FARE2013 WINNERS
Sorted By Study Section

Biochemistry - General and Lipids
Jimenez-Gomez, Yolanda
Visiting Fellow
NIA
Resveratrol improves adipose insulin sensitivity and inflammation in Rhesus monkeys on a high fat diet
Obesity is recognized as a chronic low-grade systemic inflammation that contributes to the development of insulin resistance and type 2 diabetes mellitus. Lifestyle changes including healthier diet and exercise are the primary line of intervention to halt the development of obesity and associated diseases. Resveratrol, a natural compound with anti-inflammatory properties, has been shown to improve glucose tolerance and insulin sensitivity in rodents. We tested the effect of resveratrol on the pro-inflammatory profile and insulin resistance caused by a high-fat, high-sugar diet (HFS diet) in white adipose tissue (WAT) from Rhesus monkeys. Long-term resveratrol supplementation decreased adipocyte size and reversed the majority of the transcriptional changes induced by HFS diet in visceral WAT. Furthermore, resveratrol up regulated SIRT1 and IkappaBalpha protein levels, decreased NF-kappaB phosphorylation and expression for IL-6 and IL-1beta in visceral WAT, and reduced CRP levels in circulation. Furthermore, resveratrol increased IRS-1 protein levels and decreased Akt phosphorylation. These effects observed in visceral WAT were reproduced in 3T3-L1 adipocytes cultured in media containing serum from HFS diet-fed monkeys without and with resveratrol supplementation. In these 3T3-L1 adipocytes, resveratrol reduced the prolonged insulin-induced Akt activation and increased the cell number expressing GLUT-4 at plasma membrane as well as cell surface GLUT-4 labeling after insulin treatment in 3T3-L1 adipocytes. In conclusion, resveratrol supplementation reduced chronic inflammation and improved insulin sensitivity in visceral WAT from Rhesus monkeys fed a high fat diet.

Biochemistry - General and Lipids
Taha, Ameer
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NIA
Dietary omega-6 polyunsaturated fatty acid deficiency reduces lipopolysaccharide-induced changes in behavior sickness and brain arachidonic acid inflammatory response
The omega-6 polyunsaturated fatty acid (n-6 PUFA), arachidonic acid (AA, 20:4n-6), is a precursor of prostaglandins, leukotrienes, and related compounds that have important roles in mediating the brainâ€™s innate inflammatory response to cytokines, which have been implicated in neuropsychiatric and neurodegenerative disorders. AA is released from the stereospecifically numbered-2 position of membrane phospholipids by AA-selective calcium-dependent cytosolic phospholipase A2 (cPLA2). The AA cascade can be activated in rodents with the bacterial endotoxin, lipopolysaccharide (LPS), which causes neuroinflammation and a characteristic depressive-like sickness behavioral profile. This study tested the hypothesis that dietary n-6 PUFA deprivation, which limits the supply of AA to the brain, would reduce LPS-induced AA-mediated neuroinflammation and associated changes in behavioral sickness. Rats were fed an n-6 PUFA adequate diet (control, 6% of energy) or an n-6 PUFA deficient diet (0.5 % of energy) for 15 weeks, after which, they were infused with LPS (250 ng / hr) intracerebroventricularly into the 4th ventricle for 2 days using a mini-osmotic pump. Two days after infusion, behavioral sickness was scored, and the incorporation rate of intravenously infused unesterified 14C-AA into brain phospholipids (an established marker of neuroinflammation), and cPLA2

mRNA and activity were measured postmortem. Dietary n-6 PUFA deprivation attenuated LPS-induced increases in the rate of 14C-AA incorporation into brain phospholipid (P<0.05 for main effect of LPS and diet by two-way ANOVA), as well as activity of AA-releasing cPLA2 (P<0.05 for diet-LPS interaction) without altering cPLA2 mRNA. This was associated with a significant decrease in behavioral sickness scores, which were increased by LPS infusion in control rats (P<0.05 for interaction). Because the AA incorporation rate is a quantitative marker of neuroinflammation, our results suggest a direct anti-inflammatory effect of dietary n-6 PUFA deprivation, in association with reduced behavioral sickness, via a mechanism involving post-translational reductions in AA-releasing cPLA2. Thus, lowering dietary n-6 PUFA consumption in humans from estimated current intakes of 7% of energy to clinically achievable levels of 0.5~1% of energy, might increase resistance against pro-inflammatory insults to the brain or reduce the pathological neuroinflammation reported in neuropsychiatric and neurodegenerative diseases.

Biochemistry - General and Lipids

Wassif, Christopher
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NICHD

27-hydroxy-7-dehydrocholesterol is a novel endogenous teratogen in Smith-Lemli-Opitz syndrome (SLOS) that decreases cholesterol levels and increases phenotypic severity

Smith-Lemli-Opitz syndrome (SLOS) is an autosomal recessive, multiple malformation, cognitive impairment syndrome caused by mutations in the 7-dehydrocholesterol reductase gene (DHCR7). DHCR7 catalyzes the reduction of 7-dehydrocholesterol (7-DHC) to yield cholesterol (CH) in the final step of CH synthesis. This defect leads to a deficiency of CH and increased 7-DHC and the isomer 8-DHC. The SLOS phenotype is broad and variable. 27-hydroxycholesterol is formed from CH by the action of CYP27A1. I have identified two novel oxysterols, 27-hydroxy-7-dehydrocholesterol and 27-hydroxy-8-dehydrocholesterol, derived from 7-DHC and 8-DHC respectively. Further investigation of the relationship between serum CH levels and 27-hydroxydehydrocholesterol (27-OH-DHC) levels, revealed a strong correlation between CH levels and 27-OH-DHC levels in SLOS patients. To test the hypothesis that 27-OH-DHC could suppress total sterol synthesis during development and thus contribute to the SLOS phenotype, Dhcr7+/?3-5 was crossed with CYP27Tg+ to isolate Dhcr7?3-5/?3-5:CYP27Tg+ pups. Dhcr7?3-5/?3-5 is a null model of SLOS. CYP27Tg+ mice have elevated 27-hydroxycholesterol levels but normal CH levels. Dhcr7?3-5/?3-5:CYP27Tg+ embryos are significantly more affected than Dhcr7?3-5/?3-5 embryos. Phenotypically these findings include craniofacial abnormalities, umbilical hernia, limb defects, heart defects, and agenesis of the corpus collosum. Total sterol levels were decreased 2-fold in liver and 20-fold in brain tissue in Dhcr7?3-5/?3-5:CYP27Tg+ compared to Dhcr7?3-5/?3-5 E18.5 embryos. The role of 27-OH-7DHC in SLOS teratogenesis was confirmed by showing that intrauterine growth retardation was decreased in Dhcr7?3-5/?3-5:Cyp-/- embryos which can not synthesize this novel oxysterol. Interestingly the mutant Dhcr7?3-5/?3-5:CYP27Tg+ pups seem to have an endocrine-like effect on other pups within the litter in that tissue sterol levels are decreased in CYP27TG- embryos. Consistent with this hypothesis, a significant increase in 27-OH-7DHC levels in maternal serum is noted when one or more Dhcr7?3-5/?3-5:CYP27Tg+ pups were present in the litter. This increase of 27-OH-7DHC was noted in human maternal serum of a SLOS pregnancy. This new mouse model better recapitulates the malformation spectrum found in SLOS patients and underscores the toxic role of 7-DHC in SLOS. Modulation of CYP27 may provide a therapeutic approach for SLOS.

Biochemistry - Proteins

Gao, Rui
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NCI-CCR
Substrate specificity and biochemical characterization of Tdp2: a divalent cation-dependent phosphodiesterase specific for the repair of Top2 cleavage complexes
Topoisomerase II (Top2) is a ubiquitous enzyme that disentangles the DNA during replication, transcription and chromosome segregation. It is the target of widely-used anti-cancer drugs, including etoposide, doxorubicin, mitoxantrone and amsacrine. Top2 can also be poisoned by endogenous and carcinogenic DNA lesions. The abortive activity of Top2 caused by its poisoning produces a tyrosine covalently bound to the 5â€™-terminus of a DNA strand break. Tyrosyl DNA phosphodiesterase 2 (Tdp2) is a newly discovered enzyme that specifically removes the tyrosine covalently attached to the 5â€™-end of the break, leaving a phosphate group ready for religation. Genetic inactivation of Tdp2 confers hypersensitivity to Top2 inhibitors. Overexpression of Tdp2 also confers resistance to etoposide. In this study, we characterized the biochemical activities of Tdp2, showing that its optimal substrate is a DNA duplex with a 4-base overhang at the 5â€™-terminus that corresponds to physiological Top2 abortive intermediates or single-stranded DNA. In addition, we demonstrate that Tdp2 only processes the 5â€™-terminus when a tyrosine is directly linked to the end but not a biotin or fluorescein. Also, when bulky groups, such as digoxigenin, are attached to tyrosine, Tdp2 displays enhanced catalytic activity suggesting direct recognition of the Top2 catalytic residue by Tdp2. Tdp2 requires divalent cations for activity with strong preference for Mg2+ (Mg2+=Co2+>Mn2+>Ca2+=Zn2+). Its kinetic profiles were determined (kcat/Km = 4.3 x 105 S-1M-1) in comparison with Tdp1 (kcat/Km = 9 x 107 S-1M-1). Sequence alignment analysis, structural prediction (I-TASSER), and site-specific mutations demonstrate that Tdp2 possesses four conserved catalytic motifs shared by other Mg2+-dependent endonucleases, such as APE1 and exonuclease III. Site-directed mutations at each of the four motifs (including novel mutations at histidine 351 to alanine in the C-terminus and asparagine 120 in the N-terminus) abolish Tdp2 enzymatic activity. Our results provide novel insights into the key catalytic motifs, substrates and metal requirements for Tdp2 activity. Additionally, the current study provides important knowledge on Tdp2 catalytic mechanisms, therefore lays the foundation for future screening of chemical inhibitors of Tdp2.

Biochemistry - Proteins
Pickard, Frank
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NHLBI
Coarse grained modeling of thermal protein denaturation
To function properly, proteins must fold. Therefore, as a precondition to fully understanding a protein's function, one must first acquire thorough knowledge of a protein's structure and its associated biochemical properties. Additionally, several age onset degenerative diseases have been linked to protein misfolding and aggregation, such as Alzheimer's and other various prion-related diseases. Determining the assembly mechanism of amyloid fibrils is an active area of both experimental and computational research. Computationally modeling the aggregation process presents several challenges, among them is: the spatial extent of aggregates, the abundance of meta-stable protein conformations and the potential for ergodic simulations. To address these issues, we are developing an off-lattice coarse-grained (CG) protein model, where, each amino acid is modeled by two interaction sites: one at the alpha-carbon position and one at the side-chain center of mass position. This model alleviates the challenges listed above by dramatically reducing the computational cost of simulation (by about two orders of magnitude versus standard all-atom models) and by efficiently sampling the protein's full

conformational space. This CG model is being used in conjunction with replica exchange Langevin dynamics, a proven method to enhance conformational sampling, to simulate the kinetics and thermodynamics of two small, fast-folding proteins during the process of thermal denaturation. We model the protein G-related albumin binding (pdb: 1prb) and beta-1 immunoglobulin binding (pdb: 2qmt) domains. These small proteins serve to validate our model for both alpha-helix and beta-sheet systems. For each protein we utilized 20 replicas between 300K and 400K, with each replica running for 3 microseconds. Melting curves, in conjunction with a two-state folding model, were then used to derive thermochemical properties [melting point (K), folding enthalpy (kJ/mol), change in heat capacity J/(mol K)]. For 1prb, our simulation results (355.2, 170.0, 2.94) compare favorably with experiment (345.3, 170, 1.1+/-0.1). Similarly, our 2qmt results (354.2, 253.6, 4.18) closely follow experiment (360.7+/-0.1, 258+/-3, 2.6+/-0.3) as well. This encouraging result paves the way for future work incorporating inter-molecular interactions into the model. In turn allowing us to model oligomers. Simulations of HIV-1 protease are currently underway.

Biochemistry - Proteins

Campos-Chagas, Andrezza
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NIAID

LJL138 a salivary endonuclease from Lutzomyia longipalpis allows Leishmania parasites to escape from neutrophil extracellular traps.

In the present study, we identified and characterized the main neutrophil-extracellular-trap-destroying molecule (LJL138) from the salivary glands (SG) of the Leishmania vector Lutzomyia longipalpis Leishmaniasis is a vector-borne disease initiated by the bite of an infected sand fly. The enhancing effect of sand fly saliva on cutaneous leishmaniasis has been well documented in the literature. Neutrophils are considered the host's first line of defense against infections and have been implicated in the immunopathogenesis of Leishmaniasis. Previous analysis of the SG transcriptome of L. longipalpis showed the potential presence of an endonuclease. Indeed, not only was the cloned cDNA (LJL138) shown to encode a highly active ss- and dsDNAse, but also the same activity was demonstrated to be secreted by SG of L. longipalpis . Recombinant LJL138 was expressed in mammalian cells and purified by affinity chromatography followed by size exclusion chromatography and its purity ascertained by N-terminal sequencing and LC-MS/MS analysis. LJL138 hydrolyzes both ss- and dsDNA with little sequence specificity with a Dnase activity of 300000 Kunitz units per mg of protein (U/mg). LJL13 also showed a marginal RNase activity of 0.05U/mg. The NET's major structural components, DNA and elastase, were detected in traps induced by either PMA or Leishmania parasites by immunofluorescence. Disruption of PMA- or parasite-induced NETs by treatment with LJL138, SG extracts or commercial Dnase-I during Leishmania-neutrophil interaction increased parasite survival indicating that the parasites can escape from the leishmanicidal activity of activated neutrophils. Moreover, active-site mutagenized LJL138 (mLJL138, RGH197AAA) abrogates its Dnase activity and the NET-destroying activity. In vivo experiments (murine model) showed that co-inoculation of LJL138 with Leishmania parasites significantly increased the cutaneous lesion size and it also resulted in higher parasite load when compared with parasite alone or in the presence of mLJL138. Polyclonal antibodies against LJL138 raised in mice blocked the Dnase activity of recombinant LJL138 and SG extracts indicating that LJL138 is the major NET-destroying molecule in L. longipalpis salivary glands. Our results demonstrate that this female-specific secreted endonuclease is an important factor for the establishment of Leishmania infection by increasing the parasite burden in the Leishmania inoculation site.

Biochemistry - Proteins
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Biophysics
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Activation of different functionalities inside cells through auto-recognizing therapeutic R/DNA chimeric hybrids: a novel approach in therapeutic RNA nanotechnology
Using RNA interference (RNAi) as a therapeutic agent it is routinely possible to knock down the expression of target genes in diseased cells. We developed a new strategy based on therapeutic RNA/DNA hybrids which can be generally used for triggering the RNAi pathway as well as other functionalities inside the diseased cells. Individually, each of the hybrids is functionally inactive and the therapeutic siRNA representation can only be activated by the recombination of at least two cognate hybrids simultaneously present in the same cell. This approach allows (i) the triggered release of siRNAs inside cells, (ii) activation of different functionalities intracellularly, (iii) higher control over targeting specificity, (iv) tracking of the delivery and recombination of these hybrids in real-time inside cells, (v) introduction of additional functionalities without direct interference with therapeutic siRNA processivity, (vi) increasing the retention time in biological fluids by fine-tuning chemical stability by having or substituting the DNA strands with chemical analogs, (vi) conditional release of therapeutic functionalities. We comprehensively analyzed several pairs of cognate R/DNA hybrids in vitro and ex vivo using human breast cancer cells as a model system. Using degradation assays, we demonstrated significantly higher chemical stabilities in human blood plasma for hybrids (half-life of ~2 hours) compared to pure siRNAs (half-life ~20 mins) and the inability of RNA/DNA hybrids to be processed by human Dicer was confirmed by native-PAGE experiments. Using FRET experiments, we examined the kinetics and affinities of hybrid recombination and determined the lowest concentration of hybrids to recombine to be at least as low as 1 nM. Several different ex vivo experiments allowed the tracking in real time the recombination of several split functionalities (e.g. activation or deactivation of FRET) inside the target cells. The successful release of therapeutic moieties (siRNAs) was confirmed by the significant level of suppression (>95%) of the target gene expression. Interestingly, for siRNA release, cognate hybrids can be co-delivered to the cell either on the same or even on two different days and the extent of silencing efficiency will depend on structural characteristics of individual hybrids. Overall, this novel approach opens a new route in development of nucleic acids based â€œsmartâ€ nanoparticles for biomedical applications.

Biophysics
Avram, Alexandru
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NICHD
In vivo measurement of inter-hemispheric axon diameters on a clinical scanner using multiple pulsed-field gradient diffusion MRI
Quantifying microarchitectural features of neurons, such as axon diameters, can provide valuable neuropathological and functional information. Multiple pulsed-field gradient (mPFG) diffusion MR has been recently proposed as an alternative to biopsy-driven histology due to its non-invasive nature and

its ability to image large regions of tissue in vivo. An extension of conventional diffusion NMR - mPFG MR applies multiple diffusion encoding blocks with arbitrary orientations and measures the signal as a function of the angle psi between consecutive gradient pulses. The psi-dependence exclusively characterizes diffusion of water trapped in microscopic compartments providing a measure of average pore geometry. While mPFG MRI has been extensively validated in microimaging MRI scanners, its translation to in vivo MRI applications on clinical whole-body scanners is challenging due to the lower gradient strengths and lack of a suitable theoretical framework. We implemented a novel mPFG MR pulse sequence on a standard clinical MRI scanner and evaluated its ability to measure axon diameters in vivo. Following validation in a calibrated diffusion phantom, we acquired mPFG images with 2x2x6mm3 resolution from healthy volunteers using mid-sagittal slices through the corpus callosum (CC). Fiber orientations in the CC were measured with high resolution DTI 2x2x2mm3 and incorporated in our numerical model for myelinated axons, approximated as parallel impermeable cylinders. Using our recently developed mathematical framework, we fitted angular mPFG profiles in a minimum least-square-error sense to a family of curves numerically generated for a range of axon diameters. Maps of interhemispheric axon diameters provide information complementary to DTI and reveal significant heterogeneity along the anterior-posterior CC. Moreover, this topography matches fiber tracking based functional CC parcellation. Mean axon diameters measured in fiber bundles connecting specialized brain regions - visual (3.86um), auditory (2.71um), sensory-motor (4.61um), prefrontal (1.86um) - are in remarkable agreement with ex vivo studies. Our results establish the clinical feasibility of acquiring high quality artifact-free mPFG MRIs in vivo for quantitative microstructural assessment with uL resolution. mPFG holds the promise of a non-invasive whole brain histological assessment that can prove transformative to neuropathology and neuroimaging.

Biophysics

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NICHD

Virus-mimic DNA nanoparticles have hollow shell architecture

DNA, an extended negatively-charged polymer, condenses into compact nanoparticles in the presence of polymeric cations. We report the detailed organization of DNA within nanoparticles condensed with mannose-modified polyethyleneimine. Mannose-modified DNA nanoparticles have been used in gene therapy for targeting the dendritic cells of the immune system. Dendritic cells endocytose the nanoparticles via surface receptors for mannose, a mechanism exploited by viruses to enter these cells. Mannose-modified nanoparticles are currently in clinical trials as a DNA-based vaccine against HIV. We probed the three-dimensional arrangement of DNA within the mannose-modified nanoparticles by nano-indenting them in fluid with Atomic Force Microscopy (AFM). At small indentations, the nanoparticles behaved like a rubbery elastic material. At larger indentations, however, they exhibited a classic buckling-like response, which was reversible and not due to particle damage. The rate of buckling decreased with indentation. Such poroelastic mechanics can be explained by high fluid content in the particle core. We confirmed the relative absence of DNA in the particle core by imaging with Transmission Electron Microscopy. We also estimated the fluid content of each nanoparticle by correlating their volumes in solution against that in dried state. The volumes were measured with Dynamic Light Scattering and AFM. The water content varied with nanoparticle size in a manner expected for hollow shells with wall thickness of 2 - 3 DNA. The water content was ~95% for nanoparticles in the 50 â€“ 300 nm diameter range (virus-like dimensions effective for gene therapy). We probed the DNA arrangement in the wall of the nanoparticles by using 1 kb DNA to slow down nanoparticle assembly and imaging the intermediate stages with AFM. Extended DNA initially folded

into rod-like structures. The rods assembled into interwoven networks, which compacted around condensation loci to form nanoparticles. The interwoven network arrangement could be visualized on the surface of fully formed nanoparticles with AFM phase imaging. The study showed that the mannose-modified DNA nanoparticles are hollow, with a water-filled core and woven-DNA shell. The implication of this architecture for gene therapy needs to be studied. Our study also presents new strategies for improving the gene-therapy efficacy by entrapping drugs and biomolecules in the nanoparticles for co-transport along with the DNA vaccine.

Biophysics
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NIDDK
Fractional composition of endocrine cells determines the architecture of pancreatic islets
Islets of Langerhans, consisting of alpha, beta, and delta cells, play a central role for glucose homeostasis by secreting counter-regulatory hormones, glucagon and insulin. Human and rodent islets have similar size ranges from a few cells to several thousand cells, but they have distinctive architectures of cellular fraction and arrangement. Rodent islets have beta cells in the core and non-beta cells on the periphery. However, human islets have more alpha cells (20-30% vs. 10-15% in rodents), and non-beta cells distributed throughout islets. The spatial organization of endocrine cells may play an important role for islet function. For example, the gap junctions between beta cells help to induce robust electrical activities of beta cells. Furthermore, recent advances have highlighted the roles of paracrine interactions between endocrine cells for glucose control. In this study, our goal is to characterize islet architectures and find the origin of the structural difference between the species. First, using immunohistochemistry and a computer-assisted imaging method, we quantified centroid position of every endocrine cell in each islet in human cadaveric pancreas sections, and calculated the probabilities of contact between cell types. We found that human islets were not a random mixture of endocrine cells, but beta cells preferred to be in contact with beta cells as in rodent islets. Therefore, we hypothesized that the unique architecture of human islets (vs. rodent islets) might result from differences in the cellular fraction rather than differences in the cellular adhesion. We tested the hypothesis with a computer simulation where we varied the fraction of alpha cells but kept the relative attraction between cell types (stronger attraction between beta cells). As the alpha-cell fraction increased, cell clustering resulted in spatial structures reminiscent of human islets that have subdomains of beta-cell core structures. We then confirmed the hypothesis with an in vitro pseudo-islet formation experiment with alpha and beta cell lines (alpha-TC and MIN6) with different fractions. Thus we conclude that the preferential contact between beta cells was preserved across species, and the structural difference of islets resulted not from different cellular adhesion, but from different cellular fractions. This finding may explain the functional degeneration in type 2 diabetes where islet structures are altered by preferential loss of beta cells.

Carcinogenesis
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NCI-CCR
IKK1 links inflammation and tumorigenesis in lung squamous cell carcinoma
Lung cancer is the leading cause of cancer deaths worldwide. Various genetically engineered and chemical induced lung adenocarcinoma mouse models have been established. However, spontaneous

lung squamous cell carcinomas (SCC) mouse models are very rare. IkB Kinase subunit1 (IKK1) mutations have been reported in many types of human cancer. Our recent study established a mouse model of lung squamous cell carcinoma (SCC) by inactivating IKK1 in vivo, and found EGFR/ Ras/p38 MAPK pathways were activated and played important roles in lung SCC tumorigenesis. However, IKK1â€™s attributes relating to linkage between inflammation and lung SCC have not yet been well-defined. We here show that inactivation of IKK1 in mice causes systemic whole-life chronic inflammation, which consequently promotes lung SCC initiation and progression. In mice with mutated IKK1, CD4 T cells, dendritic cells, and macrophages are all significantly increased in the spleen and lung. Proinflammatory cytokines and chemokines are also remarkably elevated in the lungs of these mice. An M2 macrophage phenotype is observed only in the lungs of old IKK1 mutant mice, not in younger mice, suggesting the M1/M2 polarization may be driven by the proinflammatory microenvironment in these mice. Production of Th2 cytokines and IL-10 are elevated in CD4 T cells from the lungs of mice with mutated IKK1, and may contribute to M2 polarization during the chronic inflammation. In addition, depletion of macrophages in these mice can significantly reduce Th2 cytokine production, oxidative DNA damage, Ras/p38 MAPK pathway activation and completely shut down SCC development in the lungs, suggesting their important roles in inflammation-associated SCC tumorigenesis. Taken together, our study generates, for the first time, a spontaneous mouse model of inflammation-associated lung SCC, and suggests that IKK1 plays important roles in linking inflammation and carcinogenesis of lung squamous cell carcinoma.

Carcinogenesis
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NCI-CCR
Integrated Genomic and microRNOmic Analysis Identifies miR-32 and MCL-1 as Drivers of Melanomagenesis
Cutaneous malignant melanoma is considered one of the most deadly human cancers, based on both its penchant for metastatic spread and its typical resistance to currently available therapy. We hypothesize that understanding events driving melanomagenesis will not only help biology but also provide better prevention and therapeutic targets. However, general mechanisms driving melanomagenesis are not fully understood. Here we develop and exploit integrated in vitro and in vivo model systems, utilizing untransformed and NRas transformed immortalized primary melanocytes and melanoma tumors, to characterize genes and microRNAs (miRNAs) driving melanomagenesis for a more effective anti-melanoma therapy. Using this model we identified 3208 regulatory relationships involving 72 miRNAs and 1031 target genes driving melanomagenesis. A substantial down-regulation of the miRNA signature and up-regulation of target genes was observed during melanomagenesis. Of interest, miR-32-MCL1 miRNA-mRNA interactions were among the top interactions identified by the integrated analysis. Of interest we find that miR-32 acts as a melanoma tumor suppressor by inducing apoptosis, pigmentation, differentiation and by reducing tumorigenicity of malignant melanoma cells. Furthermore, we identify that overexpression of the anti-apoptotic gene MCL1 is an oncogenic event in wtBRAFV600E, CRaf and PI3K overexpressing primary melanocytes. Of interest, MCL1 is not only a target of miR-32 but also its levels are regulated by tumor-suppressor ARF via proteosomal degradation. Notably, we found that treatment with an MCL-1-specific inhibitor is effective not only in different human melanoma subtypes but also is highly synergistic in combination with the BRAFV600E inhibitor vemurafenib in preclinical melanoma models in vivo. We demonstrate that MCL1 inhibition can be an effective anti-melanoma therapy, alone or in combination, in multiple different human melanoma subtypes that have no treatment options available in the clinic at present. Our results demonstrate that losses of â€˜two

tumor suppressorsâ€™ (mir-32 and ARF), along with overexpression of oncogenic MCL1 are key events driving melanomagenesis.

Carcinogenesis
Gallardo, Viviana
Visiting Fellow
NHGRI
Migration of the zebrafish lateral line as a model for metastasis
The transition of a cancer from benign to metastatic form requires the acquisition of various cellular properties, including migratory propensity and invasiveness. To date, most of our understanding of cell migration and cancer metastasis is based on in vitro studies of single cells. However, in vivo models of collective cell migration have been less intensely studied because of the inherent difficulty in undertaking such analyses. Recently, the migrating primordium of the zebrafish posterior lateral line (PLLp) has emerged as a powerful model to investigate the molecular mechanisms of cell migration and for understanding how this process is controlled. The PLL is a mechanosensory system that detects water movements. Its individual organs, the neuromasts, are formed by regular deposition from a group of migrating cells that travel along the body of the fish. The collective migration of the primordium cells has been the focus of intense study due to the parallels of this cellular behavior with that of invasive cancer cells. Through the combination of transgenic fish, FACS and microarray analysis we identified a repertoire of key genes expressed in the migrating primordium. Interestingly, mostly of the genes captured in this study have demonstrated roles in collective cell migration and cancer progression. Given the cellular and molecular equivalences between both processes, we carried out a high-throughput cell migration assay to identify drug candidates that inhibit the invasive/metastatic phenotype. Using the PLLp as a readout for migratory inhibition, we tested the LOPAC 1280 library to identify compounds that blocked PLLp migration. We used the cldnb:egfp transgenic line in combination with automated fluorescent microscopy, and identified 66 compounds interfering with primordium migration without overt toxicity. Selected compounds were confirmed in their migration blocking activity in in vitro melanoma invasion assays. One of the genes of interest arising from these studies was c-Met. We found that c-Met and downstream proteins of the HGF/Met signaling pathway such as PI3K and Mmp9 were involved in the migration of the primordium. We also identified that inhibition of the Src pathway, disrupted primordium migration. Finally, we envision that the use of high-throughput screens to identify drugs that inhibit cancer progression using zebrafish, may be crucial to create efficient targeted therapies to treat human cancers.

Carcinogenesis
Kelly, Melissa
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NHGRI
Identifying the Interaction Between Hippo and Beta-catenin Signaling in Liver Cancer Progression
Liver cancer is the third leading cause of cancer-related deaths worldwide; therefore, identifying signaling pathways, which mediate liver tumorigenesis is of the upmost importance. Recently our lab and others have demonstrated a causative role of the Hippo signaling pathway in liver cancer. The Hippo pathway, originally identified in Drosophila, plays a pivotal role in controlling cell proliferation and cell death. It consists of two serine threonine kinases, Mst1 and Mst2, which act to initiate a signaling cascade leading to the inactivation and cytoplasmic retention of the oncogene, Yap. In the absence of Hippo signaling Yap is active in the nucleus promoting transcription of cell proliferation genes. Yap has

been linked to various cancers including prostate, breast and now liver. We have generated Hippo null mice, wherein hepatocellular carcinoma (HCC) liver tumors arise as early as 3 months of age. These liver tumors are characterized by a significant increase in overall liver size as well as an expansion in the liver stem cell compartment. In the Hippo null HCCs, along with Yap protein levels and activity being enhanced, a well-known oncogene, beta-catenin, is also upregulated. Beta catenin has an integral role in the adherens junction complex as well as in mediating the Wnt signaling pathway. Like Yap, beta catenin activation has been observed in a variety of cancers. Therefore we reasoned that Yap could be signaling through beta catenin to induce HCC formation in Hippo null mice. To test this we removed beta catenin in the Hippo null mice and found that the phenotype was enhanced characterized by accelerated tumor formation and increased liver stem cell expansion. This surprising phenotype was addressed in vitro and we found that beta catenin can inhibit Yap activity by sequestering it in the cytoplasm. Thus, it would appear that in this context of Yap overexpression in the liver, beta catenin does not function as an oncogene but as a tumor suppressor. This knowledge has provided novel insight in personalized HCC treatment in that reduction of beta catenin is not always going to be beneficial in treating HCC patients.

Carcinogenesis
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NIEHS
A reporter system for identifying mutagens acting preferentially on single-strand DNA
For most carcinogens, the underlying mechanisms of action are poorly understood. Our recent results show that very strong mutagenesis can be specific to single-strand DNA, and thus can be overlooked by conventional mutagenesis assays. Given the common occurrence of ssDNA in cells, we sought to develop a reporter system in yeast that readily identifies carcinogens that are strongly mutagenic specifically to ssDNA. The reporter genes ADE2, URA3, and CAN1 were inserted into the left subtelomeric region of chromosome V, in cdc13-1 temperature-sensitive yeast. Shifting to 37C causes telomere uncapping followed by 5â€™ to 3â€™ resection, exposing a long 3â€™ ssDNA overhang containing the reporter. We induced DNA damage by expressing human APOBEC3G or by acute treatment with sodium bisulfite. These agents were not known to be strong mutagens. However, since they deaminate cytosine in ssDNA, multiple uracils are formed in the ssDNA overhang. Upon return to permissive temperature, the complementary strand of subtelomeric DNA is resynthesized. The presence of lesions in the ssDNA overhang, i.e. abasic sites from excision of uracil or the 6-sulfonyluracil intermediate of the bisulfite reaction, forces the use of error-prone translesion DNA synthesis (TLS), creating a distinctive strand-coordinated, multi-mutation signature. Treatment with either agent caused a >1,000-fold increase in simultaneous loss of CAN1 and ADE2 function. In contrast, neither agent induced mutations in controls where the reporter was maintained in a dsDNA state. Sequencing of 114 multiply-mutated reporter isolates revealed 541 mutations, of which >97% originated from cytosines in the ssDNA overhang. Deletion of the uracil-DNA N-glycosylase gene UNG1 resulted in a 5-fold increase in APOBEC3G-induced mutagenesis, and all mutations were C to T transitions, in agreement with Ung1-catalyzed removal of uracil from DNA. In contrast there was no effect on bisulfite mutagenesis, consistent with Ung1 being inefficient at excising 6-sulfonyluracil. Finally, Pol zeta is the main TLS polymerase for mutagenic bypass, since deletion of REV3 decreased the mutation frequency by 90%. Altogether, our results show the strong in vivo ssDNA mutagenic activity of a ubiquitous physiological factor (APOBEC3G) and a known food contaminant (bisulfite). In turn, this successful application of the reporter system provides validation for our approach to identifying other ssDNA-specific mutagens in a high throughput manner.

Cell Biology - General
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NCI-CCR
Suppression of tumorigenicity by a truncated form of lamin A
Advanced age is a strong risk factor for cancer and many human premature aging diseases are characterized by increased tumors susceptibility. However, patients with the premature aging disorder Hutchinson Gilford Progeria Syndrome (HGPS) do not develop tumors despite highly elevated levels of DNA damage. We demonstrate here that the disease-causing protein, a truncated form of the structural protein lamin A named progerin, is responsible for cancer resistance in HGPS patient cells. HGPS fibroblasts are refractory to experimental transformation as assessed by in vitro and in vivo tumorigenicity assays, despite showing similar proliferation kinetics as wild type cells in culture. Ectopic expression of progerin in transformed normal fibroblasts nearly abrogates their tumorigenic potential. Using a genome-wide shRNA screen, we have identified the bromodomain binding protein BRD4 as a mediator of the progerin-dependent tumor suppression mechanism. Knockdown of BRD4 in HGPS cells reverses their resistance to transformation, promoting efficient tumor-initiation ability. With regards to mechanism, we demonstrate that BRD4 acts by preventing cellular reprogramming of HGPS cells in response to oncogenic insults. While wild type fibroblasts de-differentiate upon transformation and acquire properties of cancer-initiating cells, HGPS cells resist oncogenic reprogramming, in a BRD4-dependent manner. We are currently assessing the tissue- and cancer-type specificity for the tumor suppressor mechanism mediated by BRD4.

Cell Biology - General
Mercken, Evi
Visiting Fellow
NIA
Skeletal muscle SIRT1 is necessary for the bodyâ€™s metabolic response to a high fat diet and the beneficial effects of resveratrol.
Skeletal muscle insulin resistance plays a key role in the pathogenesis of type 2 diabetes. The NAD+-dependent deacetylase SIRT1 regulates mitochondrial biogenesis and has been implicated in glucose metabolic homeostasis. Resveratrol, a proposed SIRT1 activator, has been shown to improve health and lifespan in mice fed a high fat diet (HFD). However, there is still controversy on the role of SIRT1 in these processes and whether the beneficial effects of resveratrol are due to direct SIRT1 activation. Interestingly, most studies have shown the role of SIRT1 at the whole-organism level. We generated SIRT1(i)skm-/- mice in which SIRT1 ablation is selectively induced in adult skeletal muscle. We investigated whether SIRT1 is involved in the metabolic benefits of resveratrol on a standard (SD) and under HFD conditions. SIRT1(i)skm-/- mice were metabolically similar to wild type (WT) mice under SD; however, after 24 weeks of HFD, SIRT1(i)skm-/- mice were more susceptible to diet-induced obesity and developed glucose intolerance and peripheral insulin resistance. Moreover, SIRT1(i)skm-/- mice also displayed decreased fatty acid and mitochondrial metabolism in the liver further supporting the importance of SIRT1 in skeletal muscle in response to HFD. Consistent with previous reports, resveratrol prevented most of the metabolic effects induced by HFD in WT mice while it had no benefits in SIRT1(i)skm-/- mice, demonstrating that SIRT1 in skeletal muscle plays a pivotal role in resveratrolâ€™s whole-body health effects. In addition, WT mice treated with resveratrol showed increased mitochondrial biogenesis and function in skeletal muscle; these adaptations were abrogated in SIRT1(i)skm-/- mice. These data highlight the importance of SIRT1 in skeletal muscle mitochondria in preventing the development of insulin resistance and glucose intolerance. Furthermore, we found that

resveratrol increased nicotinamide-phosphoribosyltransferase (Nampt) levels, the rate-limiting enzyme of the NAD+ salvage pathway, in a SIRT1-dependent manner. These data suggest a positive feedback loop between SIRT1 and Nampt, perpetuating the effects of resveratrol. Together, these data show for the first time that SIRT1 in skeletal muscle is required for whole body improvements in metabolism upon resveratrol treatment, and suggest that SIRT1 and Nampt act together in a positive loop that allows the sustained effects of resveratrol.

Cell Biology - General
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NIAAA
Regulation of axonal morphogenesis by docosahexaenoic acid and its ethanolamide derivative, Synaptamide.
Although the functions of spheroids, bulbs or varicosities on continuous axons are not clear it has been shown that often these structures arise during pathological conditions. It is believed that focal blockade of axonal transport initiated during stretch, inflammation or neurodegenerative conditions may induce swelling with gradual formation of bulbs and spheroids. Axonal swelling, which may occur without apparent axonal disintegration may contribute to functional impairment and present an early sign of disorders such as multiple sclerosis and brain injury. The purpose of this study is to evaluate the effects of neuron-enriched omega-3 fatty acid docosahexaenoic acid (DHA, 22:6n-3) and its endogenous metabolite N-docosahexaenoylethanolamide (synaptamide) on the structure development. Mouse cortical neurons in culture were treated with fatty acids or synaptamide and axonal spheroids were visualized by staining with axonal marker Neurofilament NF-H. In parallel, axon length was evaluated by staining with axon-specific SMI-312 antibody and quantification using automated neurite quantification module of the Metamorph. We found that average area of the spheroids in vehicle treated neurons (126sq micrometer) was significantly reduced by 48hr supplementation with 1 micromolar DHA or 10 nanomolar DHA ethanolamide, synaptamide (87 and 84 sq micrometers, respectively (P<0.001). Interestingly, non-omega-3 fatty acids, docosapentaenoic (22:5n-6) and oleic acid (18:1n-9), at 1 micromolar concentration had no effect on axonal spheroid area, when compared to control (127 and 113 sq micrometer, respectively P>0.05, ns). In addition, axonal length evaluated in at least 200 neurons indicated that DHA at 1 micromolar and synaptamide at 10 nanomolar similarly induced 1.6-1.7 fold increase of the average axon length compared to control (P<0.0001). Based on our current results we conclude that DHA and synaptamide regulate axonal morphogenesis by reducing spheroid formation and increasing average length of the axon. Further research is required to reveal mechanisms and potential implications of these findings.

Cell Biology - General
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Visiting Fellow
NIDA
Cocaine hijacks sigma-1 receptors from the ER to the nuclear membrane to interact with lamina A/C: Effect on MeCP2 gene expression
The sigma-1 receptor (Sig-1R) is an endoplasmic reticulum (ER)-resident non-opioid receptor that is expressed in the nervous system and can regulate cellular differentiation, neuroplasticity, and neuroprotection. Nuclear lamins in the nucleus are essential for the nuclear envelope assembly, DNA synthesis, and gene transcription. We tested here the hypothesis that cocaine, via Sig-1Rs, may affect

the gene expression of methyl CpG binding protein 2 (MeCP2) which relates to cocaine addiction. In glial and NG108-15 cells, cocaine increases the nuclear envelope invaginations (NEI) and grooving, suggesting that the nuclear-cytoplasmic exchange is enhanced by cocaine. Confocal microscopic data show that cocaine and another Sig-1R agonist DHEAS cause the translocation of Sig-1Rs from the ER into the nuclear membrane. After cocaine treatment, Sig-1Rs localize mainly in the outer membrane of the nucleus and could increase the chance to interact with lamin A/C. Cocaine is known to cause addictive behavior by upregulating (MeCP2) in the brain. In rat primary cortical neurons, cocaine causes an increase in MeCP2. This effect is antagonized by Sig-1R antagonist BD1063. Our results suggest that cocaine increases MeCP2 gene expression by translocating Sig-1Rs into the nuclear outer membrane to increase the NEI and grooving to facilitate the Sig-1R-lamin A/C interaction to regulate the MeCP2 gene expression.

Cell Biology - General
Ghosh, Swati
Visiting Fellow
NIEHS
An integrated approach reveals that Tet1 maintains mouse embryonic stem cell identity partly by regulating LIF dependent Stat3-mediated gene activation
Embryonic stem cells (ESCs) maintain an unique epigenetic state that enables both self-renewal and differentiation into all embryonic lineages. Because of their ability to differentiate into any of over 200 cell types in adult body, ESC-based therapies have been proposed for regenerative medicine and tissue replacement after injury or disease. The development of such therapies, however, largely depends on our complete understanding of the genes that maintain the self-renewal and pluripotency properties in ESCs, which define the ESC identity. With the goal of predicting novel regulators of ESC identity, we developed a meta-analytic approach that integrated over 60 previously published microarray gene expression datasets in mouse ESCs and differentiated cells. Our analysis revealed Tet1, an enzyme proposed to promote DNA demethylation, as one of several genes to have a previously unknown role in the maintenance of ESC identity. Using RNAi, we validated that Tet1 indeed is required to main ESC identity as Tet1 knockdown (KD) resulted in the differentiation of ESCs. To determine what role Tet1 plays in regulating transcriptional networks influencing self-renewal and pluripotency, we performed microarray expression profiling in control and Tet1 KD cells to identify genes differentially expressed upon Tet1 KD. Bioinformatics analysis of global gene expression changes upon Tet1 KD versus expression changes observed after KD/knockout of any of over 20 other pluripotency-associated factors (gathered from published reports) revealed that genes that are differentially expressed upon Tet1 KD underwent similar expression changes upon leukemia inhibitory factor (LIF) withdrawal. This led us to predict a possible functional interaction between Tet1 and LIF signaling. LIF signaling, mediated by downstream transcription factor Stat3, is known to promote self-renewal and pluripotency in mouse ESCs. Withdrawal of LIF from the culture media leads of differentiation of mouse ESCs. To explore the possible functional interaction between Tet1 and LIF/Stat3 signaling, we examined the ability of Stat3 to bind to its target sites on chromatin in the absence of Tet1. ChIP experiment using Stat3 antibody revealed the loss of Stat3 binding upon Tet1 KD, which was accompanied by loss of expression of Stat3 target genes. These data support that Tet1 is indispensable for maintaining the ESC identity, and that LIF induced Stat3-mediated gene activation in mouse ESCs is dependent on Tet1.

Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology
Chen, Pei-Wen

Visiting Fellow
NCI-CCR
ARAP2 stabilizes focal adhesions through regulation of Arf6 and Rac1
Cell migration is a dynamic process that requires cells to adhere to and detach from the extracellular matrix (ECM) in a coordinated fashion. Adhesion to the ECM is mediated through a continuum of adhesive structures starting with the small and short-lived nascent adhesions that mature to focal complexes, which give rise to the larger and more stable focal adhesions (FAs). These adhesions share some common components such as clustered integrin receptors, paxillin, talin, and vinculin. Retraction of the cell rear, necessary for cell movement, depends on the disassembly of FAs. The molecular basis for regulation of assembly and disassembly of FAs is still being discovered. Rac1 and RhoA stimulate the formation of focal complexes and FAs respectively. Several Arf GTPase-activating proteins (GAPs) associate with FAs. ArfGAPs are multidomain proteins that catalyze the conversion of Arf-GTP to Arf-GDP. Here, we have examined the possibility of one of the FA-associated ArfGAPs, ARAP2, coordinates the activity of Arf and Rho family proteins to regulate FAs. ARAP2 contains a sterile alpha-motif, 5 PH, Ank repeat, Ras association domains, an ArfGAP and a RhoGAP domain. Using confocal microscopy, time-lapse total internal reflection fluorescence (TIRF) and fluorescence recovery after photobleaching (FRAP) analysis, we found reducing ARAP2 expression in cells decreased the lifetime and the size of FAs whereas overexpression of ARAP2 had the opposite effects. Biochemical approaches revealed that ARAP2 siRNA-treated cells had higher levels of Arf6-GTP and Rac1-GTP, but had similar levels of RhoA-GTP to control cells. Constitutively active Arf6 and Rac1 mimicked and dominant negative Arf6 and Rac1 suppressed the effect of reduced ARAP2 expression on FAs. In addition, the effects of ARAP2 on FAs and Rac1 activity require its ArfGAP activity since an ArfGAP deficient mutant of ARAP2 behaved like ARAP2 knockdown. Finally, two reagents that block FA disassembly, nocodazole and [K44A]dynamin2, reversed the phenotype caused by reduced ARAP2. Conversely, cells overexpressing ARAP2, or dominant negative mutants of Arf6 or Rac1 retained more large FAs during microtubule-induced FA disassembly. Taken together, these data support a model in which ARAP2 attenuates FA disassembly and stabilizes FAs by decreasing Rac1 activity consequent to its Arf6GAP activity that lowers Arf6 activity.

Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology
Kutys, Matthew
Doctoral Candidate
NIDCR
Selective Activation of the Rac1/Cdc42 Regulator Beta-Pix Governs Cell Migration in 3D Collagen Microenvironments
Cellular adhesion to the extracellular matrix (ECM) and subsequent migration are essential components of embryonic development, tissue repair, and immune response, and are often deregulated in atherosclerosis and cancer. In each of these processes, cells must migrate in different ECM microenvironments, where their modes of migration are known to differ. However, the fundamental mechanisms that drive their ECM-specific migratory response are unknown. Differential activation of the Rho family of GTPases, such as Cdc42, Rac1, and RhoA, is implicated in governing the distinct morphological and migratory phenotypes downstream of adhesion to ECM fibers such as fibronectin and collagen. We therefore hypothesized that adhesion to specific ECM fibers triggers selective regulation of different guanine nucleotide exchange factors (GEFs), a class of molecules that activate the Rho GTPases. To investigate if the activity of certain GEFs were increased during migration in different ECM microenvironments, we utilized an affinity precipitation-based mass spectrometry screen to isolate active GEFs from primary human fibroblasts migrating in homogenous type I collagen, fibronectin, and ECM-free environments. Among the GEFs identified, we found that the Rac1/Cdc42 GEF beta-Pix was

specifically and robustly activated during migration in collagen matrices. Stable beta-Pix knockdown lines were generated using lentiviral shRNA delivery and assayed for intracellular GTPase activity levels in different ECM conditions. We observed a 60% decrease in Rac1 and Cdc42 activity levels with beta-Pix knockdown only in collagen matrices. These activity decreases led to inhibited cellular spreading and nearly complete failure of the cells to migrate in 3D collagen matrices, with no observable phenotypes in other ECM microenvironments. This migratory defect was characterized by cells exhibiting hyper-protrusive activity and an impaired ability to form a stabilized leading edge. In contrast to fibroblasts migrating on fibronectin, live cell imaging reveals that beta-Pix does not localize to focal adhesions when cells migrate on collagen, but instead accumulates on the membrane adjacent to areas of cellular protrusion, allowing for localized Rac1/Cdc42 activation. These results establish ECM-dependent regulation of a specific GEF as a fundamental mechanism of migration in different microenvironments, and that beta-Pix is critical for the cellular migratory response in collagen environments.

Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology

Maeda, Azusa
Other
NIDCR

WISP-1/CCN4: Potential Regulation of Mineralized Tissue through Modulation of Wnt Signaling

WISP-1/CCN4 (Wnt inducible secreted protein 1/CCN family 4: WISP-1) was originally identified as an oncogene that is up-regulated by wnt activation. Later studies showed WISP-1 is highly expressed in mineralized tissues and during fracture healing however its function in bone was unclear. To determine the precise function of WISP-1 in mineralized tissues, we generated mice unable to express WISP-1 (WISP-1 KO) and evaluated their bones and osteogenic cell potential using bone marrow stromal cells (BMSCs). First, femur bones were examined using Dual Energy X-Ray Absorptiometry (DEXA) and micro computerized tomography (micro-CT). At 1 month of age there was little difference in the bone mineral density (BMD) between the normal (wild type/WT) and WISP-1 KO bones, however by 3 months the BMD of the WISP-1 KO bones were significantly lower than that of WT bones. Micro-CT showed the WISP-1 KO bones from females had decreased trabecular bone mass and that bone from WISP-1 KO males and females had decreased cortical bone thickness and cortical BMD. Further, 4-point bending tests to analyze the mechanical strength of the bones showed that stiffness, yield force and ultimate force of femurs were also significantly lower in both male and female WISP-1 KO mice at 3 months of age. To understand the molecular basis for this decreased bone mass, we next isolated osteogenic progenitors from the bone marrow (BMSCs) and tested their ability to proliferate and differentiate. When tested in vitro, BMSCs from WISP-1 KO mice had higher levels of BrdU incorporation compared to WT cells indicating they proliferated faster. In contrast, mRNA levels of genes used a measure of the osteodifferentiation such as alkaline phosphate, bone sialoprotein and osteocalcin were significantly down-regulated in WISP-1 KO cells and were accompanied by a significant reduction in calcium accumulation by Alizarin Red S staining. Consistent with these data, mineralization and bone formation of BMSCs into immunocompromised mice were lower in transplant with WISP-1 KO cells. Interestingly, we found the mRNA expression of multiple canonical Wnt target genes including Axin2 and MMP14 were significantly down-regulated in WISP-1 KO. These findings suggest that wnt and its target WISP-1 could, potentially, regulate each other during osteodifferentiation. We propose that WISP-1 regulates BMD, cortical bone thickness and strength potentially by modulating the canonical wnt pathway in osteogenic cells.

Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology

Milberg, Oleg
Doctoral Candidate
NIDCR
Assembly of an actomyosin scaffold during regulated exocytosis in the salivary glands of live animals
Regulated exocytosis is a fundamental process that regulates the physiology of several secretory organs and whose impairment results in the onset of several diseases, such as pancreatitis. The events involved in regulated exocytosis are controlled by a highly dynamic cytoskeleton and its molecular motors, which coordinate how the secretory vesicles or granules travel in a timely and well orchestrated manner from the trans-Golgi network to the plasma membrane where they dock, fuse and collapse to release their contents into the extracellular space. To better understand how these events occur and how they are regulated in vivo, an intravital imaging system was developed in our lab. As a model system, we used the submandibular salivary glands of live mice and rats where regulated exocytosis is elicited upon stimulation of the ß-adrenergic receptors. We have previously shown that a contractile actomyosin scaffold is recruited onto the granules after their fusion with the plasma membrane and it drives this process to completion. Our current goal is to determine how the actomyosin scaffold is assembled, what molecular components are involved in its recruitment, and how these components are regulated in live animals. To this aim, we developed a series of transgenic mice expressing a fluorescently labeled marker for F-actin (Life act) and two isoforms of nonmuscle myosin II, NMIIa and NMIIb. We showed that after stimulation of the ß-adrenergic receptors, NMIIa and NMIIb were specifically recruited from the cytosol onto the granules following the onset of actin polymerization around the granules. To further gain insight into the machinery regulating this process, we performed a proteomic analysis of the isolated secretory granules and found that the GTPase Septin 2, a molecule previously described to interact and regulate the activity of NMIIa during cytokinesis, was present in the granule preparation. We validated the expression of Septin 2 in the salivary glands by western blot and its localization to the secretory granules by immunofluorescence. Moreover, by using intravital time lapse imaging and transient expression of Septin 2-GFP in live rats, we showed that Septin 2 is recruited after actin. Our results suggest that Septin 2 may be a good candidate for regulating the formation and function of the actomyosin complex during regulated exocytosis. Currently, we are investigating how Septin 2 functions in regulating myosin recruitment using our novel approach.

Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology
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Postdoctoral Fellow
NIEHS
Testis Expressed Actin-like 7b (Actl7b) is Required for Mouse Spermatid Morphogenesis and Male Fertility
Human male infertility is often associated with a high incidence of abnormally shaped sperm heads, suggesting that cytoskeletal regulation is important for male fertility. During spermiogenesis round spermatids differentiate into elongated and then condensed spermatids followed by spermiation. The involvement of filamentous actin (F-actin) has been suggested for several aspects of spermatid differentiation, including acrosome formation and attachment to the nucleus, formation of tubulobulbar complexes, cytoplasmic removal, and spermiation. Although the structural components and morphological changes in the complex process of spermiogenesis have been described in detail, relatively little is known about the mechanisms that drive these structural changes. Actin-like 7b (Actl7b) is an orphan actin related protein (ARP) family member. Actl7b is an intronless gene expressed in spermatids and conserved in mammals. Immunohistochemistry and indirect immunofluorescence were utilized to investigate the expression of ACTL7B. It is localized in the cytoplasm of round and elongating spermatids and co-localizes with phalloidin labeled F-actin in or around the forming acrosome,

suggesting a role in this process. To determine the functional relevance of Actl7b, knockout mice were generated from targeted ES cells, obtained from the KnockOut Mouse Project (KOMP) Repository, in which the Actl7b coding region was replaced with a LacZ reporter sequence. X-gal staining of tissues from heterozygous animals revealed that Actl7b is expressed in the testis and, unexpectedly, the brain. While Actl7b knockout mice develop to adulthood and appear normal, breeding studies revealed that Actl7b knockout males mate and produce vaginal plugs, but are infertile. Testis and epididymal weights and sperm counts are lower in knockout males than in wild type males. In addition, sperm heads are misshapen with a rounded appearance. Most of these sperm are immotile, with less than three percent showing minimal flagellar movement. These results indicate that Actl7b is required for spermatid morphogenesis, sperm motility, and male fertility. They also suggest that ACTL7B is either the F-actin recognized by phalloidin or is required for F-actin assembly in spermatids. This research was supported by the Intramural Research Program of the NIH.

Cell Cycle-General, Regulators and Checkpoints, Apoptotic Mechanisms
Leo, Elisabetta
Postdoctoral Fellow
NCI-CCR
Analysis of the sensitization of cancer cells to DNA damaging agents by SLFN11.
It is critically important to identify the molecular determinants and predictive molecular markers of cancer sensitivity to different anticancer drugs to personalize treatments and rationalize combination therapies. To identify genes that, at the expression level, could predict the cytotoxicity profiles of antiproliferative agents in the NCI-60, we correlated the expression profile of more than 17,000 genes of treated and untreated cells, finding that a single gene, SLFN11 showed very high correlation with most classes of the DNA damaging agents (TOPI, TOPII inhibitors, alkylating agents, DNA synthesis inhibitors). To test the relationship between SLFN11 expression and the sensitivity to these drugs in human cancer cells, we silenced SLFN11 expression in four cells lines, (two with high and two with low endogenous level of this protein), and tested for cell proliferation after different drug treatments. We found that the cellular response to Camptothecin (CPT), Cisplatin, Etoposide and irradiation was greatly affected by the presence or absence of SLFN11 expression level: the knockdown of this protein induced a remarkable resistance to these agents only in cells endogenously highly expressing SLFN11, whereas it did not change the response after treatment with other classes of chemotherapeutic agents such as microtubule-targeting compounds or kinase inhibitors, nor in cells that have an endogenous low level of SLFN11. To elucidate the function of this protein we performed survival assays, cell cycle studies, investigations on DNA replication, DNA damage and transcription in cells upon knockdown of SLFN11 and CPT treatments, finding remarkable differences in the way the cells responded in presence and absence of our protein of interest. In conclusion, the identification of SLFN11 modulating effects towards response to DNA damaging agents lays the ground for the study of a previously unexplored player of cancer cell response to the most used classes of anticancer agents. Moreover, SLFN11 expression could potentially become an invaluable biomarker as predictor for the response of human cancer samples to chemotherapeutical regimens.

Cell Cycle-General, Regulators and Checkpoints, Apoptotic Mechanisms
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Postdoctoral Fellow
NICHD

Using high throughput methods to identify small molecules and target genes to selectively induce DNA re-replication and apoptosis in cancer cells

In normal mitotic cells, DNA replication is highly regulated to ensure that the genome is duplicated only once per cell cycle. Geminin is a key regulator protein in this process and when suppressed in human cancer cells will cause an unregulated form of DNA replication called DNA re-replication and leading to apoptosis. Of 23 cell lines tested, only those derived from cancers were sensitive to suppression of geminin (colon, breast, lung, brain, kidney, bone). Cells derived from normal tissues have additional mechanisms to prevent DNA re-replication, providing a unique strategy for selectively targeting cancer cells that could be applied to the discovery of potential anticancer therapeutics. To determine if geminin is the only protein whose suppression selectively kills cancer cells, we completed a genome-wide siRNA high throughput screen (HTS) using cancer and normal cells. Preliminary data reveals that geminin has by far the strongest, most cancer-selective effect among the 21,584 genes tested. While several other genes can cause re-replication when suppressed, their effects were much less dramatic, suggesting a more indirect involvement in the re-replication process. This confirmed our speculation that geminin might be the Achillesâ€™ heel of cancer cells. Because of the technical difficulties with current in vivo siRNA delivery mechanisms, we completed an HTS with 343,078 small molecules to identify potential drugs that would mimic the effects of geminin suppression. From this screen, we identified 127 drugs that have selective activity on cancer cells. We are currently in the process of validating these results and determining their mode of action. Once these candidate drugs are shown to be active and selective, they will be introduced in mice containing human tumor xenographs.

Cell Cycle-General, Regulators and Checkpoints, Apoptotic Mechanisms

MO, MIN
Visiting Fellow
NICHD

Breaking the break: The role of Nup188 in the spindle assemly checkpoint silencing

The spindle assembly checkpoint (SAC) ensures faithful separation of sister chromatids during mitosis by monitoring interactions between kinetochores (KTs) and microtubules (MTs). In the absence of MT attachment or tension between sister KTs, the SAC is turned on and blocks cells from undergoing the metaphase-anaphase transition by promoting the formation of the mitotic checkpoint complex (MCC). The MCC inhibits the anaphase promoting complex/cyclosome (APC/C), a multi-subunits ubiquitin ligase complex whose activity is required for mitotic exit. Once all KTs are attached to MTs and tension is generated, the MCC becomes inactivated through a poorly understood process and the APC/C is free to promote mitotic exit by degrading key mitotic proteins such as Cyclin B. The nuclear pore complex (NPC) is the conduit for transport of molecules between the interphase nucleus and cytosol. Components of the NPC, nucleoporins, have unexpectedly also been implicated in SAC regulation, and many SAC components associate with the NPC during interphase. Nup188 is a nucleoporin that has not previously been implicated in SAC regulation. Here we show that it can be co-precipitated with the APC/C and with SAC component Mps1 from lysates of mitotic HeLa cells. Interactions between Nup188 and the APC/C were stronger during anaphase, after the APC/C became active as a ubiquitin ligase, than during SAC inhibition. Interestingly, we observed an inverse pattern of interaction between Nup188 and Nup153, another nucleoporin whose overexpression has been shown to disrupt SAC signaling. Depletion of Nup188 from Xenopus Egg Extract (XEE), an ex vivo system that recapitulates many aspects of mitotic control, did not apparently compromise spindle assembly or MT-KT interactions, a condition satisfies SAC. However, Nup188 depleted XEEs showed a SAC-dependent mitotic exit delay, suggesting that Nup188 has an important role in silencing the SAC. We are currently testing this hypothesis through direct measurement of APC/C activity and MCC integrity, two essential indicators of SAC activity. In

summary, our findings reveal an unexpected role of Nup188 in SAC regulation. These findings suggest a complex and multidimensional level of SAC regulation to assure the fidelity of chromosome segregation and prevent aneuploidy.

Chemistry
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Postdoctoral Fellow
NCI-CCR

The mechanisms of cytotoxicity of O2-arylated diazeniumdiolate anticancer agents

The study of nitric oxide (NO) as central player in a variety of physiological processes and disease states has culminated in the pursuit of NO prodrugs as potential therapeutic agents. The diazeniumdiolate functional group has been used as a way of caging NO, providing a tool to control its delivery to various tissues. O2-arylated diazeniumdiolates are a sub-class of NO prodrugs activated by nucleophiles such as glutathione (GSH). They have been widely studied for their use as anticancer agents. This study highlights features of the chemical/biological cross-talk of O2-arylated diazeniumdiolate signaling mechanisms and their contribution to drug efficacy. JS-K, an O2-arylated diazeniumdiolate prodrug, has demonstrated pronounced cytotoxicity and antitumorigenic properties in a variety of cancer models both in vitro and in vivo. A study of the metabolic fate of JS-K was undertaken to understand the origin of its cytotoxicity. Consistent with model chemical reactions, the first step in the metabolism of JS-K is the de-arylation of the diazeniumdiolate by GSH and various protein thiols via nucleophilic aromatic substitution reactions, ultimately releasing NO. A consequence of this metabolism is the depletion of reducing equivalents, GSH, leading to a rise in the cellular oxidation potential as viewed through perturbations of the GSH/GSSG redox couple. This chemical event initiates a myriad of biological events resulting in cell cycle arrest and apoptosis: a pronounced rise in the steady state levels of endogenous reactive oxygen species, the initiation of stress signaling pathways such as the p38 and SAPK/JNK cascades, mitochondrial/metabolic dysfunction, protein nitration and nitrosation, and DNA strand breaks. To exacerbate the increase in oxidative stress upon treatment, O2-arylated (bis)diazeniumdiolates were synthesized effectively doubling the payload of NO. In concert with this feature we found that these compounds are potent protein glutathionylating agents as compared to their monovalent analogues. Surprisingly the observed modification is longer lasting than the disulfide bond formed from traditional glutathionylating agents. As divalent electrophiles these compounds have been shown to crosslink molecules of GSH to protein thiols through the aromatic ring resulting in non-reversible modifications to the cellular proteosome and thus adding an alternative mechanism of cytotoxicity to the O2-arylated diazeniumdiolate family.

Chemistry
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NCI-CCR

Synergistic TRAIL sensitizers from Barleria alluaudii and Diospyros maritima

Tumor necrosis factor-alpha-related apoptosis-inducing ligand (TRAIL/Apo2L) is a member of the tumor necrosis factor (TNF) family of apoptosis triggering proteins. TRAIL is particularly important because it selectively induces apoptosis in cancer cells, while showing little to no effect in normal cells. However, TRAIL resistance has been widely documented, and there is evidence to suggest that combination chemotherapy regimens may be more effective than traditional cytotoxic mono-chemotherapy. In this manner, TRAIL activity may be restored by sensitizing tumor cells with certain chemical agents.

Therefore, a high-throughput screen was developed to identify compounds that could sensitize tumor cells to the killing effects of TRAIL. Extracts from Barleria alluaudii and Diospyros maritima showed promising activity in the initial screen and were further investigated. As a result of this study, two naphthoquinone epoxides, 2,3-epoxy-2,3-dihydrolapachol (1) and 2,3-epoxy-2,3-dihydro-8-hydroxylapachol (2), both not previously isolated from natural sources, and the known 2-methyl anthraquinone (3) were identified from B. alluaudii. Time-dependent density functional theory (TD-DFT) calculations of electronic circular dichroism (ECD) spectra were utilized to establish the absolute configuration of 1 and 2. Additionally, five known naphthoquinone derivatives, maritinone (4), elliptinone (5), plumbagin (6), (?)-cis-isoshinanolone (7), and ethylidene-6,6'-biplumbagin (8) were isolated from D. maritima. Compounds 1, 2, and 4-6 showed varying levels of synergy with TRAIL. Maritinone (4) and elliptinone (5) showed the highest synergistic effect, with more than a three-fold increase in activity observed with TRAIL than with compound alone. No previous investigations of B. alluaudii, endemic to Madagascar, have been reported in the chemical literature. Compounds 1 and 2 are members of the lapachol family of quinones. Beta-Lapachol was evaluated by the NCI in the clinic during the 1970s, but was later withdrawn due to high levels of toxicity. The closely related beta-lapachone is currently in Phase II clinical trials for advanced solid tumors.

Chemistry
Seo, Seung Kee
Postdoctoral Fellow
NIDDK
Dynamic O-GlcNAcase cycling: Synthesis and Applications of a novel Halotag bioprobe
Hexosamine signaling pathway is one of the key cellular responses to nutrient excess. O-GlcNAc is present on many intracellular proteins and appears to have a role in the etiology of several diseases including cancer, Alzheimerâ€™s diseases, and type II diabetes. To monitor flux through HSP, we devised and employed a novel synthetic route to create a bioprobe specific for O-GlcNAc cycling. Our labeling strategy utilizes an engineered bacterial enzyme, haloalkane dehalogenase- the HaloTag protein, and a novel fluorogenic bioprobe substrate containing the HaloTag ligand which was recently developed by our group. The native enzyme is a monomeric protein (MW = 33 kDa) that cleaves carbon halogen bonds in forming an ester bond between the new HaloTag ligand and the protein. The HaloTag protein contains a critical mutation in the catalytic triad (His272 to Phe) so that the ester bond formed between the HaloTag protein and ligand cannot be further hydrolyzed. Novel HaloTag ligands labeled with a small organic dye have been developed for in vivo labeling of target proteins. We designed and synthesized a potent bioprobe that is highly selective for O-GlcNAcase activity. This bioprobe contains two crucial components 1) a HaloTag reactive linker, and 2) a functional reporter such as a fluorescent dye. Here, we will leverage this technology for the specific chemical conjugation of the HaloTag ligand to a fluorogenic O-GlcNAcase-specific substrate. This bioprobe in conjunction with the HaloTag will enable us to measure the spatial and temporal activity of O-GlcNAcase as well as purify O-GlcNAc-modified targets.

Chromatin and Chromosomes
Deng, Tao
Visiting Fellow
NCI-CCR
HMGN proteins modulate neural differentiation by regulating the chromatin structure of promoters containing CpG islands

The orderly progression of development and differentiation is associated with dynamic changes in chromatin structure and gene expression. These changes are mediated by the combined action of numerous nuclear proteins, including members of the HMGN protein family. HMGN proteins bind specifically to nucleosomes, the building block of chromatin, and affect the fidelity of transcription by modulating the structure of chromatin and the levels of histone modifications. HMGN expression is down regulated during embryogenesis; however, their role in differentiation processes is not understood. By immunofluorescence analysis we now find that HMGN1 and HMGN2, the major variants of this protein family, are highly expressed in the subventricular zone (SVZ) of mice brains, which serves as a source of neural stem/progenitor cells during adult neurogenesis. Significantly, immunofluorescence analyses of brains sections and quantitative PCR of isolated SVZ regions of Hmgn1+/+ and Hmgn1-/- littermates, indicate that loss of HMGN1 leads to a significant reduction of Nestin positive cells suggesting a decreased number of neural progenitors in the brain of Hmgn1-/- mice. Furthermore, in vitro neural differentiation studies of Hmgn1+/+ and Hmgn1-/- embryonic stem cells (ES cells) revealed that the expression of 152 genes was significantly altered in Hmgn1-/- neural progenitors. ChIP-seq analysis of the genome-wide organization of HMGN1 in ES cell chromatin reveals HMGN1 preferentially binds to promoters containing CpG islands. Significantly, loss of HMGN1 alters the nucleosome organization surrounding the gene transcription start sites at promoters containing CpG islands, but not at promoters that do not contains CpG islands. We suggest that the interaction of HMGN proteins with nucleosomes on CpG island-containing promoters maintains transcriptional fidelity during ES cell differentiation along the neural lineage, thereby modulating the level of neural progenitors in the SVZ region of the brain. Our studies identify a new factor that affects neural progenitors differentiation and provide insights into the molecular mechanisms whereby chromatin architectural proteins regulate transcriptional fidelity.

Chromatin and Chromosomes

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NCI-CCR

Insights into the architecture of the nucleosome-linker histone H1 complex

The dynamic nature of chromatin is essential for many cellular functions such as cell differentiation. Chromatin dynamics are regulated by histone modifying enzymes, chromatin remodeling proteins, and other chromatin factors. The structural unit of chromatin is the nucleosome, consisting of histones H2A, H2B, H3, and H4 octomer wrapped around by 146 bp of DNA. Histone H1 binds to nucleosomes and linker DNAs and condenses the chromatin, which interferes with gene expression. Although the crystal structures of the nucleosome and the global domain of the linker histone H1 have been determined, the nucleosome-H1 complex has been resistant to crystallization, and is too large for structural determination by conventional NMR methods. Using a recently developed methyl-based NMR method, we assigned chemical shifts of the methyl groups in the Leucine, Valine, and Isoleucine residues of nucleosome histones. Using these assignments along with paramagnetic relaxation enhancement (PRE), mutation-isothermal titration calorimetry (ITC), and computer modeling, we determined the architecture of the nucleosome-HMGN2 complex. This method has opened a new avenue for studying the structure of the nucleosomes in complex with chromatin factors in solution without the need of crystallization. Here, we have investigated the architecture of the H1-nucleosome complex by combining the above approach with conventional NMR. Our results have revealed several new features of the nucleosome-H1 complex. (1) In addition to the global domain, a 10-residue region at the C-terminus of H1 also binds to the nucleosome. (2) Upon H1 binding, the peak intensities of residues in the unstructured H2A C-terminus decrease by exactly 50%, indicating one of the H2A C-tails in the

nucleosome is folded within the H1-nucleosome complex. (3) PRE analysis has revealed that H1 is close to the folded H2A C-tail in the nucleosome-H1 complex. (4) Finally, mutation-ITC results indicate that the residues involved in interacting with the nucleosomal DNA are in one of the three helices and two loops of the globular domain of H1. Based on these results, we have built a structural model for the H1-nucleosome complex using the computer program HADDOCK. Our results suggest that H1 preferentially binds to one of the linker DNA regions near the H2A C-tail and H3 N-tail of the nucleosome in an asymmetric manner. This work provides important insights into how H1 condenses the chromatin fiber in the cell nucleus.

Chromatin and Chromosomes
Brick, Kevin
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PRDM9 directs genetic recombination away from functional genomic elements
Genetic recombination during meiosis is the key driver of genetic diversity however the molecular determinants of where recombination takes place in the genome remain a mystery. Meiotic recombination begins with the formation of DNA double strand breaks (DSBs) which occur at a small subset of genomic loci called hotspots. Elegant recent studies have determined that a meiosis-specific histone H3 methyl-transferase (PRDM9) plays a key role in DSB hotspot site determination via DNA binding of its zinc-finger domain. We have recently generated the first metazoan genome-wide map of DSB hotspots and have shown that the majority of mouse DSB hotspots are associated with testis-specific H3K4me3 chromatin marks, potentially formed by PRDM9. Despite these discoveries, Prdm9 knockout mice remain proficient at initiating recombination and therefore, the role of PRDM9 in determining the sites of DSB hotspots remains unclear. In this work, we describe several simple, informative experiments that elucidate the nature and extent of the role of PRDM9 in determining DSB hotspots locations. We used a novel ChIP-Seq variant developed by our group to pull down single stranded DNA bound by the meiotic recombinase DMC1. Using this method, we precisely mapped the genome wide distribution of DSB hotspots in several congenic mouse strains and in Prdm9 knockout mice. While hotspots in mice sharing a Prdm9 allele were at virtually identical genomic loci, those in mice with different Prdm9 alleles and in the Prdm9 knockout were at completely different locations. This definitively illustrates that PRDM9 determines the location of virtually all DSB hotspots in the mouse genome. Intriguingly, the only Prdm9-independent hotspots were found at and around the pseudo autosomal region, the site of an obligate recombination event in every meiosis. This is the first evidence that alternate mechanisms of meiotic recombination initiation exist in mammals. DSBs were still found to accumulate in hotspots in Prdm9-knockout mice, however, in the absence of PRDM9 the majority of recombination (>60%) is initiated at promoters, enhancers and at other functional elements. Such sites are infrequently targeted in wild-type mice (4%) illustrating an important, unexpected role of the PRDM9 protein in sequestering the recombination machinery away from functional genomic elements where efficient repair of DSBs might be problematic.

Chromatin and Chromosomes
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Title: Developmental regulation of the murine beta-globin locus

Two non-exclusive models may explain activation of genes by distant enhancers: (1) RNA polymerase II (pol II) tracking from the enhancer to promoter and (2) chromatin looping to bring the elements into proximity. Chromosome conformation capture (3C) experiments support the looping model, however, pol II tracking cannot be excluded. The mouse ß-globin locus is a classical model system to investigate the function of distant regulatory elements. The locus contains embryonic ey and beta-h1 genes and adult beta-major and beta-minor genes. Activation of all the genes requires the locus control region (LCR) 60 kb upstream of beta-major but only 6 kb from ey. Chromatin looping occurs between the LCR and beta-major but whether this mechanism also activates the more proximal ey gene is unknown. We investigated the hypothesis that pol II tracks along chromatin from the LCR to ey by inserting the cHS4 insulator between them on one allele (D) of mouse ES cells by homologous recombination. We differentiated WT ES cells and cHS4 ES cells and determined D-allelic specific globin gene expression. In cHS4 ES cells, embryonic and adult globin transcripts were reduced 50%-80% compared to WT. If pol II tracks from the LCR to ey, intergenic transcripts should be detected in the region between them. At different time points during differentiation, intergenic transcripts were determined for WT and cHS4 ES cells using D-allele specific primers that amplify sequences in the region between the LCR and ey promoter. Unlike the result in human cells, intergenic transcripts were not observed between the LCR and ey in WT mouse ES cells; transcripts accumulated at early days of differentiation only at the ey promoter. However, in cHS4 ES cells, intergenic transcripts accumulated near the CTCF binding site in cHS4, suggesting that pol II stalls at this region. These results argue against a tracking mechanism for LCR/ey gene regulation. However, the observation of substantial transcription within cHS4 is intriguing. Possibly, pol II is recruited to the insulator. Alternatively, pol II in transit from the LCR might be blocked at the insulator and intergenic transcription might be occurring at levels too low to be detectable in our assay. To extend these observations, we are currently determining pol II occupancy and histone modifications in the LCR/ey region in these clones and in control ES cells carrying a CTCF site-deleted cHS4 or a transcription terminator.

Clinical and Translational Research

Qiu, Ping
Postdoctoral Fellow
CC

NO-Donor Agents as Potential Inhibitors of Anthrax Lethal Toxin

Introduction: Anthrax remains a health threat today capable of infecting large numbers of individuals in a relatively brief period. Defining effective but inexpensive therapies that can be quickly administered on a large scale would be of great value. Production of anthrax lethal toxin (LeTx) is central to the pathogenesis of anthrax infection. LeTx is composed of protective antigen (PA) necessary for toxin uptake by cells, and lethal factor (LF), the toxic moiety. We previously found that LeTx was potent inhibitor of endogenous nitric oxide (NO) production in lethal lipopolysaccharide (LPS) and E. coli challenged rats. We also observed that pretreatment with nonlethal doses of LPS capable of rapidly increasing NO levels were actually improved survival during subsequent lethal LeTx challenge. These findings suggested that administration of NO donor agents may be of therapeutic benefit during anthrax infection. Methods and Results: In vitro, NO donors were found to inactivate LF. Tyrosine nitration of LF was associated with its inactivation and 3-morpholinosydnoime (SIN-1), a NO and O2- donor, was the most potent agent tested. In vivo, survival and hemodynamic effects were compared in rats challenged with 24h LeTx infusions composed of LF incubated for 60 min ex vivo with either SIN-1 or diluent alone (control). The SIN-1 concentration (10mM) employed was based on in vitro experiments testing the agentâ€™s effects on LF activity. Compared to mortality measured over 168h in controls [12 nonsurvivors of 28 total, with a mean (±sem) time to death in nonsurvivors of 32±11h], animals

challenged with SIN-1 treated LT had significantly improved survival [5 nonsurvivors of 26 total, mean (±sem) time to death in nonsurvivors of 130±17h] (p= 0.018, Wilcoxon). During the 24 h toxin infusion period, mean arterial blood pressure (MBP, mmHg) was lower and heart rate (HR, BPM) and NO levels (as measured by nitrate/nitrite) were higher with SIN-1 compared to control treated LT (p<0.0001 for each averaged over the time period). Conclusion: Despite decreasing MBP, SIN-1 has potential therapeutic benefit during anthrax infection. LeTx composed of LF incubated with SIN-1 before combination with PA, was less lethal than LeTx composed of untreated LF. Further defining the mechanisms by which SIN-1 appeared to inactivate LT may provide insights into both the pathogenesis and management of anthrax.

Clinical and Translational Research
Yarmolenko, Pavel
Doctoral Candidate
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Large volume, conformal mild hyperthermia with magnetic resonance-guided high intensity focused ultrasound: implementation and application in drug dose painting with image-able, temperature-sensitive liposomes
Background: Chemotherapy of solid tumors may be improved by spatio-temporal control of drug delivery, a concept known as dose painting, where a desired concentration of drug may be deposited in a specific region, often with image guidance. Dose painting may be achieved with a combination of image-able low temperature-sensitive liposomes (iLTSL) and local mild hyperthermia (HT) using magnetic resonance-guided high intensity focused ultrasound (MR-HIFU). Clinical translation of this drug-device combination largely depends on development of algorithms that enable conformal heating of large volumes in a variety of shapes. Objectives of this study were to: 1) develop a mild hyperthermia (~41°C) algorithm for MR-HIFU to conformally heat large volumes (>2cc) of tissue, 2) characterize the algorithm's performance in vitro and in vivo, and 3) examine its utility in drug dose painting with iLTSL. Methods: Large volume conformal HT was implemented on a clinical MR-HIFU platform (Sonalleve, Philips Medical Systems, Vantaa, Finland) with an algorithm that combined electronic and mechanical HIFU steering. Performance was evaluated in terms of temperature accuracy and stability, as well as spatial accuracy (spatial offset = average distance to target from all heated voxels [>40°C]), in a phantom and in vivo (Vx2 tumor and thigh muscle in a rabbit). iLTSL release was triggered with MR-HIFU mild hyperthermia and monitored in real time. Results: In vitro, the system quickly increased mean temperature in the targeted volume to the 41°C target (heat-up =1.3-5.5min, target cross-sectional area=1.9-8.8cm²). Mean temperature was stably maintained (±1°C) for the prescribed duration (10min). Cross-sectional area of the heated volume tightly conformed to the prescribed area (spatial offset=0.04±0.51mm). Performance was similar in vivo (1.9-2.2min heat-up; area=1.3-2.9cm²; stable mean temperature [±1.6°C]; spatial offset=1.0±2.2mm). After injection of iLTSL and a 10min baseline observation, subsequent HT for 25min (area=3.3cm²) resulted in contrast agent concentration of 0.43±0.07mM in heated tumor, compared to 0.20±0.04mM in adjacent unheated muscle. Conclusion: Large volume conformal hyperthermia with a clinical MR-HIFU platform enabled HT of variable shapes and sizes that are typical of lesions encountered in clinical oncology. The system allowed real-time monitoring of iLTSL content release, suggesting that this drug-device combination may be useful in drug dose painting.

Clinical and Translational Research
Jang, Sung-Wook

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Identification of Drug Modulators Targeting Gene-Dosage Disease CMT1A
Charcot-Marie-Tooth (CMT) disease is a heterogeneous group of neurological disorders that affect approximately 1 in 2,500 people. In particular, the most prevalent type of CMT called CMT type 1A (CMT1A) is genetically linked to the duplication of the PMP22 gene which leads to its overexpression, thus manifesting itself as a typical case of dose-sensitive diseases. The PMP22 gene encodes a small tetraspan 22-kDa membrane glycoprotein whose adequate expression is required for the structural integrity of myelin formed by Schwann cells critical for proper nerve conduction in the peripheral nervous system (PNS). Although previous studies in rodent models of CMT1A have identified transcription-based strategies that ameliorate the disease by reducing levels of PMP22, one of the major limitations in therapeutic research for CMT1A has been the lack of viable assay systems that can broadly and effectively screen small molecules for their biological relevance to CMT1A. Our study is the first attempt to develop a cellular transcription-based assay portfolio for CMT1A which we functionally validated using siRNA-induced depletion of a key transcription factor known to regulate the expression of PMP22. We utilized a novel intronic element of the PMP22 gene to drive expression of two reporters, firefly luciferase (FLuc) and beta-lactamase (b-Lac), expressed in separate Schwann cell lines which function as a cross-validating orthogonal screening platform to address non-specific activity intrinsically derived from the reporters themselves. Successful miniaturization of the assays for quantitative high-throughput screening (qHTS) yielded rich data sets immediately from the primary screens and streamlined the triage process of actives for subsequent characterization. Our findings from screening the National Center for Advancing Translational Sciences (NCATS) Pharmaceutical Collection, a comprehensive library of approximately 3,000 approved drugs, identified several drugs among which fenretinide, olvanil, and bortezomib markedly induced reduction of endogenous PMP22 mRNA and protein. Animal studies are currently ongoing to evaluate their clinical significance for the treatment of CMT1A. Overall, our study presents an assay development and drug repurposing strategy for dosage-related genetic disorders, highlighting our screening design to address fundamental issues in the use of surrogate reporters often plagued by artifactual outcomes.

Clinical and Translational Research
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NCI-CCR
MOLECULAR PROFILING OF THORACIC MALIGNANCIES
Molecular profiling i.e. the prospective analysis of tumor molecular characteristics to guide treatment, offers the promise of translating the current understanding of tumor mutational landscape to clinic, thereby improving patient outcomes, minimizing toxicity and streamlining drug development. However, clinical application of molecular profiling has proven challenging due to the limitations of available tumor tissue and the methodological issues related to real-time, multiple-gene tumor genotyping within a reasonable turnaround time. In an ongoing pilot trial, we are evaluating the feasibility of performing molecular profiling on tissue from new biopsies and the efficacy of molecular-profile matched targeted therapies in patients with advanced stages of non-small cell and small cell lung cancers (NSCLC, SCLC) and thymic malignancies (TM). Oncogenic mutations and gene amplifications/translocations of 12 genes detected using a multi-platform approach (pyrosequencing, fluorescence in situ hybridization, next generation sequencing, comparative genomic hybridization) are used for to assign patients to one of five experimental treatment arms: EGFR sensitizing mutations-erlotinib; KRAS, NRAS, HRAS, BRAF mutations-AZD6244; PIK3CA, AKT, PTEN mutations, PIK3CA amplification-MK2206; ERBB2 mutations or

amplification-lapatinib; KIT mutations, PDGFRA mutations and amplification-sunitinib, or a 'standard of care arm', if none of the above biomarkers are identified. Between February 2011 and February 2012, 186 patients enrolled [NSCLC 128, SCLC 10, TM 45, Others 3; males 86, females 100; median age 59 years (range, 24-84)]. Molecular profiling was performed on fresh tissue in 107 (58%) and archival tissue in 77 (41%) patients. New biopsies were associated with an acceptable rate of non-severe complications (14%) and provided adequate tissue to perform all proposed studies in 79 (74%) patients. At least one genetic alteration was found in 80 (45%) patients (mutations: EGFR 28, KRAS 31, PIK3CA 2, BRAF 1; amplifications: HER2 4, PDGFRA 2, PIK3CA 1; ALK translocation 13, PTEN deletion 1). Based on molecular profiling results, 23 patients were assigned to biomarker-matched therapy. Interim results of this ongoing trial with a target accrual of 600, demonstrates the feasibility of performing new biopsies for molecular profiling of patients with advanced thoracic malignancies and the potential clinical benefit of this approach.

Clinical and Translational Research
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The development of a macrophage model of Gaucher disease for testing new small molecular therapies
Gaucher disease (GD), the most prevalent lysosomal storage disorder, is caused by mutations in the gene encoding glucocerebrosidase (GBA1). The disease is characterized by accumulation of glucosylceramide (GlcCer)-laden macrophages in the liver, spleen and bone marrow. Although patients with GD are deficient in glucocerebrosidase (GCase) in all cell types, the phenotype manifests primarily in macrophages. GD presents with a wide range of symptoms of varying severity, affecting the skeletal, hematologic and central nervous systems. However, the exact mechanism of GlcCer accumulation and its effect on macrophage function is still unknown. More than 300 missense mutations have been identified in GBA1, which result in misfolding, instability and mistrafficking of the enzyme to lysosome. Enzyme replacement therapy (ERT) is currently used to treat Gaucher disease. However, ERT is costly, and the enzyme does not cross the blood-brain barrier. Chemical chaperone therapy is an alternative treatment strategy. Chaperones are small molecules that bind to misfolded proteins, refold the enzyme, and facilitate trafficking of the enzyme to the lysosome. High throughput screening of large compound libraries has led to the identification of a novel lead GCase activator, NCGC0578. We studied macrophages from eight patients with three different Gaucher genotypes and demonstrated GluCer storage and phagocytosis. The small molecule (NCGC0578) increased the enzyme activity in human Gaucher macrophages eight-fold. We showed, for the first time, that NCGC0578 specifically reduced GlcCer storage in human Gaucher macrophages. The effect of the chaperone on macrophage function was evaluated by testing phagocytosis and efferocytosis (the phagocytosis of dead cells). Following the expression of FcgammaRI/II in Gaucher macrophages by flow cytometery, we found that the high phagocytic index and lower efferocytic index (<40%) in Gaucher macrophages reversed to basal level after treatment with NCGC0578. Intracellular levels of reactive oxygen species (ROS), which were significantly lower in Gaucher cells, were also restored with the administration of NCGC0578 .Thus, treating human Gaucher macrophages with NCGC0578 reduced GlcCer storage, and improved the function of these cells. Both this new macrophage model and this lead compound demonstrate great promise for the development of new therapies for Gaucher disease and related disorders.

Clinical and Translational Research
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Propofol Decreases In Vivo Binding of [11C]PBR28 to Translocator Protein (18kD) in Human Brain
The positron emission tomography (PET) radioligand 11C-PBR28 targets translocator protein (TSPO) and is a potential marker of neuroimmune activation in vivo. While several patient populations have been studied using 11C-PBR28, no reports exist on more cognitively impaired patients who would require anesthesia for the PET procedure, nor on any effects that anesthetic may have on radioligand uptake. The purpose of this study was to determine whether propofol alters brain uptake of 11C-PBR28 in healthy subjects. Methods: Ten healthy subjects (five men; five women) each underwent two dynamic brain PET scans within the same day, first at baseline then with intravenous propofol anesthesia. Subjects were injected with 680 ± 14 MBq (mean ± SD) of 11C-PBR28 for each PET scan. Brain uptake was measured as total distribution volume (VT) using an unconstrained two tissue compartment model and metabolite-corrected arterial input function. Results: Propofol decreased VT, which corrects for any alteration of metabolism of the radioligand, by about 25%. Furthermore, brain and plasma time activity curves (TACs) showed the expected decreases in brain uptake despite a 12% increase in plasma AUC with propofol. Reduction of VT with propofol was observed across all brain regions. Conclusion: Propofol anesthesia reduces VT of 11C-PBR28 by about 25% in brains of healthy human subjects. Given this finding, we recommend that future studies using 11C-PBR28 and comcomitant propofol anesthesia, including our study on neuroimmune activation in autism, include an appropriate control arm to account for effects of propofol on brain measurements of translocator protein (TSPO).

Cultural Social and Behavioral Sciences
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Self-reported versus accelerometer measured physical activity & cardiometabolic biomarkers among youth in NHANES
INTRO: Research on the beneficial health effects of moderate to vigorous physical activity (MVPA) has been based on self-report. Youth report higher MVPA levels than is objectively measured with accelerometers. The discrepancy in these estimates may impact observed relationships between MVPA & obesity-related biomarkers. The relationship between these two measures & biomarkers in youth has not been examined in a nationally representative sample. PURPOSE: To compare associations between self-report & accelerometer MVPA & cardiometabolic biomarkers. METHODS: Youth ages 12-19 (N= 2174; 1110 boys) from the combined 2003-6 National Health & Nutrition Examination Surveys with self-report MVPA & four 10-hour days of accelerometry (objective measure; Actigraph 7164) were included. The biomarkers were: body mass index (BMI, kg/m2), BMI percentile, height & waist circumference (WC, cm), triceps & subscapular skinfolds (mm), high-density lipoprotein (HDL, mg/dl), total cholesterol (mg/dl), systolic & diastolic blood pressure (BP, mm/hg), triglycerides (mg/dl), glycohemoglobin (%), insulin (uU/ml), & C-reactive protein (mg/dl). In separate models, each biomarker was regressed on accelerometer MVPA (min/day) & on self-report MVPA (min/week), with analyses stratified by sex. Covariates were age, race, SES, physical limitations, & asthma. RESULTS: Girls recorded less activity [self-report MVPA: 160.0 (16.1) vs. 214.4 (12.2) min/week; accelerometer MVPA: 18.2 (0.9) vs. 33.6 (1.3) min/day) (p<0.05 for all)]. Boys had stronger correlations between self-report & accelerometer MVPA (boys: r=0.16; girls: r=0.095; p<0.002). In boys, accelerometer MVPA was inversely associated with BMI, BMI percentile, height, WC, & triceps skinfold; & positively associated with HDL & glycohemoglobin (p<0.05 for all); the self-report measure of activity was inversely associated with diastolic BP (p=0.001). In girls, accelerometer MVPA was inversely associated with systolic BP (p=0.046); the self-

report measure of activity was not associated with any of the biomarkers. CONCLUSIONS: Accelerometry showed stronger associations with biomarkers than self-report, & relationships were stronger in boys. These findings contribute to current efforts aimed at improving measurement of physical activity in youth. If self-reported MVPA is not related to commonly-used measures of cardiometabolic health in youth, then objective measures may be a necessary component of etiological obesity studies.

Cultural Social and Behavioral Sciences
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Pain in Long-term Breast Cancer Survivors: The Effect of Body Mass Index
Purpose: Pain is common among post-treatment cancer survivors, yet the course of pain over time and factors affecting pain in specific cancer populations are not well understood. This study described pain in a cohort of breast cancer survivors (BCS) 10 years (y) after diagnosis, identified longitudinal patterns in pain, and examined associations between body mass index (BMI) and pain. Methods: BCS enrolled in the Health, Eating, Activity, and Lifestyle (HEAL) Study were surveyed at approximately 3, 5 and 10 y post-diagnosis (n=559). Pain was assessed with the SF-36 bodily pain scale (BP; lower scores indicate more pain) transformed based on national norms (T-scores: mean=50, SD=10). Multiple linear regression was used to assess associations between self-reported BMI and pain, and between changes in pain (3-10 y) and changes in BMI (% change,5-10 y). Demographic and clinical variables, including comorbidities, were evaluated as confounders. Results: At 10 y post-diagnosis, 24.9% of BCS reported BP scores <40 (>1 SD below the population mean). After adjustment, BCS who were obese (BMI >=30.0 kg/m2) at 10 y reported greater pain than normal weight (BMI < 25 kg/m2) survivors (ß=-2.94, p=0.04). From 5 to 10 y, BMI increased by >5% for 18.5% and decreased >5% for 27.0% of BCS. Change in BMI was not associated with pain at 10 y (ß=-0.04, p>0.05). Over the 7 y period, 52.9% of the sample maintained low pain (BP>40), 12.5% maintained high pain (BP<40), 13.0% reported improved pain (BP increased > 1 SD), and 21.7% reported worsened pain (BP decreased > 1 SD). These pain change categories also were not associated with change in BMI (p>0.05). Conclusion: Pain remains a significant complaint among BCS 10 y post-diagnosis. Excess body mass contributes acutely to pain and pain related functioning in long-term BCS, independent of comorbidities and previous BMI trajectory.

Cultural Social and Behavioral Sciences
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Pre-diagnosis lifestyle factors and colorectal cancer survival in the NIH-AARP diet and health study
Background: Colorectal cancer is the 3rd most commonly diagnosed cancer in the United States, and the number of survivors is large and growing. Research into modifiable factors that may impact survival is of public health importance. However, few studies have addressed this question specifically among colorectal cancer survivors. Methods: Lifestyle factors were assessed at study baseline in 1995-1996. A total of 6,303 colorectal cancer cases were identified between 1996 and 2006 through linkage to state cancer registries. Colorectal cancer cases were followed through 2008 for date and cause of death by linkage to the National Death Index. We examined relationships of healthy diet (upper two quintiles of Healthy Eating Index scores), body mass index (BMI), physical activity (=20 minutes moderate to vigorous activity = 3 times/week), alcohol intake, and smoking, to cancer-specific and total mortality. We estimated relative risk (RR) of death and 95% confidence intervals (CI) with Cox proportional hazards

models adjusting for age at diagnosis, sex, education, marital status, stage, surgery, radiation, chemotherapy, and family history of colon cancer (colon cancer models only). Results: Among 4,629 colon cancer survivors, 1,597 died during follow-up, including 911 colon cancer deaths. Among 1,674 rectal cancer survivors, 611 deaths occurred, including 193 rectal cancer deaths. Compared to smokers, nonsmokers at baseline had a 26% and 37% lower risk of colon cancer death (RR 0.74; 95% CI 0.62-0.89) and total death (0.63; 0.55-0.71), respectively. Compared to obese individuals, overweight individuals at baseline had significantly reduced risk of colon cancer mortality (0.81; 0.69-0.95), whereas those with normal BMI had non-significantly reduced risk (0.90; 0.76-1.07). Physical activity was associated with lower total mortality among colon cancer cases (0.87; 0.78-0.96). Pre-diagnosis lifestyle factors were not associated with rectal cancer-specific mortality, but healthy diet (0.79; 0.66-0.96) and nonsmoking (0.71; 0.57-0.88) were associated with lower total mortality among rectal cancer cases. Conclusion: Several modifiable pre-diagnosis lifestyle factors were related to survival among colorectal cancer cases in this longitudinal cohort study. Further studies are warranted to examine the effect of changes in lifestyle factors after diagnosis on colorectal cancer prognosis and survival.

Cultural Social and Behavioral Sciences

Jobes, Michelle
Postdoctoral Fellow
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Development and field demonstration of software for delivery of contingency management - an empirically supported behavioral treatment for addiction

Contingency management (CM) is a type of treatment frequently used in substance abuse populations to reinforce behaviors such as drug abstinence and counseling attendance. Although hundreds of studies have shown CM to be one of the most effective behavioral treatments for substance dependence, adoption of CM for delivery by community clinics has been slow. The main barriers to implementation in community clinics are cost and technical complexity. We developed software called Motivational Incentives Implementation Software (MIIS) that could be easily implemented in community clinics. As part of the development process, 25 outpatient volunteers were recruited for a field test of the software. All participants were currently enrolled in other addiction-treatment studies in our clinic. In their primary studies, participants provided urine samples 3 times weekly and attended individual counseling once a week; this study was conducted in conjunction with these activities. Feedback on usability, functionality, and simplicity from staff and participants was used to modify the software iteratively until a final version was created. Counseling staff used MIIS to reinforce participantsâ€™ attendance to 12 regularly scheduled counseling sessions over a period of 14 weeks. Participants earned opportunities to draw for prizes for each on-time attendance to their weekly counseling session. For the present analyses, their attendance was compared to that of controls who were matched on primary study and counselor and who had been enrolled in the clinic in the months concurrent with the field trial. Controls attended 82% of their scheduled counseling sessions; MIIS CM-reinforced participants attended 91% of their scheduled counseling sessions, significantly more than controls (p<.007). Increased counseling attendance is associated with positive outcomes in addiction treatment. In our study, MIIS robustly increased an already high level of counseling attendance. The complete MIIS software tracks and helps reinforce behaviors such as drug abstinence and counseling attendance using CM. The reinforcement schedule, target behaviors, and prizes can be tailored to the specific needs of the clinic and its patients. MIIS is now available to clinics across the country at no cost. Availability of MIIS as a user-friendly stand-alone program designed for even the most basic community clinic settings may improve technology transfer of a proven effective treatment to the community.

Developmental Biology
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Visiting Fellow
NHGRI
A large-scale zebrafish gene knockout resource for the genome-wide study of gene function
With the zebrafish genome project completed,it becomes possible to analyze the function of all the zebrafish genes in a systematic way. The first step in such an analysis is to inactivate each protein-coding gene by targeted or random mutation. Currently, there is no high-throughput technology to knockout zebrafish genes in a stable and targeted manner. To address this shortcoming, we developed a streamlined platform using proviral insertions coupled with high-throughput sequencing and mapping technologies to mutagenize essentially all of the identifiable genes in the zebrafish genome. Once saturation is reached, any desired mutant line can readily be generated through in vitro fertilization of the frozen sperm sample containing the mutation of interest and the F2 fish would be available for inbreeding in 3 â€“ 4 months to generate homozygous mutant fish. Previously, we have shown that retroviral insertions can be mapped efficiently using a combination of linker mediated-PCR, shotgun cloning and Sanger sequencing. This method is not only labor intensive and time consuming but also recovers a limited number of integrations. In order to use our existing retroviral mutagenesis method as a reverse genetic tool to build a knockout library, it was essential to create new methodologies to recover a higher number of insertions at a significantly reduced cost. We developed a highly multiplexed and efficient mapping strategy; the modified method starts with the use of three sets of restriction enzymes to digest genomic DNA samples in parallel. The digested samples are ligated to 6-base DNA barcode containing linkers and amplicons are generated using LM-PCR. Barcoding allows us to index individual F1 fish, and thus a pool of 500+ fish can be sequenced together. In order to make our method high-throughput and cost efficient, we utilized the massively parallel sequencing Illumina platform into our pipeline to replace conventional shotgun cloning and Sanger sequencing. We recovered ~60% more integrations using a next-gen sequencing platform while reducing both cost and labor. We report the first 2,688 mutagenized and archived F1â€™s carrying predicted mutations in 2,161 genes. Insertions mapped to the zebrafish genome and mutagenized fish lines are freely available to the public through the zebrafish stock center. This resource thus establishes an important milestone for zebrafish genetics research and should greatly facilitate functional studies of the vertebrate genome.

Developmental Biology
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Postdoctoral Fellow
NIDCD
Guidance of spiral ganglion neuron peripheral axons by secreted Semaphorins
During cochlear development, spiral ganglion neuron (SGN) peripheral axons precisely navigate through an array of cell types before selectively synapsing with inner or outer hair cells. Projections from type I SGNs (90% of the total population) form synapses with inner hair cells, whereas projections from type II SGNs (the remaining 10%) are permitted to extend past inner hair cells and pillar cells before innervating the outer hair cells. The guidance mechanisms that spatially distribute type I and type II SGN peripherals axons in this manner are poorly understood. Class 3 secreted Semaphorins bind to Neuropilin/Plexin (Nrp/Plxn) co-receptor complexes to regulate diverse aspects of axon motility across the nervous system. By in situ hybridization, we show that several Sema-3s are regionally expressed within the cochlear epithelium at embryonic day 16.5, when type II SGNs have begun to extend their processes toward the outer hair cells. By immunostaining, Nrps 1 and 2, and PlxnA3 are expressed by the SGNs, suggesting that these receptors may respond to secreted Semaphorins expressed by cells in the cochlear

epithelium. However, PlxnA3 protein appears to be down-regulated in type II fibers once they traverse the pillar cells. Genetic loss of secreted Semaphorin signaling in either Nrp1sema- knock in (KI) or Nrp2 knock out (KO) mice leads to excessive outgrowth of SGN peripheral axons into the outer hair cell region. In addition, postnatal Nrp1sema- KI mice show an increased number of outer hair cell ribbon bodies in the apex of the cochlea, indicating that exuberant outgrowth leads to ectopic synapse formation. Overall, these data suggest that secreted Semaphorins may normally activate Nrp/Plxn receptors to restrict type I SGN processes to the inner hair cell region during the formation of the cochlea. In ongoing studies, we are using live imaging to investigate specific aspects of secreted Semaphorin signaling on SGN peripheral axon motility during cochlear development. For these experiments we have established a two-color genetic model where sparse numbers of SGNs and all hair cells (NgnCreERT2;R26R-tdTomato; Atoh1-nGFP) are imaged simultaneously in a semi-intact cochlear explant using spinning disk confocal microscopy. We are also currently using standard audiology techniques to investigate how the absence of secreted Semaphorin signaling in Nrp1sema- KI and PlxnA3 KO mice impacts hearing function.

Developmental Biology
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Doctoral Candidate
NIDCR
Rapid Expansion of Embryonic Epithelia Requires Extensive Basement Membrane Perforation
Organs such as lung, kidney, mammary and salivary glands undergo rapid epithelial expansion and remodeling during embryonic development to generate the maximal surface area needed for adsorption or secretion. This expansion must be coordinated with basement membrane (BM) remodeling, since the BM is a continuous sheet of tough extracellular matrix that encapsulates epithelia. While many laboratories have investigated how cells invade through a BM in pathological processes such as tumor metastasis, the fundamental question of how epithelia normally expand rapidly during development while remaining confined by the surrounding BM is poorly understood. We hypothesized that the BM must be weakened at sites of high expansion to permit smooth and rapid outgrowth. To investigate this, we imaged several BM components of ex vivo embryonic mouse lung and submandibular gland (SMG) and discovered extensive tiny perforations in the BM, which make it appear "lace-like" around the tips of rapidly expanding end buds, yet the perforations are absent around ducts where there is little expansion. These perforations give the usually continuous, stiff BM a mesh-like quality, likely increasing its elasticity to permit expansion of the end buds. The perforations increase in number and size with epithelial expansion and gradually disappear as the gland matures and expansion slows. Next we examined the mechanisms behind BM perforation formation. Proteases were a logical candidate for BM remodeling, and treatment with protease inhibitors resulted in a buildup of BM around buds, disappearance of perforations, and significant inhibition of bud extension. We also found that non-muscle myosin IIA and IIB, key motor proteins in mechanical remodeling of the matrix, localize in puncta within the BM perforations. Live-organ imaging of embryonic lung and SMG from myosin IIA-GFP or myosin IIB-GFP transgenic mice revealed that the peripheral epithelial cells bleb through the BM. Because myosin II and protease inhibition had similar inhibitory effects on the presence of blebs, BM perforations, and epithelial expansion, we suggest that the blebs might provide local physical force to help punch small holes through protease-weakened BM at the tips of end buds to permit tissue expansion without single-cell invasion. In summary, we have discovered a new mechanism facilitating epithelial expansion associated with mechanical and proteolytic remodeling of the BM necessary for organ development.

Developmental Biology
Avella, Matteo
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NIDDK
The role of ZP1 in the induction of sperm acrosome exocytosis
The mouse acrosome is a Golgi-derived subcellular organelle that underlies the anterior plasma membrane of the sperm head. Prior to sperm-egg fusion at fertilization, the acrosome must undergo exocytosis, but the site of this event has been controversial. The â€˜fertilizingâ€™ sperm penetrates through a cumulus oophorus composed of hyaluronan interspersed with residual somatic cells and binds to the extracellular zona pellucida (ZP1, ZP2, ZP3) surrounding ovulated eggs in the oviduct. After binding at the zona surface, the â€˜fertilizingâ€™ sperm penetrates the zona matrix to enter into the perivitelline space and fuse with the egg. Initial sperm binding had been thought to trigger acrosome exocytosis. However, the recent observation that acrosome-intact sperm persist on the surface of the zona pellucida makes this less likely and it has been suggested that acrosome exocytosis is induced either prior to encountering the zona pellucida or during initial penetration of the zona matrix. To distinguish between these two hypotheses, we established transgenic mice in which EGFP is tethered to the plasma membrane (mid-piece and tail) of sperm and soluble mCherry is released upon acrosome exocytosis (Acr-mCherry/FEIC). Using high speed, time-lapse confocal microscopy for 2 h after insemination, we recorded sperm (139 sperm, 10 movies) traversing the cumulus oophorus, contacting the zona pellucida and remaining acrosome-intact. Using Cd9 null eggs that accumulate multiple sperm in the perivitelline space, we document acrosome-intact sperm on the zona pellucida, acrosome-reacted sperm mid-way through the zona matrix and acrosome-reacted sperm in the perivitelline space. No acrosome-reacted sperm were observed on the surface of the zona pellucida (0/30 eggs, 3 replicates). ZP1 is the least abundant protein in the zona pellucida and the only one that forms a disulfide-bonded homodimer. Strikingly, Zp1 null eggs that form a looser zona matrix failed to induce acrosome reaction. Acrosome intact sperm were found mid-way through the zona matrix (n=72) and in perivitelline space (n=40). Replacing mouse Zp1 with the human homologue rescued this phenotype and no acrosome intact sperm were found mid-way though the zona or in perivitelline space. These results support the hypothesis that exocytosis occurs during initial penetration of the zona matrix and emphasize the importance of the protein composition of the zona pellucida in the induction of sperm acrosome exocytosis.

Developmental Biology
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NIEHS
Regulation of APA site choice in the maintenance of ES cells
More than half of mammalian genes have multiple or â€œalternativeâ€ polyadenylation (APA) sites, primarily within the 3â€™UTR. APA site choice, either proximal or distal, results in respectively a shorter or longer 3â€™UTR, which often contains cis elements, negatively impacting mRNA stability or translation. Proximal site preference and shorter 3â€™UTRs have been observed in embryonic stem cells (ESCs), whereas distal site and longer 3â€™UTRs have been observed during embryonic development. Control of APA site choice and the biological significance for site preference is not fully understood. We hypothesize that a preference for proximal APA sites in ESCs have implications in their ability to self-renew and differentiate. Using RNAi screens in ESCs, we identified Fip1L1 and Cpsf3 as candidates involved in self-renewal. Fip1L1 and Cpsf3 are known members of the cleavage and polyadenylation specificity factor (CPSF) mRNA 3â€™ processing complex, which is required for 3â€™ cleavage and

polyadenylation. Fip1L1 and Cpsf3 are down-regulated during ESC differentiation. We discovered that silencing of these factors leads to decreased Oct4 expression and ES cell differentiation, indicating their requirement in self-renewal. Given the prerequisite for CPSF complex to specify the cleavage site, we hypothesized a role for the CPSF complex in the regulation of APA site choice. Global polyadenylation site sequencing and differential 3â€™UTR expression analyses revealed that Fip1L1 and Cpsf3 depletion results in distal APA site usage and longer 3â€™UTRs for many genes. Many of these putative CPSF targets are highly expressed in ESCs compared to other cell types. This is consistent with 3â€™UTR-lengthening during ES cell differentiation and embyronic development, accompanied by reduced gene expression. Our data supports a model that ESC maintenance is regulated by high expression of FIp1L1 and Cpsf3, which specify proximal APA site choice and shorter 3â€™UTRs in ESCs to maintain mRNA transcript stability. De novo sequence analysis within the proximal and distal APA sites for validated targets revealed putative sites for several miRNAs. Currently, we are exploring the role of these miRNAs in conjunction with longer 3â€™UTRs in ESC self-renewal and differentiation commitment. Our findings provide answers to how APA site choice is controlled in ES cells and contextualizes the significance of proximal APA site choice in ES cell self-renewal, pluripotency, and in early development.

DNA-binding Proteins/Receptors and DNA Repair

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Physical and Functional Interaction between Fanconi Anemia Group J Helicase and MRE11 Nuclease

FANCJ mutations are associated with Fanconi anemia (FA) and breast cancer. FANCJ helicase confers cellular resistance to DNA interstrand cross-link (ICL) agents or ionizing radiation (IR); however, its precise functions are not well understood. MRE11-RAD50-NBS1 (MRN) complex functions in double-strand break (DSB) repair to recognize and process DNA ends, and mediate checkpoint signaling. NBS1 and MRE11 mutations result in genomic instability diseases Nijmegen breakage syndrome and ataxia telangiectasia-like disorder, respectively. In this work, FANCJ was found to be associated with RAD50/MRE11 as demonstrated by reciprocal co-immunoprecipitation and size exclusion chromatography of cell extracts. A direct interaction between FANCJ and MRE11 was confirmed using purified recombinant proteins, and mapped to the FANCJ C-terminal noncatalytic domain (residues 881-1249). FANCJ strongly inhibited MRE11 3â€™-5â€™ exonuclease activity in a specific manner, suggesting that FANCJ regulates MRE11 during DSB repair resection. As shown by confocal microscopy, FANCJ recruitment to laser-induced Psoralen (Pso)-ICLs or DSBs occurred shortly (1-2 min) after MRE11. Cellular exposure to MRE11 exonuclease inhibitor MIRIN impaired FANCJ recruitment to DSBs, suggesting that limited DNA resection promotes FANCJ loading. FANCJ failed to recruit to DSBs in MRE11 exonuclease deficient cells. Exogenous expression of WT-MRE11, but not exonuclease defective MRE11H129N, restored FANCJ recruitment to DSBs. FANCJ is differentially recruited to DSBs compared to Pso-ICLs in a manner governed by MRE11 nuclease activity and FANCA, respectively. FANCJ or MRE11 depletion rendered HeLa cells IR-sensitive; however, co-depletion of FANCJ and MRE11 caused cells to become IR-resistant, similar to control siRNA cells. Thus, IR sensitivity and chromosomal instability associated with FANCJ or MRE11 deficiency may be at least partly a consequence of aberrant helicase or nuclease activity when the interacting partner is absent. Co-depletion of FANCJ and MRE11 resulted in reduced IR-induced gH2AX and RAD51 foci compared to cells depleted of either FANCJ or MRE11. In contrast to gH2AX and RAD51 foci, DNA-PKcs foci were elevated in cells deficient of FANCJ and MRE11, suggesting that DSBs are repaired by nonhomologus end-joining. Collectively, these studies provide evidence for a novel interaction between FANCJ and MRE11 that is important for chromosomal stability and DNA repair.

DNA-binding Proteins/Receptors and DNA Repair
Huang, Jing
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NIA
Single molecule analysis of the encounter of replication forks with absolute blocks to DNA synthesis
DNA synthesis in the large genomes of mammalian cells encounters many obstacles that can stall replication forks. Failure to resolve blocked forks can result in cell death, or genomic rearrangements which may be causally linked to cancer development. DNA interstrand crosslinks (ICLs), which can be formed by endogenous lipid peroxidation products or chemotherapy drugs, are absolute blocks to DNA synthesis. Despite many years of study the pathways through which cells overcome these blocks are poorly understood. Some models propose that repair follows the encounter of a single replication fork, after which DNA synthesis resumes (replication-repair-replication). Others argue that repair is initiated only after two replication forks have converged on the ICL (replication-replication-repair). Progress has been limited by the lack of approaches for the direct analysis of DNA synthesis in the vicinity of ICLs. Additionally, most crosslinking agents generate primarily monoadducts, with only a small fraction of actual ICLs, complicating interpretation of experiments with these compounds. We have developed a novel approach for single-molecule analysis of replication in order to study the encounter of individual replication forks with absolute blocks, such as ICLs. We introduced ICLs into genomic DNA by treating cells with psoralen, which forms a high frequency of ICLs following photoactivation by long wave ultraviolet light (UVA). Cells were exposed to an antigen tagged psoralen and UVA followed by pulses of halogenated thymidine derivatives. The location of replication tracts in the vicinity of ICLs was determined by immunofluorescent visualization of the thymidine analogues and the antigen tag on the psoralen ICL on DNA fibers. While single fork collisions with the ICLs do occur, replication on either side of the ICLs is observed more frequently. The double sided replication patterns are formed by existing replicons, rather than the activation of nearby dormant origins. Similar replication patterns were obtained in both repair proficient and deficient cells, suggesting that replication decisions are made independently of repair competence. Furthermore, in repair proficient cells, removal of the ICLs is slow relative to the resumption of replication. Our results argue that cells follow a replication-replication-repair model in response to replication blocks.

DNA-binding Proteins/Receptors and DNA Repair
Jacob, Kimberly
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NIA
Characterization of OGG1 Polymorphisms Associated with Alzheimerâ€™s Disease
Alzheimerâ€™s Disease (AD) is the most common form of dementia in adults, effecting 18 million individuals. Approximately 5% of AD cases are early-onset caused by mutations in known genes. However, the causes of the remaining cases may in part arise from accumulation of spontaneous mutations, possibly as a consequence of oxidative stress. 8-oxoguanine (8-oxoG), a mutagenic DNA base lesion, produced by oxidative stress, is linked to aging and age-related disease. In humans, the base excision repair protein 8-oxoguanine-DNA glycosylase (OGG1) is the major enzyme that recognizes and excises 8-oxoG lesions. Analysis of brain tissue from AD patients shows increased levels of oxidative DNA damage in both nuclear and mitochondrial DNA. Specifically, there was an increased accumulation of 8-oxoG lesions, suggesting that defective OGG1 activity may account for increased levels of 8-oxoG lesions observed. Recently, two polymorphisms of OGG1, A53T and A288V, have been identified in brain tissues

of AD patients. However, little is known about how these polymorphisms may contribute to AD. We have characterized the A53T and A288T polymorphic variants of OGG1 and found that OGG1-/- mouse embryo fibroblast cells expressing the A53T and A288V polymorphisms were significantly more sensitive to the DNA damaging agents H2O2 and menadione than cells expressing WT OGG1. In addition, both variants had significantly decreased long-term cell survival. Using purified proteins, we detected a ~75% reduction in the catalytic activity for both polymorphic proteins. The A53T polymorphism had significantly decreased activity at physiological temperatures and its activity was less stimulated by AP endonuclease 1, a known enhancer of OGG1 function. In addition, we found that the polymorphic proteins were less able to bind to lesion containing substrates than WT OGG1. We observed that both variants have significantly decreased binding to known OGG1 binding partners PARP-1 and XRCC1 and that they were less able to activate PARP-1. Our results provide both cellular and biochemical evidence that A53T and A288V polymorphic proteins have deficiencies in both glycosylase and protein binding properties that could explain the increase in oxidative damage to DNA found in AD patients with these polymorphisms. This data suggests that OGG1 may play an important role in AD susceptibility and progression related to these specific genetic polymorphisms.

DNA-binding Proteins/Receptors and DNA Repair

Lukasiewicz, Kara
Other
NICHD

Crm1 regulates RNA Pol-I transcription through a novel, transport-independent mechanism

Dysregulation of ribosome biogenesis, the processing of ribosomal RNA (rRNA) and protein subunits into mature ribosomes, contributes to progression of several diseases, including ribosomopathies and cancer. Crm1 (chromosome region maintenance 1) has traditionally been characterized as a Ran-GTP-dependent nuclear export receptor for proteins and RNAs. We now report a novel nuclear transport-independent cellular function linking Crm1 to regulation of ribosome biogenesis. Pulldown assays using tagged, recombinant Crm1, combined with mass spectrometry analysis, identified a small group of proteins that bind Crm1 with high affinity in the presence of Ran-GTP, which included putative histone demethylase KDM3B. Co-immunoprecipitation confirmed binding of KDM3B to Crm1 in the presence of Ran-GTP. In vitro demethylation assays and immunofluorescence coupled with fluorescence microscopy confirmed that KDM3B acts as a histone 3 lysine 9 (H3K9) specific demethylase. While most Crm1 binding proteins reside primarily in the cytoplasm as a result of Crm1-mediated export, immunofluorescence studies revealed that Crm1 and KDM3B co-localize to novel nucleolar structures called intranucleolar bodies (INBs). Treatment with Crm1-specific inhibitor leptomycin B (LMB) blocks localization of Crm1 and KDM3B to INBs indicating that binding to Crm1 is necessary for KDM3B nucleolar localization. Inhibition of RNA polymerases with actinomycin D also causes loss of KDM3B from INBs, demonstrating that KDM3B nucleolar localization is transcription dependent. We hypothesized that Crm1 and KDM3B interact in the nucleolus to regulate rRNA transcription. Chromatin immunoprecipitation (ChIP) experiments followed by qPCR with primers detecting multiple sequences along the large ribosomal genes revealed that KDM3B binds to the promoter region of the ribosomal genes. We also demonstrate that knockdown of KDM3B caused decreased rRNA transcription as measured by qPCR as does LMB treatment. We conclude that Crm1 controls the localization of KDM3B to the nucleolus where KDM3B functions to regulate ribosomal gene transcription. Interestingly, KDM3B localizes to a chromosomal region that is commonly deleted in acute myelogenous leukemia (AML), a disease characterized, in part, by ribosome biogenesis dysfunction. The identification of this novel interaction may provide novel therapeutic opportunities for diseases like AML and ribosomopathies.

Endocrinology
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NCI-CCR
Targeting the Src signaling pathway using a new inhibitor, SKI-606, for the treatment of metastatic thyroid cancer in a preclinical mouse model
Currently, no effective treatment for patients with radioiodine-refractory, advanced thyroid cancer is available. Aberrant activation of the Src pathway is frequent in aggressive thyroid cancer. We hypothesized that Src is critical for thyroid carcinogenesis and inhibition of Src could prevent cancer progression and distant metastasis. We evaluated whether the Src pathway was activated in our ThrbPV/PV mouse model of metastatic thyroid cancer. This mouse harbors a knock-in mutation of the thyroid hormone receptor ß gene (denoted as ThrbPV) and spontaneously develops metastatic thyroid cancer. Overactivation of the Src pathway was evident by increased phosphorylation of Src (3-fold) and its downstream target, focal adhesion kinase (FAK; 2â€“10-fold), in the thyroids of ThrbPV/PV mice compared with wild-type mice. To shorten testing time, we generated ThrbPV/PV Pten+/- mice with additional haploid deficiency of the tumor suppressor gene Pten, leading to accelerate cancer progression and metastasis with decreased survival. Using this novel mouse model for aggressive thyroid cancer, we evaluated the effect of SKI-606 (bosutinib), a novel potent inhibitor of the Src signaling pathway, in thyroid carcinogenesis. SKI-606 treatment effectively inhibited aberrant activation of Src and FAK to inhibit the growth of thyroid tumors by 37%, thereby significantly increasing the survival of SKI-606-treated mice by 1.2 months as compared with vehicle-treated mice. Src inhibition reduced cell proliferation as evidenced by the 50% reduction in Ki-67 nuclear staining and decreased expression of key regulators of cell cycle progression. Importantly, SKI-606 treatment decreased vascular invasion (26%) and lung metastasis (45%) of thyroid cancer. These effects were mediated by inhibition of the epithelial-mesenchymal transition via increased E-cadeherin and decreased vimentin, slug, matrix metalloproteinase-2 and -9. SKI-606 treatment also dramatically prevented de-differentiation by maintaining the normal follicular structure and expression of PAX8 and NIS, thyroid-specific differentiation markers, via down-regulation of MAPK pathways. This preclinical study found that Src and its downstream effectors are potential molecular targets for treatment of thyroid cancer. For the first time, the present study shows that blocking of Src activity inhibits thyroid cancer progression and metastasis, suggesting oral SKI-606 is a promising agent for treatment of refractory thyroid cancer.

Endocrinology
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Obesity-related hormones and endometrial cancer risk among postmenopausal women: The BFIT cohort
BACKGROUND: Obesity is an established risk factor for endometrial cancer yet the mechanisms underlying this association remain unclear. Leptin and adiponectin, secreted from adipose tissue, reportedly play a role in such carcinogenic processes as cell proliferation, angiogenesis, and insulin regulation. Only two studies have prospectively measured total adiponectin in relation to endometrial cancer, with inconsistent results, while none have assessed pre-diagnostic leptin or high molecular weight (HMW) adiponectin, the biologically active isoform. METHODS: In this case-control study, nested within the BFIT cohort (n=15,595), a follow-up to recruitment of volunteers screened for the bisphosphonate Fracture Intervention Trial (FIT), we assessed pre-diagnostic serum leptin, adiponectin, and HMW adiponectin in relation to endometrial cancer among postmenopausal women not currently on hormone therapy. During follow-up (~10 years), 62 incident endometrial cases were diagnosed and

matched on age and time of fasting blood draw (1992-1993) to 124 controls. Adipokine and c-peptide levels were measured using enzyme-linked immunosorbent assays; estradiol was measured by liquid chromatography-mass spectrometry. Odds ratios (OR) and 95% confidence intervals (CIs) were estimated via conditional logistic regression, with exposures categorized in tertiles (T). Multivariable models considered estradiol (E2), c-peptide (insulin secretion), and body mass index (BMI, kg/m2) as potential confounders. RESULTS: Endometrial cancer risk was significantly associated with higher leptin levels, adjusted for E2 and c-peptide (OR T3vsT1=2.96 (95% CI: 1.21, 7.25; P-trend<0.01). This elevated risk persisted after further adjustment for BMI, another strong risk factor (P-trend=0.02); however, estimates were attenuated and no longer statistically significant (OR T3vsT1=2.11, 95% CI: 0.69-6.44). No significant associations were observed with adiponectin or HMW adiponectin; however, increased HMW adiponectin was suggestive of reduced risk (OR T3vsT1=0.63, 95% CI: 0.27, 1.44). Additional adjustment for a marker of circulating insulin, c-peptide, attenuated this result. CONCLUSIONS: Pre-diagnostic leptin levels appear to be a risk factor for endometrial cancer independent of other obesity-related markers, including E2 and c-peptide. Continued exploration of leptin and HMW adiponectin in larger prospective studies may help elucidate mechanisms underlying observed obesity-cancer associations.

Endocrinology
Jourdan, Tony
Visiting Fellow
NIAAA

Peripheral CB1 receptor blockade reverses ß-cell Loss, hyperglycemia and improves renal function in Zucker diabetic fatty rat.

Obesity and type 2 diabetes are often associated with increased activity of the endocannabinoid system, and cannabinoid-1 (CB1) receptor blockade is effective in reducing body weight and the associated metabolic complications. We have analyzed the effects of a novel, peripherally restricted CB1 receptor inverse agonist (JD-5037) in Zucker diabetic fatty (ZDF) rat, a commonly used model of type 2 diabetes. ZDF rats are obese, steatotic, dyslipidemic and develop type II diabetes, the natural progression of which closely replicates the human disease as it advances from a hyperinsulinemicâ€“euglycemic (insulin-resistant) state to a hyperglycemic insulin-deficient state reflecting ß-cell decline. Indeed, in our study, vehicle-treated 4 month old ZDF rats had severe hyperglycemia associated with moderately elevated plasma insulin, had severe nephropathy indicated by a dramatic 80% decrease in glomerular filtration rate associated with polyuria and polydipsia, had elevated plasma cholesterol and triglyceride (TG), had hypoadiponectinemia, had elevated plasma levels of the pro-inflammatory cytokine TNFa, and were hyperphagic despite being hyperleptinemic, as a sign of leptin resistance. Treatment of an age-matched group of ZDF rats for 28 days with JD-5037 (3mg/kg) normalized glycemia, glucose tolerance and HbA1c without causing a reduction in body weight. At the same time, plasma insulin levels were higher and the ratio of insulin/c-peptide was greater compared to the vehicle-treated group suggesting a prevention of ß-cell loss. Moreover, glomerular filtration rate, urine osmolarity, urine output and fluid intake were normalized. Furthermore, JD-5037 treatment partially reversed the hypercholesterolemia and hyperleptinemia without affecting food intake, moderately reduced hepatic TG levels and normalized plasma adiponectin, and TNFa levels. In conclusion, JD-5037 exerts strong, weight-independent anti-diabetic effects likely by attenuating ß-cell loss in a model of progressive ß-cell dysfunction. In parallel, CB1 blockade also reverses/attenuates the complications of obesity and diabetes, including nephropathy, dyslipidemia, hepatic steatosis, hypoadiponectinemia and leptin resistance.

Endocrinology
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NICHD
Identification of peptides that bind growth plate chondrocytes by phage display: a first step toward targeted therapy
Mutations in genes that regulate the growth plate cause skeletal dysplasias, in which the bones are short and malformed, leading to serious disability. In the past decade, our understanding of growth plate biology has remarkably expanded, and yet translation of the experimental findings into effective treatment strategies for skeletal dysplasia still remains very limited. This is largely because most regulators of the growth plate involve local, paracrine growth factors, which do not lend themselves to therapeutic applications.However, we envision that these locally-acting molecules could be linked to cartilage-targeting peptides and administrated systemically, thus augmenting treatment efficacy and lowering off-target effects. We therefore sought to identify peptides with high binding affinity and specificity for growth plate cartilage to allow targeted therapy for skeletal dysplasias. Phage display has been proven to be a powerful technology for selection of peptides that bind to molecules of interest. We used a phage display library, in which 12-amino acid peptide variants encoded by random DNA sequences are expressed and displayed on the surface of bacteriophage. The phage library was incubated with cultured growth plate chondrocytes. After repeated washing, bound phage were eluted, amplified, and taken through two additional binding/ amplification cycles to enrich the pool in favor of cartilage-binding sequences. Resulting phage clones demonstrated increased affinity to cultured chondrocytes by ELISA, when compared to a phage lacking the peptide-encoding insert (insertless phage). Furthermore, those selected phage showed little preferential binding to other cell types, including primary fibroblasts, myocytes and adult mouse hepatocytes cultures in comparison to insertless phage, suggesting a tissue-specific interaction. Preliminary immunostaining result revealed that the peptide-displaying phage bound to the cell surface of cultured chondrocytes. Further investigation is now ongoing to determine the binding affinity, tissue specificity and precise molecular targets of the free peptides. Peptides with the highest affinity and specificity will be linked to growth factors that regulate the growth plate, such as bone morphogenetic proteins, to assess targeting efficiency and therapeutic efficacy.

Endocrinology
Hamel, Brant
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NIEHS
The N-terminus of the glucocorticoid receptor regulates its nucleocytoplasmic localization
The glucocorticoid receptor (GR) is a nuclear receptor that binds to the hormone cortisol, released in response to stress, to mediate the activation and repression of downstream genes controlling diverse physiological processes from glucose metabolism to suppression of the immune response. GR is a major target of many steroidal anti-inflammatory drugs in clinical use. Unlike related nuclear receptors, GR is predominantly located in the cytoplasm in the absence of hormone and translocates to the nucleus only upon ligand binding. The controlled localization of GR is critical for its ability to activate target genes in response to stimulation from exogenous or endogenous ligands. Multiple motifs have been discovered that influence the subcellular localization of GR including nuclear localization, export, and retention sequences. However, all of the known motifs are located within or between the DNA and ligand-binding domains, while the N-terminal domain has not been shown to play a role in controlling the localization of GR. Using both confocal microscopy and a novel image-based flow cytometric assay, we analyzed a

series of nested deletion mutants within the N-terminal domain of GR and identified a deletion, d277-297, that showed a significant increase in basal nuclear localization in the absence of hormone (nuclear localization similarity score of 1.02 versus 0.09 wild-type, p< 0.0001). Sequence conservation analysis using a multiple sequence alignment of GR from 35 species illustrated that the residues 277-97 comprised the most highly conserved region in the N-terminus, supporting an important and conserved function for these residues. Furthermore, secondary structure prediction algorithms predict the formation of a beta-sheet in this area that could be important for mediating protein-protein interactions necessary for proper localization of GR. Targeted mutagenesis of this motif lead to the discovery that when 4 residues, K277, E279, K280, and E281, were mutated to alanine, GR had almost complete nuclear localization in the absence of hormone (nuclear localization similarity score of 2.05 versus 0.09 wild-type, p< 0.0001). This study is the first to implicate a specific motif in the N-terminal domain of GR as being critical for its proper localization. The mutants will be used in order to evaluate the physiological significance of altered GR localization.

Epidemiology/Biostatistics - Etiology, Risk, and Prevention

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Circulating levels of inflammatory markers and survival in early stage lung cancer

Lung cancer mortality is the leading cause of cancer death worldwide. About 15% of lung cancers are diagnosed at early stages, when the disease is more likely to be successfully treated. However, 5-year survival rates are lower than 40% for those with stage I and II lung cancer. There is substantial heterogeneity in survival outcomes for reasons that are not well understood, though some have hypothesized that inflammation may play a role. Some small studies have looked at the association of inflammatory markers with survival, but they included subjects diagnosed at any stage and who received varied treatments, which might have confounded the results. Further, these studies have only looked at a few immune markers. New multiplexed technologies enable investigators to simultaneously evaluate large numbers of circulating inflammatory markers in epidemiological settings. We used one such panel to analyze 77 inflammatory circulating markers measured in patients selected from a population-based study of lung cancer (EAGLE). Specifically, 249 patients with stage I and II lung cancer, diagnosed with either squamous cell carcinoma or adenocarcinoma, and surviving either less than 79 weeks (1.5 years) or more than 156 weeks (3 years) were selected for this analysis. Choosing patients at the extremes of the survival distribution maximizes the statistical power to detect associations. Odds ratios (OR) and 95% confidence intervals (CI) were computed to assess associations between markers and long vs. short survival. All analyses were adjusted for age, sex, smoking status, pack-years, stage, histology, surgery, chemotherapy and radiation treatments. A Bonferroni correction was used to adjust p-values for multiple comparisons. Six markers increased the odds of short survival by 2- to 5-fold when comparing the highest quartile (Q4) to the lowest quartile (Q1). After adjusting for multiple testing, CCL15, a chemokine highly expressed in lung leukocytes and chemotactic for T-cells and monocytes, remained significant (OR=4.93, 95% CI: 1.90-12.8, comparing Q4 to Q1). In this first comprehensive study of circulating inflammatory markers and survival in lung cancer, we found that CCL15 increased the odds of short survival in early stage patients. These results suggest that deregulation of chemokines may promote rapid progression and could be used as a prognostic classifier. Further, our results suggest that treatment guidelines and prognosis should consider immune markers.

Epidemiology/Biostatistics - Etiology, Risk, and Prevention

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NCI-DCEG
Prospective Evaluation of Baseline Serum Sarcosine and Risk of Incident Prostate Cancer in the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial
A recent report (Sreekumar et al. Nature 2009) on the application of metabolomics in the discovery of a potential new prostate cancer biomarker identified, sarcosine, a derivative of the amino acid glycine, as a metabolite to pursue. Since this report additional studies have attempted to replicate these findings, some supporting the association and some contradicting the findings. Unfortunately it is difficult to compare and draw a conclusion from these studies, since they use different study designs, small subject populations (all <150 subjects/samples), different sample types (tissue, urine, serum), and different analytical methods. Thus, we prospectively examined the association between baseline serum sarcosine and risk of prostate cancer in 1,122 cases and 1,112 controls in the Prostate, Lung, Colorectal and Ovarian cancer screening trial. Logistic regression was used to calculate Odds Ratios (OR) and 95% Confidence Intervals (CI) for the association between sarcosine and risk of prostate cancer. We observed a significantly increased risk of prostate cancer with increasing levels of sarcosine (OR for the highest quartile of exposure (Q4) versus the lowest quartile (Q1) = 1.30, 95% CI: 1.02, 1.65; P-trend 0.03). When we stratified by disease aggressiveness we observed a stronger association for nonaggressive cases (OR for Q4 vs. Q1 = 1.44, 95% CI: 1.11, 1.88; P-trend 0.006) but no association for aggressive prostate cancer (OR for Q4 vs. Q1 = 1.03, 95% CI: 0.73, 1.47; P-trend 0.89). Interestingly, for men who reported having diabetes, which is typically associated with decreased prostate cancer risk, the risk of prostate cancer was 3 times as likely in those with the highest levels of sarcosine (OR for Q4 vs. Q1 = 3.02, 95% CI: 1.26, 7.25; P-trend = 0.02; P-interaction=0.01). Temporal analyses indicate that risks are stronger when sarcosine was measured closer to diagnosis suggesting that sarcosine may be an early biomarker of disease; however, this needs to be examined further.

Epidemiology/Biostatistics - Etiology, Risk, and Prevention
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Prospective cohort study of medical diagnostic x-ray procedures and thyroid cancer risk
Medical diagnostic x-ray procedures are the fastest growing source of exposure to ionizing radiation in the United States. While diagnostic x-rays provide important medical benefits, future cancer risks associated with their exposure are also possible. The thyroid gland is the most radiosensitive organ, and radiotherapy to the head and neck is associated with an increased risk of thyroid cancer. However, it is not known whether diagnostic x-rays, which yield a lower dose of ionizing radiation to the thyroid gland than radiotherapy, also increase the risk. The U.S. Radiological Technologists study is a nationwide, prospective cohort of 146,022 radiologic technologists with extensive data on self-reported medical histories of radiation exposure collected prior to developing thyroid cancer. This study provides a unique opportunity to investigate the risk of thyroid cancer related to several different types of medical and dental diagnostic x-ray procedures including x-rays to the skull, spine, neck, and chest, and upper gastrointestinal (GI) series, angiograms, mammograms and dental x-rays. We estimated the risk of thyroid cancer related to the number and type of diagnostic x-ray procedures, adjusting for exposures to all other procedures as well as age, gender, body mass index, smoking status, and history of benign thyroid conditions, using Cox proportional hazards models. We also assessed the modifying effects of age and year of first x-ray procedure, as well as of the frequency of examinations received per unit time. The most common procedures were chest and dental x-rays, followed by upper GI series and upper

spine x-rays. The procedure with the greatest number of x-rays reported was dental x-rays (mean=14 among cases, 12 among non-cases), followed by chest x-rays (mean=8 among cases, 9 among non-cases.) We found that dental x-rays, if first received before 1970, were associated with a 20% increased risk of thyroid cancer for every 10 additional x-rays received (hazard ratio=1.2, 95% confidence interval: 1.0, 1.3, P-value for trend = 0.04), and if received frequently. We found no clear evidence of risk related to other medical diagnostic x-ray procedures, and no risks related to dental x-rays received after 1970. Although doses from dental x-rays have been decreasing over time, emerging technologies and increasing use of x-ray procedures in dentistry should be treated with caution by the consistent use of a thyroid collar in addition to a lead apron.

Epidemiology/Biostatistics - Etiology, Risk, and Prevention
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Birth size and late-life brain volumes. The AGES-Reykjavik study
Background: It has been proposed that the risk of developing chronic diseases is influenced not only by genetic and adult life-style factors but also by environmental factors acting in early life. Barkerâ€™s 'fetal origins of adult disease' hypothesis proposes that fetal undernutrition, indicated by small birth size, increases the susceptibility for chronic diseases in later life. The question remains as to whether the Barkerâ€™s hypothesis can be extended to diseases of the aging brain. This is the first study investigating this hypothesis by associating birth size measures to late-life brain volumes on MRI. Methods: Within the AGES-Reykjavik Study (2002-2006), a prospective population-based study, analyses were performed in 1348 older individuals (mean age 75 +/- 5 yrs). Birth size was extracted from the original midwife records. Computational algorithms were used to quantify volumes of white matter (WM), gray matter (GM), cerebrospinal fluid (CSF), and white matter lesions (WML). Total brain (TB) volume was defined as the sum of GM, WM, and WML, and intracranial volume (ICV) as the sum of TB and CSF. Linear regression analyses relating birth size to brain volumes were adjusted for age, sex, education, midlife weight and height. To obtain a measure of brain atrophy, regression analyses were further adjusted for ICV. Results: Mean (range) birth weight, birth length, and Ponderal index (PI; indicator of fetal wasting during late pregnancy) were 3.7 kg (1.9-5.5), 52 cm (39-62), and 26 kg/m superscript-3 (15-44), respectively. Lower birth weight, birth length, and PI, were associated with smaller ICV and absolute brain volumes. Adjusting for head size (ICV), showed that lower PI, and not lower birth weight and length, was significantly associated with smaller relative TB and WM volumes and larger relative peripheral CSF volume: mean (95%CI) differences between the lowest and highest PI categories (<21 kg/m superscript-3 vs. >29 kg/m superscript-3) were -15.2mL (-28.8; -1.5), -9.5mL (-16.6; -2.4), and 12.7mL (1.3; 24.1), respectively. Conclusion: This finding that small birth size is associated with smaller ICV and with more brain atrophy later in life might suggest that fetal undernutrition, particularly during late pregnancy, is involved in both growth and loss of brain tissue during life-span.

Epidemiology/Biostatistics - Etiology, Risk, and Prevention
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Epigenome-wide association study identifies DNA methylation differences in cord blood related to in utero tobacco smoke exposure

Maternal smoking during pregnancy causes adverse health outcomes in offspring. The 2006 US Surgeon Generalâ€™s report concluded that the evidence is sufficient to infer a causal relationship between in utero tobacco smoke exposure and low birth weight, sudden infant death syndrome, and reduced lung function and suggestive for a causal relationship with preterm delivery and some childhood cancers (leukemias, lymphomas, and brain tumors). However, the mechanisms involved in these relationships are not well understood. Data from mouse models has shown that some in utero exposures cause epigenetic changes in the offspring but data in humans are limited and doses given in mouse models are often substantially higher than human exposures. Evaluating the effects of in utero exposures on DNA methylation in cord blood in humans can help elucidate epigenetic mechanisms relevant to childhood and adult disease. In this study, we evaluated the relationship between tobacco smoke exposure during pregnancy and epigenome-wide DNA methylation in cord blood, using the recently released Illumina Infinium HumanMethylation450 BeadChip. We examined maternal plasma cotinine (an indicator of tobacco smoke exposure) measured at gestational week 18 in relation to DNA methylation at 473,844 CpG sites (CpGs) in 1,062 newborn cord bloods from the Norwegian Mother and Child Cohort Study (MoBa). We observed differential DNA methylation at 26 CpGs mapped to 10 genes at epigenome-wide significance (p-value<1.06x10-7). We observed replication in 36 US cord blood DNA samples, most notably for three CpGs in genes involved in the detoxification of components of cigarette smoke. Our findings also identified altered methylation status among three of the top genes that represents a novel pathway of fetal response to in utero tobacco smoke exposure. Our study of maternal smoking in relation to epigenetic modifications in infants in the MoBa cohort is the largest and most extensive to date. In addition to the large sample size and the use of a platform with comprehensive genome-wide coverage, our study included assessment of maternal smoking by means of a highly sensitive assay for the nicotine metabolite cotinine, a well-validated biomarker. Our findings suggest epigenetic mechanisms for the effects of in utero exposure to tobacco smoke.

Epidemiology/Biostatistics - Prognosis and Response Predictions

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The association of coffee intake on liver cancer incidence and liver disease mortality in male smokers

Background: Liver cancer is the 6th most commonly diagnosed cancer and the 3rd leading cause of cancer death in the world, with only 15% of patients surviving five years after diagnosis. Previous studies have suggested benefits of coffee intake on liver disease and liver cancer. For example, increased coffee consumption has been reported to be inversely associated with liver disease in cross sectional studies, with lower rates of liver disease progression in a cohort of patients with pre-existing liver disease, and with incident liver cancer in several case-control and cohort studies. Yet, most prospective cohort studies of the association were conducted in Asian populations. Therefore, we evaluated the association of coffee intake with incident liver cancer and mortality from chronic liver disease in a prospective cohort of Finnish men with low prevalence of HBV and HCV infection. Methods: The Alpha-Tocopherol and Beta-Carotene Cancer Prevention (ATBC) Study was a double-blind, placebo-controlled trial of a-tocopherol (a form of Vitamin E) and ß-carotine supplementation, set in a population of Finnish male smokers aged 50-69. At baseline, 27,086 men completed a food frequency questionnaire assessing coffee and other items and were followed for incident liver cancer or mortality from chronic liver disease among other outcomes for up to 24 years. Relative risks (RRs) and 95% confidence intervals (CIs) were estimated by Cox proportional hazard models, adjusting for age, body mass index, education, smoking duration and intensity, alcohol and tea intake, and serum cholesterol levels. Results: Compared to men who drank up to one cup per day, men who drank four or more cups per day had a reduced risk

of incident liver cancer (RR per cup per day=0.84, 95% CI=0.75-0.95; p-trend across increasing categories=0.002) and mortality from chronic liver disease (RR per cup per day=0.56, 95% CI=0.49-0.64; p-trend<0.0001). These inverse associations persisted in nondiabetics, moderate alcohol drinkers, HBV and HCV negative cases, lighter smokers, and cases occurring both early and late in follow-up. We observed similar associations among those drinking boiled or filtered coffee. Discussion: Intake of both boiled and filtered coffee was associated with a reduced risk of liver cancer and liver disease in the cohort. Coffee could affect liver disease and liver cancer through several mechanisms including antioxidants, liver cell proliferation, and insulin resistance.

Epidemiology/Biostatistics - Prognosis and Response Predictions

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Dietary patterns and colorectal cancer incidence: a classification and regression tree analysis

Background: Classification and regression tree (CART) analysis is a nonparametric statistical technique used to explore data, assess hierarchical patterns, identify high-risk subgroups, determine risk factors, and examine multilevel interactions. CART can handle continuous, dichotomous, ordinal, and nominal variables and does not require the distributional assumptions necessary with more commonly used dietary pattern identification methods. Although it is used with increasing frequency in other disciplines, to date CART has not been applied to examine the complex relationship between diet and colorectal cancer (CRC). Objective: To model the relationship between diet and CRC incidence using CART. Design: Participants (n = 491,817) aged 50-71y from the NIH-AARP Diet and Health Study were followed for 10y, during which time 6,746 incident CRC cases were ascertained. Dietary intake data were generated from a 124-item food frequency questionnaire administered at baseline. CART analysis was performed on 28 candidate socio-demographic, dietary, and health-related variables separately for men and women. Results from CART analyses are presented in the form of inverted trees; the root node comprises the entire sample, which is subsequently partitioned into binary subgroups (nodes) based on shared characteristics. The procedure continues until all subgroups can no longer be subdivided. Results: Ten terminal nodes determined the tree structure among women; relevant variables included age, added sugar, red meat, saturated fat, whole grains, and empty calories (solid fat, alcohol, and added sugar). The two highest-risk subgroups were defined by 1) age (60-67y) and empty calories (greater than 50% of total calories) and 2) age (>67y) and higher red meat intake. In contrast, the lowest-risk subgroup was younger (<58y), with lower added sugar intakes. Among men, the tree structure had 8 terminal nodes; relevant variables included age, empty calories, diabetes, and body mass index. The highest-risk subgroup was defined by older age (>61y) and greater intake of empty calories; the lowest-risk subgroup was younger (<58y), with fewer empty calories. Age had multiple splits in both men and women, suggesting interactions between age and diet. Conclusions: CART may be a useful method to identify dietary components most relevant for given health outcomes. The multiple splits on age suggest CART may facilitate identification of interactions among key variables.

Epidemiology/Biostatistics - Prognosis and Response Predictions

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Epstein-Barr virus positivity as a prognostic indicator in gastric cancer: An international pooled analysis

Background: Epstein-Barr virus (EBV) is present in the tumor cells of up to 10 percent of gastric carcinomas, the second leading cause of cancer death worldwide. Previous small studies addressing whether viral presence influences clinical progression have been inconclusive. We therefore examined the association of EBV with survival after gastric cancer diagnosis in a large multi-center case series, accounting for surgical stage and other established prognostic factors. Methods: Thirteen studies conducted in Asia (n=8), Europe (n=3) and Latin America (n=2) recruited a total of 4,526 patients with gastric cancer diagnosed between 1976 and 2011 (71% male, age range 18-90 years). The presence of EBV was assessed by in situ hybridization with an average prevalence of 8%. Study-specific hazard ratios (HRs) for overall survival were estimated using Cox regression models, adjusted for age (linear), sex, surgical stage (American Joint Committee on Cancer classification, treated as an ordinal variable), degree of differentiation (ordinal), and anatomic subsite (categorized as cardia, noncardia, overlapping, post-surgical remnant or unspecified). The 13 HRs were pooled for a summary estimate by a random-effects model. Between-study heterogeneity was quantified with the I2 metric and statistical significance assessed by the Q statistic. Geographic region was evaluated as a source of heterogeneity by meta-regression. Results: As expected, surgical stage was strongly associated with risk of death (pooled HR= 2.5 per stage; 95% confidence interval = 2.0 to 3.1). Surgical stage was inversely associated with EBV positivity (p <0.001). Adjusted for stage and the other potential confounders, study-specific HRs of mortality for patients with EBV-positive vs. EBV-negative tumors ranged from 0.2 to 1.8. For all studies combined, there was a significant survival advantage of EBV-positivity (pooled HR= 0.7; 95% confidence interval = 0.5 to 0.9), with low heterogeneity across studies (I2 =26%; p=0.18). There was no significant variation among the three continent-specific HRs (p=0.29). Conclusion: Our findings indicate that EBV positivity is an additional prognostic indicator in gastric cancers. Further studies are warranted to identify the mechanisms underlying this protective association with mortality.

Epidemiology/Biostatistics - Prognosis and Response Predictions

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Temporal variability of pesticide concentrations in homes and implications for exposure misclassification in cancer epidemiology

Background: Residential pesticide use has been linked to childhood cancers in epidemiologic studies. Most studies estimated childrenâ€™s exposure from parentsâ€™ self-reported pesticide use, which could be subject to recall bias. Therefore, high-quality exposure estimates are critical for confirming these associations. Carpet dust serves as a reservoir for pesticides and other household chemicals, and pesticide concentrations in dust (single sample) have been used as a more objective indicator of residential pesticide exposure. However, if concentrations vary over time, this approach could substantially misclassify exposure and attenuate risk estimates. Methods: To better understand the temporal variability of residential pesticide concentrations, we collected up to 7 carpet dust samples from 21 homes in Fresno County, California from 2003-2005. Four classes of pesticides were analyzed including carbamates, organophosphates, pyrethroids, and organochlorines, using gas chromatography/mass spectrometry. In interviews, participants were asked about housing characteristics and pesticide use. We used mixed-effects models to estimate between- and within-home variance, with and without adjustment for housing characteristics and pesticide use. We computed intraclass correlation coefficients [ICC=between-home variance/(between-home variance + within-home variance)] and the potential attenuation of odds ratios in a hypothetical case-control study in which a single dust sample was collected. Results: Housing characteristics and pesticide use explained 4 to 49% of the between-home and 0 to 23% of the within-home variability in pesticide concentrations. The

median ICC was 0.79 (range: 0.43-0.95), demonstrating that the between-home variability was greater than the within-home variability for most pesticides. Given this relatively high repeatability, the attenuation in observed odds ratios for 6 of the 9 pesticides would be less than 25%. Conclusions: For most pesticides studied, use of one dust sample in an epidemiologic study of participants in our study homes would not substantially attenuate odds ratios. Self-reported treatment practices and housing characteristics explained much of the between-home variability in pesticide concentrations, but provided modest insight into with-in home variability. Significance: These results suggest that use of a single dust sample may be a useful exposure indicator for future cancer epidemiology studies.

Epidemiology/Biostatistics - Prognosis and Response Predictions

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The relationship of folate and alcohol to hepatocellular carcinoma incidence and liver disease mortality

Introduction: Excessive alcohol consumption is a well established risk factor for liver disease and hepatocellular carcinoma (HCC). Previous studies have found that increased alcohol consumption can lead to lower absorption of folate and folate deficiency. Conversely, higher folate intake has been inversely associated with liver damage and HCC. In the current study, we investigate the effect of alcohol consumption and folate intake, independently and together, on HCC incidence and liver disease mortality in the NIH-AARP Diet and Health Study. Methods: The NIH-AARP study enrolled 566,399 men and women between 1995 and 1996. Participants reported their diet over the previous year via a food-frequency questionnaire. For analysis, the number of alcoholic drinks consumed per day was categorized as: none, <1, 1-3 and >3 drinks/day and total daily folate intake was divided into tertiles. After exclusions, 494,744 individuals were analyzed to calculate hazard ratios (HR) and 95% confidence intervals (CI) using a multivariate Cox proportional hazards regression model adjusted for age, sex, race, education, smoking, body mass index and diabetes. HCC incidence (N=435) was determined through 2006 and liver disease mortality (N=789) was determined through 2008. Results: Using intake of <1 drink/day as the referent group, both liver disease mortality (HR=5.84; 95%CI=4.81-7.10) and HCC incidence (HR=1.92; 95%CI=1.42-2.59) were significantly associated with intake of more than 3 drinks/day. Neither outcome was associated with folate intake. When folate and alcohol intakes were analyzed together, folate had no effect on the relationship between alcohol and liver disease mortality (P-heterogeneity=0.25). In contrast, folate intake significantly affected the relationship between alcohol and HCC risk (P-heterogeneity<0.01). HCC risk increased 2.5 fold among persons who consumed more than 3 drinks/day and whose folate intake was in the lower 2 tertiles. Among persons whose folate intake was in the highest tertile (>737 ug/day), however, alcohol was not associated with HCC, HR=0.95; 95%CI=0.49-1.88. Conclusion: Liver disease mortality was associated with increased alcohol consumption regardless of folate intake. In contrast, HCC incidence was not related to increased alcohol consumption among persons with the highest intakes of folate. These results suggest that high folate intake may ameliorate the effect of moderate alcohol consumption on the development of HCC.

Epigenetics

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Rap2b, a novel p53 downstream target, promotes cell survival after DNA damage

The tumor suppressor p53 is a critical regulator of apoptosis and cell survival. It evokes both pro-survival and pro-apoptosis programs upon DNA damage. However, the mechanisms underlying this cell fate decision are largely unclear. To identify p53 targets in a global and unbiased manner, we performed gene expression microarray and ChIP-chip assays using mouse embryonic fibroblasts (MEFs) and mouse embryonic stem (mES) cells. I treated MEFs and mES cells with adriamycin, a DNA damage agent, for 8 hours. Comparing to the untreated cells (negative controls), I obtained common genes that were dramatically induced both in MEFs and mES cells. Subsequently, we further analyzed these genes and obtained a list of 90 common genes. Then, I compared these 90 genes with published p53 targets in human cell lines U2OS and HCT116, and found 10 genes (Alox5, Eda2r, Btg2, Mdm2, Adrb2, Cdkn1a, Tnfrsf10b, Rap2b, Ddit4, Bbc3) that are conserved p53 targets between mouse and human. Most of these genes have been well studied except for Rap2b. Both mouse and human Rap2b share the same amino acid residue sequence, which indicatingRap2b might have important conserved functions both in human and in mouse. Using conventional chromatin immunoprecipitation (ChIP) assay and luciferase reporter assay, I identified one p53 binding motif in Rap2b promoter region. After DNA damage, p53 binds to the promoter of Rap2b and activates its transcription. The reduction of Rap2b levels by small interference RNA increases the apoptosis of cells under the damaged condition, suggesting that Rap2b helps cell survive upon DNA damage. This pro-survival role of Rap2b is very similar to the function of another well-known p53 target, Mdm2. Bioinformatic analysis revealed that Rap2b is over-expressed in many types of tumors, consistent with its pro-survival function. Anchorage independent growth assay showed that Rap2b only has weak transformation activity, suggesting that it is not a typical oncogene. Importantly, Rap2b activates RalGDS-Ral survival pathway after DNA damage. Therefore, our results identified a novel player, Rap2b, in the pro-survival program conducted by p53 and revealed a connection between the p53 signaling and the RalGDS-Ral cell survival pathway Future studies will focus on investigating whether the inhibition of Rap2b may increase the apoptosis of tumor cells.

Epigenetics

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A Functional Interaction between Alpha-fetoprotein and miRNA-29 Modulates the HCC Epigenome

Globally, hepatocellular carcinoma (HCC) accounts for 70-85% of primary liver cancers and ranks second in the leading cause of male cancer death. Serum alpha-fetoprotein (AFP) is a key HCC biomarker associated with poor patient outcome. We hypothesize that AFP+ and AFP- tumors may differ biologically and have different prognosis in HCC patients. Identifying the molecular mechanisms underlying these differences may improve our understanding of HCC progression and lead to new molecular targets for therapy. In this study, we performed microarray-based global microRNA (miRNA) and mRNA profiling analyses of 242 HCC cases that differ in AFP expression. We found that members of the miRNA-29 family are the most significantly (p<1E-5) down-regulated miRNAs in AFP+ tumors. In parallel, we found that a member of the DNA methyltransferase (DNMT) family, DNMT3A, is one of the most significantly (p=8E-6) up-regulated genes in AFP+ HCC. In addition, there is a significant inverse correlation (p<1E-4) between miR-29 and DNMT gene expression, suggesting that they may be functionally antagonistic, which is consistent with the recent finding showing that DNMT3A and DNMT3B are direct targets of miRNA-29. Moreover, HCC cases with elevated AFP and low miRNA-29 expression have an increased global DNA methylation. Experimentally, we found that increased AFP expression inhibits miRNA-29a expression in HCC cells. In addition, inhibiting AFP expression increases miR-29 levels in HCC cells. Most recently, we found that AFP transcriptionally regulates the promoter of miR-29a by a luciferase assay. Further DNA methylation array profiling shows increased methylation in

patients with high levels of serum AFP. These results support our hypothesis that AFP inhibits miRNA-29, which modulates epigenetic changes that contribute to poor outcome in HCC. Taken together, this study elucidates the molecular mechanisms that link AFP and miRNA-29 expression to epigenetic alterations in HCC and may aid in the development of novel therapeutic agents to improve survival of HCC patients.

Epigenetics
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Chromatin state primes stress specific p53-regulated gene responses
In genotoxic stress, the tumor suppressor p53 plays a crucial role in cell fate, regulating cell-cycle arrest, senescence, or apoptosis by transactivating its myriad target genes. It is well established that p53 recognizes specific DNA sequences, yet little is known about factors determining its occupancy in vivo on chromatin. Understanding how chromatin states regulate p53 access to DNA and drive the diversity in p53 binding and transactivation may lead to novel targets to harness the apoptotic potential of p53. Here, we mapped p53 binding and dynamic chromatin changes in lymphoblastoid cells by ChIP-seq and studied the relevance of chromatin state to stress-specific p53 occupancy and gene expression. Doxorubicin (Doxo), ionizing radiation, or Nutlin-3 was used to induce p53 through DNA damage or MDM2 inhibition. ChIP-seq analysis detected >3000 p53 binding regions with distinctive patterns between exposures. Untreated cells exhibited p53 binding at a small portion of its binding sites, however, the presence of p53 was associated with higher RNAPol II levels (p<0.0001), open chromatin states (ENCODE H3K27Ac, H3K4me3, etc.), and was predictive of time-dependent gene expression kinetics after Doxo exposure. We examined exposure-induced chromatin status and observed that global H3K4me3 changes were distinctive between exposures and were correlated with gene expression (r2=0.42, p<0.0001). H3K4me3 levels within the gene body prior to exposure were positively correlated with gene expression but negatively correlated with fold-change gene expression, with known p53 targets overrepresented among the low expression, high fold-change genes. Comparing ENCODE transcription factor (TF) binding profiles (e.g. POU2F2, TCF12, p300, etc.) with p53 binding sites, these TFs occupy many of the accessible chromatin regions prior to p53 binding (32%), suggesting that clustered TF sites may regulate DNA access for p53. The p53 peaks in TF clusters display significantly higher average sequence conservation with syntenic mouse regions (47% vs 31%, p<0.0001), suggesting the possibility of evolutionary selection for TF binding clusters that include p53. Dynamic changes of chromatin insulator protein CTCF were also analyzed by ChIP-seq/PCR. We observed distinct changes in CTCF at p53 target genes (e.g.GDF15) between exposures. Together these data identify stress-specific dynamic chromatin changes, which underline the epigenetic mechanisms of p53-dependent transactivation.

Gene Expression
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Histone demethylase Jmjd3 contributes to epigenetic regulation during T cell development
T cell development in the thymus involves an orderly sequence of gene expression programs that control differentiation and survival decisions at each developmental stage. In addition to transcription factor-based circuitries, stage-specific gene expression is thought to involve specific chromatin modifications. Of particular interest is the tri-methylation of Histone 3 Lysine 27 (H3K27Me3). This mark

is associated with gene repression, and it is therefore presumed that its removal is necessary to initiate gene expression. We have tested this hypothesis by conditionally deleting the H3K27Me3 demethylase Jmjd3 at the CD4+CD8+ double positive (DP) stage of T cell development in the thymus. We show that disruption of the gene encoding Jmjd3 results in a substantial increase in total H3K27Me3 in DP and CD4 single positive (SP) thymocytes, indicating a genome-wide role for Jmjd3 in the regulation of H3K27Me3 marks. In addition, ChIPseq analyses of H3K27Me3 distribution document that Jmjd3 disruption compromised the removal of H3K27Me3 at specific gene loci, including the gene coding for S1P1, a surface receptor necessary for thymocyte egress. Accordingly, Jmjd3-deficient mature CD4 SP thymocytes with impaired S1pr1 expression accumulated in the thymus. Finally, Jmjd3 deletion severely impairs the development of invariant NK T cells. This study demonstrates that Jmjd3-mediated removal of H3K27Me3 is essential for proper T cell development, and that Jmjd3 is involved in both genome-wide prevention of inappropriate H3K27Me3 deposition and gene-specific removal of this epigenetic mark.

Gene Expression
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Activation of hypoxia-inducible factor 2alpha promotes colorectal carcinogenesis
Oxygen concentrations are known to vary substantially in a solid tumor including colorectal cancer, owing to the rapid cell division and aberrant tumor angiogenesis and blood flow. Cellular adaptive response to hypoxia is mainly mediated by hypoxia-inducible factor (HIF), which consists of a ubiquitously expressed beta subunit and an oxygen sensitive alpha subunit. Under normoxia, HIFalpha subunits are rapidly degraded via the von Hippel-Lindau tumor suppressor protein (VHL) E3 ubiquitin ligase complex. In the present study, western blotting and immunohistochemistry staining demonstrated an increased expression of HIF2alpha in colorectal cancer but not normal colon epithelium from human patients and mice. Intestinal epithelium-specific disruption of VHL resulted in constitutive activation of HIF signaling, and increased HIF expression augmented colon tumorigenesis in the Apcmin/+ intestinal tumor model. Intestine-specific disruption of VHL increased colon tumor multiplicity and progression from adenomas to carcinomas. These effects were completely ablated in mice with compound knockout of VHL and HIF2alpha, thus demonstrating that HIF2alpha plays an essential role in the development of colon cancer. Mechanistically, activation of HIF signaling led to increased cell survival in colon tissue, however, tumor apoptosis was not affected. Interestingly, a robust activation of cyclin D1 was observed in tumors of Apcmin/+ mice in which HIF2alpha was activated. Consistent with this result, BrdU incorporation indicated that cellular proliferation was increased in colon tumors following HIF activation. Microarray and further analysis identified intestinal iron absorption transporter divalent metal transporter 1 (DMT-1) as a HIF2alpha target in colorectal tumors from human patients and Apcmin/+ mice, which is responsible for the dysregulation of iron homeostasis in colon cancer. These data provide a mechanistic basis for the widely reported link between iron accumulation and colon cancer risk. Together, these findings demonstrate that HIF2alpha activation in the colon initiates pro-tumorigenic signaling and may have important implications in colon cancer prevention and therapeutics.

Gene Expression
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NHGRI

RRP1B, a novel metastasis suppressor, regulates alternative mRNA splicing

RRP1B (ribosomal RNA processing 1 homolog B) is a poorly characterized metastasis suppressor. A large number of RRP1B binding candidates identified by tandem affinity purification and mass spectrometry are involved in alternative mRNA splicing (AS). AS is an essential method of regulating eukaryotic gene expression that has become increasingly prominent in cancer progression and metastasis. We have therefore investigated the role of RRP1B in AS in this study. A shRNA was used to stably knockdown Rrp1b in the highly metastatic Mvt-1 cell line. Rrp1b knockdown increased in vitro cell invasiveness and in vivo metastasis, confirming RRP1B as a metastasis suppressor. RNA-seq was performed with two control and two stable knockdown cell lines to examine the role of RRP1B in AS. With the knockdown of Rrp1b, a significant change in isoform expression was observed in over 200 genes. This was verified by qRT-PCR using isoform-specific primers. In addition, we designed minigene plasmids containing the genomic sequence spanning several exons for target genes identified through our RNA-seq analysis, and transfected them in control and Rrp1b-overexpressing cell lines to perform splicing assays. An isoform of Rps19 (ENSMUST00000108430), a ribosomal protein associated with tumor progression, increased with the overexpression of Rrp1b, upholding the RNA-seq data. Among the binding candidates of RRP1B, SRFS1 (SF2/ASF) is a well-known splicing regulator that has also been shown to be an oncogene. The interaction of RRP1B and SRFS1 was verified by co-IP and co-IF analyses of 293ET cells transfected with HA-tagged full length RRP1B or various RRP1B deletion mutants, as well as non-transfected MDA-MB-231 cells to confirm the interaction between the endogenous proteins. Recent studies have shown that AS occurs co-transcriptionally and that the two events can regulate each other. To examine whether this was the case for splicing events mediated by RRP1B and SRSF1, 293ET cells were treated with DRB to inhibit transcription. Interaction between RRP1B and SRFS1 increased significantly with DRB 4 h treatment, demonstrating that transcription affects the association of the two proteins. Earlier microarray analyses demonstrated that ectopic expression of Rrp1b has profound effects on global gene expression. Based on our current findings, we conclude that RRP1B functions as a metastasis suppressor by regulating gene expression, most likely at the mRNA splicing stage.

Gene Expression

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NHLBI

Gender-specific miR-222 expression regulates eNOS levels in the heart

A role for endothelial nitric oxide synthase (eNOS) in ischemic preconditioning has long been recognized, and increased eNOS expression in female hearts is thought to contribute to gender-specific cardioprotection. Although miRNAs are thought to participate in the regulation of cardioprotective proteins, the specific miRNAs that may be responsible for gender-specific changes in cardiac risk have not been elucidated. We hypothesized that male and female hearts have different miRNA expression patterns and that these miRNAs directly affect expression of key cardiprotective proteins, including eNOS. miRNA was isolated from male and female mouse hearts and expression levels were quantitated by Affymetrix GeneChip miRNA array. Principle component analysis revealed strong clustering of female, but not male, miRNA expression patterns in principle component 1 vs. 2 and 2 vs. 3. Array analysis revealed a significant decrease in miR-222 expression in females. Using TargetScan software, the transcription factor, ets-1, which enhances eNOS expression, was identified as a putative target of miR-222. Protein and mRNA extracted from male and female hearts confirmed increased levels of ets-1 in females. Direct inhibition of ets-1 by miR-222 was established using a luciferase reporter containing the putative ets-1 3â€™UTR target transfected into HEK cells treated with either a miR-222 inhibitor or

mimic. As a target of ets-1, eNOS levels were measured in male and female hearts. Similar to previous studies, eNOS mRNA levels were increased in females. Additionally, either a miR-222 inhibitor or mimic altered expression of eNOS protein in isolated adult rat ventricular myocytes. Taken together, these data indicate that miR-222 is differentially expressed in male and female hearts and could play an important role in cardioprotection through the differential expression of eNOS.

Gene Expression
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NICHD
Brd4 orchestrates recruitment of pause-release factor P-TEFb and the pausing complex NELF/DSIF to coordinate transcription elongation of interferon stimulated genes
Transcription is a highly ordered, regulated multi-step process in eukaryotic gene expression. NELF and DSIF cooperatively bind to elongating RNA polymerase II (Pol II) and induce transcriptional pausing, which is alleviated by pause-release factor, P-TEFb mediated phosphorylation of Pol II C-terminal domain at serine 2 position. However, the mechanism of P-TEFb recruitment and regulation of NELF/DSIF during inducible gene expression is not fully understood. We addressed this question in interferon (IFN) stimulated transcription, focusing on Brd4, a BET family protein that interacts with P-TEFb. Besides P-TEFb, Brd4 binds to acetylated histones through the bromodomain. In this study, we examined the assembly of transcription pausing and elongation machinery for several IFN stimulated genes (ISGs) in NIH3T3 fibroblasts with detailed time kinetic and chromatin immuno precipitation studies. Specifically, we found that IFN stimulation triggered inducible recruitment of Brd4 at the transcription start sites of multiple ISGs and binding patterns of Brd4 correlated to, (1) mRNA induction kinetics, (2) acetylation of histone H4 and H3 at lysine 9 and 14, (3) P-TEFb recruitment and (4) phosphorylation of serine 2 Pol II. Interestingly, NELF and DSIF were hardly detectable on ISGs prior to stimulation, but recruited robustly after IFN treatment, indicating their role in elongation, but not pausing prior to ISG transcription. In addition, shRNA-based knockdown of NELF revealed that it negatively regulates the passage of Pol II and DSIF across the ISGs during elongation, which reduces total ISG transcript output. To dissect functional interplay of Brd4, P-TEFb and NELF/DSIF, we employed targeted inhibition of bromodomain binding to acetylated histones through recently developed small molecule inhibitor of BET family bromodomains. Inhibition of Brd4 binding by inhibitor sharply reduced ISGs expression by declining P-TEFb mediated transcription elongation. Moreover, analyses with inhibitor showed that IFN-induced recruitment of NELF/DSIF was also under control of Brd4. Together, our data indicates that ISGs expression is finely balanced by two opposing control mechanisms: (1) Brd4 dependent P-TEFb mediated productive transcription elongation and (2) transcription dependent recruitment of NELF and DSIF driven negative regulation. Moreover, we suggest a model where Brd4 helps to coordinate both positive and negative regulation of ISG transcription elongation.

Genetics
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NCI-CCR
Dissecting BRCA2-dependent tumorigenesis network
Breast cancer is the most prevalent cancer in women worldwide and mutations in breast cancer susceptible genes, BRCA1 and BRCA2 are the genetic factors conferring highest risk (40-70%) of developing breast cancer. Although BRCA proteins are known to maintain genomic stability mainly by

homologous recombination-mediated DNA repair, detailed mechanisms of how BRCA loss induces tumorigenesis remain unclear. Interestingly, while inactivation of both alleles of BRCA1 or BRCA2 genes is necessary for tumor development, their loss in embryonic cells affects cell viability. To explain this paradox, we hypothesize that cells lacking BRCA1 or 2 are able to survive and are predisposed to tumorigenesis due to mutations in other genes such as those involved in cell cycle regulation or DNA damage response. In the current study, we propose to identify such genes using a mouse embryonic stem (mES) cell-based insertional mutagenesis screen. In mES cells with one mutant allele of Brca2 and the other flanked by loxP sites, cell death can be induced by Cre-mediated loss of the conditional allele. To identify genes whose up-regulation can rescue BRCA2 loss-induced cell death, we transduced these mES cells with murine stem cell retrovirus (MSCV) expressing Cre recombinase. We obtained Brca2-null ES cells that were rescued by the viral insertion and used them to clone the viral insertional sites. We identified several candidate genes that were upregulated in viable ES cells. One of these genes encodes a PDZ domain-containing protein, GIPC3. Expression of Gipc3 was elevated 3 to 4-fold in the rescued clone. Furthermore, we rescued the lethality of Brca2-null ES cells by overexpressing Gipc3. The rescued cells were hypersensitive to DNA damaging agents and exhibited an overall increase in genomic instability. Mechanistically, we found DNA damage-induced p21 expression to be attenuated in GIPC3 over-expressing clones. Because p21 is a key mediator of BRCA2 loss-induced cell death, we hypothesize that GIPC3 overexpression may inhibit p21 expression resulting in viable Brca2-null ES cells. We are now studying the detailed signaling pathway of how GIPC3 affects p21 expression. In addition, we are examining if Gipc3 overxpression can rescue the lethality of Brca2ko/ko embryos and contribute to tumorigenesis in Brca2ko/+ mice. We are also examining the expression of GIPC3 in human BRCA2-deficient tumors.

Genetics
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NCI-CCR

Genome-wide analysis of host-derived metastasis regulators in breast cancer

Metastasis is the leading cause of death in breast cancer patients. Still, the molecular mechanisms underlying metastasis remain largely elusive. Many genes in cancer cells have been identified that regulate critical steps of the metastatic cascade (including invasion, EMT, or self-renewal). In addition to these tumor cell-autonomous factors, it has been increasingly recognized that the host (i.e. the non-tumor cells and compartments including vasculature, immune cells, extracellular matrix, or bone marrow-derived cells) plays a critical role in metastasis as well. Unfortunately, to date no experimental systems exist that allow for the unbiased identification of host-specific metastasis regulators. We have set up a genetic approach, using mice of different genetic background to find variants in host genes associated with metastasis. In this system, we injected a genetically diverse mouse population with a metastatic mouse mammary carcinoma cell line, and quantified lung metastasis. We then looked for associations of the metastasis phenotype with allelic variants in a genome-wide Quantative Trait Loci (QTL) analysis. As a genetic panel, we used the recently created Diversity Outbred (DO) heterogeneous stock, developed by the Jackson Laboratory. These mice have a highly mosaic genetic background from eight different strains. To avoid immune rejection of the implanted breast cancer cell line, we first crossed 35 DO female mice with FVB males, and injected 118 F1 female progenies via tail vein with FVB-derived Mvt-1 cells. Mice were euthanized 21 days later, and the lungs dissected. Lung metastases were quantified on histology slides. DO F1 genotypes were reconstructed using a hidden Markov model, and QTLs calculated with R-software. The QTL analysis of this cohort of 118 mice identified a peak on chromosome 18 with a LOD score of 6.91. Strikingly, a cluster of genes coding for cell junctions proteins

lies precisely under that peak. We are currently performing follow-up studies to validate these findings and to test if these cell junctions proteins are functionally involved in metastasis in a host-dependent manner. Our approach represents a novel model system that screens for host-specific regulators of metastasis in an unbiased manner. We demonstrate that QTL mapping is feasible to identify host-specific metastasis regulators, and our approach could help identify prognostic markers and novel therapeutic targets for metastatic breast cancer.

Genetics
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Susceptibility to NF1-associated pheochromocytoma is modified in females by Pheom1 on mouse chromosome 16
Pheochromocytoma (Pheo) is a rare catecholamine-secreting neuroendocrine tumor of the chromaffin cells in the adrenal medulla, for which few mouse models exist. These potentially malignant neoplasms can occur sporadically or in conjunction with several familial tumor syndromes including Neurofibromatosis type I (NF1). We previously reported adrenal tumors in the NPcis mouse model for NF1, albeit with limited characterization. NPcis mice harbor dual heterozygous mutations in the Nf1 and Trp53 genes on the same chromatid (in cis) of chromosome 11. In the current study, we provide more detailed histological characterization of the NPcis adrenal tumors which displayed zellballen nests of cells, fibrovascular networks, cytosolic granularization, and Chromogranin A immunoreactivity consistent with features commonly seen in human Pheo. Furthermore, features associated with malignancy were common, such as atypical mitotic figures, necrosis, profound nuclear pleomorphism, and capsular and vascular invasion. These findings, in addition to observations of secondary lesions in the lung and liver, suggested that the adrenal tumors in NPcis mice were likely malignant Pheos. In addition to histological characterization, we also demonstrate altered risk to Pheo where inbred NPcis females on the C57BL/6J (B6) background were more susceptible to adrenal tumors than their male siblings. Moreover, B6-NPcis females were significantly more prone to adrenal tumors than 129S4/SvJae (129) NPcis females, revealing strain and sex specificity of the phenotype. Backcross mapping and binary trait linkage analysis of 129x(B6x129)-NPcis animals revealed a female specific linkage peak on distal chromosome 16, corresponding to a 32 Mb region we refer to as Pheochromocytoma modifier 1 (Pheom1). Female susceptibility to Pheo was significantly altered in response to variations in the background genotype of Pheom1, whereas males were not affected by Pheom1 regardless of the genotype. Interestingly, Pheom1 overlaps the mouse Ts65Dn locus (syntenic with the human Down Syndrome region) which was previously reported to cause increased susceptibility to adrenal tumors in female Ts65Dn;NPcis mice, further supporting our conclusion that Pheom1 modulates female risk to Pheo. Overall, these data indicate that NPcis is an appropriate mouse model for Pheo studies, and that sex and strain specific modifiers in Pheom1 dictate susceptibility to adrenal medullary tumorigenesis.

Genetics
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NCI-CCR
Interdigit Bone Morphogenetic Protein Signaling is Essential for Programmed Cell Death and is Implicated in Digit Formation

Shaping of the embryonic limb involves many processes including growth, differentiation, and programmed cell death (PCD). These processes integrate complex information from multiple signaling cascades such as the Bone Morphogenetic Protein (BMP) and Fibroblast Growth Factor (FGF) pathways. Mus musculus (mouse) limb development is a useful model to study these dynamic processes and pathways due to the high degree of homology with humans and the available tools for genetic manipulation. During mouse development, the limb bud mesenchyme differentiates into digital rays connected by interdigital (ID) tissue, which is later removed by PCD. Absence of PCD results in the retention of ID tissue and webbed limbs in adults, a phenotype referred to as syndactyly. BMPs have been implicated in initiating PCD in the ID cells. Furthermore, Bmp2, 4, and 7 are expressed in the ID mesenchyme and are up-regulated prior to the onset of PCD. However, our previous work showed that BMP signaling regulates PCD indirectly by modulating the secretion of FGFs from neighboring tissue, which act as a cell survival factor to the ID mesenchyme. Nevertheless, this indirect model does not exclude a direct role for BMPs in PCD. Due to lack of genetic evidence, it is still unclear whether ID BMPs directly trigger PCD. To genetically test whether BMPs play a role as direct triggers of ID PCD, we inactivated the gene encoding the Bmpr1A receptor within the ID tissue with Osr1-Cre. The resulting mutants are syndactylous and show a decrease of ID PCD at embryonic day 13.5. To test redundancy between BMPR1 receptors in PCD we inactivated ID Bmpr1A in a Bmpr1B null background. This compound mutant has a further decrease in PCD and surprisingly rescues a subset of the skeletal malformations observed in Bmpr1B null mice: a complete rescue of the most anterior digit. Marker analysis for RNA expression of various components of the BMP signaling pathway revealed a dramatic upregulation of Growth Differentiation Factor 5 (GDF5), a potential alternate ligand, in the ID mesenchyme. Currently, GDF5 has not been implicated to be involved in limb PCD. To fully understand the role of ID BMP signaling on normal limb development, we are also inactivating Bmp2, 4, and 7 with Osr1-Cre. Preliminary analysis of these mutants reveals a syndactylous phenotype and an ectopic posterior skeletal element. This work will clarify the role of ID BMP signaling in directly regulating PCD and digit formation.

Genetics
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NEI

Exome Sequencing and Association Study Implicates Extracellular Matrix Gene, FBN2 in Early and Late Onset Macular Degenerations.

Inherited macular dystrophies are a collection of clinically and genetically heterogeneous group of disorders characterized by progressive loss of visual acuity due to macular dysfunction. Age related macular degeneration (AMD) represents the most common late-onset heritable macular disease, affecting nearly 0.2% of the population between 55 to 64 years of age and 13% of those above 85 years of age in developed countries. Stargardt macular dystrophy, Best macular dystrophy, Sorsbyâ€™s fundus dystrophy and Doyne honeycomb retinal dystrophy are some of the early onset macular dystrophies. Both early and late forms share clinical features and thus identification of causative genes in various early-onset forms has often provided important genetic insights into pathogenesis of AMD. Using a combination of whole genome linkage analysis and exome sequencing, we identified novel heterozygous segregating mutation, p.Glu1144Lys in FBN2 in a two-generation family with early form of dominant macular dystrophy. FBN2 encodes for cysteine-rich glycoprotein, which form the principal component of extracellular matrix. This variant was absent from 1000 genome, dbSNP or 500 control chromosomes. Structural modeling suggested that Lys1144 might introduce negative impact on the packing due to van der Walls forces leading to loss of backbone rigidity and flexibility. Immunohistological studies in

monkey and human fetal eye localized the FBN2 protein in Bruchâ€™s membrane, choroid and sclera. Bruchâ€™s membrane is multilayered structure located between the choroid and retinal pigment epithelium and extra¬cellular deposits within or adjacent to Bruchâ€™s membrane are often clinically associated with AMD. Genes of extracellular matrix component have been implicated in both early and late macular degenerations and recent genomewide association studies have identified variants in extracellular/collagen matrix pathway genes (TIMP3, COL8A1, COL10A), conferring susceptibility in AMD patients. This prompted us to explore the possible role of rare and common variants in FBN2 in AMD patients. We sequenced 96 AMD patients and identified a rare mutation. We also observed an association of a non-synonymous SNP rs154001 (p value=0.0001; odds ratio= 0.85) with AMD in analysis of 1988 cases and 2182 population matched controls. Taken together our findings indicate the importance of FBN2 in developing AMD and may contribute to our understanding of the pathomechanism of this sight-threatening disorder.

Genetics
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NHGRI
Identification of biomarkers for a human treatment trial to rescue hyposialylation in Hereditary Inclusion Body Myopathy
Hereditary Inclusion Body Myopathy (HIBM) is a rare, debilitating adult-onset progressive myopathy, which leaves most patients wheelchair-bound 10-20 years after onset. HIBM is caused by mutations in GNE, encoding the bifunctional key enzyme in sialic acid (SA) synthesis, UDP-GlcNAc 2-epimerase/ManNAc kinase. Although the exact pathology of HIBM remains unknown, symptoms seem to occur due to hyposialylation of a select group of (sialo-)proteins. We demonstrated that oral administration of the SA precursor N-acetyl-mannosamine (ManNAc) could rescue hyposialylation in HIBM mice. These findings incited us to design a human HIBM treatment trial, for which we explored biomarkers/outcome parameters. While isofocusing of the standard sialylation markers transferrin (N-linked) and APO-CIII (O-linked) showed no hyposialylation in HIBM serum, glycan profiling by mass spectrometry indicated hyposialylation of O-linked glycans. To quantify O-linked hyposialylation, we calculated the ratio of O-linked T-antigen (Gal-GalNAc-Ser/Thr) versus its sialylated form ST-antigen (SA-Gal-GalNAc-Ser/Thr). This T/ST ratio in normal plasma was consistently <0.06, while HIBM plasma showed ratios >0.06. Plasma from a HIBM patient treated with highly sialylated immunoglobulins (IVIG) or from a patient self-administering ManNAc (off label), we saw the T/ST ratios shifting back to the normal range. We confirmed mass spec results by Western blotting, where T-antigen was more intensely expressed in HIBM than in control. These findings justify the T/ST ratio to be employed for responsiveness of HIBM patients to ManNAc treatment. Clinical trial data will reveal whether favorable T/ST ratios after treatment can be correlated to increased/stable muscle function. Our findings led us to postulate that in HIBM patients (who have decreased SA synthesis due to their GNE defect), a shortage of total SA occurs, likely later in life. Some glycans (N-linked) will then be preferably sialylated over others (O-linked) due to differential substrate affinity of sialyltransferases for CMP-SA. Proteins with significant O-linked glycosylation will be affected and contribute to the phenotype, one of such proteins affected in HIBM is muscle alpha-dystroglycan, others remain to be identified. These findings can explain both the pathology and adult onset of HIBM.

Genomics
KOhaar, Indu

Visiting Fellow
NCI-DCEG
Association of CCNE1 splicing forms with risk to bladder cancer
A recent genome-wide association study (GWAS) identified a single nucleotide polymorphism (SNP), rs8102137, located 6 Kb upstream of the cyclin E1 gene (CCNE1) on chromosome 19q12, as a risk factor for bladder cancer (OR=1.13, p=1.7x10-11, Rothman et al, Nat Gen, 2010). CCNE1, which encodes a cell cycle protein, functions as a regulator of cyclin dependent kinases (CDK) and therefore is an important cancer susceptibility gene. mRNA expression analysis for bladder normal and tumor tissue samples showed higher expression for total CCNE1 mRNA in bladder tumors (n=42) compared to adjacent normal bladder tissue samples (n=41, 3.7 fold, p=2.7x10-12). By RNA-seq analysis in normal and tumor bladder tissue samples, we have identified two alternative splicing forms of CCNE1 of the potential importance for bladder cancer â€“ with deletions of exon 5 (CCNE1_5del) and exon 7 (CCNE1_7del) respectively. CCNE1_5del form lacks 49 aa within cyclin box, and is predicted to be non-functional due to its inability to form complex with cdk2. CCNE1_7del form has a deletion of 45 aa downstream of cyclin box and its functional significance remains unclear. Expression analysis with the splicing form-specific TaqMan assays validated the presence of these forms and showed correlation with several bladder cancer associated genetic variants in this region. Confocal microscopy analysis of recombinant CCNE1 isoforms also revealed that these variants are expressed in cytoplasm while wild type CCNE1 expresses in nucleus. Also, cell cycle analysis of synchronized, transiently transfected HeLa cells with these expression constructs reveal that more cells were arrested in G0/G1 phase after transfection with alternative forms compared to transfected wild type CCNE1. To functionally characterize these protein isoforms ChIP-seq is being carried out to identify molecular targets of all CCNE1 isoforms. In conclusion, our results suggest that bladder cancer associated genetic variants within the CCNE1 gene may affect mRNA splicing and protein structure of the gene, contributing to altered regulation of cell cycle and risk of bladder cancer.

Genomics
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NIDDK
Identifying direct targets of the DMRT transcription factor Doublesex
Identifying target genes of a transcription factor is essential for understanding how it directs developmental or disease processes. Few studies of transcription factor-induced genetic pathways attempt to understand the contribution of individual target genes to the global phenotype yet these data are important for full comprehension of a genetic pathway and for identifying promising candidates for drug targeting. In order to understand a complete genetic pathway from transcription factor genomic binding sites to phenotype, we are identifying the target genes of Drosophila Doublesex (DSX), a DMRT (DSX/Mab-3-Related Transcription Factor) transcription factor family member that regulates sexual development in Drosophila and higher eukaryotes including mammals. In order to identify DSX target genes we are determining DSXâ€™s genomic binding sites, identifying genes with transcriptional dependency on DSX and elucidating putative target geneâ€™s individual contributions to the global dsx phenotype. We have used multiple genome-wide approaches to assay DSX occupancy across diverse developmental and chromatin contexts where dsx is expressed including chromatin immunoprecipitation-sequencing (ChIP-seq) and DNA Adenine Methylation-Identification (DamID). These studies have yielded thousands of sites that are concentrated near transcriptional start sites and within introns. Furthermore, the DNA captured beneath the peaks is enriched for previously identified DSX binding sequences. To identify genes whose transcription depends on DSX we have performed RNA-sequencing on multiple dsx-expressing tissues in which we have used the power of Drosophila genetics

to alter DSX protein status between female and male isoforms that direct female or male sexual development, respectively. Collectively, these approaches identify hundreds of DSX target genes. In order to make connections between target genes and phenotype, we are knocking down these genes specifically in dsx-expressing cells to evaluate their role in sexual differentiation. DSX target gene knockdown phenotypes reveal defects affecting specific aspects of sexual differentiation from sex-specific bristle structure and pigmentation, gonad morphogenesis to egg-laying capability, indicating that we can understand how DSX directs sex-specific differentiation target gene by target gene.

Genomics
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NIEHS
A permanent record of transient hyper-mutation associated with single-strand DNA in human cancers
Frequency, location and timing are key parameters determining biological outcomes of mutations. Recent sequencing of human tumors has enabled us to address how these parameters contribute to a cancer mutator phenotype. While inactivation of DNA repair can lead to persistent, high mutation rates, few such examples are reported in cancers. Transient acquisition of multiple mutations in one or a few cell generations is an alternative and could produce synergistic or compensatory changes that provide growth advantage. We have shown that chronic DNA damage can generate clusters of simultaneous multiple mutations via transient mutagenesis. Genome sequencing of methyl methanesulfonate treated yeast revealed mutation clusters composed of "strand-coordinated" changes of either cytosines or guanines in the same strand, indicating the mutations were induced in the same generation. Mutation patterns and genetic controls suggested these mutations resulted from alkylations in long single-strand (ss)DNA formed at double-strand breaks and replication forks. Here, we report simultaneous clustered mutations in human cancers. We designed a bioinformatics approach to search for mutation clusters in large datasets of somatic mutations. Among 328,040 mutations identified in whole-genome sequencing of 23 multiple myelomas, 2 head-and-neck squamous cell carcinomas, 7 prostate and 9 colorectal cancers, we identified 635 clusters within the 4 cancer types. Similar to our yeast findings, both mutated A:T and G:C base pairs in clusters were highly strand-coordinated, indicating simultaneous occurrence of the mutations. One unusual class of clusters was composed entirely of mutated cytosines or guanines and resided close to chromosome rearrangement breakpoints. Surprisingly, nearly all of these mutations occurred at a motif targeted by APOBEC family cytosine-deaminases. These enzymes specifically modify ssDNA, suggesting that mechanisms similar to those in yeast may contribute to mutation cluster formation in human cancers. Mutation at the same motif was enriched throughout the genomes of all tumors analyzed, accounting for up to 50 percent of total cancer-specific mutations and suggesting that even scattered mutations could occur simultaneously, potentially through APOBEC activity on multiple exposed ssDNA regions. Thus hyper-mutation via multiple simultaneous changes in randomly formed transient ssDNA may be an important mechanism for rapid production of genetic variation in cancer.

Genomics
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NIEHS
Identification of a novel component of the self-renewal circuitry conserved in mouse and human ES cells
Embryonic stem (ES) cells have two unique characteristics: self-renewal and pluripotency. Understanding the molecular basis of these defining features of ES cells will provide insights to

mammalian embryonic development and facilitate the use of pluripotent stem cells for disease modeling, drug discovery, and stem cell therapies. To systematically study self-renewal and pluripotency, we previously carried out a genome-wide RNAi screen in mouse ES cells. We identified over 100 genes whose down-regulation caused differentiation, including genes encoding members of the Ccr4-Not protein complex. The Ccr4-Not complex is known for the regulation of transcription and mRNA stability, and has been implicated in various cellular activities such as DNA repair, spindle positioning, microtubule length regulation, and histone methylation. However, its role in self-renewl is not understood. We found that three components of the Ccr4-Not complex, Cnot1, Cnot2, and Cnot3, are important for maintaining mouse ES cell identity. Genes corresponding to these three proteins are highly expressed in ES cells and down regulated during differentiation, and they are also enriched in the inner cell mass of the blastocyst stage embryos. In mouse ES cells, global gene expression analysis indicated that silencing Cnot1, Cnot2, or Cnot3 induces differentiation predominantly into the trophectoderm lineage. Cnot3 ChIP-on-chip showed that it occupies a unique set of gene promoter regions. Consistently, gene expression analysis showed that the Cnot genes do not impinge on previously known self-renewal transcription factors or pathways. Instead, genetic epistasis analysis demonstrated that they maintain mouse ES cell self-renewal by repressing the expression of early trophectoderm transcription factors such as Cdx2. Importantly, we found that Cnot1, Cnot2, and Cnot3 are also required for the maintenance of human ES cells, and silencing them in human cells mainly lead to trophectoderm and primitive endoderm differentiation. Together, our results indicate that Cnot1, Cnot2, and Cnot3 represent a novel component of the core self-renewal and pluripotency circuitry conserved in mouse and human ES cells, and our approach illustrates the power of RNAi and forward genetics for the systematic study of ES cell biology.

Genomics
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NIMH

WHOLE EXOME SEQUENCING IN FIRST DEGREE COUSIN PAIRS WITH EARLY AGE-AT-ONSET BIPOLAR DISORDER

Background: Individuals with bipolar disorder (BD), a top 10 WHO burden in 2000, are afflicted with suicides and disabilities, amounting to $151 billion costs in 2009. Though BD and notably early-onset BD are highly heritable, common BD single-nucleotide polymorphism (SNP) markers identified so far by genome-wide association studies (GWAS) account for only a small portion of the heritability. Identification of rarer genetic variants by Next-Generation Sequencing (NGS) technologies may help to explain more of the heritability and lead to the identification of causal genes. Family design enriched for BD may be a good approach. First cousins share ~12.5% of their genomes, reducing the search space for shared risk variants. Objective: In this pilot study, we identified 4 first-cousin pairs of European-ancestry with bipolar I disorder beginning before age 16 for whole-exome NGS, previously ascertained by the NIMH Genetics Initiative. Methods: Whole-exome NGS was performed with SOLiD technology. Fragment libraries & exome capture was performed with Agilent SureSelect50MB array. Duplicate removal and re-calibration were performed using PICARD & GATK, respectively. Roughly 65-80M reads were mapped to the reference genome (hg19). These reads were filtered for minor allele frequencies>10% in the 1000 Genomes Database. About 10K reads were mapped using BEAGLE IBD to genomic regions shared identical-by-descent (IBD) between cousins. Genotyping for relatedness was performed using the Illumina OmniExpressSNP array. Results: The proportion of variants shared IBD by cousin-pairs exceeded that predicted by relatedness alone by 150 to 176%. About 5000 variants were shared within each pair. Variants shared across multiple cousin-pairs diminishes, with 147 shared in all 4 pairs, 100% of which

could be phased. Among these were 13 uncommon non-synonymous damaging variants in 8 genes: ACACB, ALPK2, CR1L, GRK7, KCNMB1, PRB4, TRIM22, OR7A2P. KCNMB1 is located on chromosome 5q34, near a previously identified top hit from our early-onset BD GWAS. KCNMB1, a regulatory subunit of the calcium activated potassium maxiK channel, appears consistent in gene function with the calcium channel gene, CACNA1C, previously implicated in BD. Conclusions: NGS of first-cousins with early-onset BD suggests possible targets for future studies. Family-based NGS may be an efficient approach for discovering rare alleles involved in common, complex disorders.

Hematology/Oncology, Tumor Immunology, and Therapy

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NCI-CCR

Mice with myelodysplastic syndrome respond to treatment with azanucleosides; a drug trial model for new MDS therapies.

Therapies for the treatment of myelodysplastic syndrome (MDS) are very limited. Until recently, best supportive care strategies were the main treatments available to MDS patients, or allogenic stem-cell transplantation in a very small group of patients. In 2005, the DNA demethylating agent 5â€™azacitidine (5â€™AC) was approved for clinical use in the treatment of MDS, followed recently by approval of 5-aza-2â€™-deoxycytidine (Decitabine, DAC). Trials over the past two decades have shown improved hematological responses and a survival benefit in patients treated with these azanucleosides. However, responses are temporary and variable. Further characterization of response to treatment is required, as is the identification of new drugs to better treat MDS. Using bone marrow cells from the NHD13 mouse model shown to faithfully recapitulate key features of (MDS), we co-transplanted MDS/NHD13 and wild type bone marrow cells into wild type irradiated recipients. The mixed bone marrow transplant mimics the bone marrow environment of the human MDS patient comprised of MDS clones and normal cells. Wild type and MDS cells in the mice were distinguishable by using differential CD45 alleles (Ly5.1 and Ly5.2, respectively), which enabled analysis and purification of the MDS and normal cells, a feat not easily achieved with human MDS patients. Successful treatment of the chimeric MDS BMT mice resulted in increasing levels of wild type cells in the peripheral blood with a concomitant reduction or eradication of the MDS/NHD13 cells. Chimaeric WT/MDS mice showed improved hematologic indices with DAC therapy and a significant survival benefit compared to saline treated mice. Response to treatment varied depending on donor mouse, analogous to individual based variability seen in human patients. The chimaeric WT/MDS(NHD13) BMT mouse is a useful representative model of human MDS, on which response to current therapies can be better characterized and new MDS therapies tested.

Hematology/Oncology, Tumor Immunology, and Therapy

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Tumor Suppressor p15Ink4b Determines Cell Fate of Hematopoietic Progenitors: Implications for Development of Human Blood Disorders

Red blood cells (RBCs) are a vital component of mammalian blood. Since they are short lived, they must be continuously replenished by erythropoiesis, a stepwise commitment of blood stem and progenitor cells to mature erythrocytes. The anemia due to the loss of RBC is a life threatening condition, often accompanying blood diseases such as leukemias and myelodysplastic syndromes (MDS). A striking 60-80% of these diseases have deleted or silenced expression of p15INK4B. An increased understanding of

the factors that drive erythroid lineage commitment in progenitor cells is critical for developing new treatments for blood disorders. Previous examination of p15Ink4b knock-out mouse models, revealed skewing of hematopoietic progenitor differentiation towards myeloid lineage (granulocytes, macrophages). Here, we demonstrate a novel function for p15Ink4b in driving commitment to the erythroid lineage. Mice lacking p15Ink4b have lower numbers of primitive RBC progenitors and died shortly after induction of hemolytic anemia by phenylhydrazine injection. Expression of p15Ink4b in blood progenitors induced dynamic changes at the molecular level that rendered multi-lineage cells more permissive to erythroid commitment and less permissive to myeloid commitment. Noticeably, we found that p15Ink4b regulates a switch that controls the balance between myeloid and erythroid differentiation through activation of MEK/ERK signaling. In a time-coordinated manner expression of p15Ink4b induced rapid phosphorylation of MEK/ERK that led to rapid degradation of GATA-2 and activation of the GATA-1 transcription factors. Subsequently, the active GATA-1 executed lineage commitment through activation of the Erythropoietin receptor (EpoR), the â€œmaster regulatorâ€ of erythroid differentiation. The p15Ink4b mediated increase in GATA-1 expression, also resulted in decreased expression of the myeloid specific transcription factor PU.1, suppressing myeloid differentiation. In summary, we have defined a framework that determines how multipotent progenitors coordinate the balance between myeloid and erythroid differentiation. Central to this activity is p15Ink4b, which promotes erythroid fate while suppressing myeloid cell formation, a function that is particularly important in rapid RBC replenishment following stress. Our finding has implications not only for MDS and myeloid leukemia, where loss of tumor suppressor p15INK4B is a common event, but also for other forms of human refractory anemia.

Hematology/Oncology, Tumor Immunology, and Therapy
Klebanoff, Christopher
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NCI-CCR
Memory CD8+ T cells induce precocious effector differentiation of naïve CD8+ T cells in a FasL-Fas dependent manner: a new mode of T-T lymphocyte interaction and cross talk
The ability of Thelper cells to directly communicate with and deliver pro-differentiation signals to B cells through the TNFa superfamily members CD40L/CD40 has been well characterized; however, whether memory T cells (Tmem) can provide an analogous differentiation cue to naïve T cells (Tnaïve) has not been studied. Herein, we describe a previously unrecognized interaction between Tnaïve and Tmem CD8+ T cells that directly enhanced the effector differentiation of Tnaïve through a separate set of TNFa superfamily members, the FasL-Fas network. Using congenic markers as a means of indelibly tracking the fate of CD8+ T cell subsets, we found that the presence of antigen experienced CD8+ T cells during priming caused Tnaïve to differentiate more rapidly than Tnaïve activated in isolation, a process we have termed precocious differentiation. In addition to an accelerated loss of the lymphoid homing markers CD62L and CCR7, Tnaïve primed in the presence of Tmem acquired higher levels of IFNg, granzyme B, and the effector-associated transcription factors T-bet and Blimp-1. Further investigations using microarray analysis of re-isolated Tnaïve and Tmem cells demonstrated the pervasiveness of this phenomenon as Tnaïve transcriptionally associated with memory T cells by hierarchical clustering within 18 hours of activation. This process was TCR-ligation dependent, cell-dose dependent, and required cell-cell contact as it was entirely abrogated by physical separation of Tmem from Tnaïve using a semipermeable membrane. Mechanistically, disruption of FasL-Fas signaling either by antibody blockade of FasL or use of Tnaïve CD8+ T cells deficient in the Fas receptor prevented precocious differentiation while provision of exogenous FasL trimer in the absence of Tmem recapitulated this phenomenon. In order to interrogate the biologic significance of precocious differentiation, we adoptively transferred

Tnaïve activated alone or in the presence of Tmem to treat hosts bearing B16 melanoma tumors, a model in which less differentiated T cells mediate more efficient tumor regression. Naïve cells primed in vitro with Tmem cells acquired a terminally differentiated phenotype upon transfer, as evidenced by low levels of CD27 and high KLRG1 expression, and exhibited substantially diminished persistence and antitumor activity compared with Tnaïve primed alone. These findings have major implications for the design and execution of current adoptive immunotherapy clinical protocols.

Hematology/Oncology, Tumor Immunology, and Therapy
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NHLBI
PPAR-gamma in adipocytes plays a negative regulatory role in immune-mediated bone marrow failure
Aplastic anemia (AA), one of the well-known bone marrow (BM) failure diseases, is characterized by a decrease in the number and function of hematopoietic stem cells (HSCs) and progenitors, and by a massive expansion of adipocytes in BM cavities. A functional role of adipocytes in AA is unclear. In this study, we demonstrate that inhibition of adipogenesis by peroxisome proliferatorâ€“activated receptor-gamma (PPAR-g) inhibitor (BADGE or GW9662) can improve hematopoiesis in a mouse model of immune-mediated BM failure. We induced BM failure by injection of B6 lymph node cells to sublethally irradiated C.B10 recipient mice that were matched at major histocompatibility (MHC) antigens (Ags) but differed in multiple minor histocompatibility Ags. In this setting, all mice exhibit progressive and fatal pancytopenia, closely resembled human BM failure. To define a role of adipocytes in BM failure, we treated recipient mice with BADGE, GW9662, or control vehicle from day -1 to day 14. On day 14, mice in the BADGE- and GW9662-treated group showed higher levels of leukocytes, neutrophils, red blood cells, and platelets in peripheral blood (PB), and higher total nucleated cells and Lin- Sca1+ c-kit+ stem cells in BM than did animals in the control group. In PPAR-g inhibitor-treated groups, there was significantly less CD8+ cell infiltration in BM, suggesting that inhibition of adipogenesis might affect activation of cytotoxic T cells and/or prevent their migration from PB into marrow cavities. Both confocal microscopic imaging and HE staining of BM also showed significantly higher numbers of nucleated cells in the treated groups. These inhibitors neither altered T cell proliferation and expression levels of activation markers on T cells, nor caused hematopoietic expansion in vitro. PPAR-g inhibitors had no effects on hematopoiesis in autologous BM transplantation mice. These results suggest that PPAR-g inhibitors do not directly affect the functional status of HSCs, progenitors, and activated T cells. We are currently investigating the efficiency of combination therapy with PPAR-g inhibitors plus conventional immunosuppressive drugs in an MHC Ag-mismatched condition which can lead to more severe pancytopenia. This is the first report that reveals a negative role of adipocytes in immune-mediated BM failure, and this study suggests PPAR-g can be a novel therapeutic target in AA.

HIV and AIDS Research
Do, Thao
Doctoral Candidate
NCI-CCR
3D visualization of HIV transfer at the virological synapse between the T cell and astrocyte
Human immunodeficiency virus (HIV) infects the central nervous system causing encephalopathy such as HIV-associated dementia. The presence of HIV proteins activate the release of neurotoxins resulting in cognitive and motor dysfunctions due to widespread synaptic loss. Understanding how HIV is transferred to the glial cells provides insight on how to inhibit virus propagation. HIV often exploit

immunological cell-cell interactions to facilitate transmission across the virological synapse, a mechanism that is up to 100 fold more efficient than free viral transfer. To visualize HIV transmission to glial cells, we co-cultured human fetal astrocytes with chronically HIV-1-IIIb-infected H9 T cells. The architecture of the virological synapse was imaged with a focused ion beam scanning electron microscope, which iteratively abrades thin 15 nm slices of the specimen with a gallium ion beam and raster each newly exposed surface with a scanning electron beam at 3 nm pixel size, creating a 2D image stack. The 2D images showed budding virions and a high concentration of virus particles on one cell membrane and very few virions on the other cell membrane, which confirmed the identification of the infected T cell and the uninfected astrocyte. The dark densities of the virion cores and the average virion diameter of 104 nm confirmed the identification of HIV virus particles. The 2D images were reconstructed into a 3D model to provide a global visualization of the structure of virological synapse, which is not possible with light microscopy or 2D electron microscopy images. The 3D volume revealed that although the T cell and astrocyte were separated by a distance of 1-3 um, there was complex interdigitation between both cell membranes. The astrocyte extended filopodial bridges of 100-180 nm width and 1-3 um length toward the T cell where clusters of HIV are found. Virions localized within the center of the virological synapse. The semi-enclosed T cell-astrocyte virological synapse suggests that it is possible to block the transmission of HIV by targeting small inhibitors through the maze of filopodial interdigitations between the two cells. The HIV clusters found along the astrocyte filopodial bridges indicate that the bridges play a fundamental role in ensuring effective HIV transfer. Mechanisms that disrupt the polarization and formation of filopodial bridges by the target cell could significantly reduce the efficiency of HIV transmission to the brain.

HIV and AIDS Research
Kononchik Jr., Joseph
Postdoctoral Fellow
NIAID

Inhibition of HIV-1 infection on CD4+ T cells by blocking adhesion molecule mediated virus adsorption

HIV-1 infection of primary T cells is well characterized on the basis of CD4 and co-receptor recognition. The mechanism of entry involves protein-protein interaction between these cell receptors and gp120, the outer glycoprotein of the virus. This interaction is specific and required for infection. Adsorption of the virus to the cell surface, however, has been largely ignored. We have previously shown that viral envelope-associated sialic acids are important for HIV-1 adhesion to and thus infection of monocyte derived macrophages. There, it was shown that blocking host Siglec receptors, the sialic acid binding immunoglobulin-like lectins, with specific compounds and antibody significantly decreases surface bounds gp120 and HIV-1 infection. This inhibition was seen for both pseudo-typed luciferase virus and replication competent X4 and R5 strains. Here, we present data examining the importance of gp120 glycosylation with respect to HIV-1 infection of primary T cells. Using techniques including surface plasma resonance and ELISAs we show that gp120 binds specifically to L-selectin, a host adhesion molecule present on naïve and central memory T cells. We developed a novel Qdot-gp120 binding assay and showed that gp120 bound to primary T cells expressing endogenous L-selectin, and anti-CD62L significantly blocked both gp120 binding to and HIV-1 infection of CD4 T cells. These data suggests an important role of L-selectin in facilitating HIV-1 infection. Combined with the previously published data regarding macrophages, we present a new paradigm in HIV-1 infection in which cellular lectin receptor-mediated viral adhesion precedes CD4 mediated viral entry. These data provide insights to previous findings that HIV-1 preferentially infects central memory T cells and has also been shown to infect naïve T cells.

HIV and AIDS Research
Mendoza, Daniel
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Cytotoxicity Capacity of SIV-Specific CD8+ T Cells Against Primary Autologous Targets Correlates with Immune Control in SIV-Infected Rhesus Macaques
Background: we evaluated rhesus macaques infected with Simian Immunodeficiency Virus (SIV), a lentivirus closely related to HIV. Similarly to HIV-infected humans, most SIV-infected rhesus macaques progress to immunodeficiency. However, a small proportion of them named long-term nonprogressors or elite controllers (LTNP/EC) are able to control SIV replication and remain healthy for prolonged durations. The mechanism underlying effective immune responses in LTNP/EC is not well understood but evidence indicates that CD8+ T cells play a role. In this study, CD8+ T-cell cytotoxic capacity was examined to determine whether it is a correlate of immune control in the rhesus macaque (RM) SIV infection model. Methods: rhesus macaque CD8+ T cells (effectors) were co-incubated with autologous SIVmac251-infected telomerase-transduced CD4+ T-cells (targets) to measure infected CD4+ T-cell elimination (ICE) and effector capacity to deliver active granzyme B (GrB) to targets. Results were evaluated by flow cytometry. Twenty-three SIV-infected rhesus macaques with widely varying plasma viral RNA levels were evaluated in a blinded fashion. Results: 19 of 23 subjects (83%) were correctly classified as LTNP/EC, slow progressors, or progressors based on measurements of ICE (weighted Kappa 0.75). LTNP/EC had higher median ICE than progressors (67.3% vs. 23.7%, p=0.002). In addition, significant correlations between ICE and viral load (R= -0.57, p=0.01), and between GrB delivery and ICE (R=0.89, p<0.001) were observed. Furthermore, the CD8+ T cells of LTNP/EC exhibited higher per-cell cytotoxic capacity than those of progressors (p=0.002). Conclusions: these findings support that greater lytic granule loading of virus-specific CD8+ T cells and efficient delivery of active GrB to SIV-infected targets are associated with superior control of SIV infection in rhesus macaques, consistent with observations of HIV infection in humans. Therefore, such measurements appear to represent a correlate of control over SIV replication and their role as predictors of immunologic control in the vaccine setting should be evaluated.

HIV and AIDS Research
Sette, Paola
Visiting Fellow
NIAID
Deubiquitination of HIV-1 sites of assembly: an anti-viral approach to control virus release and spread
To release virions from cells, HIV-1 and most human enveloped viruses utilize members of the cellâ€™s Endosomal Sorting Complex Required for Transport (ESCRT). The latter is comprised of heteromeric complexes necessary for catalyzing membrane-modeling events critical for the budding of viruses and endosomal vesicles as well as completion of cytokinesis. HIV-1 structural protein Gag gains access to ESCRT members via two peptidic motifs: PTAP and LYPXnL, that recruit Tsg101 and Alix, respectively. Both proteins function in the ESCRT pathway and drive two independent virus release pathways, which ensure efficient virus production synergistically. ESCRT-mediated membrane fission requires ubiquitin modification and evidence suggesting the involvement of ubiquitin transfer in virus release has been reported. However a direct role for ubiquitin in HIV-1 release is yet to be shown. To address this question, we used a new strategy to permanently remove ubiquitin molecules from HIV-1 budding sites by fusing the active domain of the Herpex Simplex Virus deubiquitin enzyme UL36 (Dub) to either Gag (Gag-Dub), or to Gag binding proteins Tsg101 (Dub-Tsg101) or Alix (Dub-Alix). Remarkably, although Dub-Alix and Dub-Tsg101 proteins became ubiquitin-resistent (deubiquitinated), the fusion did not

affect their functions (incorporation in the ESCRT pathway or binding to Gag). Conversely, ectopic expression of Dub-Tsg101 or Dub-Alix exerted a strong inhibition on HIV-1 release. The inhibitory effect was specific to HIV-1 since the Murine leukemia virus (MLV), a model retrovirus that does not utilize Tsg101 or Alix remained insensitive to deubiquitin activity. These findings provide the first direct evidence for a critical role for ubiquitin transfer in virus release and demonstrate that removal of ubiquitin from the sites of HIV-1 budding is an efficient and powerful approach to prevent virus release and spread. We are currently testing the effect of deubiquitin activity on the budding of HIV-1 particles from primary and T-cells lines in vitro. Our ultimate goal is to apply this approach in in vivo studies. To achieve this, we are designing and testing lentiviral vectors expressing Dub-Gag fusion proteins that retain the ability to co-assemble within nascent HIV-1 particles, thus efficiently and specifically delivering deubiquitin activity to sites of virus assembly and budding. Our studies identified a novel therapeutic strategy to control HIV-1 release and spread.

HIV and AIDS Research

Francica, Joseph
Postdoctoral Fellow
NIAID-VRC

Adjuvants improve the magnitude and quality of humoral responses to HIV envelope protein vaccination in non-human primates.

An effective preventive vaccine against HIV-1 will require the generation of potent antibody responses against the HIV envelope (Env). Accordingly, a recent efficacy trial using a viral vaccine encoding Env and Gag followed by an Env protein/alum boost showed 30% protection. As the Env antibody titer and efficacy appeared to wane after the final immunization, the data suggested that improving the magnitude, quality and durability of Env immunity could further enhance protection. Because innate immune activation through a variety of TLR, NLR and RIG-I signaling pathways can improve both humoral and cellular immunity, we investigated how adjuvants that activate distinct DC subsets and B cells through such signaling pathways influence Env-specific humoral immunity in non-human primates (NHP). NHP were immunized with Env clade C protein alone, or with alum+/- a TLR 4 or TLR 7 agonist, MF59+/- a TLR 4 or TLR 7 agonist, or poly I:C. We found that Env-specific antibody binding and neutralization titers against a panel of tier 1 HIV viruses were increased approximately one log when Env protein was given with alum+ TLR 4 or TLR 7 agonist, MF59 alone, or poly I:C. Antibody-dependent cellular cytotoxicity titers were also significantly enhanced with alum+ TLR 7 agonist, MF59 or poly I:C. Notably, we observed that immunization with alum+ TLR 7 agonist resulted in a focusing of the antibody response to the Env CD4 binding site, a critical site of vulnerability on the viral surface. To obtain insight into the mechanism of these improved antibody responses and to further establish qualitative differences in such responses, we developed a method to identify and sort Env-specific B cells. Indeed, robust Env-specific B cell responses- as high as 20% of memory B cells- were found across multiple adjuvant groups. As recent evidence shows that potent antibodies from HIV+ individuals have unique characteristics such as long CDR3 regions and extensive somatic mutation, the Ig loci of the Env-specific B cells were characterized. We developed a deep sequencing method to access affinity maturation and other physical antibody characteristics induced by the adjuvants. Taken together, our data suggest improvements in magnitude and quality of immune responses to Env can be achieved by the use of different adjuvants and formulations; these studies should be useful in guiding future HIV vaccine efforts towards the elicitation of broad, potent and protective antibody responses.

Immunology - Autoimmune

Hong, Changwan
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NCI-CCR
A novel mechanism of regulating common gamma chain (gc) cytokine signaling by a soluble form of gc
The gc is the central signaling unit for a number cytokines, collectively known as gc cytokines. gc cytokine signaling had been thought to be mainly regulated by cytokine-specific receptor a chain expression levels, with little effects by gc levels because gc expression is presumed to remain unchanged on T cells. Extensive studies have demonstrated that gc is essential for lymphocyte development and homeostasis, while little information exists about mechanisms of gc expression and regulation. In contrast to existing data, we here describe that gc expression is actively modulated during T cell activation and development. Specifically, gc expression is downregulated in DP thymocytes during thymocyte development but re-induced upon positive selection. Importantly, we found that such lower gc expression is also partially regulated by a novel post-transcriptional mechanism. Specifically, we identified a soluble form of gc (sgc), which is generated by alternative splicing, and which seems as an endogenous regulator of gc cytokine, as evidenced by 1) real-time PCR assay, ELISA and immunoprecipitate Western blot, 2) as well as by performing cytokine signaling assay using recombinant sgc. The existence of such sgc mRNA and proteins were confirmed in humans as well. Moreover, we found that sgc expression is significantly upregulated in autoimmune disease bearing mouse models (IL2-/-, CD25-/- and CTLA4-/-) and increased in Experimental Autoimmune Encephalomyelitis (EAE)-induced mice sera. Interestingly, sgc enhanced in vitro Th17 differentiation by blocking IL-2 signaling, as observed by Th17 differentiation assays in the presence of anti-IL-2, recombinant IL-2 and sgc or of IL2-/- CD4 T cells with sgc. To identify its in vivo role, we generated sgc-overexpressing transgenic mice (sgcTg), in which naïve:memory T cell ratio was reduced and activated-CD4+ T cells skewed to Th17 cell type. These data indicate that sgc negatively regulates T cell homeostasis but positively regulates Th17 differentiation. In EAE, more severe autoimmune disease in sgcTg mice was induced compared to wildtype mice, with 1) faster progression of the disease, 2) higher disease score, and 3) delayed spontaneous remission. Collectively, we identified sgc and its biological role in regulation of gc cytokine signaling. Our studies provide a new model in which sgc is an important player in regulating T cell development and autoimmune responses.

Immunology - Autoimmune
Roychoudhuri, Rahul
Postdoctoral Fellow
NCI-CCR
The transcriptional repressor Bach-2 regulates effector differentiation in T cells
Humans with polymorphisms in the gene encoding transcriptional repressor Bach-2 are at increased risk of developing a variety of T-cell mediated autoimmune conditions including multiple sclerosis and type I diabetes mellitus. Despite this, a function of Bach-2 in peripheral T cells has not been defined. Here, we show that Bach-2 is expressed in T cells, its expression is lost rapidly upon activation in response to antigen. Bach-2 was required for the maintenance of immune quiescence in T cells since its deletion in mice resulted in the induction of a lethal multiorgan inflammatory syndrome involving the lung and gut with massive infiltration of T cells and macrophages secreting inflammatory cytokines specifically in inflamed tissues. When a low-affinity melanocyte self-antigen specific TCR Pmel-1 was introduced into these mice, rapid induction of autoimmune vitiligo was observed, an observation that could be recapitulated by the transfer of naïve T cells into wildtype recipients, thus demonstrating that Bach-2 plays a non-redundant role in the maintenance of T cell quiescence. Consistent with its role in preventing effector differentiation in T cells, we found that loss of Bach-2 was accompanied by the

acquisition of markers terminal differentiation, and the rapid induction of interferon gamma expression. Conversely, overexpression of Bach-2 was able to suppress acquisition of these characteristics. We found that Bach-2 prevented effector differentiation by repressing the gene encoding the master regulator transcription factor of effector differentiation Blimp-1 by binding its promoter (EMSA and ChIP assays). Given its ability to suppress effector differentiation, we enquired whether the function of Bach-2 could be regulated in a context dependent fashion. Consistent with the literature, we found that the mTOR kinase was necessary for effector differentiation induced by IL-2 signalling. We found that effector differentiation could only occur as a result of inactivation of Bach-2 through its phosphorylation downstream of IL2-induced mTOR signaling, specifically as a result of its interaction with S6K1. The context dependent regulation of effector function in T cells is critical to the prevention of autoimmunity. Our work reveals that Bach-2 is a context-dependent fate-switch that prevents autoimmunity by permitting effector differentiation to occur only in the presence of inflammatory signals.

Immunology - Autoimmune
Askenase, Michael
Doctoral Candidate
NIAID
Runx3 controls dendritic cell development and maintenance of tolerance in mucosal tissue
The mammalian gut is perpetually exposed to immunogenic stimulus from food, environmental antigens, and gut-resident bacteria. Therefore, the intestinal immune compartment has evolved mechanisms for immune tolerance and regulation of inflammation. Disruption of these regulatory systems can lead to chronic inflammation and disease. Specialized populations of gut-resident antigen presenting cells are conditioned by the tissue to shape the adaptive immune system and maintain tolerance at this critical site. Specifically, we have previously demonstrated that a specialized population of CD103+ dendritic cells (DCs) found in the gut lamina propria and gut-associated lymphoid tissue (GALT) induces the differentiation of naïve T cells into regulatory T cells essential for the maintenance of intestinal tolerance. However, this population of DCs is capable of generating both inflammatory and regulatory responses depending on environmental stimuli. The transcriptional programs induced in DCs by these environmental signals are not well characterized. Runx3 is a transcription factor known to participate in the transcriptional program induced by TGF-beta, a cytokine expressed highly in the gut and critical for maintaining tolerance in this tissue. Mice deficient in Runx3 develop spontaneous intestinal inflammation, suggesting that this transcription factor participates in maintenance of intestinal tolerance. Although Runx3 has been shown to play a critical role in CD8 T cells and B cells, its role in dendritic cell function has not been explored. Using a fluorescent protein reporter system, we observed that CD103+ DCs express high levels of Runx3 in the GALT. To explore the role of this transcription factor in murine intestinal DCs, we utilized a Cre-LoxP system to delete Runx3 specifically from CD11c+ cells and found that the absence of this transcription factor in CD11c+ cells leads to a substantial decrease in the number of these cells in the small intestinal lamina propria and their accumulation in the draining lymph nodes of the intestine. This was associated with a dramatic increase in the number of activated T cells in these lymph nodes, suggesting that deletion of Runx3 in CD103+ DCs disrupts their function and contributes to increased T cell activation. These results suggest a novel role for Runx3 in the control of CD103+ DC development and function and the maintenance of peripheral tolerance.

Immunology - Autoimmune
Voynova, Elisaveta

Visiting Fellow
NIAID
TLR7 induces an IL15-dependent killer dendritic cell population with multiple functionalities affecting the onset and severity of autoimmune disease
Interferon-producing killer dendritic cells (IKDC) are a recently discovered innate cell subset that shares morphological and functional characteristics of NK and DC cells. The aim of this study is to investigate the role of IKDCs in terms of initiating autoimmune disease. We found that these cells are expanded in mice with multiple copies of the TLR7 gene and that the number of these cells correlates with disease activity. By making bone-marrow chimera we observed that expansion of this population is due to their cell intrinsic sensitivity to TLR7 stimulation. Development of IKDCs is dependent of IL15, but is not dependent of IL18 and is partly dependent of IFN type I. The gene expression profile of IKDCs isolated from TLR7tg mice compared to wt NK cells indicates upregulation of genes involved into MHC processing pathway, and downregulation of most of the NK-specific receptors. IKDCs purified from TLR7tg mice produce high levels of IFN type I and type II upon stimulation with a TLR7 agonist. They also express surface MHC class II, can present and activate naïve CD4 cells as well as cross-present antigen to CD8 cells. Moreover, IKDCs overexpressing TLR7 are cytotoxic to cells lacking MHC I molecules in vitro. Adoptive transfer of IKDCs to wild type mice causes activation of naïve T cells, expansion of inflammatory monocyte populations, granulocytes and IFN-dependent CD8 activation. IKDC adoptive transfer also induces elevated inflammatory cytokine and IgG levels in the serum of transferred mice. We found that IFN type I and CD40/CD40L interactions are essential for the inflammatory effect of these cells. In the absence of IL15 and IKDCs, autoimmune disease in TLR7 tg mice is delayed. IL15 deficiency reduces many pro-inflammatory subsets, delays the development of glomerulonephritis and increases the survival of TLR7tg mice. Taken together these results uncover a potential role for this killer dendritic cell population in the development of autoimmune pathologies.

Immunology - General
Simhadri, Venkateswara
Other
FDA/CDER
CD300A BINDS TO PHOSPHATIDYLSERINE AND PHOSPHATIDYLETHANOLAMINE AND INHIBITS THE UPTAKE OF APOPTOTIC CELLS BY MACROPHAGES.
INTRODUCTION Adequate immune responses are balanced between a multitude of activating and inhibitory signals. Disruption of this balance may lead to immunodeficiency or autoimmunity. Activation signals can be negatively regulated by receptors bearing immuno-receptor tyrosine based inhibitory motifs (ITIMs) in their intra-cytoplasmic tail. CD300a is an ITIM containing molecule that belongs to the CD300 family of paired activating/inhibitory receptors. Ligation of this receptor with monoclonal antibodies inhibits activation signals on cells of both myeloid and lymphoid lineages. Here, we sought to elucidate the ligand/s for CD300a. METHODS The extracellular domain of CD300a fused to the Fc-fragment of human Ig was used to identify the ligands. Surface plasmon resonance, ultracentrifugation, reporter cell assays, ELISA and flow cytometry experiments were used to identify the ligands. We explored the functional role of CD300a in macrophage-mediated phagocytosis. RESULTS We show that CD300a-Ig specifically binds to evolutionary distant apoptotic/necrotic cells thus suggesting that the ligands are much conserved across species. We have identified phosphatidylserine (PS) and phosphatidylethanolamine (PE), two amino-phospholipids that translocate to the outer leaflet of the plasma membrane of dead cells, as the ligands for CD300a. Structural modeling and mutational studies identified a cavity in the CD300a IgV like domain where the hydrophilic heads of PS and PE can penetrate. Furthermore, we show that a non-synonymous polymorphism (R94Q) that is associated with

the development of psoriasis exhibits a lower binding affinity for PS and PE, suggesting that this receptor has a role in the pathogenesis of this disease. In vitro functional experiments show that CD300a inhibits the uptake of apoptotic cells by both human macrophages and by L929 cells ectopically expressing CD300a. CONCLUSIONS Our data suggest that CD300a, through its interaction with PS and PE, has a role in the clearance of dead cells. Abnormalities in the removal of dead cells can lead to different forms of pathology, from autoimmunity to cancer development. Furthermore, because PS (and probably PE) is not only exposed on apoptotic cells, but also on activated cells, addressing the role of CD300a in modulating other immune functions such as cell mediated cytotoxicity, cell differentiation and inflammation, during health and disease, should be the matter of future research.

Immunology - General
Hogg, Alison
Research Fellow
NCI-CCR
Colonic DCs but not small intestinal DCs instruct CD8+ T cells to home to the colon
Specialized dendritic cells (DCs) present in mesenteric lymph nodes, peyers patches and lamina propria (LP) of the small intestine induce preferential homing of T cells to the small intestine. Small intestinal DCâ€™s express high levels of RALDH enzymes that metabolize retinol into retinoic acid, which acts on T cells to induce expression of gut homing receptors CCR9 and alpha4 beta7. The ligands for CCR9 and alpha4beta7, CCL25 and MADCAM-1 respectively, are highly expressed along the small intestine. Whereas MADCAM-1 is expressed on colonic tissue, CCL25 is not. We hypothesized that the molecules and DCs involved in homing of T cells to the colon maybe different from those of the small intestine. To test this we isolated DCs from small intestine LP and colon LP and used these DCs to stimulate CD8+ T cells in vitro. Activated CD8+ T cells were then adoptively transferred to recipient wild-type (wt) mice and trafficking of these cells to the small intestinal LP and colonic LP was examined by the flow cytometry. Whereas CD8+ T cells cultured in the presence of small intestinal DCs homed to the small intestinal LP, CD8+ T cells cultured with colonic DCs homed more efficiently to the colonic LP. Ex vivo analysis of colonic DCs showed that these cells express RALDH enzymes and induce alpha4beta7 expression on CD8+ T cells. However, colonic DCs express lower levels of RALDH enzymes compared with small intestinal DCs and did not induce CCR9 expression on activated CD8+ T cells. We observed a similar finding in vivo, in which we immunized animals intracolorectally (IR) or subcutaneously with vaccinia virus and analysed expression of alpha4beta7 and CCR9 on the Ag-specific CD8+ T cells. IR immunization but not subcutaneous induced expression of alpha4beta7 on Ag-specific CD8+ T cells, whereas neither route induced CCR9 expression. To determine whether alpha4beta7 is required for homing to the colon, we isolated CD8+ T cells from beta7 deficient or wt mice and adoptively transferred these cells to recipient wt mice. We observed that beta7 deficient CD8+ T cells are defective in their ability to home to the colon LP. Our current data indicate that different molecules are involved in the homing to the large and small intestine and that homing of T cells to the large intestine may require alpha4beta7 expression but not CCR9. Whether unique chemokines/chemokine receptors are involved in colon tropism of T cells is currently under investigation.

Immunology - General
Parish, Stanley
Postdoctoral Fellow
NCI-CCR
Identification of sulfatide reactive type II NKT cells using CD1d dimers

Natural killer T cells (NKT) are a unique subset of lymphocytes whose niche is to bridge the gap between innate and adaptive immunity. Unlike conventional T cells that recognize MHC presented antigen, NKT cells recognize lipid antigen presented by CD1d. Each subset of NKT cells recognizes non-overlapping set of glycolipids. Type I NKT cells have been extensively studied by using CD1d tetramers or dimers loaded with a-galactosylceramide (a-GalCer), a type I specific lipid antigen. In contrast, studying type II NKT cells has been severely hindered by lack of a method to identify these cells, since methodology used to load a-GalCer onto CD1d has failed to provide reproducible outcomes when used to load sulfatide, a type II specific lipid antigen. In this study, we have developed sulfatide loaded CD1d dimers that can accurately identify type II NKT cells. Using the knowledge of the cellular machinery behind lipid presentation onto CD1d, we developed a new method to load lipid antigens onto CD1d dimers for flow cytometry. By using both saposin C, a chaperone protein necessary for lipid loading, and acidic conditions, as found in the endosome in which lipid loading occurs, we can reliably create sulfatide-loaded CD1d dimers to identify type II NKT cells. Sulfatide-loaded CD1d dimers specifically identify type II NKT cells in both WT and type I NKT deficient mice, and are not observed in CD1d -/- mice that lack both types of NKT cells, as expected. The specificity of the binding of dimers through TCRß is also confirmed by TCRß blockade. Consistent with previous findings in the liver, the sulfatide-reactive type II NKT cell population encompasses about 2% of all lymphocytes, and is completely distinct from its type I counterpart, which represents approximately 10% of liver lymphocytes. All these data suggest that the method we developed can create sulfatide-loaded CD1d dimers in a highly reproducible manner that can identify type II NKT cells. This method to load glycolipids antigens onto recombinant CD1d molecules has enabled us to not only identify a type II NKT cell subset based on previous knowledge of these cells, but also expand on that by identifying other markers. Ultimately, this method of loading previously unloadable lipids onto CD1d dimers may allow for their use to identify many more subsets of NKT cells across multiple models.

Immunology - General

Gerner, Michael
Postdoctoral Fellow
NIAID

Multiplex Static and Dynamic Imaging Reveals the Role of Lymphatic Sinus-Associated Dendritic Cells in Inducing Immunity to Particulate Antigens

Within seconds after peripheral tissue immunization with soluble proteins, antigens (Ags) are delivered via the lymphatics into draining lymph nodes (dLNs) and are efficiently taken up by LN-resident dendritic cells (DCs) that directly sample small proteins (<70kDa) from LN conduits. This in turn leads to Ag processing and presentation to naïve T cells, which is essential for effector T cell differentiation and expansion, and importantly for pathogen clearance and life-long immunity. In contrast, it is thought that large particulate Ags, such as bacteria, viruses, and particle-based vaccines, do not passively drain to the LN due to size-restricted, inefficient lymphatic vessel entry in the peripheral tissues. It is believed that in these conditions, only the peripheral tissue DCs migrating to the dLN with a cargo of antigen days after the initiation of infection are the principal mediators of T cell activation. However, such reliance on peripheral DC migration would necessitate a relatively long period of time before adaptive immune responses are initiated, thus potentially allowing for infectious spread. By utilizing high-resolution, multi-parameter confocal microscopy and multi-photon intra-vital imaging, we demonstrate that large particulates, including particle-based vaccines and bacteria, do in fact rapidly access the lymphatics and drain to the dLN, where they are immediately sampled by a specialized population of LN-resident DCs lining the lymphatic sinuses (LS). These DCs (LS-DCs) tightly integrate their cell bodies into the endothelial/macrophage meshwork lining the LS, extend highly motile dendrites through the openings in

the basement membranes directly into the LS lumen, and are thus poised for surveying the draining lymph fluid. Importantly, LS-DCs efficiently induce rapid CD4 T cell activation and functional effector differentiation independently of migratory DCs. These results suggest that the lymphatic architecture is specifically designed for rapidly communicating with the innate sensory cells in the dLN, which during infection of peripheral tissues allows for a prompt and efficient generation of adaptive immune responses independent of Ag size.

Immunology - General
Kindrachuk, Kristen
Visiting Fellow
NIAID
Involvement of MUC5AC in Signaling Through the Type II IL-4 Receptor Complex
MUC5AC and MUC5B are the two major secreted mucins in healthy airways. In Th2-mediated inflammatory lung diseases such as allergic asthma MUC5AC is upregulated in response to IL-13. IL-13 signals through the Type II IL-4 receptor, a heterodimer formed by IL-4Ra chain and IL-13Ra1 chain. IL-13 binds IL-13Ra1 to drive complex formation and phosphorylation of STAT6 to mediate downstream effects such as upregulation Fizz1, Ym1 and Arg1, as well as increased eosinophilia, airway remodeling and mucus hypersecretion. IL-13 is distinct from IL-4 in that it is only capable of signaling through the Type II receptor, while IL-4 can use both the Type I and Type II receptors. The purpose of this study was to investigate the role of MUC5AC in a model of allergic asthma. A mouse model of allergic lung inflammation using house dust mite was performed in wildtype C57BL/6 (WT) and MUC5AC knockout (KO) mice. A significant reduction in airway eosinophilia in conjunction with reduced expression of Fizz1, Ym1 and Arg1 was observed in KO compared to WT mice; however, no differences in the expression or production of IL-4, IL-5 and IL-13 were observed. To determine if the KO mice were capable of responding to IL-13, 3 doses of rIL-13 were delivered i.t. to WT and KO mice. No tissue inflammation, airway eosinophilia or increase in IL-13 regulated genes was observed in the KO mice in response to rIL-13. Alveolar macrophages and lung fibroblasts were isolated from naive WT and KO mice. Cells were grown in culture and treated with either IL-4 or IL-13. Both the macrophages and the fibroblasts of KO mice had significantly reduced expression of Fizz1 and Ym1 compared to WT in response to IL-13, however did not have reduced levels in response to IL-4. Phosphorylation of STAT6 in response to both IL-4 and IL-13 in WT and KO macrophages was assessed. KO macrophages did not phosphorylate STAT6 in response to IL-13 despite the ability to phosphorylate STAT6 in response to IL-4. The ability of KO, but not WT, cells to phosphorylate STAT6 in response to IL-4 was ablated after pretreatment with a blocking antibody against common gamma chain which blocks the Type I IL-4 receptor but does not affect the Type II receptor phosphorylation. No difference in the mRNA expression or protein levels of IL13Ra1 was observed in KO cells compared to WT cells. These data identify MUC5AC as a novel component of the Type II IL-4 receptor and a novel target to disrupt IL-4/IL-13-mediated airway inflammation.

Immunology - General
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Erythropoietin signaling: A novel regulator of white adipose tissue inflammation during diet-induced obesity
Obesity-induced white adipose tissue (WAT) inflammation and insulin resistance are associated with macrophage infiltration and phenotypic shift from â€œanti-inflammatoryâ€ M2-like F4/80+MGL-1+ to

predominantly â€œpro-inflammatoryâ€ like M1 F4/80+MGL-1- cells. Erythropoietin (EPO), discovered for its indispensable role during erythropoiesis, is a glycoprotein hormone whose biological activities extend to non-erythroid tissues and include anti-apoptotic and anti-inflammatory effects. We observed high levels of EPO receptor (EPO-R) expression in WAT and investigated EPO involvement in the regulation of obesity-induced inflammation and insulin resistance. Using the murine model of diet-induced obesity, and based on in vivo and ex vivo qRT-PCR, flow cytometry, immunofluorescent microscopy, ELISA and functional analyses, EPO treatment was found to inhibit WAT inflammation, normalize insulin sensitivity and reduce glucose intolerance. Remarkably, and prior to any detectable changes in body weight or composition, EPO treatment reduced M1-like macrophages and increased M2-like macrophages in WAT, while decreasing Ly6ChiCCR2+ inflammatory monocytes in the circulation. Obese mice lacking EPO-R expression in WAT exhibited more metabolic impairment and elevated WAT inflammation, with higher circulating inflammatory monocytes, increased M1-like and reduced M2-like macrophages, than wild type controls confirming an anti-inflammatory role for endogenous EPO. Collectively, our findings identify EPO/EPO-R signaling as a novel regulator of obesity-induced WAT inflammation, and extend its non-erythroid activity to encompass effects on both the infiltration and subset composition of macrophages in the WAT.

Immunology - Infectious Disease

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Airborne Transmission and Host Immune Response in a Nonhuman Primate Model of Bordetella pertussis Infection

Pertussis is a highly contagious, acute respiratory illness caused by the bacterial pathogen Bordetella pertussis. Despite near universal vaccine coverage, pertussis rates in the U.S. have been steadily rising over the last twenty years. Gaps in our knowledge of pertussis disease and mechanisms of vaccine-mediated protection have hindered our ability to comprehend and counteract this important public health problem. A major reason that many important questions remain unanswered is because the current animal models do not replicate the full spectrum of human disease, including coughing and transmission to naïve hosts, hallmarks of clinical pertussis. With this problem in mind we set out to develop a nonhuman primate model. We found olive baboons (Papio anubis) provide an excellent model of human pertussis: 9 of 9 challenged baboons developed clinical disease characterized by nasopharyngeal colonization, leukocytosis, and paroxysmal coughing fits. When naïve animals were housed in separate cages so that physical contact was prevented, 3 of 3 became infected thus providing the first demonstration that pertussis is transmitted via aerosolized respiratory droplets. We also found that convalescent baboons are resistant to re-infection suggesting that a careful analysis of the immune response to pertussis in this model may uncover protective mechanisms that could be exploited for designing therapeutics and improved vaccines. The innate immune response to pertussis was characterized by measuring cytokines in the nasopharynx of infected baboons. Within 5-10 days post-infection we observed strong induction of IL-6, IL-23, and transient IL-1b. This group of cytokines is implicated in the development of IL-17-producing T-cells (Th17 cells). Consistent with this role, there was a significant induction of IL-17 and several IL-17-associated cytokines and chemokines over the same period of infection. To explore the adaptive immune response, peripheral blood mononuclear cells from convalescent animals were stimulated with heat-killed Bordetella pertussis. Using ELISPOT we detected the presence of IL-17 secreting cells, consistent with the induction of a Th17 response. Importantly these cells were not found in naïve animals, suggesting they are related to immunological

memory to pertussis. Collectively, these data shed important light on the immune response to pertussis and suggest a Th17 immune response likely contributes to immunity observed in convalescent animals.

Immunology - Infectious Disease
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Dengue Virus Core Protein Affects Induction of Interferon-beta
Dengue virus (DENV) is a member of the Flaviviridae family of viruses and is of significant global importance, infecting an estimated 50 to 100 million people each year. Due to the geographic expansion of the insect vector as well as increasing urbanization and travel, dengue is of increasing importance to global health. Dengue infection can range anywhere from subclinical illness to life threating hemorrhagic disease. Currently, there are no approved vaccines for DENV and treatment consists of supportive therapy. The innate immune response plays an important role in controlling DENV infection. Previously it has been shown that a number of DENV genes are involved in evasion of the immune response by interfering with signaling of the type-I interferon receptor. However, little is known about how DENV may interfere with induction of type-I interferon. Here we show for the first time that the DENV Core (DENV-C) protein plays a role in evasion of the innate immune response by interfering with the induction of interferon-beta. To examine the effect of DENV-C on induction of type-I interferon, expression vectors were constructed containing the DENV-C gene. After overexpression of DENV-C or an empty vector control, HEK293 cells were treated with poly(I:C) and monitored for induction of interferon-beta by qRT-PCR and ELISA, as well as for antiviral activity by measuring virus titers after infection with Vesicular stomatitis virus (VSV). Cells expressing the DENV-C protein showed decreased levels of interferon-beta and decreased antiviral activity when compared to mock transfected controls. Mutations were introduced into the DENV-C gene using site-directed mutagenesis. DENV-C with mutations at the N-terminus showed decreased ability to influence induction of interferon-beta when compared to wild-type counterparts. Interaction partners for DENV-C were identified by Co-Immunoprecipitation (Co-IP) followed by mass spectrometry (MS). MS identified a number of potential host interaction partners, including the nuclear factor NF45. Knockdown experiments demonstrated a role for NF45 in antiviral activity. Results were confirmed by Co-IP followed by western blot analysis. Virus-host protein interactions were also verified by examining co-localization by confocal microscopy. These data indicate that DENV-C protein plays a role in virus evasion of the innate immune response.

Immunology - Infectious Disease
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Commensally induced regulatory monocytes control acute inflammation
Surviving a virulent pathogen challenge requires a rapid yet regulated inflammatory response. Appropriate regulation is especially important at mucosal sites, such as the gut, where a poorly controlled immune response can swiftly lead to host mortality. At steady-state, a network of gut resident regulatory dendritic cells (DCs) and macrophages are responsible for modulating the threshold of immune activation at this site. In response to pathogen challenge this network is dramatically reorganized as inflammatory cells are recruited into the gut. How regulation is maintained after influx of these inflammatory cells remains unclear. Toxoplasma gondii infection triggers a strong immunopathology in the gastrointestinal tract characterized by massive influx of parasite-killing Ly6Chi

inflammatory monocytes and neutrophils. Here we show that surprisingly, Ly6Chi inflammatory monocytes, acquire an immunoregulatory capacity in this setting. The phenotype was restricted to the gut and strongly driven by commensal-derived signals. Unexpectedly Ly6Chi inflammatory monocytes directly inhibit neutrophil activation in vitro, via production of prostaglandin E2 (PGE2). Moreover, in vivo, immunopathology was enhanced in mice lacking Ly6Chi inflammatory monocytes as a result of uncontrolled neutrophil activation. In this setting treatment of mice with agonists of PGE2 signaling controlled neutrophil function and limited immonopathology. Collectively these data demonstrate a previously unappreciated dual action of Ly6Chi inflammatory monocytes, to control parasite expansion while providing local regulation during acute inflammatory responses in the gut. Further, our results place PGE2 as a central player in the dialog between inflammatory cells at mucosal sites.

Immunology - Infectious Disease
Naik, Shruti
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Commensally driven IL-17A mediates barrier function in the skin
The skin, our bodyâ€™s external barrier, is colonized by diverse communities of microorganisms known as commensals. An immunological arsenal comprised of antimicrobial molecules and potent inflammatory cells reinforces the skin's physical barrier to help guard against pathogenic invasion. We have recently demonstrated that cutaneous commensals are necessary for appropriate cutaneous immune cell homeostasis and function. Specifically, in the absence of commensals inflammatory pathways are dampened and immunity to dermal pathogens is compromised. However, the mechanisms underlying the immune commensal dialogue at the cutaneous interface have not been examined. The IL-1 pathway is the central driver of pathology in several cutaneous inflammatory disorders such as psoriasis, therefore we hypothesized that commensals utilize this pathway to direct immune cells in the skin. Using mice deficient in IL-1R1 as well as downstream signalling molecules MyD88/TRIF, we identified a novel requirement for this pathway in directing IL-17A cells in the skin. Next we examined if commensals utilize the IL-1 pathway to heighten IL-17A production in the skin. To address this, we developed a novel model of commensal colonization by associating specific pathogen free mice with human cutaneous commensal Staphylococcus epidermidis. Using bacterial 16s rDNA fluorescence in situ hybridization we confirmed that colonization of S. epidermidis was restricted to the surface of the skin and that these organisms did not penetrated past the epidermal barrier causing an invasive infection. Remarkably, the addition of S.epidermidis to skin of mice elicited a potent IL-17A response in the underlying dermis. Indeed, association with S.epidermidis increased cutaneous levels of IL-1 confirming that even in the absence of overt infection commensals augment both innate and adaptive immune pathways. Importantly, penetration of the epidermal barrier with needle inoculation elicited a dramatically higher acute neutrophil response in mice associated with S. epidermidis in an IL-1/ IL-17 dependent manner suggesting that commensals induce immunological pathways that are critical for promoting protection upon breach of barrier. These data reveal that levels of cutaneous IL-17A relate to the density of bacteria on the skinâ€™s surface and underscore the importance of commensally driven IL-17A in immune surveillance at the skin barrier.

Immunology - Infectious Disease
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Development of genetically engineered TCR-transduced T cells for immunotherapy of chronic HBV and HCV infections

Chronic HBV and HCV infections continue to be a significant health problem worldwide with more than 350 million people chronically infected. Although a preventive vaccine exists for HBV, there is still no cure for those patients who are chronically infected with HBV, because treatment with antivirals does not eliminate HBV from the host cell nucleus where it persists in the form of closed circular DNA. Moreover, no preventive vaccine exists for HCV, which establishes chronic infection in a high percentage of infected patients and 40% of the chronically infected patients do not respond to treatment with pegylated interferon and ribavirin. A vigorous CD8 T cell response is required to spontaneously clear both HBV and HCV. However, in both chronic HBV and chronic HCV infection, the number of virus-specific T cells is low and their function is impaired. Here, we set out to use adoptive T cell therapy with TCR-engineered lymphocytes, a technique that has shown substantial success in cancer immunotherapy, to restore HBV- and HCV-specific immune responses in chronic infection. Specifically, we identified and isolated MHC class I-restricted HBV and HCV-specific T cell receptors (TCRs) with high avidity and anti-viral activity from patients and chimpanzees who mounted successful T cell responses and cleared acute HBV and HCV infections. These TCRs (3 TCRs for HBV and 4 TCRs for HCV) were cloned into retroviral vectors and re-expressed in blood lymphocytes of chimpanzees with chronic HBV and HCV infection. The transduced T gained a broad spectrum of effector functions (secretion of IFN-g, cytotoxicity and proliferation), that were specific to the cognate antigens of the re-expressed HBV and HCV-specific TCRs. The TCR-transduced T cells were expanded to high numbers with anti-CD3 plus IL-21/IL-15 stimulation, which resulted in a preservation of their differentiation status and a lower frequency of immunosuppressive regulatory T cells than conventional stimulation with anti-CD3 plus IL-2. Collectively, our results demonstrate that the combination of retroviral TCR gene transfer together with IL-21/IL-15 stimulation can efficiently redirect the antigen specificity of resting primary T cells and generate a large number of functional effector T cells. These expanded effector T cells will be used in adoptive immunotherapy protocols to restore HBV- and HCV-specific immune responses of chimpanzees with chronic HBV- and HCV infection.

Immunology - Infectious Disease

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Memory T cells Convert Brain Resident Myeloid Cells Into Antigen Presenting Cells During Clearance of a Persistent Viral Infection

Persistent viral infections are often very difficult to treat in humans. Adoptive immunotherapy is an approach that involves administration of anti-viral T cells and has shown some promise in the clinic. Our laboratory models adoptive immunotherapy by transferring anti-viral memory T cells into mice persistently infected from birth with lymphocytic choriomeningitis virus (LCMV). Here, we demonstrate that memory T cells can completely purge the brain of persistently infected mice without causing immunopathology or blood brain barrier breakdown. Memory T cells accomplish this by inducing a tailored release of chemoattractants that recruit adaptive immune cells, but few pathogenic innate immune cells (e.g. neutrophils and inflammatory monocytes) into the nervous system. Memory T cells also enlist the support of nearly all brain resident myeloid cells (referred to as microglia) by converting them into CD11c-expressing antigen-presenting cells (APCs). Interestingly, we observed morphologically similar CD11c+ microglia in the brain of a virally infected human, suggesting that conversion of microglia into APCs is conserved across species. Following conversion, CD11c+ microglia release CCL5 and promote interactions with therapeutic memory T cells in the brain parenchyma. Two photon imaging

studies revealed that anti-viral CD8 T cells are more likely than CD4 T cells to decelerate and form stable interactions with brain-resident APCs. These APCs are protected from effector T cell induced damage, as they do not undergo cell death following T cell engagement. We propose that successful non-cytopathic viral clearance from the brain by therapeutic memory T cells results from tailored chemokine production and conversion of resident myeloid cells into CD11c+ APCs that can then be engaged locally by anti-viral T cells.

Immunology - Innate and Cell-mediated Host Defenses

IIDA, NORIHO
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Gut commensal bacteria promote anti-tumor innate immune responses in distant tumors after immunotherapy and chemotherapy

Immune system has evolved to fight pathogenic microbes. However, 100 trillion of microbes inhabit in hostâ€™s gut, skin and urogenital organs without being eliminated and are considered as commensal bacteria. Recent evidences indicated that gut commensal flora give us a benefit by influencing innate and adaptive immune responses. For example, mice treated with oral antibiotics show impaired immune response against respiratory influenza virus and the subsequent increase in the viral load. Thus, commensal flora especially in gut has important roles in development of systemic host immunity. Tumor tissues have unique inflammatory environment in themselves, and targeting tumors with anti-tumor immune cells is a promising strategy to conquer the cancers. The evidences that gut bacteria positively affect immunity against pathogens prompt us to examine the role of the flora in anti-tumor immunity. This study aimed to determine the effect of gut flora on tumor-associated immune responses after immunotherapy and immunogenic chemotherapy. To deplete gut commensal flora, C57BL/6 mice were administered an antibiotic cocktail including neomycin, vancomycin and imipenem in the drinking water, resulting in a 3-log reduction in fecal bacteria. Because CpG oligonucleotide stimulates innate immune cells to produce inflammatory molecules, intratumoral injection of CpG causes destruction of tumors. In mice bearing subcutaneous MCA38 colon carcinoma or B16 melanoma, the antibiotic treatment suppressed the CpG treatment-induced tumor necrosis. TNF is a key effector molecule for tumor destruction in CpG-based therapy. Antibiotic-treated mice showed reduced frequency of tumor-infiltrating TNF-producing myeloid cells after the therapy. Germ-free animals also showed the impaired TNF production. Administration of various single antibiotics revealed that tumor TNF production correlated with the number of gut bacteria. Oral administration of bacterial componet lipopolysaccharide (LPS) restored TNF production in antibiotics-treated mice, so LPS is one of the mediators between gut bacteria and the treatment-induced inflammation in the tumors. Finally, the cytotoxic oxaliplatin-induced immunogenic death of tumor was also impaired in antibiotic-treated mice in Myd88, adaptor molecule sensing bacterial products, -dependent manner. Altogether, these results demonstrate that optimal immunotherapeutic and chemotherapeutic responses against cancer require an intact commensal flora.

Immunology - Innate and Cell-mediated Host Defenses

Zhang, Yan
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NCI-CCR

Induction of Autophagy is Essential for Monocyte-Macrophage Differentiation

Macrophages are chief participants in host inflammatory responses. Deregulation of macrophage differentiation and function may lead to diseases including autoimmune disease and cancer. The molecular processes that occur during monocyte-macrophage differentiation, particularly in the human system, are not completely understood. In the absence of differentiation, circulating monocytes are programmed to undergo apoptotic cell death. Stimuli that promote monocyte-macrophage differentiation not only cause cellular changes but also prevent the default apoptosis of monocytes. Autophagy is a ubiquitous process believed to occur in all eukaryotic cells which regulates both cell survival and cell death. There is some evidence that autophagy is an important event for differentiation in many kinds of cells, however, there have been no reports on the role of autophagy during monocyte-macrophage differentiation. In this study, we demonstrate that autophagy is induced when monocytes are triggered to differentiate and that the induction of autophagy is pivotal for the survival and differentiation of monocytes. We find that autophagy and its protection from cell death occur irrespective of whether monocytes are differentiated with GM-CSF or M-CSF. Both GM-CSF and M-CSF induce a punctate LC3 pattern and the characteristic autophagic vacuoles surrounded by double membranes with partially degraded intracellular material inside in human primary monocyte. Inhibition of autophagy by autophagy inhibitors (3-methyladenine and chloroquine) or knocking-down autophagy genes (Beclin1 and Atg5) results in the apoptosis of cells that are engaged in differentiation. We also made similar findings in mouse bone marrow monocytes and when triggering monocyte-macrophage differentiation by treating mice with thioglycolate in vivo, suggesting that our discovery is a general phenomenon during monocyte-macrophage differentiation. Mechanism study show that the differentiation signal releases Beclin1 from Bcl-2 through activating JNK and blocks Atg5 cleavage, both events are critical to the induction of autophagy. Importantly, preventing autophagy induction hampers differentiation and cytokine production. Thus, autophagy is an important transition from monocyte apoptosis to differentiation. Our findings support a novel function of autophagy in monocytes-macrophages differentiation, which will be helpful in understanding the role of autophagy in infectious, autoimmune, and inflammatory diseases.

Immunology - Innate and Cell-mediated Host Defenses

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A Proteomics Approach to Study Signaling by Beta2-Integrin LFA-1

Natural killer (NK) cells provide a unique opportunity to study integrin signaling in the absence of inside-out signals. In contrast to T cells where inside-out signaling is required to activate beta2 integrin LFA-1, LFA-1 on primary NK cells binds directly to ICAM-1. LFA-1 binding to ICAM-1 is sufficient to induce granule polarization in NK cells, but not degranulation. Conversely, the binding of Fc receptor CD16 to IgG1 results in unpolarized degranulation. Surprisingly, given these different functional outcomes, LFA-1 and CD16 induce similar signals, such as phosphorylation of Syk and phospholipase C (PLC)-gamma. We took a proteomics approach to examine signaling by LFA-1 and determine how it signals for granule polarization. Primary human NK cells were stimulated on plates coated with ICAM-1 or IgG1. Tyrosine-phosphorylated proteins and their associated proteins were pulled down with a mAb to phospho-tyrosine, and eluted with phenyl-phosphate. Proteins in the total eluate were identified by mass spectrometry. Proteins that were enriched after stimulation by LFA-1 but not CD16 were validated by immunoblotting and their importance in signaling for granule polarization was tested by siRNA-mediated silencing. Integrin-linked kinase (ILK), gamma-parvin and RhoGEF7, all known to be involved in establishing polarity during cell migration, were critical for LFA-1-dependent granule polarization. Downstream signals for polarity in migrating cells require Par6 and phosphorylation of kinase GSK3beta

at Ser9, which relieves inhibition of the microtubule-stabilizing molecule APC. Par6 and APC silencing blocked granule polarization. Binding of NK cells to ICAM-1 alone induced an ILK-dependent GSK3beta Ser9 phosphorylation. Our work reveals that granule polarization induced by LFA-1 in NK cells uses a signaling pathway similar to that used to establish polarity during cell migration.

Immunology - Innate and Cell-mediated Host Defenses
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An unexpected role for p53 in NF-kappaB-mediated inflammatory responses
The transcription factors p53 and NF-kappaB are key mediators of cellular stress such as genotoxic stress. While NF-kappaB is important in inflammation, little is known about the role of p53 in this process. Here, we show that p53 activation in human primary macrophages by the genotoxicants 5-Fluorouracil (5-FU) and Doxorubicin (DXR) or by the p53 stabilizer nutlin-3 leads to expression of several pro-inflammatory genes. This effect is specific to human primary macrophages. Although some pro-inflammatory genes are induced to levels typical of p53 targets such as p21, there is much greater induction of IL-6. IL-6 mRNA is induced to high levels within 1 hr after treatment, followed by accumulation of secreted protein. ATM, an upstream regulator of both p53 and NF-kappaB in the DNA Damage Response (DDR), as well as p53 and NF-kappaB are required for IL-6 induction based on responses to their respective inhibitors KU55933, Pifithrin-alpha and Bay-11-7082. While DXR and 5-FU are known to activate NF-kappaB, we show that NF-kappaB is also activated by nutlin-3, presumably via the DDR since nutlin-3 induces gamma-H2AX foci. In response to nutlin-3, IkappaBalpha is phosphorylated and degraded, and the NF-kappaB subunit p65 is transported to the nucleus. Additionally, we show that nutlin-3 induces p65 phosphorylation at S276, which is required for IL-6 induction. Thus, p53 and NF-kappaB can cooperate to induce IL-6 in primary human macrophages in an ATM-dependent manner. Interestingly, the kinetics and magnitude of IL-6 expression by nutlin-3 mirrors that of TLR-dependent IL-6 expression suggesting an equally important biological response. Microarray analyses of RNA from primary macrophages demonstrate limited similarity in gene expression after brief treatments with nutlin-3 and LPS (2 hr), and most genes that are highly induced by both treatments are pro-inflammatory. Contrary to commonly held views, these results show that the relationship between p53 and NF-kappaB may not always be inhibitory and shed new light on the function of p53 in innate immunity. The positive feedback between p53, NF-kappaB and rapid cytokine production may assure innate immune protection under conditions of genotoxic stress. These findings also have important health implications since many environmental agents can induce p53 and NF-kappaB and a derivative of nutlin-3 along with 5-FU and Doxorubicin are currently used or being tested as chemotherapeutic agents.

Immunology - Innate and Cell-mediated Host Defenses
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NINDS
Type I Interferon Deficiency Completely Abolishes the Innate Immune Sentinel Response to a Persistent Viral Infection
Viral infections of central nervous system (CNS) often trigger an inflammatory response that gives rise to a wide range of pathological outcomes. The CNS is equipped with an elaborate network of innate immune sentinels (e.g. microglia, macrophages, dendritic cells) that routinely serve as first responders

to these infections. To gain novel insights into how the brain responds to a virus as it establishes persistence, we utilized genomic and two-photon imaging approaches to study a pure innate immune response to a lymphocytic choriomeningitis virus (LCMV) infection. LCMV is a natural pathogen mouse and humans that can invade both the CNS and peripheral tissues. LCMV is non-cytopathic so viral pathogenesis following infection are mediated by the innate and adaptive immune responses. Intracerebral inoculation of mice with LCMV Armstrong results in severe meningitis and convulsive seizures due to influx of cytotoxic lymphocytes (CTL) and myelomonocytic cells into the CNS. In the absence of virus-specific CTL, mice become asymptomatic, lifelong carriers of the virus; thus provide an interesting model to study how brain resident cells innately sense and respond to an RNA virus as it establishes persistency. We generated CX3CR1-GFP mice lacking LCMV-specific T cells and recorded myeloid cell dynamics in the brains of living mice by two-photon imaging. Our data revealed that CNS myeloid sentinels responded significantly to acute infection by sequestering DsRed-labeled virus. Microglia (brain resident macrophages) but not peripheral myeloid cells proliferated following acute infection and responded directly to viral antigen by reducing process complexity, suggesting a localized response to the presence of virus. At the genomic level, acute infection triggered massive changes in CNS gene expression that were attenuated over time (likely due to inhibition by viral nucleoprotein) and were all directly linked to type I interferon (IFN-I) signaling. Surprisingly, in the absence of IFN-I signaling, no genes were differentially expressed in the nervous system despite the presence of persistent virus. In addition, two-photon imaging studies revealed that IFN-I receptor deficient innate immune sentinels were completely unresponsive to the establishment of a persistent viral infection. These data demonstrate that IFN-I drives all innate immune activity in the brain following LCMV infection, and a deficiency in IFN-I signaling renders LCMV invisible to its murine host.

Immunology - Lymphocyte Development and Activation

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Enhanced T cell activation in lymphocytes from transgenic mice expressing LAT molecules resistant to ubiquitination

Linker for activation of T cells (LAT) is critical for the propagation of T cell signals from the plasma membrane to the cytoplasm upon T cell receptor (TCR) activation. Previous studies demonstrated that substitution of LAT lysines with arginines (2KRLAT) resulted in a decrease in LAT ubiquitination and elevated T cell signaling, indicating that LAT ubiquitination is a molecular checkpoint for attenuation of T cell signaling. To investigate the role of LAT ubiquitination in vivo, we have generated transgenic mice expressing WT and ubiquitin-defective 2KRLAT. Upon TCR stimulation of T cells from these mice, proximal signaling was elevated in lymphocytes expressing 2KRLAT versus WT as evidenced by higher levels of phospho(p)-tyrosine proteins, p-LAT, p-phospholipase C-gamma and cytosolic calcium flux. Evaluation of various cell surface markers also revealed an enhanced memory T cell profile in 2KR lymphocytes. Moreover, upon TCR stimulation, a higher percentage of T cells expressing greater levels of effector molecules such as intracellular interferon (IFN)-gamma, tumor necrosis factor (TNF)-alpha and interleukin-2 was found in 2KR versus WTLAT mice. A highly cytotoxic T cell-polarized immune response was observed in the mesenteric lymph nodes of 2KRLAT mice orally infected with a low dose of Toxoplasma gondii, as demonstrated by higher frequency of activated/proliferating CD8(+) population producing T-bet, IFN-gamma and TNF-alpha. We have thus identified enhanced proximal T cell signaling, an enhanced memory T cell profile and a higher expression of cytokine effector molecules, both in vivo and in vitro, in lymphocytes from 2KRLAT animals. These results suggest that 2KRLAT transgenic mice

may respond more efficiently to pathogenic challenges, a hypothesis that will be tested with additional experimental models of mouse infection and carcinogenesis.

Immunology - Lymphocyte Development and Activation

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Glycolytic flux regulates memory CD8+ T cell Differentiation

The molecular mechanisms regulating whether CD8+ T cells are programmed to die or persist as long-lived memory T cells remain active area of intense investigation but there is very little data characterizing the metabolic changes that might ultimately influence these T cell fate decisions. The mammalian target of rapamycin (mTOR) is a nutrient-sensitive intracellular kinase that regulates cell growth and metabolism that has emerged as a key regulator of CD8+ T cell memory formation. A recent report by Pearce et al. makes a claim that inhibition of mTOR mediates its profound effect on CD8+ memory formation by enhancing fatty acid oxidation (FAO) and the notion that rapamycin potentiates FAO has entered into the immunologic canon. Through a global metabolomic analysis, we found that the mTOR inhibitor rapamycin profoundly suppressed glycolytic metabolism in activated CD8+ T cells but did not significantly affect medium chain fatty acid, long chain fatty acid or essential fatty acid levels as previously proposed. Consistently, although fatty acid oxidation constituted the bulk of resting mitochondrial respiration in CD8+ T cells, this pathway was not altered by mTOR blockade. Conversely, rapamycin treated T cells exhibited decreased glucose uptake and lactate production confirming that mTOR regulates glycolysis in CD8+ T cells. Using NBDG, a dye that measures the glucose uptake in CD8+ T cells, we define a new method to distinguish and isolate memory precursors and short-lived effector cells based on intrinsic cellular metabolism rather than surface markers. To directly target the glycolytic metabolic pathway in T cells, we used 2-DG (2-deoxyglucose), a glucose analog that inhibits hexokinase, a rate limiting enzyme in the glycolytic pathway. Treatment with the glycolytic inhibitor, 2-DG recapitulated the phenotype, gene expression and function of rapamycin-treated T cells. Furthermore, 2-DG treatment significantly augmented the in vivo anti-tumor activity of pmel-1 T cells resulting in increased tumor regression and prolonged survival compared to mice receiving T cells grown in the vehicle control. Our data represents the first report that a single agent directly targeting a specific metabolic pathway (2-DG) is sufficient to generate long-lived CD8+ T cell memory. We describe a paradigm shift of how glucose metabolism can be manipulated to enhance vaccines and T cell-based immunotherapies for the prevention and treatment of infectious disease and cancer.

Immunology - Lymphocyte Development and Activation

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NIAID

Persistent exposure to endogenous LPS programs gut dendritic cells to impair CD4+ Th17 immunity in vivo

Lipopolysacchraide (LPS), a cell wall component of gram-negative bacteria, drives adaptive CD4+ helper type-17 (Th17) immunity via the activation of Toll-like receptor 4 (TLR4). However, excessive TLR4 stimulation can lead to a period of â€œLPS tolerance,â€ during which immune cells become non-responsive to LPS. Acyloxyacyl hydrolase (AOAH) is a mammalian enzyme that deacylates the lipid A moiety of LPS and inactivates LPS in vivo, thereby preventing LPS non-responsiveness. Although it has been described that AOAH deficiency leads to impaired innate immunity, the effect of AOAH function on

the generation of T cell responses remains unexplored. We hypothesized that AOAH function, by influencing LPS exposure on dendritic cells (DCs), alters CD4+ T cell polarization. To test this hypothesis, we used flow cytometric and bioplex analysis to compare CD4+ T cell polarization in age-matched littermate mice deficient (-/-) and sufficient (+/+) in AOAH expression. Polyclonal and antigen (Ag)-specific stimulation of CD4+ T cells isolated from the gut mucosa showed that AOAH-/- mice had diminished Th17 immunity. The absence of TLR4, or the depletion of gram-negative flora with in utero and post-partum antibiotic treatment, reversed the decreased mucosal Th17 responses observed in AOAH-/- mice. This suggests that the skewed T cell responses in AOAH-/- mice depend on TLR4 signaling by endogenous microflora. Since CD4+ T cells do not express AOAH, we investigated whether AOAH expression in DCs modulates the altered Th17 phenotype in AOAH-/- mice. qRT-PCR analysis demonstrated that gut DCs from the colonic lamina propria (cLP) express elevated levels of AOAH. Interestingly, AOAH expression was selective to a cLP aldehyde dehydrogenase (ALDH)-CD103+CD11b+ DC subset. AOAH deficiency significantly reduced the ability of this DC subset to polarize Ag-specific Th17 cells ex vivo, but enhanced their ability to generate Ag-specific CD4+ T regulatory cells. These data demonstrate that AOAH deficiency, which increases exposure to LPS, conditions cLP DCs to impair Ag-specific Th17 immunity. Furthermore, these results show that host pathways to handle microflora-derived products can influence T cell polarization, and suggest that these pathways may be involved in susceptibility to infectious and immune-mediated diseases.

Immunology - Lymphocyte Development and Activation

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NIAID

A New Robust Method for Measuring Protein Translation Rates in Individual Cells Ex Vivo Reveals Complex Regulation of Protein Synthesis in Naïve and Viral Antigen Activated Lymphocytes

Protein translation is an essential and intricately regulated cellular process. Currently, measuring protein translation in animals relies on laborious methods that utilize radioactive amino acids. We recently described the RiboPuromyclation Method (RPM) to measure protein translation in cultured cells. RPM is based on ribosome catalyzed addition of puromycin to nascent chains followed by detection of ribosome-bound nascent chains with a monoclonal antibody specific for puromycin in fixed and permeabilized cells. To apply RPM in vivo, we administered puromycin intravenously and measured puromycin staining ex vivo via flow cytometry in various immune cell subsets. We were able to detect a robust signal that was blocked by ribosome targeting antibiotics that prevent nascent chain puromycylation. Seven days after infection with vaccinia virus (VACV), we find profound increases in protein translation in activated T (CD62L-, CD44+, and CD25+) and B cells (CD38-). In mice with adoptively transferred OT-1 T cells we find that translation of individual cells peaks at 3 days after infection, i.e. several days before T cell number peak. Intriguingly, resting (CD44-) host T cells decrease protein translation relative to baseline levels at times when OT-1 T cells are translating at twice their baseline levels. The RPM method also uncovered a complicated pattern of translational regulation in lymphocytes adoptively transferred into congenic recipients. Taken together, these findings illustrate the ability of in vivo RPM to provide novel information regarding the participation of translational regulation in workings of the immune system.

Immunology - Lymphocyte Development and Activation

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NIAID-VRC

Priming protective CD8 T cell immunity by replication deficient Adenoviral vectors inversely correlates with innate responses but correlates with expression of antigen.

CD8 T cell responses play an important role in eliminating infected cells and controlling pathogen load during infections such as human immunodeficiency virus (HIV) and malaria. The replication defective Adenoviral vector (rAd) based on serotype 5 (rAd5) is a potent vaccine for inducing CD8 T cell responses and has been used in clinical trials. Unfortunately, prior immunity to Ad5 due to natural infection can reduce the potency of rAd5-vectored vaccination. Therefore, we need an rAd vector of comparable potency to rAd5 but with low sero-prevalence in human target populations. These include vectors based on low sero-prevalent human serotypes (rAd28 and rAd35) but also vectors based on adenoviruses isolated from closely related species such as apes (chAd3 and chAd63). We performed comprehensive analyses of the adaptive response using a dose titration of these vectors; including tracking magnitude, quality and phenotype of vaccine-induced CD8 T cell responses and challenge with a Listeria monocytogenes-based infection model. These criteria revealed a hierarchy across the five vectors for protective CD8 T cell responses. rAd5 and chAd3 induced robust CD8 T cell responses that were protective at all doses, rAd28 and chAd63 induced lower level CD8 T cell responses that were partially protective and rAd35 rapidly lost both detectable CD8 T cell responses and protection. We then assessed factors such as innate immunity and antigen expression that might give rise to this hierarchy. Microarray analysis of innate responses in the draining lymph node and analysis of serum cytokines showed that rAd5 and chAd3 induced lower type I and II IFN responses and lower expression of apoptosis-related genes, such as TRAIL, Fas and caspases 1,3 and 7. Additionally, rAd5 and chAd3 infected more than 10% of DCs and distributed to all DC subsets, with the highest expression of antigen at both the transcriptional and protein level for at least 14 days. These data show that rAd5 and chAd3 vectors share the ability to express large amounts of target antigen for a prolonged period of time but do not activate a robust innate response. This suggests that potency of Ad vectors is linked to lower innate responses, which may result in less apoptosis of Ad-infected cells and increased antigen expression to facilitate robust adaptive responses. In addition, it illustrates that chAd3 is an appropriate vector to substitute for rAd5 in vaccines that aim to prime robust CD8 T cell immunity.

Immunology - Lymphocyte Development and Activation

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Novel sources of Interleukin-9 in lung inflammation

Interleukin-9 (IL-9) is a pleiotropic cytokine that is expressed at elevated levels in the lungs of asthmatic patients; blocking antibodies to IL-9 are currently in clinical trials as potential therapies for atopic disease. Similarly, specific over-expression of IL-9 in lungs results in the induction of an asthma-like phenotype and blockage of IL-9 signalling reduces airway inflammation in mouse models. An important function attributed to IL-9 in lung physiology is the induction of mucus production, goblet cell hyperplasia and other features of airway remodelling, functions that were also ascribed to IL-13 and IL-5. T cells are believed to represent the main source of IL-9 in the context of airway inflammation. However, reliable data identifying the dominant cellular source of IL-9 in vivo is still missing and it is not clear how IL-9 mediates its function in the context of airway inflammation. Our aim was first, to identify the dominant sources of IL-9, and second, to reveal the target cell type for IL-9 provoked function in airway inflammation. To achieve the first goal, we generated an IL-9 specific reporter mouse strain, enabling the permanent labelling of cells, which have expressed the IL-9 gene. Strikingly, analysis of cells expressing the reporter gene revealed that a subset of innate but not T cells was the main source of IL-9,

but also IL-5 and IL-13 in a model of papain-induced lung inflammation. Such cells were only recently discovered to arise during helminth infections and were subsequently termed innate lymphoid cells (ILC). Timepoint analysis by intracellular cytokine staining showed that IL-9 production in ILC was transient and dependent on IL-2 produced from adaptive immune cells. Intranasal application of IL-33 induced ILC poised for high IL-9 production. Although IL-9 expression waned quickly, ILC continued to produce IL-13 and IL-5. Recent data suggested high IL-9 receptor expression on ILC rendering them a possible target for autocrine IL-9 regulation. Indeed, IL-9 stimulation of ILC enhanced their IL-5 and IL-13 expression, while blockade of IL-9 via neutralizing antibodies substantially reduced IL-13 and IL-5 in the lungs of papain challenged mice. In conclusion we identified ILC as a previously unrecognized source of IL-9 induced via IL-2 from adaptive immune cells. Since many features of IL-9 mediated airway inflammation depend on the expression of IL-5 and IL-13 our data suggests ILC as the key mediator of such responses.

Informatics/Computational Biology
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Phosphorylation Site Assignment for Mass Spectrometry Data
Protein phosphorylation is one of the most studied post-translational modifications due to its critical role in cellular regulation, protein function and physiological responses to stimuli. Accurately determining which specific amino acids in a protein get phosphorylated is an important goal for large-scale phosphoproteomic studies. These studies often rely on mass spectrometry (LC-MS/MS) to pinpoint exact sites of modification and quantify the extent of phosphorylation. Systematic errors in phosphorylation site assignment could undermine these studies as well as the biological conclusions that arise from them. Traditionally, identifying the correct phosphorylation site(s) within a phosphopeptide has been done by manual validation of LC-MS/MS spectra. However, with the advent of high throughput mass spectrometers, the number of spectra generated is vast and manual site assignment is no longer practical. To automate this process we designed, implemented and tested a novel dynamic programming strategy for phosphorylation site assignment, called PhosSA. The algorithm maximizes an objective function based on the sum of the peak intensities that match to the theoretical peptide spectrum and is differentially optimized for widely used LC-MS/MS fragmentation strategies (both CID and HCD). A quality post-processing is then introduced that exploits the specific characteristics of the mass spectrometry data to stratify the assigned sites based on the scoring function and associated parameters. The algorithm is able to correctly assign phosphorylation sites with > 99% accuracy with > 90% sensitivity for experimentally generated data sets of peptides with known phosphorylation sites. PhosSA outperformed other leading tools, such as Ascore and Phosphoscore, and has the added capability of accepting iTRAQ and SILAC datasets. Dynamic programming allows us to devise a linear time and space strategy making PhosSA a highly efficient algorithm that can accomplish millions of site assignments for large data sets within minutes.

Informatics/Computational Biology
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Genome-wide Regulatory Roles of Non-B DNA Structure in Escherichia coli

Usually DNA exists as standard right-handed B form, which is double helix with Watson-Crick base pairing. But several other non-B DNA structures can also be formed and sequence signatures compatible with formation of such alternative structures are abundant in all species. Non-B DNA structures include tetraplexes (G-quadruplex), left-handed Z-DNA (Z-DNA), stress-induced DNA duplex destabilization (SIDD), cruciform (Cruciform), triplex (H-DNA) and slipped structure (S-DNA). Most of those non-B DNA structures are postulated to be formed in part during transcription or protein binding, and play important roles in regulation. Otherwise, the non-B DNA structures are believed to be involved in genetic instability and thus strongly associated with human diseases. Currently, with few notable exceptions, most evidences regarding the roles of non-B DNA structures are computational and indirect. Such analyses are mainly in eukaryote, and typically look for enrichment of characteristic sequence signatures in regulatory regions based on individual genes. In prokaryote, functionally related genes are grouped as operon and transcribed together, which gives us a unique opportunity to test the hypothesis about regulatory roles of non-B DNA structures: if they paly a role in regulation, the distribution of such structures should be different in regulatory regions of the operon as compared to the middle genes of the operon. In this study, for the first time, we sought to systematically investigate the regulatory roles of non-B DNA structures in E. coli based on operon. We found that there is indeed a significant enrichment (SIDD, Cruciform, H-DNA and S-DNA) or avoidance (G-quadruplex and Z-DNA) of non-B DNA structures in the promoter region of operon compared to the upstream region of middle genes. Additionally, we found that, after correction for Rho-independent terminator, cruciform is still highly enriched in downstream of operon compared to middle genes. Also, non-B DNA structures are enriched in intergenic regions separating either divergently oriented operons (SIDD, H-DNA and S-DNA) or convergent oriented operons (Cruciform). Above all, all the results are consistent with the hypothesis that non-B DNA structures play important regulatory roles in the activity of operon. The enrichment of cruciform downstream even after correction for standard Rho-independent terminator indicates that role of cruciform in regulation of termination has been underappreciated in E. coli.

Informatics/Computational Biology

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Allele specific compatibility of interactions underlying yeast DNA repair phenotype

Recent studies suggest that many phenotypes are complex and are likely governed by interactions between multiple loci. When such interactions are disrupted, the phenotype is changed as well. For example, it is often observed that after crossing two parental strains their common phenotype is lost in some progenies. In this case, the genotype of a single locus, defined by which parent it is inherited from, cannot explain the phenotype of both parents and progenies but interacting loci could. To uncover such interacting loci underlying a phenotype, we propose a model and associated computational approach. Our model assumes that interacting loci co-evolve to acquire allele specific compatibility to maintain interaction. For example, if a pair of interacting loci has the same genotype (inherited from the same parent), they are compatible to interact and hence the phenotype is the same with parental strains. But when their genotype is different, their interaction might be compromised, causing phenotype change. We have developed a novel computational method, LoCAp (Locus Compatibility Approach) to efficiently detect pairs of loci whose genotypes fit for the above patterns without considering all possible locus pairs. In the method, 1) we comprehensively represent genotype of loci, progeny strains and their phenotype in a graph. 2) We use a set of graph structure related parameters to measure how well a locus pair fits a certain pattern. 3) We develop an efficient algorithm to search for locus pairs with parameters exceeding threshold from the graph. 4) Rigorous statistical test is performed to evaluate the

p-value of findings. We applied our approach to the data of drug resistance to DNA damaging agents of 123 haploid meiotic progeny of BY/RM diploid. For each of 4-NQO (4-Nitroquinoline 1-oxide), bleomycin and caffeine, we were able to detect 4 interacting locus pairs out of millions of pairs (p < 0.05). We then carefully scan areas close to those loci to locate potential causal genes. For 4-NQO, we identified Rad5 as a potential hub for genetic interaction, which was verified by an independent experiment. For bleomycin, we identified Rad28 as a potential hub. In addition, we found that DNA damage checkpoint genes Mec1 and Mec3, multidrug transporter genes Qdr3 and Pdr5, are among potential causal genes. These findings serve as a proof of principle for exploring allele specific compatibility in predicting interactions between genetic loci.

Informatics/Computational Biology
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A comprehensive census of horizontal gene transfers from prokaryotes to unikonts
Horizontal gene transfer (HGT) is a dominant factor in the evolution of prokaryotes (bacteria and archaea). However, in eukaryotes, the impact of HGT is generally assumed to be much lower but has not been thoroughly analyzed, with the exception of the massive HGT from the endosymbionts. Here we report the most comprehensive census of likely HGT events in different groups of unikonts and an in depth analysis of a conservatively defined minimal set of transfer events. We analyzed complete proteomes from 36 species of unikonts (2 Amoebozoa, 18 Fungi, 13 Metazoa and 1 Choanoflagellida). These proteomes were manually selected to widely represent the unikont supergroup. Candidate horizontally transferred genes were initially identified by analyzing each proteome using the DarkHorse method. This method identifies sequences that are compositionally different from the genome average, i.e. potentially acquired genes. Several tests were performed to rule out contamination of eukaryotic genome sequences with prokaryotic ones including analysis of the genomic neighbors and exon-intron structures of each candidate gene. Subsequently these candidates were analyzed in detail using taxonomic breakdown of database search results and maximum likelihood (ML) phylogenetic analysis followed by cutting-edge statistical tests on tree topology. These tests include an analysis of similarity between candidates and prokaryotes (nodal and patristic) and the AU-test (the current most stringent method for testing phylogenetic hypotheses). Overall, we detected 1768 highly probable HGT events from prokaryotes to unikonts. Approximately 90% of the detected transfers were from bacteria and only 2% from archaea. The transfer events are non-uniformly distributed in the evolution of unikonts, e.g.: 196/197 HGT detected in Amoebozoa occurred after the divergence of Archamoeabe and Mycetozoa. There are also many more apparent HGT events in Fungi (1030) than in Metazoa (350). Moreover, the distributions of the probable bacterial donors of transferred genes were substantially different for different unikont taxa. Examination of the known and predicted functions of the acquired genes from prokaryotes reveals a clear preponderance of genes encoding various enzymes. In conclusion, although HGT is not as pervasive in eukaryotes as it is in prokaryotes, the amount of HGT detected in this study implies that acquisition of genes from bacteria played a major role in the evolution of the unikonts.

Informatics/Computational Biology
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A Machine Learning Approach for Identifying Novel Cell Type-Specific Transcriptional Regulators

Elucidating the structure-function relationship of transcriptional enhancers is essential to understand how complex spatiotemporal genetic programs are established. We combined a machine learning method with comparative genomics to develop a DNA-based classifier that distinguishes enhancers from genomic background, and applied our approach to muscle development in Drosophila. We first assembled a set of enhancers active in Drosophila melanogaster muscle founder cells (FCs) and other mesodermal cell types. Because this set comprised only 16 sequences, we then devised a phylogenetic profiling approach to extract diverged orthologs (50-80% sequence identity) in 11 other Drosophila species for each D. melanogaster FC enhancer. Transgenic reporter assays confirmed that, despite extensive evolutionary reshuffling of critical transcription factor binding sites (TFBSs), the orthologs directed expression in patterns comparable to those of their D. melanogaster counterparts. We also showed that including the orthologs in the analysis increased our statistical power to detect subtle patterns and associations in the original data. Finally, we used the set of FC enhancers and their orthologs to train a SVM classifier able to distinguish FC enhancers from genomic background based on the presence of TFBSs. We assessed the performance of the classifier in a cross-validation setup, obtaining an area under the receiver operating characteristic curve of 0.89. We used the classifier to achieve two main aims. First, we created a genome-wide map of 5,500 putative FC enhancers. Predicted FC enhancers were 4-fold over-represented in proximity to known FC genes. Moreover, in situ hybridization showed that many genes with unknown functions flanking FC enhancer predictions are indeed expressed in FCs (13-fold enrichment, P-value=0.0002). Transgenic reporter assays validated 75% of our enhancer predictions. Second, we examined the TFBSs exhibiting the greatest power in discriminating FC enhancers from genomic background, confirming that many of the associated transcription factors are involved in muscle development, and identifying POU homeodomain, Myb, Ets, Forkhead, and T-box motifs as critical for FC transcription, a role subsequently validated through reporter and mutagenesis assays. In summary, we designed a readily generalizable strategy to effectively unravel the rules governing particular genetic programs starting with an extremely small number of enhancer sequences.

Intracellular Trafficking

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The Tubby Protein Is Essential for Transport of a Subset of G-Protein Coupled Receptors to the Neuronal Cillia

The primary cilia are increasingly being recognized as a vital signaling organelle in diverse tissues and cell types, having important roles in development and homeostasis. In the adult CNS, at least two 7-pass transmembrane G-protein coupled receptors (GPCR), somatostatin receptor 3 (Ssrt3) and melanin concentrating hormone receptor 1 (Mchr1), are localized to neuronal primary cilia where they sense peptide hormones in the environment and connect the extracellular cues to the cAMP signaling cascade which mediates downstream cellular responses. Disruption of this signaling cascade leads to cognitive deficits and altered feeding behavior. The tubby protein is the founding member of the tubby-like family of proteins, which includes TULP1, 2, and 3 (for Tubby-like protein 1, 2 and 3). Their functions are not fully understood, but two have been linked to neurosensory disorders. The naturally occurring tubby mutation in mice causes retinitis pigmentosa, hearing loss and obesity. Tubby proteins have been proposed to function either as nuclear transcription factors or as interacting partners for IFT particles important for transciliary trafficking. We show here that the tubby protein is essential for the cilliary trafficking of a subset of GPCRs. In the retina, lack of tubby protein is associated with partial failure of rhodopsin and cone opsins (also GPCRs) to be transported to the photoreceptor outer segments,

manifesting as shortened outer segments, mislocalization of rhodopsin and cone opsins in the cell bodies, and photoreceptor degeneration. In the brain, lack of tubby specifically abolished the ciliary localization of Sstr3 and Mchr1. In contrast, loss of tubby function does not appear to affect ciliogenesis or maintenance, as evidenced by the normal length of neuronal cilia, nor does it affect trafficking of adenylate cylcase III, a non-GPCR transmembrane protein, to the cilia. These defects are remarkably similar to those found in certain Bardet-Biedle syndrome (BBS) mutants. Our data provide novel insights into the mechanism of tubby protein functions and explain the tubby mouse phenotype. We propose a model in which the tubby proteins function in the trafficking of GPCR proteins in different tissues and at different developmental stages, by serving as linker proteins between select cargoes and IFT particles.

Intracellular Trafficking
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A conserved ER-membrane protein complex facilitates phospholipid exchange between the ER and mitochondria.
Mitochondria are critical cellular components that are needed for energy production, lipid metabolism, and apoptosis. Mitochondrial membrane biogenesis requires the import of proteins and lipids. Most lipids are synthesized in the endoplasmic reticulum (ER) and transferred to different cellular compartments by vesicular trafficking. Interestingly lipid trafficking from the ER to mitochondria has been found to be non-vesicular as it is energy independent and doesnâ€™t require any cytosolic protein. It has long been thought that lipids are exchanged between the ER and mitochondria at regions of close contact between the membranes of these organelles. However, the mechanism of transport and the proteins responsible for tethering the ER and mitochondria has not been determined. Here, we devised a novel genetic screen in S. cerevisiae to identify proteins needed for lipid exchange between the ER and mitochondria. Among the proteins we found were all six members of an evolutionarily conserved complex named ER-membrane protein complex (EMC). Knockout of multiple EMC proteins resulted in a massive reduction in the transport of phsophatidylserine (PS) from the ER to mitochondria. The EMC-mutants showed severe growth retardation and loss of mitochondrial function, as they were unable to grow in nonfermentable carbon source. Cells lacking EMC proteins and a protein that is a member of a recently described complex that tethers ER and mitochondria (the ER-mitochondria encounter structure, ERMES) were not viable. Moreover in this strain, for the first time, we observed ER to mitochondria PS transfer to be almost completely abolished. The PS transport defect along with the growth defect could be rescued by the expression of an artificial ER-mitochondrial tethering protein. Together, these findings suggest that the EMC-proteins along with the EREMS-complex mediate ER to mitochondria PS transport by promoting membrane tethering between these two organelles. They also conclusively establish that lipid transport from the ER to mitochondria is an essential cellular process. Whether the EMC and ERMES complexes directly facilitate lipid exchange between the ER and mitochondria or simply tether these organelles remains a topic for future investigation.

Metabolomics/Proteomics
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Untargeted Metabolomics Profiling Identifies Putative Biomarkers For Early Detection of Lung Cancer

Lung cancer remains the most common cause of cancer deaths world-wide. Despite the intensive research over many years, the prognosis of this deadly disease is still very poor, with fewer than 15% of the patients surviving 5 years after primary diagnosis. While there are several methodologies described and proposed for early detection of lung cancer (spiral CT, circulating pro-inflammatory cytokines IL6, IL8 and CRP), the specificity and robustness remains to be achieved. What we readily know is that cancer cells have a distinguishable metabolic fingerprint compared to normal cells. Metabolomics holds promise to be able to detect and capture subtle shifts in multiple metabolic paths and cellular modifiers that will enable identification of critical components of cancer risk and tumor behavior. We conducted a first of its kind effort using mass spectrometry-based untargeted metabolic profiling of 1005 urine samples obtained from 469 lung cancer patients and 536 healthy population controls. We identified four putative biomarkers, high levels of which are associated with lung cancer diagnosis and poorer survival (ORs ranging from 2.3 to 5.5; HRs ranging from 1.39 to 2.31). They all passed the Bonferroni correction and were associated with diagnosis and prognosis independent of stage, smoking status, race and gender. The combination of the four metabolites resulted in better predictions, indicating that they are independent diagnostic and prognostic putative biomarkers. We then profiled the metabolome of 73 adenocarcinoma and squamous cell carcinoma stage I lung tumor samples and compared them to the matched adjacent normal tissues. Levels of one of the four putative biomarkers, Met C, were more highly prevalent in tumor compared to adjacent normal tissues (FC=9.03, p-val <0.0001). Moreover, Met C was detected in 80% of lung tumor, while it was only detected in 30% of adjacent normal tissues, albeit at lower levels compared to those in the tumor. These findings indicate that Met C might be a direct bi-product of tumor metabolism and is detectable in stage I tumors. Met C, along with the other putative biomarkers identified in this study, may serve as diagnostic and prognostic indicators in early detection of lung cancer in non-invasively collected urine samples. The structure of Met C is being confirmed by synthesizing the predicted compound and mechanistic studies illuminating the abrogation of the Met C pathway in lung cancer are ongoing.

Metabolomics/Proteomics

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Urinary metabolomics identifies mechanistically associated noninvasive biomarkers for colorectal cancer

Colorectal cancer is the third leading cause of cancer incidence and mortality in both sexes. Since advanced stages of the disease show metastasis and poor prognosis, early diagnosis is of pivotal importance in increasing survival. Unfortunately, the current diagnostic tools, such as endoscopy, biopsy, CT scan and PET scan, are either too invasive or costly and suffer from low throughput thus making them unsuitable for screening and diagnosis of large populations. This makes a high-throughput noninvasive diagnostic tool of high value to the medical community. In this study, the ApcMin/+ mouse was used to explore potential alternative diagnostic approaches using metabolomics as well as to investigate the underlying molecular mechanism. ApcMin/+ mice bear a mutation in the Adenomatus polyposis coli (APC) gene that is frequently mutated in human sporadic colorectal cancer and also associated with a major hereditary form of colon cancer. The temporal evolution of the urinary metabolic profile of wild-type and ApcMin/+ mice was monitored from 1.5 to 6.5 months of age using ultra-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry. Multivariate data analysis showed a progressive divergence of the metabolic trait during tumorigenesis. Consequently, a number of differentially regulated metabolites were identified through chemoinformatics, tandem mass spectrometry analysis, and comparison with authentic standards. The changes in abundances of a number of these metabolites, particularly, those of

two acylglycine conjugates of an amino acid, one progressively elevated and the other attenuated during tumorigenesis, were found to be highly correlated with tumor burden. Interestingly, elevation in the abundance of the acylglycine was not only significant, but also correlated with the final tumor burden starting from as early as 3.5 months until 6.5 months. Microarray analysis revealed significant upregulation of a gene involved in the production of this metabolite that is also a putative target of c-MYC, a key oncogene involved in neoplastic transformation, thus indicating a novel and coordinate disregulation of amino acid metabolism associated with intestinal tumorigensis. This study demonstrates the prowess of metabolomics not only to find biomarkers for noninvasive high-throughput screening, diagnosis and prognosis but also to capture snapshots of key molecular events involved in colorectal cancer.

Metabolomics/Proteomics
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Discovery of urinary biomarkers for nonalcoholic steatohepatitis using metabolomics
The prevalence of nonalcoholic fatty liver disease (NAFLD) is increasing worldwide due to sedentary lifestyle and overnutrition. NAFLD is histologically classified into two clinical entities, simple steatosis and steatohepatitis. Nonalcoholic steatohepatitis (NASH) is characterized by hepatocyte injury, inflammation and fibrosis, in addition to steatosis in the absence of alcohol consumption. Unlike simple steatosis, NASH can progress into cirrhosis and hepatocellular carcinoma as well as alcoholic liver disease, thus reliable non-invasive methods for early diagnosis of NASH would be of great value. Some serum biomarkers associated with NASH have been reported, but such biomarkers have not been examined in urine, which can be more easily and less invasively obtained than blood. It is well known that a choline-deficient (CD) diet causes steatosis while methionine and choline-deficient (MCD) diet induces NASH in mice. Therefore, urinary biomarkers related to NASH were investigated using these mouse models and metabolomics. Male C57/B6 mice (8-10 weeks of age) were treated with a control diet, CD diet, and MCD diet for 2-8 weeks and urine metabolites analyzed using ultra-performance liquid chromatography coupled with electrospray ionization quadrupole time-of flight mass spectrometry. Metabolomic analyses revealed that urinary levels of 21-steroid carboxylic acids, 11-beta-hydroxy-3,20-dioxopregn-4-en-21-oic acid (HDOPA) and 11-beta, 20-dihydroxy-3-oxo-pregn-4-en-21-oic acid (DHOPA), were significantly increased in MCD diet-treated mice. These increases were correlated with hepatic up-regulation of 21-hydroxysteroid dehydrogenase (Akr1c18), which is responsible for synthesis of HDOPA/DHOPA. These increases in hepatic Akr1c18 expression and urinary HDOPA/DHOPA concentrations were specific for steatohepatitis. Since the mRNA levels of Akr1c18 were increased by oxidative stress in primary hepatocytes, urinary excretion of HDOPA/DHOPA seemed to reflect enhanced oxidative stress generation in the liver. Furthermore, Akr1c18 expression was induced in galactosamine-treated ob/ob mice and dioxin-treated mice, both of which are also representative NASH mouse models. Collectively, these results provide a possible urinary metabolomic approach that could identify novel and unexpected biomarkers of NASH.

Metabolomics/Proteomics
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Alterations of Mitochondrial Acetylation Profile by Loss of or Inhibition of Cyclophilin D

Heart disease associated with myocardial ischemia/reperfusion (IR) injury is the most frequent cause of lethality worldwide. Mitochondria play a critical role in mediating cell death during IR injury through mitochondrial permeability transition pore (mPTP) opening. Cyclophilin D (CypD), a mitochondrial chaperone protein, has been shown to be the main mPTP regulator. Genetic deletion of CypD has been shown to reduce IR injury in vivo. Acetylation/deacetylation of proteins on lysine residues has emerged as an important post-translational modification of many proteins. Recent data show that CypD activity is regulated by acetylation; therefore, this led us to investigate whether CypD might also regulate protein acetylation. We hypothesized that a loss of CypD or addition of cyclosporine A (CsA), an inhibitor of CypD, might alter protein acetylation. We have confirmed that genetic ablation of CypD or 5 min CsA administration in isolated mouse hearts prior to 20 min ischemia and 2 hr of reperfusion improved post-ischemic contractile dysfunction and reduced myocardial cell death (n=4, p<0.05). To identify the changes in lysine-acetylated proteins and to map acetylation sites following chronic ablation of CypD or 5 min acute treatment of CsA, we subjected tryptic digests of isolated mitochondria to immunoprecipitation using agarose beads coupled to anti-acetyl lysine antibody followed by mass spectrometry. In the CypD-/- mitochondria, we identified 845 acetylated peptides with 750 acetylated peptides common between WT and CypD-/- samples. We used label-free analysis for relative quantification of the common acetylated peptides between WT and CypD-/- samples and found 11 peptides are decreased while 89 peptides are increased in the CypD-/- samples. Following 5 min CsA treatment, we identified 794 identified acetylated peptides, with 644 peptides common between CsA-treated and untreated samples. Of the common acetylated proteins, 65 showed a significant increase and none were decreased. We found 4 peptides that showed increased acetylation in both acute CsA treatment and CypD-/- mitochondria; these included ADP/ATP translocase 1, ATP synthase subunit O, trifunctional enzyme subunit alpha, and isocitrate dehydrogenase. These results implicate a role for CypD in modulating protein acetylation. Taken together, these results suggest that ablation of CypD or acute treatment of CsA leads to changes in the acetylation profile of the mitochondrial proteome.

Microbiology and Antimicrobials

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Whole genome sequencing to track a hospital outbreak of carbapenem-resistant Klebsiella pneumoniae

Klebsiella pneumoniae is a major cause of hospital infections, primarily among immunocompromised patients. The recent emergence of strains resistant to carbapenems has left few treatment options, making infection containment critical. Complicating infection control is the propensity for K. pneumoniae to cause outbreaks in healthcare institutions. Recognizing and controlling these outbreaks in their early stages is essential, but lack of understanding into how these outbreaks manifest has hindered the design of optimal infection control procedures. In 2011 the National Institutes of Health Clinical Center experienced an outbreak of carbapenem-resistant K. pneumoniae that affected 18 patients, 5 of whom died as a result of their infection. To gain insight into the events underlying outbreak propagation we performed whole-genome sequencing on K. pneumoniae isolates taken from the 18 patients colonized during the outbreak. Patterns of shared sequence variants among the outbreak genomes and epidemiological data were used to systematically construct a putative transmission map. Genome sequence analysis demonstrated that the outbreak could be traced to a single index patient, who was discharged three weeks before the next case became clinically apparent. Genomic data revealed that, despite isolation of the index patient immediately upon admission, three independent transmission events stemmed from this patient. These unexpected transmissions indicated a major role of silent colonization in the outbreak spread. Genomic analysis also uncovered several

instances where there was strong evidence for transmission between patients, whom had no known link in terms of shared location, staff or equipment, indicating complex routes of transmission. Additional genomic comparisons linked a patient to contaminated medical equipment and revealed mutations unique to colistin-resistant isolates. Whole genome sequence analysis of carbapenem-resistant K. pneumoniae isolates provided sufficient molecular resolution to infer likely patient transmission events. Through analysis of these transmission events we were able to gain insight into how the outbreak unfolded, and thereby provide specific suggestions for how future outbreaks may better be contained. Furthermore, our analysis suggested several ways in which the real-time application of genomic sequencing to hospital pathogens can provide actionable insights and facilitate the control of nosocomial transmission.

Microbiology and Antimicrobials
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NIAID
Ultrastructural and biochemical effects of meropenem-clavulanate on Mycobacterium tuberculosis
The cell wall core of M. tuberculosis (Mtb) is a triple-layered structure made of covalently linked mycolic acids (MA), arabinogalactan (AG), and peptidoglycan (PG) layers and is essential to survival and virulence of the pathogen. Isoniazid (INH) and ethambutol (EMB) are front-line drugs that target MA and AG biosynthesis respectively. In contrast, beta-lactams such meropenem inhibit biosynthesis of the PG scaffold that anchors the arabinogalactan-mycolyl complex. The study described here evaluated the effects of targeted-disruption of each cell wall core component on the ultrastructure of Mtb cell envelope. This work provides the first high-resolution cryo-electron microscopy images of untreated and drug-treated Mtb. Among the three layers, disruption of PG (by meropenem-clavulanic acid; MCA) was found to be most damaging to the integrity of the cell envelope as well as to the viability of the bacillus. Inhibition of PG synthesis prevented incorporation of precursors into MA and AG leading to immediate collapse of the cell envelope. Treatment with MCA resulted in leakage of internal contents within hours as measured by release of cytoplasmically-expressed GFP. Additionally, a rapid 4-log kill of bacilli occurred within 24 hours. This loss of viability and leakage of internal contents was sustained over 7-days at which point no viable bacilli could be recovered anymore. A similar treatment with INH led to rise of resistant mutants within 2 days while EMB took at least 3 days to cause a 4-log kill. Furthermore, scanning and transmission electron microscopy showed that MCA selectively damaged the poles and septa of actively-dividing cells confirming these as sites of nascent PG biosynthesis. These regions also showed accumulation of peptidoglycan pentapeptide precursor indicating inhibition of D,D-carboxypeptidase(s). Transcriptional profiling confirmed dysregulation of PG-biosynthetic machinery within 3hrs of treatment with increased expression of D,D-carboxypeptidases. These results indicate that the PG-anchor is crucial for proper positioning and assembly of the cell envelope. Thus our findings support a polar model of growth for Mtb and provide convincing evidence for the use of drugs targeting the integrity of peptidoglycan in general and the enzyme(s) targeted by meropenem in particular as potential therapeutic agents against tuberculosis.

Microbiology and Antimicrobials
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NIAID

Identification of Host Cell Proteins Required for Salmonella enterica serovar Typhimurium Invasion of Nonphagocytic Cells

Nontyphoidal Salmonella gastroenteritis remains one of the most common foodborne illnesses in the world. A crucial component of Salmonella pathogenicity is their ability to actively invade nonphagocytic intestinal epithelial cells. To do this, Salmonella use a type III secretion system to translocate a cohort of bacterial effector proteins into the host cell cytosol where they drive actin polymerization at the entry site. Rapid rearrangement of the actin cytoskeleton leads to the localized formation of prominent plasma membrane ruffles and internalization of the bacteria. Several aspects of Salmonella invasion are well understood but we still lack a complete understanding of which host cell proteins are required. Taking a multipronged approach, which includes live-cell fluorescence microscopy of the HeLa epithelial cell model of Salmonella invasion, we have observed previously unrecognized phases of invasion and identified novel host cell proteins required for this process. Specifically, we determined that the giant phosphoprotein AHNAK is enriched at the plasma membrane in response to Salmonella invasion. This ~700kDa membrane-associated protein interacts with actin, acts as a scaffolding protein during membrane signalling events, and has been implicated in vesicle-mediated plasma membrane repair processes. To examine the role of AHNAK in Salmonella invasion, we performed siRNA-mediated knockdown of AHNAK in HeLa cells and consequently observed a ~75% decrease in Salmonella invasion. To validate these results, we examined the ability of Salmonella to invade mouse embryonic fibroblasts (MEFs) isolated from AHNAK+/+ and AHNAK-/- mice and found that Salmonella invasion was decreased by ~80.0% in the AHNAK-/- MEFs. Next, we examined the potential role of known binding partners of AHNAK. AHNAK interacts with the Annexin A2/p11 heterotetramer at the cytoplasmic face of the plasma membrane and this multiprotein complex is implicated in the regulation of actin cytoskeleton reorganization and plasma membrane remodeling. Salmonella invasion was decreased by ~63% and ~71% in HeLa cells following siRNA-mediated knockdown of Annexin A2 and p11, respectively. These results clearly demonstrate that AHNAK, Annexin A2, and p11 are required for efficient Salmonella invasion. These host cell proteins may represent novel therapeutic targets and our next objective is to determine the mechanism by which these host cell proteins contribute to Salmonella invasion.

Molecular Biology - Eukaryotic

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Two modes of transcription termination by RNA polymerase III

How the active center of any eukaryotic RNA polymerase switches from elongation to termination with transcript release is unknown. Eukaryotic RNA polymerase III (pol III) terminates transcription while transcribing a stretch of T residues on the non-template strand. This is the simplest termination signal for any RNA polymerase known. Also, pol III termination is factor independent. These properties make pol III suitable model to study transcription termination. The inherent instability of rU.dA hybrid formed by transcription of the terminator is known to destabilize elongation complexes and is proposed to be the driving force for termination by pol III. Recently it was suggested that a subcomplex of pol III (C53/C37) is involved in pol III termination by reducing the elongation rate, allowing pol III to stay longer on terminator. Reduction in elongation rate on the terminator can also increase propensity for backtrack induced arrest. Another subunit of pol III, C11, is required for hydrolytic cleavage of the 3â€™ end of nascent transcripts during termination, also allows pol III to overcome arrest due to mismatches and possibly other conditions. A version of pol III referred to as pol III? which lacks C53, C37 and C11 can be purified from yeast cells and shown to have transcript cleavage deficiency as well as transcription termination defect. Our results show that compared to WT pol III, the transcripts released from an

oligo(dT) terminator, pol III? have longer U tracts. In addition, pol III? retains a significant fraction of transcripts with shorter 3' U tracts that are released by WT pol III. These data suggest a dual defect of pol III?: (i) longer U stretch is required for transcript release (supports the termination defect observed previously), and (ii) release defect of transcripts with shorter terminal U tract. These observations led us to propose that pol III can terminate transcription by two mechanisms: (i) a principal or core mechanism that causes release of transcripts with =6Us, and (ii) an auxiliary mechanism that involves C11/53/37 which prevents terminator arrest by promoting release of RNAs with fewer Us. Further analysis of the arrested complexes suggests that they are extensively backtracked complexes. Taken together, we propose that C53/37 has two different roles in pol III termination: (i) Reduction in elongation rate, and (ii) prevention of backtracking at the terminator.

Molecular Biology - Eukaryotic
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NICHD
Identification of novel regulatory surfaces in the HisRS-related and pseudokinase domains of the protein kinase GCN2 of budding yeast.
The regulation of the activity of kinases is a central aspect of intracellular signal transduction. One striking example is the regulation of the multidomain protein kinase GCN2. In response to amino acid starvation, GCN2 phosphorylates the alpha subunit of eukaryotic initiation factor 2, which leads to the inhibition of translation that allows cells to conserve resources. GCN2 is present in a latent form in nonstarved cells and is activated by binding of uncharged tRNA to the histidyl-tRNA synthetase (HisRS)-related domain located adjacent to its protein kinase (PK) domain. It is thought that tRNA binding provokes a stimulatory association of the HisRS region with the PK domain. To probe the molecular basis of this mechanism, we isolated mutations in the HisRS domain that constitutively activate GCN2 kinase function and suppress the need for a conserved tRNA binding determinant in the HisRS region of GCN2. Those mutations are located in the conserved, predicted active site of the HisRS domain. In authentic synthetases, substrate binding evokes conformational changes in the active site essential for the catalytic activity of the enzyme. Our finding that altering conserved residues of the predicted active site of the HisRS domain impairs GCN2 activity leads us to suggest that tRNA binding alters the conformation of this conserved binding pocket to mediate allosteric activation of the PK domain by the HisRS region. In addition to the HisRS and PK domains, GCN2 contains a degenerate kinase, or pseudokinase (YK) domain that is required for kinase activity. By mutagenesis of evolutionarily conserved residues predicted to be exposed on the surface of the YK region, we identified novel regulatory mutations that impair GCN2 activity. These mutations identify three key surfaces in the YK domain essential for GCN2. Because the isolated YK and PK domains can physically interact, we hypothesize that the affected residues mediate an interaction between the two domains that stimulates GCN2 activity. Interestingly, we also isolated mutations in same surface patches of the YK that constitutively activate GCN2, reducing the requirement for tRNA binding determinants in the HisRS region for kinase activation. Presumably, these latter YK mutations enable constitutive activation of the PK domain by the YK moiety in the absence of bound tRNA. Our results provide the first molecular insights into the role of the enigmatic YK domain in activation of GCN2 by unchanged tRNA.

Molecular Biology - Eukaryotic
Rijal, Keshab
Visiting Fellow

NICHD

RNA polymerase III mutants in the TFIIFα-like Rpc37p subunit impair transcription termination and RNA 3' end cleavage

RNA polymerase III (pol III) is a multisubunit enzyme that transcribes noncoding RNAs such as tRNA, 5S rRNA, U6 spliceosomal snRNA, 7SK RNA and others including a subset of microRNAs. Pol III is deregulated in tumorigenesis. Termination is the least understood of the complex multistep process of transcription. The pol III subunits C53/37 which are homologous to pol II initiation factors TFIIFß/a are known to reside near the pol III active center, are required for open complex formation as well as termination, and together with C11 promote pol III recycling, a poorly understood process that has been proposed to be mechanistically dependent on normal termination in vitro. Our laboratory had developed different strains of S. pombe that phenotypically (red/white) report two different pol III termination defects on a suppressor-tRNA gene and used them to isolate distinct classes of C11 mutants. For this work, randomly mutagenized libraries of C53 and C37 were created and screened in a strain that reports if the polymerase fails to stop at the terminator and instead reads through. The C53 library yielded very few mutants of very weak phenotype with multiple mutations. The C37 library yielded a wealth of single-mutation mutants. A minority of mutants map to the C37-C53 dimerization domain and exhibit mild phenotypes, whereas the majority have strong phenotypes and map to a short C-terminal tract previously localized in the pol III active center. Quantification of terminator readthrough transcripts suggests as much as 40% terminator readthrough in some of the mutants. These and other analyses indicate that these mutants readthrough the pol III terminator because they have increased elongation rate. We also monitored intron-containing nascent pre-tRNA levels as a reflection of relative transcription rate in vivo. Notably, these mutants and a rpc2-termination mutant do not show decrease in pol III transcription in vivo as would be expected if they were impaired for pol III recycling. Further analysis reveals that the nascent transcripts that end within functional dT7 terminators in the mutants have normal 3' terminal lengths of 3-4Us, appreciably shorter than in C11 mutants defective for RNA 3' cleavage. These data provide another important new insight, that termination, even from a fast-elongating pol III, involves 3' end shortening or pol III retraction.

Molecular Biology - Eukaryotic

Krivega, Ivan

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NIDDK

Homo-dimerization of Ldb1 forms the basis for long-range enhancer looping of the beta-globin LCR in vivo

Long-range interaction, or chromatin looping, between the beta-globin LCR enhancer and gene involves a multi-protein complex that includes Ldb1, LMO2 and DNA-binding partners GATA-1 and TAL1. Ldb1 stabilizes occupancy of GATA-1, TAL1 and LMO2 at LCR HS2 and the beta-globin promoter, even though it does not bind DNA, and is required for long-range chromatin looping and beta-globin gene activation. Ldb1 can homo-dimerize in vitro through its N-terminal dimerization domain (DD). We tested whether the homo-dimerization of Ldb1 plays a key role in LCR/beta-globin looping and transcription activation in erythroid cells. Full-length Ldb1, truncated forms and fusion proteins were expressed in the background of Ldb1 knock-down (KD) MEL cells and their ability to rescue Ldb1 complex occupancy, chromatin looping and beta-globin expression was examined by 3C, ChIP and RT-qPCR. Full-length, shRNA-immune Ldb1 occupied HS2 and the beta-globin promoter and rescued GATA-1/Tal1/LMO2 occupancy, long-range LCR/beta-globin interaction and gene expression. Ldb1 proteins with deletions of short conserved sequences throughout the DD domain expressed in the background of Ldb1 KD MEL cells were unable to fully rescue beta-globin expression. All deleted proteins were capable of occupying

HS2 and the beta-globin promoter, but only partially rescued GATA-1/Tal1/LMO2 occupancy and failed to rescue chromatin looping. To further test Ldb1 homo-dimerization in vivo, DD alone was expressed in wild type MEL cells. DD acted as a dominant negative inhibitor of beta-globin expression by interfering with homo-dimerization of endogenous Ldb1. Co-immunoprecipitation experiments confirmed that the DD homo-dimerize with endogenous Ldb1 and, through it, interacts with other members of the Ldb1 complex. DD domains with deletions of short conserved sequences were unable to homo-dimerize with endogenous Ldb1and did not negatively affect beta-globin gene expression. Finally, a fusion protein of DD with LMO2 (LMO-DD), the protein with which Ldb1 interacts directly within the Ldb1 complex, was expressed in Ldb1 KD MEL cells. LMO-DD occupied HS2 and the beta-globin promoter, stabilized GATA-1/Tal1 occupancy at these sites and rescued beta-globin gene expression to wild type level. These results support the hypothesis that homo-dimerization of Ldb1 provides the link between LCR and beta-globin bound complexes that mediate chromatin looping and gene activation.

Molecular Biology - Prokaryotic
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Role of sRNAs in Biofilm Formation
In response to stress conditions, bacteria can form communities of surface adherent cells embedded in a polysaccharide matrix called biofilm. Biofilms are ubiquitous, and can create severe problems for human health as biofilms provide a barrier to the immune response and antibiotics. We are studying the role of small noncoding regulatory RNAs (sRNAs) in biofilm development since sRNAs have been shown to be important in stress responses. These sRNAs require the Hfq chaperone and regulate the expression of specific proteins by pairing with the mRNA. To investigate how sRNAs influence biofilm formation by Escherichia coli, we tested whether the overexpression of these sRNAs had an effect on biofilm formation. For those sRNAs that showed significant results in biofilm formation (MicA, ArcZ, ChiX, and MicF downregulate it, while McaS, CsrB, and DsrA upregulate it), we tested whether deleting these sRNAs influenced biofilm development. Interestingly, DsrA sRNA, an activator of RpoS (master regulator of general stress response) and an inhibitor of H-NS (global transcriptional regulator), showed the most drastic result; deleting the dsrA gene resulted in a strain that was unable to form biofilms. To determine whether the disregulation of either of the two known targets of DsrA, RpoS or H-NS, resulted in the elimination of biofilm formation in the dsrA mutant, we made two different mutants of DsrA, one that regulates rpoS but not hns (DsrA H-NS mutant), and another that regulates hns, but not rpoS (DsrA rpoS mutant), and tested these mutant DsrAs for their effect on biofilm formation. Mutation in the region of DsrA that is involved in binding to H-NS showed decreased levels of biofilm formation (1/20th of Wildtype) suggesting that DsrA is activating biofilm formation by inhibiting H-NS, while the mutation that disrupts rpoS binding had no effect. Currently, an hns compensatory mutant is being created in order to observe if biofilm formation is restored. In addition, we are evaluating possible downstream targets of H-NS that may be influencing biofilm formation. pgaA, a gene involved in the synthesis of polysaccharide, and flhDC, the principle regulator of bacterial flagellum biogenesis, were identified as potential downstream targets of hns. Our study provides new insights into sRNAs role on biofilm formation and the results of this study may help open a new era for the treatment of biofilms.

Molecular Biology - Prokaryotic
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NICHD

Factors that dictate mRNA target selection by small regulatory RNAs in bacteria

Small regulatory RNAs (sRNA) in bacteria play critical roles in the adaptation to environmental stress including the stresses encountered by pathogens in the host cell environment. Most sRNAs are rapidly induced in response to specific environmental signals and base pair with specific mRNAs resulting in increased or decreased mRNA stability and/or translation. Most sRNAs depend on the Hfq protein for stability and base pairing with mRNA targets. Hfq enhances sRNA-mRNA interactions by a mechanism that is not fully understood. It is also unclear how an sRNA efficiently and rapidly targets a specific set of mRNAs among thousands. This study focuses on identifying the determinants of the sRNA Spot42 that dictate mRNA target selection with the aim of understanding the mechanism governing sRNA-mRNA interactions in bacteria. The spf gene codes for Spot42 and is up regulated in response to the presence of a preferred carbon source like glucose. The Spot42 sRNA in turn represses genes involved in the catabolism of non-preferred carbon sources. The bioinformatic program TargetRNA was used to predict targets of Spot42 based on sequence complementarity near the mRNA ribosome-binding site. Predicted mRNA targets of Spot42 were confirmed by constructing lacZ fusions with the target mRNA and assessing ß-galactosidase activity in the presence and absence of Spot42 overexpression. Predicted mRNA-sRNA binding sites were confirmed by generating Spot42 mutations that disrupt regulation and compensatory mutations in the mRNA that restore regulation. Upon analysis of all tested and confirmed Spot42 mRNA targets the following general themes were found: only unstructured regions of Spot42 and its mRNA targets are involved in base pairing and an Hfq binding sequence motif needs to be close to but not overlapping the predicted sRNA binding site in the mRNA. The importance of these features was confirmed by converting non-targets of Spot42 into targets by the addition of unstructured sequences complementary to Spot42 and the addition of an Hfq binding site near the sRNA binding site. In summary, we identified critical features of mRNAs that allow for target recognition and repression by Spot42 and factors that contribute to the strength of regulation. Such features are likely to be involved in all mRNA and sRNA interactions that require Hfq, and can be used as guidelines to increase the accuracy of sRNA target predictions and the design of synthetic sRNA targets.

Neuropharmacology and Neurochemistry

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Postdoctoral Fellow

NIA

Resveratrol Supplementation Improves Cerebrospinal Fluid Neuroprotective Biomarkers in Rhesus Macaquesâ€™ Fed with a High-Fat High-Sugar Diet

Diets rich in saturated fat and refined sugar contribute to the development of obesity and diabetes. These metabolic disorders are linked to an increased rate of cognitive decline, neuroinflammation, increased occurrence of neurological defects and the development of Alzheimerâ€™s disease (AD). Supplementing the diet with specific nutraceuticals is a putative therapeutic for counteracting many of the detrimental effects of poor dietary habits. Resveratrol, a plant derived polyphenol abundantly found in the seeds and the skin of grapes, is one such neutraceutical agent demonstrating cardioprotective, anti-diabetic, anti-carcinogenic and anti-inflammatory effects in rodents. However, the efficacy of resveratrol supplementation in primates and on brain health is not known. Hence, we sought to investigate resveratrolâ€™s effects on cognitive health using potential AD biomarkers in a non-human primate model. Twenty four adult male Rhesus monkeys (age 7-13 years) were randomly assigned to one of three dietary regimens: standard diet, high fat and sugar (HFS) or HFS + resveratrol supplemented with 40 mg resveratrol twice daily for one year then 240 mg for the second year. After 24 months, the cerebrospinal fluid (CSF) and brain tissue from the temporal neocortex were collected and

examined for inflammatory cytokines, neurotrophic factors (BDNF and NGF), amyloid-beta proteins (Abeta-40 and Abeta-42), and beta seceretase by ELISA and qRT-PCR. Resveratrol and its metabolites were detected in the CSF via mass spectroscopy. HFS diet caused significant decreases in BDNF and NGF in CSF and brain mRNA. A corresponding decrease of growth factor receptors, TrkB and TrkC was also observed in brain mRNA. Additionally, HFS diet led to increased expression of CSF inflammatory markers and AD biomarkers, Abeta-42/Abeta40, beta amyloid and beta seceretase. Remarkably, resveratrol prevented the decrease of neuronal growth factor expression caused by a HFS diet and improved inflammatory and AD markers. These results suggest that resveratrol supplementation can ameliorate the detrimental effects of a diet rich in high fat and sugar on cognitive health by improving neuronal vitality and function. This study is the only non-human primate investigation into resveratrolâ€™s effects on brain health and demonstrates that resveratrol can cross the blood-brain barrier.

Neuropharmacology and Neurochemistry
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Enhanced endocannabinoids and fear extinction
Endocannabinoids are released â€˜on-demandâ€™ on the basis of physiological need, and can be pharmacologically augmented by inhibiting their catabolic degradation. The endocannabinoid anandamide is degraded by the catabolic enzyme fatty acid amide hydrolase (FAAH). Anandamide is implicated in the mediation of fear behaviors, including fear extinction, suggesting that selectively elevating brain anandamide could modulate plastic changes in fear. Here first tested this hypothesis with preclinical experiments employing a novel, potent and selective FAAH inhibitor, AM3506. Systemic AM3506 administration prior to extinction decreased fear during a retrieval test in a mouse model of impaired extinction. AM3506 had no effects on fear in the absence of extinction training, or on various non-fear related measures. Anandamide levels in basolateral amygdala were increased by extinction training and augmented by systemic AM3506, while application of AM3506 to amygdala slices promoted long-term depression of inhibitory transmission, a form of synaptic plasticity linked to extinction. Further supporting the amygdala as effect-locus, the fear-reduced effects of systemic AM3506 were blocked by intra-amygdala infusion of a CB1R antagonist and were fully recapitulated by intra-amygdala infusion of AM3506. Based on these preclinical findings, we hypothesized that variation in the human FAAH gene would predict individual differences in amygdala threat processing and stress-coping traits. Consistent with this, carriers of a low-expressing FAAH variant (C385A; rs324420) exhibited quicker habituation of amygdala reactivity to threat, and had lower scores on personality trait stress-reactivity. Our findings show that boosting amygdala anandamide enables extinction-driven reductions in fear in mouse and may promote stress-coping in humans.

Neuropharmacology and Neurochemistry
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NIDA
New allosteric modulators of mGluR5 receptors display therapeutic effects in rodent models of anxiety and drug addiction
The metabotropic glutamate receptor subtype 5 (mGluR5) is a G protein-coupled receptor highly expressed in distinct brain circuits. Excess stimulation of mGluR5 signaling in mesocorticolimbic circuits is implicated in brain diseases such as anxiety and drug abuse. Thus, attenuation of mGluR5 signaling is a

promising pharmacological approach to treatment of these diseases. In order to develop new molecular tools--and potential lead medications--we have designed, synthesized and tested a library of novel mGluR5 ligands that bind to an allosteric site on the receptor and inhibit mGluR5 signaling. Targeting an allosteric site, which is separate from the orthosteric site in which endogenous glutatmate binds, provides a mechanism to improve selectivity at mGluR5 versus other glutamate receptor subtypes. We sought to improve binding affinity and selectivity over the prototypic mGluR5 negative allosteric modulators (NAMs), MPEP (Ki=16 nM; IC50=31 nM) and MTEP (Ki=42 nM; IC50=111 nM). A novel series of aryl-substituted alkynyl analogues of MPEP was synthesized, employing Sonagashira coupling conditions, to evaluate structure-activity relationships and identify modifications to the MPEP pharmacophore that optimize binding affinity and selectivity for mGluR5. Of the nineteen novel compounds developed and tested, six had improved affinity for mGluR5 compared to MPEP, using [3H]MPEP in rat brain membranes. Each of the high-affinity ligands was a potent inverse agonist in an ELISA-based immunocompetitive assay of agonist-evoked second messenger accumulation in rat mGluR5-transfected HEK293 cells. Two novel high-affinity NAMs, MFZ 10-7 (Ki=0.67 nM; IC50=2.4 nM) and ZP 3-74 (Ki=2.8 nM; IC50=9.7 nM), were chosen for further evaluation in vivo. In two mouse models of anxiety-like behaviors, MFZ 10-7 and ZP 3-74 produced anxiolytic effects 10-300 times more potently than MPEP or MTEP. MFZ 10-7 potently inhibited cocaine self-administration and cocaine-seeking behavior in rodent models of reinstatement and incubation of craving. Moreover, MFZ 10-7 is highly selective for mGluR5, producing no significant binding (<13% inhibition) at any of 69 other receptor targets tested at a concentration of 100 nM. In conclusion, the design and testing of these novel mGluR5 ligands has identified new highly selective and potent NAMs with great promise as tools for in vivo investigations of addiction and other neurological conditions.

Neuropharmacology and Neurochemistry
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Serotonergic terminals co-release glutamate into the VTA and SNc
The dorsal raphe nucleus (DRN) is the largest nucleus of serotonergic neurons and major source of ascending serotonin in the mammalian brain. Although animals will vigorously self-administer for electrical stimulation of this brain region, drugs that acutely enhance serotonergic function are generally non-rewarding or aversive. These two observations seem paradoxical; however, one theory to reconcile them is that serotonergic neurons also corelease another neurotransmitter that carries rewarding value. While it has been shown that serotonergic neurons express a vesicular glutamate transporter gene, there is no direct physiological evidence that this results in synaptic release of glutamate in mature neurons. In the present study we used a combined optogenetic-electrophysiological approach to provide the first direct evidence for serotonin-glutamate corelease. To selectively activate serotonergic neurons, we injected a virus encoding a cre-dependent channelrhodopsin2-YFP fusion protein into the DRN of transgenic mice (SERT:cre) with cre expression limited exclusively to serotonergic neurons. 10-12 weeks after surgery, we prepared horizontal brain slices containing midbrain dopamine neurons in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc). We used whole-cell voltage clamp to examine neurons throughout the VTA and SNc, using morphology and the presence of Ih current as markers for putative dopamine neurons. Optogenetic stimulation of serotonergic terminals in the VTA and SNc resulted in excitatory post-synaptic potentials (EPSCs) in 14/64 cells. EPSC responses were comprised primarily of AMPA currents, with a small NMDA component. Average EPSC magnitude was larger in the SNc compared to the VTA, indicating greater number of release sites and/or larger postsynaptic current per vesicle. Paired pulse depression was also stronger in the SNc, suggesting

greater probability of release. Thus, the SNc appears to receive stronger innervation than the VTA through this circuit. In conclusion, here we show that serotonergic neurons originating in the DRN corelease glutamate and directly excite midbrain dopamingic neurons, with particularly strong effects in the SNc. This provides a novel mechanism to account for observations of brain reward in the DRN, and opens a new avenue of research in the study of the brain circuitry of reward.

Neuroscience - Cellular and Molecular
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Rbfox3-regulated Alternative Splicing of Numb Promotes Neuronal Differentiation in the Developing Spinal Cord
Alternative splicing, which is a major mechanism for generating diversity of gene products, occurs frequently in neural tissues. However, the biological consequences of alternative splicing events during development are largely unknown. Our laboratory focuses on a neuron-specific RNA binding protein, Rbfox3 that we recently identified as the antigen of the widely used anti-NeuN antibody. To make use of the chicken neural tube as a model to study neuronal development, we have cloned and characterized chicken Rbfox3. Two splice variants for the Rbfox3 coding sequence, Rbfox3-full and Rbfox3-d31 which lacks 31 amino acids in the RNA recognition motif, were found. Rbfox3-full and Rbfox3-d31 have different protein stabilities as well as different activities with respect to splicing regulation. Only Rbfox3-full expression is confined to late postmitotic neurons of the embryonic chicken spinal cord. To explore whether Rbfox3-full is involved in spinal neuronal development we carried out neural tube electroporation of siRNA and plasmid in ovo for loss-of- and gain-of-function studies. Significant reductions in differentiated interneurons and motor neurons with concomitant increases in undifferentiated neurons are observed in Rbfox3 knock-down neural tubes. The gain-of-function studies show that, while Rbfox3 is not sufficient to promote cell cycle exit when misexpressed in ventricular progenitors, it appears to nevertheless accelerate the timing for differentiation of interneurons and motor neurons. We further sought the target gene for which splicing is regulated by Rbfox3 in the developing spinal cord. The data show that Numb pre-mRNA which encodes a signaling adapter protein is a direct target of Rbfox3 action. Rbfox3 is responsible for exclusion of exon12 in Numb mRNAs via its direct binding to the upstream intronic Rbfox3 binding element (UGCAUG) in chicken embryos. Specific knock-down of the exon12-excluded Numb isoform reduces the number of differentiated interneurons and motor neurons, as we observed in the Rbfox3 knockdown embryos. In contrast, knock-down of the exon12-included Numb isoform does not affect the differentiation of these neurons. Importantly, the exon12-excluded Numb isoform rescues, in a splice isoform-specific manner, the phenotype due to Rbfox3 knock-down. These data provide the first evidence that Rbfox3-dependent alternative splicing of Numb indeed plays a critical role during neuronal differentiation in the developing spinal cord.

Neuroscience - Cellular and Molecular
McGuire, James
Postdoctoral Fellow
NIAID
Astrocytes Facilitate the Intercellular Spread of Prions in the CNS
Prion diseases are a group of invariably fatal neurodegenerative diseases caused by prions, infectious agents primarily comprised of misfolded prion protein (PrPres). PrPres propagates by inducing conformational conversion of a cellular, normal form (PrPc) into PrPres. During infection, PrPres spreads

throughout the CNS, often along defined neural pathways. Unfortunately, there is a significant gap in our understanding of the mechanisms that mediate the intercellular spread of PrPres in the brain. This includes potential roles for glial cells such as astrocytes, which propagate prions, outnumber neurons 10:1, and have been shown by our lab to efficiently internalize PrPres. To characterize the spread of PrPres in the CNS, we used compartmented microfluidic neuronal culture devices that allow for the separation of axonal termini from soma and the separation of cell populations, which is valuable for co-culturing neurons and astrocytes. After inoculation of somal or axonal compartments with fluorescently tagged PrPres, we analyzed PrPres transport in these cultures by live cell imaging. In neuronal cultures, uptake and transport of PrPres was evident 3 days post inoculation. The number of axons actively transporting PrPres increased at 5 and 7 days post inoculation, which is consistent with slow axonal transport. Internalized PrPres moved bidirectionally with axonally inoculated neurons demonstrating net retrograde movement of PrPres and somally inoculated neurons demonstrating net anterograde movement. To determine if PrPres could spread from astrocytes to neurons, astrocytes pre-loaded with labeled PrPres were introduced to the axonal chamber. Remarkably, transfer of PrPres from astrocytes to axons was observed after 3 days of co-culture. After 5 days, PrPres had been transported to neuronal soma. The number of soma containing PrPres particles increased for up to 9 days of co-culture. These data show that prions can be internalized by axons and soma and transported retrogradely or anterogradely, recapitulating the spread of prion infectivity in vivo. Importantly, we have demonstrated for the first time that PrPres can spread from astrocytes to neurons. Our observations suggest that pathways allowing the exchange of molecules between neurons and astrocytes may facilitate the spread of prions in the CNS, thereby drawing attention to an as yet unappreciated role for astrocytes in prion disease.

Neuroscience - Cellular and Molecular
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NIDA

A non-invasive post-treatment strategy for stroke by intranasal delivery of cocaine- and amphetamine-regulated transcript

Current treatment strategies for stroke primarily focus on reducing the size of ischemic damage and on rescuing dying cells early after occurrence. Pharmacological treatments are often limited by a narrow therapeutic time window, and no agent has shown effectiveness when therapy is initiated 3 days after stroke in patients. Recent studies have demonstrated that cocaine- and amphetamine-regulated transcript (CART) is protective against ischemic brain injury when given before the onset of stroke in mice. Its neural regenerative action in post-stroke brain has not been investigated. Utilizing a classic stroke model in rodents, middle cerebral artery occlusion (MCAo), we describe a new therapeutic approach entailing the intranasal administration of CART starting from day 3 post-stroke by enhancing the functional recovery of injured brain. Animals were subjected to a 90-min MCAo and were balanced between two groups to receive CART or vehicle according the size of lesion measured by T2WI at 2 days after MCAo. Animals receiving CART treatment showed improved behavioral recovery, examined by rotarod test, Bedersonâ€™s neurological test, and an elevated body asymmetry test. In the subventricular zone (SVZ), CART enhanced immunolabeling of bromodeoxyuridine, the neuroprogenitor cell marker Musashi-1, and the proliferating cell nuclear antigen, as well as upregulated brain derived neurotrophic factor (BDNF) mRNA. Overexpression of BDNF by local administration of AAV-BDNF in SVZ enhanced migration of SVZ cells toward the ischemic cortex and induced behavioral improvement. In SVZ culture, CART increased neurosphere formation and cell migration. CART-mediated cell migration was significantly antagonized by anti-BDNF blocking antibody. Using diffusion-tensor imaging and 1H-

magnetic resonance spectroscopy, significant increases in the fractional anisotropy ratio and N-acetylaspartate levels were found in the lesioned cortex after CART treatment. CART significantly increased the expression of growth-associated protein 43 (GAP43) in the lesioned cortex. In summary, our study has demonstrated that delayed intranasal treatment with CART modifies endogenous neural repair in stroke brain by facilitating neuroprogenitor cell proliferation and migration, enhancing reinnervation, and improving the functional recovery. Our results may provide a new treatment strategy for stroke patients, enabling a non-invasive and longer treatment window of days after stroke occurrence.

Neuroscience - Cellular and Molecular
Zhang, Shiliang
Research Fellow
NIDA
Ultrastructural and optogenetic evidence for dual neuronal signaling by dopamine neurons of the ventral tegmental area (VTA)
Dopamine signaling neurons expressing tyrosine hydroxylase (TH) play a role in movement, motivation, learning and reward. The VTA contains a subset of TH neurons that co-express transcripts encoding the vesicular glutamate transporter 2 (VGluT2). As VGluT2 is involved in the accumulation of glutamate into synaptic vesicles for glutamate signaling, the dual TH-VGluT2 neurons potentially could release dopamine, glutamate or both. We set up 3 studies to investigate whether the dual TH-VGluT2 neurons could mediate synaptic co-release of dopamine and glutamate. (1) We performed in vivo tagging of VTA neurons in the rat by either region specific injections of tract tracing molecules or viral vectors. Findings from these studies indicate that TH and VGluT2 do not co-exist in the same axon terminal. (2) To determine whether lack of detection of co-existence of TH and VGluT2 protein was due to lack of translation of VGluT2 mRNA in these TH-VGluT2 neurons or due to subcellular segregation of TH and VGluT2-protein, we next used transgenic mice and viral injections to induce in TH or in VGluT2 neurons expression of the light activated channelrhodopsin-2 tethered to m-Cherry. Ultrastructural analysis of these brains showed that all axon terminals from VTA tagged neurons making asymmetric synapses contained VGluT2 protein but lacked TH, and that those making symmetric synapses contained TH but lacked VGluT2. We also found that TH axon terminals lacked VGluT2, however, TH was often observed in the contiguous axons of axon terminals containing VGluT2. From here, we conclude that TH-VGluT2 neurons synthesize VGluT2 proteins; these transporters are in synaptic vesicles within axon terminals, as such allowing the accumulation of glutamate into these vesicles. (3) By combination of ex vivo electrophysiology and optogenetics (light induced activation of TH- or VGluT2-fibers expressing ChR2-mCherry), we found that axon terminals from VTA VGluT2 neurons with or without TH mediate glutamatergic signaling. Thus, we provide for the first time evidence indicating that a brain area involved in addictive behaviors has a unique set of neurons with the unanticipated capability to co-release two different signaling molecules from two distinct subcellular compartments: glutamate from axon terminals, and dopamine mostly from axons. Further studies are necessary to determine the role of this novel dual signaling mechanism in brain function.

Neuroscience - Cellular and Molecular
de Marchena Powell, Jacqueline
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NIEHS
A novel approach to isolate the function of the galanergic subpopulation of the locus coeruleus

The neuropeptide galanin (GAL) is released as a co-transmitter by a variety of neuronal cell-types throughout the central and peripheral nervous system, including a reported 80% of noradrenergic neurons of the locus ceoruleus (LC). Interestingly, GAL has been implicated in a variety of neurological disorders, including Alzheimerâ€™s disease. To better understand where GAL-expressing noradrenergic neurons are located and to which brain areas they project, we are using a dual recombinase-based strategy to specifically label this noradrenergic subpopulation. Using this genetic strategy, we have examined the noradrenergic nuclei of the murine hindbrain and found that GAL is primarily expressed in the LC and that the subpopulation of LC neurons expressing GAL increases significantly between postnatal days (P)7, P28, and P56. We are also determining where these GAL-expressing neurons project throughout the brain, with particular emphasis on the hippocampus, an area of the brain critically important in learning & memory. While global GAL knockout mice show deficits in hippocampal-dependent learning & memory, it is unclear how GAL from the LC specifically contributes to this phenotype. To overcome this limitation we are taking advantage of the unique LC-specific overlap of dopamine ß-hydroxylase (Dbh) and Engrailed-1 (En1) gene expression to design an intersectional strategy that selectively knocks out GAL in the LC. To target this population, we have designed a completely novel knockout strategy that makes cre expression contingent upon Dre-mediated recombination. For this strategy, we have generated two new knock-in alleles: (1) En1::Dre and (2) Dbh::Dre dependent cre. Crossing these two alleles results in cre expression that is restricted to LC neurons. When En1::Dre; Dbh::Dre dependent cre mice are then crossed to a conditional Gal allele, GAL will be knocked out exclusively in the LC. This genetic scheme allows us to circumvent secondary effects that come from knocking out GAL in all cell-types. Excitingly, these same mouse lines can also be used to conditionally knock out any floxed allele in the LC. This model will allow us to be the first to discern how the specific loss of GAL in this neuronal subpopulation affects neurodevelopment and learning & memory.

Neuroscience - Cellular and Molecular

Gu, Qinhua
Visiting Fellow
NIMH

Functions of microRNAs in long-term synaptic potentiation

Synaptic plasticity is the ability of synapses to change in strength. Long-lasting forms of synaptic plasticity, such as long-term potentiation (LTP), are believed to be important cellular mechanisms for learning and memory. Functional changes of synapses are often accompanied by structural changes, such as enlargement of dendritic spines in LTP. The maintenance of LTP requires protein synthesis, but how it is regulated in LTP is still largely unknown. microRNAs are small non-coding RNAs which act as important post-transcriptional regulators of gene expression. Circumferential evidence suggests that microRNAs may regulate protein synthesis-dependent late phase synaptic plasticity, but has not been experimentally tested. To address this question, first we used deep sequencing to analyze microRNA expression profiles following LTP induction, and selected those miRNAs changed in LTP as candidate â€œLTP microRNAsâ€ for detailed characterization. Second, we investigated the role of two candidate microRNAs, miR-26a and miR-384-5p, in LTP. Both of them are down-regulated in neurons undergoing LTP, and interestingly, predicted to target to ribosomal s6 kinase (RSK) 3. To test whether miR-26a and miR-384-5p are involved in LTP, we transduced cultured hippocampal slices with lentivirus expressing these two miRNAs and recorded LTP in the CA1 region. Remarkably, although LTP can still be induced in the microRNA expressing virus transduced slices, the protein-synthesis dependent late-phase LTP was impaired. Likewise, RSK inhibitor also blocked late phase LTP. Inhibition of late-phase LTP by miR-26a was abolished by transduction of RSK3 expressing lentivirus, suggesting that the effect of miR-26a on

LTP is mediated by reduction of RSK3 expression. In addition, we examined whether miR-26a and miR-384-5p are involved in spine enlargement in LTP. We treated primary hippocampal neurons with tetraethylammonium (TEA), a chemical LTP induction protocol that induces enlargement of dendritic spines and increase of spine number. Overexpression of miR-26a and miR-384-5p did not affect the size and number changes of dendritic spines in early-phase LTP, but dramatically reduced spine changes in late-phase LTP. A pharmacological RSK inhibitor also selectively blocked spine changes occurring in late-phase LTP. Our findings elucidate a new miRNA-mediated mechanism for LTP maintenance and identify two miRNAs and their primary target gene that are crucial for late-phase LTP.

Neuroscience - General

Navarathna, Dhammika
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Magnetic resonance and Two photon microscopic imaging of brain in a mouse model of disseminated candidiasis

Disseminated candidiasis primarily targets kidney and brain. Invasion of the brain during acute infection can result in meningitis. Studies using cultured endothelial cells suggested that C. albicans penetrates the endothelial cell barrier through transcellular migration. Nevertheless, the integrity of the blood-brain barrier during this process has not been directly studied. In order to investigate damage caused to the integrity of the blood-brain barrier by candidia invasion in the CNS, we employed MRI imaging of brain using a vascular contrast agent Gd-DTPA in a mouse model of disseminated candidiasis. We found in-vivo evidence for breaching in the blood-brain barrier during disseminated Candida infections. Breaching of blood-brain barrier increased during the post inoculation period showing higher leakage of Gd-DTPA in a time point infection model. Then we employed Two-Photon microscopy and a Candida strain expressing green florescent protein to monitor real-time dynamics of C. albicans in mouse brain. We found that C. albicans escapes from the brain vasculature and penetrates brain tissue as early as 2 hours post inoculation. The same method visualized filamentous growth of C. albicans, a known virulence attribute of candidiasis, inside the brain tissues. These imaging methods could be useful tools to further study pathogenesis of C. albicans infections and to assess new therapeutic measures and drug development.

Neuroscience - General

Hao, Hong
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NEI

Mapping transcription regulatory networks in rod photoreceptors and implications for retinal degeneration

Precise control of gene expression is fundamental to the function and survival of neurons. Rod and cone photoreceptors are light-sensing neurons that are responsible for night vision and day light vision, respectively. The transcription factor NRL determines and maintains a rod photoreceptor cell identity by initiating transcription regulatory networks to activate the expression of rod-specific genes and simultaneously suppress cone-specific genes. The transcription factor CRX is required for photoreceptor terminal maturation but not for cell fate determination. Together with NRL, CRX co-regulates several phototransduction genes. Mutations in human NRL, CRX and a few target genes have been identified in patients with inherited retinal degeneration and blindness. Here, we identified important components of NRL-centered transcription regulatory networks: genome-wide direct NRL target genes, cofactors and

secondary regulatory nodes, by interrogating NRL in vivo occupancy using ChIP-Seq, global expression profiling using microarray analysis. We identified 281 direct NRL transcriptional target genes. Mutations in 22 of the identified NRL targets are known to cause human retinal degeneration, whereas 95 targets are mapped to regions of retinal disease loci where the disease genes remain to be identified. In vivo knockdown of 16 target genes by shRNAs led to photoreceptor cell death and/or abnormal morphology, suggesting that NRL target genes are excellent candidates for retinal disease gene screening. About 70% of the NRL ChIP-Seq peak regions overlap with published CRX ChIP-Seq peak regions. Furthermore, the photoreceptor-specific genes are largely co-occupied by NRL and CRX, suggesting global co-regulation of these genes by the two factors. In silico analysis of NRL ChIP-Seq peak regions revealed an enrichment of distinct sets of binding sites for additional transcription factors. Using In vivo knockdown, gene expression profiling and in vivo functional assays, we identified histone demethylase Kdm5b and myocyte enhancer factor 2c as novel downstream regulators in the NRL transcriptional hierarchy. Mapping NRL-centered transcription regulatory networks facilitates the identification of retinal disease genes and therapeutic targets.

Neuroscience - General

Gu, Zhenglin
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Cholinergic coordination of pre- and postsynaptic activity induces timing-dependent hippocampal synaptic plasticity

Correlated pre- and postsynaptic activity is the core element in inducing Hebbian plasticity, including spike timing-dependent plasticity (STDP). However, little is known about the physiological events that could mediate such correlation. Recently we have found that correlated cholinergic input can induce STDP-like alpha7 nicotinic acetylcholine receptor (nAChR)-dependent hippocampal synaptic long-term potentiation (LTP) or short-term depression (STD). Alpha7 nAChRs, as well as other neuromodulator receptors, are localized at both pre- and postsynaptic sites of glutamatergic synapses, and thus providing a potential mechanism to coordinate pre- and postsynaptic activities to induce synaptic plasticity. To test this, we directly monitored the pre- and postsynaptic activities of hippocampal CA3 Schaffer collateral (SC) to CA1 synapses with genetically-encoded calcium indicators (green GCaMP3 and red R-GECO1) in septo (providing cholinergic innervation)-hippocampal co-cultures. R-GECO1 (in neuron specific synapsin promoter-driven AAV virus) was microinjected into hippocampal CA1 neurons and the dendritic spines in the CA1 SR (stratum radiatum) layer were monitored as postsynaptic activity; GCaMP3 was microinjected into CA3 neurons and the projecting SC axons in CA1 SR layer were monitored as presynaptic activity. We found that the alpha7 nAChR-dependent LTP (induced by pairing cholinergic pathway 100 ms before SC pathway) involves prolonged enhancement of both pre- and postsynaptic activities, and both were abolished in alpha7 nAChR knockout slices. Restoring alpha7 nAChRs to either pre- or postsynaptic sites in knockout slices only resulted in short-term potentiation at either site. Restoring alpha7 nAChRs to both sites of knockout slices was required to restore the LTP. On the other hand, the alpha7 nAChR-dependent STD (induced by pairing cholinergic pathway 10 ms before SC pathway) involves transient depression of both pre- and postsynaptic activities, and both were abolished in knockout slices. Restoring alpha7 nAChRs to both sites was also required to restore the STD in knockout slices, while there was no effect on either site if only restored to one site. These results suggest that correlated cholinergic input, through the alpha7 nAChRs, can coordinate pre- and postsynaptic activities to induce synaptic plasticity, providing a novel mechanism for neuromodulators to precisely modulate network activity during higher brain functions.

Neuroscience - General

Liu, Ning
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NIMH

Hierarchical organization of face-selective regions in macaque cortex as revealed by fMRI and pharmacological deactivation

One of the most remarkable properties of the visual system is the ability to recognize and discriminate a wide variety of faces effortlessly. Neuroimaging studies in humans and monkeys have reported several face-selective regions (â€˜face patchesâ€™) in the inferior temporal (IT) cortex, which respond more strongly to faces as compared to non-face objects. In addition, there are several face-responsive regions (e.g. the amygdala), which respond more strongly to faces as compared to scrambled faces. By using fMRI combined with electrical microstimulation, it has been demonstrated that these regions are interconnected. However, the functional relationship among these regions remains unclear. To clarify this functional network, we used fMRI combined with pharmacological silencing of specific nodes in this network. First, we mapped the face-selective regions. The expected set of face-selective regions was found in IT cortex: the posterior and anterior face patches. Next, by using a novel variable angle guide grid, we infused the GABAA agonist muscimol into the given face patches to silence them, and then repeated the initial localizing fMRI experiments. Different from the classic deactivation method, MRI contrast agent Gadolinium was added into muscimol solution so that the infusion results (e.g., location and size) were visualized in MRI images. We found that after deactivation of the posterior face patches, the responses to both faces and non-face objects in the anterior patches were eliminated. After deactivating the anterior face patches, the posterior face patches still but less responded to faces compared to vehicle infusion sessions. However, deactivation of the anterior face patches did not change the responses to non-face objects in the posterior face patches. In both conditions, the responses to faces and non-face objects were as same as vehicle infusion sessions in the object-selective regions (which responded more strongly to non-face objects as compared to faces), whereas reduced in the amygdala. These results demonstrate that the functional network among the face-selective and face-responsive regions is hierarchically organized and specifically processes face-related information. The anterior face patches in IT cortex require inputs from the posterior face patches for their activation. However, face selectivity in the posterior face patches arises in part from top-down inputs from the anterior face patches.

Neuroscience - General

Stevens, William
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Category Dissociations in Ventral Temporal Cortex are Associated with Different Patterns of Intrinsic Functional Connectivity

The topographical organization of category-specific regions in the human ventral temporal cortex (VTC) is remarkably consistent across individuals, including regions showing preferential activation for conceptual categories such as faces, animals, bodies, objects, tools, scenes, and words. To date, the determinants of this organization are largely unknown. One possibility is that it is driven, in part, by differential intrinsic connectivity of these category-regions with other functionally relevant brain areas critical for processing and representing different types of category-relevant properties. This preferential intrinsic connectivity would then facilitate task-related co-activation of these brain regions underlying perceptual and conceptual processing. Numerous studies have shown that analysis of spontaneous low-frequency fluctuations of fMRI BOLD signal at rest (rs-fcMRI) can be used to reveal intrinsic functional

brain networks. We used fMRI and a multi-category functional localizer to identify dissociable regions of VTC showing preferential responses to different categories of stimuli (e.g, tools, scenes, faces) in healthy young adults. We then used these ROIs as seeds in whole-brain rs-fcMRI analyses of independent rest-runs in these same participants to explore the differential intrinsic connectivity of these regions across the brain. Our results demonstrate that across participants, different category-preferential regions in VTC show strong intrinsic rs-fcMRI with other regions of the cortex that represent information about the properties associated with these different categories. For example, a tool-responsive region in the left medial portion of the fusiform gyrus showed strong connectivity with brain regions associated with: 1) perceiving non-biological motion (posterior middle temporal gyrus); 2) storing and/or retrieving â€œmotor programsâ€ (ventral premotor cortex); and 3) executing complex hand gestures (inferior parietal lobe). By contrast, strong intrinsic connectivity was observed between scene-preferential posterior parahippocampal cortex and retrosplenial cortex involved in contextual associations, and between face-preferential lateral fusiform cortex and superior temporal sulcus involved in perception of biological motion. These results are consistent with the idea that the topographical organization of category-specialized regions in VTC depends on connectivity with other domain relevant regions of the brain.

Neuroscience - General

McNeil, Stephanie
Doctoral Candidate
NINDS

Identification of a novel dynamin family large GTPase that regulates density of dendritic spines

Large GTPases of the dynamin family are important regulators of membrane dynamics, as they target to specific membranes and mediate their fission or fusion. The dynamin family is relatively small, and no new family members have been identified in nearly 10 years. Here we report the identification of graduatin, a novel large GTPase, through both sequence homology to the dynamin family and functional assays. We found graduatin can hydrolyze GTP, and that a single point mutation of a critical lysine in the G1 domain (GTPase mutant) diminished its catalytic activity. We have shown that graduatin exclusively localizes to endosomes, making it the only large GTPase with that subcellular distribution. Graduatin is brain specific, with its expression restricted to particular types of neurons in the postnatal brain, including the pyramidal neurons of the hippocampus and cortex. Pyramidal neurons have complex dendritic arbors coved in spinesâ€”small membranous protrusions containing the post-synaptic sites of neuronal communication. AMPA-type ionotropic glutamate receptors are concentrated on these spines, and are critical for receiving and relaying excitatory signals. Over-expressing the GTPase mutant in cultured hippocampal neurons resulted in significantly reduced spine density on the pyramidal neurons. Fitting with those findings, over-expression of the GTPase mutant also decreased AMPA receptor (AMPAR) surface expression in these neurons. Graduatin was first identified in a screen for proteins containing a RING domain. To investigate the importance of this domain, we generated a RING domain mutant of graduatin, but found no effect on spine density or AMPAR levelsâ€”suggesting that the GTPase domain of graduatin is the critical region for proper spine maintenance. We established a graduatin knock-out (KO) mouse and found that the brains of KO animals were smaller and contained reduced levels of synaptic proteins, including AMPARs and PSD-95, compared to their wild type littermates. Using Golgi staining to visualize spines in brains of KO mice, we observed a decrease in spine density compared to wild type littermates, suggesting a physiological role for graduatin. By culturing hippocampal neurons from KO mice, we found that over-expressing wild type, but not the GTPase mutant, graduatin completely rescued the spine density back to wild type levels; thereby confirming that spine defects are not developmental and that graduatin is required to maintain spine density.

Neuroscience - Integrative, Functional, and Cognitive

Cui, Guohong
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NIAAA

In-Vivo Optical Measurement Reveals Concurrent Activation of Striatal Direct- and Indirect-Pathway Neurons in Freely Moving Mice

The basal ganglia are a group of subcortical nuclei that that control voluntary actions and are affected by a number of debilitating neurological disorders. The classical model of the basal ganglia proposed that information from cortical inputs is processed in the basal ganglia via two opposing projection systems: the so-called direct and indirect pathways, originating from two populations of medium-sized spiny neurons (MSNs) in the striatum. According to this classical model, activation of direct-pathway MSNs facilitates movement and activation of indirect-pathway MSNs inhibits movement. However, due to the lack of methods to selectively record from direct- and indirect-pathway MSNs in freely moving animals, the activities of these two groups of neurons during voluntary movements still remain unknown. Here we developed a novel in-vivo method to measure direct- and indirect-pathway MSN neural activities using Cre-dependent viral expression of the genetically encoded calcium indicator (GECI) GCAMP3 in the dorsal striatum of D1-Cre (direct-pathway specific) and A2A-Cre (indirect-pathway specific) mice. Using fiber optics and time-correlated single photon counting (TCSPC), we observed frequent transient increases in neural activities in both direct- and indirect-pathway MSNs in freely moving mice. These transients were most frequent during active states when animals were actively engaged in lever-pressing and magazine checking behaviors, but rarely occurred during inactive periods when they were resting or consuming food reward. Sensory stimuli caused time-locked activation in both direct- and indirect-pathway MSNs. Initiation of contraversive movement was preceded by co-activation of MSNs in both pathways. These results demonstrated that our new in-vivo method is a valuable tool for exploring subcortical cell type-specific neural activities in freely moving animals. Our observations in direct- and indirect-pathway MSNs contrast with the classical model and suggest a collaborative rather than antagonistic relationship between these two pathways.

Neuroscience - Integrative, Functional, and Cognitive

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NICHD

Investigating neuronal circuit modulation of startle behavior using larval zebrafish

Startle behavior is observed across vertebrates in response to sudden aversive environmental stimuli. Several neuropsychiatric disorders including Schizophrenia cause disruptions in startle behavior and reduced prepulse inhibition (PPI) of the startle response. PPI suppresses the startle response and occurs when a non-startling stimulus precedes a startling one by a few hundred milliseconds, a period defined as the interstimulus interval (ISI). The development of and mechanisms by which neuronal circuits modulate startle remains to be fully elucidated. Larval zebrafish demonstrate PPI at similar ISI to mammals. With its comparatively simple nervous system and established high throughput methods for analyzing behavior, it is a desirable model with which to examine startle. To begin these analyses, we generated over 200 enhancer trap lines with Gal4 expression in various CNS regions. Gal4 was used to drive expression of UAS-Nitroreductase in 46 of these lines to conditionally ablate defined CNS areas by exposing larvae to the prodrug metronidazole. Ablated larvae were then examined for defects in acoustic startle responsiveness and PPI. We identified line xa213 to be more responsive to weak

acoustic stimuli and to have reduced PPI at long ISI (500ms). At shorter ISI (30-100ms), xa213 has increased PPI suggesting that at least two distinct mechanisms exist to generate PPI dependent on the ISI. Examination of UAS:GFP expression in xa213 reveals columns of Gal4 positive neurons in the hindbrain, reminiscent of Glutamatergic and GABAergic neurotransmitter columns that form during zebrafish brainstem development. Analysis of UAS:synaptophysin expression in xa213 hindbrain shows that presynaptic punta lie in close proximity to both the eighth nerve which transmits signals from the ear to the CNS and the Mauthner neuron which fires to produce a startle maneuver. This positioning suggests that xa213 neurons are within the same circuit known to control startle. To determine a genetic basis for PPI, we mapped the gal4 integration site in xa213 by LM-PCR to a region ~20kb upstream of the homeobox transcription factor gsx1. Consistent with our mapping, in situ hybridization and immunohistochemical analyses show that gsx1 expression is nearly identical to that of Gal4 in xa213. Intriguingly, gsx1 expression is conserved between zebrafish and mouse, suggesting that this population of neurons might be analogous in form and function across vertebrates.

Neuroscience - Integrative, Functional, and Cognitive

Yokogawa, Tohei
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The dorsal raphe modulates sensory responsiveness during arousal in zebrafish

Animals are not constantly vigilant, but rather modulate their readiness to respond to sensory stimuli. This is particularly apparent when comparing states of sleep and wakefulness, however while awake, short-term behavioral states enable the organism to adapt to changing environmental demands. One such state is arousal, where animals anticipate challenges by showing heightened activity and readiness to respond to sensory stimuli. The serotonergic system is strongly linked to regulation of arousal states across the sleep-wake dimension. To understand the specific role of the serotonergic system during arousal, we developed a behavior based arousal assay using zebrafish. Zebrafish are a genetically modifiable vertebrate species with a transparent body with a simple brain structure relative to mammals. We first challenged fish with several types of stimulus including water flow, visual patterns and vibration. Among these stimuli only water flow induced elevated locomotion. During the hyperactive period, fish showed heightened sensitivity to perceived motion, suggesting that flow triggers a state of arousal. We generated a stable transgenic line (tph2:Gal4) with specific expression of Gal4 in serotonergic dorsal raphe (DR) neurons for further anatomical and functional analysis. Calcium imaging of neuronal activity using UAS:GCaMP3 in aroused fish showed increased activity in DR neurons after a water flow stimulus. Genetic ablation of these neurons using UAS driven toxin abolished the increase in sensory responsiveness without affecting locomotion. These experiments show that DR neurons are activated by the water flow stimulus and specifically required to modulate sensory responsiveness during arousal. To understand how the DR modifies the arousal state we traced its projection using a UAS driven TagRFP reporter. DR neurons projected to the optic tectum, an area important for processing visual information. Laser ablation of the optic tectum demonstrated that this structure, like the DR, is required for improved visual sensitivity during arousal. Based on these experiments we propose a model in which the DR improves performance during arousal by modulating activity within the optic tectum.

Neuroscience - Integrative, Functional, and Cognitive

Britt, Jonathan
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NIDA

Hippocampal afferents to the nucleus accumbens convey reinforcement signals

The nucleus accumbens (NAc) facilitates reward seeking by identifying and capitalizing upon reward-associated cues. This region is thought to integrate dopaminergic reinforcement signals with the glutamate-encoded descriptive features that characterize any given moment in time. Recent work found inconsistencies with this characterization, since mice would work for the selective stimulation of glutamatergic amygdala inputs to the NAc. To assess the implications of this, we asked if there was something unique about these amygdala afferents or if this was simply a general feature of strong inputs to the NAc. Brain slice electrophysiology combined with pathway-specific optogenetic techniques showed that larger postsynaptic responses could be elicited from hippocampal fibers in the medial NAc shell than from amygdala or prefrontal cortex inputs. Consistent with this, viral-mediated yellow fluorescent protein expression showed hippocampal afferents were uniquely concentrated in the medial NAc shell. Due to the importance of the NAc in drug addiction, we also examined each afferent pathway following repeated cocaine injections. Hippocampal synapses were selectively potentiated following this drug treatment and optogenetic manipulations to this pathway in vivo could attenuate or exaggerate acute cocaine-induced locomotor responses. In drug-naïve mice, the selective activation of hippocampal afferents to the NAc could generate a place preference and elicit motivated responses. These findings challenge the notion that these glutamatergic afferents convey motivationally-neutral descriptive information. Without any direct provocation of dopamine signals, the activation of strong glutamate afferents to the NAc shell is rewarding and sufficient to reinforce behavior. This suggests these glutamatergic fibers encode value and, in light of our current understanding of this system, probably convey the incentive properties of a given situation. Accordingly, dopamine might not be the signal that bestows qualities of reinforcement. Those qualities are inherent in the glutamate signals. This reasoning relegates the dopamine signal to the position of arbiter of reinforcement, where it can gate and regulate the influence of the glutamatergic incentive signals clamoring to direct behavior.

Neuroscience - Integrative, Functional, and Cognitive

Pagani, Jerome
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NIMH

Oxytocin Decouples Aggression from Anxiety through its Influence on the Serotonergic System

Our understanding of oxytocin (Oxt) has moved beyond a role in lactation and parturition to neuronally mediated effects on anxiety, social and reproductive behaviors, and learning and memory. Recent work in mice suggests that Oxt may influence behavior by interaction with other neurotransmitter systems: Oxt appears to functionally regulate serotonergic tone and anxiety through the large number of Oxt receptors (Oxtr) in the serotonergic raphe nuclei. We hypothesized that Oxtr in the raphe nuclei might effect aggression given the strong link between the serotonergic system and reactive aggression. We created mice with loxP sites flanking the gene for the Oxtr (Oxtr flox/flox) that when crossed with mice expressing Cre recombinase under control of cell- or region-specific promoters, have selectively inactivated Oxtr. For this experiment, we crossed Oxtr flox/flox mice with mice expressing Cre recombinase under control of the serotonin transporter promoter (Slc6a4-cre) to selectively inactivate Oxtr in the dorsal raphe subset of the serotonergic system. The knockout (KO) mice generated by this cross are normal across a range of behavioral measures: there are no effects on locomotion in an open field, olfactory habituation/dishabituation or anxiety behaviors in the zero and plus mazes. We did find a profound deficit in aggression, however. Only 1 out of 12 DR Oxtr KO showed any aggressive behavior, compared to 8 out of 12 wildtype (WT). The single attacking KO mouse had fewer attacks, tail rattles and shorter attack duration than WT mice. This demonstrates a profound effect on a social behavior

(aggression) by selectively eliminating one type of receptor in a specific class of cells, and that Oxt is a key regulator of reactive aggression via its effects on the serotonergic system. Moreover this is the first demonstration of neurobiological mechanisms that segregate aggression and anxiety. This has implications not only for the treatment of anxiety disorders where aggression is not frequently comorbid, but also borderline personality disorder and combat-related PTSD in which it frequently is.

Neuroscience - Neurodegeneration and Neurological disorders

Rothman, Sarah
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NIA

Chronic Mild Sleep Restriction Exacerbates Biochemical and Cognitive Deficits in a Mouse Model of Alzheimerâ€™s Disease

Age-related disruption of the sleep-wake cycle and circadian rhythms are well documented. However, neurodegenerative diseases are often associated with sleep disturbances beyond what is observed in normal aging. Alzheimerâ€™s disease (AD), characterized by impaired memory, is associated with sleep abnormalities, including increased sleep time and sleep fragmentation, that cannot be explained by aging alone. Nighttime sleep fragmentation, decreased slow wave sleep and frequent daytime napping occur in 25-35% of AD patients. Very few studies have described sleep disturbances in animal models of AD, and despite the prevalence of sleep loss in AD, the possible effects of sleep loss on AD cognitive decline and underlying brain pathology are unknown. Here we describe sleep patterns in a mouse model of AD and determine how clinically-relevant chronic mild sleep restriction (SR) affects behavioral and pathological outcomes in an animal model of AD. Male AD (3xTgAD) mice and wild-type controls were placed in a Comprehensive Lab Animal Monitoring System (CLAMS) which uses infrared beam breaks to outline activity patterns and extrapolate sleep estimates over 48 hrs. A separate set of 3xTgAD mice were exposed to SR using a platform-over-water technique for 6 hrs/day for 6 weeks to test for an affect of chronic mild SR on plasma corticosterone (a measure of stress), cognition, and cortical hippocampal Abeta and ptau levels. Results were compared to non-SR controls. AD mice display a dampening of circadian activity patterns compared to wild-type controls at 9 months of age, prior to the onset of cognitive symptoms or pathology. SR induces an increase in circulating corticosterone at both 1 and 4 weeks after the start of the SR period. SR also causes a worsening of contextual memory as measured by the fear conditioning paradigm and a worsening of AD pathology in the cortex including increases in the levels of both Abeta and ptau. Circulating corticosterone is positively and significantly correlated with both hippocampal and cortical Abeta levels, potentially indicating a role for corticosterone signaling in mediating observed changes after SR. Our findings suggest that sleep abnormalities may accelerate disease progression in AD.

Neuroscience - Neurodegeneration and Neurological disorders

Wang, Zhifei
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NIMH

Chronic Valproate Treatment Enhances Post-ischemic Angiogenesis and Promotes Functional Recovery in a Rat Model of Ischemic Stroke

Stroke is the third leading cause of death in the US and still lacks effective treatments. Angiogenesis is a process in which new capillaries are formed through directed proliferation and migration of endothelial progenitor cells from pre-existing blood vessels. Post-stroke angiogenesis restores blood supply to the injured tissue, provides neurotrophic support to concurrent neurogenesis and synaptogenesis, and

ultimately leads to functional recovery. Therefore, enhancement of post-stroke angiogenesis holds great promise for the treatment of stroke. Previous studies from our laboratory demonstrated that valproate (VPA), a histone deacetylase (HDAC) inhibitor, protects against experimental brain ischemia. The present study investigated whether VPA could enhance angiogenesis and promote long-term functional recovery after ischemic stroke. Male rats underwent middle cerebral artery occlusion (MCAO) for 1 hour followed by reperfusion. VPA (200 mg/kg, i.p.) was administrated immediately after ischemic onset, 12 hours later, and once daily for up to 14 days. Post-ischemic VPA treatment robustly improved the rotarod performance of MCAO rats on days 7 and 14 after ischemia, and significantly reduced brain infarction on day 14, as assessed by T2-weighted MRI. Concurrently, VPA markedly enhanced microvessel density and facilitated endothelial cell proliferation. VPA robustly increased relative cerebral blood flow in the ipsilateral cortex, as detected by perfusion-weighted imaging. The transcription factor hypoxia-inducible factor (HIF)-1alpha and its downstream pro-angiogenic factors, vascular endothelial growth factor (VEGF) and matrix metalloproteinase (MMP)-2/9, were upregulated after MCAO and significantly potentiated by VPA treatment in the ipsilateral cortex. Histone-H3 acetylation was robustly increased by chronic VPA treatment, indicating HDAC inhibition. Moreover, the beneficial effects of VPA on rotarod performance and microvessel density were abolished by HIF-1alpha inhibition. Our findings suggest that (1) chronic VPA treatment enhances post-ischemic angiogenesis and promotes long-term functional recovery in an experimental model of ischemic stroke, and (2) the angiogenic effects of VPA likely involve HDAC inhibition and upregulation of HIF-1alpha and the downstream pro-angiogenic factors VEGF and MMP-2/9. These findings lead to a better understanding of the beneficial effects of VPA against ischemic stroke and pave the way for potential clinical trials.

Neuroscience - Neurodegeneration and Neurological disorders

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NINDS

Translational technologies for neurological disease: Targeting PINK1 and Parkin to modulate mitochondrial quality control

Parkinsonâ€™s disease (PD) is a complex disorder that is poorly understood on the molecular level. Currently, there are no agents that can prevent the neural pathology of PD. To develop therapeutics that will aid millions with PD, we must understand and modulate the molecular basis of the disorder. Mitochondrial dysfunction is increasingly recognized as a key contributor to neurodegenerative disease. Recently, the putative kinase PINK1 has been shown to act as a sensor of mitochondrial dysfunction. Upon mitochondrial insult, Pink1 translocates to the outer membranes of mitochondria where it recruits the E3-ubiquitin ligase Parkin by an unknown mechanism. Activated Parkin initiates signaling to eliminate damaged mitochondria by an autophagic process (mitophagy). Mutation of PINK1 and Parkin appears in familial PD, highlighting mitochondrial dysfunction as a core pathological mechanism. My hypothesis is that mitophagy is a useful point of intervention to prevent dopaminergic neuron loss, a hallmark of PD. To discover drug-like compounds that induce clearance of damaged mitochondria from neurons, I built a high quality (Zâ€™ > 0.5) cell-based assay for Pink1 expression. I have screened the assay against a library of ~400,000 compounds to find those that increase Pink1 expression in a mitophagy-relevant manner. Screening is being followed with a battery of diverse cell and biochemical assays for profiling of putative hits. By analyzing drug action with gene mutants, knockouts, and iPS cells generated from PD patients, I am evaluating specificity and toxicity. Rodent models of PD are being employed for pharmacokinetic analysis. To date, I have discovered a handful of drug-like molecules that can modulate PINK1 expression without reducing cell viability. My second research aim utilizes high content imaging with genome-wide RNAi screening to map the genetic pathways of mitophagy. By

identifying genes that regulate mitophagy, I will be opening the door to future therapeutic interventions. To this end, I developed a robust cell-based assay (Zâ€™ > 0.5) that reports both Parkin activation and elimination of damaged mitochondria. After executing a genome-wide siRNA screens, hits were confirmed with rigorous secondary assays. I have revealed a number of novel genes that modulate mitophagy. The siRNA screens revealed that Tomm7 is likely a key receptor for PINK1 translocation and the NF-?B pathway converges with PINK1/Parkin-mediated mitophagy.

Neuroscience - Neurodegeneration and Neurological disorders

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Manganese Enhanced MRI of APP-induced neurodegeneration and recovery in an olfactory-based AD model

Olfactory dysfunction is an early symptom of Alzheimerâ€™s disease (AD), suggesting that olfactory sensory neurons (OSNs) are more sensitive to AD related factors then neurons in other brain parts. A reversible olfactory-based AD model recently established that degeneration of OSNs can be rapidly induced by simply over expressing amyloid precursor protein (APP), a protein linked to AD. With this transgenic model, we sought to follow the progression of neural pathology and its recovery using MRI. This would enable direct tracking of neurodegeneration through a non- invasive in vivo measurements and the ability to correlate them with functional assays. Manganese enhanced MRI (MEMRI), provides a unique contrast in the rodent brain. It can detect layers in different areas of the brain, including olfactory bulb (OB), cortex and cerebellum and highlight discrete anatomical features such as glomeruli in the OB. Since OSNs regenerate continuously throughout life and project their axons directly to OB glomeruli, we performed MEMRI in mutant mice to detect both the degeneration and recovery of OSNs with particular focus on the glomerular layer. We infused, 100 mM MnCl2 isotonic solution i.v. in the tail vein (88 mg/kg at a rate of 0.25 ml/h) of both control and transgenic mice (BW: 15-27 gr). 3D T1-weighted images of 50 um isotropic resolution (TR/TE=40/4.4 ms, flip angle 25 degrees, scan time = 50 min) were acquired on 11.7 Tesla scanner, 24h later. Our findings showed that in 3-4 week old mutant mice, OB volumes decreased by ~50% compared to controls. OB shape and structure was also altered with the glomerular layer virtually eliminated. After APP expression was turned off by feeding 3 week old mutant mice doxcycline containing chow for a week, we observed a striking recovery of OB layers, and reappearance of the glomerular layer but no significant increase in OB volume. This study demonstrates that MEMRI can detect specific anatomical changes associated with both APP-induced neurodegeneration and recovery in the OB. Currently we are extending this approach to assess the ability of different pharmacological reagents to block olfactory neural loss. Ultimately, we believe that MEMRI can serve as a unique in vivo screening tool to both identify potential therapeutics and to test their efficacy.

Neuroscience - Neurodegeneration and Neurological disorders

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Investigating Axonal Damage in Multiple Sclerosis by Diffusion Tensor Spectroscopy

Multiple sclerosis (MS) is a chronic, immune-mediated demyelinating and neurodegenerative disease of the central nervous system. As axonal damage is an important determinant of clinical status in MS, sensitive and specific in vivo measures of axonal damage might greatly benefit prognostication and

therapy assessment. The long-term effects of neurodegeneration can be detected in the form of tissue loss (atrophy), whereas ongoing neurodegeneration, manifested as neurons that are damaged but still intact, is exceedingly difficult to measure in vivo. Diffusion tensor spectroscopy (DTS) combines features of diffusion tensor imaging and magnetic resonance spectroscopy, allowing measurement of the diffusion properties of intracellular, cell-type-specific metabolites. As such, it may provide specific information about axonal microstructure and might consequently serve as a useful marker of axonal integrity in the complex mixture of pathologies that characterizes MS (inflammation, demyelination, edema, gliosis, axonopathy). In this cross-sectional pilot study, diffusion of the neuronal metabolite N-acetylaspartate (NAA) was measured in the human normal appearing corpus callosum on a 7 tesla MRI scanner, comparing 15 MS patients and 14 healthy controls. NAA diffusion was compared to MRI measures of water diffusion and brain atrophy and clinical measures of disease severity. We found that NAA parallel diffusivity (D||) is lower in MS (p=0.030) and inversely correlated with both water D|| (p=0.020) and clinical severity (p=0.015). Based on histological characterizations of diffuse WM injury in MS and simulated diffusion models of axon damage in complex geometries, we expect that the structural disruptions described in axonal degeneration would restrict the diffusion of NAA along the length of axons and would be associated with lower NAA D||, as seen in our data. Decreased NAA D|| may confirm the presence of axonopathy in the face of overall increased diffusion of water, which is commonly observed in MS but pathologically nonspecific. This is highlighted by the correlation between clinical status and NAA D|| (but not water D||), since disability may be more closely related to neuroaxonal pathology than to inflammation. Interpreted in the context of previous experiments, our findings provide preliminary evidence that DTS can distinguish axonopathy from other processes such as inflammation, edema, demyelination, and gliosis.

Neurotransmission and Ion Channels

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NIMH

Target-specific Suppression of GABA Release from Parvalbumin-Interneurons in Basolateral Amygdala by Dopamine

Activation of dopamine receptors in basolateral amygdala (BLA), the major input site of amygdala nucleus, is necessary for acquisition and expression of fear memory. At the cellular level, it has been shown that dopaminergic signaling facilitates sensory input from cortex to BLA by attenuating feedforward inhibition of excitatory principal neurons (PNs). However, the synaptological mechanisms underlying dopaminergic disinhibitory effects remain unclear. Given high heterogeneity of BLA interneurons, it is essential to determine how specific interneuronal subtypes in BLA contribute to dopaminergic disinhibition of PNs. The parvalbumin-expressing interneurons (PV-INs) control firing of PNs through robust perisomatic inhibition and form the largest interneuronal subpopulation in BLA. Here, we hypothesized that DA disinhibits amygdala by targeting PV-INs. To test this hypothesis, we selectively expressed channelrhodopsin 2, a heterologous light-activated cation channel, in PV-INs of BLA and evoked GABA release from PV-INs by photostimulation of amygdala slice with pulses of blue light. Using whole-cell patch-clamp recording, we found that DA suppressed light-induced inhibitory postsynaptic currents (IPSCs) in BLA PNs. The suppression was accompanied by an increase in the paired-pulse ratio, which indicates that DA inhibits presynaptic release of GABA from PV-INs. At the same time, DA did not affect GABAa receptor-mediated currents evoked by local puffing of GABAa receptor agonist muscimol, which indicates that DA does not inhibit postsynaptic response to GABA in PNs. Furthermore, the inhibitory effect of DA was blocked by D2 receptor antagonist raclopride but not by D1 receptor antagonist SCH23390. Consistently, D2 receptor agonist quinpirole, but not D1 receptor

agonist SKF38393 mimicked the inhibitory effect of DA. Taken together, these findings show that DA suppresses GABAergic transmission between PV-INs and PNs by activating presynaptic D2 receptor. To examine how DA modulates synaptic transmission between PV-INs and other INs, we repeated same experiment while recording from INs visualized in GAD67-GFP transgenic mice. In contrast to its effect in PNs, DA did not suppress IPSCs in INs. In conclusion, the present results provide direct evidence that DA targets PV-INs for amygdala disinhibition and that effect of DA on GABA release from the same PV-IN is determined by the nature of its postsynaptic targets.

Neurotransmission and Ion Channels
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NIMH
Are the enzymes of the catecholamine biosynthetic pathway locally synthesized in the axon?
Catecholamines control a wide range of brain functions, and altered levels of these neurotransmitters have been implicated in conditions such as Parkinsonâ€™s disease, schizophrenia and depression. Tyrosine hydroxylase (TH) catalyzes the rate-limiting step of catecholamine synthesis, the conversion of tyrosine to L-DOPA. In noradrenergic neurons, TH has been shown to be synthesized in the cell soma and shipped to the axon through anterograde axonal transport. However, recent studies have shown that axons contain a heterogeneous population of mRNA, and that local protein synthesis is important for axonal function. In this study, we tested the hypothesis that TH can be synthesized in axons. To isolate pure axonal mRNA and protein, rat superior cervical ganglion (SCG) neurons were cultured in compartmentalized Campenot chambers. RT-PCR and fluorescence in situ hybridization (FISH) analyses showed that TH mRNA is present in axons. A significant increase in the relative abundance of TH mRNA was observed when axons were grown under conditions that led to an increase in the number of synaptic terminals. Metabolic labeling of axonally synthesized proteins was performed by incubating the axonal compartments of Campenot chambers with the methionine analogue L-azidohomoalaine (AHA). AHA-labeled TH was detected in axonal protein extracts but not in those from the corresponding neuronal somas, indicating that labeled TH was specifically synthesized in axons. Furthermore, AHA-labeled TH was detected in axonal protein extracts from distal axons that were severed from the cell bodies prior to metabolic labeling with AHA. SILAC-based mass spectrometric (MS) analysis confirmed that TH is locally synthesized. Preliminary data on dopamine beta hydroxylase (DBH), the enzyme that catalyzes the conversion of dopamine to norepinephrine, suggest that DBH may also be locally synthesized in SCG axons. Our results show that the mRNA encoding TH is present and locally translated in axons. A higher relative abundance of TH mRNA correlated with an increase in the number of synaptic terminals indicating that axonal TH mRNA expression is inducible, and suggesting that it may be regulated during neuronal differentiation. Taken together, these results show that axonal TH mRNA is functional. Finally, preliminary data on DBH point to the possibility that multiple components involved in catecholamine synthesis are axonally synthesized.

Neurotransmission and Ion Channels
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NINDS
Syntaxin 1 modulates activity-dependent inhibition of voltage-gated calcium channels at a central synapse

Repetitive neuronal firing triggers short-term synaptic depression that is critical for information processing within brain circuitry. Multiple lines of evidence suggest that activity-dependent inactivation of voltage-gated calcium channels (VGCCs) plays a major role in mediating such short-term synaptic depression. Despite in vitro studies demonstrating that SNARE proteins, which are essential in mediating vesicle fusion, are able to bind and inhibit VGCCs and therefore transmitter release, it remains unclear how VGCC inactivation is regulated by the release machinery in the synapse. We recorded the presynaptic calcium current at the calyx of Held, a large glutamatergic synapse, while injecting various Botunlinum neurotoxins to specifically cleave distinct SNARE proteins. Here we show that cleavage of syntaxin 1 by injection of Botunlinum neurotoxin C caused a slight but significant increase in calcium currents, suggesting that syntaxin 1 normally induces a steady-state inhibition of VGCCs at the synapse. More importantly, calcium-dependent inactivation of VGCCs during high frequency train stimulation was remarkably alleviated by injection of Botulinum neurotoxin C. In contrast, cleavage of SNAP-25 by Botulinum neurotoxin E had no effect on calcium currents or activity-dependent inactivation of VGCCs, despite a similar blockade of exocytosis. Our findings suggest a novel mechanism by which syntaxin-VGCC interaction promotes activity-dependent depression of VGCCs, in addition to the well-established steady-state inhibition of VGCCs. Therefore, we propose that syntaxin-VGCC interaction not only regulates the baseline strength of transmitter release at the synapse, but also activity-dependent synaptic plasticity.

Pharmacology and Toxicology/Environmental Health
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NIAAA

ROLE OF JNK-MEDIATED PHOSPHORYLATION IN MITOCHONDRIAL DYSFUNCTION AND LIVER INJURY

Background and aims: c-Jun N-terminal protein kinase (JNK), activated by a variety of apoptosis stimulants, modulates cell death pathways. However, it is unknown how activated JNK promotes cell/organ damage. In fact, the target proteins of JNK-mediated phosphorylation and their roles in organ damage are poorly understood. We aimed to study the role of JNK-mediated phosphorylation of mitochondrial proteins in mitochondrial dysfunction and organ damage. Methods: We used a mouse model of acute liver injury by hepatotoxic carbon tetrachloride (CCl4), which potently activates JNK. Male 129/Svj mice were injected with a single dose of CCl4 (0.4 g/kg, ip, n=4/group) and killed at 1, 2, 4, 8 and 24 h post-injection. Liver histology, blood alanine aminotransferase (ALT), and other enzyme activities were measured in CCl4-exposed mice without or with a new JNK inhibitor, which blocked JNK. Results: JNK was activated as early as 1 h and it lasted up to 4 h post-CCl4 injection, while liver damage assessed by histology and ALT levels was maximal at 24 h. Activated (phosphorylated) JNK was translocated to mitochondria and JNK-mediated phosphorylation of many mitochondrial proteins were observed at 2, 4 and 8 h in CCl4-exposed mice. Pretreatment with new JNK inhibitors, BI78D3 and SU3327, significantly reduced CCl4-mediated liver damage evidenced by ALT activity and liver histology. We also observed markedly reduction of JNK activation and phosphorylated mitochondrial proteins by SU3327 pretreatment, suggesting a causal relationship between JNK-related phosphorylation and liver damage in CCl4-exposed mice. Thus we purified the phosphorylated mitochondrial proteins by metal-affinity chromatography and determined their sequences. Mass spectral data showed that more than 180 mitochondrial proteins including ATP synthase (complex V), aldehyde dehydrogenase (ALDH2), glutathione peroxidase, 3-ketoacylCoA thiolase, NADH-ubiquinone oxidoreductase (complex I), and pyruvate dehydrogenase (PDH) were phosphorylated in CCl4-exposed mice. Similar to JNK-mediated phosphorylation and suppression of PDH, ALDH2, a key defensive enzyme, was phosphorylated and inhibited in CCl4-exposed animals while the suppressed ALDH2 activity was restored by SU3327

pretreatment. Conclusion: These data demonstrate for the first time that activities of many key mitochondrial proteins are modulated by JNK-mediated phosphorylation, contributing to robust mitochondrial dysfunction and acute liver damage.

Pharmacology and Toxicology/Environmental Health
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NIDCR
A novel positive allosteric modulator of TRPV1 with improved potency
The transient receptor potential ion channel, family V, number 1 (TRPV1) protein is a nonselective cation channel playing a key role in physiological and patho-physiological pain sensation. Side effects produced by the systemic inhibition of TRPV1 seriously limited the use of orthosteric vanilloid antagonists as analgesics. Positive allosteric modulators (PAMs) could circumvent these problems by acting in a state-dependent fashion, provoking selective inactivation of nociceptive nerve endings through calcium overload only if they express active TRPV1. We performed a high-throughput screen (HTS) of the MLSMR library collection to identify novel PAMs of TRPV1 with improved potency. The HTS identified 19 candidate PAMs, of which we chose one for further structure-activity relationship studies. Testing of 40 analogs identified a novel positive modulator, NPT-32, with increased potency when compared to our previously described proof-of-concept PAM, MRS1477. NPT-32 reduces the EC50 value of capsaicin from 110nM +/-11 to 25.26nM+/-5, compared to a reduction to 62.44nM+/-9 in the presence of MRS1477. The efficacy of TRPV1 activation is also enhanced with a comparable 50% increase in calcium uptake at saturating agonist concentrations for both compounds. NPT-32 also positively modulates pH activation of TRPV1, reducing both the extracellular proton concentration necessary for activation and increasing efficacy by 100% at saturating pH. MRS1477 achieved a comparable reduction in the EC50 of pH activation with only 50% efficacy modulation at pH5.4 or below. Co-injection of NTP-32 or MRS1477 with capsaicin into the hind paws of rats resulted in significant thermal analgesia in vivo when compared to capsaicin-only treated animals. Although the effect of the PAMs in vivo was comparable, NPT-32 caused thermal analgesia at markedly lower concentrations, confirming the increased potency over MRS1477 observed in vitro. In summary, through HTS and subsequent SAR we identified a novel PAM of TRPV1 with improved potency. We demonstrated both in vitro and in vivo activity, the later resulting in thermal analgesia. These results suggest the possibility of developing novel analgesics that target TRPV1 in a state-dependent manner and would yield a long duration localized effect. These characteristics will avoid the side-effects associated with TRPV1 antagonists and may allow the full exploitation of TRPV1 as a target for analgesic drug development.

Pharmacology and Toxicology/Environmental Health
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Glucocorticoid Receptor (GR) Regulation of P-glycoprotein (Pgp) at the Blood-Brain Barrier (BBB)
The blood-brain barrier (BBB), comprised of the brain capillary endothelium, provides a major obstacle to drug delivery to the brain. An important element of barrier function is the ATP-driven drug efflux transporter, Pgp, which exhibits broad substrate specificity and high luminal plasma membrane expression in brain capillaries. Previous studies demonstrated that certain ligand-activated nuclear receptors upregulate ATP-driven transporter expression at the BBB. Here, we examined whether GR, a ligand-activated nuclear receptor targeted by an extensive class of synthetic glucocorticoids (GCs),

regulates Pgp at the BBB. The effects of synthetic GCs on BBB properties are poorly understood. However, given the potent anti-inflammatory properties of synthetic glucocorticoids, these drugs are routinely used for the treatment of cerebral edema during brain-tumor chemotherapy. Therefore, it is critical to understand how synthetic GCs modulate BBB properties. We hypothesize that synthetic GCs increase the activity and expression of Pgp, thereby limiting the efficiency of drug delivery to the brain. The basal expression of GR in rat brain capillaries was confirmed by western blotting. Adrenalectomized (depleted of endogenous GCs) and intact rats were injected with the synthetic glucocorticoid dexamethasone and 24 hours later brain capillaries were isolated for Pgp transport activity analysis (confocal imaging of accumulation of fluorescent substrate in the capillary lumen) and Pgp protein expression (western blots). In-vivo dexamethasone treatment of both intact and adrenalectomized rats significantly increased Pgp activity and protein expression in brain capillaries. Exposing capillaries (from adrenalectomized and intact rats) to dexamethasone in vitro increased Pgp activity and protein expression in a concentration-dependent manner. These increases were abolished by co-treatment with the GR antagonist RU486. These results show that dexamethasone treatment, in the presence and absence of endogenous GCs, increases the activity and expression of the potent drug efflux pump, Pgp, effectively tightening the barrier for a large number of therapeutic drugs. Thus, the use of synthetic GCs as adjuvants may hinder delivery of other therapeutic drugs to the brain.

Pharmacology and Toxicology/Environmental Health
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Differential susceptibility to ozone-induced lung inflammation maps to mouse chromosome 17: role of Notch receptors
Ozone (O3) is a highly toxic air pollutant and worldwide public health concern. O3 exacerbates preexisting lung conditions and causes airway hyperresponsiveness and inflammation. Mechanisms of genetic susceptibility to O3-induced airway inflammation are not completely understood. We identified a significant quantitative trait locus on mouse chromosome 17 (Inf2) that contains candidate susceptibility genes for O3-induced airway inflammation, including histocompatibility genes and the Tnf (tumor necrosis factor) gene cluster. Also located in Inf2 is Notch4, and proximally adjacent to Inf2 is Notch3. Notch receptors are cell surface receptors important in development and immune cell differentiation. We hypothesized that Notch3 and Notch4 are determinants of susceptibility to O3-induced airway inflammation. To test this hypothesis, wild type (B6129SF1, WT), Notch3 (Notch3-/-) and Notch4 (Notch4-/-) knockout mice were exposed to O3 (0.3 ppm) or filtered air continuously for 6-48 hr. Immediately after exposure, airway inflammation and injury was assessed using protein concentration (a marker of lung permeability) and inflammatory cells in bronchoalveolar lavage fluid (BALF). O3 significantly increased BALF protein in all genotypes, but greater concentrations were found in Notch3-/- compared to WT (24, 48 hr), and concentrations were greater in Notch4-/- mice compared to Notch3-/- (24, 48 hr). Greater mean numbers of BALF neutrophils were found in Notch3-/- and Notch4-/- mice compared to WT (24, 48 hr). Expression of whole lung Tnf was significantly increased after O3 (24 hr) in all genotypes, and was greater in Notch3-/- and Notch4-/- compared to WT. Pre-treatment with the TNFa inhibitor etanercept significantly attenuated the enhanced O3-induced BALF neutrophils in Notch3-/- and Notch4-/- relative to WT. O3-induced transcript expression of other Inf2 genes was not different between genotypes. We then used mRNA transcriptomics analyses to further investigate the role of Notch3/4. Statistical and visual data mining approaches identified differentially expressed genes basally [e.g. Gbp1, Cntn1] and after O3 [e.g. Ccl7, Il33] between WT and KO mice. Results are consistent with the hypothesis that Notch3 and Notch4 are susceptibility genes for O3-induced airway

inflammation. Furthermore, results suggest an important interaction between Notch3, Notch4, and Tnf. These novel findings suggest Notch receptors protect against the innate immune inflammatory response to O3.

Pharmacology and Toxicology/Environmental Health
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Substance P exacerbates neurotoxins-induced nigral dopaminergic neurodegeneration through activation of microglial NADPH oxidase
Dysregulation of substance P (SP), a major endogenous neuropeptide present in the striatonigral projecting pathway, has been linked with Parkinsonâ€™s disease (PD). However, roles of SP in regulating long-term survival of dopaminergic neurons in the substantia nigra (SN) remain unstudied. Here, we demonstrate that SP exacerbates dopaminergic neurodegeneration in rodent PD models through augmenting microglia-mediated neuroinflammation. Two in vivo rodent PD models were employed: 1) a single dose of LPS or 2) repeated MPTP regimen in SP-deficient (TAC1-/-), neurokinin-1 receptor (conventional G-proteinâ€“couple receptor for SP) knockout (NK-1R-/-) or wild type (WT) mice. We found that lack of endogenous SP significantly decreased LPS- or MPTP-induced dopaminergic neurotoxicity in both SN and striatum and brain neuroinflammation. Surprisingly, no difference of the above-mentioned changes were observed between NK-1R-/- and WT mice, suggesting that these synergistic effects of SP and neurotoxins are not mediated through the conventional NK-1R. In midbrain neuron-glia cultures, SP enhanced both LPS- and MPP+-induced dopaminergic neurodegeneration and neuroinflammation with an interesting bimodal dose-response curve: effective in nanomolar (10-8~10-7 M) and subpicomolar (10-14~10-13 M) concentrations, but not in between. Further studies revealed that subpicomolar SP, which is released from terminals of the striatonigral neurons, diffused out synaptic junction and acted on the surrounding microglia. We identified NADPH oxidase, an important superoxide-producing enzyme in microglia, was a novel NK1R-independent target responsible for the synergistic effects of SP and neurotoxins. Additional mechanistic studies showed that SP directly bound to gp91phox, the membrane subunit of NADPH oxidase, through C-terminal PHE-GLY-LEU-MET domain and then either alone or with LPS induced membrane translocation of cytosolic subunits p47phox and p67phox, resulting in NADPH oxidase activation. Signaling studies further showed that SP was capable of enhancing production of TNFa by potentiating LPS-induced activation of MAPK and NF-?B pathway through NADPH oxidase-dependent manner. Altogether, we have demonstrated a GPCR-independent novel mechanism whereby SP displays its pro-inflammatory effect by directly binding to the gp91phox and increasing superoxide production, which is critically linked with synergistic neurotoxic effects between SP and neurotoxins in PD.

Physiology
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NCI-CCR
A novel mouse model of airway epithelium remodeling
The epithelial lining of the respiratory tract protects against harmful aerosol particles by mucociliary clearance. It consists of Clara, goblet, ciliated and basal cells that work in concert. In response to inhaled stimuli, the goblet cells increase in number and secrete mucins to form a mucus layer whereas the ciliated cells clear the mucus from the airways. With this regard, excessive airway mucus secretion is a

reflection of certain respiratory pathologic conditions, e.g. asthma and chronic obstructive pulmonary disease (COPD). Goblet cells may be derived from subsets of Clara cells or from ciliated cells via transdifferentiation. A recent in vitro study showed that goblet cells are derived from Foxj1-expressing airway epithelial cells. The expression of the transcription factor Foxj1 is regulated by Wnt pathway. We and others have shown that in vitro the transcription factor ASCL1 is able to regulate Wnt pathway. In the current study we examined whether ASCL1 can alter airway differentiation in vivo by activating Wnt and Foxj1 expression. We generated transgenic mice in which Clara cell-specific CC10 promoter drives constitutive ASCL1 expression. Airway epithelial cells were identified by immunostaining of lineage-specific cell markers, including CC10 for Clara cells, Foxj1 for ciliated and goblet precursors, beta-tubulin for ciliated cells, and MUC5AC (mucin) for goblet cells. The wild type littermates were used as controls in this study. At 9 months, the ASCL1-transgenic mice showed massive increase in MUC5AC-positive cells in the airways, consistent with goblet cell hyperplasia that extended into terminal bronchioles. In addition, the number of Foxj1-positive progenitors was increased by 90 % in the peripheral airways. The increase of Foxj1-positive cells was accompanied by up-regulation of Sox2, a critical gene for airway homeostasis. Also, ciliated cell population was increased by 33%. In contrast, Clara cell number was reduced to 50%. Our data suggests that ASCL1 can promote the differentiation of both goblet and ciliated cells through increasing the precursor population. The underlying mechanism may involve up-regulation of Foxj1 and Sox2. We conclude that ASCL1 transgenic mice provide a novel mouse model for airway pathology that greatly mimics the characteristic conditions that seen in human respiratory diseases such as cystic fibrosis, asthma, and COPD. This model has potential therapeutic implications on common lung diseases.

Physiology
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NIDCR

Coordination of mitochondrial activity across the rat salivary glands epithelium imaged by intravital two-photon microscopy

In individual cells, levels of the reduced form of nicotinamide adenine dinucleotide (NADH) have been reported to oscillate periodically. These oscillations are linked to mitochondrial activity that is regulated through reactive oxygen species and are thought to correlate with the spatio-temporal redox state of the cell. In order to investigate whether this phenomenon occurs in vivo, we imaged the salivary glands (SGs) of live rats using intravital microscopy, exploiting the fact that NADH emits upon two-photon excitation. Fast scan imaging allowed us to capture periodic oscillations of NADH levels under normal physiological conditions in vivo. Strikingly, we observed for the first time, that NADH oscillations are temporally and spatially synchronized across the SGs epithelium. To confirm that these oscillations were linked to mitochondrial activity, we used cationic dyes, such as TMRM and Rhodamine123, which are sensitive to mitochondrial membrane potential. Maximal mitochondrial activity was initially observed in specific areas of the epithelium and propagated throughout the tissue in a wave-like pattern. This phenomenon was not observed in confluent cell cultures, in explanted SGs, or in vivo, when the blood flow was reduced by ligation of the vessels. As expected, disruption of mitochondrial membrane potential led to the disruption of wave propagation. We hypothesized that synchronized oscillations might be related to basal secretory activity of the SGs that is mediated by several receptors. To test this hypothesis, we used a pharmacological approach. Inhibition or stimulation of muscarinic, alpha-adrenergic and purinergic receptors did not affect the propagation of the waves, whereas stimulation of beta-adrenergic receptors led to a sharp and uniform increase in NADH fluorescence across the tissue and the loss of the oscillations. Interestingly, both the oscillations and the propagation of the waves were restored 20 minutes after stimulation, consistent with the reported clearance of the agonist from

the tissue. Based on our findings, we speculated that NADH oscillations and their synchronization might have implications in the coordination of tissue function under physiological conditions. Ongoing work is focusing on identifying the mode of signal propagation (e.g. through gap junctions or small diffusible molecule), on better understanding of its regulation, and the alteration of oscillations and signal propagation under pathological conditions.

Protein Structure/Structural Biology
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Computational separation of conformational heterogeneity using cryo-electron tomography and 3D sub-volume averaging
Cryo-electron tomography (CET) is a three-dimensional (3D) imaging method, which enables the structural study of biological specimens frozen in vitreous ice. Consequently, cryo-electron tomography constitutes a structural analysis tool able to resolve the organization of macromolecular assemblies close to their native, physiological context. The low signal to noise ratio (SNR) imposed by the data collection conditions of cryo-electron tomography limits the resolution that can be discerned in individual tomograms. Averaging of accurately aligned 3D images (sub-tomograms) collected from many instances of the same molecular entity enables the determination of density maps at resolution of ~20 Angstrom. We have previously used cryo-electron tomography combined with sub-volume averaging and classification to obtain 3D structures of macromolecular assemblies in cases where a single dominant species was present, and applied these methods to the analysis of a variety of trimeric HIV-1 and SIV envelope glycoproteins (Env). Here, we extend these studies by demonstrating automated, iterative, missing wedge-corrected 3D image alignment and classification methods to distinguish multiple conformations that are present simultaneously. We present a method for measuring and representing the distribution of conformational states found in a specimen and identifying data processing strategies that allow clear separation of the different conformations. We validate these procedures for computational separation by successfully separating and reconstructing distinct 3D structures for unliganded and antibody-liganded as well as open and closed conformations of Env present simultaneously in mixtures. We show that identifying and removing spikes with the lowest signal-to-noise ratios improves the overall accuracy of alignment between individual Env sub-volumes, and that alignment accuracy, in turn, determines the success of image classification in assessing conformational heterogeneity in heterogeneous mixtures. This development extends the sub-tomogram averaging capabilities to heterogeneous samples. Furthermore, it turns cryo-electron tomography into a powerful analytical tool that can directly determine the relative amount of the different conformations found in the specimen.

Protein Structure/Structural Biology
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NCI-CCR
Structural Basis for NusB in the Initiation of Transcription Antitermination and dsRNA Supercoiling
Processive transcription antitermination by bacteriophage lambda is initiated by the formation of a ternary complex of bacterial host proteins, NusB and NusE, and the phage RNA sequence, BoxA. We have elucidated the structure of the NusB-NusE-BoxA ternary complex by X-ray crystallography, demonstrating that the BoxA RNA sequence is composed of eight nucleotides recognized by the NusB-

NusE heterodimer. The protein sequences taken from the thermophilic bacterium, Aquifex aeolicus, were cloned using the Gateway cloning system, overexpressed in E. coli, and purified using standard protein isolation and chromatographic methods. Supporting functional and biophysical data confirmed the relative significance of key protein-protein and protein-RNA interactions. Further crystallographic investigation of a NusB-NusE-dsRNA complex revealed a heretofore-unobserved dsRNA-binding site contiguous with the BoxA-binding site. We propose that the observed dsRNA represents the phage BoxB stem-loop, as both single-stranded BoxA and double-stranded BoxB components are present in the classical lambda antitermination site. Together, these data provided a specific model for the assembly of the complete antitermination complex, thus furthering our understanding of this viral regulation of gene expression. Through temperature variation and further crystallographic screening, we have implicated a novel role for NusB in the higher-order assembly of dsRNA. Genome packaging is an essential housekeeping process in virtually all organisms for proper storage and maintenance of genetic information. Although the extent of packaging and its associated mechanisms vary, the process involves the formation of nucleic-acid superstructures. However, such superstructures have not been revealed for RNA. We have provided the first atomic-resolution structure of protein-mediated RNA supercoils, which displays one level higher molecular organization than previously reported structures of DNA coiled coils. In this case, two interlocking coiled coils of dsRNA, a â€œcoil of coiled coilsâ€, form a plectonemic supercoil. The crystal lattice exists as an ordered array of these RNA supercoils, whose packaging is mediated and stabilized by NusB via the proteinâ€™s dsRNA-binding site. This study provides novel structural information for protein-mediated assembly of nucleic-acid superstructures, and is particularly useful in understanding essential genome packaging mechanisms of dsRNA viruses.

Protein Structure/Structural Biology
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Antigenic Switching of HBV by Alternative Dimerization of core- and e-Antigens
Hepatitis B virus remains a major public health threat: 360 million people worldwide are chronically infected, resulting in one million deaths annually from liver cancer and cirrhosis. Two key aspects of HBV infection remain poorly understood: (1) how HBV evades the immune system in chronic infection and (2) the function of HBV e-antigen (eAg). Recent evidence suggests that the two may be related. eAg is a variant of the capsid protein (cAg; core-antigen) that does not assemble into capsids, is secreted into sera, and has different antibody reactivities than cAg. These observations are mysterious upon considering that eAg and cAg share the same protein sequence, save for 10 extra propeptide residues at the N-terminus of eAg. Nevertheless, eAg is used as a clinical marker for HBV replication and disease severity, and is present in all members of Hepadnaviridae, altogether suggesting it has an important and conserved function. I aimed to explore the long-standing question of how modest alteration of sequence termini can so profoundly affect the assembly and antigenic properties between cAg and eAg. To this end, I have crystallized and determined the structure of a complex of eAg and an anti-eAg monoclonal antibody fragment (Fab), exploiting a separately determined structure of the Fab. The antigensâ€™ monomer folds are very similar; however in eAg, the propeptide induces a radically altered mode of dimerization (relative 140 deg. rotation) compared to cAg, locked into place through formation of a novel intramolecular disulfide bridge. To test the importance of the disulfide on eAg structure, negative-stain electron microscopy and analytical ultracentrifugation were used to assay eAg assembly state vs. oxidation state. Under reducing conditions, the eAg disulfide is disrupted and the subunits revert to a cAg-like association mode, forming capsid-like particles. The eAg structural switch reveals how capsid assembly is prevented and a distinct antigenic repertoire is created, permitting evasion of the robust

cytotoxic response that cAg evokes upon interaction with B cells. Despite this, the antigens remain highly cross-reactive at the T-cell level due to sequence identity. This duality is likely to be central to eAgâ€™s role in establishing immune tolerance for cAg. The crystal structure now provides a framework upon which further study can fully elucidate the role of eAg in HBV persistence, both as an antigenic decoy and a direct modulator of the immune system.

Protein Structure/Structural Biology
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Biogenesis of beta-barrel membrane proteins
Membrane proteins are important therapeutic targets due to their role in nutrient import, signaling, motility and survival. While the mechanism by which membrane proteins are integrated into the membrane has been well established for alpha-helical membrane proteins, the mechanism for integration of beta-barrel membrane proteins has remained elusive. Beta-barrel membrane proteins are a unique type of membrane protein that is found in the outer membranes of Gram-negative bacteria, mitochondria, and chloroplasts. The complexes responsible folding and inserting the nascent beta-barrel proteins into the membrane have been identified and are conserved across species. For Gram-negative bacteria, a five-component complex called the beta-barrel assembly machinery (BAM) complex is required for membrane integration. It consists of an integral membrane protein called BamA (a beta-barrel membrane protein itself) and four lipoproteins called BamB-E. The crystal structures of all the components of the BAM complex have been reported, except for the structure of full length BamA, but they have failed to give clues that might help postulate a plausible mechanism for membrane integration by the BAM complex, suggesting that the structure of BamA will be required in order to understand this unique mechanism. To this end, we have produced crystals of two different homologs of BamA diffracting to 3.1 and 3.2 angstroms, respectively. Preliminary analysis indicates that the beta-barrel domain of BamA consists of 16-strands with a large periplasmic domain (known to interact with other Bam proteins) sitting in close proximity to the periplasmic face of the beta-barrel domain. This domain has been shown to be very flexible and may directly interact with the nascent beta-barrel protein. In addition, a long extracellular loop (Loop 6) appears to be folding down within the inside of the beta-barrel domain and could potentially interact with nascent beta-barrel proteins or even other Bam proteins. Based on these and other studies, our working model for the mechanism of membrane protein integration by the BAM complex involves initial recognition of the nascent beta-barrel protein via periplasmic chaperones, a pre-folding event involving the periplasmic domain of BamA and the other Bam proteins, and a final integration step that requires the beta-barrel domain of BamA, including Loop 6. This work represents a crucial step towards understanding this unique and important mechanism.

Psychiatry
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NIMH
Functional metabotropic glutamate receptor subtype 5 (mGluR5) levels are altered in the brain cortex of Fragile X syndrome
Purpose: Fragile X syndrome (FXS) is a common inherited form of mental retardation caused by loss of function of the fragile X mental retardation protein (FMRP). Recent preclinical rodent studies suggest that dysregulation of downstream signaling by metabotropic glutamate receptor subtype 5 (mGluR5)

may be a key factor in the multiple cognitive and behavioral abnormalities associated with FXS. However, there are no reports on alterations in functional mGluR5 levels in brain tissue of FXS. Methods: To investigate the functional mGluR5 levels in brain, the current study used in vitro radioligand binding and immunoblotting to measure the binding parameters (receptor density (Bmax); dissociation constant (KD); specific binding (SB); binding potential (Bmax/KD) and expression of mGluR5. Measures were obtained from the postmortem prefrontal cortex (PFC) of FXS subjects (n = 15) as well as matched controls (n = 17), and from the neocortex of FXS model (Fmr1 knock-out) mice (n = 10) and control mice (n = 11). Results: Bmax was significantly increased (19%; P < 0.05) in the postmortem PFC of individuals with FXS compared to controls; this was associated with significantly elevated (36%; P < 0.03) mGluR5 expression, equivalent KD, and a trend toward elevated SB and Bmax/KD. In contrast, in the neocortex of Fmr1 knock-out mice, mGluR5 Bmax tended to decrease (7%; P = 0.15), with a significant reduction (14%; P < 0.03) in mGluR5 expression and equivalent levels of KD, SB, and Bmax/KD, compared to control mice. Conclusions: The results suggest that alterations in upstream mGluR5 levels may contribute to the pathophysiology associated with FXS. The opposite trend noted in FXS model mice may indicate that mGluR5 signaling is differentially regulated in mice and humans. Detecting changes in functional mGlur5 levels could be of potential diagnostic and therapeutic value for FXS patients treated with mGluR5 modulators.

Psychiatry
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Exploring the Anxious Brain at Rest: Increased Subcortico-Frontal Coupling Associated with an Anxious State

Neuroimaging research has traditionally explored fear and anxiety through the use of discrete threat cues, such as those in fear conditioning paradigms. However, a more relevant approach to understanding clinical anxiety comes from paradigms that elicit sustained anticipatory anxiety. Here we implement a translational method of anxiety-induction (unpredictable threat of shock) that serves as a robust model of anxiety disorders. Anxiety creates an emotional, cognitive and physiological context that prepares organisms for a rapid defensive response to potential threat. No prior investigations have examined such neural context. Resting-state fMRI is uniquely suited to examine intrinsic, task-independent differences in neural networks. We used this technique to probe how anticipatory anxiety alters amygdala-prefrontal cortical (PFC) connectivity in order to support a defensive response. Twenty healthy participants (13 m) were scanned under threat of shock (no shock given) and safe (no shock) conditions. Subjects viewed a single word (THREAT or SAFE) during two 6-minute scans. Respiration and pulse data were collected and used to control for physiological and scanner-related variations in the MR signal. Neural connectivity during threat and safety was assessed with AFNI. Findings show differential amygdala-prefrontal connectivity during threat versus safe. Under threat (where subjects reported more anxiety) versus safe, amygdala activity was positively correlated with insula, dorsomedial PFC, and thalamus. Conversely, under safe versus threat, amygdala activity was positively correlated with ventromedial PFC (vmPFC) and precuneus. Results suggest anticipatory anxiety increases connectivity between the amygdala and regions involved in threat appraisal (medial PFC), as well as autonomic nervous system control (thalamus), indicating this network may facilitate preparatory behaviors (e.g., vigilance, avoidance). In contrast, safety was associated with increased connectivity between amygdala and regions implicated in fear reduction (vmPFC) and introspection (precuneus). These results are underscored by similar findings in anxiety patients, suggesting that pathological and induced anxiety both create a specific preparatory context for responding to threat that is distinct from the default

mode network. Future research may use this preparatory context to identify biomarkers of anxious pathology and target these circuits for therapeutic intervention.

Radiology/Imaging/PET and Neuroimaging
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CC
Exploring Novel Associations Between Clinical and Imaging Features for Predicting Abnormal Imaging Patterns of Pulmonary Infections
Radiology serves as a primary diagnostic method for assessing pulmonary infections, monitoring disease progression and determining response to therapy. Computed tomography (CT) is a widely utilized imaging modality that provides detailed anatomical information for identifying potential sites of disease. On the other hand, clinical laboratory measurements elucidate underlying biological mechanisms of a certain disease without any anatomical information. However, there is a lack of effective computational and statistical methods integrating image data in quantified usable formats and clinical laboratory information for diagnosing disease, assessing disease severity, and generating scientific observations. In this study, we designed and tested a novel hybrid statistical model that integrates radiologic image and clinical features in order to automatically predict abnormalities in chest CT scans and identify potentially important infectious disease biomarkers. Our novel data exploration method and experimental study demonstrates that certain variables, such as hemoglobin level, mean corpuscular volume, summation of average intensities, and run-lengths features, have potentially useful and novel diagnostic relationships with anatomic pulmonary abnormalities. In 156 patients, 116 with various pulmonary infections and 40 healthy controls, we extracted 34 clinical variables from laboratory tests, and 25 textural features from CT scans. The proposed system included two important steps: i) global identification of abnormal imaging pattern by adaptively selected features, and ii) local selection of the most important features from the previous step, and assign them as biomarkers depending on their prediction accuracy. By the proposed method, we can reliably identify the following abnormal lung patterns with high accuracy: consolidations (86.02%), pulmonary nodules (63.44%), tree-in-bud (70.97%), ground glass opacities (84.95%), pleural effusions (82.8%), and linear thickening (64.52%). Results demonstrate that systematically revealing quantitative and statistical relationships between seemingly unrelated information spaces can facilitate early detection of infectious pulmonary disease by uniquely integrating clinical and radiologic imaging data. We demonstrated that quantified texture features in radiologic images could be statistically integrated with clinical variables to improve mathematical modeling of disease in identifying potentially important biomarkers.

Radiology/Imaging/PET and Neuroimaging
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Human Breast Cancer Cells Are Sensitized to Ionizing Radiation by a Novel Multi-targeted Kinase inhibitor RGB 286638.
A majority of patients with cancer will have therapeutic radiation as a component of their treatment. Radiation sensitizers are compounds that act synergistically with radiation to increase killing of malignant cells. One focus of our lab is the evaluation of select chemotherapeutic agents in pre-clinical development as potential radiation sensitizers, in hopes of improving patient treatment and outcome. RGB-286638 is a multi-targeted kinase inhibitor that has been shown to result in tumor regression in

preclinical models of solid and hematological malignancies. Therefore, we evaluated this compound for potential sensitization to ionizing radiation (IR) in breast tumors. Radiosensitizing effects of RGB-286638 on the in vitro human breast cancer cells, MDA MB231 and others control cells were evaluated using standard clonogenic assays. The Dose Enhancement Factor (DEF) was determined from standard survival curves. DNA damage, repair and mitotic catastrophe were examined using fluorescence microscopy. In addition, flow cytometry and western blot assays were performed to understand the cell cycle distribution, apoptotic fraction, and the mechanism of observed effects. Pre-incubation of tumor cells with a low dose of 40 nM RGB-286638 (IC50 is >>10 μM) for 24 H followed by exposure to IR (2, 4, 6, and 8 Gy) showed significant sensitization with a DEF of 1.48 (48% increase in effective radiation dose yielding 90% killing). Treatment with RGB-286638 (40 nM) alone for 24 hours induced apoptosis in ~29% of cells. However, our data indicate that the RGB-286638-mediated radiosensitization of MDA MB231 cells does not involve enhanced susceptibility to apoptosis but resulted in enhancement of mitotic catastrophe. As a measure of DNA double strand breaks (DSBs), ?-H2AX foci were determined as a function of time after drug pre-treatment followed by IR. After 24-H pre-treatment with RGB-286638, greater ?H2AX foci formation and increased delay in dispersal of foci was observed relative to after IR alone. Cell cycle analysis after 24-H pre-incubation with RGB-286638 with or without subsequent IR showed a proportion of cells in G2 delay. In conclusion, at low concentrations more than 500-fold below its IC50, RGB-286638 effectively enhanced the radiosensitivity of MDA MB231 cells with a remarkable DEF of 1.48. Radiation-induced DNA damage repair is the underlying primary mechanism involved in radiosensitization of RGB 286638.

Radiology/Imaging/PET and Neuroimaging

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Imaging the needle and the haystack - Simultaneous Correlative 3D imaging of nanoscale objects within large biological volumes by Focused Ion Beam Scanning Electron Microscopy

Electron microscopic (EM) imaging of small regions of interest within a larger field of view, such as a 100 nm diameter virus inside a 1000 cubic micron cellular volume, with high fidelity and efficiency poses a significant challenge in biology. Serial section transmission EM or tomography have been employed, but these are slow and often performed manually; in addition, these methods suffer from a crippling trade-off between image resolution and image size. Focused Ion Beam Scanning Electron Microscopy (FIB-SEM) is a recently developed technique for 3D EM imaging of resin-embedded biological samples. Here, image stacks are generated via iterative cycles of milling or â€œslicingâ€ of the sample by a focused ion beam followed by SEM imaging of the newly revealed face. The 2D stacks are then computationally converted to a 3D image volume. Here we describe advances in FIB-SEM imaging that allow for routine and automated high-quality imaging cells and tissue samples as large as 20,000 cubic microns. We have determined optimal imaging conditions and milling protocols to generate image stacks with xy pixel sampling as high as 3 x 3 nm and z slices consistently as thin as 5 nm. Crucially, we show that we can now rapidly image a large field of view at intermediate resolution and simultaneously image a selected sub-region of interest at higher resolution in x,y and z, thereby resolving the problematic trade-off between resolution and volume. We tested our system by attempting to image a single HIV core in the cytoplasm of an entire mammalian cell. Using fluorescence microscopy, we first produced a â€œtarget mapâ€ of a HeLa cell transfected with TRIM-5a-YFP and infected by VSV-g pseudotyped HIV virions containing Vpr-GFP. We then generated a correlated high-quality 3D EM volume of the entire cell, and simultaneously, targeted high-resolution 3D images of cytoplasmic TRIM bodies. We also localized and imaged an individual HIV core associated with a TRIM body, thus demonstrating the ability to image

volumes across a scale of 10^9 in a single dataset. The ability to perform selective high-resolution imaging reduced the time required to image this volume from 28 days of continuous imaging to a single overnight run. These results represent significant progress toward â€œpoint-and-clickâ€ imaging where one can efficiently visualize the nanoscale local structure as well as mesoscale global context of biological objects of interest.

Radiology/Imaging/PET and Neuroimaging
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PET imaging of serotonin transporter in monkeys: effects of maternal separation, and chronic fluoxetine treatment during adolescence
Major depressive disorder (MDD) is a serious disorder that often begins following stress during adolescence. Selective serotonin reuptake inhibitors (SSRIs) are a common treatment for both adolescent and adult MDD. While MDDâ€™s early onset and available efficacy data support use of SSRIs in adolescents, concerns about safety have arisen, based on associations with suicidal behavior in adolescents, coupled with minimal data on long-term effects on the developing brain. This study used rhesus monkeys as a model to study the long term effects of both early life stress and chronic antidepressant treatment during adolescence (2-year of age) on the serotonin transporter. Thirty-two monkeys were randomly assigned to one of four groups (8 monkeys / group). They were peer-reared (PR) vs. mother-reared (MR), and with or without fluoxetine treatment. For PR, monkeys were separated from mothers at birth and nursery-reared until 6 months of age and thereafter housed with their peers. Chronic fluoxetine treatment began at 2-year of age for one year. One to two years post-washout, monkeys (average age of 5) were scanned with Positron Emission Tomography (PET) using [11C]DASB for serotonin transporter (SERT). Our data from 32 monkeys (8 in each group) show 1. A significant global decrease in SERT binding in PR compared to MR monkeys, and 2. SERT binding is reversed in PR monkeys that received fluoxetine treatment. Our study demonstrates serotoninergic alterations in PR monkeys, and chronic flouxetine treatment may reverse deficits in SERT density that is persistent more than one year after medication discontinuation.

Signal Transduction - General
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NIAID
A truncated splice-variant of FceRIß confers calmodulin binding critical for microtubule formation and degranulation following Ca2+ influx in human mast cells
Mast cells play a key role in allergic diseases, such as asthma, through the sustained release of a plethora of proinflammatory mediators. Mast cells present in the asthmatic lung display a hyper-responsive phenotype with evidence of on-going mediator release. Importantly, the gene locus containing the MS4A2 gene (encoding FceRIß) has strong linkage to allergy and asthma susceptibility. Since FceRIß expression is specific to mast cells, MS4A2 was considered as a candidate gene, but studies on the functional consequence of mutations in FceRIß have been disappointing. We have identified a truncation of FceRIß (t-FceRIß) in humans and in this study we aimed to identify the function of this variant. We used lentiviral shRNA to silence the FceRIß variants and identified that t-FceRIß is required for human mast cell degranulation and cytokine production, but not for eicosanoid production or Ca2+ influx. We demonstrate that the requirement for t-FceRIß in degranulation is downstream of Ca2+ influx

and upstream of cytoskeletal reorganization. We also demonstrate that t-FceRIß forms a complex with Fyn kinase; the adaptor molecule Gab2; the p85 subunit of PI3K, and the microtubule-forming component, a-tubulin: all critical signals, or in the case of a-tubulin, components for microtubule formation. Molecular modeling revealed that the truncation of FceRIß exposes a putative calmodulin binding domain. Thus in the presence of Ca2+, calmodulin binds to the t-FceRIß complex conferring phosphorylation of the complex components. Confocal microscopy demonstrated translocation of the t-FceRIß complex to a tubulin-rich region after stimulation which was followed by microtubule formation and F-actin depolymerization. Knockdown of t-FceRIß eliminated microtubule formation, but F-actin depolymerization was unaffected. 3D reconstruction of the imaging data revealed that the translocated t-FceRIß complex surrounded the centrosome and microtubules. The translocated t-FceRIß complex co-localized with the cis-Golgi protein GM130 which has been shown to play an important role in anchoring the Golgi to the centrosome. Thus we demonstrate the mechanism for a novel function of a novel protein which acts to propagate Ca2+ signals, linking Ca2+-influx to microtubule formation, granule translocation to the membrane and degranulation. These data represent important findings since the t-FceRIß complex represents an excellent drug target which would, presumably, be mast cell specific.

Signal Transduction - General

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p53 cooperates with MAP kinase and NFkB signal transduction pathways to potentiate human immune/inflammatory response

The p53 tumor suppressor can regulate transcription of genes associated with a wide range of cellular functions including apoptosis, growth arrest, DNA repair, differentiation and glycolysis. Recently, we extended this list to include the Toll-like receptor (TLR) human innate immunity genes. TLRs are highly conserved integral membrane glycoproteins that recognize a variety of chemically distinct pathogen-associated molecular patterns (PAMPs). Upon stimulation, TLRs recruit adaptor molecules that lead to activation of NF?B, interferon responsive factors (IRFs) and MAP kinases resulting in distinct patterns of gene expression essential to immune/inflammatory responses and elimination of pathogens. To determine the extent to which p53 dependent upregulation of the receptors could influence downstream response, we utilized a pair of isogenic cell lines with very different levels of p53: wild type p53 MCF7 breast adenocarcinoma cells or MCF7 cells stably transfected with p53 shRNA. Cells were pre-treated with Nutlin-3 to induce p53 and subsequent induction of TLR receptors (Nutlin-3 blocks the p53 inhibitor MDM2) and then exposed to TLR5 ligand flagellin. We found a dramatic (over 10-fold) p53-dependent amplification of cytokines IL-6 and IL-8 mRNA and protein production in response to flagellin. This change in response was accompanied by a specific increase in phosphorylation of p38 MAP kinase; p38 inhibitor SB203580 prevented the p53 dependent increase in IL-6 mRNA levels following flagellin exposure. On the contrary, IL-8 levels were not affected by p38 inhibition. To delineate the signaling pathway(s) linking p53 expression to p38 activation and enhanced cytokine production we performed gene expression analysis. The expression of over 200 genes was synergistically increased by a combination of flagellin and Nutlin-3 in a p53-dependent manner. Genes associated with Gene Ontology terms such as immune, defense and inflammatory response were significantly (p-value <0.0001) over represented. In addition, promoter analysis of the synergistic group demonstrated enrichment for NFkB binding sites (p-value <0.0001). Interestingly, Nutlin-3 increased IL-6 and IL-8 expression also in response to TNFalpha that similarly utilize MAP kinase and NFkB pathways demonstrating, therefore, that p53 can interact with these signaling pathways in a broad context. Our findings reveal a novel role for p53 to promote immune/inflammatory response.

Signal Transduction - G-proteins and Ion Channels

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CC

Peroxisome Proliferator-activated Receptor (PPAR) gamma and G-protein Coupled Receptor 40 (GPCR40) Function as an Integrated Two-receptor Signaling Pathway

PPARgamma is a type II nuclear receptor (NR) that regulates fatty acid storage and glucose metabolism. Ligand/agonists of PPARgamma such as rosiglitazone (RGZ) are insulin sensitizers used in the treatment of type 2 diabetes mellitus, but an incomplete understanding of PPARgamma signaling has hampered the development of safer, more effective drugs. Recently, RGZ and other PPARgamma ligands were shown to activate stress kinase pathways through specific binding to G-protein coupled receptor 40 (GPCR40), a free fatty acid Gq receptor linked to glucose homeostasis. Notably nitric oxide (NO) has been shown to initiate PPARgamma signaling in the absence of exogenous ligand through a p38 mitogen-activated protein kinase- (MAPK) dependent mechanism. Here, PPARgamma ligand/agonist activation of GPCR40/p38 MAPK was found to control downstream PPARgamma signaling. Like NO, RGZ activated p38 MAPK in EA.hy926 cells, a human endothelial line, and p38 inhibitor, siRNA knockdown (KD) and dominant-negative (DN) mutant over-expression impaired RGZ induction of PPARgamma signaling as measured by DNA binding and a PPAR response element-driven reporter gene. Unlike NO, RGZ activation of p38 MAPK was GPCR40 dependent; GPCR40 inhibitor, siRNA KD, and over-expression of a DN Gq mutant all blocked RGZ activation of p38 MAPK and substantially inhibited RGZ-induced PPARgamma signaling. RGZ/GPCR40/p38 MAPK signaling led to ATF2 phosphorylation and subsequent PPARgamma acetylation, as assessed by co-immunoprecipitation, which were shown to control downstream ligand-dependent PPARgamma signaling. DN ATF2 blocked both NO- and RGZ-induced PPARgamma activation, while a constitutively active ATF2 construct alone was associated with PPARgamma acetylation and activation, which fully abrogated or markedly blunted PPARgamma signaling by either NO or RGZ, respectively. Likewise, over-expression of histone deacetylase 1 inhibited NO- and RGZ-induced PPARgamma activation and trichostatin A, a deactylase inhibitor, increased PPARgamma acetylation, which worked synergistically with RGZ to activate the PPARgamma reporter. Thus, GPCR40 and PPARgamma can function as an integrated two-receptor signal transduction pathway with p38 MAPK, ATF2 and acetylated PPARgamma serving as intermediary components. Unexplored effects through cognate GPCRs, as exemplified by this GPCR40/PPARgamma signaling pathway, could explain important safety and efficacy differences among NR-directed drugs.

Signal Transduction - G-proteins and Ion Channels

Balenga, Nariman
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NIAID

The role of regulators of G-protein signaling 4 and 5 in the pathophysiology of asthma

Asthma is a chronic inflammatory disease manifested by increased mucus secretion, smooth muscle hyperplasia, eosinophilia and reversible airways obstruction/remodeling. Asthmatic patients suffer from hyper-responsiveness (elevated contraction) of airways to inflammatory mediators i.e. histamine. The overriding contracting cells in the airway lumens are airway smooth muscle cells (ASM), equipped with contractile components such as myosin heavy and light chains and alpha-actin. Histamine triggers a G protein coupled receptor (GPCR), expressed on the surface of ASM, leading to Galphaq-mediated release of Ca2+. This enables the cross-bridging of myosin and actin filaments in ASM leading to

contraction and culminating in airway luminal narrowing. Regulators of G-protein Signaling (RGS) proteins switch off GPCR-mediated downstream signaling by accelerating the hydrolysis of GTP bound to Galpha proteins. We investigated the role of RGS4 and RGS5 (two highly expressed RGS proteins in human ASM) in the function of ASM and pathophysiology of asthma. We found that RGS5 expression was increased in ASM isolated from patients who died of asthma compared to ASM from non-asthmatic donors. Bronchial smooth muscle of BALB/c mice that were sensitized and challenged with Aspergillus fumigatus (Af) extract had increased RGS5 expression compared to that of naïve mice. Human asthmatic ASM demonstrated reduced intracellular Ca2+ release upon stimulation with histamine compared to normal donors. Lentiviral overexpression of RGS5 reduced Ca2+ responses in ASM and decreased contraction of small airways in precision cut lung slices (PCLS) from healthy donors. Therefore, higher expression of RGS5 might impair contractility of ASM in severe asthma, which could contribute to fixed narrowing of airways seen clinically in fatal asthma. Similarly, bronchial smooth muscle bundles of asthmatics had increased RGS4 expression, and the number of RGS4+ cells correlated with the degree of clinical pulmonary dysfunction. Surprisingly, lungs of Rgs4-/- mice sensitized and challenged with Af contained more goblet cell hyperplasia, eosinophilia, and increased peribronchial collagen deposition than those from WT mice. The impact of RGS4 and 5 deficiencies on ASM contractility in vivo is currently being investigated. Collectively, RGS4 and RGS5 are likely to play major roles in the function of ASM and might consequently be potential targets for asthma therapy.

Signal Transduction - G-proteins and Ion Channels

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TrpA1 channels are signal amplifiers of itch responses

Itch is an important, though often overlooked, aspect of many skin, systemic and nervous system disorders. Indeed, antihistamine-resistant itch is the most commonly cited reason for non-compliance with the anti-malarial drug, chloroquine. Current therapies for treating itch are often ineffective, leading our search to better understand the mechanisms behind the coding of itch in sensory neurons. The non-specific cation channel, TrpA1, has been known for its role as the mustard oil receptor, but recent studies have also demonstrated its role in itch. TrpA1-deficient mice have been shown to exhibit significantly decreased responses to pruritogens (itch-inducing agents) such as chloroquine and BAM8-22. Here, we use a transgenic mouse model that overexpresses TrpA1 to further examine its role in itch pathways. TrpA1 overexpressing mice have 50% more neurons that respond to capsaicin, histamine, and mustard oil compared to wild type littermates in calcium imaging experiments. These mice also display increased scratching behavior after intradermal choloroquine, SLIGRL-NH2 (both non-histamine dependent) and histamine injections. In agreement with previous reports, TrpA1 -/- animals showed dramatically reduced scratching in response to the same chloroquine injections. As expected, there were no significant differences in scratching behavior with serotonin, whose signaling pathway is thought to be independent from TrpA1 and TrpV1. The TrpA1 antagonist, HC 030031, inhibits virtually all responses to histamine in calcium imaging experiments in both wild type and TrpA1 overexpressing neurons. This can be explained because TrpA1 channel activation is likely required for amplifying the response of histamine receptors. Though TrpA1 -/- mice still respond to some pruritogens, our TrpA1 overexpressing animals demonstrate a gain of functionâ€” cells can be recruited and signal strength increased with altered ion channel expression, highlighting TrpA1â€™s role as a signal amplifier in sensory neurons.

Signal Transduction - G-proteins and Ion Channels

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Selective activation of Gq signaling in pancreatic beta cells in vivo improves beta cell function and whole body glucose homeostasis

Obesity and type 2 diabetes (T2D) have emerged as major threats to human health worldwide. Impaired function of pancreatic beta-cells and decreased beta-cell mass are two key features of T2D. Thus, strategies aimed at enhancing beta-cell function and maintaining beta-cell mass should prove useful in the treatment of T2D. Beta-cell function is modulated by various nutrients, hormones and neurotransmitters most of which act through G-protein coupled receptors (GPCRs). GPCRs are linked to different functional classes of heterotrimeric G proteins, primarily Gq, Gs, and Gi. Pancreatic beta-cells express several GPCRs that are selectively coupled to Gq including receptors for biogenic amines, fatty acids, and various peptide ligands. At present, it remains unclear to which extent chronic activation of these receptors in vivo can modulate beta-cell function and beta-cell mass as well as whole body glucose homeostasis. To address this issue, we generated transgenic mice that expressed a Gq-coupled designer GPCR (Rq) in pancreatic beta-cells only (b-Rq mice). Importantly, this designer receptor can only be activated by an exogenously administered drug, clozapine-N-oxide (CNO), an otherwise pharmacologically inert compound. Prolonged activation of beta-cell Gq signalling by chronic CNO treatment of b-Rq mice was associated with elevated serum insulin and decreased blood glucose levels, increased pancreatic insulin content, increased beta-cell mass and rate of beta-cell proliferation, and elevated expression of several genes important for the maintenance of beta-cell function and mass, including IRS-2 and the transcription factors Pdx1, MafA, NeuroD1 and Ngn3. Chronic activation of beta-cell Gq signalling also protected b-Rq mice against hyperglycemia and glucose intolerance induced by consumption of a high-fat diet or treatment with low doses of streptozotocin. Studies with b-Rq-mice lacking IRS2 strongly suggested that IRS2 plays a central role in mediating the beneficial metabolic effects resulting from prolonged activation of beta-cell Gq signalling. In vitro studies demonstrated that the enhanced expression of IRS-2 triggered by activation of beta-cell Gq signaling required PKC-dependent ERK activation. These results suggest that agents aimed at enhancing Gq signaling in pancreatic beta-cells could become clinically useful as antidiabetic drugs.

Stem Cells - General

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NHGRI

Sox10 plays a dual role in maintaining the melanocyte lineage and establishing the melanocyte stem cell.

During embryogenesis SOX10 functions within neural crest cells to upregulate â€˜master switchâ€™ transcription factors needed for specifying different neural crest sublineages. Melanocytes, being a neural crest derivative, use SOX10 to initiate the expression of the transcription factor, Mitf. SOX10 and MITF together then drive the survival and differentiation of the melanocyte lineage embryonically. Postnatally, melanocytes are incorporated into the hair follicle and those that reside in a region called the hair bulge give rise to the melanocyte stem cells (McSCs). These McSCs replenish the melanocyte system of the hair follicle throughout adult hair cycling, however it is unknown how this population is established. We hypothesize that through the differential regulation of Sox10, postnatal melanocytes can maintain their lineage specification while also allowing a portion of them to acquire the role of a McSC. In support of this idea, we find that McSCs express SOX10 and MITF but remain undifferentiated. By knocking out Sox10 in the melanocyte lineage (Sox10fl; Tg(Tyr::CreER)), we also show that McSCs

need Sox10 for their survival. However, by gain-of-function analysis (Tg(DctSox10)) we demonstrate that while McSCs may require Sox10 for their survival, overexpression of Sox10 results in their premature differentiation, eventual loss, and consequently leads to early hair graying. This suggests that Sox10 must be downregulated in order for the McSC to be established. In an attempt to dissect whether SOX10â€™s role in McSCs is simply to regulate Mitf we asked whether haploinsufficiency for Mitf (Mitfvga9) can rescue hair graying in Tg(DctSox10) animals. Surprisingly, the combination of Mitfvga9 and Tg(DctSox10) exacerbates hair graying and suggests that MITF participates in repressing Sox10 in McSCs. Together these data suggest a mechanism where SOX10 can be present to support the maintenance of the melanocyte lineage while also be inhibited from driving differentiation in the McSC population. These data illustrate how tissue-specific stem cells can arise from lineage-specified precursors, and how this can occur through the regulation of the very transcription factors important in defining that lineage. Understanding the etiology of McSCs will aid in our approach to studying diseases with stem cell-like characteristics, namely melanoma, as well as have broader implications in how we might use adult stem cells in regenerative medicine.

Stem Cells - General
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Instability in maintenance of X-chromosome inactivation in female human iPSCs: implications for treatment of X-CGD female carriers
X-linked chronic granulomatous disease (X-CGD) is characterized by neutrophils that lack production of microbicidal reactive oxygen species (ROS), caused by CYBB gene mutations. Due to random X-chromosome inactivation during embryonic development, female carriers of CYBB mutations are mosaic for both functional (ROS+) and non-functional (ROS-) neutrophils. In some carriers, extreme skewing of X-inactivation results in clinical X-CGD. To study X-inactivation during iPSC reprogramming and differentiation, we established iPSCs from peripheral blood CD34+ mononuclear cells of X-CGD female carriers who exhibit extreme skewing of the inactive X-chromosome (Xi) towards silencing of their normal CYBB allele. At the time of cell collection for iPSC reprogramming, carriersâ€™ peripheral blood contained =6% normal ROS+ neutrophils (down to 0.04% in one carrier severely afflicted with the disease phenotype) by DHR assay, with similar Xi skewing in mononuclear cells by HUMARA assay. Normal female and X-CGD carrier iPSCs each exhibited a clonal Xi by SNP expression assays. Upon in vitro neutrophil differentiation of iPSCs, all clones exhibited increased percentages of ROS+ neutrophils (18-72% ROS+ by DHR; >10-fold increase; p<0.03) over those originally present in the carriersâ€™ peripheral blood, with a 455-fold increase for the carrier afflicted with severe clinical X-CGD. In X-chromosome SNP expression assays, iPSC-derived neutrophils from female carriers and normal clones expressed both SNP alleles equally, indicating activation of both X-chromosomes. This correlated with loss of expression of XIST, the major effector of X-inactivation. Additionally, we observed loss of Xi-associated histone markers in some normal and carrier clones during iPSC culture, indicating general Xi instability in female human iPSCs. Our results demonstrate that Xi instability during female iPSC differentiation enables production of ROS+ neutrophils without gene therapy for extremely lyonized carriers of X-CGD, a finding with implications for treatment of female carriers of X-linked diseases manifesting severe clinical phenotypes.

Stem Cells - General
Lin, Fumin

Visiting Fellow
NIEHS
Role of GLIS3 in the generation of pancreatic beta cells from ES and iPS cells
Diabetes mellitus is a major health concern presently affecting 10% of the US population. State-of-the-art insulin therapy does not prevent long-term complications from diabetes, therefore, development of beta cell replacement therapy might become an attractive alternative strategy. Recent studies have developed protocols to induce differentiation of (embryonic) stem cells into pancreatic endocrine cells, including ß cells. However, many details of the mechanism controlling this differentiation process are still lacking. Our lab identified Krüppel-like zinc finger transcription factor Gli-similar 3 (Glis3) as a critical factor in the regulation of pancreatic ß cells. Study of Glis3 knockout mice showed that these mice develop neonatal diabetes and lack the presence of beta cells. In addition, Glis3 plays an important role in the regulation of insulin transcription in matured ß cells. Combined with the fact that mutations in Glis3 have been linked to type I/II diabetes in humans, we hypothesize that Glis3 may be critical for the development of pancreatic ß cells. The goal of this study is to determine the role of Glis3 in the regulation of the differentiation of (embryonic) stem cells into pancreatic endocrine cells. We successfully induced differentiation of human adipose-derived stem cells (hASC), human embryonic stem cells (hESC), and induced pluripotent stem (iPS) cells into pancreatic endocrine cells and monitored the expression of various differentiation markers, including Pdx1, Ngn3, MafA, insulin, and glucagon, by real-time PCR and immunostaining. In these cell models, Glis3 mRNA expression was significantly induced during differentiation. We demonstrated that the expression of Glis3 was up-regulated as early as the definitive endoderm stage, but was greatly induced at the pancreatic progenitor cell stage. These observations suggested that Glis3 plays a role early in the differentiation of embryonic stem cells into pancreatic endocrine cells. To study the role of Glis3 in the differentiation of iPS cells into pancreatic endocrine cells, we generated iPS cells from wild type and Glis3 null mice. Our results suggest that Glis3 plays an important role in the differentiation of stem cells into ß cells and expression of Glis3 might benefit cell replacement therapy against diabetes.

Stem Cells - General
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MicroRNA-mediated regulation of the BRG1 chromatin remodeling complex underlies the balance between pluripotency and differentiation in human embryonic stem cells
Embryonic stem (ES) cells hold great promise for regenerative medicine because of their unique characteristics of self-renewal and pluripotency. Balance between ES cell pluripotency and lineage commitment is maintained by gene expression networks that are largely dictated by chromatin structure. The BRG1 chromatin remodeling complex is required for mouse ES cell self-renewal and pluripotency and lineage specification during early murine development. Its role in human ES (hES) cells and early development, however, remains unclear. The complex is comprised of a central ATPase (BRG1) and multiple BRG1-associated factors (BAFs), which are assembled in a combinatorial fashion to dictate context-dependent functional specificity. Here we address the mechanisms by which this complex promotes pluripotency and early differentiation events in hES cells through changes in complex composition. We utilized in vitro culture and differentiation of hES cell lines to explore the regulation of BAFs in early human development. Through gain- and loss-of-function experiments we identified a microRNA-mediated regulatory event critical for BAF regulation. The ES-cell specific miR-302 family directly represses BAF170 in hES cells. This repression is relieved upon differentiation and miR-302 inhibition. The importance of BAF170 repression for gene expression was explored through genome-

wide microarray and RNA-seq studies. 352 genes were significantly affected at least 1.5 fold by BAF170 KD with 63% also misregulated upon miR-302 inhibition. Functional analysis revealed enrichment in Nodal signaling. qRT-PCR confirmed that miR-302 and BAF170 conversely regulate endodermal differentiation markers and targets of Nodal signaling, a pathway known to regulate both ES cell pluripotency and endodermal differentiation. Our data support a role for miR-302-mediated BAF170 repression in maintaining pluripotency through suppressing endodermal differentiation and suggest that relief of this inhibition is important for human endodermal lineage specification. This places the BRG1 complex at the center of cell fate decisions during early human development and provides mechanistic insight into the essential role of this complex in balancing stem cell pluripotency and differentiation. As the endodermal lineage gives rise to cells of the liver and pancreas, understanding these mechanisms will aid in the use of stem cell therapies for liver disease and diabetes.

Stem Cells and Cancer
Adhikari, Amit
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Dedifferentiation of mature astrocytes by abrogation of Rb tumor suppression leads to Astrocytoma initiation in GEMs
Astrocytomas are the most common malignant brain tumors which maintain a poor survival rate despite decades of research effort. Understanding the initiation process and identifying astrocytoma cell(s) of origin have been of immense interest. Genetically engineered mouse models (GEMMs) are ideal for studying the initiation process compared to evolved human tumor cells. Our lab has developed an adult inducible GEMM that alters key human glioblastoma (Grade IV) pathways. Inactivation of Rb tumor suppression (TS) by expressing N-terminal 121aa of large T antigen (T121) under GFAP promoter is sufficient to initiate grade II astrocytoma. Addition of mutant KrasG12D leads to grade III and further PTEN deletion to glioblastomas. KrasG12D activation and PTEN deletion alone or in combination failed to initiate astrocytomas, suggesting that Rb TS inactivation is essential for its initiation. Studies from several cancers have identified tumor-initiating cells, a subset of tumor cells with stem cell-like properties responsible for generating the entire tumor mass. Their origin remains a mystery. We hypothesize that Rb TS inactivation dedifferentiates astrocytes into stem/progenitor-like state generating potential cell(s) of origin for astrocytoma. Immunostaining of progenitor and proliferation markers (e.g. Sox2, nestin and Ki67) showed their co-expression in cortex with T121, suggesting a possible dedifferentiation of mature astrocytes. Moreover, astrocyte differentiation marker S100beta was downregulated in T121 cells. To rule out the possibility of migration resulting in the observed cortical progenitor cells, focal induction of T121 in cortex using lenti-cre injection was performed. We found expression of Sox2 and nestin and reduced expression of S100beta, suggesting that the dedifferentiation was independent of the brain germinal zones. Furthermore, cortical T121 expressing astrocytes were able to generate neurospheres but astrocytes from wildtype cortex failed to do so. These cells also showed self-renewability and multilineage ability, emphasizing their dedifferentiated status upon Rb-TS inactivation. Our preliminary results show that even 200 T121 neurosphere cells have tumor forming ability. Molecular analysis is underway to decipher the mechanism behind dedifferentiation. With the Rb pathway known to be altered in more than 75% of glioblastomas, our results are a step in the direction of unraveling the process of tumor initiation for this deadly disease.

Stem Cells and Cancer
Sun, Lei

Postdoctoral Fellow
NCI-CCR
Epigenetic Regulation of CpG Promoter Methylation by the NF-kB Signaling Pathway in Pancreatic Cancer Stem Cells
Pancreatic cancer is one of the most common causes of cancer death internationally. The diagnosis of early-stage pancreatic cancer often has a poor prognosis and the survival rate is quite low once it becomes advanced or metastatic. Epigenetic modifications such as DNA methylation play a significant role during both normal human development and cancer progression. We sought to investigate which genes are epigenetically regulated in the invasive population of pancreatic cancer cells that are also termed cancer stem cells (CSCs). We conducted epigenetic arrays in both PANC1 and HPAC pancreatic cancer cell lines and compared the global DNA methylation status of CpG promoters in invasive cells (CSC population) to their non-invasive counterparts (non-CSC population). The differentially methylated genes were applied into Ingenuity pathway analysis and our results showed that the NF-kB pathway is one of the top activated pathways in invasive cells. In line with this, we determined that upon treatment with NF-kB pathway inhibitors, the invasive and migratory ability of total cells are significantly disrupted. Moreover, the SRY-box transcription factor SOX9, which is demethylated in invasive cells, is shown to play a crucial role in invasion of both cell lines. In addition, we found a potential NF-kB binding site located in the SOX9 promoter in Genomatix database. Interestingly, the NF-kB subunit p65 positively regulates SOX9 expression by binding to its promoter directly, which can be efficiently blocked by NF-kB inhibitors. Thus, our work establishes a link between the classical NF-kB signaling transduction pathway and the invasive properties of pancreatic CSCs. We believe our data can result in the identification of novel signals and molecules at an epigenetic level that can potentially be targeted in pharmaceutical investigations and clinical trials.

Stress, Aging, and Oxidative Stress/Free Radical Research
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The NADPH oxidase NOX4 is a critical mediator in the Radiation-Induced Bystander Effect (RIBE)
During radiation therapies, special care is taken to ensure that as much normal tissue as possible is spared from radiation exposure. Understanding and minimizing the side effects of radiation to normal tissues has remained a long-standing challenge over the years. One of the therapeutic biological consequences of radiotherapy for normal tissues may come from the radiation-induced bystander effect (RIBE). The RIBE, studied by our group and others, concerns the responses of unexposed cells to signals received from nearby cells damaged by radiation. These responses include the induction of point mutations, chromosomal abnormalities, micronuclei and apoptosis and are accompanied by the formation of DNA double-strand breaks (DSBs) in the unirradiated bystander cells. Mechanistic studies aimed at providing insight into the nature of the RIBE signaling pathways will be instrumental in assessing their medical importance as well as means to mitigate their effects in patients. It has become clear that reactive oxygen species (ROS) are crucial players in the RIBE. Because nicotinamide adenosine dinucleotide phosphate (NADPH) oxidases produce ROS as part of their primary function in a large array of cells and tissues, we raise the question whether these oxidases might be involved in radiation-induced ROS and subsequent DNA damage. Our data indicate that irradiation leads to activation of NADPH oxidase NOX4 expression along with an increase in DNA DSB incidences in human cancer cells. In addition, medium transferred from irradiated cell cultures to bystander cell cultures results in the positive up-regulation of NOX4 in these bystander cells. Knockdown of NOX4 by small interference RNA (siRNA) prevents irradiation-induced DSBs as detected by 53BP1/gamma-H2AX foci in irradiated cells.

Finally, we show that siRNA knockdown of NOX4 in the unirradiated bystander cells abrogates, at least partly, the RIBE. In conclusion, our study shows for the first time a role for the ROS-generating NADPH oxidase NOX4 in both irradiation-induced DNA damage and the RIBE. These findings support the hypothesis that NOX4 may be included as therapeutic target to mitigate the radiation-induced damage in normal tissues during cancer therapies.

Stress, Aging, and Oxidative Stress/Free Radical Research
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NIA
NEIL 1 glycosylase promotes short-term spatial memory retention and protects from ischemic stroke induced brain dysfunction and death in mice.
Stroke is a leading cause of adult disability and the third most frequent cause of death in United States with 140,000 stroke-related deaths per year. Following ischemic stroke, reperfusion causes acute oxidative stress and brain injury. Normally, neuronal cells encounter relatively more oxidative DNA damage due to their higher metabolic rates and lower antioxidant proteins and this is exacerbated by stroke. Recent evidence suggests that neuronal cells are capable of repairing oxidative DNA damage. The role of DNA repair may be significant in stroke survivors and has not been well studied. Oxidative DNA damage is mainly repaired by base excision repair (BER), a process initiated by DNA glycosylases that recognize and remove damaged bases. Endonuclease 8-like 1 (NEIL1) is a DNA glycosylase that recognizes a broad range of oxidative lesions. To understand the role of NEIL1 in normal brain function and in ischemic stroke induced stress resistance, we used behavioral tests and middle cerebral artery occlusion/ reperfusion (MCAO/R). There was a 2.3-fold higher death rate in NEIL1-/- mice (37.5%) compared to WT mice (16.7%) after MCAO/R. Similarly, we observed increased neurological deficits and motor dysfunctions after ischemic stroke in NEIL1-/- mice compared to WT. In addition, NEIL1-/- coronal brain sections displayed (~14%) greater infarct volumes and ~2.8-fold more apoptosis as measured by TUNEL staining. To understand the role of NEIL1 in normal brain function, we performed a battery of behavioral tests (open field, rota-rod, novel object recognition, fear conditioning and water maze) on unstressed NEIL1-/- and WT mice. We found significant loss of short-term spatial memory retention during cued-water maze probe trials in NEIL1-/- mice. The NEIL1-/- and WT mice performed similarly in the remaining tests. To corroborate the defective functional outcome with loss of DNA repair, we performed incision assays that probe the specific biochemical activity of NEIL1 in the brain tissue lysates. The incision capacity on a 5-hydroxyuracil containing bubble substrate was lower (~40% at 15 min time point; p<0.05) in the ipsilateral side of the stroked brains and (~33%; p=0.026) in the mitochondrial lysates of unstressed old NEIL1-/- mice. These results indicate that NEIL1 plays an important role in normal brain function and its response to stress-resistance after stroke.

Stress, Aging, and Oxidative Stress/Free Radical Research
Sun, Yaning
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NIA
Cytochrome b5 reductase overexpression increases lifespan and oxidative stress resistance in fruit fly
Free radicals, mainly reactive oxygen species (ROS), have been proposed as a main proximate cause of aging. The removal of free radicals by cellular free radical defense systems can minimize the free radical-induced oxidative damages to DNA, protein, and lipid. Cytochrome b5 reductase (Cytb5R) has been shown to play important roles in the free radical defense system on both plasma membrane and

mitochondrion. Studies in yeast demonstrate that overexpression of NQR1, a yeast homologue of Cytb5R, significantly extends both chronological and replicative lifespan in yeast. However, no lifespan study has been reported in higher eukaryotes and the mechanism is still unclear. We hypothesize, Cytb5R, a conservative enzyme in eukaryotes, also mediates lifespan in more complicated organism than yeast. In this study, we used Drosophila melanogaster (fruit fly) as our model to verify our hypothesis and try to understand the mechanism of cytb5R mediating lifespan. Using the Gal4-UAS system, we overexpressed Cytb5R in Drosophila, and measured the lifespan, stress resistance, and gene expression changes. We found that overexpression of Cytb5R significantly increased mean lifespan (>10%) of female but not male flies fed with the standard diet (10% sugar, 10% yeast). We also found that overexpression of Cytb5R increased mean lifespan (>10%) of both male and female flies fed with the calorie restriction diet (2.5% sugar, 2.5% yeast). Moreover, Cytb5R overexpression increased fliesâ€™ resistance to free radical-induced oxidative stress, but rendered to flies sensitivity to starvation. Our mechanistic studies indicated that Cytb5R overexpression induced the expression of stress response genes, including genes in Jun kinase (JNK) signaling, such as JNK, puckered (puc) and lethal (2) essential for life (l(2)efl), and in the Nrf2 pathway, such as mitochondrial peroxiredoxins (Prx5037 and Prx5) and glutathione S-transferase D1 (GstD1), and in the sirtuin pathway, including dSir2. In summary, our findings verified our hypothesis and found a connection between cytb5R and JNK, Nrf2 and/or sirtuin pathways, which suggests Cytb5r could be a novel target for pro-longevity interventions and a therapeutic target for the development of calorie restriction mimetics.

Tumor Biology and Metastasis

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Chronic myeloid leukemia (CML) exosomes promote angiogenesis in a Src-dependent fashion in vitro and in vivo

CML is an uncontrolled proliferation of bone marrow myeloid cells driven by the constitutively active fusion product tyrosine kinase BCR/ABL. Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is newly recognized as a factor in CML progression. Exosomes are microvesicles that play an important role in cell-to-cell communication both in physiological and pathological conditions. The role of exosomes released by CML cells in angiogenesis is emerging; however, little is known about the mechanisms involved in this process. We isolated and characterized exosomes released by K562 CML cells and we demonstrated their ability to stimulate human vascular endothelial cells (HUVECs) tube differentiation on Matrigel. K562 exosomes induced an increase of the cumulative tube length in a dose-dependent manner, with a maximum effect at 10μg/ml (p=0.003). Next, we evaluated the effect on exosome behavior of imatinib and dasatinib, two tyrosine kinase inhibitors in use in CML treatment. K562 CML cell treatment with either imatinib or dasatinib reduced exosome release by 58% and 56%, respectively (p<0.01). Dasatinib treatment of HUVECs strongly reduced exosome-induced vascular differentiation (p=0.0002). On the contrary, little effect was observed following treatment with imatinib. Vascularization of an exosome containing Matrigel plug in vivo was markedly inhibited by oral administration of dasatinib (p<0.01), but not imatinib. Immunofluorescence analysis showed increased exosome-induced Src and FAK phosphorylation in HUVECs. Both FAK and Src phosphorylation were increased at points of membrane-matrix contact. Immunoblot analysis confirmed that K562 exosomes induced a dasatinib-sensitive phosphorylation of Src and FAK and their downstream effectors, Erk and Akt. Again, imatinib was minimally active against exosome stimulation of HUVEC cell signaling. Thus, K562 CML exosomes stimulate angiogenesis in vitro

and in vivo in a dasatinib-sensitive fashion. This credentials exosomes and angiogenesis as molecular targets in CML via activation of Src both in leukemia and its microenvironment.

Tumor Biology and Metastasis
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Disruption of the HIF-1a/p300 complex with select epidithiodiketopiperazines (ETPs) has anti-angiogenic effects
Solid tumors develop regions of hypoxia because of an imbalance in oxygen supply and consumption. Thus, adaptation of cancer cells to hypoxia is critical for tumor survival. The most important mediator of the cellâ€™s response to reduced oxygen is the hypoxia inducible factor-1 (HIF-1) transcription factor. HIF-1 is a heterodimeric transcription factor composed of an O2-regulated HIF-1a subunit and a constitutively expressed HIF-1ß subunit. Due to the involvement of HIF in tumor progression and angiogenesis or the formation of new blood vessels from preexisting ones, inhibition of HIF-mediated transcription has the potential for treatment of cancer. Since p300 is a crucial coactivator of hypoxia-inducible transcription, disruption of the HIF-1a/p300 complex is desirable as a selective mechanism of inhibiting HIF activity. Previous data from our laboratory showed that several members of the epidithiodiketopiperazine (ETP) family of natural products are able to block the interaction between HIF-1a and p300. We performed a series of rat aortic ring assays, which are widely used to assess anti-angiogenic compounds, to determine the effects of the ETPs on neovascular outgrowth. Chetomin and chaetocin concentrations of 50 nM inhibited approximately 90% of outgrowth, while 500 nM of gliotoxin was needed to achieve a similar effect. The aortic rings were immunostained for HIF-1a to confirm that vessel outgrowth was due to HIF-1a upregulation. Next, we immunoprecipitated the endogenous HIF-1a/p300 complex from cells in the absence or presence of the aforementioned ETPs, and found that the ETPs that had anti-angiogenic effects in the rat aortic ring assays, also blocked co-immunoprecipitation of HIF-1a and p300. The downstream effect of blocking the HIF-1a/p300 interaction was demonstrated by ELISA, which showed a dose-dependent decrease in secreted vascular endothelial growth factor, the primary mediator of angiogenesis and a prominent downstream target of HIF. Treatment with ETPs in mice bearing prostate tumor xenografts resulted in significant inhibition of tumor growth and a reduction in tumor vascularization, as determined by analysis of tumor sections via immunohistochemistry for CD31, a histomorphological marker of tumor angiogenesis. Together, these results suggest that targeting the HIF-1a/p300 complex with these ETPs may be a very effective approach for inhibiting angiogenesis and tumor growth, thus representing promising novel agents for cancer therapy.

Tumor Biology and Metastasis
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Ras-driven colorectal cancers require protein SUMOylation
Ras is a prototypical member of Ras small GTPase family and the most common proto-oncogene in human. Oncogenic mutations switch Ras into a permanent GTP-bound â€œonâ€ status, which constitutively activates its downstream pathways, such as the mitogen-activated protein (MAP) kinase pathway, leading to proliferation and transformation of tumor cells. Oncogenic Ras mutations are found in ~30% human tumors, most prevalent in lung, colorectal and pancreatic cancers. This is a subset of

cancers often associated with poor prognosis and yet without any effective targeted therapy. To date, Ras itself is pharmacologically intractable and inhibitors against major Ras downstream effectors have not been proved effective in clinical trials. Thus, to better characterize the genetic dependencies of Ras-driven tumors and seek for potential therapeutic targets, we conducted a genome-wide RNAi screen for genes Ras mutant cancer cells depend on and unexpectedly identified ligases of SUMOylation, the small Ubiquitin-like protein modification. We found that blocking protein SUMOylation by knocking down SUMO E1 ligase SAE1 or E2 ligase Ubc9 with shRNAs modestly inhibited growth, but dramatically blocked colony formation of Ras mutant cells. In a panel of colorectal cancer cell lines, similar SUMOylation dependency was observed in Ras mutant cells, but not in Ras wild type ones. Furthermore, inhibition of SUMOylation blocked anchorage-independent growth, the in vitro tumor transformation in engineered Ras-transformed cells. A focused siRNA screen on SUMO pathway genes indicate Ras mutant cells preferentially depend on SUMO2 rather than SUMO1 modification for their growth. To further address the functional roles of SUMOylation in Ras mut cells, we compared global protein SUMOylation profiles between Ras mutant and wild type cells with quantitative proteomics and found 40 differentially SUMOylated proteins. This Ras-related SUMOylation signature contains proteins regulating gene expression and cellular signaling. Overall, these results support a critical contribution of protein SUMOylation in sustaining Ras mutant colorectal cancer cells. This study offers a potential therapeutic approach for Ras mutant cancers by targeting the SUMOylation enzymes.

Tumor Biology and Metastasis

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Ribosomal RNA Processing 1 Homolog B (Rrp1b) is a novel metastasis suppressor that modulates progression-related gene expression through regulation of MYC expression

Previous studies have demonstrated that Rrp1b activation suppresses the growth and metastatic capacity of the highly aggressive Mvt-1 mouse mammary tumor cell line, and induces a gene expression signature that predicts survival in human breast cancer. However, the mechanism by which RRP1B modulates transcription is unclear. The aim of this study is two-fold: to clarify the role of Rrp1b in metastasis; and to ascertain the mechanisms by which Rrp1b regulates transcription. Rrp1b BAC transgenic mice were developed to clarify the role of Rrp1b in metastasis. These mice were crossed to the PyMT mouse model of mammary tumorigenesis, and tumor growth and metastasis quantified in Rrp1b Tg+ vs Rrp1b Tg- F1 mice. While Rrp1b Tg+ mice had no difference in average tumor weight, a significant decrease in pulmonary surface metastasis count was observed (p=0.048). These data conclusively demonstrate that Rrp1b is a metastasis suppressor. ChIP-seq analysis of endogenous RRP1B was performed in MDA-MB-231 and Hela cells to define RRP1B-occupied genomic loci. Peaks were called using MACS with a genome-wide significance level of p<10^6. We identified 680 RRP1B binding peaks in the Hela cells and 339 peaks in the MDA-MB-231 cells, with 136 peaks overlapping between datasets. The consequences of RRP1B binding were investigated by quantifying the expression levels of 40 genes with peaks either within 5kb up- or downstream in cells over-expressing RRP1B. Seven genes had increased and 13 had decreased expression on q-RT-PCR analysis. We focused on genes with decreased expression since RRP1B physically interacts with various inducers of heterochromatin and gene silencing including TRIM28 and HP1a. One particularly interesting region of RRP1B occupancy was immediately downstream of the MYC oncogene. Many metastatic prognostic signatures reflect the level of MYC activity, with decreased expression of MYC in MDA-MB-231 cells inhibiting cell growth, invasion and metastasis. We found that ectopic expression of RRP1B reduced MYC expression in MDA-MB-231 cells. ChIP-reChIP assays using antibodies against RRP1B and either TRIM28 or HP1a reveal that RRP1B-

occupied regions, including downstream of MYC, are bound by a complex containing all of these proteins. Our data indicate that RRP1B is directing the TRIM28/HP1a complex to discrete genomic loci to regulate transcription. These findings strongly support that RRP1B suppresses metastasis through down-regulation of MYC expression.

Vascular Disease and Biology
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Doctoral Candidate
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Evaluation of the Role of Tetrahydrobiopterin Bioavailability in the Pathogenesis of Experimental Cerebral Malaria
The WHO estimates that malaria caused 655,000 deaths in 2011. Improved anti-malarial therapies have been a key factor in reducing overall deaths but are not always effective in patients presenting with severe complications, including organ failure or involvement of the central nervous system. Patients with severe malaria exhibit reduced bioavailability of the signaling molecule nitric oxide (NO) and dysregulation of several NO-dependent mechanisms of vascular homeostasis. We hypothesized that malaria infection impairs endogenous NO synthesis by reducing bioavailability of the nitric oxide synthase (NOS) cofactor tetrahydrobiopterin (BH4) and that BH4 supplementation may reverse vascular dysfunction associated with severe malaria, thereby improving outcomes. In order to test this hypothesis, we used a mouse model of severe malaria in which NO availability is low (C57BL/6 mice infected with Plasmodium berghei ANKA). We found that BH4 and total biopterin levels were reduced in both plasma and red blood cells at day 6 post-infection, but not in highly vascularized tissue (including aorta, lung, kidney, liver and brain). We then sought to assess the effect of increased tissue BH4 concentration on disease progression. Endothelium-specific transgenic over-expression of GCH1, an enzyme required for BH4 synthesis, improves BH4 bioavailability and NO production in uninfected mice. In our model of severe malaria, we found that mice expressing transgenic GCH1 demonstrated increased BH4 concentrations in aorta, lung, kidney and spleen tissue on day 6 post-infection compared to wild-type littermates. Six days after infection, plasma BH4 concentrations in transgenic mice were not significantly different from wild-type controls, but plasma BH4 correlated with disease severity across transgenic and wild-type groups. GCH1 over-expression did not improve survival. In subsequent analyses, we found that infection also resulted in low plasma levels of L-arginine, the NOS substrate. The combined depletion of BH4 and L-arginine in the blood compartment may impair NO synthesis by NOS expressed in red blood cells and may explain why red blood cell deformability, which is regulated by NO, is reduced in patients with severe malaria. The ability of improved BH4 bioavailability to restore endogenous NOS activity may also be limited by low L-arginine. In future work, we will assess the efficacy of combined supplementation of BH4 and L-arginine.

Vascular Disease and Biology
Pan, Weijun
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Utilizing tumor-produced VEGFa to block tumor growth with CDP-diacylglycerol synthase inhibition
VEGFA is the predominant pro-angiogenic factor in most types of human solid tumors, and almost all anti-angiogenic cancer therapies in clinical use or in development target this factor. Yet despite a great deal of initial excitement, massive investment, and a huge amount of effort at many, many pharmaceutical companies around the globe, the clinical benefits of current anti-VEGF therapies have

been relatively modest. A major reason for this is that vascular failure-induced tumor hypoxia triggers greatly increased production of VEGF and other pro-angiogenic cytokines by the tumor, overcoming vessel inhibition and resulting in revascularization of tumors after a period of anti-angiogenic therapy. Using genetic and experimental studies in zebrafish and in human endothelial cells in culture, we have uncovered a central role for CDP-diacylglycerol synthetase (CDS) in the regulation of VEGFA signaling and angiogenesis. CDS activity maintains phosphatidylinositol 4,5-bisphosphate (PIP2) availability through re-synthesis of phosphatidylinositides, while VEGFA, mainly through phospholipase C gamma-1 (PLCG1), consumes PIP2 for signal transduction. Loss of CDS2, one of two vertebrate CDS enzymes, results in vascular-specific defects in zebrafish in vivo and failure of VEGFA-induced angiogenesis in endothelial cells in vitro. In CDS-deficit conditions VEGFA stimulation results in dramatic reduction in PIP2 levels, collapse of VEGF signaling, and failure of vascular formation. CDS2 deficit-caused phenotypes can be successfully rescued by artificial elevation of PIP2 levels, and excessive PIP2 or increased CDS2 activity can promote excess angiogenesis. We hypothesize that by inhibiting CDS2 enzymatic activity, we can trap tumors into a VEGFa-induced endothelial specific vascular collapse circuit. Tumor-produced VEGFa would trigger PtdIns(4,5)P2 depletion in endothelium and limit tumor angiogenesis. The tumor, in turn, would produce more VEGFA to reinforce angiogenesis, further enhancing the collapse of VEGFA signaling and the blockage of angiogenesis and tumor progression. We are currently performing tumor angiogenesis assays in CDS-deficient zebrafish and mice to test this hypothesis. The results of these experiments could have profound implications for the treatment of cancer.

Vascular Disease and Biology
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NIEHS

The deficiency of beta-arrestin2 attenuates abdominal aortic aneurysm formation in mice.

Abdominal aortic aneurysms (AAAs) are an increasing health concern, particularly in the aging male population. AAAs begin as a dilation of the abdominal aorta, which gradually expand over time due to inflammation and vascular remodeling. Currently, there are no pharmacological treatments for AAAs. A widely used mouse model to study AAAs involves chronic infusion of angiotensin II (AngII), and this model displays many characteristics of human AAAs. AngII mediates its effects primarily by activating the G-protein coupled angiotensin type 1 receptor (AT1). Recent studies have shown that the multifunctional scaffolding protein Beta-arrestin2 (Barr2) forms a complex with AT1 to initiate G-protein-independent signaling, which contributes to many pathophysiological conditions. To examine the role of Barr2 in AngII-induced AAA formation, Barr2-deficient (Barr2-/-) and wild-type (Barr2+/+) mice were infused with AngII (1000ng/kg/min, minipumps) for 28 days. AngII induced a 71% incidence of AAAs in Barr2+/+ mice, whereas only 15% of Barr2-/- mice developed AAAs. AngII is a potent inducer of cyclooxygenase-2 (COX-2), an inflammatory enzyme that we have previously shown to be involved in AAA formation. Therefore, we hypothesized that increased COX-2 expression may be a mechanism by which Barr2 contributes to AAA pathology. AngII induced abundant COX-2 expression in the abdominal aortas of Barr2+/+ mice, whereas COX-2 expression was significantly attenuated in the aortas of Barr2-/- mice, indicating that Barr2 is involved in the induction of COX-2 in response to AngII. An extensively studied pathway mediated by the Barr2-AT1 complex is ERK1/2 signaling, and ERK1/2 activation independently has been shown to be important in AAA development. Indeed, activated ERK1/2 was observed in the abdominal aortas of Barr2+/+ mice, but not in the abdominal aortas of Barr2-/- mice. To determine if ERK1/2 activation was a mechanism for Barr2-AT1-induced COX-2 expression, Barr2+/+ mice were treated with the ERK1/2 inhibitor, CI1040 (100mg/kg) together with AngII and examined for

COX-2 expression. ERK1/2 inhibition significantly attenuated AngII-induced COX-2 expression in the abdominal aortas of Barr2+/+ mice. Thus, Barr2 may contribute to AAA formation by activating the ERK1/2-COX-2 pathway, resulting in increased inflammation and vascular remodeling. These studies have important implications for the design of therapeutics that target Barr2-mediated signaling for the treatment of AAAs.

Virology - DNA
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NIAID
A model system for the study of Kaposiâ€™s Sarcoma-associated herpesvirus (HHV-8) infection of B cells.
Kaposiâ€™s Sarcoma-associated herpesvirus (KSHV/HHV-8) is an HIV-associated oncogenic gamma-herpes virus. In areas of Africa, Kaposiâ€™s Sarcoma is the most common form of cancer. KSHV is strongly linked to two B cell lymphoproliferative disorders: multicentric Castlemanâ€™s disease (MCD) and primary effusion lymphoma (PEL). KSHV is widely believed to be transmitted via the saliva, probably shed from tonsillar B-cells. Latently infected B-cells are also believed to be a major â€œreservoirâ€ for KSHV. Paradoxically, while several "irrelevant" cell lines (e.g. 293 HEK) are highly permissive for KSHV infection, human cultured B-cell lines are notoriously resistant; with human lymphoid tissue, KSHV infects only around 2% of primary B cells. With the absence of a KSHV-susceptible B cell line, study of KSHV in this critical target cell type has been greatly impeded. Such a system would facilitate elucidation of the mechanisms of KSHV entry and replication, as well as evaluation of disease treatments. Reports analyzing lymphoid tissue slices from MCD patients as well as ex vivo infection of lymphoid cells have indicated preferential infection of B cells with the IgM/lambda phenotype. We used this information to guide a strategic search to aid identification of a B cell line susceptible to KSHV infection. We used a recombinant KSHV encoding EGFP under the constitutive EF-1alpha promoter; in infected cells EGFP is expressed even during KSHV latency. This recombinant KSHV also contains a puromycin resistance marker. We identified a novel IgM/lambda positive B cell line that is susceptible to KSHV infection. Following exposure to the recombinant KSHV, EGFP was expressed in up to 20% of cells. Viral DNA replication, latency associated transcription, and viral protein synthesis were also detected. Addition of puromycin yielded cultures in which the entire cell population was KSHV infected. Upon long-term serial passage, in the absence of selection, low-level latent KSHV infection was detected. Consistent with other cell lines harboring latent KSHV, the lytic replication promoter RTA was induced by sodium butyrate treatment. These data indicate that KSHV can undergo both latent and lytic replication in this cell line. The cell line is phenotypically characteristic of transitional B cells based on flow cytometric analysis. These results provide initial steps in the establishment of a much-needed B cell line model to study the biologically relevant steps of KSHV infection.

Virology - DNA
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Other
NIAID
In vitro reconstitution of early vaccinia virus membrane assembly intermediates
Poxviruses are large, enveloped DNA viruses that assemble in the cytoplasm by a unique mechanism of membrane acquisition and growth. Most enveloped viruses acquire membranes from the host cell by budding through membranes or by being wrapped by cellular cisterna. In contrast, vaccinia virus (VACV) and other poxviruses acquire a membrane by a distinct mechanism whereby the membrane is added in

a stepwise manner, likely by vesicle fusion or rupture. VACV membrane is grown until a spherical immature virion (IV) is formed; an intermediate of this process appears as a crescent shape by electron microscopy (EM) and is cup-shaped in three dimensions. It was proposed that small vesicles are added to the growing edge of a crescent, however, the mechanism of membrane addition and the proteins sufficient for crescent formation are not characterized. Previous studies have shown that decreased expression of the VACV protein, A14, prevents crescent formation and is characterized by a buildup of small vesicles and tubules (25-50 nm diameter) that contain the VACV membrane protein A17; it was also shown that in the absence of A17, no discernible viral membranes are formed. Together, this leads to a hypothesis that A17 is required for VACV membrane biogenesis. Based on the considerable efforts to understand the mechanism of virus growth in vivo, we can now begin testing a minimal set of VACV proteins to determine which are necessary and sufficient to form crescents in vitro. We noted that A17 shares homology with the membrane domains of cellular proteins that are responsible for the tubular structure of the endoplasmic reticulum and we proposed that A17 alone could cause membrane curvature. To this end, we purified A17 and reconstitututed the protein into liposomes. By EM, liposomes without A17 appeared heterogenous in size while liposomes containing A17 appeared predominantly as homogenous 25-50 nm vesicles. Increasing the concentration of A17 led to extensive tubulation of lipids while the 25-50 nm diameter remained constant. These data suggest that A17 induces membrane curvature, is sufficient for the small vesicle phenotype observed during infection in the absence of A14, and may also be responsible for the curvature at the ends of growing crescents. Further work will use in vitro assays to determine the steps and proteins necessary to fuse the small vesicles and tubules into crescents and will also examine the effect of drugs that inhibit VACV morphogenesis.

Virology - DNA

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Poxvirus superinfection is halted at the stage of virus-cell membrane fusion independently of primary virus gene expression

Vaccinia virus (VACV) â€“ the prototypic member of the Poxviridae â€“ encodes ~200 proteins within its 195-kbp dsDNA genome. Unlike most large DNA viruses, poxviruses replicate exclusively in the cytoplasm of infected cells and display rapid spread in tissue culture. However, poxvirus entry into already infected cells â€“ superinfection â€“ was shown to be potently blocked by cell surface expression of viral A56 and K2 proteins. Here we describe an additional superinfection exclusion mechanism that does not require viral gene expression. Utilizing a self-quenching fluorescent probe, we demonstrated that inhibition of the superinfecting virus occurred at the initial lipid mixing (hemifusion) step of viral entry. Inhibition of VACV superinfection was dependent on the multiplicity and length of time following the primary infection, but entirely independent of viral protein synthesis. Additionally, primary infection with virions devoid of genomic DNA or rendered transcription-deficient both induced similar levels of superinfection exclusion as wildtype virus. Nevertheless, binding of primary virus was required but not sufficient to inhibit superinfection as entry-impaired virions did not induce superinfection exclusion of secondary virus. Superinfecting secondary virions largely remained on infected cell surfaces and upon their retrieval found to be fully functional and capable of infecting uninfected cells. Cells infected with A56/K2-deletion virus were equally non-permissive to superinfection compared to wildtype controls and the block of secondary virus entry also occurred at the virus-cell hemifusion step. Although viral core entry into uninfected cells stably expressing A56/K2 on their surface was indeed inhibited, virus-cell hemifusion proceeded with normal kinetics, indicating that

A56/K2 acts at a later step in entry. The specificity of superinfection exclusion was demonstrated by the ability to superinfect poxvirus-infected cells with flavivirus pseudovirions. Our results indicate the existence of alternative and redundant mechanisms to prevent superinfection at successive steps in viral entry. We suggest that cellular membrane changes triggered by the primary infection with VACV render the cell incompetent to mediate a subsequent poxvirus infection. Given the promising use of poxviruses as vaccine platforms and gene therapy vectors, the further study and elucidation of such poxvirus-host cell interactions are clearly warranted.

Virology - DNA
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Human genome-wide siRNA screen reveals the importance of the nuclear pores for the cytoplasmic life cycle of vaccinia virus
Poxviruses are large, double strand DNA viruses which complete their life cycle in the host cytoplasm. Variola virus and the closely related monkeypox virus, the causative agents of smallpox and an emerging disease in Africa, indicate the threats of the poxviruses family. While an effective orthopoxvirus vaccine is available, its numerous counter-indications restrict its general use. Moreover, its efficacy after exposure to the virus is limited. Therefore, there is a critical need for anti-viral drugs against poxviruses. Understanding virus-host interactions during the replication cycle will help us understand the biology of the virus and provide potential drug targets to impede virus replication. In order to study the cell biology of the poxviruses, we used genome-wide siRNA libraries to systematically screen for host genes that either reduce or increase vaccinia virus spread. Multiple libraries in different formats were utilized to maximize genome coverage and increase the accuracy of the predictions. We used a Zâ€™-score to quantify the level of activity of each siRNA. Gene Set Enrichment Analysis (GSEA) was performed using Gene Ontology terms across the genome. The detection of numerous genes that were previously reported to inhibit vaccinia virus demonstrated the biological relevancy of the screen. For example, several ER-to-Golgi transport proteins, previously shown to be essential for the wrapping of mature virions, were selected as hits. Since vaccinia virus replicates independently of the nucleus, we were surprised to find that the nuclear pores and the import/export machinery appear to be required for virus spread. We rejected an off-target explanation by further validations and reconstitution experiments. The attenuation of spread correlated with reduced late gene expression rather than entry or early gene expression. The inhibition of virus spread by Leptomycin B, a drug that blocks export through the nuclear pores, was consistent with the siRNA data. However, our screens indicated that knocking down Pol II, its transcription factors and supportive complexes (such as the Mediator complex) actually resulted in increased viral spread suggesting other nuclear proteins may be involved in vaccinia virus replication. Thus, our screen holds promise for generating new insights into the biology of poxviruses and new targets for drug development.

Virology - RNA and Retroviruses
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Functional Redundancy in HIV-1 Assembly
The human immunodeficiency virus type I (HIV-1) affects millions of people worldwide. Virus particle assembly is a potential therapeutic target and, therefore, a better understanding of the process is

necessary. In mammalian cells, assembly can be achieved by solely expressing Gag, a polyprotein encoded by the viral genome that is the building block of the virus particle. Retroviral Gag proteins consist of three common domains: 1) the matrix (MA), which binds the plasma membrane mainly via a myristate moiety; 2) the capsid (CA), in which a dimerization interface has been identified; and 3) the nucleocapsid (NC), which is the main genomic RNA-binding domain. RNA, or any nucleic acid, is required for Gag molecules to assemble in vitro. However, in vivo, deletion of NC in HIV-1 does not affect the ability of Gag to form virus-like particles. Interestingly, several studies have shown that Gag chimeras, in which the NC domain is replaced by dimerizing or trimerizing zippers, assemble efficiently into particles lacking RNA, suggesting that oligomerization of Gag is an essential first step in assembly and that RNA contributes to assembly by promoting Gag oligomerization. We have investigated the role of RNA in assembly in vivo by analyzing the content of HIV particles lacking NC. We found that they contain very little total RNA (~12% or less compared to wild type). To understand how HIV-1 assembles in vivo with very little dependence on RNA, we designed several mutants that disrupt i) the Gag dimer interface on the CA domain, ii) Gag-membrane interactions, iii) NC-RNA interactions, and iv) combinations of the three types of interactions. Assembly was evaluated by electron microscopy and immunoblotting. We found that disruption of any one type of interaction is not sufficient to disable assembly. However, combination of any two mutants completely abolishes normal particle formation. Thus, in vivo, HIV-1 assembles with some functional redundancy that renders the participation of RNA unnecessary. We propose that in HIV-1 Gag molecules can come together into oligomers in several ways: cooperative binding to nucleic acid; direct CA-CA interactions; and Gag-membrane interactions, which increase local Gag concentration by targeting Gag to the plasma membrane. None of these interactions is sufficient by itself, but any two can mediate assembly.

Virology - RNA and Retroviruses

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Modeling the Nipah virus transmission cycle

Nipah virus (NiV) was discovered during an outbreak of respiratory and neurological disease in Malaysia with 276 human cases. Since 2001, outbreaks of NiV have occurred almost yearly in Bangladesh with case-fatality rates up to 90%. Epidemiological data suggest that in Bangladesh, NiV is transmitted from the natural reservoir, fruit bats, to humans via consumption of date palm juice contaminated by bats during collection, with subsequent human-to-human transmission in ~50% of cases. To model the transmission cycle of NiV in Bangladesh, we determined the viability of NiV, strain Bangladesh, in artificial date palm juice. At 22°C and 30°C virus titers remained stable for >3 days, indicating that it perfectly preserves NiV, thus potentially allowing food-borne transmission. Next, we modeled food-borne NiV infection by supplying Syrian hamsters with artificial date palm juice containing NiV instead of drinking water. Hamsters were monitored for signs of disease; sequential necropsies were performed to analyze virus distribution in 17 tissues and swabs were collected daily for up to 12 days to analyze virus shedding, a prerequisite for efficient transmission. Drinking of NiV resulted in neurological disease in 25% of hamsters, indicating that food-borne infection with Nipah virus can indeed occur. However, virus shedding was hardly observed in these hamsters. In comparison, intranasal (i.n.) inoculation with the same dose of NiV resulted in lethal respiratory disease in all animals with high amounts of virus shedding from the nose and throat. A 50x higher dose of NiV was then supplied in artificial date palm juice, resulting in 63% of hamsters with neurological signs and virus shedding in all hamsters on several days after drinking. To model human-to-human transmission, we used the hamster transmission model we recently established for NiV, strain Malaysia. Based on virus shedding and seroconversion,

transmission of NiV, strain Bangladesh occurred through direct contact, but not via aerosols or fomites, in 25% of hamsters upon i.n. inoculation. Experiments to study contact transmission of NiV upon drinking of contaminated date palm juice are currently underway. Understanding the NiV transmission cycle is essential for limiting NiV outbreaks. The limited potential for medical intervention in the resource-poor outbreak areas highlight the need for preemptive strategies like educational outreach focused at preventing zoonotic and human-to-human transmission.

Virology - RNA and Retroviruses
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Real Time Visualization of Influenza Viral RNA Intracellular Trafficking
Influenza viruses are composed of eight negative sense viral RNA (vRNA) segments that must be packaged together to produce infectious virus. Influenza vRNA is synthesized in the nucleus and must travel from the nucleus to the plasma membrane (PM) for packaging. As with most viruses, influenza virus hijacks the host machinery for its replication. Much is still unknown about this process, including the mode of transport from the nucleus to the PM and whether the 8 vRNA segments traffic together or separately. We developed a fluorescent virus to visualize the processes involved in the export and movement of the vRNA to the PM in real time during a productive infection. This virus stably expresses a GFP tagged viral polymerase protein (PA-GFP). The polymerase complex, composed of PB1, PB2 and PA proteins, is attached to each vRNA segment; thus the PA-GFP virus can be used to visualize the intracellular movement of vRNA. The fluorescent influenza virus, WSN PA-GFP, is replication competent and GFP localizes to the nucleus 4 hours post infection (hpi) and forms GFP cytoplasmic foci around 8 hpi. These cytoplasmic foci move toward the PM and co-localize with vRNA segments as determined by fluorescent in situ hybridization (FISH). Using leptomycin B and nocodazole treatments we found that transport of PA-GFP cytoplasmic foci to the PM is CRM1 but not microtubule dependent, as has been previously suggested. In addition we found that the movement of the PA-GFP cytoplasmic foci is rapid and directional; using selective plane illumination microscopy (SPIM) we are able to visualize and track the 3-dimensional movement of individual foci in real time and with higher resolution than conventional spinning disk microscopy. We are using SPIM to visualize both PA-GFP cytoplasmic foci and fluorescently labeled actin or tubulin to elucidate the mechanism by which vRNA is transported to the PM. Using FISH to visualize multiple vRNA segments in a single cell, we have found that some vRNA segments co-localize in cytoplasmic foci, demonstrating for the first time that vRNA can be transported to the PM as complexes rather than as individual segments. This GFP encoding influenza virus can be used to screen antiviral therapeutics and to study host range restriction by visualizing the movement of vRNA in the nucleus and cytoplasm in real time in different cell types.

Virology - RNA and Retroviruses
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Antibody Pressure by a Human Monoclonal Antibody Targeting the Influenza Hemagglutinin Drives the Emergence of a Virus That is More Virulent in Mice
Protection against influenza virus infection is most efficiently mediated by neutralizing antibodies targeting the influenza hemagglutinin (HA) protein. In 2009, a novel H1N1 influenza A virus (2009 pH1N1) emerged causing a pandemic. As this virus encounters immune pressure it will undergo

antigenic drift; however, which mutations will arise, their location, and how they may affect viral pathogenesis are unknown. The purpose of this study was to map the epitope of a human monoclonal antibody (hMAb, EM4C04) highly specific for the 2009 pH1N1 HA isolated from a severely ill 2009 pH1N1 virus-infected patient. We postulated that under immune pressure, the 2009 pH1N1 virus would mutate at the antibody binding site. We infected MDCK cells in the presence of EM4C04 and generated 11 escape mutants, displaying 7 distinct amino acid substitutions in the HA. Six substitutions greatly reduced mAb binding (K123N, D131E, K133T, G134S, K157N, G158E). Residues 131, 133 and 134 are contiguous with 157 and 158 in the globular domain structure and contribute to a novel pH1N1 antibody epitope that includes residues from the Sa antigenic site and residues previously believed to be non-antigenic. Detection of the K123N and G134S substitutions identified positions of two potential glycosylation sites that may emerge as a result of antigenic drift. One mutation near the receptor binding site, S186P, increased binding affinity of the HA to the receptor. 186P and 131E are present in the HA of the highly virulent 1918 pandemic influenza virus and were recently identified as virulence determinants in a mouse-passaged 2009 pH1N1 virus. We found that pH1N1 escape variants expressing these substitutions enhanced replication and lethality in mice compared to wild-type 2009 pH1N1 virus. Increased virulence of these viruses was associated with an increased affinity for a2,3 sialic acid receptors. Thus, antibody pressure by a hMAb targeting a novel epitope in the Sa region of 2009 pH1N1 HA can inadvertently drive the development of a more virulent virus with altered receptor binding properties. Our study identifies the previously unappreciated potential for antibody escape to enhance pathogenicity. This novel finding makes us rethink pathogenesis and broadens our understanding of antigenic drift.

Virology - RNA and Retroviruses

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Mutational Analysis of the Rotavirus RNA-Dependent RNA Polymerase Active Site

Rotaviruses are the primary cause of severe acute dehydrating gastroenteritis in infants and young children worldwide. The rotavirus RNA-dependent RNA polymerase (RdRp; VP1) is a hollow enzyme that contains canonical RdRp motifs A through F and a priming loop (PL). VP1 is inactive in the absence of the viral inner core shell protein. Accordingly, crystallized VP1 fails to interact stably with divalent cations or nucleotides, and its PL bends away from the active site. Thus, mechanisms by which VP1 interacts with substrates to achieve initiation and elongation are unclear. We hypothesized that comparison with other RdRps could provide insight into VP1 catalytic mechanisms. For the closest structural homolog of VP1, reovirus lambda3, conserved aspartic acids in motifs A and C coordinate divalent cations and interact with the ribose of an active-site nucleotide, and conserved basic residues in motif F interact with the nucleotide triphosphates. The extended lambda3 PL supports the priming nucleotide during initiation and undergoes conformational rearrangements to permit elongation. To identify conserved residues in the VP1 catalytic site, we made sequence-based alignments of VP1 from phylogenetically divergent rotaviruses and structure-based alignments with reovirus lambda3. Based on the alignments, we engineered, purified, and characterized the RNA synthetic capacity of VP1 mutants in vitro to gain insight into the functions of VP1 catalytic site residues and structures. We found that highly conserved aspartic acids in canonical RdRp motifs A and C were required for RNA synthesis. Mutants of conserved arginines in motif F exhibited significantly diminished RNA synthetic capacity. Mutation of several individual PL residues diminished levels of RNA synthesis, but for S495A and Q496A mutants, they were enhanced. Increased PL flexibility due to loss of side chain interactions with the rest of the molecule might explain this phenotype. These studies represent the first biochemical assessment of contributions

of PL residues and basic residues in motif F to RdRp function. They suggest that highly conserved aspartic acids in VP1 motifs A and C perform canonical functions and that interactions of motif F with nucleotides are important for RNA synthesis. These findings also suggest that the PL is a dynamic regulatory element, with some residues promoting and others hindering RNA synthesis.

Virology - RNA and Retroviruses
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Analysis of Maturation Inhibitor Treated HIV-1 Reveals Potential Maturation Intermediate
Retroviral maturation, involving the ordered cleavage of the polyprotein Gag into its components, MA (matrix), CA (capsid), SP1 (spacer) and NC (nucleocapsid) by the viral protease (PR), is essential for infectivity. The lack of intermediates has made the mechanism of maturation difficult to study in detail, but interference with PR activity results in particles that fail to mature and are non-infectious. Maturation inhibitors are a new class of HIV-1 antivirals that block viral replication by inhibiting the final PR cleavage event, preventing separation of the CA and SP1 domains of Gag. Maturation inhibitors appear to work through a novel mode of action, directly interacting with immature assembled Gag to block the CA/SP1 cleavage site, preventing its recognition by PR. In order to examine the structural effects of maturation inhibition, I have used cryo-electron tomography to analyze the three-dimensional structure of HIV-1 virions produced in the presence of two chemically unrelated maturation inhibitors, BVM and PF-46396. Particles treated with either compound contain an incomplete crescent-shaped shell of density beneath the viral membrane, as well as a small irregular core-like structure. To integrate the data from many particles and enhance viral features with local order or symmetry, I employed a strategy of sub-tomogram averaging. The averages reveal the observed shells are a protein lattice exhibiting 6-fold symmetry and 8-nm spacing. I conclude this is a remnant of the immature Gag lattice, due to the matching symmetry and spacing of the lattices. Particles with a genetic block in cleavage have no such lattice, but can be induced to contain similar structures by treatment with either drug, suggesting that both drugs actively stabilize the immature lattice. The ability of both tested drugs to block CA/SP1 cleavage and stabilize the immature lattice suggests that an effective maturation inhibitor requires both functions. Additionally, these particles provide a rare opportunity to examine a potential maturation intermediate; we observe hybrid shells within some treated particles that contain contiguous areas of both immature and apparently mature CA lattice. This previously unobserved structure has broad implications for the mechanism of mature capsid formation, suggesting that the transition from the immature to mature lattice can occur in-place, without CA lattice disassembly and re-assembly as depicted in current predominant models.