and ErbB2 Mediated ERK1/2 Activation Is Required for Neisseria gonorrhoeae Invasion of Genital Epithelial Cells

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Neisseria gonorrhoeae, the causative agent of the sexually transmitted disease gonorrhea, adhere to and invade genital epithelial cells. Confocal microscopy revealed that gonococcal microcolony formation on the surface of either HEC-1-B or ME180 cells caused redistribution of both EGFR and ErbB2, a related family member. EGFR localization was primarily at cell-cell junctions, but also localized beneath the gonococcal microcolonies. Initially ErbB2 was evenly distributed on the cell surface, but redistributed to primarily encircle the gonococcal microcolonies. Inhibitors of the kinase activity of EGFR (AG1478) and ErbB2 (AG825) had no effect on their redistribution. We were able to block invasion of HEC-1-B cells without changing the adherence ability of gonococci by inhibiting activation of the ERK1/2-MAPK pathway. PD98059 and U0126, inhibitors of MEK1/2 activation, inhibited invasion by 50% and 95% respectively. SB203580, an inhibitor of p38 MAPK activation, had no effect on invasion. Also invasion was inhibited by 75% with AG1478. AG825 however, caused a 12-fold increase in invasion. The addition of N. gonorrhoeae to genital epithelial cells caused a sustained 2-3 fold increase of pERK1/2 levels in HEC-1-B cells and 10 fold increase of pERK1/2 levels in ME180 cells. This increase began by 2 hr post infection, peaked by 4 hr and remained maximal until 6 hr, the last times tested. These increases in pERK1/2 levels were completely abrogated by inhibiting EGFR activation with AG1478. In contrast to this, inhibition of ErbB2 with AG825 enhanced the gonococcal mediated increase of pERK levels. These data indicate that N. gonorrhoeae invade genital epithelial cells by modulating EGFR and ErbB2 distribution and activity leading to sustained ERK1/2 activation, which is indispensable for invasion.

A Range of Ready-to-Use Kits to Analyze the Localization, Post-translational Modification, and Function of Proteins in Cell Biology Studies

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The sheer complexity of the cellular environment is a major problem facing cell biologists, with isolation and quantification of low-abundance proteins being especially problematic. This problem can only be addressed through sample preparation or fractionation procedures. Where possible such procedures should be standardized to ensure reproducibility and allow comparison of results between labs. A range of fractionation products based on spin and gravity-flow columns and syringe-compatible FPLC cartridges enables fast, standardized fractionation of proteins from complex samples. These kits can be used to localize or characterize proteins in cells grown under different conditions (e.g., with or without siRNA, inhibitors, etc) or isolated from different tissues. In these studies we demonstrate efficient and reproducible separation of active proteins into distinct fractions, ready for further downstream analysis. Easy-to-use kits are available that separate proteins on the basis of post-translational modification (glycosylation, phosphorylation), their subcellular localization (cytoplasm, nucleus, mitochondria, membrane), and their physicochemical properties (affinity to nucleic acids, and overall solubility). The Qproteome portfolio contains additional kits for the depletion of albumin and IgG from serum or plasma samples and for extraction of proteins from FFPE samples.

A Cell Culture Model to Investigate the Invasive Pathway of Neisseria meningitidis

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Neisseria meningitidis is a major cause of gram-negative septicaemia and meningitis. However infections usually result in asymptomatic colonisation of the human nasopharynx. While mechanisms that mediate bacterial attachment to epithelial cells are understood, little is known about subsequent steps, which include translocation across the nasopharyngeal epithelium. Investigation of the translocation process is hindered by the lack of appropriate animal models, and the technical complexity of human organ culture models. In this project we have established a physiologically relevant cell culture model of the upper respiratory epithelium and used it to investigate translocation of N. meningitidis. The human bronchial epithelial cell line ‘Calu-3’ is widely used in the aerosol medicine field to investigate drug delivery across respiratory epithelia, but has not been used to investigate pathogen invasion. Calu-3 cells were grown over the course of five days on a porous membrane where they formed a differentiated, polarised monolayer. Using transmission electron microscopy we demonstrated that the gross morphology of these monolayers closely resembles that of the respiratory epithelium. The integrity of the monolayers was confirmed by: the development of high levels of transepithelial electrical resistance
Dissection of c-Met Dynamics and Signalling

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Host-pathogen interactions are frequently accompanied by induced reorganizations of the host cell actin cytoskeleton. A prominent example for these events certainly constitutes InlB-mediated host cell entry of the gram-positive food-borne pathogen *Listeria monocytogenes*. InlB is known to interact with the hepatocyte growth factor / scatter factor (HGF/SF) receptor c-Met, a prominent receptor tyrosine kinase, which has been shown to be essential for this route of bacterial internalization. Signalling through c-Met has also been implicated in the regulation of development, tissue regeneration and tumor invasiveness. However, the precise molecular mechanisms driving c-Met dynamics at the plasma membrane and its signalling are still elusive. To achieve a better understanding of the dynamic interactions between c-Met, components of endocytic machineries or regulators of the actin cytoskeleton, which have all previously been implicated in c-Met internalization and signalling, we generated a fluorescently-labelled c-Met variant (c-Met-EGFP). Expression and maturation of the fluorescently-tagged receptor was virtually identical to the wildtype protein. Furthermore, the signalling capability of c-Met-EGFP could be confirmed by restoration of HGF- and InlB-induced ruffling upon expression of c-Met-EGFP in c-Met-deficient cells. To get more insight into c-Met dynamics at the plasma membrane, we performed total internal reflection fluorescence (TIRF) microscopy of cells co-expressing c-Met-EGFP and markers for clathrin- or caveolin-mediated endocytosis. Interestingly, c-Met-EGFP was highly dynamic and frequently accumulated in discrete pit-like structures, which never coincided with clathrin-coated pits. Instead, significant overlap and co-internalization could be observed when comparing c-Met dynamics with caveolin. Moreover, transient and reversible interference with clathrin pit turnover by treatment of cells with primary alcohols affected neither caveolin nor c-Met dynamics. Future studies are aimed at unravelling the detailed timing of signalling and association of prominent receptor tyrosine kinases with different endocytic machineries, and clarifying the functional relevance of these interactions in normal physiology and disease.

Infection of Insect Cells with *Choristoneura fumiferana* Defective Nucleopolyhedrovirus (CfdefNPV): A TEM Study of Spindlin Proteins

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The eastern spruce budworm, *Choristoneura fumiferana*, is one of the most destructive forest insect pests in Canada and the north eastern United States. Wild-type *C. fumiferana* nucleopolyhedrovirus is composed of two distinct viral species, CMNPV and CfdefNPV that appear to have mutualistic association within the host larva. The virus is a potential candidate in insect pest control. While CfdefNPV is capable of replication across the membrane; confocal and electron microscopy showing tight junction formation between cells; and the inability of non-invasive bacteria to penetrate the monolayer. The apical surface of model epithelia was infected with a 1:1 ratio of wild-type and various mutants of *N. meningitidis*. Analysis of the ratio of bacteria erecting the basal surface of the monolayer allowed identification of bacterial factors which are required for this step in pathogenesis. The results indicate that the bacteria traverse the layer through (not between) the epithelial cells and experiments are underway to define the pathway used. We will use the model to define both the host and pathogen genes involved in the translocation process.

**2008**

Direct Deciphering of *Plasmodium falciparum* Proteins Destined to the Surface of the Infected Erythrocyte

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Malaria, continues to be a major public health concern causing a huge toll of morbidity and mortality in young children and pregnant women in sub-Saharan Africa. The major culprit, *Plasmodium falciparum*, has a complex life cycle and passes through several developmental stages with different protein expression profiles. Pathogenesis in the human host is attributed to the erythrocytic stages of infection via sequestration of parasite infected red blood cells (pRBCs) in the microvasculature of various organs such as the placenta in pregnant women. The parasite remodels the RBCs extensively upon invasion. Many antigens are destined to the RBC-surface for insertion/secretion and take part in the disease pathology. Analyzing freshly isolated pRBCs from the placenta of Ugandan women post-partum we have in a recent study characterized the nature of host receptors involved in the placental sequestration of pRBCs. To substantiate our understanding of sequestration and identity potential vaccine candidates, identification of pRBC surface proteins is the next important step. **Aim:** To develop a direct approach providing a snapshot of the surface associated/secreted signature parasite peptides in infected erythrocytes. **Methods:** Mild trypsinization was performed on live pRBCs. Released peptides were pre-fractionated based on pl, using an OFFGEL Fractionator. Massspectrometric analysis was performed on each fraction by nanoflow-liquid chromatography/electrospray ionization. **Results & Conclusions:** To date, direct analysis of the pRBC surface sub-proteome has been a technical challenge owing to low abundance, variability and poor solubility of these proteins. In addition, analysis of pRBC lysates is impeded by the presence of the highly abundant hemoglobin. Adopting two unique approaches, surface trypsinization and OFFGEL pl-fractionation, we obtained a less complex peptide-mixture identifying a number of parasite-derived proteins. Such approach may in continuation prove to be universally applicable in the study of surface proteome of other cell types than pRBCs. Rasti N et al. PNAS 2006 12;103(37).
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**Brucella Intracellular Replication Requires Trafficking through the Late Endocytic/Lysosomal Compartment**

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Upon entry into mammalian cells, the intracellular pathogen *Brucella abortus* resides within a membrane-bound compartment, the *Brucella*-containing vacuole (BCV), the maturation of which is controlled by the bacterium to generate a replicative organelle derived from the endoplasmic reticulum (ER). Prior to reaching the ER, *Brucella* is believed to ensure its intracellular survival by inhibiting fusion of the intermediate BCV with late endosomes and lysosomes, although such BCVs are acidic and accumulate the lysosomal glycoprotein LAMP-1. Here we have further examined the nature of intermediate BCVs using confocal microscopy and live cell imaging. We show that BCVs rapidly acquire several late endocytic markers, including the GTPase Rab7 and its effector RILP, and are accessible to fluid phase markers either delivered to the whole endocytic pathway or preloaded to lysosomes, indicating that BCVs traffic along the endocytic pathway to lysosomes. Consistently, intermediate BCVs are acidic and display proteolytic activity up to 12 h post infection. Furthermore, expression of dominant alleles of Rab7 or overexpression of RILP significantly impaired the ability of bacteria to convert their vacuole into an ER-derived organelle and replicate, indicating that BCV maturation requires functional late endocytic/lysosomal compartments. In cells expressing dominant negative Rab7[T22N], BCVs displayed decreased fusion with lysosomes, demonstrating that BCV traffic through late endocytic compartments is required for intracellular trafficking and eventual replication. These results demonstrate that BCVs traffic along the endocytic pathway, interact with lysosomes and that such interactions are required for further maturation of BCVs into a replication- permissive organelle.

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**Infection of Cerebral Endothelial Cells and Alteration of BBB Functions by HTLV-1**

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The blood-brain barrier (BBB), which constitutes the interface between blood and cerebral parenchyma, has been shown to be disrupted during retroviral associated neuromyelopathies. Human T cell Leukemia Virus type 1 (HTLV-1) retroviral infection is associated with a slowly progressive neurodegenerative disease called HTLV-1 Associated Myelopathy/ Tropical Spastic Paraparesis (HAM/TSP). BBB alteration has previously been demonstrated during the course of HAM/TSP by showing both fibrinogen leakage, IgG deposits in the CNS parenchyma, and lymphocytic passage through the thoracic cord endothelium. Besides, we have previously shown Tight Junctions alterations in spinal cord sections obtained from HAM/TSP patients, this was then confirmed in vitro (Afonso et al. J Immunology 2007). Infection of the endothelial cells (ECs) by HTLV-1 could also play a significant role in the pathogenesis of HAM/TSP. In fact, HTLV-1 encodes several proteins, among which the Tax transactivator, that can alter a number of cellular signaling pathways. Until now, there was no evidence of a possible infection of human brain ECs. This was mainly due to the scarcity of HAM/TSP autopsy material but also to the low level of HTLV-1 expression in tissues in general. In the present study, we first demonstrate that the Glut-1 and Neuropilin-1 proteins which participate in the entry of the virus are expressed in the ECs on spinal cord sections of uninfected donors as well from HAM/TSP patients. Using our previously characterized in vitro human model of BBB, we also established that ECs can be productively infected and that this infection leads to an alteration of the BBB functions as exemplified by a modified endothelial permeability and by lymphocytes transmigration. In conclusion, infected ECs could therefore facilitate the HTLV-1 transmission to the CNS, either by facilitating the entry of infected cells into the CNS of by producing virions that could infect astrocytes.

2912

**Molecular Regulation of Actin Rearrangements Accompanying Salmonella Invasion**

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The enteric pathogen *Salmonella typhimurium* activates host cell signalling cascades by type III-mediated delivery of bacterial virulence proteins into the host cell cytosol, which promotes uptake of the bacteria into normally non-phagocytic cells. The cocktail of translocated bacterial factors modifies host cell signaling, accompanied by activation of small GTPases of the Rho family. Members of these such as Cdc42 and Rac1 are well established to drive the nucleation of actin filaments, catalyzed for instance by the Arp2/3 complex, which has previously been implicated in many cellular processes including the engulfment of different bacteria. Consistently, the uptake of *Salmonella* is accompanied by extensive membrane ruffling, which is considered an essential pre-requisite for the translocation of the bacteria into the host cell cytosol. In this study, we investigated *S. typhimurium* invasion in cell lines ablated by RNA interference for or genetically deficient of components of Cdc42- and Rac1 signaling pathways to Arp2/3-mediated actin polymerization. Surprisingly, neither WAVE-complex nor N-WASP, prominent activators of Arp2/3-complex-mediated actin assembly were observed to function in bacterial entry, since individual and collective removal of both type I nucleation promoting factors did not reduce invasion. Yet, actin and Arp2/3-complex accumulation below bacteria was observed in both the presence and absence of WASP/WAVE family members. Consistently, Arp2/3-complex contributed to efficient bacterial entry. Interestingly, interference with WAVE-complex function abrogated *Salmonella*-induced ruffling without affecting entry efficiency, suggesting that ruffling and bacterial invasion may be separable processes. Scanning electron microscopy of WAVE-complex-defective cells revealed entry events, which appeared to be mediated by small leaflets of plasma membrane zipper around attached bacteria. Thus, we conclude that *Salmonella* induced ruffling is driven by WAVE-complex but not required for invasion. Future experiments aim at unraveling the molecular mechanisms driving this entry pathway, including identification of the elusive Arp2/3-complex activator and of the Arp2/3-complex-independent actin assembly activity.
Endoplasmic Reticulum Stress Is Activated by Enterovirus 71 and Possibly Contributes an Alternative Pathway of Translation Attenuation Caused by Picornavirus Infection

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EV71 infection induced endoplasmic reticulum (ER) stress in human rhabdomyosarcoma cells (RD) manifested by BiP and calreticulin protein induction, eukaryotic initiation factor 2alpha (eIF2α) phosphorylation, XBP1 splicing, and a general reduction in protein synthesis as assessed by [35S]methionine incorporation. However, transcriptional activation of XBP1 target gene EDEM was inhibited. We showed that p90ATF6 was cleaved by EV71 infection but its transcriptional active product was degraded and thus not seen in the nucleus. BiP and calreticulin, normally activated by ATF6, were activated by a virus-induced, ATF6-independent mechanism at protein but not mRNA level. In addition, transactivating activity of ATF6 was not increased by viral infection. To further increase our understanding of protein synthesis attenuation caused by UPR induced by EV71, we have stably overexpressed BiP epigenetically in RD cells and examined the effect on stress response and viral replication. BiP overexpression mitigated EV71-induced eIF2α phosphorylation and thus substantially rescued translational attenuation. Alleviated UPR resulted in reduced viral protein expression and replication, suggesting UPR is required during infectious cycle. BiP overexpression did not rescue ATF6 cleavage during infection, suggesting that the disappearance of ATF6 induced by E71 is not due to reduced protein expression. The time course for eIF2α phosphorylation correlates with that for inhibition of cellular protein synthesis. Host protein synthesis was restored by BiP overexpression. Collectively, UPR induced by EV71 can regulate host protein synthesis. We thus established that EV71 encodes yet another function, distinct from those required to counteract initiation factors in the translation mechanism, to reduce translation in the presence of ER stress. These results suggest that, although EV71 infection induces the unfolded protein response, it regulates the outcome to combat cellular response to benefit viral replication.

Chlamydia trachomatis Infection Suppresses Centrosome Clustering

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Chlamydia trachomatis is an obligate intracellular bacterium responsible for four million new cases of sexually transmitted disease each year. We have previously reported that C. trachomatis interacts with the cell’s centrosome in a dynein dependent manner. This interaction induces supernumerary centrosomes in infected cells, which leads to multipolar spindles and can inhibit accurate chromosome segregation during mitosis. Positioning of the microtubules and centrosomes is critical for cell cycle control. Pre-cancerous cells possess the capability to cluster centrosomes suppressing the multipolar spindle phenotype, in a dynein dependent manner. We hypothesize that Chlamydia not only causes defects in centrosome numbers, but by interfering with dynein can inhibit centrosome clustering. The N1-e115 neuroblastoma cancer cell line normally exhibits a multipolar centrosome phenotype; however, these cells are able to coalesce the centrosomes into two functional spindle poles necessary for completion of the cell cycle. We utilized the inherent multipolar suppression mechanism of these cells to determine if chlamydial infection inhibits centrosome clustering. Our findings indicate that although neuroblastomas infected with Chlamydia show little difference in centrosome numbers, we see a dramatic decrease in centrosomal clustering compared to uninfected controls. Subsequently, neuroblastomas demonstrate a multipolar spindle phenotype at 36 hours post-infection. This suggests that the intimate interaction between Chlamydia and dynein affects the cell’s ability to suppress multipolar spindles via the clustering mechanism.

Implication of RtxA Toxin from V. vulnificus in Intestinal Epithelial Cell Death

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Vibrio vulnificus is a gram-negative bacterium that inhabits marine or estuarine areas, and is a causative agent of food-borne diseases, such as human life-threatening septicemia, and wound infection. Although a variety of factors that contribute to the pathogenesis of V. vulnificus infection have been proposed, the contribution of repeats-in-toxin (Rtx)A toxin has not been examined. In this study, we investigated the role of V. vulnificus toxin in intestinal epithelial cell (CMT-93) death. ROS was significantly generated in response to V. vulnificus infection of CMT-93 cells via NAD(P)H oxidase 1, which resulted in eventual host cell death. Epithelial cells infected with a mutant strain of V. vulnificus that was defective for RtxA failed to induce ROS, which significantly attenuated cell death. Pretreatment of cells with diphenylene iodonium (DPI), an NAD(P)H oxidase inhibitor, reduced the level of ROS in infected cells, and host cell death. Activation of matrix metalloproteinases-2 and -9 was also noted following V. vulnificus infection, which might contribute to rapid destruction of host tissues following the infection. However, mutant strain of V. vulnificus that was defective for RtxA infected or DPI pretreated epithelial cells were completely abolished activation of MMPs. Taken together, these results indicate that V. vulnificus induces host cell death by inducing the generation of ROS, and that RtxA plays an important role in this process. This work was supported by grant No. RTI 05-01-01 from the Regional Technology Innovation Program of Ministry of Commerce, Industry and Energy (MOCIE). These authors are supported by Research Center for controlling Microbial Virulence.

Identification and Characterization of a Putative T. gondii IκB Kinase

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Toxoplasma gondii is an obligate intracellular parasite that grows in a specialized compartment, the parasitophorous vacuole (PV) delimited from the host cytoplasm by the parasite-modified PV membrane (PVM). T. gondii infection causes the inhibition of host apoptosis in an NF-κB dependent manner. Activation of the NF-κB pathway by T. gondii correlates with the localization of phosphorylated IκB at the PVM, which is mediated by a T. gondii-derived kinase activity (TgIKK). TgIKK is capable of phosphorylating serines 32 and 36 of IκBα which are well-established targets for the mammalian IKK complex and are critical for the activation of NF-κB. TgIKK phosphorylation of IκB at the PVM is independent of the host IKK since p-IκBα is detected in IKK−/−, β−− infected cells. On the other hand TgIKK and host IKK complex are both required to sustain NF-κB activity.
in the infected host cell. Biochemical studies revealed the conditions for optimum activity of the TgIKK allowing the use of kinase assays for monitoring its activity. Using the fusion protein GST-IkB, encoding full length IkB and a GST-IkB<sub>1-54</sub> truncation construct as substrates in pulldown experiments, we were able to enrich for the TgIKK activity. Multidimensional Protein Identification Technology (MuDPIT) was used to identify a putative TgIKK based on the pulldown of the activity on GST-IkB<sub>1-54</sub> beads. The putative kinase (583.m05729 in the Toxoplasma genome, www.ToxoDB.org) has homology to mammalian IKK particularly in the kinase domain. We have subcloned and are characterizing the predicted full length cDNA and are in the process of generating a targeted deletion which will enable us to confirm it as the TgIKK and define its function and role in pathogenesis.

2917

**Interactions between Bacillus anthracis Spores and Host Epithelial Cells**

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Internalization by host non-phagocytic cells can be advantageous to bacterial pathogens for dissemination, immune evasion and nutrient acquisition. Investigations into the mechanisms by which bacteria induce their own uptake by non-phagocytic cells (i.e. epithelial cells) have not only improved the understanding of how bacteria exploit host machineries, but have also revealed how the different cellular machineries cooperate to allow the entry of microbes. *Bacillus anthracis* is a Gram-positive, aerobic, spore-forming pathogen that causes anthrax. Infections are usually initiated by the entry of the spore form into the host through the gastrointestinal tract or cuts in the skin. After entry, spores germinate to become vegetative bacilli which release virulence factors and replicate to high titers in the blood. Systemic infections usually lead to host death. Recent animal studies indicated that in inhalational anthrax, dissemination of spores from the lung is a critical step in the establishment of infection. The proposed mechanism of spore dissemination has been via alveoli macrophages. We have recently reported that *B. anthracis* spores were able to attach to and subsequently be internalized by fibroblasts and intestinal epithelial cells (Russell et al., Cell Micro., 2007). These studies raised the possibility that epithelial cells may provide a means for *B. anthracis* spore dissemination. We have initiated studies to further investigate this topic using a combination of gentamicin protection assays, electron microscopy and fluorescence microscopy. The results showed that 1) both cultured A549 cells (a human lung epithelial cell line) and primary human small airway epithelial cell can support *B. anthracis* spore attachment and internalization; 2) the internalization was an actin-dependent process, 3) internalized spores could survive in the lung epithelial cells and possibly replicate intracellularly, and 4) *B. anthracis* could cross an epithelial cell barrier without disrupting the barrier integrity.

2918

**Macrophages and Dendritic Cells Infected with Leishmania amazonensis Express Hypoxia-inducible Factor-1α**

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Increasing evidence indicates that hypoxia-inducible factor 1α (HIF-1α) can be up regulated in different cell types by nonhypoxic stimuli such as growth factors, cytokines, nitric oxide, lipopolysaccharides and a range of infectious microorganisms. In this study, the ability of the following mononuclear phagocytes to express HIF-1α is reported: mouse macrophages (mMΦ), human macrophages (hMΦ) and human dendritic cells (DC), parasitized in vitro with *Leishmania amazonensis*; as assessed by immunofluorescence microscopy. A logical explanation for HIF-1α expression might be that the mononuclear phagocytes became hypoxic after *L. amazonensis* infection. Using the hypoxia marker pimonidazole observation revealed that *L. amazonensis*-infected cells were not hypoxic. In addition, experiments using a HIF-1α inhibitor, CdCl<sub>2</sub>, to treat *L. amazonensis*-infected macrophage cultures showed reduced parasite survival. These studies indicated that HIF-1α could play a role in adaptive and immune responses of mononuclear phagocytes presenting infection by the parasite *L. amazonensis*.

2919

**Response of Dictyostelium discoideum Amoeboid Cells to Microbial Ligands**

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Mammalian innate immune cells rely on pattern-recognition receptors, such as the Toll-like receptors, to detect pathogen-associated molecular patterns (PAMPs) on microbial invaders. The pattern-recognition receptors used by mammalian immune cells are conserved in a variety of organisms, and thus can be studied using simple experimental model systems. The slime mold *Dictyostelium discoideum* is a unique model organism that exists for part of its lifecycle as single-celled amoebae, but is induced to form a multicellular sporulating body upon starvation. As amoeboid cells, *D. discoideum* phagocytose bacteria for nutrient uptake, and the mechanisms underlying this process are similar to those used by mammalian innate cells to eliminate invading pathogens. It has not been appreciated, however, whether *D. discoideum* detect bacterial prey using the same types of pattern-recognition receptors as do mammalian immune cells. Here we show that *D. discoideum* cells indeed respond to known PAMPs, as exposure to the bacterial cell wall products LPS and peptidoglycan induces the production of reactive oxygen species. Furthermore, pretreatment of starving cells with LPS delays the development of sporulating bodies, suggesting that *D. discoideum* may identify the cell wall products as potential bacterial prey. Finally, as measured by qRT-PCR, exposure of amoeboid cells to LPS and peptidoglycan upregulates the expression of genes with homology to those involved in microbial recognition by mammalian immune cells. Upregulated genes include a putative scavenger receptor and a gene containing the toll/interleukin-1 receptor domain present in Toll-like receptors. We are currently in the process of creating *D. discoideum* cells that overexpress or are deficient for these gene products. The characterization of such genes in *D. discoideum* should provide insight into conserved molecular mechanisms underlying microbial recognition by both *D. discoideum* and mammalian immune systems.
Late Abstracts Wednesday

Cell Biology of the Immune System (2920-2924)

2920
Positive Regulation of T Cell Activation by Swiprosin-1
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Antigen-independent adhesiveness of T lymphocytes and antigen-presenting cells (APCs) is essential for scanning for specific antigens on the APC surface and for initiating immune response. Here we show, through time-lapse imaging of live cells, that a newly discovered protein swiprosin-1 is involved in the interaction between T cells and APCs. Swiprosin-1 was associated with F-actin and enriched in the membrane-ruffling region although it was presented primarily in the cytoplasmic region. Swiprosin-1 was also over-induced during T cell activation stimulated by CD3/CD28 or PMA/A23187. We observed that swiprosin-1 was highly clustered to the region distal to the outside of pSMAC in immunological synapse, a specialized junction between T lymphocytes and APCs. Accordingly, over-expression of swiprosin-1 increased IL-2 production with SEE-loaded APC stimulation. Conjugate formation assay revealed that swiprosin-1 has no significant effect on the stabilization of immunological synapse after T cell contact with APCs. Interestingly, however, swiprosin-1 dramatically increased the initial conjugate formation of T lymphocytes and APCs. Time-lapse video microscopy revealed that the conjugate formation was also prominent even in the absence of SEE on APC, thereby suggesting that swiprosin-1 may play important roles for the initial scanning of the APC surface by T cells before antigen recognition. Further study is required to understand the molecular mechanisms how swiprosin-1 increases initial contact of T cells and APCs.

2921
TNF-α Suppresses the Expression of Clock Genes by Interfering with E-box-mediated Transcription
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Production of TNF-α in infectious and autoimmune diseases is associated with fever, fatigue, and sleep disturbances, which are collectively referred to as sickness behavior syndrome. In mice TNF-α increases non-rapid eye movement sleep. Because clock genes regulate the circadian rhythm and thereby locomotor activity which may alter sleep architecture we assessed the influence of TNF-α on the circadian timing system. Here, we show that TNF-α suppresses the expression of the PAR bZip clock-controlled genes Dhp, Tef, and Hlf and of the period genes Per1, Per2, and Per3 in fibroblasts in vivo and in vivo in the liver of mice infused with the cytokine. Furthermore, using clock reporter genes we demonstrate that TNF-α inhibits the CLOCK-BMAL1-induced activation of E-box regulatory element-dependent clock gene promoters. The inhibitory effect of TNF-α on Dhp occurs very rapidly. In contrast and surprisingly, the expression of Per1 and Per2 is first induced before it is repressed later, suggesting different mechanisms at least at early time points. Different hypotheses may account for the mechanism of TNF-α action on clock gene promoters. Among these are different posttranslational modifications such as phosphorylations of CLOCK and/or BMAL1 leading to relocalisation of the proteins within the cell and reduced DNA-binding affinity, and a reduction in histone acetylation in the promoters of affected genes. So far, reduced binding activities of BMAL1 to the dhp promoter can be observed and although there is less acetylated histone H3, active deacetylation seems not to be involved. In addition we can show that early TNF-α dependent Per1 and Per2 induction, but not Dhp inhibition, is due to a rise in intracellular Ca²⁺. The mechanism of TNF-α interference with well orchestrated clock gene expression and with locomotor activity in mice will be assessed further.

2922
Epidermal Growth Factor Increases Prostaglandin E₂ Production via ERK1/2 Mitogen Activated Protein Kinase and Nuclear Factor kappa B Pathway in Rheumatoid Arthritis Synoviocytes and Osteoarthritic Chondrocytes
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Background: High concentration of epidermal growth factor (EGF) is present in the synovial fluid of rheumatoid arthritis (RA), implicating the involvement of EGF in the pathogenesis of rheumatoid diseases. Objective: To investigate if EGF is involved in the regulation of prostaglandin-endoperoxide synthase 2 (PTGS2) and the prostaglandin E₂ (PGE₂) production in human RA fibroblast like synoviocytes (FLS) and osteoarthritic chondrocytes (OC). Methods: The levels of PTGS2 and microsomal prostaglandin E synthase-1 (mPGES-1) were evaluated using RT-PCR and immunoblot analysis. Electrophoretic mobility shift assay (EMSA) was performed to investigate EGF mediated DNA binding activity of nuclear factor-xB (NF-xB). 6-keto-prostaglandin F₁α (6-keto-PGF₁α) and prostaglandin E₂ (PGE₂) levels were analyzed by ELISA. Results: EGF enhanced both PTGS2 protein and mRNA expressions. mPGES-1 mRNA level was also increased by EGF treatment. EGF also stimulated ERK1/2 MAPK activity on the APC surface and for initiating immune response. Here we show, through time-lapse imaging of live cells, that a newly discovered protein swiprosin-1 is involved in the interaction between T cells and APCs. Swiprosin-1 was associated with F-actin and enriched in the membrane-ruffling region although it was presented primarily in the cytoplasmic region. Swiprosin-1 was also over-induced during T cell activation stimulated by CD3/CD28 or PMA/A23187. We observed that swiprosin-1 was highly clustered to the region distal to the outside of pSMAC in immunological synapse, a specialized junction between T lymphocytes and APCs. Accordingly, over-expression of swiprosin-1 increased IL-2 production with SEE-loaded APC stimulation. Conjugate formation assay revealed that swiprosin-1 has no significant effect on the stabilization of immunological synapse after T cell contact with APCs. Interestingly, however, swiprosin-1 dramatically increased the initial conjugate formation of T lymphocytes and APCs. Time-lapse video microscopy revealed that the conjugate formation was also prominent even in the absence of SEE on APC, thereby suggesting that swiprosin-1 may play important roles for the initial scanning of the APC surface by T cells before antigen recognition. Further study is required to understand the molecular mechanisms how swiprosin-1 increases initial contact of T cells and APCs.

2923
Anti-inflammatory and Neuro-protective Effects of Andrographolide on Microglial and Neuronal Cells
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Andrographolide (Andro) is one of the active components of Andrographis paniculata, a Chinese official herbal medicine used as an anti-inflammatory drug and hypoglycemia. Andro is known to display anti-inflammatory activity, but its effects and mechanisms in microglia and neuron are not clear. Microglial cells become activated and play deleterious roles during brain injury and various neurodegenerative diseases such as ischemic stroke by over-responsiveness or improper regulation through producing excessive pro-inflammatory mediators including nitric oxide (NO), cytokines (e.g., interleukin-1β (IL-1β) and tissue necrosis factor-α (TNF-α), and reactive oxygen species (ROS) in CNS. In the present work, we examined the effects of Andro on LPS- and IFN-γ-stimulated microglia cells (BV-2) and in oxygen-glucose deprivation (OGD)-activated microglia and neurons. Andro concentration-dependently reduced iNOS and NO production in LPS- and IFN-γ-stimulated BV-2 possibly through inhibition of nuclear factor-κB by suppressing IkB activation and ERK activation. Andro also inhibited LPS-induced ROS and cytokine (TNF-α and IL-1β) production in BV-2 through modulation of intracellular calcium mobilization. Furthermore, Andro decreased OGD-induced protein nitrotyrosin formation in BV-2 by reducing the iNOS expression. OGD triggered a mitochondria dysfunction-dependent ROS production leading to neuronal cell death. Andro prevented OGD-induced neuronal death but did not reverse the OGD-triggered mitochondria dysfunction. Based on these results, we conclude that Andro is a potent anti-inflammatory drug in compromising microglia activation during inflammation and hypoxia condition. It is also protective against hypoxia-induced neuronal death. These effects confer Andro beneficial properties for the treatment of inflammation-related CNS disease.

2924
Evaluation of Kinase Inhibitors In Vitro using Biomarkers for Mechanism of Action and Efficacy
R. R. Subramanian; Cell & Tissue Biology, Pfizer Inc, Cambridge, MA
Biomarkers are used to evaluate whether clinical treatments affect the correct pathway and target. These biomarkers can be for mechanism of action or for efficacy of pharmacological agents. To determine whether biomarkers for mechanism of action and efficacy are correlated, and which biomarker is best suited for clinical results, we evaluated the effect of compounds on the phosphorylation status of Inducible T-cell kinase (ITK) and p38 kinase, and the subsequent release of cytokines (e.g. TNF-α, IL-1β and IL-2). p38 kinase plays a critical role in regulating inflammatory cytokine production (TNF-α and IL-1β) in Rheumatoid arthritis, while ITK is a kinase target in asthma whose activation results in increased IL-2 production. We used Jurkat T-cells treated with anti-CD3 antibody to stimulate T-cell receptor signaling and thus activate ITK phosphorylation and signaling. To evaluate p38 kinase activation we used PMA-differentiated U937 cells which were stimulated with LPS to induce p38 activation and signaling. Briefly, the cells were incubated with kinase inhibitors for 1h prior to treatment with anti-CD3 antibody or LPS. Phosphorylation kinetics was determined so as to evaluate cellular phospho-p38 and phospho-ITK status at peak phosphorylation times. The phosphorylation of ITK and p38 kinases was measured at 30min post-stimulation using FACS and multiplex ELISA respectively. Release of cytokines from the above cell types were measured using multiplex ELISA 24h post-stimulation. Our results indicate that decreased phospho-p38 or phospho-ITK (mechanism of action biomarker) correlates with decreased TNF-α, IL-1β and IL-2 secretion respectively (efficacy biomarker). Thus, biomarkers for mechanism of action and efficacy are closely related for these two targets, and biomarkers of efficacy may provide a broader window to assess clinical value of kinase inhibitors.

Imaging Technology (2925-2929)

2925
Model Convolution: A Computational Approach to Digital Image Interpretation
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Digital fluorescence microscopy is commonly used to track individual proteins and their dynamics in living cells. However, extracting molecule-specific information from fluorescence images is often confounded by the noise and blur intrinsic to the cell and the imaging system. Here we present a method called “model-convolution,” which uses experimentally measured noise and blur to simulate the process of imaging fluorescent proteins whose spatial distribution cannot be resolved. We then compare model-convolution to the more standard approach of experimental deconvolution. In some circumstances, standard experimental deconvolution approaches fail to yield the correct underlying fluorophore distribution. In these situations, model-convolution removes the uncertainty associated with deconvolution and therefore allows direct statistical comparison of experimental and theoretical data. Thus, the model-convolution method better utilizes information gathered via fluorescence microscopy, and naturally integrates experiment and theory. In addition, it can be used to validate and test image processing algorithms. Development of a freely available ImageJ “model-convolution” plug-in, which will be demonstrated, allows for straightforward integration of model-convolution as a standard method for interpretation of digital fluorescence images.

2926
Quantitative 3-D Imaging of Eukaryotic Cells Using Soft X-ray Tomography
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An enormous amount of effort has been devoted to imaging and classifying organelles. We have developed a microscope that is especially well suited to this endeavor. We use an x-ray microscope to perform tomography of fully-hydrated, intact cells that have not been subjected to dehydration, sectioning, or staining - the cells are simply flash frozen. The soft x-rays used are more strongly absorbed by organic material than by water by an order of magnitude, and can penetrate specimens up to 10 microns thick. The natural contrast yields a quantitative measurement of the x-ray density of specific cellular components, based on the amount of carbon and nitrogen they contain, with a spatial resolution of 40 nm or better.
This allows identification of organelles based on comparisons with expected x-ray absorption values. A full tomographic data set with images of several cells can be collected in less than three minutes, so relatively large numbers of cells can be imaged and analyzed in a short period of time. We present soft x-ray tomography results on the fission yeast Schizosaccharomyces pombe. After collecting data on four cells at different stages of the cell cycle, the 3-dimensional images were digitally segmented to allow identification and quantification of individual organelles. We were able to identify the nucleus, lipid vesicles, vacuoles, and mitochondria based on their x-ray density and their shape, and were then able to measure the numbers, volumes, and spatial distributions of these organelles in the cells.

2927
Image Tiling vs. Cell Segmentation: A Case Study
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Segmentation is a common pre-processing step in image analysis used to identify cells in an image and perform subsequent processing steps only on these isolated cells. In contrast, tiling is simply dividing the image into equally sized sub-images. In this work, we compare the effectiveness of these two approaches in accurately classifying phenotypes generated in a RNAi screen. We have utilized a subset of a commercially available dsRNA library (Open Biosystems) to cause single gene knockdown in cultured insect cells. We employed an automated system to capture images of the resulting phenotypes which were either segmented or tiled 4x4 into 16 sub-images. The cells and tiles were then analyzed using a multi-purpose image classifier (WND-CHARM). Surprisingly, the results showed that the phenotype classification accuracy after tiling was ~20% higher than when the cells were segmented from the image background. We conclude that while segmentation is often used by biological image analysis applications, in some cases better results can be achieved using simple tiling of the cell images.

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Measuring FRET by Acceptor Photobleaching and Sensitized Emission Using the Nipkow Spinning Disk Confocal Microscope
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Forster’s Resonance Energy Transfer (FRET) is a process in which a donor fluorophore in the excited state non-radiatively transfers energy to an acceptor molecule. Although a number of methods to measure FRET by microscopy have been devised and implemented, the accuracy of these methods is still unknown which makes interpretation of FRET efficiency values difficult between the methods and instruments. Common methods for measuring FRET include sensitized emission and acceptor photobleaching. During this study the methods of sensitized emission and acceptor photobleaching were compared using a Nipkow Spinning Disk Confocal Microscope (SDCM) coupled with a novel FRAP accessory module. Also a comparison of two different confocal systems, SDCM fitted with the FRAP accessory module and the Point Laser Scanning Confocal Microscope (LSCM) for acceptor photobleaching was investigated. Results for FRET efficiency showed that using the Spinning Disk Live Cell Imaging system FRET efficiency for the control, ECFP-EYFP a common standard FRET pair was 32.12±8.30% for sensitized emission and 30.02±6.68% for acceptor photobleaching. Results for ECFP-EYFP from the LSCM showed that the FRET efficiency for the pair was 33.8±2.83% and 8.36±1.49% for the negative control whereas for the SDCM these were 30.68±6.68% and 1.81±1.13%, respectively, for acceptor photobleaching. In conclusion this study shows that sensitized emission and acceptor photobleaching using the SDCM fitted with a FRAP accessory module produced comparable results for ECFP-EYFP. In addition, the SDCM with the FRAP module also produced comparable FRET values as the LSCM system when negative control values were taken into account.

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CTO (Confocal/Tem Overlay) Microscopy: Bridging the Gap between Confocal and Electron Microscopy of GFP/YFP Fusion Proteins in Cultured Cells
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Cartilage oligomeric matrix protein (COMP) is a large pentameric glycoprotein found in the extracellular matrix of cartilage, tendon and ligament. While its precise function remains unclear, it is thought to act as a structural protein in the matrix via its interactions with other matrix components. The importance of COMP in normal joint development and function is highlighted by the fact that COMP mutations can cause pseudoachondroplasia and multiple epiphyseal dysplasia, two autosomal dominant human skeletal disorders characterized by dwarfism and osteoarthritis. Central to their pathogenesis is disturbed COMP secretion that results in accumulation of mutant COMP in the endoplasmic reticulum of growth plate chondrocytes. While it is clear that mutant COMP is retained within the ER of cells, there is currently no detailed understanding of the processes involved in the retention of mutant COMP or of the precise consequences that this retention and accumulation has on protein trafficking in the ER and on the physiology of the cell as a whole. As such we have used a cell culture model system to perform a detailed study of the effects and consequences of accumulation of mutant COMP in the ER of chondrocytes. To generate a model cell line for study, rat chondrosarcoma (RCS) cells were transfected with a construct encoding cartilage COMP harboring the pseudoachondrodysplasia causing mutation del D469 and fused via its C-terminus to YFP. Stably transfected cells were selected by growth and repeated passage over approximately 2 weeks. Cells were sorted by FACS to enrich for brightly fluorescent cells and the sorted cells were then expanded. For Confocal/TEM Overlay Microscopy, cells were seeded onto thermonox cover slips in the wells of a 12 well culture plate at 2.5 x10^5 cells per well and cultured for 48 hours to allow the cells to adhere and spread.
Rewiring of JIP-1-dependent JNK Signaling Pathway Using Heterologous Scaffold Assembly
J. Moon, P. Sang-Hyun; Seoul National University, Seoul, Republic of Korea
In a range of MAP Kinase pathway module, scaffold proteins play an important role in enhancing the efficiency and specificity of signaling pathways. In case of Yeast, it is known that the stereochemical perturbation of yeast scaffold protein, Ste5 and rewiring kinase to scaffold by alternative linkage, but not by original docking does not disrupt signal transduction, thus tethering of pathway components is the fundamental mechanism of scaffold proteins. However, it is not clear whether mammalian scaffold protein simply assemble signaling proteins of MAP Kinase pathway or actively participates in MAP Kinase cascade. We would examine whether the main role of scaffold protein is also conserved in the mammalian protein, JIP1. To begin with, we made JNK(MAPK) docking mutation of JIP1 that disrupted signal transduction as well as the interaction between JNK and JIP1. Then, we fused JNK and JNK docking mutant respectively to PDZ domains-nNOS PDZ and Syn PDZ- in order to bind to each other. The interaction between PDZ domains help JNK to bind to the JNK docking mutant. We confirmed that the connection of them could restore JNK pathway, although the level of JNK phosphorylation is lower than the activation of JNK dependent on wild type, JIP1. Furthermore, we would investigate the recruiting of signaling pathway by alternative assembly in the case of MKK7 as well as JNK. It could demonstrate that the role of tethering the components of the pathway is conserved in mammalian scaffold, JIP1 and that the assembling of the components could deliver the signaling, but not at the original kinase docking site in scaffold protein.

Analyses of Threonine and Tyrosine in Dual Phosphorylation of MAP Kinases in Saccharomyces cerevisiae by Mass Spectrometry
M. Choi, S. Park; Seoul National University, Seoul, Republic of Korea
The mating and high osmolality responses in the budding yeast, Saccharomyces cerevisiae, depend on the MAP kinase signaling cascade. The dual phosphorylation of MAP kinase is known to be essential for generating signaling flux. Fus3 is dually phosphorylated at threonine-180 and tyrosine-182 residues by MAP kinase kinase Ste7 when simulated by mating pheromone, while Hog1 is dually phosphorylated at threonine-174 and tyrosine-176 residues by MAP kinase kinase Pbs2 in response to increases in extracellular osmolarity. Previous study has been done with Fus3 and Hog1 to demonstrate the order and magnitude of dual phosphorylation using quantitative mass spectrometry. Since, both the tyrosine and threonine phosphorylations are necessary for full enzymatic activity, we performed functional assays and quantitative mass spectrometry with mutations in threonine and tyrosine residues of Fus3 (Fus3<sup>WT</sup>, Fus3<sup>T180F</sup>, Fus3<sup>T182F</sup>) and Hog1 (Hog1<sup>WT</sup>, Hog1<sup>T174A</sup>, and Hog1<sup>T176F</sup>) to verify the order and magnitude of phosphorylation of threonine and tyrosine residues in dual phosphorylation. Fus3 and Hog1 were TAP-tagged to isolate them out of cell lysate at different time points after stimulation, and the phosphorylation of threonine and tyrosine residues were quantitatively analyzed using mass spectrometry with reference phospho-peptides.

Bimolecular Fluorescence Complementation Analysis System for In Vivo Detection of Protein-Protein Interaction in Saccharomyces cerevisiae
M. Sung, W. Huh; School of Biological Sciences, and Research Center for Functional Cellulomics, Seoul National University, Seoul, Republic of Korea
Bimolecular fluorescence complementation (BiFC) assay has been widely accepted for studying in vivo detection of protein-protein interaction in several organisms. To facilitate the application of BiFC assay to yeast research, we have created a series of plasmids that allow single-step PCR-based, C- or N-terminal tagging of yeast proteins with yellow fluorescent protein fragments for BiFC assay. By examination of several interacting proteins (Sis1-Sis1, Net1-Sir2, Cet1-Cet1, and Pho2-Pho4), we demonstrate that BiFC assay can be used to reliably analyze occurrence and subcellular localization of protein-protein interaction in living yeast cells.

Absolute Quantification and Profiling of MAPK Phosphorylation by Mass Spectroscopy
N. Lee, S. Park; Dept. of Biological Science, Seoul National University, Seoul, Republic of Korea
Mitogen activated protein (MAP) kinase signaling is critical for various cellular responses including cell proliferation, differentiation and cell death. The MAP kinase cascade is conserved in eukaryotic kingdom as three-tiered kinase module; MAPKKK, MAPKK and MAPK and transduces signals via phosphorylation. In mammalian cells, extracellular signal-regulated kinases (ERKs) pathway plays an important role in cell growth including cell proliferation and differentiation, and is known to be activated by dual phosphorylation of ERK at threonine and tyrosine residues. The dual phosphorylation of MAP kinase is known to be essential for catalytic activity and for signal activation, but the mechanism by which the two residues are phosphorylated remains elusive. In this study, we attempted to elucidate the order and magnitude of dual phosphorylation of endogenous MAP kinases in mammals and profiled MAP kinase phosphorylation patterns using absolute quantification (AQUA) by mass spectrometry.
CelluSpots represent a new method [1] that allows the production of hundreds of identical peptide arrays from a single synthesis run on modified individual membrane disks. The peptides are synthesized on a modified cellulose support which is dissolved in a cleavage-mixture after the synthesis. The resulting solutions of peptide-cellulose-conjugates are then spotted onto coated microscope slides by conventional spotting techniques. The identical arrays are useful tools to screen human sera, characterize antibodies, enzyme substrates or sequence specificities of interaction partners with given peptide sequences (e.g. SH2, SH3 and other domains). Here we present the use of CelluSpots arrays for screening of 200 human sera samples against overlapping peptides representing different borrelia antigens. Clustering results of positive and negative control sera show immunodominant epitopes that are of interest for diagnostic tests and vaccine development. As second application example we present results obtained by kinase incubations on CelluSpots arrays containing annotated kinase substrates and consensus sequences of kinase targets. The new array format offers several major improvements compared to the well known SPOT membranes [2]: Smaller volumes (only 100μl) for incubations and high number of identical array copies enable large scale, parallel screening experiments. It is possible to use detection methods like autoradiography, chemiluminescence or enzymatic color development which can be performed without expensive instrumentation. References I. Dikmans, A. et al. (2006) QSAR Comb. Sci. 25(11), 1069-1080. 2. Frank, R. Tetrahedron 48, 9217 (1992)

Cell-Phone Radiation Might Alter Protein Expression in Human Skin
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Our in vitro studies have shown that cell phone radiation causes changes in protein expression and activity in human endothelial cell line EA.hy926. The objective of this study was to determine whether similar changes will take place in the skin of human volunteers. Ethical permit was obtained from the Ethics Committee of Department of Surgery of Hospital District of Helsinki and Uusimaa, Finland. A small skin area of a forearm of 10 female volunteers was irradiated with cell phone signal for 1 hour at specific absorption rate (SAR) of 1.3W/kg which is below the safety limit (SAR=2.0W/kg) recommended by the International Commission on Non-Ionizing Radiation Protection. Immediately after the exposure, a punch biopsy of the exposed area of skin was taken to a physician. Another punch biopsy was taken from the non-exposed forearm. Proteins from punch biopsies were extracted, separated with 2-DE (pH 4–7; 9%SDS-PAGE), and differential protein expression was analyzed using PDQuest 7.2. The ratio of exposed and control sample expression was analyzed spot by spot, after logarithm transformation, with variance analysis. Due to small numbers and potential violations of model assumptions, the ratios were also studied by spot with the Wilcoxon test. The statistical analysis has identified 8 differentially expressed proteins among the 579 identified proteins. The so far conducted human volunteer studies have focused on cognitive responses to cell phone radiation. This is the first study where human response was examined on molecular level. Our results suggest that human skin might respond to cell phone radiation by changes in protein expression. However, without further testing (study underway), it is not possible to predict whether these changes have impact on skin physiology. Finally, our study confirms that proteomics approach can identify protein targets of cell phone radiation and that larger study is needed to strengthen our pilot observations.

Study of Stochasticity in MAP Kinase Signaling Using Single-Cell Analyses
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The mitogen-activated protein kinase (MAPK) signaling pathways is essential for cell growth, cell differentiation and survival in eukaryotes. The MAPK signaling pathways transmit signals from the cell surface to nucleus. In the budding yeast, Saccharomyces cerevisiae, the mating and high osmolarity responses depend on the MAPK signaling pathways. In this study, we have shown the real-time gene expression patterns of the mating and high osmolarity responses at single-cell level using cell chips. We used mating and the high osmolarity MAPK pathways in yeast as a model system, which are highly relevant to ERK and p38 MAPK pathways, respectively, in mammals including in humans. The fluorescent responses we monitored in the study reflect the immediate output of MAPK signaling from living cells and thus can be used as a direct and dynamic indicator of cell responses. Our result of the mating and high osmolarity MAPK signaling showed a non-uniform, stochastic flux with alternative kinetics in the population of yeast cells analyzed. Furthermore, the stochastic response behavior did not propagate into daughter cells and therefore, seems to be a non-genetic and integrated cellular program. We postulate that the stochastic response program facilitates evolutionary fitness during natural selection by broadening the window of cell survival upon fast changing environments.

Bioinformatics/Biological Computing (2937-2943)

Functional Annotation of the MyTH4 Domain Using Bioinformatics Tools
S. Tsoi, M. Lai, S. Singh, Y. Leon; Biology, Brooklyn College, Brooklyn, NY

Myosins constitute a large super family of proteins with diverse properties adapted for a variety of important cellular activities. The relevance of myosins for mammalian physiology and pathology is underscored by the finding that many pathological conditions and genetic diseases are associated with mutations in myosins. Some important questions that remain unanswered in the field of myosin pertain to the role of the domains present in a particular region of the proteins called the tail. This present work investigates one such myosin tail domain called the MyTH4 domain, a domain whose structure and function is essentially unknown but has been implicated in pathological conditions associated with mutations in myosins.
and is also found in another class of motor proteins called Kinesins. Using a computational approach that integrates various bioinformatics and computational tools, for the first time, we propose a structural fold for the MyTH4 domain called the “alpha alpha superhelix” which is often found in proteins that are involved in protein-protein interactions. MyTH4 domains modeled on this fold allow us to predict biological function for this domain and provide a starting point for understanding why mutations in these domains in myosin and other proteins result in pathogenic conditions.

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Simulated Diffusion of CaMKII in Dendritic Spines

Z. Yixiao, 1 T. Reese, 1 S. M. Khan 1, 2 NINDS, NIH, Laboratory of Neurobiology, Bethesda, MD, 2 Molecular Biology Consortium, Chicago, IL Calcium calmodulin dependent kinase II (CaMKII) translocates within seconds after stimulation into dendritic spines in hippocampal organotypic slices or dissociated neuronal cell cultures. Translocation of CaMKII is central to early long term potentiation (LTP), and it is of interest to understand the mechanistic basis of its translocation. As a first step we model the mobility of CaMKII as revealed in fluorescence photobleaching recovery experiments from our and other laboratories utilizing green fluorescent protein tagged CaMKII fusions. SMOLDYN, a program that simulates single molecule Brownian dynamics at the Smoluchowski level of approximation (Phys. Biol. 1:137-51, 2004), provides a powerful tool to model diffusion in cells with complex morphology such as neurons. We find that (i) spines render CaMKII diffusion along the dendritic stalk anomalous (mean square displacement grows non-linearly with time); (ii) the diffusion between stalk and spine head is limited by spine neck diameter; and (iii) simulated recovery upon photobleaching is more rapid than that observed experimentally. We are currently investigating whether this discrepancy arises from modulation by the actin cytoskeleton of the apparent cytoplasmic viscosity or volume, or CaMKII diffusion directly.

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Proteomic Analysis of Brain Calmodulin-binding Proteins of the Forager and Nurse Workers Honeybees Apis mellifera L.

L. Calabria, 1 L. Hernandez, 2 R. Roland, 3 M. Souza, 4 F. S. Espindola 1, 1 Instituto de Genética e Bioquímica, Universidade Federal de Uberlandia, Uberlandia, MG, Brazil, 2 Dep. de Biologia Celular, Universidade de Brasilia, Brasilia, DF, Brazil Calmodulin is a Ca²⁺-binding protein important in a variety of cell functions. The Ca²⁺/calmodulin complex interacts with and regulates various enzymes and target proteins, known as calmodulin-binding proteins (CaMBPs). In this study, we revealed a comparative identification of the CaMBPs composition in the worker honeybee (Apis mellifera) brain, aiming to correlate the composition with the behavior of these bees in the colony. To this end, the CaMBPs of forager and nurse workers were purified by affinity chromatography, separated in 1D gel, digested and submitted to peptide mass fingerprinting (PMF). In the PMF analysis, 17 different proteins, considered behavior-specific proteins, were identified, one of them only in forager workers and 12 in nurse. All the proteins were classified in terms of their function and cell localization, revealing a greater expression of metabolism-related CaMBPs in both worker subcastes. In addition, the sequences were analyzed for the presence of the calmodulin-binding site. The results presented here indicate that behavioral changes in the colony lead to alterations of the CaMBPs composition and, possibly modify the function of these proteins in the worker honeybee brain.

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Genome-wide Characterization of Two Phosphoinositide Binding Domains of the Arabidopsis thaliana Using High-Throughput and Manual Computational Analyses

E. Wywial, 1 A. Silkov, 2 D. Murray, 2 S. M. Singh 1; 1 Biology, Brooklyn College, Brooklyn, NY, 2 Pharmacology, Columbia University, New York, NY Phosphoinositide binding domains have emerged as molecules responsible for trafficking and anchoring of membrane oriented proteins from yeast to mammalian cells. Two such domains, i.e. the Pleckstrin homology (PH) and FYVE domains, are of special interest because of their unique characteristics and genome wide prevalence. Structurally, the PH domains share a fold of seven β sheets followed by a C-terminal α-helix whereas the FYVE domains comprise two small double-stranded β sheets, a C-terminal α-helix and eight Zn²⁺ ion-binding cysteines. Regardless of their structural similarity, the PH and FYVE domains constitute functionally diverse families as yet largely uncharacterized for plant proteins. Here we carried out extensive searches of Arabidopsis databases of protein sequences using an automated pipeline and manual inspection to verify previously known and identify unknown instances of PH and FYVE domain-containing proteins. Then, we integrated experimental and predictive data on sequence and structure to propose a genome-wide domain-based classification of both Arabidopsis PH and FYVE proteins separately. Consequently, we have re-defined forty nine Arabidopsis PH and fifteen Arabidopsis FYVE proteins into fourteen and five classes, respectively. Our results show the exclusive presence of plant specific variant PH and FYVE domain-containing proteins, such as the PRAF proteins. When possible, phospholipid-binding partners and in vivo functions have been proposed for members of each of the classes based on their molecular models and their biophysical properties.

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The NCI-Nature Pathway Interaction Database: A Cell Signaling Resource

S. Krupa, 1 K. Anthony, 1 J. Bucholtz, 2 M. Day, 2 T. Hannay, 1 C. Schaefer 2; 1 Nature Publishing Group, Cambridge, MA, 2 National Cancer Institute Center for Bioinformatics, Rockville, MD, 3 Nature Publishing Group, London, United Kingdom The Pathway Interaction Database (PID, http://pid.nci.nih.gov) is a freely available collection of expert-curated and peer-reviewed signaling pathways composed of human biomolecular interactions and cellular processes. Created in a collaboration between the U.S. National Cancer Institute and Nature Publishing Group, the database is a research tool for cell biologists, biochemists, computational biologists and bioinformaticians. As of October 2007, the database contains 55 pathways encompassing 3034 interactions, 3905 proteins, 688 small biomolecules and 2740 complexes. The PID offers a range of tools to facilitate pathway exploration. Users can browse pre-defined pathways and create interaction network maps centered on a single molecule or Gene Ontology (GO) biological process of choice. Alternatively, users can input extensive lists of molecules or GO biological
Simultaneous Measures of Structure, Function, and Evolution from Primary Amino Acid Sequence
R. L. Patterson, D. B. van Rossum, K. D. Hong, Y. Hong, C. S. Gue; Biology, The Pennsylvania State University, University Park, PA
We present here a quantitative, scalable, and interoperable platform for structural, functional and evolutionary annotation of protein sequences across phylogenetic boundaries. It is based on a unified, comparative approach which amplifies, encodes, and decodes heretofore unrecognized biologically relevant information from low-identity alignments (<25%). This information may be used for the simultaneous inference of evolutionary relationships, functional assignments, and structural homology from primary amino acid sequence. As proof of the increased informational content provided by the Gestalt Domain Detection Algorithm (GDDA)-pipeline we present a detailed computational study, validated by experiment, for multiple functional, structural, and evolutionary domains within members of the transient receptor potential channel (TRP) superfamily, and in other ion channels as well. Overall, our findings suggest that the information contained in alignments <25% identity far exceeds what is currently considered significant and that the GDDA-pipeline enables access to this information.

OCLC, a Novel Organism Centric Literature Curation Database
V. Nataraj Dongre, S. M. Singh; Brooklyn College, Brooklyn, NY
Here, we present an expert curated organism centric literature database, capturing various aspects of an organism published in peer reviewed journals. Sometimes, it is necessary to have a whole picture about an organism, especially in the case of a pathogen, to draw conclusions about its pathogenicity and counter measures. These include the information about genes, genetic organization, protein structure, host-pathogen interactions etc. As a case study, we have showcased Hepatitis E literature curation to capture protein-protein interactions, structural information, current status of drug/vaccines and various other relevant information. The curated data is stored as a relational database and can be accessed using a web-based search engine provided on our portal. We also present this database as a model system for addressing the curation needs of varying biological problems. We show how the basic framework can be easily customized to incorporate information generated using Bioinformatics tools, at sequence, structure and systems biology level in addition to the curated literature and generate a protein-domain centric database. This small scale example demonstrated here allows for the inclusion of modeled proteins when structure is not known, predicted protein-protein, protein-lipid interactions etc to address the needs of the particular biological problem being addressed.

RNAi Technology (2944-2945)

siLentMer™ Dicer-Substrate siRNAs Mediate More Potent Gene Silencing
E. Hefner, T. Rubio, L. Ugozzoli; GD, Bio-Rad Laboratories, Hercules, CA
RNAi is a powerful tool used to silence genes and determine gene function in mammalian cells. The activation of an RNAi pathway via delivery of small interfering RNA’s (siRNA) into cells catalyzes sequence-specific degradation of a messenger RNA (mRNA) and reduction of its corresponding protein product. Key considerations for a successful RNAi experiment are: (1) the use of an effective siRNA, and (2) delivery of siRNAs with high transfection efficiency and low cytotoxicity. Recent findings demonstrate that a new class of double-stranded RNA molecules with increased length and altered end structure are capable of initiating an enhanced and, likely, more specific silencing response when compared to standard (21-mer) siRNAs. This new class of longer, end-modified siRNAs (known as dicer-substrate siRNAs or siLentMer siRNAs) is bound and cleaved by the RNA endonuclease, Dicer, before entering the RISC complex. Specific siLentMer end modifications promote the binding of Dicer to only one of the two ends of the siLentMer molecule. This in turn leads to greater RISC incorporation of the RNA strand with complimentarity to the mRNA of the gene of interest. The siLentMer mRNA duplexes deliver highly potent gene silencing, often at concentrations as low as 100 picomolar. The siLentMer siRNA duplexes also potentially reduce off-target effects, do not activate the Protein Kinase R (PKR) pathway or induce an interferon response and have been functionally tested via RT-qPCR to guarantee >85% reduction in target mRNA levels. The siLentMer siRNA’s effectiveness is enhanced when coupled with an efficient delivery method, such as the siLentFect lipid reagent that is used for validation of the gene targets. Determining and validating the optimal delivery method for each cell line is critical to the success of RNAi experiments because despite continued improvements in siRNA design, still experiments sometimes fail due to poor transfection efficiency.

Cell-based Screening Applications Utilizing Cryo-preserved siRNA-Transfected Cells
D. E. Hughes, S. J. Hong, B. L. Webb; ThermoFisher Scientific, Rockford, IL
Targeted gene silencing through RNA interference (RNAi) has become an established method to probe gene function. However, siRNA transfection optimization and demonstration of successful mRNA and protein knockdown adds significant time to siRNA experiments. Here we demonstrate the feasibility of a validated and storable siRNA-transfected cell population. This methodology provides access to aliquots of validated ready-to-use cells for general molecular biology laboratory experiments, profiling, pharmacology or screening applications. Using Thermo Scientific ON-TARGETplus™ SMARTpool™ siRNA Reagents we investigated the hypotheses that siRNA-transfected cells can be stored frozen and then used in
cell-based assays in the future. Our experiments demonstrate that cryo-preserved siRNA-transfected cell populations frozen greater than 10 months retain their gene silencing profile and stable protein knockdown. To assess potential screening applications, we used cryo-preserved cell populations previously transfected with siRNA against several genes in the EGF pathway. ERK1/2 phosphorylation was monitored by Western blot to determine the effect of siRNA-mediated gene silencing on EGFR pathway activation. In addition, cell-based screening applications were evaluated using Thermo Scientific Cellomics® HCS Reagent Kits designed to monitor EGF-mediated phenotypic changes. These screening assays demonstrated comparable results between cryo-preserved and non-cryo-preserved cells. Furthermore, cell viability and morphology appear normal in cryo-preserved cell populations. Our results demonstrate the feasibility of using cryo-preserved siRNA-transfected cells in both classic molecular biological techniques and high-throughput cell based assays.

**Single Molecule Technologies (2946)**

**A Magnetic Tweezers Apparatus for Concurrent Force and Fluorescence Measurements of Protein-DNA Interactions**

J. S. Graham, J. F. Marko; Northwestern University, Evanston, IL

We have developed a magnetic tweezers device to perform concurrent force and fluorescence measurements of protein-DNA interactions. The design allows extension of a DNA molecule in the focal plane of an inverted microscope objective. This configuration exposes the full length of the DNA allowing direct length measurement as well as direct visual observation of protein-DNA interaction dynamics via fluorescence microscopy techniques.

**New and Emerging Technologies for Cell Biology (2947-2957)**

**Quantitative Similarity of Induced Phenotypes: A New Method of Comparing Genes**

D. Eckley, L. Shamir, T. Macura, N. Orlov, I. Goldberg; Image Informatics and Computational Biology Unit, Laboratory of Genetics, National Institute on Aging, National Institutes of Health, Baltimore, MD

Single-gene knockdown via a commercially available RNAi library was used to generate phenotypes in *Drosophila melanogaster* Kc167 cells. Multiple images of the resulting phenotypes were collected using a restoration microscope (DeltaVision) and maximum-intensity projection prepared for further processing. The multi-purpose pattern analysis tool WND-CHARM, was used to measure similarity between groups of images resulting from a single dsRNA treatment. A simple set of landmark genes was targeted to assay nuclear and mitochondrial morphologies including several kinases (polo, cdc2c, chk1, chk2, p38MPK2, MPKAK2), ubiquitin ligases (diap1, hyd and cul-4), members of the Fanconi Anemia protein complex (FANC-M and FAAP24) and genes with a demonstrated role in cytokinesis (pebble, pav, RacGAP 50C). Genes encoding interacting proteins (FANC-M/FAAP24 and p53/chk1) were expected to produce phenotypes much more similar to each other than to other phenotypes in the set. Broader functional classes (e.g. cytokinesis genes) were expected to cluster by phenotype similarity. Results from pattern-based image analysis showed that the closest phenotype similarities were between p53/chk1, and FANC-M/FAAP24. The cytokinesis genes formed a larger-order similarity cluster, while genes expected to have unique phenotypes (e.g. diap1, chd1) were morphologically distinct from the others. Similarity relationships between groups of genes can be effectively visualized using phylogenetic trees where the basis of comparison is phenotypic similarity rather than gene sequence.

**Incorporating Tissue Architecture into Cellular Microchannel Assays Using Droplet-based Passive Pumping**

V. M. Echeverria, A. Skoien, S. Hayes, C. Lamers, I. Meyvantsson; BellBrook Labs, Fitchburg, WI

The advantages of microfluidics have been difficult to fully realize in drug discovery and biomedical research because highly parallel operation requires complex networks of tubing and pumps. An alternative approach using the surface tension in droplets to create and control fluid flows in microchannels is presented. Operated by standard automated or manual liquid handlers, arrays of these microchannels can be readily used to study cell biology in 3D matrices. We show here that complex biological models can be recapitulated in these devices. When mammary epithelial cells embedded in laminin-rich extracellular matrix were loaded into these microchannels, acinar morphogenesis occurred normally. Furthermore, laminar flow patterning was used to create separate stromal and epithelial compartments. Under these conditions, mammary fibroblasts were shown to delay the growth arrest of mammary organoids, resulting in larger clusters that are more representative of terminal ductal lobular units in vivo.

**Fluorescence Lifetime Imaging to Measure Fluorescence Resonance Energy Transfer Using Novel “Tag” Fluorescent Proteins**

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FLIM enables the measurement of the excited state lifetime of a particular fluorophore at each spatially resolved element in an image and can thereby provide a map of the molecular environment within each cell. A prime application of FLIM is the detection of FRET events, whereby a particular
fluorescence species (the donor) can undergo non-radiative energy transfer from its excited state to a second chromophore (the acceptor) with accompanying decrease in the mean excited state lifetime of the donor. Fluorescent proteins have been successfully used as FRET pairs, commonly used FRET pairs are ECFP (donor) and EYFP (acceptor). However, there are new probes that are brighter, fold faster and are less toxic. In this study a novel set of monomeric fluorescent proteins, which include TagCFP, TagGFP, TagYFP and TagRFP were used to produce tandem FRET pairs to investigate the different methods for measuring FRET. TagCFP-TagYFP, TagYFP-TagCFP, TagGFP-TagRFP and TagCFP-TagRFP were constructed with a 23 amino acid linker. To make reliable measurements of FRET the four different “Tag” fusion proteins were compared to the ECFP-EYFP positive control. ECFP-EYFP had been constructed with a 7 amino acid linker. FLIM-FRET data was collected using wide field time gated technology combined with a CSU10 Nipkow Spinning Disc Microscope head to provide optically sectioned fluorescence lifetime images. Results showed that TagGFP-TagRFP was the better pair of the Tag FRET pairs constructed, 29±1.72% compared to 13±1.53% for the control ECFP-EYFP. To conclude, this study demonstrated the application of an optically sectioning fluorescence lifetime microscope to measure FRET of a novel set of fluorescent proteins. In addition, TagGFP-TagRFP showed the highest FRET efficiency value, 29±1.72% among the 4 Tag FRET pairs.

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Site-Specific Incorporation of a Novel Amino Acid Analog
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Study of protein conformation and protein-protein interactions is a fascinating but difficult task. One approach is to site-specifically link a fluorophore to a chosen amino acid residue in the protein of interest. In order to do so, that specified amino acid must be chemically unique within the protein. Using a suppressor plasmid that expresses an altered tRNA and tRNA synthetase cognate pair from the archaeal species Methanococcus jannaschii, certain desired unnatural amino acids can be incorporated at an amber stop codon. In our research, we are experimenting with the efficiency of four suppressor plasmids designed for incorporating amino acids p-isodophenylalanine, p-acetylphenylalanine, m-acetylphenylalanine, and tyrosine at incorporating a novel amino acid analog that is of interest to us. We are performing the test in E. coli strain BL-21 carrying one of the four suppressor plasmids, and a plasmid with an expressible test gene carrying an amber stop codon mutation, and that is grown in the presence of the novel amino acid analog. We have tested these suppressors to determine which will incorporate this novel amino acid analog at a site-specific position in the desired protein. One of these suppressors has allowed for production of the full molecular weight of the test protein, without truncation, indicating the successful incorporation of our amino acid analog. Future work will be directed toward confirmatory methods to show the presence of the amino acid in the test protein. If we confirm successful incorporation of the amino acid analog we will have demonstrated a broadly applicable new approach to studying protein conformation and interaction.

2951
Cyclic Stretch Induces Reorientation of Actin Stress Fiber and Cellular Axis of Randomly Oriented Human Dermal Fibroblast in a Serum Dependent Manner
Y. Son, E. Lee; Genetic Engineering, Kyung Hee University, Yong-in, Republic of Korea
The cells in the tissue receive a variety of mechanical stimuli such as tension, shear, and compression. According to the previous reports, when the cyclic stretch was applied to the cells, they tend to align away from the stretch direction and actin stress fibers are also reoriented to longitudinal axis of the cell probably to resist the pulling force given by the cyclic stretch. In this study, we explored whether serum in culture medium is important to the stretch-dependent reorientation of cellular axis as well as actin stress fiber and signaling molecules in the stretch dependent reorientation. We applied equibiaxial cyclic stretch at 0.5 Hz frequency with 20% maximum elongation with FLEXCELL4000 system to the randomly arranged human dermal fibroblasts (hDFs) with serum (2%) or without serum for 24hr. After stretch application, the cells were stained with TRITC-phalloidin and stained with anti-vinculin. Then the angles (θ) between the stretch direction and the longest axis of cells or stress fibers were measured. Without serum, the cells reoriented their stress fibers at angles about 70 to 90 degrees to the direction of stretch but the longest cellular axis did not change. However with serum, both the longest cellular axis and the actin stress fibers were reoriented to the angle about 70 to 90 degrees to the stretch direction. When the early signaling molecule initiated by cyclic stretch such as AKT and JNK1/2 were blocked by treatment of their specific inhibitors, the reorientation of cells under culture medium with serum was inhibited. In conclusion, serum existence in culture medium was important to reorientation of cell dimension about stretching direction, but was not affect the stretch-induced rearrangement of actin stress fiber.

Acknowledgements This work was supported by the Musculoskeletal Bioorgan Center Project of Korean Ministry of Health and Welfare given to Dr. Youngsook Son.

2952
The Propagation of Quorum Sensing Gene Responses in High Density Colonies
H. Cho,1 J. K. Campbell,2 J. W. Williams,3 A. Groisman,2 A. M. Stevens,1 A. Levchenko1; 1Biomedical Engineering, The Johns Hopkins University, Baltimore, MD, 2Department of Physics, University of California, San Diego, San Diego, CA, 3Department of Biological Science, Virginia Tech, Blacksburg, VA
Biofilms are not just passive accretion of bacterial cells, but highly organized complex structures. Genetic analyses on biofilms have led to propose the extracellular signals and quorum sensing regulatory systems may be closely associated with the biofilm development. To investigate the role of quorum sensing in biofilm formation, it will be critical to monitor the signaling process in situ in highly dense colonies and correlate the quorum sensing related gene regulation to any changes in the biofilm development such as differentiation, structural changes and an increase in antibiotics resistance. In this study, we develop the microfluidic device in which real time monitoring of gene regulation at single cell level and at population level for prolonged periods of time is feasible combined with automated image acquisition. In particular, we investigate the expression of the truncated lux operon of quorum sensing system in Vibrio fischeri at high density colonies in the chemostatic growth condition. Not only can we observe the switch from the basal state to the fully induced rate of quorum sensing gene expression for the collective behavior of the cell population,
but we can also characterize the spatial distribution of quorum sensing response within the clonal population. Interestingly, we find that the heterogeneity of gene expression is present through the colony growth upon the onset of quorum sensing response. Furthermore, using two different engineered cells containing autoinducer-receiving component and autoinducer-producing component, we observe the signal propagation between two populations. This experimental design allows us to investigate the signal response propagation across the populations when the extracellular signal is noisy or non-uniformly distributed, mimicking the complex structures of biofilm found in nature. The proposed experimental systems will provide us tools for mapping the spatiotemporal behavior of quorum sensing response in various bacterial species and that of interspecies communication.

2953

**Acoustic Droplet Ejection and Laser Scanning Platforms Enable High-Throughput Cell Dispensing and Quantitation**

S. Pickett,¹ J. Shieh,² S. C. Miller²; ¹Labcyte Inc., Sunnyvale, CA, ²Blueshift Biotechnologies, Inc., Sunnyvale, CA

Objective: Assess performance of acoustic droplet ejection and whole-well fluorescence imaging for quantitative high-throughput cellular dispensing and measurement. Methods: Source plates contained CMFDA (Invitrogen)-labeled CHO cells resuspended in PBS from 1 to 2x10⁶ cells/mL. Cells were dispensed over a range of 0-400 droplets of 2.5 nL each (i.e. final dispensed volumes of 0-1000 nL) using acoustic droplet ejection (ADE; Echo® 555 liquid handler, Labcyte Inc.). The cell concentration curve was dispersed in both forward and reverse direction across the receiving plate to determine whether cells settled in the source wells over the dispensing time. The receiving 384-well plates contained 40 μL/well growth medium + 10% FCS and after dispensing, the plates were incubated at 37°C/5% CO₂. Whole-well images of the 384-well plate were acquired using a 488 nm laser scanning platform (IsoCyte™, Blueshift Biotechnologies, Inc.). Total cell counts per well was determined using the IsoCyte™ software. A solution of saponin and propidium iodide (PI) was added at 40 μL/well and the plates were scanned again to determine nuclear counts. Results: These tests demonstrated that the cell counts per well increased linearly with increasing dispense volume with both CMFDA and PI stains. A starting concentration of 1x10⁶ cells/mL yielded about one cell per 2.5 nL droplet across all dispense volumes, with the expected doubling at 2x10⁶ cells/mL. Among 24 replicates for each dispense volume above 5 nL, the average CV for the cell counts was 19%. Conclusions: The Echo® ADE liquid handling system can be used to dispense live cells into high-density microplates in a quantitative manner. Total cell counts in each well can be rapidly determined using fluorescent dyes and the IsoCyte™ laser scanning platform. Some applications for high-throughput low-cell-number dispensing and fluorescent quantitation include cell health measurements, cell cloning, and RNAi studies.

2954

**Formation of Artificial Bone Scaffolds with Surface-modified Hydroxyapatite Crystals and Polyamine Organic Polymer for Bone Regeneration**

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To make osteo-conductive and osteo-inductive bone biomaterials, it is ideal to have both organic and inorganic materials in one composite. However, it is not easy to mix the inorganic crystal and the organic polymer to the homogeneous state. To solve this problem, we applied several types of molecular linkers that can covalently bind to the surface of hydroxyapatite (HA) crystals. By chromogenic chemical analysis, we confirmed that the linker was successfully bound on the surface of HA crystals through covalent linkage. The chemical groups on the molecular linker can be used to make covalent linkage with organic polymer of choice and construct bone sponge. When hBMScs (human Bone Marrow Stromal Cells) were seeded into this bone sponge, there was good cell survival and distribution. By MTT assay, it was confirmed that the prepared composite showed good cell affinity and no cytotoxicity. hBMScs-seeded bone sponges were transplantation into immuno-compromised mice, and sacrificed according to the time point at 4, 8, 10, 12, 15 weeks after surgery. The histology demonstrated good bone-like structure formation at earlier stage. At the later stage, lamellar bone formation was observed, which was confirmed by polarized microscopy. This result indicated that this artificial bone material provides an appropriate environment for hBMSCs to survive and undergo bio-mineralization and can be used for purpose of bone regeneration.

2955

**Myoblast Differentiation on Cell Adhesive Nanostructured Biointerfaces**

T. Wolfram, C. Gojak, T. Schoen, D. Aydin, J. P. Spatz; New Materials and Biosystems, Max-Planck Institute for Metals Research, Stuttgart, Germany

Micro- and nanostructured bioinstructive interfaces are now a widely used tool to investigate complex cellular behavior like cell adhesion and to mimic cell-cell contacts and cell-substrate interactions. So far, all these studies are restricted to biomolecules which show cell adhesion functions, like fibronectin, laminin, cadherins or oligopeptide cell binding motifs from those proteins. Polypeptides such as growth factors or bioinstructive transmembrane proteins like Delta and its receptor Notch regulate and modulate key cell function with respect to cell differentiation and tissue development but most of these molecules show little or no cell adhesion affinity and no cytotoxicity. hBMScs-seeded bone sponges were transplantation into immuno-compromised mice, and sacrificed according to the time point at 4, 8, 10, 12, 15 weeks after surgery. The histology demonstrated good bone-like structure formation at earlier stage. At the later stage, lamellar bone formation was observed, which was confirmed by polarized microscopy. This result indicated that this artificial bone material provides an appropriate environment for hBMSCs to survive and undergo bio-mineralization and can be used for purpose of bone regeneration.
controls. Our approach provides a possibility for micro- and nanostructured biomolecules on synthetic biomaterials to mimic closely the cellular microenvironment.

2956

**Combining Microfluidics and Protein Microarray for Complex Cell Signaling Study**

Z. Yin, S. Tao, R. Cheong, A. Levchenko; 1Department of Biomedical Engineering, Johns Hopkins University, Baltimore, MD, 2High Throughput Biology Center, Johns Hopkins School of Medicine, Baltimore, MD

Microarray technology has been the standard for large-scale DNA/RNA screening. However functions of organisms are carried out by proteins encoded by DNA. During the last decade, protein microarrays have been developed. Ultimately, proteins carry out their functions in cells, where thousands of protein molecules exist in a small volume and multiple reactions take place at any given moment. It is thus a natural extension to use protein arrays to study functioning of live cells. In reference, the authors reported an extracellular matrix (ECM) microarray platform. Thirty two different combinations of 5 extracellular matrix were printed on a single slide to probe cellular differentiation. Besides ECM components, there are far more protein ligands that affect cell fate and functions by binding to cell surface proteins. Here, we present a new platform to perform complex cell signaling analysis in a high-throughput manner by combining microfluidics with protein microarray technologies. Ligands at different concentrations and combinations were spotted on a glass substrate with electrode array and N-hydroxysuccinimide coating. Cells were seeded into a Polydimethylsiloxane (PDMS) chip bonded on the substrate and patterned exactly on the spots by dielectrophoretic force. Cells then attach and spread on the spots coming into contact with ligands. TNFα and IGF1 at 16 combinations were printed on the chip to study NFkB pathway as a demonstration.

2957

**Interspecies Adhesion Forces Measured by Atomic Force Microscopy in Bacterial Biofilms**

S. Sharma, A. Palmer, E. Sokurenko, M. Parsek, W. Thomas; 1Department of Bioengineering, University of Washington, Seattle, WA, 2Department of Microbiology, University of Washington, Seattle, WA

Objective: We report on the potential use of AFM as a tool to measure the direct adhesion forces between bacterial cells and biofilm surfaces which are of great significance in cell-to-cell communication, aggregation and biofilm formation processes. Method: In this paper we present 1. A method for covalent, viable cell immobilization and 2. Use this to investigate forces of cell-cell interaction between bacteria and a mature biofilm surface using AFM. We examine two medically important microbes, *E.coli* (related to urinary tract infections and implant infections via adhesion of type 1 fimbria) and Pseudomonas (the most common studied pathogen causing infections) in order to characterize the cell interactions that interplay between bacterial adhesion and biofilm formation. FimH is an ubiquitous mannose-specific adhesin of *E. coli* located on the tip of type 1 fimbriae. The effect of fimbriated *E.coli* attaching to mannose rich Pseudomonas biofilm matrix was has been investigated. Results: Our AFM studies on the intercellular forces between strains of *E.coli* (differing in their ability of FimH to bind mannose) and the Pseudomonas biofilms surface demonstrated that FimH-mannose specific interactions occur at the biofilm interface. Conclusions: The method provides an approach for directly measuring interfacial and adhesion forces between bacterial and other cells/ biofilms or surfaces in fluid and can be tailored to accommodate variety of cell surface interactions.

### Pre-College and College Science Education (2958-2959)

2958

**The Geneticist-Educator Network of Alliances (GENA) Project: An NSF-sponsored Math and Science Partnership Grant to ASHG**

K. Shaw; ASHG, Bethesda, MD

The Geneticist-Educator Network of Alliances (GENA) Project will provide the partnering scientific societies involved with tools to instruct, facilitate and measure the meaningful engagement of science, technology, engineering and mathematics (STEM) faculty members in secondary science education. The GENA Project is exploring ways that an ASHG-sponsored secondary science education outreach effort can play a positive role in the career development of both junior (pre-tenure) and senior (post-tenure) level genetics faculty. Exemplary inquiry-based educational materials in genetics will be utilized to design methods to facilitate meaningful interactions between scientists and their local education community. Development of a network of geneticist-educator alliances will be used to design teaching strategies relating to standards and misconceptions in genetics that can to decrease time required for scientists to prepare for outreach, thus maximizing the effective and meaningful interaction between the geneticists and students. To date, 13 geneticist-educator alliances have been selected and trained as part of this program. Over the next two years the GENA project will recruit another 80 geneticists to participate and assist in the development of this model program that will become an integral part of the strategic development plan for the education efforts of both ASHG and GSA, thus making K-12 education outreach a truly systemic aspect of society activities.

2959

**The Use of Digital Photography to Enhance Learning of Biotechnology**

F. N. Norflus, K. Szeniawski; Natural Sciences, Clayton State University, Morrow, GA

The objective of this poster is to demonstrate how the use of digital photography can be used to enhance the learning of biotechnology. This project was performed at Clayton State University in a biotechnology class of 18 students. There were no teaching assistants for this class making it difficult for the instructor to explain the techniques individually to each of the students. Therefore, digital photography was used. At the beginning of the semester, pictures of the equipment to be used were taken and posted on the class web site. For example, the volumes of the different micropipettes...
were adjusted, photographed and posted on an interactive web site enabling the students to learn their proper use. Pictures were also taken of the centrifuge and balancing of the rotor. On the first exam, there were questions based on learning the material in these photographs. The students scored from 94.4-100% correct on all of the pipettes shown except the p100 pipette. All of the students also correctly answered the question on balancing the rotor correctly. Students also examined some of the other equipment used in this class and pictures of this equipment obtained from the Internet were posted on the class web site and on the exam. However, the success rate on these questions was less than for the digital photography. Only 55.6% of the students could identify an ethidium stained gel and only 66.7% could identify the white light box used for viewing the gels. Gel electrophoresis was also analyzed by photography in an attempt to better explain the experiments. In conclusion, by supplementing the class with photography, class discussion and learning by the students was enhanced. The data could more easily be analyzed by the class than if everyone had to huddle around one gel.
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