

FARE2017 WINNERS

Sorted By Study Section

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Chad Brocker

Research Fellow

Biochemistry - General and Lipids

Liver-specific peroxisome proliferator-activated receptor alpha (PPARA) knockout reveals a role for extrahepatic PPARA in glucose homeostasis

Peroxisome proliferator-activated receptor alpha (PPARA) is a ligand-activated transcription factor and a key mediator of systemic lipid homeostasis. PPARA is activated in response to fasting and promotes the uptake, utilization, and catabolism of fatty acids by regulating fatty acid transport and peroxisome and mitochondrial beta-oxidation. Moreover, constitutive agonist-induced PPARA activation causes hepatocellular carcinoma in mice. PPARA has profound effects on the fasting response, and PPARA activation protects against fasting-induced oxidative stress. After fasting, full-body PPARA knockout (KO) mice exhibit hepatic lipid accumulation leading to steatosis and elevated oxidative stress, lipid peroxidation and lipotoxicity. In order to elucidate the relationships between PPARA, fasting-induced hepatosteatosis, and oxidative stress, a liver-specific PPARA knockout (LKO) mouse was generated. Serum chemistry panels revealed markedly elevated serum phospholipids, cholesterol and triglycerides in fasted LKO mice when compared to KO. These endpoints were confirmed by LC/MS-based lipidomics, which showed significantly higher levels of circulating medium- and long-chain fatty acids. H&E and Oil Red O staining indicated significantly less hepatic lipid accumulation in LKO when compared to KO. These data were supported by TBARS assays revealing that lipid peroxidation was significantly lower in LKO mouse livers. Unlike KO mice, LKO mice do not display hypoglycemia in response to fasting. Furthermore, only KO mice exhibited insulin resistance as revealed by glucose tolerance tests. Alterations in glucose metabolism were supported by PAS staining which showed that impaired glycogen mobilization in KO was dramatically reduced in LKO mice. Differential glycogen metabolism was further supported by analysis of glycogenolytic gene expression that indicated slight but significant changes in expression of rate-limiting enzymes. Taken together, these studies provide strong evidence that extrahepatic PPARA expression plays a major role in regulating lipid and glucose homeostasis during the acute fasting response. Moreover, these studies serve as the foundation for future work utilizing cell-type specific knockout mice to expand our current understanding of PPARA's role during physiological response to fasting and agonist-induced carcinogenesis.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Cen Xie

Visiting FellowVisiting Fellow

Biochemistry - General and Lipids

Intestinal farnesoid X receptor signaling modulates hepatic gluconeogenesis

Enhanced hepatic gluconeogenesis is a major contributor to hyperglycemia in type 2 diabetes. To date, the underlying molecular mechanism of increased hepatic gluconeogenesis in type 2 diabetes remains largely unknown. Farnesoid X receptor (FXR) belongs to the nuclear receptor superfamily and is a crucial modulator that senses and regulates hepatic glucose levels and lipid metabolism. Recent studies revealed that gut microbiota influences metabolic disease by metabolizing bile acids into metabolites that inhibit intestinal FXR signaling. Manipulation of the gut microbiota-FXR signaling axis profoundly impacts glucose intolerance, but the molecular mechanism for this effect was poorly understood. Here, a combination of lipidomics, biochemical assays and use of intestine epithelial cell-specific FXR knockout (FXR-dIE) mice were employed to dissect the precise mechanism by which inhibition of intestinal FXR signaling improves glucose homeostasis. Absence of intestinal FXR in FXR-dIE mice was correlated with lowered intestinal-derived serum ceramides due to the direct control by FXR of genes involved in ceramide synthesis in the intestine. Lower serum ceramides resulted in decreased hepatic mitochondrial acetyl-CoA levels and pyruvate carboxylase activities, which attenuated hepatic gluconeogenesis. These processes were found to be independent of body weight change and hepatic insulin signaling. Further, ceramide administration to the FXR-dIE mice and primary hepatocytes substantially attenuated hepatic mitochondrial citrate synthase activities, primarily through the induction of endoplasmic reticulum (ER) stress, ER-mitochondria tethering, and calcium influx, which triggers increased hepatic mitochondrial acetyl-CoA levels and pyruvate carboxylase (PC) activities. Collectively, this study revealed a novel mechanism by which intestinal FXR regulates hepatic gluconeogenesis through a novel intestinal FXR-ceramide pathway, leading to decreased hepatic mitochondrial acetyl-CoA levels and PC activities, without affecting hepatic insulin signaling. Similar results as were obtained with FXR-dIE mice, could be achieved by the direct inhibition of intestinal FXR using an FXR antagonist, thus demonstrating that FXR is a potential target for the treatment of metabolic diseases such as type 2 diabetes.

National Institute on Alcohol Abuse and Alcoholism (NIAAA)

Janos Paloczi

Visiting FellowVisiting Fellow

Biochemistry - General and Lipids

Novel clinically relevant model of alcohol-induced cardiomyopathy associated with impaired cardiovascular function and cardiometabolic dysregulation

Chronic alcoholism ranks among the top five risk factors for disease and premature death. Binge drinking is the most common pattern of excessive alcohol use in the United States, particularly among young adults. Long-term, heavy alcohol consumption impairs vascular function and leads to development of cardiomyopathy and heart failure. However, reproducible and clinically relevant models of alcohol-induced cardiomyopathy in mice are lacking. Here we describe new mouse models of alcoholic cardiomyopathies induced by chronic and binge ethanol (EtOH)-feeding and characterize detailed hemodynamic and metabolic alterations, mitochondrial dysfunction, and impaired redox signaling. Mice were fed with 5% EtOH for 10, 20, 40 days (d) combined with single/multiple EtOH-binges. Isocalorically pair-fed mice served as controls. Left ventricular (LV) function was assessed by

sophisticated pressure-volume approach (allowing characterization of multiple load- and heart rate-independent indices of left ventricular performance) and by echocardiography. Mitochondrial complex (I, II, IV) activities, mitochondrial biogenesis, oxidative stress markers and histopathology were also investigated. Chronic and binge EtOH-feeding was characterized by contractile dysfunction (decreased slope of end-systolic pressure-volume relationship and preload recruitable stroke work), impaired relaxation (decreased time constant of LV pressure decay and dP/dt_{min}) and vascular dysfunction (impaired arterial elastance and lower total peripheral resistance). This was accompanied by enhanced myocardial oxidative/nitrative stress and deterioration of mitochondrial complex I, II, IV activities and mitochondrial biogenesis, myocardial hypertrophy, and excessive cardiac steatosis. The above described deleterious effects of alcohol consumption were dramatically more pronounced in groups exposed to chronic drinking combined with alcohol binges. Our results demonstrate that chronic plus binge EtOH-feeding in mice leads to alcohol-induced cardiomyopathy and vascular dysfunction characterized by increased myocardial oxidative/nitrative stress, impaired mitochondrial function and biogenesis, enhanced cardiac steatosis and hypertrophy. These results also suggest that combination of regular drinking with binges even within a short period of time may profoundly affect cardiac and vascular function and metabolism.

National Institute of Environmental Health Sciences (NIEHS)

Erin Romes

Postdoctoral Fellow

Biochemistry - Proteins

The Crystal Structure of the Ubiquitin-like Domain of Ribosome Assembly Factor WDR12 and Characterization of Its Interaction with the AAA-ATPase Midasin

The synthesis of eukaryotic ribosomes is a complex, energetically demanding process requiring the aid of numerous non-ribosomal assembly factors such as the PeBoW complex, Midasin, and Nle1. Ribosomal RNA (rRNA) transcription accounts for 60 percent of total cellular RNA transcription in growing cells and can lead to aberrant cell growth when improperly regulated. Although the structure and mechanics of the fully assembled ribosome are well understood, much less is known about the process of assembly. The mammalian PeBoW complex, comprised of Pes1, Bop1, and WDR12, is essential for processing the 32S pre-rRNA. Without removal of the PeBoW complex from pre-60S particles by the large dynein-like AAA-ATPase, Midasin, ribosome maturation is stalled, which activates the p53 response and ultimately leads to cell death. Our objective is to determine how Midasin recognizes its substrates; WDR12 and Nle1, and removes them from pre-60S particles. We solved the crystal structure of the ubiquitin-like (UBL) domain of the WDR12 homolog from *S. cerevisiae* at 1.7 Angstrom resolution, which revealed that the domain contains a beta-grasp fold and a well-conserved hydrophobic core. Through pull-down assays in HEK293 cells, we were able to demonstrate that Midasin contains a C-terminal metal ion-dependent adhesion site (MIDAS) domain that specifically interacts with the N-terminal UBL domain of WDR12. Subsequent pull-downs revealed that the MIDAS domain of Midasin also binds to the UBL domain of Nle1, at a later step of the ribosome maturation pathway. The interaction between the MIDAS and UBL domains is dependent upon metal ion coordination as removal of the metal or mutation of residue E78 of WDR12, which coordinates the metal ion, diminishes the interaction. We also identified that Midasin contains a well-conserved extension region upstream of the

MIDAS domain that is uniquely required for binding WDR12 and Nle1. Mammalian WDR12 displays prominent nucleolar localization that is dependent upon active rRNA transcription while Midasin displays prominent nucleoplasmic localization suggesting that the interactions between Midasin and WDR12 are transient. We conclude that the release of the PeBoW complex and subsequent release of Nle1 by Midasin is a well-conserved step in the ribosome maturation pathway and that Midasin recognizes its substrates; WDR12 and Nle1 through metal ion coordination with a conserved glutamate residue and a conserved extension domain.

National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS)

Aishe Angeletti Sarshad

Postdoctoral Fellow

Biochemistry - Proteins

Investigating the nuclear functions of Argonaute protein

The Argonaute (Ago) proteins form the core components of the RNA induced silencing complex. In RNA interference (RNAi) processes, Ago proteins are guided by bound small RNAs, microRNAs and siRNAs, to (partially) complementary sequences on target RNAs, resulting in target mRNA destabilization or translational repression. In metazoans, post-transcriptional gene silencing by cytoplasmic RNAi processes has been well documented. Recent reports suggest that components of the RNAi biogenesis pathway, including Ago protein, are nuclear and have additional functions beyond canonical miRNA processing. Here, we aim to combine biochemical and high throughput methods to validate the nuclear Ago findings and identify any regulatory roles Ago may have in the nucleus. We have performed nuclear and cytoplasmic fractionations in a panel of cell lines including HEK293, HeLa, P19, SHSY5Y and C2C12. Our results indicate a nuclear localization of Ago2. However, this phenomenon is dynamic and the levels of endogenous Ago2 fluctuate in a cell line dependent manner. We found that embryonic teratocarcinoma P19 cells and muscle specific myoblast C2C12 cells have high levels (~50%) of nuclear Ago2, whereas in HEK, HeLa and SHSY5Y Ago2 does not localize to the nucleus. P19 and C2C12 cells can differentiate to neurons and myotubes (MT), respectively. Therefore we investigated if Ago levels change upon differentiation. We observed that in differentiated P19 cells the levels of Ago2 decrease whereas endogenous Ago2 significantly increase in MTs. Nuclear localization of Ago is therefore a dynamic and highly regulated event. We next aim to investigate how nuclear Ago localization and function is regulated. We are performing Ago2 immunoprecipitation coupled to mass spectrometry to identify potential co-factors and possible protein modifications. These assays will give us a global understanding of processes nuclear Ago2 may be involved in. Since Ago2 is a RNA binding protein we will also characterize possible nuclear RNA targets by performing Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP). In summary, our work will shed light on whether in metazoan cells cytoplasmic RNAi function extends into the nucleus and how specific these events are. Furthermore, we aim to understand how Ago moves into the nucleus and what possible functions it may have there since our data confirm that these events are highly specific.

National Institute of Dental and Craniofacial Research (NIDCR)

Zulfeqhar Syed

Visiting Fellow

Biochemistry - Proteins

Functional analysis of Drosophila mucins during development

The mucosal barrier in the gut, lungs and other vital tubular organs consist primarily of mucins and protects the underlying cells against pathogens, dehydration and physical or chemical injury. Mucins are large, high molecular weight glycoproteins which contain a central polymorphic domain composed of tandem repeats, rich in amino acids serine, threonine and proline. These highly O-glycosylated proteins form densely arrayed structures that provide multivalency and high stoichiometric power. In *Drosophila*, twenty-three mucins and mucin-related proteins are identified which are expressed dynamically during different stages of the life cycle. To understand the role of *Drosophila* mucins during development, we used the transgenic RNA interference (RNAi) system to systematically knockdown mucins. Ubiquitous knockdown of mucins expressed in the gut, resulted in larval and pupal lethality, indicating that they are essential and suggesting their requirement for protective barrier formation in these tissues. Interestingly, tissue-specific knockdown of one mucin (Muc26B), which is expressed in the digestive tract showed 1st instar larva lethality along with a dramatic up-regulation of genes encoding antimicrobial peptides. Additionally, loss of this mucin resulted in activation of the JAK/STAT signaling pathway. Immunostaining for this mucin showed that it is expressed in specific cells of the digestive tract that are responsible for producing the mucosal membrane. Indeed, MUC26B staining could be seen in this protective membrane in wild type flies. We therefore hypothesize that MUC26B serves as a crucial component of this membrane. We are currently investigating the cellular processes and signaling cascades activated in response to the loss of MUC26B. We are generating transgenic flies that endogenously express Muc26B lacking mucin-domains and associated chitin-binding domains to investigate the role of MUC26B in mucous membrane formation and barrier function. These generated transgenic flies will aid in elucidating changes in mucous membrane structure and function upon modulation of different MUC26B domains. In addition, we will investigate the role of MUC26B glycosylation in the packaging, secretion and formation of the mucous membrane using correlative light and electron microscopy (CLEM). These studies will ultimately advance our understanding of mucins and their role in the protection of epithelial cell surfaces.

National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)

Rakesh Sikdar

Visiting Fellow

Biochemistry - Proteins

Characterizing the mechanism of assembly and secretion of the trimeric autotransporter UpaG

Trimeric Autotransporter Adhesins (TAAs) are prominent pathogenicity factors found in Gram-negative bacteria, which are associated with host colonization. UpaG is a TAA derived from the uropathogenic *E.coli* strain CFT073, which promotes adhesion of the pathogen to human bladder epithelial cells with specific affinity to fibronectin and laminin. Additionally, UpaG promotes cellular aggregation and attachment of the bacteria to abiotic surfaces. TAAs, like classical autotransporters, are composed of a C-terminal β -barrel embedded in the outer membrane and an N-terminal passenger domain, which is

secreted to the cell surface. However, unlike classical autotransporters, the passenger domain of TAAs remains attached to the β -barrel embedded in the outer membrane, after translocation is completed. TAAs are translated as monomeric species with a cleavable signal peptide in the cytoplasm and are transported across the inner membrane to the periplasm via the general secretory pathway (Sec pathway) and finally escorted by periplasmic chaperones to the outer membrane, where it undergoes trimerization and assembly into the functional adhesin by an unknown mechanism. In our studies, we have attempted to characterize the molecular mechanism by which monomeric forms of UpaG are transported to the outer membrane of Gram-negative bacteria and assembled into a functional homotrimeric adhesin. Our preliminary results using 35-S pulse-chase and immunoprecipitation techniques demonstrate the spatial and temporal dynamics of trimerization of UpaG, assembly of the C-terminal β -barrel at the outer membrane and translocation of the N-terminal passenger domain to the cell surface. Additionally, using site-specific photo-crosslinking techniques, we have identified the formation of a transient intermediate in the assembly and secretion process, which demonstrates that the assembly of UpaG proceeds via the formation of a possible unstable trimeric form. By incorporating major truncations and disordered modifications to the passenger domain, we demonstrate that the folding of the N-terminal passenger domain of UpaG is dispensable for its translocation as well as the assembly of the C-terminal β -barrel at the outer membrane. Lastly, we have observed that the Bam (Barrel Assembly Machine) multi-protein complex, which is essential for the folding and insertion of β -barrel proteins at the outer membrane, is required for efficient assembly and secretion of UpaG.

National Institute of Child Health and Human Development (NICHD)

Gregory Holmes-Hampton

Postdoctoral Fellow

Biochemistry - Proteins

Use of Antisense Oligonucleotides to Correct the Splicing Error in ISCU Myopathy Cell Lines

Iron-Sulfur clusters (ISCs) are protein cofactors found in essentially every living organism. They are used in processes such as DNA replication and repair, electron transfer, and cellular respiration. ISC production involves several proteins; central to this is the scaffold protein ISCU. Individuals have been described with ISCU myopathy wherein patients harbor a G>C point mutation in the fourth intron that amplifies a polypyrimidine tract. This leads to the inclusion of an aberrant exon that includes a premature stop codon and leads to synthesis of a rapidly degraded, truncated form of the protein. Phenotypes of life-long exercise intolerance wherein mild physical activity can lead to severe fatigue and muscular degeneration and cellular level defects in several ISC containing enzymes have been noted especially in skeletal muscle. Currently no treatment has been established for ISCU myopathy, so patients must learn to deal with the disease by not exerting themselves physically. We have collaborated with Ionis Pharmaceuticals and received antisense oligonucleotides (ASOs) targeted to this mutation. Ionis has an FDA approved drug with similar chemistry (targeted at blocking protein synthesis rather than correcting a splicing error) and has shown that ASOs can be delivered throughout the body (including skeletal muscle) via subcutaneous injection. Northern blot analyses of ASO treated patient cells demonstrate decreased levels of the abnormal transcript and increased levels of the normal transcript. Furthermore western blot analysis has shown that protein levels of ISCU which are drastically decreased in cells from patients return to levels found in healthy control cells with ASO treatment. We

have also demonstrated that aconitase activities, which are diminished in patient cells, increase following ASO treatment. Western blots for SDH subunit B (a subunit that is destabilized in the absence of ISCs) show diminished levels in patient cells, but they increase following ASO treatment. Additionally we have demonstrated decreased activity of SDH in patient cells and a corresponding increase in succinate levels; following ASO treatment we see an increase in SDH activity and a decrease in succinate levels to levels similar to those of healthy controls. Taken together these results present a promising strategy for correcting a previously untreatable disease, and perhaps a more general strategy for treatment of other diseases caused by splicing errors.

National Institute of Allergy and Infectious Diseases (NIAID)

Oded Yaakobi

Visiting Fellow

Biophysics

A three-dimensional, time-dependent computational model of a whole-cell: coupling the biomechanics to the signaling network

A main factor hindering progress to a more quantitative cell biology is the lack of computational tools enabling experimentalists to explore detailed models of cellular behavior. One of the challenges is that realistic descriptions of cell signaling networks often comprise molecular complexes including many components in various states, leading to systems with up to thousands of complexes, that usually have important spatial aspects. Writing by hand the thousands of partial differential equations that describe the three-dimensional (3D) network dynamics is impractical. In order to overcome this hurdle, our group has developed software that guides the user via the specification of multiple hierarchical scales of cellular behavior, and automatically generates the mathematical formalism needed for computer simulations. A specific powerful feature of our tool is that it can simulate time-dependent cell morphologies. However, a coupling between cell biochemistry and mechanics has still been missing. My goal is to add critical biological aspects that are absent in our software, such as the cytoskeleton and membrane dynamics in motile cells. In the past year, I developed a C++ code that implements a physical model of a two-phase (cytoskeleton and cytosol) fluid flow interacting with the cell membrane. The model couples between the cell mechanics and biochemistry by (1) the local net rate of cytoskeletal polymerization as a source term in the mass conservation law of each phase, and (2) moving integration domain boundaries of the reaction-diffusion equations due to 3D membrane deformation. The code simulates the following effects: Cell membrane surface tension; Diffusion of a messenger that is emitted from the cell membrane into the cytoplasm; Polymerization of actin in a rate that depends on the local messenger concentration; Drag between the flowing two phases. My code supports parallel computation, in order to allow simulating complex phenomena in reasonable runtime period. I validated my code by testing its results in well-known cases e.g. diffusion in a box and sessile drop flattening. My current model assumes that the cytoskeleton is an isotropic continuum phase, i.e. neglects the actin fibers directivity, but I intend to modify this description to capture directivity soon. Next, I plan to integrate my code with our existing signaling pathways software, and test our integrated software by comparison to experimental data of cell migration (e.g. chemotaxis).

National Institute on Alcohol Abuse and Alcoholism (NIAAA)

Youngchan Kim

Visiting Fellow

Biophysics

Photon antibunching and fluorescence correlation spectroscopy reveal remarkably strong coupling between fluorescent proteins in Venus oligomers

Fluorescent proteins (FPs) have revolutionized biomedical research by enabling genetic protein tagging and the visualization of cellular interactions in living cells. Interpretation of these experiments typically assumes that FP photo-physics are the same as those observed for organic fluorophores. Surprisingly, anomalous FP behavior has been observed in several studies suggesting that these fluorophores might have unique photo-physical behaviors that complicate experimental interpretation but may be exploited to enable quantum computing. Thus, a better understanding of FP photo-physics is warranted. Here, we present results of fluorescence correlation spectroscopy (FCS) and photon antibunching (AB) for six specific fluorescent protein constructs composed of between 1 and 6 concatenated Venus molecules (V1-V6). FCS was used to accurately and precisely measure the concentration of V1-V6 constructs, and to confirm the number of linked Venus molecules comprising these constructs (1 through 6 respectively). AB measures coincidences, an indicator of the probability for detecting two emitted photons, as a function of the time between when each photon is detected. Quantum mechanics and the particle nature of light dictate that a single fluorophore or quantum entity can only give off one photon at a time. In contrast two or more independent fluorophores should be able to give off two photons at the same time. In this experiment AB was used to determine if any of the Venus oligomers could emit more than one photon at a time by monitoring the number of coincidences observed at times shorter than the Venus lifetime (3 ns). V1-V6 constructs were diluted to a concentration where ~ 0.3 molecules were in the microscope observation volume (determined by FCS). Remarkably, AB revealed that Venus oligomers all behave like a Venus monomer, they emit only one photon at a time regardless of how many Venus fluorophores are in the complex. We conclude that Venus fluorophores in V2-V6 are not independent, but are strongly coupled. Two apparently incredulous explanations for the strong AB observed for V2-V6 are: 1. Venus fluorophores in V2-V6 act as a single quantum entity (thus, only one excitation event can occur), or 2. When more than one Venus is excited in these oligomers their excitation energy efficiently hops from Venus to Venus until singlet-singlet annihilation occurs resulting in the emission of a single photon. Experiments to test these hypotheses are being conducted.

National Institute of Neurological Disorders and Stroke (NINDS)

Anowarul Amin

Visiting Fellow

Biophysics

Role of Counterions in Acidification in Mouse Liver Lysosomes

The lysosome is a membrane-bound compartment that digests macromolecules and cellular debris. To function properly, lysosomes must maintain an acidic environment (pH 4.5-5.0), generated by the proton-pumping action of a v-type H⁺ ATPase. However, since the ATPase is highly electrogenic, the

voltage generated by its action must be dissipated by another ion movement, known as the ‘counterion pathway.’ The mechanism of counterion movement is controversial, with proposals that it involves Cl⁻ ions entering the lysosome or cations exiting. The CIC-7 Cl⁻/H⁺ antiporter, a CLC gene family member which is the primary pathway for Cl⁻ movement across the membranes of rat liver lysosomes, has been proposed as an important contributor to the counterion pathway. However, several studies suggested that lysosomal pH is unaffected in cultured cells from CIC-7 knockout mice, hinting that other ion conductances contribute to the counterion pathway. Here, we sought to comprehensively analyze the role of CIC-7 and the Cl⁻ movement it mediates in a single well-characterized system: liver lysosomes isolated from mice with tissue specific knockouts of the transporter. We loaded lysosomes with the pH-sensitive fluorescent probe FITC-dextran by intraperitoneal injection of mice, then isolated lysosomes or liver cells for further characterization. Acidification in mouse liver lysosomes is strongly dependent on the presence of Cl⁻ in the bathing buffer, and is minimally affected by varying buffer concentrations of K⁺, suggesting that Cl⁻ indeed serves as the counterion. As expected, knocking out the CIC-7 gene in these cells abolishes the previously described H⁺-coupled Cl⁻ transport. Lysosomes from the knockout mouse liver are strongly reduced in ATP-driven acidification, consistent with CIC-7 acting as the primary counterion pathway in this system.

National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)

pengfei Tian

Visiting Fellow

Biophysics

Structural determinants of misfolding in multidomain proteins

Protein misfolding and aggregation are well-known for their association with amyloidosis and other diseases. Proteins with two or more domains are abundant in higher organisms, accounting for up to 70% of all eukaryotic proteins, and domain-repeat proteins in particular occupy a fraction up to 20% of the proteomes in multicellular organisms, therefore their folding is of considerable relevance. Recent single molecule experiments, using either atomic force microscopy (AFM) or Förster resonance energy transfer (FRET) have shown that multidomain proteins containing tandem repeats may form stable misfolded structures. Topology-based computational models have been used successfully to generate models for these structures with domain-swapped features, fully consistent with the available data. However, it is also known that some multidomain protein folds exhibit no evidence for misfolding, even when adjacent domains have identical sequences. Here we pose the question: what factors influence the propensity of a given fold to undergo domain-swapped misfolding? Using a coarse-grained simulation model, we can reproduce the known propensities of multidomain proteins to form domain-swapped misfolds, where data is available. Contrary to what might be naively expected based on the previously described misfolding mechanism, we find that the extent of misfolding is not determined by the relative folding rates or barrier heights for forming the initial intermediates leading to folded or misfolded structures. Instead, it appears that the propensity is more closely related to the relative stability of the folded and misfolded intermediates. We show that these findings can be rationalized if the folded and misfolded domains are part of the same folding funnel, with commitment to one structure or the other occurring only at a relatively late stage of folding. Nonetheless, the results are still fully consistent with the kinetic models previously proposed to explain misfolding, with a specific

interpretation of the observed rate coefficients. Finally, we investigate the relation between interdomain linker length and misfolding, and propose a simple alchemical model to predict the propensity for domain-swapped misfolding of multidomain proteins.

National Institute of Environmental Health Sciences (NIEHS)

Ming Ji

Postdoctoral Fellow Postdoctoral Fellow

Carcinogenesis

Haploinsufficiency of SIRT1 promotes cancer development through enhanced glutaminolysis

SIRT1, the most conserved NAD⁺-dependent protein deacetylase, is an important cellular metabolic and stress sensor. However, the role of this critical factor in cancer development remains unclear and inconclusive. SIRT1 has been shown to directly maintain genome stability and repress inflammation, thereby decreasing tumor growth. On the other hand, SIRT1 also has been reported to inhibit activities of tumor suppressors, promoting growth and survival of cancer cells. As a result, whether SIRT1 is an oncogene or tumor suppressor remains controversial. Here, we explored the possibility that SIRT1 regulates cancer development in a quantitative dose-dependent manner. We hypothesized that different SIRT1-regulated cellular pathways have distinct sensitivities to changes of SIRT1 dosages. These distinct sensitivities may differentiate the outcomes in cancer cell proliferation and growth, contributing to observed dual functions of SIRT1 in tumor development. To test this hypothesis, we generated immortalized mouse embryonic fibroblasts (MEFs) and human colorectal cancer cell lines carrying two copies (WT), one copy (Het), or no copy (KO) of SIRT1 gene. Consistent with our hypothesis, SIRT1 Het cells displayed elevated proliferation in culture, increased colony formation on soft agar, and enhanced tumor formation in nude mice in a xenograft model, whereas SIRT1 KO cells exhibited reduced proliferation, colony formation, and cancer formation. Further mechanistic studies revealed that deletion of one copy of SIRT1 gene was sufficient to activate NF- κ B and induce c-Myc expression, promoting cell proliferation, autophagy, and stress resistance in a glutamine-dependent manner. Deletion of both copies of SIRT1 gene, on the other hand, triggered cellular apoptotic pathways, leading to increased cell death, diminished autophagy, and reduced cancer formation. Consistently, intestine-specific SIRT1 heterozygous mice have enhanced intestinal tumor formation, whereas intestine-specific SIRT1 homozygous knockout mice have reduced development of colon cancer. Finally, expression levels of SIRT1 are reduced in human colorectal tumors, and reduced tumor SIRT1 expression correlates with poor prognosis in colorectal cancer patients. In summary, our findings reveal a crucial function of SIRT1 in dose-dependent regulation of cancer metabolism and development.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Helen Michael

Postdoctoral Fellow Postdoctoral Fellow

Carcinogenesis

Initiation and progression of UV-induced melanocytic moles and melanoma in a mouse model of human

melanoma

Melanoma is the deadliest form of skin cancer with approximately 132,000 new cases each year worldwide. Melanocytic moles (nevi) are ubiquitous and typically benign lesions that develop in childhood or early adulthood. Individual nevi rarely progress to melanoma, but up to half of melanomas arise from a pre-existing nevus. UV exposure, particularly childhood sunburn, is believed to be important for the development of melanocytic lesions, although the exact mechanism remains unknown. Mutation in MAP kinase pathway genes is common in both melanomas and melanocytic nevi, but 1/3 of melanomas have no known major driver. There are no biomarkers or histologic features that predict which nevi may progress. Studying nevus initiation and transformation in human populations is impractical due to the low frequency and long latency to progression. Wild-type mice have follicle-restricted melanocytes and are resistant to melanoma. Our HGF genetically engineered mouse model has a “humanized” junctional distribution of melanocytes and the iDCT-GFP background has melanocyte-specific nuclear GFP expression. Following a single relevant neonatal UV-exposure modelling childhood sunburn, HGF iDCT-GFP mice develop small, discrete nevi, some of which resume growth within 6-12 months and progress to melanoma with a radial growth phase (RGP) followed by a vertical growth phase (VGP). The melanocytic lesions are histologically similar to human nevi and melanoma, label strongly with melanocyte markers, and are transplantable into syngeneic mice. Exome sequencing of 16 melanocytic lesions, including stable nevi, RGP and VGP melanomas show a predominance of C to T mutations at all stages, consistent with UV exposure. The mean number of nonsynonymous coding mutations is higher in vertical growth phase tumors (299) than in radial growth phase tumors (112) or nevi (101). The increase in mutations in VGP lesions is due to increased C > T transitions. Mutations in DNA repair genes are seen only VGP tumors, suggesting a role in the increased mutations associated with progression to VGP melanoma. Genes potentially involved in initiation (mutated in nevi and melanomas) and progression (mutated only in melanoma) have been identified and compared with human melanoma sequencing data to select candidates for functional validation with in vitro skin reconstitution assays. This information could inform clinical management of nevi and identify novel drivers and therapeutic targets for human melanoma.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Hien Dang

Postdoctoral Fellow Postdoctoral Fellow

Carcinogenesis

ONCOGENIC ACTIVATION OF THE RNA BINDING PROTEIN RDBP IS MEDIATED BY C-MYC SIGNALING IN HEPATOCELLULAR CARCINOMA

Global transcriptomic alterations of both coding and non-coding RNA species are a ubiquitous feature associated with human cancers including hepatocellular carcinoma (HCC). Dysregulation of RNA-binding proteins (RBPs), the key regulators of RNA processing, is one mechanism in which cancer cells select to promote tumorigenesis. We analyzed genomic alterations amongst a family of more than 800 mRNA RBPs (mRBPs) in 1,225 clinical specimens from HCC patients and found that RBPs are significantly activated through gene amplification in a subset of tumors with poor prognosis, suggesting their potential oncogenic roles in HCC progression. Amongst the top candidates, RD binding protein (RDBP) was further characterized for its oncogenic role and effects on the HCC transcriptome. While the

activation of RDBP induced an oncogenic phenotype, the abrogation of RDBP in HCC cells significantly decreased cancer associated phenotypes such as cell proliferation, migration/invasion and tumorigenicity in vivo. Further analyses revealed that RDBP-dependent genes were tumor-related with a significant enrichment for c-Myc targets, suggesting interplay between RDBP and c-Myc signaling. Similar data were also found in HCC clinical specimens where c-Myc amplification was uncommon. Consistently, the RDBP-dependent c-Myc target gene signature was able to predict HCC patient survival in two independent cohorts of more than 400 patients. Our results demonstrate that oncogenic activation of RDBP is a novel mechanism that contributes to global transcriptome imbalance, which is selective for the activation of c-Myc oncogenic signaling in HCC. Concurrent with the current model that indicates that c-Myc can promote tumorigenesis through transcription dysregulation, our current work suggests that therapies focused on targeting RDBP may be valuable for clinical treatment of many different tumors with activated c-Myc signaling, including HCC.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Feng Zhu

Research Fellow

Carcinogenesis

Fungal Infection and Immune Dysfunction Contribute to Esophageal Carcinogenesis

Human esophageal cancer is the sixth leading cause of cancer death worldwide. More than 90% of esophageal cancer is esophageal squamous cell carcinoma (ESCC). While the etiological cause remains unclear, esophageal mucosal fungal infection is very common in esophageal cancer patients, including patients with autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) who have more than 20-fold risk of ESCC when compared to the healthy population. Here, we report that kinase-dead Ikka knock-in mice (hereafter referred to as mutant mice) develop APECED-like autoimmune disease due to developmental defects in thymus and lack of central tolerance. Similar to human APECED patients who develop esophageal mucosal fungal infection on average at the age of five, mutant mice develop fungal infection in oral cavity and esophagus as early as seven weeks old. About 20% of mutant mice develop ESCC during aging, which exhibits specific molecular signatures observed in human ESCC, such as p16 gene silencing, elevated EGFR phosphorylation and PD-L1 expression, etc. Autoreactive T cell depletion, or central tolerance reconstitution and/or normal T cell transfer prevents fungal infection and ESCC development in mutant mice. Importantly, antifungal drug treatment inhibits inflammation and ESCC development in mutant mice. However, oral inoculation of *Cladosporium*, one of the major fungal species isolated from mutant mice, increases the tumor incidence from 20% to 63% in mutant mice. These results reveal that immune dysfunction and fungal infection is associated with ESCC development, which sheds new light on ESCC etiological factors, prevention and treatment.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Andrea Simone Baechler

Visiting Fellow

Carcinogenesis

Novel role of mitochondrial topoisomerase I (Top1mt) in carcinogenesis

Mitochondria are essential cellular organelles, playing a key role in the regulation of cellular metabolism and apoptosis. Mitochondrial topoisomerase I (Top1mt) is exclusively located in the mitochondria and has been shown to be critical for mitochondrial DNA homeostasis by releasing mtDNA topological stress. We recently reported that lack of Top1mt impairs cellular proliferation under high-energy demand, as required during liver regeneration. Notably, Top1mt is highly upregulated in hepatocellular and colon carcinomas, which is accompanied with a poor survival prognosis of patients carrying an alteration of Top1mt (TCGA database). Based on these findings, we raised the question whether Top1mt could play a role in carcinogenesis, since cancer cells are highly metabolically demanding. In a xenograft model using human colon carcinoma cells (HCT116), we transplanted Top1mt-KO cells, generated through CRISPR/Cas9 genomic editing, and wild-type (WT) cells into the flank of nude mice. Lack of Top1mt resulted in a significantly slower tumor growth compared to tumors from WT cells. Additionally, tumor frequency was significantly diminished in a limiting dilution assay. In line with our hypothesis steady-state ATP-levels were decreased in tumors lacking Top1mt compared to the WT controls pointing to impaired mitochondrial function and forcing cells to exclusively utilize glycolysis to supply their energy for proliferation. To address if our findings translate to an intrinsic carcinogenesis model, we induced hepatocellular carcinoma in WT and Top1mt KO neonate mice by diethylnitrosamine injection, a liver-specific DNA alkylating agent. Lack of Top1mt led to significantly diminished tumor burden, decreased maximal tumor size and reduced multiplicity 50 weeks after drug-injection compared to the WT littermates. Thus, our data demonstrate that loss of Top1mt delays tumor growth and frequency, in both a xenograft and chemically induced carcinogenesis model. We conclude, that Top1mt is important for cancer cell growth in metabolically compromised microenvironments, in which cells have a limited ability to tolerate decreased mitochondrial function. These microenvironments represent niches for cancer stem cells, which are slowly growing and resistant to standard chemotherapy. Our study identifies Top1mt as a novel mitochondrial target for cancer therapy and could result in new combinational treatments by enhancing the efficacy against quiescent cancer stem cells.

National Institute of Child Health and Human Development (NICHD)

Carlos Guardia

Visiting Fellow

Cell Biology - General

Coupling to different kinesin motors directs regional movement of lysosomes along distinct microtubule tracks

Recent advances in live-cell imaging have revealed that the spatial organization of cytoplasmic organelles within eukaryotic cells is highly dynamic. Indeed, organelles move around the cytoplasm, change their size and shape, and establish transient contacts with one another, all under precise regulatory controls. Prime examples of such dynamic organelles are lysosomes, membrane-enclosed vacuoles that function in the degradation of biological macromolecules in the endomembrane system. Lysosomes move back and forth between the center and the periphery of the cell along microtubule tracks. This bidirectional movement is of paramount importance for the distribution of the degradative activity of lysosomes to all regions of the cytoplasm, as well as for the involvement of lysosomes in other

cellular processes such as microbial killing, plasma membrane repair, cell migration, metabolic signaling, and tumor invasion. We recently discovered a multisubunit complex named BORG that recruits the small GTPase Arl8 to promote lysosome movement toward the cell periphery. We have now found that BORG and Arl8 function upstream of two structurally different microtubule motors, kinesin-1 and kinesin-3, to drive centrifugal movement of lysosomes. Strikingly, each of these kinesins links lysosomes to a different set of microtubule tracks: more convoluted and centrally located for kinesin-1; more rectilinear and peripheral for kinesin-3. Expression of ATPase-defective “rigor” mutants of these kinesins, which can bind the corresponding microtubules but no walk along them, revealed that both kinesins can function sequentially to move lysosomes to the peripheral cytoplasm in non-neuronal cells. BORG/Arl8-dependent coupling of lysosomes to different kinesins may also explain the ability of lysosomes to move along distinct microtubule tracks in neuronal axons and dendrites. Defective lysosome transport by the mechanisms described here may contribute to the pathogenesis of hereditary spastic paraplegia syndromes caused by mutations in kinesin-1 and kinesin-3 proteins.

National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)

LI-KA LIU

Visiting Fellow

Cell Biology - General

An ER-Golgi tether facilitates ceramide transfer out of the ER and alleviates ceramide toxicity

Ceramides are the key intermediate of sphingolipid biosynthesis, but also act as messengers in a variety of signaling pathways. The amount of ceramide needs to be precisely regulated, as excess ceramide leads to cell growth arrest and apoptosis. Ceramide accumulation is associated with several human pathologies, such as cancer and neurodegenerative diseases. Several chemical-therapy drug resistant cancer cells can also result from abnormal ceramide homeostasis. Therefore, understanding how the cell responds and adapts to ceramide toxicity is of critical importance, and can provide new insights into the development of novel drug targets in several types of cancer. Here we identify a novel mechanism by which cells prevent the toxic accumulation of ceramides: through promotion of nonvesicular ceramide transfer out of their site of synthesis – the ER, to the Golgi complex where ceramides are subsequently converted to complex sphingolipids. Ceramides synthesized in the ER are delivered to the Golgi by both COPII dependent vesicular transport and COPII-independent nonvesicular transport. We find that the protein Nvj2p is a dynamic ER-Golgi tether that induces close ER-Golgi contacts and promotes the nonvesicular transfer of ceramides to the Golgi. We show that overexpression of Nvj2p results in increased nonvesicular ceramide transfer in vesicular transport-deficit mutants both in vivo and in vitro. Conversely, knockout of Nvj2p in these mutants exacerbated the block in ceramide transport. We further show that overproduction of Nvj2p induces ER-Golgi tethering, underscoring its role as an interorganelle tether. Intriguingly, whilst Nvj2p normally resides at contact sites between the ER and other organelles, we observe relocalization of this protein to the ER-Golgi junction to induce ER-Golgi contacts during ceramide accumulation or ER stress. Our findings reveal a unique mechanism by which cells can regulate ER-Golgi contacts in response to stress, in order to enhance nonvesicular ceramide transfer out of the ER and prevent toxic build-up of ceramides.

National Institute of Child Health and Human Development (NICHD)

Rui Jia

Postdoctoral Fellow

Cell Biology - General

BORC regulates autophagosome-lysosome fusion through KXD1-LC3 interaction

Autophagy is a vital degradative pathway involving the engulfment of cytoplasmic materials into autophagosomes and their subsequent fusion with lysosomes to form autolysosomes. A growing number of reports show that dysregulated autophagy is implicated in various human diseases, including infectious, neurodegenerative, metabolic and neoplastic disorders. Recently, we identified a multi-protein complex named BORC that associates with the lysosomal membrane and regulates lysosome positioning by mediating coupling to kinesin. Interestingly, we found that knock out (KO) of BORC subunits using CRISPR/Cas9 did not only cause clustering of lysosomes in the pericentrosomal area, but also elevated the levels of the early autophagy marker LC3-II and the number of autophagosomes in the cell. BORC-KO cells accumulated aggregates of a pathogenic huntingtin mutant that is normally cleared by autophagy. Furthermore, inhibition of lysosomal degradation by incubation with chloroquine increased LC3-II in BORC-KO cells to the same levels as in wild-type cells. These results indicated that BORC KO did not increase autophagy initiation but decreased autolysosomal degradation. Fluorescence microscopy analyses showed that BORC KO reduced co-localization of autophagosomes with lysosomes by 40%, and the number of autolysosomes by 50%, both consistent with a defect in autophagosome-lysosome fusion. This defect could be due to the inability of lysosomes to move toward the autophagosomes. However, we also found that the BORC subunit KXD1 interacts with LC3 via an LC3-interacting-region (LIR) motif, consistent with an additional role of BORC in autophagosome-lysosome fusion. Taken together, our results indicate that BORC promotes both the encounter of autophagosomes with lysosomes and their eventual fusion into autolysosomes, thus contributing to the function of the autophagy machinery.

National Institute of Allergy and Infectious Diseases (NIAID)

Miao Pan

Visiting Fellow

Cell Biology - General

Identification of a Chemoattractant G-Protein-Coupled Receptor for Folic Acid that Controls Both Chemotaxis and Phagocytosis

Highly motile amoeboid cells, such as neutrophils, macrophages and Dictyostelium discoideum are professional phagocytes that track down infecting microorganisms by chemotaxis and capture and destroy them via phagocytosis. Both chemotaxis and phagocytosis share two common steps: detection of bacterial targets and activation of a signaling network that controls the actin cytoskeleton to generate pseudopods for cell movement or engulfment of the targets. G-protein-coupled receptors (GPCRs) for chemokines, detect chemoattractants generated by bacteria, and activate signaling pathways to regulate actin polymerization for cell migration towards the bacteria. While GPCRs are essential for chemotaxis, their functions in phagocytosis are not clear. As an established model organism, the social

amoeba *D. discoideum* cells are known to chase bacteria via chemotaxis to folic acid and ingest them as food through phagocytosis. However, although folic acid was shown to be a chemoattractant for *D. discoideum* to seek bacteria more than 40 years ago, a folic-acid receptor(s) has not been identified. Here we developed a mass spectrometry based quantitative phosphoproteomic approach for discovering missing components in a particular signaling network. Using this methods, we discovered the long sought after folic acid receptor, fAR1, in *D. discoideum*, which exhibited a specific phosphorylation increase upon folic acid stimulation. We showed that the seven-transmembrane receptor fAR1 is required for folic acid-binding and folic acid-mediated signaling events. Consistently, fAR1-GFP is localized on the cell surface. Furthermore, fAR1 knockout (KO) cells is defective in both chemotaxis toward folic acid and phagocytosis of bacteria. Live cell imaging and flow cytometry results suggested that fAR1 is essential for pseudopod formation during chemotaxis and phagocytosis. By utilizing latex beads with fluorescently labeled folic acid crosslinked to the surface, we demonstrated that the binding of immobile folic acid to fAR1 receptor induces localized chemotactic response to form phagocytic cups, thereby promoting the engulfment of the beads. Collectively, we uncovered a chemoattractant GPCR that mediates not only chasing but also ingesting bacteria. Our discovery revealed that a phagocyte is able to internalize particles via a cellular process of “chemoattractant-mediated engulfment”. We propose that mammalian phagocytes may also use this mechanism to subdue bacterial pathogens.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Ritankar Majumdar

Postdoctoral Fellow

Cell Biology - General

Nuclear lipid microdomains as a novel niche for exosome biogenesis during relay of chemotactic signals in neutrophils.

Neutrophils migrating towards a weak chemoattractant gradient amplify their recruitment range via signal relay by releasing the secondary chemoattractant leukotriene B4 (LTB4). LTB4 is synthesized from arachidonic acid (AA) through the sequential action of the 5-lipoxygenase (5LO) and its associated activating protein (FLAP). We previously demonstrated that LTB4 and its synthesizing enzymes are packaged and released in multivesicular bodies (MVB)-derived extracellular vesicles called exosomes. Ultrastructural images of chemotaxing neutrophils show that 5LO-labeled MVBs are associated with the nuclear envelope (NE). This is unusual, as MVBs are known to be generated through endosomes. To determine the role of the NE in MVB formation, we isolated nuclei from activated neutrophils and found that FLAP and 5LO are enriched in the detergent resistant membranes (DRMs) of the NE. As the primary role of FLAP is to facilitate the transport of AA to 5LO, we investigated the distribution of FLAP mutants with high or low AA affinity. Compared to the high affinity FLAP mutant, which localized to nuclear DRMs in both activating and resting cells, the low affinity mutants showed no localization to lipid DRMs under either conditions. AA is a positive regulator of sphingomyelinase-2 (SMPD2), which converts sphingomyelin to ceramide, both of which are key constituents of ceramide microdomains on the NE. shRNA-mediated knock down of SMPD2 caused the mislocalization of FLAP and a loss of exosome production, suggesting that AA mediates ceramide-dependent nuclear microdomain formation at the NE. Two-photon imaging of chemotaxing neutrophils revealed localized lipid ordered regions around the

NE, which could be disrupted by treating neutrophils with short chain ceramides. Expressing a mutant form of VPS4, a key component of the vesicle budding machinery that prevents disassembly of the MVB, led to the formation of un-dissociated vesicular buds on the NE. Together, these findings establish that neutrophil activation causes the mobilization of AA and sphingomyelin/ceramide at the NE. This results in the formation of localized regions of high membrane anisotropy that act as seed sites for the recruitment of MVB synthesizing machinery and exosome biogenesis at the nucleus. We envision that this novel mechanism will represent an important means of MVB biogenesis for the release of NE-associated components.

National Institute of Child Health and Human Development (NICHD)

Felipe Montecinos-Franjola

Postdoctoral Fellow

Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology

Probing the multi-tubulin hypothesis: the reversible dissociation of α/β -tubulin dimers is modulated by isotype composition and posttranslational modifications

Microtubules are a major component of the eukaryotic cytoskeleton that direct chromosome segregation, intracellular traffic, and cell motility. Microtubules are assembled by evolutionarily conserved α/β -tubulin heterodimers (“tubulin dimers”). Regulation of tubulin function involves expression of different isoforms of α - and β -tubulin as well as by posttranslational modifications (PTM) of both monomers. The multi-tubulin hypothesis states that different tubulin dimers are performing specialized functions determined by isotype composition and PTM. We have resolved a long-standing controversy about the ability of brain tubulin dimers to dissociate reversibly, and also determined the thermodynamics and kinetics of the process. We used state-of-the-art analytical ultracentrifugation and fluorescence spectroscopy methods to measure the tubulin dimer dissociation constant with unprecedented accuracy ($K_d = 84 \pm 34$ nM), and measured the first-order rate of dimer dissociation ($k_{off} = 3.1 \pm 0.4 \times 10^{-3}$ s⁻¹). However, most studies of purified tubulin (including ours) used tubulin extracted from brain (or neural tissue) which is known to contain multiple isoforms of α - and β -tubulin subunits and also contain abundant PTM. Since we are able to measure tubulin dimer dissociation with high accuracy we extended our studies by comparing the reversible dissociation of other tubulins extracted from neural and non-neural sources. We obtained tubulin from brains of different animals containing multiple isoforms and high level of PTM, as well as from non-neural sources such as avian red blood cells and human epithelial cell cultures, which are mostly single isotype with low or no PTM. We found that the reversible dissociation of tubulin dimers extracted from these various sources are very different from each other. While tubulin dimers extracted from neural tissue readily dissociate in our experiments, tubulin from non-neural sources showed tighter association, including absence of detectable dissociation. These findings are absolutely novel since tubulin dimer dissociation has never been determined before for such a variety of tubulins. In addition, our findings add a new dimension to the multi-tubulin hypothesis where the stability of the tubulin dimer is determined by isotype composition and also by the level of PTM.

National Heart, Lung, and Blood Institute (NHLBI)

Pinar Gurel

Postdoctoral Fellow

Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology

The Role of Actin Structural Plasticity in Mechanosensation

The ability of a cell to respond to the mechanical properties of its environment (mechanosensation) influences almost all core cellular processes, including division, migration, differentiation, and survival. Misregulation of mechanosensory pathways has recently been implicated in malignant transformation, tumorigenesis, and metastasis, highlighting this process as a key component of cancer progression. At the foundation of this behavior lies an intricately coordinated contractile network consisting of the actin cytoskeleton, myosin motor proteins, and their myriad binding partners; however, we currently lack a basic understanding of the underlying molecular mechanisms connecting actin to cellular mechanosensation. Actin filaments are flexible polymers that can adopt multiple conformational states, and recent evidence shows that forces can influence interactions with binding partners. Here, we explore how mechanical stimuli alter the actin filament structural landscape and how this may influence downstream interactions, potentially serving as an initial signal in mechanosensation. We have developed a novel reconstitution system to place actin filaments under tension suitable for high-resolution structural studies with cryo-EM. We immobilize active myosinV (barbed-end directed motor) and myosinVI (pointed-end directed motor) onto the carbon substrate of holey EM grids, suspending motor-engaged actin filaments over holes. Using a modified gliding assay, we find that the opposing activity of the two myosins induces mechanical strain in filaments, evidenced by increased severing in the presence of ATP. In contrast, either myosin alone produces processive gliding of filaments. Low-resolution cryo-EM reveals a novel, persistent actin structural state found in the presence of force generation. High-resolution studies are currently underway to investigate the conformational changes produced in actin in detail. Resolving these structures will provide unprecedented insight into the molecular mechanisms of mechanosensation, and ultimately advance the development of targeted therapeutics against specific actin conformational states.

National Institute of Dental and Craniofacial Research (NIDCR)

Brian DuChez

Doctoral Candidate

Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology

Cancer cells exhibit directional migration in response to gradients of extracellular stiffness

Durotaxis is a mechanism of directed migration in which cells respond to the local microenvironment by moving toward increasingly stiff regions. While durotaxis has been characterized in a subset of mesenchymal cells, its role in cancer biology has not been defined. Numerous studies have demonstrated the role of diffusible factors in cancer cell migration and metastasis. However, given the gradual stiffening of many tumor microenvironments, we hypothesized that a durotactic mechanism might also contribute to the migration of cancer cells. To test for a role of local stiffness on directional migration of cancer cells, we manufactured hydrogels that mimic pathological stiffness gradients. These gradients were modified for cell culture by derivatizing the hydrogel surface with extracellular matrix proteins. We developed a graphical user interface (GUI) to automate tracking of migrating cells and to

provide an unbiased, high-throughput method to evaluate directional responses of cells to stiffness gradients. The GUI enables the user to interactively alter parameters of time-lapse images of fluorescently stained nuclei to avoid nonspecific fluorescence, locate nuclei positions, and rapidly generate and quantify cell trajectories for hundreds of cells. These tools were used to compare the durotactic phenotypes of MDA-MB-231, HT1080, and U87 cells – three commonly used cell lines that represent distinct cancer types. We found that U87 was the only cell line out of the three cancer types to exhibit durotactic migration. Interestingly, a robust durotactic response was observed only when cells migrated on the softest region of the gradient. These findings were replicated with a second glioblastoma cell line - T98G. We also observed a particularly effective durotaxing subpopulation that displayed rounded morphology with multiple tiny cellular extensions distinct from the canonical polarized, elongated morphology previously observed for durotaxing mesenchymal cells. These findings represent the first thorough, quantitative description of cancer cell durotaxis and demonstrate that glioblastoma cells exhibit durotaxis, which is optimal in a range of stiffness that mirrors that of their native brain tissue environment. Furthermore, the novel rounded morphology observed for these durotaxing tumor cells may provide greater mechanistic insight into understanding how cells sense and respond in different ways to the stiffness of extracellular environments.

National Human Genome Research Institute (NHGRI)

Senta Kapnick

Doctoral Candidate

Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology

Cortical actin regulates lytic granule secretion in CD8+ cytotoxic T lymphocytes

CD8+ cytotoxic T lymphocytes (CTLs) are an integral part of the immune response, critical for eliminating virally infected and tumorigenic cells. Killing by CTLs is initiated when T cell receptor engagement triggers adherence of CTLs to target cells, the accumulation and centralized clearance of actin into a ring at the CTL:target synapse which leads to cell polarization, and finally the release of secretory granules containing effector proteins that induce cytolysis of targets. Because a single CTL is capable of killing multiple targets, they are thought to act as “serial killers.” Thus, secretion of granules must be tightly regulated, ensuring that only appropriate cells are killed, and preserving the finite number of granules for this sequential cytolytic activity. However, how T cells regulate granule secretion during CTL:target interactions is not completely understood. Here, we used fluorescence reporters with live confocal and total internal reflection fluorescence microscopy to evaluate how actin affects lytic granule secretion at the plasma membrane. While previous work has shown that clearance of actin from the center of the synapse precedes secretion of lytic granules, by imaging throughout the duration of CTL:target interactions, we found that after granule fusion, actin recovers at the synapse, after which no further secretion is observed. Using Latrunculin A to depolymerize the recovered actin at the synapse, we found that granule secretion resumed following disruption of the actin network. Our observations suggest that recovered actin acts as a barrier to prevent further granule fusion. We also observed that CTLs from ashken mice, which are unable to secrete lytic granules due to a mutation that affects their granule fusion machinery, fail to recover actin at the synapse. This suggests that granule secretion itself triggers actin recovery at the synapse in CTLs. Finally, we correlated both the clearance of actin and its recovery with the synaptic phospholipid, PIP2, as visualized by LifeAct and Tubby-eGFP reporters. As that PIP2

binds several actin regulatory proteins, our work suggests that the distribution of phosphatidylinositols in the membrane is a potential mechanism by which CTLs regulate the density of actin at the synapse during killing. Our work therefore provides insight into actin-related mechanisms regulating secretion in CTLs that may preserve serial killing capacity during immune responses.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Anil Shukla

Postdoctoral Fellow

Cell Cycle-General, Regulators and Checkpoints, Apoptotic Mechanisms

Centriole block to reduplication is lost upon mitotic entry in Plk1-dependent manner

Cycling cells contain two centrosomes each comprised of a centriole (microtubules-based structure), which organizes a proteinaceous matrix called pericentriolar matrix (PCM) around it. Centrosomes are major microtubule organizing centers of the cell, they organize two poles of mitotic spindle during mitosis, and convert into basal bodies during ciliogenesis. Abnormal centrosome number leads to tumorigenesis, thus it is critically important that a cell precisely controls the number of centrosomes. Only one new centriole (daughter centriole) is formed in association with the preexisting centriole (mother centriole) once per cell cycle in the process of centriole duplication. Two duplicated centrioles are then evenly segregated during mitosis. Mother and daughter centrioles are thought to lose association at the end of mitosis. This association must be preserved during interphase to prevent premature centriole duplication resulting in supernumerary centrosomes. This work aims to answer when, during the cell cycle, the association between a mother and a daughter is broken, and to identify molecular mechanisms that synchronize the loss of centriole association with other cell cycle events. To answer this, we combined high-resolution light microscopy, correlative live-cell and electron microscopy, and biochemical methods. Our results show that mother and daughter centrioles irreversibly lose their association already after nuclear envelope breakdown. Once centrosomes are “primed” by Polo-like kinase 1 (Plk1) in late G2, the separation of centriole pairs cannot be reversed, and does not require further Plk1 activity, or microtubule-driven forces. Our biochemical analysis demonstrated that Plk1 activity simultaneously directs the degradation of centrosomal proteins required for initiation of centriole duplication (Sas6, STIL and CPAP), via promoting E3-ubiquitin ligase anaphase promoting complex/cyclosome (APC/C) complex activity. Specifically, the stability of APC/C co-activator Cdh1 in mitosis is positively regulated by Plk1. Centriole separation coupled with attenuated APC/Cdh1 activity in mitosis led to premature duplication of only one, older, centriole during G1, instead of coordinated duplication of both centrioles in S-phase. These novel findings illustrate that two aspects of centrosome cycle, centriole separation and the degradation of centrosomal proteins are coordinated via Plk1 activity to ensure the correct centrosome number in cells.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Lisa Ritchey

Postdoctoral Fellow

Cell Cycle-General, Regulators and Checkpoints, Apoptotic Mechanisms

Identification of YAP as a Regulator of Endothelial Cell Proliferation and Vascular Morphogenesis

The transcriptional activator YAP modulates transcription through activation of the transcription factors TEAD, AP-1 and SMAD. Cues from cell contact, cytoskeleton organization and cell polarity activate the Hippo signaling cascade resulting in the phosphorylation and subsequent nuclear-cytoplasmic shuttling of YAP leading to its degradation. Previous studies have shown organ-specific overexpression of YAP results in overgrowth of the target organ and that the YAP gene is amplified in some cancers. Other studies have shown unphosphorylated YAP translocates to the nucleus promoting cell division in epithelial cells grown at low density, whereas phosphorylated YAP is mostly retained in the cytoplasm where it is degraded in cells grown at high density. Since the role of YAP in endothelial cells is currently unclear, we examined the role of YAP in the regulation of growth and vessel morphogenesis of primary human endothelial cells (ECs). We found that YAP localizes to the nucleus in proliferating ECs. Upon ECs reaching confluency associated with contact inhibition, YAP translocates from the nucleus to the cytoplasm. We transduced ECs with control shRNA, YAP shRNA, full-length YAP or a mutant YAP lacking TEAD-binding activity. ECs that lack YAP or express the TEAD-binding deficient YAP incorporated less [3H] thymidine than control ECs, whereas cells overexpressing YAP incorporated more [3H] thymidine than control ECs. These results provide evidence that YAP plays a role in regulation of ECs proliferation. In proliferation-independent morphogenesis assays that assess formation of vessel-like tubular structures by post-mitotic ECs, immunofluorescence showed that YAP is primarily restricted to the nucleus, suggesting YAP activity. YAP-depleted ECs formed less durable vessel-like structures than control ECs, whereas YAP-overexpressing ECs produced vascular structures with increased thickness and stability overtime. ECs expression of the TEAD-binding deficient YAP mutant produced a moderate vascular phenotype compared to YAP-deficient ECs. These results provide evidence for a role of YAP in ECs vascular morphogenesis. In sum, we have unveiled that YAP has important and previously unrecognized roles in the regulation of ECs growth and formation of vascular structures. As endothelial cells in the tumor microenvironment play diverse pro-tumorigenic functions, targeting the Hippo-YAP signaling pathway provides a new avenue for reducing tumor angiogenesis.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Alexander Gorka

Postdoctoral Fellow

Chemistry

Near-IR Light-Mediated Cleavage of Antibody-Drug Conjugates Using Cyanine Photocages

Antibody-drug conjugates (ADCs) seek to enable the delivery of highly potent, but otherwise toxic, therapeutics selectively to tumors. Existing ADC linker strategies utilize cellular processes for drug cleavage (e.g. proteolysis) that have poor tumor bias. Resulting off-target release in circulation or in benign tissue is a significant liability. Seeking to address this critical issue, we have developed the first ADC linker strategy that uses tissue-penetrant, cytocompatible near-IR (NIR) light to control the antibody-drug cleavage event. Light provides a targetable external stimulus that can be applied site-specifically, thereby confining drug release to the irradiated area. Here, we take advantage of a recently discovered NIR uncaging reaction from our laboratory, which utilizes heptamethine cyanine photooxidation chemistry to release bioactive phenols in response to 690 nm light. We have developed

conjugates of cyanine-caged combretastatin A4 (CA4), a potent microtubule inhibitor, to panitumumab, a clinical anti-EGFR antibody. Upon irradiation with modest flux, photooxidative cleavage of the cyanine linker followed by hydrolysis provides useful yields of free CA4. This results in growth inhibition of EGFR-positive cells that recapitulates the activity of the native drug. Critically, the absence of irradiation, use of receptor-negative cells, and use of related conjugates that release biologically inactive molecules all result in substantially diminished (at least 70-fold) growth inhibition. The ADC exhibits excellent tumor localization and stability up to 7 days in vivo as assessed using the 800 nm emission of the cyanine. Using an A431 double xenograft model, we show that this signal can be ablated using external irradiation from a 690 nm laser, selectively in the light-exposed tumor. These results illustrate the potential theranostic utility of this approach. The NIR fluorescence of the cyanine enables assessment of tumor localization and resulting loss of that signal indicates drug release. Modifications to the cyanine core have enabled cleavage at 740 nm and improved stability in the absence of irradiation. Using an ADC constructed from this linker and the potent DNA alkylating drug, duocarmycin, we have achieved light- and antigen-selective growth inhibition in the low pM regime with a 100-fold therapeutic window. This construct forms the basis of ongoing studies aimed at addressing in vivo tumor burden using NIR light-mediated ADC cleavage.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Erin Anderson

Postdoctoral Fellow

Chemistry

A Near-IR Treatment Strategy that Transitions from Photosensitizing to Photoredox Uncaging in Response to Reduced Oxygen Tension

The ability to use tissue penetrant near-IR light to treat cancer has been a long-standing goal. Existing clinical modalities have largely addressed this goal using photodynamic therapy (PDT). In this approach, a non-toxic photosensitizer is irradiated with near-IR light to produce cytotoxic reactive oxygen species (ROS), such as singlet oxygen (1O_2). While PDT can be highly effective, the requirement for oxygen limits efficacy against hypoxic cell populations. This limitation can be significant since hypoxia is prevalent in solid tumors and can be exacerbated over the course of the O_2 -consuming PDT treatment. The use of near-IR light to site-specifically deliver a chemotherapeutic molecule (near-IR uncaging) is an appealing alternative for the targeting of hypoxic cell populations. However, most known near-IR uncaging methodologies sacrifice the benefits of conventional PDT treatment, and an agent that is able to act on both normoxic and hypoxic cell populations would be beneficial for total tumor ablation. We hypothesized that a combination of photosensitizing activity under normoxia and drug delivery under hypoxia would create a small-molecule system that is able to impart cytotoxic effects on both normoxic and hypoxic tumor cells. We have established that a silicon phthalocyanine (SiPc) photosensitizer undergoes a photoinduced electron transfer (PET) reaction to release an aryl ether drug payload upon exposure to near-IR light. This release was dependent on the presence of an electron donor (like the intracellular reductant glutathione, GSH) and the absence of oxygen. Extensive mechanistic analysis was conducted to confirm the PET mechanism of drug release. Furthermore, we show that this same molecule is efficacious as a traditional PDT agent under aerobic conditions. ROS phototoxicity was distinguished from the biological effects of drug molecule uncaging through cell cycle analysis using

fluorescence-activated cell sorting (FACS) and through photopatterned viability experiments. Our dual action photosensitizer is effective against both aerobic and hypoxic cancer cells in a mechanism that switches in response to oxygen tension. This dual treatment modality should prove advantageous for clearing hypoxic cell populations, a subset of cells known to evade traditional PDT treatment and be a significant risk for tumor recurrence.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Yuri Pevzner

Postdoctoral Fellow

Chemistry

Synthetically Accessible Virtual Inventory (SAVI)

Success of any drug discovery program largely depends on its ability to interrogate the chemical space of drug-like molecules to find suitable scaffolds and generate promising leads. When in-house synthesis capabilities and/or access to outside resources are limited, many researchers rely on existing compound collections to aid in their discovery efforts. Commercial catalogs list up to millions of compounds at a wide range of price points, albeit with varying degrees of availability and reliability. However, these libraries represent only a minuscule fraction of the space of drug-like chemistry, which, by many estimates, numbers in $>10^{40}$ structures. In order to help expand the frontiers of the known chemical universe while allowing researchers to explore this space in a robust, reliable and cost-efficient way, we have developed the Synthetically Accessible Virtual Inventory (SAVI) system. A result of an international collaboration between NCI's CADD Group and several industry and academic partners, SAVI combines richly annotated compilation of retrosynthetic chemical transforms with the database of high-availability chemical building blocks. The algorithm that we've developed adapts the retrosynthetic transforms to model the forward-synthetic reactions such that the primary driving criteria is robustness of synthesis and availability of inexpensive building blocks. Each SAVI-generated structure is annotated with a wide range of properties that include drug-likeness criteria, the compound's potential to interfere with assays, its complexity, and other properties considered relevant in the context of modern drug development. More importantly, in addition to the attributes that describe the final structure, the proposed compounds are also annotated with the specific reaction(s) via which they can be made along with the estimated cost of the starting materials. Computational infrastructure designed specifically for SAVI includes multi-CPU hardware with low latency high capacity storage necessary to process and provide quick access to a large amount of chemical data. Finally, a web-based interface will allow users to filter the SAVI database according to their specific project needs using structural and other criteria. We have released a subset of SAVI database for public evaluation while the project's ultimate goal is one billion novel and diverse chemical structures.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Andres Canela

Research Fellow

Chromatin and Chromosomes

Differential breakage, end-processing and repair in the genome measured by END-seq

DNA double strand breaks (DSBs) are essential in numerous physiological processes including adaptive immunity, where V(D)J recombination and class switch recombination are initiated by RAG and AID breaks respectively, meiotic recombination initiated by SPO11 breaks, transcription by type II topoisomerases and replication by resolvases. In addition, DSBs can arise by external factors such as ionizing radiation and chemotherapeutic drugs. Erroneous repair of all of these DSBs can also result in non-physiological chromosomal translocations and genome instability promoting tumorigenesis. We have developed a sensitive unbiased method (END-seq) for genome-wide mapping at base-pair resolution of the broken DNA ends generated by DSBs. END-seq permits calculation of absolute levels of DSBs by comparison with the number of telomeric DNA ends captured, and can detect at least 1 DSB per cell amongst 10,000 cells not harboring DSBs. We used ENDseq to study the genomic context that defines where a DSB is produced and how it is processed and finally repaired. We used two controlled systems to generate DSBs, an inducible restriction enzyme that potentially targets more than 1,000 sites in the genome, and RAG expression in vitro and physiologically in vivo during B and T cell development. We have been able to define the repertoire of breaks in both systems and surprisingly we find that breakage, processing of the DNA ends (DNA end-resection) and repair varies considerably across the genome depending of the chromatin landscape and transcription. We predict that that this will have important consequences for gene targeting. For example, the efficiency of gene targeting facilitated by CRISPR mediated breaks may be distinct in different genomic regions depending on how ends are processed. In addition and specifically for RAG-induced breaks, we find that each end of the DSB, coding and signal, follows different fate in terms of processing and repair and it is dependent of ATM activity. END-seq also allows us to detect off-target RAG induced breaks occur at a frequency of at least 1000-fold less than bona-fide RSS breaks, these off-targets could contribute to translocations, genomic instability and the initiation of leukemias. Thus END-seq enables us to understand the rules governing recruitment and efficiency of recombination enzymes to their on- and off-target sites, the mechanism of DNA repair choice and finally the etiology of chromosomal translocations.

National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)

Ezequiel Nazer

Visiting Fellow

Chromatin and Chromosomes

Argonaute2 Functions With LaminB to Mediate Transcriptional Silencing of Spermatogenesis Genes in Drosophila melanogaster

Argonaute proteins are commonly known as core components of RNA silencing pathways. However, Argonaute proteins have also been shown to possess nuclear functions, such as regulation of transcription, splicing and chromatin architecture. Previous work showed that *Drosophila* AGO2 functions directly on euchromatin to promote enhancer-promoter interaction at the homeotic *Abd-B* locus independently of the RNA interference (RNAi) pathway. CHIP-seq analysis revealed that AGO2 binds thousands of sites in the genome, raising the possibility that AGO2 could modulate global chromatin architecture. To identify factors that can function with AGO2 to regulate transcription, we performed immunoaffinity purification of AGO2 from nuclear extracts followed by mass spec analysis.

Interestingly, we found that LaminB is enriched among the top AGO2-associated proteins. Reciprocal co-immunoprecipitation validated the specificity of this interaction, and biochemical fractionation assays confirmed that both proteins reside in chromatin and nuclear matrix fractions. To directly assess the global role of both proteins in transcription, we performed nascent RNA-Seq upon depletion of either AGO2 or LaminB in Kc167 cells. We found that both proteins co-repressed a highly significant number of genes, particularly those located at the borders of Lamin-associated domains (LADs). In order to assess the physiological role of AGO2 in transcriptional regulation, we performed mRNA-Seq in null versus RNA slicing catalytic activity mutant female larvae. Strikingly, we observed de-repressed transcription of spermatogenesis genes in the absence of AGO2, independent of its catalytic activity. One of the de-repressed genes is *nht*, which encodes a key upstream activator of spermatogenesis gene expression. Null mutation of *nht* suppresses the up-regulation of spermatogenesis genes observed in AGO2 null mutants, suggesting that AGO2 acts upstream of *nht* to silence the spermatogenesis gene program. Given that *nht* is located within a LAD harboring flanking AGO2 chromatin binding sites, we hypothesized that AGO2 and LaminB could modulate chromatin topology to repress *nht*. Chromosomal conformation assays (3C) using the *nht* promoter as bait showed a decrease in the frequency of interactions within the LAD upon AGO2 or LaminB knockdown. We conclude that both proteins may repress transcription at LAD borders by regulating chromatin architecture.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Iain Sawyer

Visiting Fellow

Chromatin and Chromosomes

Oncogenes drive Cajal body formation

There is growing awareness that disease influences the regulation of cellular structures. Specifically, the relationship between gene expression and nuclear domains, including Nuclear Bodies (NBs), requires further study. NBs form at precise sites of gene activity, lack a defining membrane and concentrate specific proteins and RNAs. These structures occupy a large area within the cell nucleus and perform critical processes that contribute to genome function. Unfortunately, as NBs are continuously present during interphase it is difficult to identify factors that coordinate their reassembly after mitosis. To study these phenomena, we have described the genome-wide Cajal body (CB) interface in HeLa cells and its impact upon gene expression. This network consists of highly expressed U snRNA genes, sno/scaRNAs and histone genes. These major NBs are mostly found in transformed cancer cells and guide efficient spliceosome snRNP assembly. Therefore, it is imperative to characterize the transition from normal diploid cells (which lack CBs) to transformed CB-containing cells. Accordingly, we have identified several oncogenes, including H-RAS and MYC, which induce CB formation in primary diploid fibroblast cells 12 hours after induction. This coincides with the upregulation of 99 CB components or target genes, identified by RNA-seq. These may represent fundamental factors for CB biogenesis. Using six-color DNA FISH, we also observed a substantial reorganization of chromatin structure by de novo CBs. Many of the same higher-order interchromosomal gene clusters as HeLa-CBs were formed, indicating that this is a universal contrivance. Intriguingly, the association between de novo CBs and the major histone cluster was higher than in HeLa cells and correlated with increased histone expression. This implies that oncogene-induced transformation may rely on CB-dependent augmentation of histone RNA expression

to support increased proliferation. Finally, circular chromosome conformation capture (4C)-seq revealed a dramatic CB-induced interchromosomal pairing between U1 and U2 snRNA genes (chromosomes 1 and 17), which we previously reported as CB nucleation sites. These data suggest that CB formation and subsequent genome reorganization is an influential early step in oncogenesis by enhancing the expression of histones and essential RNA splicing factors that support transformation. Disruption of CB assembly in cancer may represent a novel and specific therapeutic opportunity.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Elizabeth Finn

Postdoctoral Fellow Postdoctoral Fellow

Chromatin and Chromosomes

Clarifying patterns of genome-wide DNA interactions via high throughput imaging

The genome is spatially organized on many levels in vivo. DNA is wrapped around nucleosomes, packaged into chromatin fibers, and folded into promoter-enhancer loops and regulatory domains which build distinct nuclear territories each representing an individual chromosome. 3D genome organization is crucial to genome function: gene position is developmentally regulated and cell-type specific, and disruptions of localization are a hallmark of both aging and cancer. While the organization of the genome has historically been studied via imaging methods such as DNA FISH, new biochemical crosslinking-based techniques such as 3C and Hi-C have allowed global analysis of DNA interactions. These methods generate high-resolution genome-wide interaction maps, but the relationship of crosslinking data to spatial position is neither well defined nor intuitive. Furthermore, biochemical methods require pooling of millions of cells and generate average maps, leaving variability between cells or alleles a completely open question. To examine cell-to-cell variation and relate sequence reads to physical orientation, I am systematically comparing biochemical interaction data with spatial distance measurements using single cell imaging. I use high-quality, 100kb-resolution Hi-C data to identify potential interactions at multiple genomic distances within a chromosome and between chromosomes. I probe these regions via FISH, using a combination of high-throughput microscopy and automated image analysis to determine inter-probe distances. At short genomic distances (5-20Mb), Hi-C capture frequency correlates well with FISH interaction frequency, although even the most enriched interactions occur at only ~30% of alleles, suggesting that the averaged biochemical signals are generated by distinct subpopulations. The correlation between biochemical interaction frequency and physical proximity degrades as genomic distance increases and associations between loci on different chromosomes occur in no more than 5% of cells. In addition, I find that the majority of inter-chromosomal pairs with high capture frequencies are likely the result of segmental duplications and repetitive regions, rather than true interactions. I conclude that 3D genome organization is highly heterogeneous among individual cells. My approach provides a powerful tool for examining Hi-C data taking into account single cell variation, and allows me to study in depth the mechanisms and function of genome organization.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Reema Railkar

Visiting Fellow

Clinical and Translational Research

Quantitative high-throughput screening as a tool to identify novel therapies in Bladder Cancer: Lessons from Flavopiridol

Introduction: Bladder cancer (CaB) is the 4th most common cancer among men in the US. It is the most expensive malignancies to treat from diagnosis to death. No new pharmacological agents have been approved for treatment of bladder cancer since the approval of BCG in 1990. Thus, there is an urgent need for development of new treatment therapies. Quantitative high throughput screening (qHTS) of representative cancer lines with oncology drugs may identify new treatments or re-purpose already existing therapies for different disease. We utilized this technique to identify new therapies in two primary bladder lines (T24 and UMUC3) and their metastatic lines (T24T, SLT3 & FL3 of T24 & LUL-2 for UMUC3). Methods: We screened 7 bladder cancer cell lines (including RT4, T24, and UMUC3) against 1,912 oncology drugs using a 48 hour cell proliferation assay with an ATP-based readout (CellTiterGlo) to determine activity and potency of compounds in a dose response manner. One of the candidate drugs inhibitory in all cell lines tested is flavopiridol, a pan CDK inhibitor. We further characterized the mechanism of action and in vivo effects of flavopiridol using cell based assays and mouse xenograft studies. Results: The initial screen identified 95 compounds active in 7 cell lines. The top 50 compounds were further analyzed for molecular size of >200 g/mol and TPSA<90. This identified mitomycin C and 8 novel compounds. Further testing revealed Flavopiridol to be most consistent with qHTS data having IC50 of 100-300nM in all the cell lines tested. Flavopiridol induces G2/M arrest; however, very little apoptosis was seen suggesting cytostatic rather than cytotoxic mechanism of flavopiridol action. Flavopiridol showed dose dependent inhibition of migration, invasion and colony formation in CaB cell lines tested. Xenograft studies in rapidly growing UMUC-3 cells showed slowing of tumor growth but not complete reduction indicating cytostatic mechanism of flavopiridol. However, in slow growing cells, 5637, 5/8 treated mice showed complete tumor reduction. Conclusions: qHTS can identify novel compounds. Flavopiridol seems to be a very effective inhibitor both in vitro and in vivo. Physical properties of Flavopiridol are most suited for intravesical use which may lead to it being an effective inhibitor of CaB in the bladder at higher doses without any/few systemic toxicities. Studies are underway to elucidate the use of flavopiridol as a single intravesical agent.

NIH Clinical Center (CC)

Zachary Lerner

Postdoctoral Fellow

Clinical and Translational Research

A Robotic Exoskeleton to Treat Crouch Gait from Cerebral Palsy: Design and Initial Clinical Evaluation

Cerebral palsy (CP) is the most prevalent childhood physical disability and adversely affects walking and other motor abilities. Crouch gait, a pathological walking pattern characterized by excessive knee flexion, is one of the most common gait disorders observed in children with CP. Effective treatment of crouch during childhood is critical to maintain mobility into adulthood, yet current interventions do not adequately eliminate or alleviate crouch in most patients. Wearable robotic exoskeletons have the

potential to improve crouch gait by providing on demand assistance during walking. However, no exoskeletons suitable to treat children are commercially available, and no evidence exists regarding the feasibility or efficacy of utilizing motorized assistance to alleviate knee flexion from crouch gait. Enhanced knowledge of neuromuscular adaptations to powered assistance in children with crouch gait is needed to optimize this treatment approach. To meet these needs, we developed the first lower-extremity exoskeleton intended to treat crouch gait by providing powered knee extension assistance at different phases of the gait cycle. We evaluated the effects of powered knee extension assistance on knee motion, and knee flexor and extensor muscle activity in four children with crouch gait from CP. The exoskeleton was effective in reducing crouch in three of the four participants compared to their baseline gait pattern. The reduction in crouch was clinically significant (greater than 10 degrees) in two of the participants. Knee extensor activity was maintained during early stance in all of the participants during walking with the exoskeleton which is a desirable outcome because we want the device to supplement voluntary control, not replace it. In two of the participants, knee extensor activity in mid-stance represented a more normal, well-modulated pattern. Modest increases in knee flexor activity were also exhibited. Therefore, additional training focusing on reducing knee flexor activity may lead to further improvements. In summary, we demonstrated the promise of a wearable robotic exoskeleton as a potential treatment for individuals with crouch gait. Our results provide novel insights into motor control strategies for individuals with CP and may enhance understanding of the neuromuscular causes underlying crouch gait. While currently a laboratory-based intervention, the ultimate goal is to prescribe the exoskeleton as a device for long-term rehabilitation.

National Institute of Neurological Disorders and Stroke (NINDS)

Mika Komori

Visiting Fellow

Clinical and Translational Research

Intrathecal Rituximab in progressive multiple sclerosis stopped for insufficient inhibition of CNS inflammation: a randomized, double-blind, placebo-controlled study

Background: The lack of efficacy of immunomodulatory treatments in progressive multiple sclerosis (MS) may be caused by the unreachable compartmentalized inflammation in the central nervous system (CNS). Objective: The double blind combination of Rituximab by IntraVenous and IntraThecal injection versus placebo in patients with Low-Inflammatory Secondary progressive MS (RIVITALISE; NCT01212094) trial was designed to answer: 1. Whether an induction dose of intravenous and intrathecal rituximab efficiently depletes CNS B cells? and 2. If so, whether this leads to global inhibition of CNS inflammation and slowing of CNS tissue destruction? Methods: Patients aged 18-65 years were randomly assigned (2:1; randomization sequence table balanced for age) to rituximab (n=18) or placebo (n=9). Protocol-stipulated interim analysis of serum and cerebrospinal fluid (CSF) biomarkers in patients who completed the induction dose of study drug quantified the efficacy of B cell depletion. Results: The selected rituximab regimen failed to reach criteria for continuation of the trial. Changes in B cell-related CSF biomarkers (soluble CD21 [sCD21] and B-cell activating factor [BAFF]) occurred only in the active-treatment arm. While the mobile pool of CSF B cells was depleted by a median of -79.71% (p= 0.0176), B cells in CNS tissue were depleted inadequately (~-10-20%, p<0.0001). Consequently, the T cell specific CSF biomarker sCD27 also decreased only slightly (-10.97 %, p=0.0005), while a marker of axonal

damage, neurofilament light chain did not change. Insufficient saturation of CD20, lack of lytic complement and paucity of cytotoxic CD56dim NK cells contribute to decreased efficacy of rituximab in the CNS. Conclusions: Biomarker studies reliably quantified complementary pharmacodynamic effects of rituximab in the CNS, exposed causes for poor efficacy and determined that the RIVITALISE trial would be underpowered to reliably measure efficacy on clinical outcomes.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Scott Medina

Postdoctoral FellowPostdoctoral Fellow

Clinical and Translational Research

Evolution of Cancer-Targeting Peptides from Hydrogel Materials

Peptide-based hydrogels are a class of injectable solid materials that can deliver encapsulated therapies to a target site via syringe. In my initial work, I demonstrated the ability of cationic self-assembling peptide hydrogels to encapsulate large quantities of plasmid DNA, and enhance its immunostimulatory potential in vivo. Interestingly, while studying these materials, we discovered that truncating the terminal residues of the gel-forming sequence afforded a new peptide, named SVS-1, which no longer undergoes hydrogelation, but instead selectively binds to, and folds at, the negatively-charged surfaces of tumor cells. This finding allowed us to develop a new class of anticancer peptides (ACPs), which showed highly potent and specific oncolytic activity. Importantly, the ability of SVS-1, and many other ACPs, to lyse tumor cell membranes through rapid and non-stereospecific mechanisms has encouraged the perception that cellular resistance towards this class of agents is unlikely to occur. Using SVS-1 as a model ACP, I demonstrate, for the first time, that cancer cells can indeed develop resistance towards oncolytic peptides, through alterations in cell-surface glycosylation. Further, when cancer cells are presented with concentrations of SVS-1 below the IC50 for its lytic action, the peptide does not kill the cells but rather rapidly penetrates across the cell membrane via mechanisms involving physical translocation and clathrin-dependent endocytosis. This activity allows SVS-1 to serve as a drug delivery vehicle capable of solubilizing hydrophobic drugs and preferentially transporting them into cancer cells in vitro and tumor tissue in vivo. Following this work, I developed a new class of cell-penetrating peptides that exclusively employs non-endocytic mechanisms to enter cells, allowing for the successful cytoplasmic delivery of a ligated molecule that is otherwise completely membrane-impermeable. Overall, this work has identified a wide range of peptides with broad utility in biomedicine; including as building blocks in biomaterials, translation into anticancer therapeutics and as drug delivery devices. Collectively, my research efforts have culminated in six publications within journals that include Nature Nanotechnology, Nature Communications, Angewandte Chemie, Biomaterials and the Journal of Controlled Release.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Xintao Hu

Postdoctoral FellowPostdoctoral Fellow

Clinical and Translational Research

Novel Prime-Boost Vaccine Regimen to Augment Breadth, Magnitude, and Cytotoxicity of Cellular Immunity to Subdominant Gag Epitope of SIV/HIV

HIV sequence diversity and potential “decoy” epitopes are hurdles in the development of an effective global AIDS vaccine. Using an HIV-derived conserved element (CE) gag DNA vaccine, we demonstrated improved breadth of immunogenicity. By analogy to HIV, we engineered DNA-based immunogens encoding conserved elements of SIV p27Gag to target immune responses towards invariable viral regions. Macaques were immunized with DNA delivered by intramuscular injection followed by electroporation. Cellular responses were measured in peptide-stimulated PBMCs by polychromatic flow cytometry. Analysis of 31 macaques vaccinated with SIV gag DNA showed that only 58% of the animals developed cellular responses against the CE regions. In contrast, all 14 macaques immunized with SIV p27CE elicited CE-specific cytotoxic T cells with memory phenotype. Both booster regimens (gag only or CE+gag pDNA) significantly increased the CE responses inducing robust CE-specific cytotoxic T cells. Interestingly, CE+gag pDNA booster vaccination elicited significantly broader epitope recognition, inducing a more potent change in the immunodominance hierarchy, resulting in improved development of subdominant responses. Importantly, we demonstrate that Gag responses targeting CE are more cytotoxic and that priming with CE pDNA is critical to efficiently induce cytotoxic T cells targeting subdominant viral epitopes, which can be poorly achieved by vaccination with p57gag DNA. The cellular responses induced by these regimens were long-lasting (up to 2 years) and could be rapidly recalled by a single CE DNA boost. In conclusion, we identified a SIV vaccine regimen (CE DNA prime-CE+gag DNA boost) as most effective in increasing the cytotoxic T cell responses to subdominant highly conserved regions with maximal response breadth. The expanded breadth of the responses targeting invariable regions could provide an advantage for preventing immune escape and suppressing viral propagation. This concept is being tested in a clinical trial of prophylactic vaccination using HIV p24CE as prime supported by HVTN/NIAID. Evaluations of the efficacy of the SIV p27CE prime/p27CE and gag DNA as boost regimen upon SIV challenge in the non-human primate model are ongoing.

National Institute of Child Health and Human Development (NICHD)

Yeyi Zhu

Visiting Fellow

Visiting Fellow
Cultural Social and Behavioral Sciences

Are you what your mother ate? Maternal dietary grain intake during pregnancy and offspring growth from birth through age 7 years

Refined grains, a major source of dietary carbohydrates, have been linked to impaired glucose homeostasis and increased adiposity. Emerging animal data suggest that in utero exposure to dietary refined carbohydrates may predispose offspring to an obese phenotype, indicating a potential role for nutritional programming in the developmental origins of obesity, but intergenerational human data are lacking. To address this critical data gap, we prospectively investigated refined-grain intake during pregnancy in association with offspring growth through age 7 years among high-risk children of women with gestational diabetes (GDM). The analysis included 918 mother-singleton child dyads from the Danish National Birth Cohort. Offspring body mass index z-scores (BMIZ) were calculated using weight and length/height measured at birth, 5 and 12 months, and 7 years. Overweight/obesity (OW/OB) was

defined by WHO cutoffs. Linear and Poisson regression were used, adjusting for maternal pre-pregnancy BMI and other demographic, lifestyle, and dietary factors. Dietary refined-grain intake during pregnancy was positively associated with offspring BMIZ (adjusted beta per serving increase/day = 0.09, 95% CI 0.02, 0.15) at 7 years. Offspring born to women who consumed refined grains in the highest quartile (≥ 4.3 servings/d) experienced a 1.8-fold (95% CI 1.1, 3.0) increased risk of OW/OB at 7 years, compared to their counterparts in the lowest quartile (≤ 1.8 servings/d). Corresponding relative risk comparing the top decile (≥ 5.3 servings/d) to the lowest quartile was 2.6 (95% CI 1.3, 5.0). The associations were more pronounced among children who were breastfed ≤ 6 months, were physically inactive (≤ 2 h/weekday), or consumed more sugar-sweetened beverages (\geq once/week) at 7 years. Substitution of one serving-per-day of refined grains with an equal serving of whole grains during pregnancy was related to a 10% (95% CI: 0.82, 0.98) lower risk of offspring OW/OB at 7 years. No associations were observed between maternal refined-grain intake and infant growth. In conclusion, higher refined-grain intake during pregnancy significantly increased offspring BMIZ and the risk of OW/OB at 7 years, even after adjustment for other factors. Additionally, our findings suggest potential benefits of substituting refined grains with whole grains during pregnancy and modifying obesogenic factors during childhood to mitigate childhood obesity risk among children born to women with GDM.

National Human Genome Research Institute (NHGRI)

Jeffrey Lienert

Doctoral Candidate

Cultural Social and Behavioral Sciences

Social influence on 5-year survival in a longitudinal chemotherapy ward co-presence network

Chemotherapy is often administered in group settings, which allows for social influence between patients. Patients can influence one another through contact or solely based on co-presence. Social influence in turn can affect health directly as well as indirectly mediated by stress response. This influence is likely strongest when patients are familiar with one another; here, we define familiarity as patients being consistently co-present (CCP) over the course of their chemotherapy cycles. We provide empirical results to support the hypothesis that patients who are CCP with one another influence one another's health in the chemotherapy ward. We use data on the population of all 4,691 out-patients in a single chemotherapy ward in Oxfordshire, UK from Jan 1, 2000 to Jan 1, 2009. We assume that it is possible for patients to influence one another only if they are CCP more than expected by chance, adjusting for chemotherapy schedules. We model 5-year survival following chemotherapy to examine social influence with an indicator for whether one is CCP with at least one other patient (alter). We also create count variables for the number of CCP alters who survive or die in the 5 years following chemotherapy. We adjust for age, sex, cancer severity, total person-hours of co-presence, and chemotherapy duration. We find that patients CCP with at least one alter (n=2,704) have their odds of 5-year survival increased by a factor of 1.59 (95% CI: 1.36, 1.85). Additionally, every CCP alter that survives for 5 years following chemotherapy increases one's own odds of survival by a factor of 1.13 (95% CI: 1.08, 1.19), and every CCP patient that dies within 5 years decreases one's odds of survival by a factor of 0.93 (95% CI: 0.89, 0.96). These results indicate that social influence on health in the chemotherapy ward is a joint function of being CCP with other patients, as well as their health status. Given the presence of both positive and negative influence effects, ethically maximizing outcomes based on these results may

not be straightforward and require further research. It is also important to note that we observe these effects based solely on co-location data, which are relatively easy and cheap to obtain, but are often considered inferior to relational data for detecting social influence. Our results suggest that data of this nature may be used to study the presence of social influence in settings where traditionally higher-quality data could not be obtained.

National Institute of Dental and Craniofacial Research (NIDCR)

Belinda Hauser

Postdoctoral Fellow

Developmental Biology

SINGLE-CELL RNA-SEQ ANALYSIS OF ADULT SALIVARY GLAND PROGENITORS

Belinda R. Hauser, Joseph C. Burns¹, Michael C. Kelly¹ Matthew W. Kelley¹ and Matthew P. Hoffman. Salivary gland hypofunction after irradiation for head and neck cancer severely impacts the oral health of patients. We propose to regenerate salivary gland function using adult salivary progenitor cells. Keratin 5-positive (K5+) and K14+ cells are progenitors during fetal development of murine submandibular glands (SMGs) as determined by genetic lineage tracing studies; however, the relationship between these progenitors in the adult gland is unknown. Here we used K5-venus and K14-RFP expressing mice to FACS sort subpopulations of K5+, K14+, K5+;K14+ and K5-;K14- from adult SMGs. We then used microfluidic-based single-cell analysis to isolate, process, and profile RNA expression in individual cells. Our goal is to further characterize these subpopulations to identify markers that could be used to isolate them to investigate their function during regeneration. In total, 156 single-cell libraries were sequenced with an average read depth of over 1 million reads. Using bioinformatic analyses we defined 5 groups based on the 60 most variable genes through unbiased clustering. Using the molecular signatures database (MsigDB) we further characterized the phenotype of the clusters, which included mesenchymal cells, cells enriched in epithelial tissue stem cell markers, epithelial ductal cells, and markers of hematopoietic/bone marrow cells. Bioinformatics analyses also suggest subpopulations within the clusters express genes involved in regulating progenitor cell lineage such as Kit, Hs3st3, Sox9, and Itga6. RNAseq analysis has identified gene regulatory networks that may be useful to direct progenitor cell lineage and expansion for the regeneration of salivary glands after either irradiation damage or for bioengineering of salivary tissue.

National Institute of Environmental Health Sciences (NIEHS)

Barbara Nicol

Visiting Fellow

Developmental Biology

From Granulosa Cells To Oocytes: The Transcription Factor RUNX1 Regulates The Stock Of Oocytes In The Ovary

Starting a family later in life has become increasingly common in the past decades. This change poses unwanted consequences to women, as their ability to conceive declines with age. The ovary contains

only a finite number of follicles, composed of a single oocyte enclosed by granulosa and theca cells. This stock of follicles is called ovarian reserve and is set around the time of birth. Overtime, this reserve is gradually exhausted, ultimately limiting women's reproductive lifespan. Understanding how the ovarian reserve is established is therefore of great importance. Here we show that RUNX1, a transcription factor expressed in granulosa cells, regulates the initial size of ovarian reserve in mice. Inactivation of Runx1 (cKO) in the fetal ovary led to a significantly larger stock of oocytes shortly after birth. At postnatal day 3 (PND3), expression of oocyte markers such as *Sohlh1* or *Nobox* became significantly higher in Runx1 cKO ovaries. At PND21, Runx1 cKO ovaries contained more follicles than control ovaries. Despite these differences, the fertility of Runx1 cKO females was similar to control females over a period of 6 months. At advanced age (8-12 months), Runx1 cKO ovaries still contained more follicles than control ovaries, suggesting that loss of RUNX1 in the fetal ovary delays the age-related exhaustion of oocytes. The ovarian reserve is set around the time of birth, after a wave of apoptosis that eliminates two-third of oocytes. Since the appearance of Runx1 cKO ovarian phenotype coincides with this critical event, we investigated germ cell death in newborn ovaries and found that Runx1 cKO ovaries contained fewer apoptotic cells, suggesting that the larger stock of oocytes in Runx1 cKO is the result of repression of germ cell apoptosis. In summary, we have uncovered a novel role of RUNX1, a granulosa cell-specific transcription factor, in the control of the ovarian reserve size. In the normal ovary, RUNX1 promotes germ cell apoptosis around the time of birth, therefore determining the number of oocytes in the initial pool of ovarian reserve. In the absence of RUNX1, a larger stock of oocytes is maintained, resulting in an increased follicle pool throughout reproductive lifespan. Since RUNX1 is only found in the granulosa cells, our findings support the hypothesis that establishment of the definitive stock of oocytes is not exclusively cell-autonomous. Instead, granulosa cells, through the action of RUNX1, play a critical role in this process.

National Institute of Child Health and Human Development (NICHD)

Gernot Wolf

Visiting Fellow

Developmental Biology

Genetic deletion of recently evolved tandem zinc finger gene clusters causes retrotransposon activation in mice

Transcriptional gene regulation is a fundamental process in all living organisms and the evolution of transcription factors and their binding sites is a major factor driving evolutionary diversity. Mammalian genomes encode several hundred Krüppel-associated box zinc finger proteins (KRAB-ZFPs) that bind DNA in a sequence-specific manner through tandem arrays of C2H2-type zinc fingers. The KRAB domain induces transcriptional silencing via its cofactor KAP1, which recruits chromatin modifiers such as histone methyltransferases. Interestingly, the KRAB-ZFP family rapidly amplified and diversified in mammals by segmental gene duplications, mutations, and zinc finger rearrangements. Recent evidence suggests that KRAB-ZFP diversification reflects an ongoing arms race between the host defense system and retrotransposons that continuously arise and amplify within host genomes. However, the vast majority of KRAB-ZFPs have not been investigated to date. To gain new insights into KRAB-ZFP gene evolution and function, we determined the genomic binding sites of approximately 10% of the more than 300 murine KRAB-ZFPs, including 17 KRAB-ZFPs that are encoded within a 2.4 Mb gene cluster that

is not conserved in other mammals. Consistent with the arms-race hypothesis, we show that the majority of these mouse-specific KRAB-ZFPs bind mouse-specific retrotransposons. To investigate the functional role of KRAB-ZFPs, we genetically deleted the entire 2.4 Mb KRAB-ZFP cluster in embryonic stem cells (ESCs) using CRISPR/Cas9 technology. Genome-wide RNA expression and histone modification analysis revealed a strong reactivation of retrotransposons, accompanied by a local loss of repressive histone modifications in KRAB-ZFP cluster knockout ESCs. Furthermore, we observed an upregulation of several genes that are located near reactivated retrotransposons, indicating that KRAB-ZFPs can act on genes by binding to nearby retrotransposons. Our data supports an arms-race model in which KRAB-ZFPs primarily evolve to repress potentially hazardous retrotransposons. However, an important consequence of this arms race is the establishment of species-specific gene regulatory patterns guided by KRAB-ZFP/target retrotransposon pairs. Finally, we show that CRISPR/Cas9 technology is suitable to genetically delete large KRAB-ZFP clusters in ESCs, opening a promising opportunity to generate mouse models in which human diseases associated with retrotransposon activity may be simulated.

National Institute of Environmental Health Sciences (NIEHS)

Yu-Wei Chen

Visiting Fellow

Developmental Biology

Mapping the Function of Genetically Defined Noradrenergic Subtypes in the Regulation of Stress-related Behavior

It is now well accepted that central noradrenergic (NE) neurons are diverse. Collectively defined by their ability to synthesize the neuromodulator norepinephrine, these neurons differ in their connectivity, physiological properties, and function. Defining molecular diversity among these neurons, and how these differences relate to neuronal circuitry, is a prerequisite for uncovering the contributions of discrete NE subtypes to behavior. Toward this goal we used an intersectional genetic strategy to resolve the NE system into molecularly separable subtypes by exploiting the differences in gene expression that distinguish NE progenitor populations in the developing hindbrain. We identified two major NE subtypes that project to the forebrain: first, a population that resides primarily in the locus coeruleus (LC) and shares a developmental history of *En1* expression; and second, a population that resides outside of the LC and shares a developmental history of *Hoxb1* expression. While both the *En1*- and *Hoxb1*-derived subpopulations project to regions modulating stress and anxiety, such as the basolateral amygdala (BLA) and bed nucleus of the stria terminalis (BNST), the relative contribution and axon morphology of each NE subtype at these target regions is quite different. We therefore hypothesize that these genetically-defined NE subtypes play functionally distinct roles in the regulation of anxiety and other stress-related behaviors. To test this hypothesis, we assessed the behavioral effect of selectively activating either *En1*- or *Hoxb1*-derived NE neurons using an intersectional chemogenetic approach. Results from light dark box and elevated plus maze show that activation of *En1*-derived NE neurons increases anxiety-like behavior. Conversely, activation of *Hoxb1*-derived NE neurons results in an anxiolytic phenotype. Consistent with this observation, activation of *Hoxb1*-derived NE neurons also results in decreased depressive-like responses in the forced swim test, and markedly reduced heart rate. Our results indicate that NE neurons of distinct genetic lineages have opposing roles in regulating stress-related behaviors.

Studies are ongoing to examine the possibility that within BLA and BNST, En1- and Hoxb1-derived NE neurons may form separate microcircuits that could lead to distinct behavioral outcome.

National Institute on Aging (NIA)

Jian Sima

Postdoctoral Fellow

Developmental Biology

Eda triggered p50/RelB recruits the SWI/SNF chromatin remodeling complex and facilitates transcription during skin appendage development

Ectodysplasin (Eda), encoded by the X-linked EDA gene, is a TNF ligand with a limited function. Eda signaling is mediated by Eda, Edar and Edaradd, which form a separated TNF ligand-receptor-adaptor family that is restricted to skin appendages. Gene mutations in Eda signaling cause anhidrotic/hypohidrotic ectodermal dysplasia, which affects morphogenesis of human skin appendages including hair, teeth, and several exocrine glands. Previous studies have demonstrated that Eda triggers NFkB function during skin appendage development. However, the molecular mechanism of gene transcription driven by Eda signaling remains unclear. To identify potential new components and their function under Eda signaling, we utilized protein affinity purification, the CRISPR/Cas9 technology, and knockout (KO) mouse models to clarify the signal transduction pathway. Firstly, we performed NFkB transactivation assays in keratinocyte culture and found that p50-RelB dimer, but not other subunits, is a major mediator of Eda function. Further experiments using wild type (WT) and Tabby (Eda deficient) mice confirmed that RelB was remarkably upregulated and activated by Eda signaling. To identify possible "specific" protein associated with p50-RelB complex after Eda stimulation, we used protein affinity purification method to show that the "SWI/SNF", a major chromatin remodeling complex, combined with the p50/RelB dimer. Further experiments in cell cultures confirmed the complex formation between p50/RelB and SWI/SNF chromatin remodeler induced by Eda. By analyzing mass spectral data, we further found Baf250a, an optional component in the SWI/SNF complex, is most strongly associated with RelB. Baf250a expression was indeed enriched in hair follicles and Meibomian gland germs in WT mice, but remained at basal expression levels in Tabby mice. Phenotypic analysis of skin-specific Baf250a KO mice is ongoing. Our recent cell culture results also showed that RelB, Brg1 and Baf250a share some common gene targets under Eda stimulation. Currently, we are using the CRISPR/CAS9 system to generate skin-specific KO cell lines for each of RelB, Baf250a and Brg1. To explore target genes triggered by Eda signaling, microarray gene expression and CHIP-seq will be performed in these cell lines. Thus, the current model is that NFkB binding to a target gene occurs in a protein complex that also remodels local chromatin to facilitate transcription.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Vivek Sharma

Visiting Fellow

DNA-binding Proteins/Receptors and DNA Repair

A long non-coding RNA regulates DNA repair by homologous recombination

Long non-coding RNAs (lncRNAs) have been shown to contribute to the cellular DNA damage response (DDR) by regulating gene expression. However, it has also been speculated that lncRNAs play key roles in regulating the DNA repair itself. We have characterized a lncRNA that regulates DNA repair by homologous recombination (HR). Using a genome-wide microarray screen we identified a novel ubiquitously expressed lncRNA, DDSR1 (DNA damage-sensitive RNA 1), which is induced upon DNA damage by several DNA double-strand break (DSB) agents. DDSR1 induction upon DNA damage is dependent on the ATM-NF- κ B pathway. Loss of DDSR1 impairs cell proliferation, DDR signaling, and reduces DNA repair capacity by HR. The HR defect upon DDSR1 knockdown is characterized by aberrant BRCA1 and RAP80 accumulation at DSB sites. Consistent with its role in regulating BRCA1 recruitment to DSB sites DDSR1 interacts with BRCA1. Interestingly, DDSR1 also interacts with hnRNPUL1, an RNA-binding protein involved in modulating HR by regulating DNA end-resection. Similar to DDSR1 depletion, loss of hnRNPUL1 also results in aberrant BRCA1 and RAP80 recruitment at DSB sites. Our results indicate that DDSR1/hnRNPUL1 depletion results in HR inhibition due to reduced end resection caused by aberrant accumulation of BRCA1 and RAP80 at DSBs. Our results establish a role for lncRNA DDSR1 in maintaining genome stability. Consistent with such a role, DDSR1 is down-regulated in triple negative breast cancer samples. Our future goal is to evaluate DDSR1 expression as a tool for predicting breast cancer prognosis.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Anthony Tubbs

Postdoctoral Fellow

DNA-binding Proteins/Receptors and DNA Repair

Lineage-specific transcriptional programs attenuate the DNA damage response in primary myeloid cells and acute myeloid leukemia.

DNA repair pathways are well conserved among all eukaryotes. Disruption of the DNA damage response (DDR) leads to genome instability and cancer in humans. Although DNA repair pathways have been intensely studied, we lack explanations for why defects in DNA repair cause tissue-specific syndromes, as opposed to global dysfunction. For example, BRCA1 is involved in several aspects of DNA repair and is essential for genome maintenance in cells, but mutations lead mainly to breast and ovarian cancer. Furthermore, the platinum-based drug Cisplatin creates DNA damage in all cell types, but it is particularly effective for the treatment of certain ovarian and testicular cancer. In order to maximize the therapeutic effects of chemotherapy and drugs that target DDR, we require a more precise understanding of tissue-specific DDR. Here, we test the hypothesis that a cell type-specific DDR is controlled by lineage-restricted transcription factors present in the transformed cell of origin. Lymphoid and myeloid cells are closely related and originate from common progenitors in the bone marrow. However, lymphoid and myeloid tumors have vastly different mutational signatures and etiology. Whereas lymphoid tumors are driven by mutations in the DDR, myeloid tumors almost never exhibit this loss. By global gene expression analysis, we demonstrate that expression of DDR genes in myeloid cells is severely impaired compared to lymphoid cells and uncommitted progenitors. This transcriptional defect leads to impaired DNA repair and DDR signaling in primary myeloid cells, and myeloid DDR attenuation is carried through to acute myeloid leukemia (AML), the most common myeloid malignancy.

We then tested whether myeloid transcription factors may actively repress the DDR. Remarkably, expression of a single myeloid-restricted transcription factor C/EBP α into non-myeloid cells shows a severe reduction in DDR gene expression and a blunted functional DDR. Additionally, AML generated in the absence of C/EBP α retain a robust DDR, suggesting C/EBP α is both necessary and sufficient to suppress the DDR. We conclude that a single lineage-restricted transcription factor regulates the DDR during hematopoiesis. This is a highly unexpected finding important for understanding chemotherapeutic responses and strategic targeting of DDR in distinct subtypes of AML and other tumors exhibiting characteristics of a lineage-specific DDR.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Diego Presman

Postdoctoral Fellow

DNA-binding Proteins/Receptors and DNA Repair

DNA-binding Triggers the Tetramerization of the Glucocorticoid Receptor in Live Cells

The glucocorticoid receptor (GR) is a ligand-regulated transcription factor and one of the most targeted proteins in the pharmaceutical industry due to its powerful anti-inflammatory actions. The search for safer, side effects-free glucocorticoids relies exclusively on a model where GR oligomerization state dictates its transcriptional output. It is still widely accepted that GR directly binds DNA as a homodimer, even though this paradigm has been exclusively established from in vitro studies. In this work we combine for the first time 1) an experimental model where GR-DNA interaction is observed in real time with fluorescence microscopy, and 2) a fluorescent technique that allows the quantification of the oligomeric state of proteins inside living cells. We used a mouse cell line harboring a tandem gene array which contains ~200 copies of a GR responsive promoter structure. Thus, a GFP-tagged version of the GR bound to the array can be directly visualized in living cells as a localized domain enriched in GFP signal. Quantification of GR's oligomeric state was performed using Number and Brightness (N&B) analysis. This novel technique provides the molecular brightness of molecules with pixel-size resolution. The brightness is obtained from fluctuations in the intensity due to the movement of molecules at each pixel of a confocal image. The higher the oligomerization state of a protein, the higher the amplitude of the fluctuations. These fluctuations could arise from diffusion in and out of the pixel or binding and unbinding to an immobile or slowly moving cellular feature such as chromatin. Thus, the relative oligomerization state of a protein can be determined accurately in live cells with the N&B assay. Our results show that while GR is mostly dimeric in the nucleoplasm, the receptor presents higher oligomerization states, most likely a tetramer, when bound to the tandem-array. Consistently, a point mutation that mimics DNA-induced conformational changes in the receptor promotes tetramerization throughout the entire nucleoplasm. Interestingly, other members of the steroid receptor family presented striking differences in oligomerization regulation. We therefore propose that DNA allosterically modulates the GR, triggering a novel quaternary structure (i.e. a tetramer), which would be the truly active form of the receptor. Our findings open new doors to the rational design of novel GR ligands and re-define the quaternary structure of steroid receptors.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Andre Stanlie

Postdoctoral Fellow

DNA-binding Proteins/Receptors and DNA Repair

Extra-telomeric functions of the shelterin component Trf2 in DNA double strand break repair

The shelterin telomeric complex is essential for preventing chromosomal ends from being recognized as DNA damage. This safeguard mechanism prevents cells from “catastrophic” end-to-end telomeric fusions. Telomeric repeat-binding factor 2 (Trf2) is the major shelterin component that ensures such end-recombination is averted. The capacity of Trf2 to regulate recombination outside of telomeres has not been reported. Mature B cells, as part of an adaptive immune response, undergo a programmed recombination called immunoglobulin class switch recombination (CSR) that occurs in the transcribed switch (S) region. Similar to telomeres, S regions contain highly repetitive DNA sequences and undergo DNA repair through non-homologous end joining pathway. Here we described that depletion of Trf2 in mouse primary B cells – achieved through CD21Cre-mediated deletion – augment ex-vivo CSR to IgG1 by about 2 fold as compared to the wild-type counterpart. Utilizing a ChIP-seq strategy to determine the distribution of endogenous Trf2 genome-wide in B cells, we demonstrated that Trf2 is recruited not only to telomeres, but also transcribed promoters including the S regions. By using a novel approach termed End-seq to label free DNA ends in-situ and subsequently subjecting the pulled-down DNA to next-generation sequencing, we further show that the increased CSR efficiency in the absence of Trf2 is attributed to enhanced DNA repair in the recombining S regions. The accumulations of Trf2 outside of telomeres suggest the possibility that TRF2 inhibits DNA repair throughout the genome. To test this, we analyzed the capacity of cells to repair irradiation-induced DNA breaks using 2 distinct DNA repair assays – comet assay and 53BP1 foci quantification (a marker of unrepaired DNA ends). Both approaches demonstrate that Trf2 does indeed block efficient DNA repair, not only in B cells, but also in MEFs. Finally, by screening various deletion mutant of Trf2, we revealed that expression of Trf2 lacking the N-terminal domain still promotes DNA repair without inducing telomeric fusion. This separation of function mutant uncouples the deleterious outcomes associated with unprotected telomere from the DNA repair phenotype at non-telomeric sites

National Institute on Aging (NIA)

HUIMING LU

Visiting Fellow

DNA-binding Proteins/Receptors and DNA Repair

RECQ4 promotes DNA end resection in repair of DNA double-strand breaks

DNA double-strand break (DSB) is a major contributor to genome instability and cell death, and it can be repaired via homologous recombination (HR) in S and G2 phases of cell cycle. HR repair is initiated with 5' DNA resection at broken ends, which generates 3' protruding single-strand DNA for RAD51-mediated strand exchange. In mammalian cells, 5' DNA resection occurs in two steps: the initial resection with MRE11-RAD50-NBS1 (MRN) and CtIP, and then the extensive resection by EXO1 or DNA2-BLM-TOP3-RMI1/2. However, the regulatory mechanisms of this process are largely unknown. Human RecQ helicase RECQ4 is associated with three genetic diseases: Rothmund-Thomson Syndrome (RTS), RAPADILINO and Baller-Gerold syndrome, as well as cancers. We previously reported that lack of RECQ4

induces persistent DNA damage and triggers senescence in primary cells, which contributes to the RTS features in the mouse model. However, it is unclear how RECQ4 participates in DSB repair. RECQ4 is highly expressed in S phase, when HR repair dominates. Thus, we investigated the possibility that RECQ4 plays a role in HR repair. Here, we found that RECQ4 co-localizes with DSB marker γ H2AX at laser-induced DSBs, and depletion of RECQ4 causes cell sensitivity to ionizing radiation. Significantly, knockdown of RECQ4 reduces 73% of HR repair and 57% of DNA resection in U2OS cells, suggesting that RECQ4 plays an unrecognized but crucial role in HR repair and DNA resection. To gain insight into RECQ4's role in DNA resection, RECQ4-interacting proteins were pulled down from irradiated cells and identified by mass spectrometry. RECQ4 forms a complex with DNA resection players including MRN, CtIP, EXO1, DNA2 and BLM. Further, we found that MRN recruits RECQ4 to DSBs and the exonuclease of MRE11 regulates the retention of RECQ4 at laser-induced DSBs. In vitro, RECQ4 also stimulates the exonuclease activity of MRE11, which is required for DNA resection. Interestingly, RECQ4 interacts with CtIP via its N-terminal domain and promotes CtIP recruitment to the MRN complex at DSBs to initiate resection. Moreover, inactivation of RECQ4's helicase activity impairs DNA resection and HR repair, indicating an important role for RECQ4's unwinding activity in the process. Thus, we identified a crucial role for RECQ4 in HR repair whereby it promotes 5' DNA end resection through MRN-CtIP. These findings promote the understanding of the molecular mechanism of RECQ4 in the maintenance of genome stability.

National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)

John Dougherty

Postdoctoral Fellow Postdoctoral Fellow

Endocrinology

Taltirelin, a thyrotropin-releasing hormone analog, alleviates fatigue in mouse models of cancer-related fatigue

Abstract removed at request of the author

National Institute of Environmental Health Sciences (NIEHS)

Bo He

Postdoctoral Fellow Postdoctoral Fellow

Endocrinology

An imbalance in glucocorticoid receptor and mineralocorticoid receptor signaling contributes to cardiac disease

Heart failure is one of the leading causes of death in the world, and stress is increasingly associated with adverse cardiac outcomes. Glucocorticoids are primary stress hormones that regulate cell and tissue homeostasis through two closely related nuclear receptors, the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). We have demonstrated before that a deficiency in glucocorticoid signaling through GR in cardiomyocytes resulted in profound alterations in gene expression profiles that lead to left ventricular (LV) systolic dysfunction, cardiac hypertrophy, heart failure, and death, revealing a crucial role for cardiomyocyte GR signaling in the function of the heart. Reinstallation of GR by Adeno-Associated Virus (AAV)-mediated gene transfer in cardiomyocytes partially rescued cardiovascular

function in adult mice. In addition to GR, cardiomyocytes also express MR. However, very little is known concerning the coordinated actions of GR and MR in heart physiology and whether an imbalance in their signaling contributes to cardiac disease. To further examine the in vivo function of glucocorticoid signaling in the heart through both these receptors, we generated mice with cardiomyocyte-specific deletion of GR (cardioGRKO), MR (cardioMRKO), or both GR and MR (cardioGRMRdKO). While the cardioGRKO mice spontaneously developed cardiac hypertrophy, heart failure, and death, the cardioMRKO mice exhibited normal heart function. Surprisingly, the cardioGRMRdKO mice were protected from the cardiac disease and early death observed for the cardioGRKO mice. At a molecular level, pathological gene changes present in the GR deficient hearts were also found in the double knockout hearts whereas cardioprotective gene changes were detected only in hearts from the cardioGRMRdKO mice. Furthermore, reinstallation of MR expression by AAV-mediated gene transfer in the cardioGRMRdKO hearts reversed the cardioprotective genes and led to systolic dysfunction. MR reinstallation by AAV at neonatal age of cardioGRMRdKO mice is expected to result in more significant phenotype seen in cardioGRKO mice. These findings reveal critical gene-regulatory roles for both GR and MR in the heart and suggest that an imbalance in these two signaling pathways leads to heart disease. Therapies designed to modulate cardiomyocyte glucocorticoid signaling to favor more GR and less MR activity may provide an improved approach for treating the failing heart.

National Institute of Environmental Health Sciences (NIEHS)

Matthew Quinn

Postdoctoral Fellow Postdoctoral Fellow

Endocrinology

Aberrant glucocorticoid signaling drives hepatic steatosis in the absence of ovarian hormones

Hallmark features of decreased ovarian function associated with female aging are steatosis and obesity. The molecular mechanism driving these metabolic disturbances in the absence of ovarian hormones still remain elusive. Interestingly, loss of ovarian function phenocopies Cushing's syndrome, a disorder of excessive glucocorticoids. To determine if glucocorticoids drive steatosis following loss of ovarian function we performed ovariectomy (OVX) on wild-type C57bl/6 mice +/- concurrent adrenalectomy (ADX) to reduce glucocorticoids. Consistent with previous reports we saw increased body weight and steatosis. Interestingly, OVX+ADX mice did not have the increased hepatic triglyceride load observed in OVX alone animals or the presence of lipid droplets within the liver. To determine if ovarian hormones alter hepatic glucocorticoid signaling we performed RNA-seq in mice that were intact or OVX treated with vehicle or the synthetic glucocorticoid Dexamethasone. Interestingly we found unique cohorts of genes regulated by glucocorticoids depending on the presence of ovarian hormones. Moreover, we see an alteration in Dex-regulated metabolic genes in OVX mice treated compared to Dex-treated intact animals. Pathway analysis revealed a shift in Dex-regulation of hepatic lipid metabolism in OVX mice. Perilipin-5 (PLIN5), a lipid droplet protein critical for the formation of lipid droplets is significantly induced by Dex-treatment in intact mice and hyper-induced in Dex-treated OVX mice. Molecular analysis of PLIN5 indicates it is a direct target of glucocorticoid action, as it was rapidly induced in response to Dex-treatment in vivo (1.5 hours) with subsequent recruitment of GR to glucocorticoid response elements within the PLIN5 gene as shown by chromatin immunoprecipitation and alterations in chromatin architecture/accessibility as shown by FAIRE-seq analysis. Moreover, analysis of PLIN5 in OVX

mouse liver indicates increased mRNA and protein, which was blocked in OVX+ADX mice. Aberrant GR regulation of the PLIN5 gene provides a potential molecular mechanism underlying steatosis in response to loss of ovarian function. Furthermore, these data indicate the glucocorticoid transcriptome is dramatically altered depending on the ovarian hormonal milieu. Taken together our data suggest glucocorticoids may underlie the metabolic syndrome associated with menopause and raises the possibility synthetic glucocorticoids behave differently between pre- and post-menopausal women.

National Institute of Child Health and Human Development (NICHD)

Annabel Berthon

Postdoctoral Fellow Postdoctoral Fellow

Endocrinology

Functional investigation of a new PMAH suppressor gene, ARMC5, using animal models

Primary Macronodular Adrenal Hyperplasia (PMAH) is an adrenal disorder characterized by bilateral adrenal nodules secreting mostly cortisol. PMAH is one of the rare causes of Cushing syndrome (CS). The main genetic cause of PMAH (being mutated in 40 to 50% of patients) has recently been identified as mutations in Armadillo repeat Containing 5 (ARMC5) gene. These mutations are found at both germline and somatic level suggesting that ARMC5 acts as a tumor suppressor gene. To uncover its function (that remains unknown), we develop two different strategies using animal models. We, first, performed a RNA-Seq experiment on zebrafish for both transient loss- (morpholino injection) and gain- (mRNA injection) of function at 30 hours post-fertilization. The comparative analysis of these two conditions suggests a role of Armc5 in apoptotic processes and steroid biosynthesis. This corroborates previously published human in vitro experiments and demonstrates a conservation of Armc5 function through vertebrates. Pathway analysis also highlights the potential involvement of Armc5 in the regulation of NAD⁺ deacetylase family, sirtuin. This has been confirmed in PMAH tumors. Indeed, the expression of 3 sirtuins, SIRT1, SIRT2, SIRT6 is significantly modified between PMAH with or without ARMC5 mutations. This is associated with an ARMC5 dependent difference in acetylation profile in the tumors. In parallel, we develop Armc5 knockout (KO) mice. Interestingly, the Armc5 heterozygote mice mimic the patient status for Armc5 and develop an adrenal phenotype at 12 months-old. Unfortunately, the adrenal phenotype in Armc5 KO mice cannot be studied due to the embryonic lethality observed between 7.5-9.5 days of development. We plan to generate an adrenal-specific KO mouse to study the adrenal phenotype further. Therefore, ARMC5 is a well-conserved protein with key roles in embryonic development and whose heterozygosity is sufficient to destabilize the adrenal function.

National Institute of Child Health and Human Development (NICHD)

Sarah Pugh

Postdoctoral Fellow Postdoctoral Fellow

Epidemiology/Biostatistics - Etiology, Risk, and Prevention

Gestational weight gain patterns in relation to short-term birth outcomes

Gestational weight gain (GWG) is a simple clinical measure used throughout pregnancy to monitor

maternal and fetal nutritional status. Gaining too much or too little is associated with adverse short- and long-term outcomes such as low birthweight, stillbirth, and childhood obesity. In an attempt to balance the risks of high and low GWG, the Institute of Medicine released guidelines in 2009 recommending a total and rate of GWG for optimal outcomes. However, at the time of review, there was limited evidence on the pattern of GWG used to inform the guidelines. Our objective was to describe patterns of GWG and examine their association with birthweight outcomes using a novel approach. We used a prospective cohort of 2,802 singleton pregnancies recruited from 12 US prenatal centers between 2009-2013. Prenatal weights were measured at study visits and abstracted from medical charts. Infant birthweights ≤ 2500 g and ≥ 4000 g were defined as low birthweight (LBW) and macrosomia, respectively. Small-for-gestational age (SGA) and large-for-gestational age (LGA) were calculated using race-specific fetal growth standards. Semiparametric, group-based trajectory models were used to estimate weight gain trajectories. Poisson regression with a robust error variance was used to calculate the relative risk of birth outcomes by the probability of trajectory membership. There were 2,795 women contributing 43,287 observations with a median (IQR) of 17(15-19) visits. Four distinct trajectories were identified with total GWG ranging from 3.6 kg to 24.0 kg and 2nd/3rd trimester rates of gain from 0.08 to 0.76 kg/week. Compared with the moderate gain group, the two highest gaining trajectories were associated with a 1.5- to 2.3-fold increased risk of LGA and a nearly 2.0-fold increased risk of macrosomia while the lowest gaining group was associated with a 1.6-fold increased risk of SGA. There was no difference in the risk of low BW by trajectory. When stratified by pre-pregnancy BMI, results trended in the same direction with one additional finding. Compared with the moderate gain group, only normal weight women with a low GWG trajectory had a 2.3-fold increased risk of low BW. Our findings support an association between GWG patterns and the risk of birth outcomes. Identifying an early high pattern of weight gain could serve as an important target to modify the risk of LGA and macrosomia.

National Institute on Aging (NIA)

Elisa Marques

Postdoctoral Fellow

Epidemiology/Biostatistics - Etiology, Risk, and Prevention

Impact of Hip volumetric bone mineral density on total mortality: A prospective cohort study

Hip fracture, the most clinically serious osteoporotic fracture in the elderly, has led to a long-standing interest in bone at the proximal femur. When measured using dual-energy X-ray absorptiometry (DXA), proximal femur areal bone mineral density (BMD) is associated not only with fracture but greater mortality. However, the proximal femur is a complex structure comprised of trabecular and cortical bone, which differ in structure, distribution and function. The relationship between trabecular and cortical BMD and mortality is unknown. We used CT-based techniques to acquire separate estimates of cortical and trabecular bone to address mortality risks. We used Cox proportional hazards models, adjusted for age, sex, hip size and other potential confounders to examine associations between baseline trabecular and cortical volumetric BMD (vBMD) with risk of all-cause mortality among 4,824 participants (56% females) of the Age, Gene/Environment Susceptibility (AGES) –Reykjavik Study who completed a detailed lifestyle questionnaire, and computed tomography in 2002-2006, who were aged 66-92 at baseline, and who were followed for mortality through 4 October 2015. Over 13.4 years of

follow-up, there were 2,222 deaths. Decreased trabecular vBMD was associated with a 9% increased mortality risk (hazard ratio (HR) for each standard deviation (SD) decrease in vBMD = 1.09, 95% confidence interval (CI) = 1.03–1.15, $p=0.002$). This association was independent of cortical vBMD (HR= 1.0, 95%CI = 0.96-1.06, $p=0.862$) and all potential confounders. Cortical vBMD and hip size (HR for each SD decrease in femoral neck cross-sectional area = 1.01, 95%CI = 0.96-1.07, $p=0.655$; and HR for trochanter cross-sectional area = 0.99, 95%CI = 0.93-1.05, $p=0.722$) were not associated with mortality. Our data demonstrate for the first time that only lower trabecular bone and not cortical bone is associated with higher mortality in older adults. The discovery of additional bone structure pathways related to mortality risk may provide better insight on underlying and potentially modifiable mechanisms and could have substantial public health impact.

National Cancer Institute - Division of Cancer Epidemiology and Genetics (NCI-DCEG)

Jessica Petrick

Postdoctoral Fellow

Epidemiology/Biostatistics - Etiology, Risk, and Prevention

Early Adulthood Adiposity, Life Course Trajectories of Adiposity, Weight Change, and Change and

Esophageal and Gastric Cardia Adenocarcinoma Risk: A Pooled Analysis of NIH-AARP and PLCO Studies

Background: Over the last two decades, esophageal and gastric cardia adenocarcinomas (EA/GCA) have been among the most rapidly increasing cancer types in the US. These tumors have overlapping risk factor profiles, anatomical proximity, and 5-year survival rates of ~26%. Elevated body mass index (BMI, kg/m^2) is a risk factor for EA and GCA. While the underlying causal mechanisms of the BMI–EA/GCA association are unclear, leading hypotheses include the ideas that high levels of adiposity may promote gastroesophageal reflux and/or metabolic sequelae. Few studies have examined early-life adiposity and weight change, and no study has assessed BMI trajectories over the life-course in relation to these cancers. As more than a third of the US population is considered obese (BMI=30), it is vital to understand when obesity interventions may be successfully implemented to reduce the risk of these lethal cancer types. Thus, we must understand the timing of obesity and weight gains in relation EA/GCA. Methods: We pooled and harmonized data on 410,699 individuals (632 EA, 416 GCA) from two prospective cohort studies: NIH-AARP Diet and Health Study and Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial. Height and weight were self-reported for ages 20, 50, and baseline. BMI trajectories were determined using latent-class mixture analysis. Hazard ratios (HRs) and 95% confidence intervals (CI) were estimated using Cox proportional hazards regression with age as the underlying time metric, adjusted for sex, race, smoking, education, and study. Results: Compared with individuals who were never overweight, first exceeding a BMI of $25 \text{ kg}/\text{m}^2$ at age 20 was associated with increased risks of EA (HR=1.77, 95%CI:1.36–2.31) and GCA (HR=1.63, 95%CI:1.17–2.27). A BMI trajectory of overweight at age 20 with later progression to obesity was associated with increased risks of EA (HR=2.98, 95%CI:1.72–5.16) and GCA (HR=4.52, 95%CI:2.62–7.81), compared with individuals always in the normal range. Similarly, weight gain of =20 kg between age 20 and baseline was associated with an increased risk of EA (HR=1.97, 95%CI:1.43–2.73) and modestly with GCA (HR=1.42, 95%CI:0.97–2.07). Conclusion: Overweight in early adulthood, increasing lifetime BMI trajectories, and weight gain were associated with increased risks of EA and GCA, underscoring the need for obesity interventions to be focused on early-life and maintenance of a healthy weight throughout the life course.

National Institute on Aging (NIA)

Eric Shiroma

Research Fellow

Epidemiology/Biostatistics - Etiology, Risk, and Prevention

Strength Training and the Risk of Type 2 Diabetes and Cardiovascular Disease

Federal physical activity guidelines recommend muscle-strengthening activities at least twice a week in addition to at least 150 minutes per week of moderate-to-vigorous aerobic physical activity for health benefits. While the primary rationale for including muscle-strengthening activities in the guidelines was musculoskeletal health, muscle-strengthening activities have recently been associated with reduced risk factors of type 2 diabetes and cardiovascular disease. However, there is little research directly examining the longitudinal associations of weight lifting and strength training with incident type 2 diabetes and cardiovascular disease risk. Therefore, to provide additional information, in a large, prospective cohort of older women, we examined the associations of strength training with incident type 2 diabetes and cardiovascular disease. We followed 35 754 healthy women (mean age, 62.6 years) from the Women's Health Study, who responded to a health questionnaire that included physical activity questions in 2000; assessing health outcomes through annual health questionnaire through 2014 (average (SD) follow-up = 10.7 (3.7) years). Strength training (minutes per week) was self-reported and updated throughout the follow-up. Incident type 2 diabetes (N cases = 2120) and cardiovascular disease (N cases = 1742) were confirmed on medical record review. Cases of cardiovascular disease were defined as confirmed cases of myocardial infarction, stroke, coronary artery bypass graft, angioplasty, or cardiovascular disease death. Compared to women who reported no strength training, women engaging in any strength training experienced a reduced rate of type 2 diabetes of 30% (hazard ratio: 0.70, 95% confidence interval: 0.61, 0.80) when controlling for time spent in other activities and other confounders. A risk reduction of 17% was observed for cardiovascular disease among women engaging in strength training (HR: 0.83, 95% CI: 0.72, 0.96). Participation in both strength training and aerobic activity was associated with additional risk reductions for both type 2 diabetes and cardiovascular disease compared to participation in aerobic activity only. These data support the inclusion of muscle-strengthening exercises in physical activity regimens for reduced risk of type 2 diabetes and cardiovascular disease, independent of aerobic exercise. Further research is needed to determine the optimum dose and intensity of muscle-strengthening exercises.

National Cancer Institute - Cancer Prevention Fellowship Program (NCI-CPFP)

Daniel Beachler

Cancer Prevention Fellow

Epidemiology/Biostatistics - Etiology, Risk, and Prevention

Trends in cervical cancer incidence in younger US women from 2000-2012

Background: US guidelines no longer recommend cervical cancer screening prior to age 21. While invasive cervical cancer (ICC) is rare among 21-25 year olds, there is some concern a lack of screening prior to age 21 could result in increasing ICC incidence rates among 21-25 year olds. We utilized US

population-based data to assess the cervical cancer screening prevalence and ICC incidence rates over time among 21-25 year old women. Methods: We compared population-based Pap testing prevalence and ICC incidence rates by year and birth cohort in women born in 1978-1990. Weighted Pap test data was examined utilizing the US Behavioral Risk Factor Surveillance System (BRFSS) and ICC incidence was evaluated using the Surveillance Epidemiology & End Results (SEER) 18 data. Annual percent changes (APCs) were estimated using Joinpoint regression. Results: The prevalence of never having a Pap test prior to age 21 increased from 22.0% in 1998-2004 to 35.4% in 2006-2010 (APC= +5.48, p<0.001). Despite this decline in screening prior to age 21, ICC incidence among 21-23 year olds significantly declined between 2000-12 (APC= -5.39, 95%CI= -8.34, -2.35), while remaining constant among 24-25 year olds (APC= +0.64, 95%CI=-2.29, 3.66). The decline in ICC incidence among 21-23 year olds was particularly pronounced between 2007-2012 (APC= -13.86, 95%CI=-21.86, -5.04). Compared to women born in 1978-1986, women born in 1987-1990 had a higher prevalence of never receiving a Pap test prior to 21 (37.4% vs. 23.9%, p<0.001), but had a lower ICC incidence at ages 21-23 (0.97 vs. 1.54 per 100,000, p<0.001). Conclusions: While US females born in the late 1980s were less likely to receive a Pap test prior to age 21, the incidence of subsequent ICC in this cohort of women was rare and lower than those born in earlier years. These data suggest that delaying cervical cancer screening initiation until age 21 has not strongly increased the subsequent risk of ICC at young ages. Better understanding of this recent decline in ICC incidence in young US women is necessary.

National Cancer Institute - Division of Cancer Epidemiology and Genetics (NCI-DCEG)

Erikka Loftfield

Postdoctoral Fellow

Epidemiology/Biostatistics - Prognosis and Response Predictions

Diabetes, insulin, and insulin resistance in relation to liver cancer and chronic liver disease mortality

Liver cancer is the 6th most commonly occurring cancer and the 2nd leading cause of cancer-related death worldwide. Traditionally, the burden of liver cancer has fallen on developing countries where hepatitis B (HBV) and C (HCV) viral infection are prevalent. Recently, rates of liver cancer have increased in developed countries. Mounting evidence suggests that increasing rates of diabetes are contributing to this upward trend. However, many of these studies have employed a cross-sectional design or had limited follow-up between diabetes assessment and cancer incidence, precluding evaluation of temporality and raising concerns about the impact of undiagnosed disease. Chronic liver disease (CLD) is often asymptomatic until late stage, yet alters glucose and insulin signaling, and is itself a strong risk factor for liver cancer. Prior studies have also had limited ability to evaluate the diabetes association in HBV/HCV negative participants. Here we evaluated associations of diabetes and insulin resistance with liver cancer and CLD mortality in a study with low HBV/HCV infection rates and extended follow-up. We conducted a nested case-control study of 138 primary liver cancer cases, 216 CLD deaths, and 681 matched controls during 22 years of follow-up in the ATBC Cancer Prevention Study. Glucose and insulin levels were measured in fasting baseline serum. Diabetes was defined by self-report or glucose ≥ 126 mg/dL. Age, alcohol use, body mass index, HBV and HCV status, education, and smoking adjusted odds ratios (OR) and 95% confidence intervals (CI) were estimated with logistic regression. Diabetes was associated with risk of liver cancer (OR=2.82, CI=1.66-4.77) and CLD mortality (OR=1.83, CI=1.08-3.09). Among those without self-reported diabetes, glucose (Quartile 4 vs. Quartile 1 (Q4/Q1): OR=2.44,

CI=1.35-4.43) was positively associated with liver cancer. Insulin (Q4/Q1: liver cancer, OR=3.45, CI=1.78-6.72; CLD, OR=2.51, CI=1.44-4.37) and HOMA-IR, a measure of insulin resistance, (Q4/Q1: liver cancer, OR=3.93, CI=2.00-7.72; CLD, OR=2.28, CI=1.32-3.95) were each positively associated with liver cancer and CLD mortality. For liver cancer, associations were particularly strong for cases occurring >10 years after blood draw, where reverse causality is less of a concern. In summary, our data provides key evidence that diabetes and insulin resistance are independent risk factors for liver cancer, and make substantial contributions to its current and future burden.

National Institute of Child Health and Human Development (NICHD)

Shristi Rawal

Postdoctoral Fellow

Epidemiology/Biostatistics - Prognosis and Response Predictions

Iron: Friend or Foe for Pregnant Women? A Prospective Study of Body Iron Status in Pregnancy and Risk of Gestational Diabetes in a Multi-racial Cohort

Iron acts as a two-edged sword in living systems. Both iron deficiency and overload cause increased oxidative stress and inflammation, which subsequently contribute to impaired glucose metabolism. Iron demands increase during pregnancy, yet longitudinal studies that examine iron status markers across gestation and their influence on gestational diabetes (GDM), are lacking. Particularly, hepcidin, a hormone recently identified as the master regulator of iron homeostasis, has not yet been examined in the context of GDM risk. We aimed to address these data gaps in a prospective case-control study of 107 GDM cases and 214 controls (matched 1:2 on age, race, and gestational age at blood draw) within the multi-racial NICHD Fetal Growth Studies. GDM diagnosis was based on medical record review. Plasma levels of hepcidin, ferritin and soluble transferrin receptor (sTfR), were measured at two visits prior to GDM diagnosis (gestational weeks 8-13 and 16-22), and weeks 24-29 and 34-37. Adjusted odds ratios (aORs) [95% confidence intervals (CIs)] for GDM were estimated using conditional logistic regression adjusting for demographics, C-reactive protein levels, pre-pregnancy BMI and other major GDM risk factors. As the pregnancy progressed, hepcidin and ferritin levels declined whereas sTfR levels increased. Levels of hepcidin, a marker of iron surplus, were significantly higher in cases than controls and were positively related to GDM risk during weeks 16-22 but not 8-13; aOR (95% CI) comparing the highest vs. lowest quartile was 2.6 (1.1, 6.4). Similarly, ferritin, a marker of body iron stores, was positively associated with GDM risk; aOR (95% CI) comparing the highest vs. lowest quartile was 2.4 (1.1, 5.3) at weeks 8-13 and 3.9 (1.4, 11.3) at weeks 16-22. Consistently, women in the highest quartile of ratio of sTfR to ferritin (an indicator of iron deficiency) had a lower GDM risk than women in the lowest quartile at both weeks 8-13 (aOR=0.3, 95% CI: 0.1, 0.8) and 16-22 (aOR=0.2, 95% CI: 0.1, 0.5). This is the first study to prospectively show that excess iron stores in pregnancy is significantly associated with a higher GDM risk using longitudinal measurements of both traditional and novel iron biomarkers. Our findings suggest that while women with elevated iron status in the second trimester are at the greatest risk for GDM, high maternal iron stores starting as early as first trimester may play a role in the development of this common pregnancy complication.

National Cancer Institute - Division of Cancer Epidemiology and Genetics (NCI-DCEG)

Andriy Derkach

Postdoctoral Fellow

Epidemiology/Biostatistics - Prognosis and Response Predictions

Fast and accurate power calculation methods for rare-variant association tests

Many statistical tests have been proposed for association studies with rare genetic variants. These methods can be divided into two classes: tests based on a linear composite statistic (e.g. burden tests) and tests based on a quadratic statistic (e.g. variance-component tests). Power calculations for these tests currently require the specification of the exact genetic architecture relating locus and trait. Therefore, power calculations require specifying a large number of parameters: the number of rare variants in a locus, the proportion of causal variants, the effect size of each causal variant, and, for linear statistics, the direction of the effects. With limited knowledge about the genetic architecture and the corresponding parameters, existing power calculations have very limited utility in practice. Here we propose fast and accurate methods to calculate the power of test statistics in both the linear and quadratic classes. Through theory and simulations, we demonstrate that power can be approximated using at most three parameters: the proportion of phenotypic variation explained by the locus, the number of causal variants, and, for linear statistics, the proportion of causal variants that are deleterious. Furthermore, we use the proposed methods to investigate whether the power of rare variant tests can be increased by restricting the set of tested variants to those predicted to be functional by bioinformatic annotation. We show that the power can be increased if the AUC for identifying functional variants exceeds 0.70. Given that current annotation offer AUCs between 0.70 and 0.75, we suggest using liberal specificity values for variant selection and focusing on removing variants that are most probable to be neutral.

National Cancer Institute - Division of Cancer Epidemiology and Genetics (NCI-DCEG)

HAN ZHANG

Postdoctoral Fellow

Epidemiology/Biostatistics - Prognosis and Response Predictions

Detailed SNP coverage information is crucial to ensure statistical validity of multi-locus tests based on GWAS meta-analysis results

In the post-GWAS era, easily accessible SNP-level summary data generated from a large-scale GWAS meta-analysis provides ample opportunities to use more powerful multi-locus methods for identifying novel susceptibility variants. Currently, the publicized meta-analysis results rarely provide the exact SNP coverage information (i.e., the list of SNPs studied by each of the participating studies). In order to estimate the correlation between a pair of SNP-level summary statistics, existing multi-locus methods usually assume one of two SNP coverage structures: the uniform structure that assumes all SNPs are studied by the same set of subjects, and the nested structure that assumes any two correlated SNPs are studied by two nested sets of subjects, with one completely covering the other. However, the two oversimplified structures are often invalid, especially when the meta-analysis is conducted by a consortium of many studies, with some using different genotype platforms. As a result, these methods tend to overestimate the correlation, and thus have an inflated false positive rate. We derive an unbiased estimate of the correlation assuming the exact SNP coverage information is known, and incorporate it

into various multi-locus methods. We illustrate the importance of getting the appropriate SNP coverage information by conducting a conditional test, gene-level test, and pathway-based test, on the summary data generated by an Asian consortium meta-analysis that integrated eight type 2 diabetes GWAS with 6,952 cases and 11,865 controls. Among over 3 million tested SNP pairs, 0.02%, 0.1% and 0.4% of the p-values of the conditional test using the exact, nested and uniform SNP coverage structures are less than $1E-4$, respectively. When analyzing over 18,000 genes across the genome, the genomic control inflation factors for gene-based tests using the exact, nested and uniform structures are 1.11, 1.20 and 1.84, respectively. In the evaluation of over 4,700 pathways, the pathway association tests using the exact, nested and uniform structures have inflation factors of 1.37, 2.13 and 4.99, respectively. These results demonstrate that multi-locus tests can have highly inflated false positive rates when an incorrect SNP coverage structure is assumed. We advocate that GWAS consortia should provide the study-specific SNP coverage information when posting their meta-analysis results so that researchers can conduct multi-locus tests with well calibrated false positive rates.

National Cancer Institute - Division of Cancer Epidemiology and Genetics (NCI-DCEG)

Hannah Oh

Postdoctoral Fellow

Epidemiology/Biostatistics - Prognosis and Response Predictions

Breast cancer risk factor associations by loss of E-cadherin tumor tissue expression: a pooled analysis of 5,896 cases in 12 studies from the Breast Cancer Association Consortium

Breast cancer comprises multiple molecular subtypes, typically defined by hormone receptor expression and histology. Efforts to provide precise risk estimates by subtypes could result in improved prevention and screening interventions. Yet, how to define these subtypes remains unclear. E-cadherin is a tumor suppressor protein and plays a critical role in epithelial cell adhesion and epithelial-to-mesenchymal transition in normal epithelium. E-cadherin loss is more frequently observed in tumors of lobular vs. ductal histology; however, it is unknown whether E-cadherin loss could define a more homogenous subgroup of cancers beyond estrogen receptor (ER) expression and histology. We examined whether established breast cancer risk factor associations differed by low vs. high E-cadherin tumor tissue expression. Data on 5,896 cancers, representing 12 studies participating in the Breast Cancer Association Consortium were obtained, the largest pooled dataset ever procured. Breast cancers were centrally stained at the NCI for E-cadherin using tumor tissue microarrays (TMA). Intensity (0-3) and percentage of tumor cells of E-cadherin expression were assessed using digital images of stained TMA cores. A final score (range 0-300) was calculated by multiplying percentage of positive cells with stain intensity. Low levels of E-cadherin were defined as tumors with score ≤ 100 . Differences in risk factor associations by E-cadherin (low vs. high) were evaluated using case-only logistic regression, adjusted for age and study site and stratified by ER status and histology. E-cadherin low tumors (20%) were more likely to be lobular, well differentiated, >2 cm, and HER2-negative (all $p < 0.003$). Among both ER+ and ER- tumors, family history of breast cancer [ER+: OR (95% CI)=1.30 (1.03-1.64), $p=0.03$; ER-: 1.37 (0.87-2.17), $p=0.17$] and ever use of oral contraceptive [ER+: 1.35 (0.85-2.14), $p=0.21$; ER-: 2.19 (1.05-4.55), $p=0.04$] were associated with low E-cadherin expression; uniparity (vs. multiparity) [ER+: 0.73 (0.57-0.93), $p=0.04$; ER-: 0.90 (0.61-1.33), $p=0.75$] was associated with high E-cadherin expression. When we further stratified ER+ tumors by ductal and lobular histology, the above associations for ER+ tumors were limited to

ductal but not lobular tumors. Our data suggest that family history and select hormonally related risk factors may enhance loss of E-cadherin expression. E-cadherin tumor expression may provide further precision for breast cancer risk estimates.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Delphine Lissa

Visiting Fellow

Epigenetics

Ultrasensitive quantification of promoter methylation in cell-free circulating DNA for early detection of lung cancer

Lung cancer is the leading cause of cancer-related death worldwide. Screening for early-stage lung cancers is essential to decrease the mortality rate. The recent incorporation of molecular biomarkers in clinical practice has improved disease management and survival outcome. In recent years, cell-free circulating DNA (cfDNA) has gained increasing attention as a surrogate for tumor DNA. In addition to providing a minimally-invasive source of tumor DNA, cfDNA reflects molecular alterations and tumor heterogeneity. Epigenetic changes, including DNA methylation, occur early in carcinogenesis. Cancer cells are characterized both by global hypomethylation and hypermethylation of CpG islands in gene promoter regions. Analysis of tumor-specific DNA methylation in cfDNA is a promising strategy for applying epigenetic biomarkers to the detection of cancers at an early-stage. In a prior genome-wide screening of DNA methylation, we identified a locus methylated de novo in tumor tissues resected from stage I lung cancer patients that had high discriminatory power to distinguish tumor from non-tumor tissues in multiple patient cohorts. High promoter methylation was also associated with shorter cancer-specific survival. The present study aims at evaluating the diagnostic and prognostic value of promoter methylation in cfDNA. We developed a methylation-specific droplet digital PCR (ddPCR) assay to detect and quantify rare methylation events. The DNA was subjected to bisulfite treatment to convert unmethylated cytosine residues to uracil. We designed specific primers and probe containing 7 CpGs to only amplify the methylated promoter. Experimental conditions were first optimized using fully methylated and unmethylated control DNAs, DNA extracted from cancer cell lines, germline cells and lung tissues (paired tumor and non-tumor). The ddPCR assay could detect as little as 30 haploid genomes equivalent of methylated promoter DNA, and count a single methylated allele present at 0.2% (i.e., 1 methylated copy among 500 unmethylated copies). Differences in methylation levels between tumors and adjacent tissues were also observed. Lastly we demonstrated the high efficiency and reproducibility of cfDNA extraction from plasma. We have thus established a robust and ultrasensitive method for standardized determination of promoter methylation status in cfDNA from plasma. We are currently evaluating its potential value for noninvasive diagnosis and prognosis of lung cancer patients.

National Human Genome Research Institute (NHGRI)

Brendan Miller

Doctoral Candidate

Epigenetics

Digital Droplet PCR Liquid Biopsy Assay for Detecting Circulating Tumor DNA in Patient Plasma

One in four deaths in the United States is due to cancer despite an emphasis on prevention, early detection, and treatment. Further improvements in survival rates are likely to come from improving the limits of detection sensitivity at earlier stages of cancer. Apoptotic and necrotic cells release their DNA as fragments into the patient's blood and can be detected as cell free DNA (cfDNA) in a "liquid biopsy", or patient plasma sample. In a similar fashion, tumor cells release circulating tumor DNA (ctDNA), which contains genetic and epigenetic signatures specific to the tumor, however, these fragments are usually diluted in the total cfDNA pool. One epigenetic hallmark of many cancer types is differential methylation status at multiple loci compared to normal tissue. Thus, detection and assessment of the methylation state of ctDNA at a specific locus, via bisulfite sequencing, could be utilized as an effective cancer diagnostic. We have recently identified the CpG island promoter region of ZNF154 as a hypermethylated locus in 15 solid epithelial tumor types from 13 different organs, making it a promising pan-cancer biomarker for use in a blood-based cancer detection assay. Because ctDNA is typically fragmented and exists at low copy numbers per milliliter of plasma we first performed a simulated dilution of the ZNF154 locus using solid tumor DNA and report robust sensitivity and specificity at fractions as low as 1 percent. To overcome the challenge of low copy number detection, we utilized digital droplet PCR (ddPCR), which can achieve superior detection sensitivity compared with real-time PCR and absolute copy number quantification without an external reference. We report the number of target molecules detected in tumor and normal plasma samples and confirm that full-length target fragments are indeed present and amplifiable in patient plasma. Moreover, significant amounts of copies per microliter were detected across tumor types and stages with 1mL of plasma or less. Total copies of target fragment in a sample can be leveraged to determine the minimum number of ctDNA fragments required to make a diagnosis. In summary, we show that ddPCR is a complementary technique for emerging cancer diagnostics and that our blood based cancer screening assay is a feasible approach to detect cancer non-invasively.

National Heart, Lung, and Blood Institute (NHLBI)

Wenfei Jin

Visiting Fellow

Epigenetics

Genome-wide detection of DNase I hypersensitive sites in single cells and FFPE tissue samples

DNase I hypersensitive sites (DHSs) provide important information on the presence of transcriptional regulatory elements and the state of chromatin in mammalian cells. Conventional DNase-Seq for genome-wide DHSs profiling is limited by the requirement of millions of cells. Here we report an ultrasensitive strategy, called Pico-Seq, for detection of genome-wide DHSs in single cells. We show that DHS patterns at the single cell level are highly reproducible among individual cells. Among different single cells, highly expressed gene promoters and the enhancers associated with multiple active histone modifications display constitutive DHS while chromatin regions with fewer histone modifications exhibit high variation of DHS. Furthermore, the single-cell DHSs predict enhancers that regulate cell-specific gene expression programs and the cell-to-cell variations of DHS are predictive of gene expression. Finally, we apply Pico-Seq to pools of tumor cells and pools of normal cells, dissected from formalin-fixed paraffin-embedded (FFPE) tissue slides from thyroid cancer patients, and detect thousands of

tumor-specific DHSs. Many of these DHSs are associated with promoters and enhancers critically involved in cancer development. Analysis of the DHS sequences uncovers one single-nucleotide variant (chr18:52417839 G>C) in the tumor cells of a follicular thyroid carcinoma patient, which affects the binding of the tumor suppressor protein p53 and correlates with decreased expression of its target gene TXNL1. In conclusion, Pico-Seq can reliably detect DHSs in single cells, greatly extending the range of applications of DHS analysis for both basic and translational research and may provide critical information for personalized medicine.

National Institute of Environmental Health Sciences (NIEHS)

MA WAN

Postdoctoral Fellow

Epigenetics

DNA methylation changes link tobacco smoke to atherosclerosis in human circulating monocytes

Cigarette smoke associates with numerous human diseases and influences DNA methylation. However, the biological links between the alteration of DNA methylation and disease are largely missing. While Human Methylation 450 BeadChip Array (450k array) has been widely used to measure smoking-associated DNA methylation changes and their association to diseases, a comprehensive assessment of the characteristics of smoking-associated differential methylated regions (SM-DMRs) is lacking. Here we reveal genome-wide SM-DMRs by Reduced Representation Bisulfite Sequencing (RRBS) using primary monocyte DNA from 58 age, gender and race matched smokers and nonsmokers selected from among 1,264 participants of the Multi-Ethnic Study of Atherosclerosis (MESA). We found that SM-DMRs preferentially occur at regulatory regions with the characteristics of enhancer, CpG Island (CGI), and/or CGI shore. SM-DMRs were enriched for transcription factor (TF) binding suggesting crosstalk between TFs, DNA methylation, and tobacco smoking exposure. These discoveries were replicated in 40 independently recruited subjects (20 smokers vs 20 nonsmokers) by RRBS. Candidate SM-DMRs were further validated in an independent group (40 smokers vs 40 nonsmokers) using either pyrosequencing or bisulfite amplicon sequencing (BSAS). SM-DMRs were significantly associated with the differential expression of corresponding enhancer RNAs (eRNAs) and their nearby genes by transcriptional analysis. For example, Aryl-Hydrocarbon Receptor Repressor (AHRR) SM-DMR represents the most significant DMR between smokers and nonsmokers. It was further validated by BSAS and a novel CpG site was uncovered to be more sensitive to smoking exposure than the most significant CpG (cg05575921) previously identified by 450K array. Functionally, the AHRR SM-DMR, which overlaps an intragenic enhancer indicated by ENCODE data sets, significantly associated with increased eRNA (>20-fold) and AHRR mRNA expression (~10-fold) in smokers. Importantly, our recent analysis of the cg05575921 CpG site within AHRR SM-DMR using 450K array data in 1264 MESA subjects revealed that methylation significantly ($p < 0.01$) mediated the association between smoking exposure and subclinical atherosclerosis indicated by ultrasound-measured carotid plaques. Our findings suggest SM-DMRs may provide a mechanistic link between altered DNA methylation, regulatory element activity, target gene expression, smoking exposure, and smoking-related disease.

National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)

Katryn harwood

Postdoctoral FellowPostdoctoral Fellow

Epigenetics

The Enzymes of O-GlcNAc Cycling: Nutrient Sensitive Readers, Writers, and Erasers of the Histone Code

Epigenetic regulation of gene expression is essential for a multitude of processes in an organism and aberrant transcription can result in disease development. Better understanding of how transcriptional profiles are regulated, particularly in response to external signals such as nutrient supply, could aid in development of methods to better detect, treat, or prevent disease. One way cells regulate gene expression is through posttranslational modification (PTM) of histones. An often-overlooked PTM is the O-GlcNAcylation of serine/threonine residues. O-GlcNAc is added and removed by a single pair of enzymes, O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA), respectively. O-GlcNAc addition is the final step of the nutrient driven hexosamine-signaling pathway, and as such it is likely that O-GlcNAc addition/removal informs upon an organism's nutritive state. Interplay between O-GlcNAcylation and other PTMs on histones may allow cells to fine-tune transcriptional activity in response to cellular cues. To examine this possibility, we have focused on the interaction of OGA and OGT with both known modifiers of histone PTMs and modified histones themselves. Previous studies suggest that OGT plays a role in down regulating gene expression by binding and O-GlcNAcyating the SIN-3 histone deacetylase complex. We demonstrated through transcriptional analysis in *C. elegans* that about half of the genes that are deregulated when OGA or OGT are absent are also deregulated when SIN-3 is absent. We went on to apply a genetic approach using these knockout *C. elegans* strains to further characterize the relationship between O-GlcNAc cycling, SIN-3, histone acetylation and PolII occupancy. These studies provide insight into how the nutritive state can be translated into changes in transcriptional profiles on the level of an entire organism. In addition, OGA is known to possess a putative histone acetyltransferase (HAT) domain, prompting us to question what role it might play in OGA/histone interactions. Using recombinant OGA protein and differentially modified histone tail peptides, we have identified specific modifications with well known roles in transcriptional regulation to which OGA exhibits binding selectivity. Further characterization of these binding interactions both in vitro and in *C. elegans* allows us to begin "decoding" the intricate relationship between PTMs, transcriptional regulation, intermediary metabolism and transgenerational inheritance.

National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS)

Tejpal Gill

Research FellowResearch Fellow

Gene Expression

Microbial Dysbiosis Implicated in Spondyloarthritis

Spondyloarthritis (SpA) represents a family of immune-mediated inflammatory diseases involving entheses, joints and the gastrointestinal tract. Host-microbe interaction in the gut is emerging as an underlying component of inflammatory diseases including SpA. We are studying a rat model where transgenic expression of HLA-B27 and human β 2-m (B27) on different genetic backgrounds, namely Dark Agouti (DA), Lewis (LEW) and Fischer (F344), leads to SpA-like inflammatory disease with variable penetrance. The DA background confers resistance to SpA and associated gut inflammation, while LEW

and F344 are permissive, but associated with differences in severity. To characterize the nature of this influence, we employed unbiased next-generation sequencing to determine the gut microbiome and host transcriptome from terminal ileum, cecum and distal colon of B27 and healthy (wild type; WT) rats. This provides a comprehensive assessment of bacterial communities and host immune modulation as a function of disease severity based on histologic scoring. Disease severity correlates with expansion of opportunistic/pathogenic bacteria like Akkermansia, Bacteroides and Sutterella with concomitant decreases in commensals producing short chain fatty acids such as Ruminococcus. Metabolic profile predictions revealed many crucial host metabolic pathways such as bile acid, glutathione and steroid hormone biosynthesis pathways affected in B27 LEW and F344 animals, while the bacterial motility pathways implicated in inflammation were decreased in B27 DA animals. LEW and F344 harbored segmented filamentous bacteria (SFB) in the ileum, whereas the DA background completely lacked SFB. Host tissue transcriptome revealed upregulation of IL-23/IL-17, IFN γ , and TNF α pathways with downregulation of metabolic and antioxidant pathways in LEW and F344 B27. In DA B27 rats, initial upregulation of these pathways was not sustained. Gene expression based immune cell prediction suggests a disease correlation with enriched $\alpha\beta$ and $\gamma\delta$ T cell, myeloid, NK cells and stromal cells. In summary, our results implicate microbial dysbiosis and disruption of metabolic pathways in the gut associated with activation of the IL-23/IL-17 axis and oxidative stress machinery as key features of the host inflammatory response in the pathogenesis of HLA-B27-associated SpA. Furthermore, we identify SpA-associated microbial phenotypes, which may aid in the diagnosis or prognosis of HLA-B27-dependent disease.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Sarah Deasy

Doctoral Candidate

Gene Expression

A systems genetics approach reveals a novel Rnaseh2c-immune response axis that affects metastasis severity in breast cancer

Metastatic breast cancer is a devastating disease with a 5-year survival rate of only 26%. This is due to a lack of effective therapies against established metastases and an inability to identify high risk patients who would benefit from specific adjuvant therapies to prevent metastatic progression. We have shown in mouse models that spontaneously arising tumors metastasize with different severity based on the mouse genetic background. Using systems genetics approaches we have identified genes correlated with metastasis and survival in both mice and humans. Rnaseh2c was identified as a novel candidate metastasis susceptibility gene. This gene encodes a scaffolding subunit of the Ribonuclease H2 enzyme which removes ribonucleotides misincorporated into the DNA. Experimentally modulating Rnaseh2c expression in a murine mammary cancer cell line resulted in significant changes in pulmonary metastasis, confirming this gene as a metastasis modifier. Mutations in Rnaseh2c are known to cause Aicardi-Goutieres Syndrome, a neurological autoinflammatory disorder that overlaps clinically with congenital viral infections and the autoimmune disease Systemic Lupus Erythematosus. Given this, we hypothesized that altered expression of Rnaseh2c in breast cancer cells affects metastasis by engaging the immune system. To investigate immune system involvement, we analyzed metastasis in immunocompromised mice. T cell deficiency ablated the effect of reduced Rnaseh2c expression on

metastasis, revealing for the first time an Rnaseh2c-immune response axis in metastasis. Gene ontology pathway analysis following mRNA-sequencing of Rnaseh2c knockdown and overexpression tumors revealed that 20% of the genes with altered expression are involved in immune system-related pathways, including T cell signaling and antigen presentation. Furthermore, genes with significant changes included Type I interferons, T cell markers, and immune regulators. These results confirm that Rnaseh2c is a novel metastasis modifier gene and validate our hypothesis that the immune system is mediating the effect of Rnaseh2c on metastasis. We are currently characterizing tumor-infiltrating immune cells to elucidate the type of immune response observed. This mechanism highlights a potential new target for combination with immune modulatory therapies to combat this devastating disease and adds to a panel of genes we identified that together could determine patients with high risk for metastasis.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Joseph Rodriguez

Postdoctoral Fellow

Gene Expression

Regulation of estrogen-responsive genes in single cells

The estrogen response in mammary epithelial cells has served as a human transcriptional paradigm for over 40 years. The estrogen receptor binds estrogen response elements (EREs) which can be located both proximal and distal to putative target genes. These target genes are both activated and repressed in response to ligands and respond to a variety of agonists, antagonists, and partial agonists. Estrogen-responsive genes also exhibit complex dynamic regulation such as oscillatory behavior and are arranged in a non-random spatial configuration within the nucleus. Recently, genome-wide measurements such as Pol2 ChIP-seq, GRO-seq and RNA-seq have indicated that EREs themselves are transcribed, resulting in enhancer RNAs (eRNAs), but the function of these non-coding RNAs is largely unknown. Here, we describe the use of single-molecule imaging to dissect the regulation of the TFF1 gene in MCF7 cells. Using CRISPR/Cas9, we have succeeded in inserting PP7 and MS2 stem loops into multiple TFF1 alleles transcribed from their endogenous loci. We are therefore able to observe the transcription of single alleles over many hours in live cells. Using this direct observation of transcription dynamics, we find behavior which is inconsistent with the prevailing stochastic models of transcription. Surprisingly, we observe refractory periods both in the ON and OFF states. Moreover there seems to be little correlation between TFF1 alleles both in steady state and induction conditions indicating that alleles transcribe independently. Using smFISH, we simultaneously measure the production of eRNA and TFF1 mRNA. Although TFF1 is off in the absence of inducing ligand, we find that the eRNA is produced under both repressed and activating conditions. Furthermore, there is a positive correlation between eRNA and TFF1 synthesis. Finally, we have used both deletion and gain-of-function experiments to precisely elucidate the role of cis-acting elements in the estrogen response.

National Eye Institute (NEI)

Aman George

Postdoctoral Fellow

Gene Expression

Zebrafish rere gene is required for optic fissure closure

Rere gene is a transcriptional co-repressor known to regulate Shh signaling and Fgf 8 expression from anterior signaling centers during mouse embryonic development. Rere mRNA is expressed in developing mouse and zebrafish eyes and its loss of function is associated with microphthalmia. We used the zebrafish rere mutants (babtb210) and morpholino mediated knockdown strategy to investigate the role of rere in eye development, specifically optic fissure closure (OFC) and visual function along with the underlying mechanisms. Homozygous rere mutant embryos exhibited OFC defects and abnormal development of neural retina tissue into the optic stalk. Visual function testing by electro-retinogram (ERG) and opto-kinectic response studies revealed significantly reduced ERG response and reduced eye movements in rere mutant zebrafish embryos. Using in situ hybridization, rere mutant embryos exhibited up-regulation of fgf8 and pax2.1 in the optic stalk and down-regulation of pax6 and nlz1 in the neural retina and ventral optic cup, respectively. Dorso-ventral patterning of the eye was not disrupted as studied by expression of aldh1a3 and aldh1a2. Increased fgf8a and pax2.1 expression, and decreased expression of pax6 and nlz1 expression have been associated with OFC defects previously. We also observed down regulation of vax1 and vax2 genes which are required for maintaining the neural retina and optic stalk boundaries, and could be associated with the penetration of the neural retina tissue in the optic stalk area. Since fgf8 is a direct target of rere mediated repression and is also involved in the patterning of the neural retina, we studied whether defects similar to rere mutants can be recapitulated by overexpressing fgf8. We used the transgenic HSP70:fgf8 zebrafish line to overexpress fgf8 at 5-10, 15-20 somite and 20 somite-prim5 stages of zebrafish development by giving a heat-shock treatment for 3 hrs. Overexpression of fgf8 at earlier stages (5-20s) caused OFC defects and at 20s-prim5 it caused severe microphthalmia along with OFC defects. In a cell culture model system of human fetal retinal pigment epithelial cells we detected strong nuclear staining and weak membrane staining of the RERE protein, suggesting that it might have some role in RPE cells of the eye also. The zebrafish is thus a good model system to study cerebral visual defects including OFC defects associated with human RERE gene mutations which we and others have observed.

National Institute of Child Health and Human Development (NICHD)

KI SOON KIM

Visiting Fellow

Gene Therapy

Wild-type macrophages reverse disease in heme oxygenase 1-deficient mice

Heme oxygenase 1 (Hmox1) is an inducible enzyme that catalyzes the degradation of heme into biliverdin, carbon monoxide and ferric iron. Loss-of-function mutations in the Hmox1 gene cause a rare and lethal disease in children, characterized by severe anemia, intravascular hemolysis and tissue damage. Macrophages of the reticuloendothelial system play a key role in recycling iron from hemoglobin of senescent or damaged erythrocytes. Macrophages were depleted from the liver and spleen of Hmox1 knockout (KO) mice, which resulted in intravascular hemolysis and severe damage to

the endothelial system and organs. Previously, we have shown that bone marrow transplantation rescues the phenotype by providing Wild-type (WT) reticuloendothelial cells in Hmox1 KO mice. To investigate whether the macrophages in the reticuloendothelial system can repopulate and divide in tissue, we injected macrophages into the tail vein of KO mice. We isolated the bone marrows from GFP-expressing mice to enable us to detect them in recipient animals. Donor bone marrow cells were extracted from femur and tibial bones of 2 month-old mice, and cells were differentiated with L-cell conditioned medium for 1 week. Subsequently, the differentiated GFP expressing macrophages were injected into recipient KO mice. The introduction of WT macrophages reversed abnormal blood parameters of KO mice. Mean cell volume was significantly improved in transplanted KO mouse (51.7 fL) compared to KO mouse (42.3 fL), greater than the mean cell volume of transplanted WT mouse (49.9 fL). The hematocrits (49%) of transplanted KO mouse increased compared to the KO mouse (44%), equal to the hematocrit of transplanted WT (48%). Furthermore, the white blood cell counts (WBC) returned to normal. The WBC of KO and transplanted KO mouse was shown 12.17 (K/ul) compared to 3.88 (K/ul). The WBC of WT and transplanted WT mouse was within the normal range, as expected (5.3 (K/ul) and 2.72 (K/ul)). This phenomenon had not been shown in previous bone marrow transplantation experiments. Our results suggest there is no barrier to repopulating the reticuloendothelial system, and the macrophages in the reticuloendothelial system may divide in tissues as needed. Our results suggest that Hmox1-/- patients could be potentially be treated by repair of their mutations in macrophages, followed by reinfusion, without resorting to bone marrow transplantation. Our preliminary results will form the basis of further experiments.

National Eye Institute (NEI)

Wenhan Yu

Postdoctoral Fellow Postdoctoral Fellow

Gene Therapy

In Vivo Rod Photoreceptor Reprogramming Using AAV-Delivered CRISPR/Cas9 Rescues Retinal Degeneration

Retinitis pigmentosa (RP) is a degenerative retinal disease and the leading cause of inherited blindness, due to gene mutations primarily impairing the dim-light sensor rod photoreceptors. As rods provide nutritional and structural support to the daylight sensor cone photoreceptors, the progressive rod loss in patients leads to a secondary cone cell death and eventual blindness. Preservation of cone function is critical to patients' quality of life and has been the focus of therapy development. NRL is a transcription factor that determines the rod cell fate during retinal development. Cre-induced ablation of Nrl was reported capable of reprogramming adult rods, rendering them resistant to the effects of rod gene mutations and consequently preventing cone loss. To advance this into a therapeutic approach, we tested the use of CRISPR/Cas9 gene editing system for direct in vivo knockdown of Nrl in postmitotic photoreceptors. We first developed an adeno-associated virus (AAV)-based CRISPR/Cas9 system, in which expression cassettes of short guide RNA and photoreceptor-specific SpCas9 were delivered by two separate vectors. The system was validated by eGFP knockdown in a mouse line with EGFP-labeled rods following co-delivery of the vectors via subretinal administration. Efficient Nrl disruption in postmitotic photoreceptors was then achieved following Nrl-CRISPR/Cas9 vector treatment, without detectable off-targeting event. A majority of the transduced rods acquired hybrid rod-cone features,

including reduced expression of rod-specific genes and enhanced expression of cone-specific genes, loss of the unique rod chromatin pattern, and diminished rod electroretinograph (ERG) response. To assess whether the reprogrammed rods can survive the effects of rod gene mutations and protect cone cells, three mouse models harboring either recessive or dominant rod-specific mutations received Nrl-CRISPR/Cas9 vector treatment. Preservation of rod cell bodies and protection of cone function and viability were achieved in all three diseased mouse lines, as revealed by remarkably thicker photoreceptor layer, higher cone cell number and cone protein expression, greater cone ERG amplitude and better visual behavior than controls. We conclude that AAV-CRISPR/Cas9 mediated Nrl knockdown can efficiently reprogram postmitotic rods into hybrid rod-cone cells and prevent secondary cone death in retinal degeneration, which could be developed into a viable treatment for RP in humans.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Ritu Chaudhary

Visiting Fellow

Genetics

Regulation of chemoresistance by a novel p53-regulated long noncoding RNA

Resistance to chemotherapy is a major challenge in cancer. Here, we show that a novel p53-regulated long noncoding RNA (lncRNA) which we named LRC (lncRNA regulator of chemoresistance), is induced >100-fold after DNA damage and plays a critical role in chemoresistance in colorectal cancer (CRC), both in vitro and in vivo. Targeted deletion of LRC in CRC cells using CRISPR/Cas9 significantly reduced G1 arrest and induced hypersensitivity to multiple chemotherapeutic drugs including 5-FU (5-Fluorouracil). Importantly, this reduced G1 arrest was significantly rescued by re-introduction of LRC in LRC^{-/-} cells. Transcriptome analysis from LRC^{+/+} and LRC^{-/-} cells suggested that LRC is necessary for the upregulation of the p53 targets and G1 regulators BTG2 and RRM2B in response to 5-FU treatment. This defect in induction of BTG2 and RRM2B after 5-FU treatment was mediated by the binding of LRC to Matrin 3, a RNA-binding protein that we identified by in vitro RNA pulldowns followed by mass spectrometry. We found that LRC binds to Matrin 3 and is necessary for the efficient binding of a Matrin 3-p53 complex to the p53-response element of BTG2 and RRM2B. To determine if endogenous LRC is associated with the p53-response element of BTG2 and RRM2B, we used CRISPR/Cas9 to knock-in in the LRC genomic locus, a S1-tag which is an RNA aptamer that binds to streptavidin with high affinity. Pulldowns from the S1-tagged cells showed that endogenous LRC binds to Matrin 3 but LRC itself is not associated with these p53-response elements of BTG2 and RRM2B. These unpublished findings demonstrate a critical role of a LRC/Matrin 3/p53 axis in chemoresistance in CRC and suggest that targeting LRC can be utilized for future translational studies.

National Cancer Institute - Division of Cancer Epidemiology and Genetics (NCI-DCEG)

Wei Jie Seow

Research Fellow

Genetics

Association between GWAS-identified lung adenocarcinoma susceptibility loci and EGFR mutation status in never-smoking Asian women

Background: Never-smoking women in Asia have among the highest rates of lung adenocarcinoma in the world. Genome-wide association studies (GWAS) have identified several loci for lung cancer susceptibility in Asia. Epidermal growth factor receptor (EGFR) mutations in lung adenocarcinomas are more frequently observed among never-smokers and in Asian populations. We extend previous findings of the associations between GWAS-identified single nucleotide polymorphisms (SNPs) and lung adenocarcinoma risk, and given that lung adenocarcinoma patients with EGFR mutations (EGFR-positive) elicit a better response to tyrosine kinase inhibitor treatment, we evaluated the differential associations by EGFR mutation status among never-smoking Asian women. Methods: We conducted the largest meta-analysis to date using GWAS data and new replication genotyping of up to 11,529 cases and 14,098 controls in never-smoking Asian women for 12 SNPs identified from previous GWAS studies of various populations and subgroups in Asia. Eight of the 12 SNPs were previously shown to be significant among never-smoking women but the other four SNPs have not yet been studied in never-smoking women. Logistic regression models were used to evaluate the associations, adjusting for age and ethnic groups (Chinese, Korean, Japanese). Results: Two SNPs achieved genome-wide significance for the first time in never-smoking Asian women, rs7216064 (17q24.3, BPTF) for overall lung adenocarcinoma risk, and rs3817963 (6p21.3, BTNL2) in the subgroup of EGFR-positive cases. We found stronger associations for cases who were EGFR-positive compared to EGFR-negative cases. Two SNPs, rs9387478 (ROS1-DCBLD1) and rs2179920 (HLA-DPB1), showed significantly stronger effects in EGFR-positive compared to EGFR-negative cases after adjusting for multiple comparisons. In addition, we confirmed eight SNPs previously identified in never-smoking Asian women with an expanded sample size. Comparisons of overall associations with the Western population revealed some degree of commonality but were mostly distinct. Conclusion: Our findings further the understanding of the genetic architecture of lung adenocarcinoma and provide evidence of differential associations by EGFR mutation status, which may have important public health implications with respect to risk stratification, screening and treatment for lung adenocarcinoma among never-smoking women in Asia.

National Human Genome Research Institute (NHGRI)

Steven Boyden

Postdoctoral Fellow Postdoctoral Fellow

Genetics

Abstract removed at request of author

National Cancer Institute - Division of Cancer Epidemiology and Genetics (NCI-DCEG)

Amy Moore

Postdoctoral Fellow Postdoctoral Fellow

Genetics

Polygenic risk score of body mass index is associated with increased risk of Diffuse Large B-Cell Lymphoma

Abstract removed at request of the author

National Cancer Institute - Division of Cancer Epidemiology and Genetics (NCI-DCEG)

A. ROUF BANDAY

Visiting Fellow

Genetics

Bladder cancer GWAS signal at 4p16.3 affects response of TMEM129 to chemically-induced endoplasmic reticulum stress

Background: SNP rs798766 at 4p16.3 locus is a known GWAS signal associated with bladder cancer risk. This SNP is located within a linkage disequilibrium block that includes TMEM129, TACC3, FGFR3 and SLBP. We aimed to explore molecular mechanisms of this GWAS signal. Methods: Expression analysis of all isoforms of TMEM129, TACC3, FGFR3 and SLBP was performed in TCGA bladder cancer dataset (N=412) by linear regression in relation to rs798766 genotypes adjusting for age, sex, race, DNA methylation and copy number variation. Additional bladder tumors and tissue microarrays were tested with isoform specific TaqMan and in situ hybridization assays, respectively. Allele-specific TMEM129 minigene exontrap assays in 5 bladder cancer cell lines were used to evaluate alternative splicing. TMEM129 isoforms were overexpressed in cell lines for functional characterization by confocal microscopy, reporter assays, RNA-seq and qRT-PCR arrays to explore their effects on cell signaling and cell viability. Endoplasmic reticulum (ER) stress was induced by treating bladder cancer cell lines with chemicals dithiothreitol (DTT), tunicamycin and MG132. Results: Of all the isoforms of four genes tested the strongest association with rs798766 genotypes was observed for TMEM129-b transcript ($P_{adj} = 1.65E-06$); expression was decreased with bladder cancer risk allele G. Exontrap analysis revealed that allele G of a SNP rs2236786, which is located within exon 3 of TMEM129 ($r^2=1.0$ with GWAS SNP rs798766), changes alternative splicing of TMEM129. Allele G decreases TMEM129-b expression and creates a new TMEM129-a2 transcript which undergoes nonsense mediated decay except when under ER stress. TMEM129 is an E3 ligase and its protein isoforms are predicted to be functionally different. Confocal imaging showed that all isoforms are located in ER and thus can compete with each other. Overexpression of TMEM129 isoforms resulted in differential activation of genes from the main ER stress response pathways - IRE1, PERK and ATF6. Chemically-induced ER stress increased expression of all TMEM129 isoforms but induction was lower with risk G allele of rs2236786. Conclusions: Our results suggest that bladder cancer risk could be modulated by a genetic variant that affects the function of TMEM129, an E3 ligase. This mechanism could be important for response to environmentally-induced ER stress and development of bladder cancer. Environmental exposures are known to increase bladder cancer risk.

National Heart, Lung, and Blood Institute (NHLBI)

Wenjing Yang

Research Fellow

Genomics

A novel single-cell polyadenylation sequencing (SPA-seq) technique reveals an extensive bimodal choice of alternative polyadenylation

Polyadenylation (PA) is an essential step in eukaryotic mRNA maturation and alternative

polyadenylation (APA) is one of the major contributors of transcriptome/proteome diversity and plasticity. To date, spatiotemporal regulation of APA events can only be investigated at the population level by averaging polyadenylation usages among individual cells. It is therefore unclear how APA is regulated at the single cell level and how differential APA regulations between seemingly homogenous cells contribute to their expression heterogeneity. Here we report a single-cell polyadenylation sequencing (SPA-seq) strategy, which to our knowledge is the first NGS application allowing for monitoring genome-wide polyadenylation in single cell. SPA-seq was employed to acquire PA profiles of 96 individual MEF cells in response to interferon beta treatment. Comparison of SPA-seq and conventional PA-seq results demonstrated that SPA-seq can reliably capture the overall expression profiles as well as polyadenylation choices of individual cells. Strikingly, majority of the alternatively polyadenylated transcripts exhibit a bimodal distribution. For genes with two or more APA sites detected in the above cell population, each cell predominately, if not exclusively, use one of the APA sites. We provided further evidence that the APA bimodality in part contribute expression bimodality. Conversely, the relative expression level of a gene among individual cells may dictate its APA choices and ultimately the length of its 3' UTR. Taken together, our data underscore an extra layer of complexity of APA regulation and pave the road towards a better understanding of its underlying molecular mechanism at an unprecedented single cell resolution.

National Institute of Child Health and Human Development (NICHD)

Josefina Ocampo

Visiting Fellow

Genomics

The ISW1 and CHD1 ATP-dependent chromatin remodelers compete to set nucleosome spacing in vivo

The DNA of eukaryotic cells is packaged into chromatin. The basic structural unit of chromatin is the nucleosome, which is composed of a histone octamer containing two molecules each of the four core histones, around which the DNA is wrapped. In yeast, most genes have a nucleosome-depleted region at the promoter and an array of regularly spaced nucleosomes phased relative to the transcription start site. The nucleosome constitutes a barrier to transcription, replication, recombination and repair, which can be circumvented by Chromatin Remodeling Complexes. They use the free energy obtained from adenosine triphosphate (ATP) hydrolysis to modify chromatin structure, by altering interactions between histones and DNA. ISW1, ISW2 and CHD1 belong to this family of remodelers and possess nucleosome spacing activity. In vitro, they form arrays with different spacing. To determine whether these enzymes space nucleosomes differently in vivo, we constructed null mutants for all three spacing enzymes and mapped nucleosomal DNA obtained by micrococcal nuclease digestion of nuclei genome-wide using paired-end sequencing. We find that CHD1 and ISW1 compete to set the spacing on most genes, such that CHD1 dominates genes with shorter spacing and ISW1 dominates genes with longer spacing. In contrast, ISW2 plays a minor role, limited to transcriptionally inactive genes. Furthermore, we have studied the connections between spacing and transcription by mapping RNA polymerase II using ChIP-seq, and between spacing and linker histone (H1) using ChIP-exo data for H1 published by others. We found that heavily transcribed genes show weak phasing and extreme spacing, either very short or very long, and are depleted of H1. Genes with longer spacing are enriched in H1, which directs chromatin folding. We propose that CHD1 directs short spacing, resulting in eviction of H1 and

chromatin unfolding, whereas ISW1 directs longer spacing, allowing H1 to bind and condense the chromatin. Thus, competition between the two remodelers to set the spacing on each gene may result in a highly dynamic chromatin structure.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Valerie Miller

Postdoctoral Fellow Postdoctoral Fellow

Genomics

Identification of a gene signature predictive of hepatocellular carcinoma (HCC) patient response to adjuvant transcatheter arterial chemoembolization (TACE)

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, and outcome is dismal, due to tumor heterogeneity and lack of effective treatment options for patients with later stage disease. Transcatheter arterial chemoembolization (TACE) is the gold standard of therapy for patients with intermediate to locally advanced tumors. TACE delivers a high dose of chemotherapy directly to the tumor via the hepatic artery, followed by an embolizing agent to restrict tumor blood supply. However, tumor hypoxia is linked to alterations in metabolism, such as increased glycolysis (the Warburg effect), and can lead to enhanced cell survival. Several randomized control trials (RCTs) showed a survival benefit with TACE, but only with strict patient selection criteria. In Asia, TACE is also commonly used as adjuvant therapy after surgical resection, yet RCTs evaluating adjuvant TACE have shown conflicting results, likely due to patient selection and stratification. We hypothesize that tumor gene expression is predictive of response following TACE, and that differential cellular metabolism resembling a hypoxic phenotype prior to treatment is responsible for TACE resistance. We retrospectively analyzed gene expression data from treatment-naive tumor tissue from a cohort of Chinese patients who received TACE (n=105). Using hierarchical clustering, followed by class comparison and survival risk prediction, we identified a 15-gene signature that is predictive of response vs. non-response to TACE, as measured by overall survival, independent of other clinical variables. We found that hypoxia-related genes are enriched among differentially expressed genes in TACE Responders vs. Non-Responders, and that hypoxia master regulator HIF-1a is significantly up-regulated in Non-Responders. We determined that a key glycolysis gene is up-regulated in Non-Responders, and conversely, two rate-limiting genes involved in gluconeogenesis, the pathway opposing glycolysis, are up-regulated in Responders. We also examined metabolomic data from the TACE cohort, and found an enrichment of glycolysis-related metabolites in Non-Responders, and gluconeogenesis-related metabolites in Responders. Further investigation will be required to connect altered glucose metabolism to TACE resistance and to determine driver genes linking hypoxia and metabolism, which, together with our 15-gene signature, may serve as a stratification tool to guide personalized treatment modalities for HCC patients.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Suntae Kim

Postdoctoral Fellow Postdoctoral Fellow

Genomics

Ewing sarcoma cells harboring a translocation that retains EWSR1 exon 8 require HNRNPH1 to express the in-frame oncogenic fusion transcript EWS-FLI1

The primary oncogenic event in ~85% of Ewing sarcomas (ES) is a t(11:22)(q24;q12) translocation. It generates a fusion gene containing the 5' end of the EWSR1 and the 3' end of the FLI1 referred to as EWS-FLI1. The exact genomic breakpoints within the EWSR1 and FLI1 genes vary, but typically occur within introns and require the splicing machinery to generate an in-frame EWS-FLI1 transcript. In particular, translocations that retain exon 8 of EWSR1, which is an estimated 40% of EWS-FLI1 driven tumors, generate an out-of-frame transcript unless this exon is removed. In this study, we demonstrate that ES cells harboring a genomic breakpoint that retains exon 8 of EWSR1 require HNRNPH1 to express an in-frame EWS-FLI1 mature mRNA. Using a genome-wide RNAi screen, we identified several proteins involved in RNA processing as required for the activity of EWS-FLI1, including HNRNPH1. The role of HNRNPH1 in alternative splicing led us to hypothesize that ES cells are dependent on HNRNPH1 for the expression of the EWS-FLI1 transcript. Analysis of the expression of EWS-FLI1 following HNRNPH1 silencing in ES cell lines representing different translocation breakpoints and transcript isoforms showed that only ES cell lines retaining EWSR1 exon 8 at a genomic level (TC32 and SKNMC) are dependent on HNRNPH1. Silencing of HNRNPH1 results in an out-of-frame EWS-FLI1 transcript, which cannot translate the EWS-FLI1 protein. This leads to the reversal of expression of EWS-FLI1 gene targets and cell death. ES cell lines that harbor a translocation upstream of EWSR1 exon 8 (TC71 and RD-ES) exhibit none of these molecular or phenotypic changes upon HNRNPH1 silencing. We next employed an RNA IP to identify putative binding sites on the EWS-FLI1 pre-mRNA. The results showed enrichment for the binding of EWSR1 exon 8 by HNRNPH1. Towards the 3' end of EWSR1 exon 8 we identified two G-rich sequences, a motif typically bound by HNRNPH1. To determine if HNRNPH1 binds these sites, we developed an in vitro protein-RNA binding assay and confirmed the binding of HNRNPH1 to both G-rich sites in EWSR1 exon 8. Current studies are focused on using this assay to fully map the interaction of HNRNPH1 with EWSR1 exon 8 and understand the molecular mechanism to target it. These results demonstrate a sequence-specific, breakpoint-dependent vulnerability in Ewing sarcoma that has the potential to be exploited as a therapeutic target and suggests a novel strategy to target fusion oncogenes.

National Human Genome Research Institute (NHGRI)

Brennan Decker

Doctoral Candidate

Genomics

Biallelic BRCA2 Mutations Shape the Somatic Mutational Landscape of Aggressive Prostate Tumors

Prostate cancer is the most common malignancy and second leading cause of cancer deaths in American men, with approximately 220,800 diagnoses and 27,540 deaths in 2015. The five-year survival for local disease is nearly 100%, compared to only 28% for metastatic disease. This outcome disparity frames the major clinical challenge associated with PCa: distinguishing those men who are likely to get metastatic disease, which may be prevented by specific and early therapy, while minimizing the iatrogenic morbidity associated with overtreatment of indolent disease. Consequently, a great deal of PCa research is focused on finding molecular and genetic biomarkers that facilitate early and accurate identification of

men with potentially high-risk tumors. To identify clinically important molecular subtypes of prostate cancer (PCa), we characterized the somatic landscape of aggressive tumors using deep whole genome sequencing. In our discovery set of 10 tumor/normal pairs with Gleason scores of 8-10 at diagnosis, coordinated analysis of germline and somatic variants, including single nucleotide variants, indels, and structural variants, revealed biallelic BRCA2 disruptions in a subset of samples. Compared to the other samples, the PCa BRCA2-deficient tumors exhibited a complex and highly specific mutation signature, featuring a 2.88-fold increased somatic mutation rate, depletion of context-specific C>T substitutions, and an enrichment for deletions, especially those longer than 10-bp. We next performed a BRCA2 deficiency-targeted reanalysis of 150 metastatic PCa tumors, and each of the 18 BRCA2-mutated samples recapitulated the BRCA2 deficiency-associated mutation signature, underscoring the potent influence of these lesions on somatic mutagenesis and tumor evolution. Among all individuals with BRCA2-deficient tumors, only about half of 21 carried deleterious germline alleles. Importantly, the somatic mutation signature in tumors with one germline and one somatic risk allele was indistinguishable from those with purely somatic mutations. Further, any test designed to leverage BRCA2 status as a biomarker for PCa must consider both germline and somatic mutations, and all types of deleterious mutations. Our observations clearly demonstrate that BRCA2-disrupted tumors represent a unique and clinically relevant molecular subtype of aggressive PCa, highlighting both the promise and utility of this mutation signature as a prognostic and treatment-selection biomarker.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Sanja Stevanovic

Visiting Fellow

Hematology/Oncology, Tumor Immunology, and Therapy

Comprehensive T-cell specificity analysis in successful immunotherapy of human papillomavirus+ cancers redefines target antigens for T-cell based immunotherapy

Human papillomavirus-associated (HPV) malignancies (cervical, anogenital and oropharyngeal cancers) are caused by HPV, and account for 300,000 deaths annually worldwide. Decades of targeting HPV E6 and E7 oncoproteins with therapeutic vaccines in these cancers have been unsuccessful. However, adoptive transfer of tumor-infiltrating lymphocytes (TIL) has demonstrated tumor regression in some HPV+ cancer patients. To gain insight into the mechanism of successful immunotherapy in HPV+ cancers, we performed a comprehensive analysis of anti-tumor T-cell specificities in TIL administered to two patients with cervical cancer who experienced durable complete tumor regression following treatment. Reactivity of T cells was assessed in immunological assays against three classes of tumor antigens, HPV antigens, non-synonymous somatic mutations and cancer-germline antigens, identified by whole-exome and RNA sequencing of patient's tumors. Tumor antigen reactivity and specificity of T cells was confirmed by T-cell receptor gene transfer, and in vivo survival was profiled by T-cell receptor deep sequencing. Intriguingly, the dominant anti-tumor T-cell reactivity identified in infused TIL from both patients was directed against non-HPV antigens. In one patient, 5, 1 and 2 CD8+ T-cell clonotypes were revealed to target mutated SETDB1, METTL17 and ALDH1A1, respectively, and 5 CD8+ and CD4+ T-cell clonotypes targeted HPV E6 or E7. Notably, the frequency of mutated neoantigen T-cell reactivity was 2.5-fold higher compared to HPV T-cell reactivity. In the other patient, a CD8+ T-cell clonotype comprising 67% of the infused TIL was found to target the KK-LC-1 cancer-germline antigen, and a CD4+

T-cell clonotype comprising 14% of the infused TIL targeted HPV E7. In both patients, the tumor antigen specific T-cell clonotypes could be detected in peripheral blood during ongoing remission. While identified immunogenic mutations appeared patient-specific, KK-LC-1 is expressed by many cancer types, and thus represents a promising target for immunotherapy approaches in various cancers. These data provide evidence that mutated neoantigens and cancer-germline antigens, rather than HPV antigens, represent the dominant T-cell targets in successful immunotherapy of HPV+ cancers. The diverse tumor antigen targeting in these effective treatments conveys a paradigm shift in the future study and development of immunotherapies for HPV+ cancers and other epithelial cancers.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Aizea Morales-Kastresana

Visiting Fellow

Hematology/Oncology, Tumor Immunology, and Therapy

Tumor-derived extracellular vesicle analyses by nanoFACS

Extracellular vesicles (EV) are heterogeneous populations of nano-sized (50-150nm) vesicles with important roles in immunity and cancer. The source and characteristics of EVs are associated with distinct functional roles, suggesting the existence of different EV subsets. To study subset-specific functions and characteristics, we developed a flow cytometric method (nanoFACS) to analyze and sort individual EVs. We demonstrated the use of nanoFACS and these labeling methods to identify, isolate and characterize subsets of prostate cancer derived EVs with Prostate Specific Membrane Antigen (PSMA). EVs from two human prostate cancer cell lines, PC3 (PSMA-negative) and PC3pip (PSMA-positive) were isolated by differential ultracentrifugation and size distribution characterized by Nanoparticle Tracking Analysis (NTA, NanoSight). We first stained EVs with anti-PSMA antibodies coupled to PE (specific-EV staining), and then labeled the bulk population of EVs with CFSE (pan-EV staining). To wash out the excess of CFSE dye and antibody, we performed an optimized size exclusion chromatography method. NanoFACS was performed with an AstriosEQ flow cytometer, triggered with a high sensitivity SSC detector for EV analyses based on light scattering, in addition to standard FSC and fluorescence parameters. EVs from both PC3 and PC3pip cell lines, demonstrated a heterogeneous distribution of EV sizes between 70 and 150 nm by NTA, and nanoFACS analyses by light scattering and CFSE fluorescence also showed a clear EV population in the 70-150 nm size range. NanoFACS with monoclonal antibody staining detected PSMA presence on PC3pip-derived EVs, but not PC3-derived EVs, and studies are ongoing to label, sort and characterize PSMA-positive subsets from patients with prostate cancer. EVs have tremendous potential as biomarkers and regulators of disease. NanoFACS offers a unique platform for the identification and isolation of tumor-specific and treatment-associated EV subsets. In this study, we established a robust workflow for EV production and isolation, and we refined pan-EV (CFSE) or specific-EV (PSMA) staining methods for EV analyses and sorting. Ultimately, we plan to study EV subsets by using specific EV markers for nanoFACS analyses, in responders and non-responders from immunotherapy trials to translate the EV subset signatures into information for personalized and adaptive treatments for our patients.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Robbie Majzner

Clinical Fellow

Hematology/Oncology, Tumor Immunology, and Therapy

Chimeric Antigen Receptor (CAR) T-cell Therapy Against Anaplastic Lymphoma Kinase (ALK) is Limited by Target Antigen Density and CAR Surface Expression

ALK is overexpressed on the surface of neuroblastoma (NB) and is associated with high risk disease. We developed a second generation CAR targeting ALK. ALK CAR T-cells significantly delay the growth of human NB cell lines in xenograft models, but animals eventually succumb to tumors. To understand how target antigen density limits the effectiveness of this CAR, we created a library of NALM-6 B-cell leukemias with variable amounts of ALK expressed on each clone. We found that there is a minimum target antigen density required for CAR T-cells to produce appreciable amounts of Th1 cytokines. This threshold of ALK expression is above the expression on NB. There is also a target antigen density at which maximum cytokine production occurs. Above this density, no additional cytokines are produced. In xenograft models, ALK CAR T-Cells are significantly more effective against high ALK than low ALK expressing leukemias. To our knowledge, this is the first report of greater in vivo efficacy of CAR T-cells against tumors with higher antigen expression. The level of surface expression of the CAR on T-cells also alters the function of CAR T-cells. We have identified a phenomenon in which both ALK and CD19 CAR T-cells lose surface expression of CAR over time in culture. Additionally, both CARs downregulate quickly after T-cells are exposed to antigen. Using a fluorescently tagged CD19 CAR, we demonstrate that CARs are rapidly internalized upon antigen encounter. We created an assay to understand the interplay of target density and CAR surface expression. We transduced T-cells with different amounts of supernatant to achieve different ALK CAR surface densities and then exposed these T-cells to varying amounts of immobilized protein-L. T-cell activation (measured by an NFAT reporter) is greatest when both CAR surface expression and target antigen density are highest. As both variables are decreased, there is loss of T-cell activity. Thus, diminished CAR surface density limits CAR T-cell efficacy. We have created a novel CAR targeting ALK that demonstrates in vivo efficacy against NB. Efficacy is limited by low target antigen density and low CAR surface expression. We have identified phenomena in which CAR T-cells lose surface expression of CAR over time and also quickly internalize their receptors in response to antigen. These factors contribute to the efficacy of other CAR T-cells and this data provides important insights into future CAR target selection.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Jasmin Leshem

Visiting Fellow

Hematology/Oncology, Tumor Immunology, and Therapy

Combining Immunotoxins Targeting Mesothelin with CTLA-4 Immune Check Point Blockade Eradicates Murine Cancer by Promoting Anti-Cancer Immunity

Immune check point blockade using antibodies to cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) benefits only a limited subset of patients. Combination therapies are being pursued in order to augment the immune activation and further improve the drug effect. SS1P and RG7787 are recombinant immunotoxins that consist of an anti-mesothelin antibody fragment genetically fused to a portion of

Pseudomonas exotoxin A (PE). SS1P and RG7787 are being tested in phase 1 trials for mesothelin expressing malignancies. We previously observed delayed onset of responses to SS1P treatment that persisted even after the discontinuation of the drug. This observation led us to hypothesize that immunotoxins can elicit anti-tumor immunity and thus be potentiated by adding anti-CTLA-4 antibodies (aCTLA-4). The immunotoxins we developed are human specific and do not target mouse mesothelin. To test our hypothesis, we transfected 66C14 murine breast cancer cells with human mesothelin (hMSLN). These cells were rejected by wild type BALB/c mice; therefore hMSLN transgenic BALB/c mice were used for the in-vivo tumor model. Treatment started after the tumors were established at a size of 80-100mm³. Immunotoxins were injected directly into the tumor and combined with intra-peritoneal injection of aCTLA-4. We found that combining aCTLA-4 with RG7787 or SS1P induced complete remission (CR) in 60% of the mice providing a significant survival benefit compared to mono-therapy (P<0.01). No tumor eradication was observed using aCTLA-4, RG7787, or SS1P alone. Furthermore, in mice reaching CR, new tumor challenge was rejected by all mice. In addition, the therapeutic effect of combining immunotoxins with aCTLA-4 was greatly diminished when CD8 depleting antibodies were administered concurrently (P<0.01), indicating that the anti-tumor effect is CD8 T cells dependent. To exclude the possibility that the anti-tumor effect is achieved due to recognition of a bacterial pattern in the PE, we combined aCTLA-4 with a mutant inactivated RG7787 and observed a significant reduction in mice survival compared to aCTLA-4 with the active RG7787 (P<0.01). This indicates that the anti-tumor activity relies on immunotoxin's cytotoxic effect. Altogether, an excellent antitumor effect was achieved by combining local immunotoxins with systemic aCTLA, overcoming resistance to each drug given alone. Our findings provide the first preclinical evidence to support use of this combination in clinic.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Liat Goldberg

Postdoctoral Fellow

Hematology/Oncology, Tumor Immunology, and Therapy

A novel mouse model recapitulating high risk acute early T cell leukemia

A high risk subgroup of T-cell acute lymphoblastic leukemia (ALL) patients has recently been described. The cell of origin for this high risk group is thought to be an early T-cell precursor (ETP), which retains the capability to differentiate into both myeloid and T-lineage cells. ETP ALL is characterized by lack of typical T-cell surface markers, aberrant expression of myeloid or hematopoietic stem cell (HSC) markers and a gene expression signature resembling that of murine ETP cells. ETP ALL patients also have a unique mutational landscape that includes acquired mutations in genes typically associated with myeloid differentiation. The ambiguous lineage attributes of ETP ALL may explain, at least in part, the ineffectiveness of current therapies, highlighting a need for pre-clinical models. Chromosomal translocations leading to generation of a NUP98-HOXD13 (NHD13) fusion are seen in a subset of MDS and AML patients, and NHD13 transgenic mice develop MDS, AML, and T-cell ALL. Mutations in IDH1/2 genes are associated with AML and T-cell ALL in humans, though, expression of a IDH2R140Q mutant did not lead to leukemic transformation in transgenic mice. However, we identified spontaneous IDH1 mutations in NHD13-driven AML, suggesting that these two aberrations may collaborate to cause leukemia. We therefore crossed IDH2R140Q mice with NHD13 mice and found that double transgenic

IDH2R140Q/NHD13 mice exhibit decreased survival and increased disease penetrance compared to all control groups. Detailed flow cytometry analysis revealed that most IDH2R140Q/NHD13 mice develop leukemias resembling ETP or double negative (DN) 2 cells. Analysis of the TCR β locus revealed that unlike NHD13 T-cell leukemias, IDH2R140Q/NHD13 leukemias did not harbor clonal VDJ rearrangements but rather clonal DJ rearrangements, implying that these leukemias emerged during an early stage in T cell development. Expression analysis revealed that the IDH2R140Q/NHD13 leukemia signature closely matched the signature of murine ETPs and human ETP ALL patients. Finally, exome sequencing showed that the mutational landscape of IDH2R140Q/NHD13 leukemias is similar to that of ETP ALL patients. These results demonstrate that IDH2R140Q/NHD13 leukemias resemble human ETP ALL in terms of gene expression, immunophenotype, and cooperative mutations. Therefore, we predict that IDH2R140Q/NHD13 mice will be an excellent pre-clinical model to study ETP ALL and evaluate potential therapies.

National Institute of Allergy and Infectious Diseases (NIAID)

Jason Yolitz

Doctoral Candidate

HIV and AIDS Research

Monoclonal antibody associated with protection from HIV infection (mAb CH58) recognizes non-mature envelope protein (ER-Env)

During HIV transmission, a genetic bottleneck occurs in which the diverse swarm of viruses in the donor contracts to one or very few transmitted/founder (T/F) viruses that establish a new infection. T/F isolates are believed to have genetic characteristics that provide them with increased transmission fitness. The best-characterized transmission signature involves a reduced number of N-linked glycosylation sites in the Envelope protein (Env), particularly gp120. A second signature involves an overrepresentation of basic amino acids in the signal peptide of T/F Envs. We recently determined that this second signature can impart a more high-mannose, less complex-carbohydrate glycan profile on the resulting Envs. These two genetic signatures suggest that post-translational processing of Env may impact the transmission fitness of T/F isolates. Therefore, we sought to characterize how altered post-translational processing can influence Env structure/function. To do this, we manipulated Env processing through the ER and Golgi by use of Brefeldin A (BreA), an inhibitor of transport between the ER and Golgi. BreA treatment of infected cells should increase the proportion of Env expressed on the surface of the cell that is processed in a Golgi-independent manner. Env processed in this way is called ER-Env for its lack of Golgi processing. Such Env should bear increased high-mannose glycans. Studies from the most efficacious HIV vaccine trial to date (RV144) show a correlation between mounting an antibody response to the V2 region of gp120 and efficacy. A non-neutralizing mAb termed CH58 may be linked to that efficacy. Therefore, we probed the surface of infected CD4+ T-cells cultured in the presence of BreA with CH58. CH58 recognizes one conformation of V2 while a second mAb, PG9, which is neutralizing, recognizes an alternative conformation. In the absence of BreA, both CH58, and PG9, recognize significant fractions of Env on the surface of infected cells. However, in the presence of BreA, CH58 reactivity is only slightly reduced while PG9 reactivity is significantly depleted. This data suggests that CH58, which may be associated with protection from HIV infection, recognizes ER-Env. This data supports the hypothesis that the mechanism of protection from a vaccine may not be through the

induction of classically defined neutralizing antibodies. This finding has major implications for the design of HIV vaccine immunogens that may provide protection from infection.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Sanath Kumar Janaka

Visiting Fellow

HIV and AIDS Research

Myxovirus Resistance Protein Mx2 Inhibits HIV-1 Replication by Aggregating Multiple HIV-1 Complexes in the Cytoplasm

The human immunodeficiency virus (HIV) genome is reverse transcribed in the cytoplasm of an infected cell in association with a viral capsid core and enters the nucleus at the nuclear envelope (NE). Mx2 is a recently identified interferon-inducible protein that restricts HIV-1 infection in a capsid-dependent manner in infected cells. Long and short isoforms of Mx2 are expressed from the same mRNA by translational initiation at the first and second initiation codons separated by 25 residues, respectively. The mechanisms by which Mx2 restricts HIV-1 have not been fully elucidated. To gain insights into Mx2 restriction, we employed fluorescence microscopy to study HIV-1 viral complexes in infected Mx2+ cells that were labeled with APOBEC3F tagged with YFP (A3F-YFP). A3F-YFP is incorporated into HIV-1 viral particles and remains associated with the viral genetic material throughout infection. First, HeLa cells stably expressing Mx2 with an N-terminal HA epitope tag were created by lentiviral transduction and shown to inhibit HIV-1 infection; in these cells, the long:short Mx2 isoform ratio was higher compared to primary CD4+ T cells. As previously reported, we found that Mx2-mediated inhibition of HIV-1 is dependent on cyclophilin A (CypA) binding to CA, and abrogated by treatment with cyclosporine A (CsA), an inhibitor of capsid-CypA interaction. Mx2- and Mx2+ cells were infected with A3F-YFP labeled HIV-1 virions, and colocalization of HIV-1 complexes with Mx2, NE association, and nuclear import were quantified. The NE association and nuclear import of HIV-1 complexes in the Mx2+ cells was inhibited. We also observed that Mx2 formed cytoplasmic puncta in infected cells that colocalized with HIV-1 complexes, while uninfected cells showed few if any Mx2 puncta. In Mx2+ cells, the YFP puncta were larger and brighter, suggesting that multiple HIV particles were present in individual Mx2 puncta. Cytoplasmic aggregation correlated with Mx2's antiviral activity as treatment of infected cells with CsA prevented their formation. We are determining how the Mx2 long:short isoform ratio affects its antiviral activity and formation of cytoplasmic aggregations with viral complexes. These results provide new insights into the antiviral activity of Mx2 and provide strong evidence that aggregation of HIV-1 complexes in the cytoplasm is a major mechanism by which Mx2 inhibits HIV-1 replication.

National Institute of Allergy and Infectious Diseases (NIAID)

Christina Guzzo

Visiting Fellow

HIV and AIDS Research

Critical Role of V2 Sulfotyrosines in Stabilizing the HIV-1 Envelope Trimer in Its Closed, Antibody-

Protected Conformation

Immune evasion is a hallmark of the HIV-1 envelope and represents a major barrier to vaccine development. We recently discovered that two conserved tyrosines (Y173, Y177) in the V2 loop of the gp120 envelope glycoprotein can be post-translationally modified by O-sulfation and functionally mimic the sulfotyrosines present in the N-terminal region of CCR5, stabilizing the intramolecular interaction between V2 and V3. To gain further insight into the functional role of the V2 sulfotyrosines, we examined the effects of tyrosine sulfation modulation and mutagenesis on HIV-1 neutralization sensitivity. Inhibition of tyrosine sulfation increased HIV-1 sensitivity to soluble CD4 and poorly/non-neutralizing mAbs; at the same time, neutralization by trimer-specific mAbs was reduced, suggesting that tyrosine sulfation contributes to stabilizing the closed trimer conformation. Reciprocal results were obtained upon enhancement of tyrosine sulfation. An even more dramatic effect was observed upon phenylalanine or alanine substitution of the V2 tyrosines, indicating that the tyrosine side-chains play a stabilizing role irrespective of their sulfation status. Strikingly, the V2 tyrosine mutants became highly susceptible to neutralization by HIV-1-infected patient sera, including those with weak/restricted neutralizing capacity, suggesting that the bulk of host-produced antibodies cannot reach through the tight protective shield of the native trimer. Altogether, these results document the key role played by the V2 tyrosines, particularly in their sulfated form, as a mechanism of HIV-1 immune evasion.

National Institute of Child Health and Human Development (NICHD)

Sang Yoon Park

Visiting Fellow

HIV and AIDS Research

Autophagy-related Gene 9A (ATG9A) is a Novel Nef-interacting Host-cell Protein that Promotes HIV-1 Infectivity

Nef is an accessory protein encoded in the HIV-1, HIV-2, and SIV genomes. Although Nef does not have enzymatic activity, it promotes viral dissemination and pathogenesis through interference with many host cell processes. Most importantly, Nef enhances the infectivity of HIV-1 up to 40 fold in a host-cell dependent manner. This enhancement is largely due to downregulation of two host-cell restriction factors, SERINC5 and SERINC3. However, at present it is not known if other host-cell factors are also involved in this process. In this abstract, I report the identification of the autophagy-related gene 9A (ATG9A) protein as a novel Nef interactor that contributes to Nef-dependent enhancement of HIV-1 infectivity. To identify Nef-interacting proteins, I performed tandem affinity purification followed by mass spectrometry of tagged Nefs from one HIV-1 strain and three SIV strains [chimpanzee (cpz), macaque (mac) and sooty mangabey (smm)] expressed in human cells. In addition to Nef, we identified the following numbers of co-purifying proteins: 72 for HIV-1, 17 for SIVcpz, 19 for SIVsmm, and 27 for SIVmac. Among these proteins, I confirmed the interaction of Nef with KIF3A, REEP5, SPTLC2, PAK3 and ATG9A using co-immunoprecipitation analysis. This latter protein is the only known multispanning membrane component of the autophagic machinery. Further analyses showed that this interaction was dependent on the N-terminal myristoylation and acidic cluster motif (62EEEE65) of Nef, and the N-terminal cytosolic tail of ATG9A. Despite several reports suggesting that Nef affects autophagy, we could not observe any alterations of the lipidation and degradation of the early autophagy marker LC3B by Nef overexpression. Instead, to investigate a possible role of ATG9 in HIV-1 biogenesis, we knocked out the

ATG9A gene in HeLa and Jurkat cells using CRISPR/Cas9, and examined the effects of this KO on viral particle release and viral infectivity. We found no significant difference in the number of viral particles released into the medium in WT and ATG9A-KO cells. However, HIV-1 produced in ATG9A-KO cells showed significantly reduced infectivity as compared to virus produced in WT cells (3-fold reduction in HeLa; 35-fold reduction in Jurkat). This reduction was almost similar to the reduction caused by deletion of the Nef gene in HIV-1. These results thus identify ATG9A as a novel host-cell factor involved in Nef-dependent enhancement of HIV-1 infectivity.

National Institute of Allergy and Infectious Diseases - Vaccine Research Center (NIAID-VRC)

Jason Hataye

Clinical Fellow

HIV and AIDS Research

Sustained HIV Release By Persisting CD4+ T Cells During Latency Disruption

Despite more than 3 decades of HIV research, the fundamental question of how much virus is released from an HIV-infected CD4+ T cell remains unanswered. In addition, while both instantaneous burst and multi-day sustained release theoretical models have been proposed, there is little experimental evidence to support one over the other. To directly observe the detailed dynamics of virus release, we performed HIV gag RNA RT-PCR to quantify HIV released from isolated primary CD4+ T cells undergoing latency disruption in ex vivo cultures in which new rounds of infection were blocked with a reverse transcriptase inhibitor. Culture supernatant sampling was performed daily followed by a virus washout to distinguish new release events from earlier releases. Twenty-three limiting dilution culture wells, each HIV RNA positive and with at least a 77% probability of having been seeded by a single virus-releasing cell, were analyzed for viral release. Half of these wells became positive within the first 3 days of culture. The accumulated virus for a well varied from less than 120 to 10500 HIV RNA copies. Although a low amplitude instantaneous burst pattern was frequently observed, 97% of detected HIV RNA was attributed to a higher amplitude virus release that was sustained for 2-6 days. The half-life of the HIV RNA signal in culture was determined to be 3 days. Applying these experimental results to a deterministic model consisting of a system of two ordinary differential equations describing the rate of change of infected cells and released virus as functions of time, we estimated an average total sustained release per seeded infected cell of 3900 HIV RNA copies. Our results are consistent with a multi-day sustained virus release model in which HIV-infected CD4+ T cells undergoing latency disruption survive and produce virus over several days without succumbing to viral cytopathic effects. Furthermore, this work provides new theoretical and experimental platforms on which to assess the effectiveness of interventions targeting infected cells that persist in HIV-infected individuals despite viral suppression with current anti-retroviral therapy.

National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS)

Keith Sikora

Clinical Fellow

Immunology - Autoimmune

Early-Onset Severe Arthritis Associated with a De Novo Gain-of-Function Germline Mutation in MYD88

Juvenile idiopathic arthritis (JIA) is a heterogeneous collection of diseases defined by the presence of arthritis of at least 6-weeks duration before 16 years of age. We evaluated a 9-year old female with progressively deforming polyarticular JIA and an intermittent erythematous rash since age 2. Synovial biopsy revealed a prominent neutrophilic infiltrate with scant mononuclear cells. We further evaluated the patient and family members by whole exome sequencing (WES), peripheral blood immunophenotyping, and functional studies of monocytes and dermal fibroblasts. WES revealed a de novo heterozygous missense mutation in MYD88 (c.666T>G, p.Ser222Arg or S222R), a critical adaptor protein of the innate immune system that connects Toll-like and IL-1 receptor signaling to activation of the IL-1 receptor-associated kinases (IRAKs), which was confirmed by Sanger sequencing and determined to be germline. Immunophenotyping showed an absence of CD16+ monocytes, an expansion of CD4+ Th17 T lymphocytes, and the presence of a previously uncharacterized CD123+CD11c+ dendritic cell population, as well as markedly increased basal and stimulated pSTAT3 (Y705) in monocytes and T and B cells in the patient. Monocytes exhibited an interferon gene expression signature and increased expression of neutrophil and monocyte chemokines, as well as increased IL-6, at baseline. Ex vivo whole blood spontaneously produced higher amounts of TNF- α , IL-8, IL-6, and IFN- γ compared to healthy controls. Fibroblasts exhibited ~10-fold greater baseline CXCL1, CXCL5, and IL-8 expression, while baseline patient protein secretion was significantly greater for IL-8 (11-fold), CXCL1 (16-fold), MCP-1 (7-fold), IL-6 (8-fold), and TNF- α (2.8-fold) compared to controls. To study the mutation in isolation, we generated MYD88-knockout THP-1 cells expressing wild type MYD88 or S222R-MYD88 GFP fusion proteins. S222R-MyD88 cells exhibited 2.1-fold greater NF- κ B reporter activity than WT-MyD88 cells at baseline, yet differences diminished upon increasing levels of stimulation with TLR2 agonist Pam3CSK4. Loss-of-function MYD88 mutations have been shown to result in immunodeficiency, and gain-of-function somatic mutations have been reported in B cell lymphoma. This is the first description of a de novo germline MYD88 gain-of-function mutation associated with severe arthritis and suggests the mutation causes a significant increase in innate immune activation through this critical adapter molecule.

National Institute of Allergy and Infectious Diseases (NIAID)

Michael Leney-Greene

Doctoral Candidate

Immunology - Autoimmune

Loss of function mutations in GIMAP5 lead to a novel Mendelian disease of autoimmunity

The incidence and prevalence of autoimmune diseases have increased in developed countries to the point of being among the top ten causes of death among young and middle aged women. The underlying causes of these diseases are poorly understood and thus prophylaxis and treatment options are often limited and ineffective. The use of whole-genome sequencing to study hereditary Mendelian autoimmune disorders shows great promise for delineating pathways involved in the development of autoimmune disease and identifying novel drug targets. To address this, we have identified a cohort of eight human patients from three pedigrees suffering from a novel Mendelian autoimmune disease characterized by lymphopenia, thrombocytopenia, autoantibodies and liver disease inherited in a

recessive manner. Whole-genome sequencing revealed mutations in a gene called GIMAP5 whose expression is primarily restricted to cells of the immune system. Murine models of GIMAP5 deficiency are phenotypically very similar to our human patients, and furthermore, polymorphisms in GIMAP5 have been linked to SLE and diabetes. Western blots reveal that the GIMAP5 mutations in our patients lead to a near complete loss of mature protein and defective protein folding has been confirmed via in vitro structural studies. Very little is known about the biochemical role of GIMAP5 at a cellular level, which is a crucial obstacle to exploiting this gene or the cellular processes it regulates as a therapeutic targets. As GIMAP5 localizes to the membrane of lysosome/late endosomes, we hypothesized that it may be involved in the trafficking or function of these organelles. Consistent with this hypothesis, electron microscopy images of activated lymphocytes from GIMAP5 deficient mice show a drastic accumulation of aberrant vesicular structures that are absent from wild type controls, and furthermore, decreased lysotracker staining intensity which suggests a failure to completely acidify the lysosomal compartment. Co-immunoprecipitation Mass spectrometry experiments revealed putative interactions with multiple Rab family members, consistent with a role for GIMAP5 in the regulation of vesicular trafficking. Our future work will focus on confirming interactions with various Rab family members and establishing confocal systems with which to study the vesicular trafficking pathway in GIMAP5 deficient lymphocytes.

National Institute of Allergy and Infectious Diseases (NIAID)

Mathilde Turfkruyer

Postdoctoral Fellow

Immunology - Autoimmune

Role of a TGF β R mutation in mast cells regulation and food allergy development

Loeys-Dietz syndrome (LDS) is an autosomal dominant aneurysmal syndrome caused by mutations in TGFBR1, which encodes one of the subunits of the TGF β receptor. LDS patients are more prone to developing nearly all forms of allergic disease, including food allergy. Here, we demonstrate that knock-in mice harboring LDS mutations known to cause severe disease in humans are more prone to developing anaphylaxis in a murine model of peanut allergy, recapitulating the human phenotype. Following peanut sensitization, LDS mice exhibited higher total and peanut-specific IgE levels, Th2 cytokine secretion following stimulation of mesenteric lymph node cells with peanut extract, and serum levels of mMCP1, a marker of mast cell degranulation, compared to their wild type (WT) littermates. Using this model, LDS mice also demonstrated greater accumulation of mast cells both in the gut and in the peritoneal cavity compared to WT mice. These data suggest that exaggerated anaphylactic responses in LDS mice may, at least in part, be due to enhanced mast cell frequency and/or function as a result of altered TGF β signaling. To further address this possibility, we differentiated mast cells in vitro from mast cell precursors in the peritoneal cavity and bone marrow by culturing them with IL-3 and stem cell factor (SCF), and then stimulated them with recombinant TGF β 1. LDS mast cells were more resistant to apoptosis and their proliferation was less suppressed in response to TGF β 1 compared to WT mast cells. Whereas WT mast cells decreased their expression of Fc ϵ RI, the high affinity receptor for IgE, and manifested reduced IgE-mediated degranulation after culture with TGF β 1, this was not seen in cultures of LDS mast cells. Finally, LDS mast cells express higher amounts of ST2, the receptor for IL-33, both before and after culture with TGF β 1. IL-33 is a key epithelial cytokine which is known to promote

Th2 immunity and is essential for the development of food allergy. Collectively, these data suggest that primary alterations in TGF β signaling are sufficient to promote Th2 immunity and food allergy, at least in part by altering mast cell responses.

National Institute of Allergy and Infectious Diseases (NIAID)

Kevin Hart

Postdoctoral Fellow Postdoctoral Fellow

Immunology - General

Distinct Roles for TGF-beta and Eosinophilic Type-2 Inflammation in NAFLD-associated Fibrosis

Worldwide incidence of obesity has attained epidemic proportions, putting an estimated 300-500 million people at risk for the myriad of health concerns termed the metabolic syndrome. This includes non-alcoholic fatty liver disease (NAFLD) and its pathophysiologic spectrum of non-alcoholic steatohepatitis (NASH) and cirrhosis, which has become the most common form of progressive liver disease in developed countries. Progression is marked by abnormal fat accumulation in hepatocytes (steatosis), immune cell infiltration, and eventual scarring, or fibrosis, of the liver. NAFLD appears to be intricately tied to chronic low-level type-1 inflammation originating in the adipose tissue, resulting in insulin resistance and altered hepatic lipid metabolism. However, the specific immunological mechanisms underlying progression to NASH and cirrhosis in the liver are poorly understood, with limited therapeutic options aside from transplant. To test the model that type 1 inflammation drives disease progression, we assessed mice deficient in type 2 regulatory cytokines IL-10 and IL-4 on a chronic high fat diet (HFD). Unexpectedly, the IL-10/4 double knockout animals were protected from liver disease, despite still being susceptible to obesity. RNA-sequencing analysis revealed a type-1 immune signature highlighted by interferon-gamma activity in liver tissue of the IL-10/4 knockouts. Consistent with this data, type-1 deficient interferon gamma (IFN-gamma) knockout mice on the HFD had increased steatosis and rapidly progressed to NASH with evidence of fibrosis. Unlike the switch to type-1 inflammation and loss of adipose resident eosinophils seen in expanding adipose tissue, we found that in both mouse and human disease, NAFLD progression was associated with mixed eosinophilic inflammation and altered regulation of the profibrotic mediators IL-13 and transforming growth factor beta (TGF- β). Antibody blockade of TGF-beta in chronic HFD or in IFN-gamma knockout mice attenuated expression of some fibrosis-associated extracellular matrix proteins including collagen, but increased markers of type-2 inflammation. These studies reveal a role for regulation of type-2 inflammation in controlling fibrosis. Thus, rather than driving metabolic disease as has been described in adipose tissue, our data reveal protective roles for some pro-inflammatory cytokines in NAFLD progression and demonstrate divergent roles for TGF-beta and type-2 immunity in NASH-associated fibrosis.

National Institute of Allergy and Infectious Diseases (NIAID)

Fanny Legrand

Postdoctoral Fellow Postdoctoral Fellow

Immunology - General

Targeting the inhibitory receptor siglec-8: a novel approach for the treatment of eosinophilic disorders

Hypereosinophilic syndromes (HES) are a heterogeneous group of disorders in which eosinophils (EOS) play a primary role in pathogenesis. Whereas conventional therapies are initially effective in a majority of patients, resistance and toxicity are common over time. Consequently, there has been increasing interest in novel therapies that specifically target EOS. Siglec-8 is an inhibitory surface receptor expressed on mature EOS, basophils and mast cells. Crosslinking of Siglec-8 on EOS in vitro causes apoptosis, that is enhanced when EOS are activated (a common feature of HES). Reduction of EOS in this way provides a theoretical advantage as a treatment approach since it is unlikely to lead to release of cytotoxic mediators. AK002 is a humanized afucosylated IgG1 antibody to Siglec-8 that induces apoptosis and enhances NK-mediated ADCC. The aim of the present study was to explore the in vitro efficacy of AK002 in targeting EOS from subjects with HES in anticipation of a phase I clinical trial. Siglec-8 expression was studied on EOS from HES and normal donors (ND) by flow cytometry (FCM). The efficacy of AK002 at inducing EOS death was assessed in vitro by FCM using 1) purified EOS only (primed 18 h +/-IL-5 then 18 h +/- AK002), 2) purified EOS and autologous NK cells (effector: target ratio=5:1) and 3) peripheral blood leucocytes (PBL). Siglec-8 was highly expressed on EOS from all donors (GM MFI 651, n=39). AK002 induced apoptosis of purified EOS after 48 h in the presence of IL-5 in 8/8 ND and 29/30 subjects with HES (GM Annexin V+ EOS 54% vs 12% for isotype control, $P < 0.0001$). Although IL-5 was required in ND, eosinophil apoptosis was observed in the absence of IL-5 in 9/15 HES subjects, consistent with in vivo eosinophil activation. In the presence of NK cells, AK002 induced significant EOS cell death at only 4 h (GM Annexin V+ EOS of 47.7% vs. 19.6%, $n=13$, $P < 0.001$, Wilcoxon matched pairs test). Finally, EOS depletion was assessed using PBL to determine the effect of AK002 without optimization of the E:T ratio. In this assay, EOS depletion was seen after 4 h only in donors with an NK:EO $> 0.25:1$, but was increased by addition of IL-5 (from 0.2% to 47%) ($P < 0.01$, $n=11$). To conclude, AK002 targeting of Siglec-8 induces eosinophil death by two complementary mechanisms, providing a novel therapeutic strategy for subjects with HES. Measurement of eosinophil mediator release and LDH as surrogates of eosinophil lysis (and potential toxicity) is ongoing.

National Institute of Allergy and Infectious Diseases (NIAID)

Rafael Prado

Postdoctoral Fellow Postdoctoral Fellow

Immunology - General

The L-arginine transporter Slc7a2 identified as critical regulator of asthma pathogenesis

Asthma is an inflammatory disease of the airway wall caused by type 2 cytokines, which is characterized by the overproduction of mucus and airway wall remodeling that leads to airway hyperreactivity (AHR) and airways obstruction. L-arginine is classified as a conditionally essential amino acid, with local competition for L-arginine between immune and non-immune cells critically regulating their function. Previous studies identified the L-arginine transporter Slc7a2 as an important regulator of cationic amino acid uptake by activated macrophage. Expression of Slc7a2 is also markedly upregulated in response to the type-2 cytokines IL-4 and IL-13. However, the role of Slc7a2 in the pathogenesis of asthma was unclear. Here, we show that during homeostasis, innate lymphoid cells and macrophages both express arginase in the lungs. However, following house-dust mite (HDM) allergen exposure, type-2 cytokine

mediated arginine metabolism is restricted to alveolar macrophages. We observed that in vivo and in vitro activation of macrophages by IL-4/IL-13 (M2) is impaired in the absence of Slc7a2. HDM exposed Slc7a2-deficient mice also exhibit increased L-arginine concentrations in plasma, less airway bronchoconstriction, and reduced pulmonary eosinophilia. Bone marrow chimera experiments revealed that amino acid transport in hematopoietic cells is required to drive AHR while expression of Slc7a2 in parenchymal tissues, likely fibroblasts, is required to mediate the recruitment of eosinophils and CD4+ Th2 cells to the lungs. Transfer of wild-type M2 cells, but not Slc7a-deficient cells, restored AHR in Slc7a2-deficient mice, confirming that among the hematopoietic compartment, macrophages function as the critical drivers of bronchoconstriction. Together, these data identify critical roles for Slc7a2 in the pathogenesis of asthma but reveal distinct and non-overlapping roles for macrophages and other non-immune cells in AHR and immune cell recruitment, respectively.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Thoma Ciucci

Postdoctoral Fellow Postdoctoral Fellow

Immunology - General

Memory fate is imprinted in CD4+ T-cells by a Thpok-dependent transcriptional program

Lymphocyte memory is the cornerstone of adaptive immunity and the basis for vaccination strategies. Memory CD4+ T-cells are critical for the generation and the maintenance of memory CD8+ T-cells and B-cells, both involved in long-lived immunity and vaccine protection. In experimental models, the pool of CD4+ memory T-cells decreases over time while memory CD8+ T-cells appear to be stably maintained suggesting that intrinsically CD4+ and CD8+ memory T-cell responses are maintained differently. Yet, what controls the maintenance of memory CD4+ T-cells in comparison of that of CD8+ memory T-cells has remained elusive. Our previous studies have shown that the zinc finger transcription factor Thpok is a key gatekeeper of post-thymic CD4+ differentiation. Based on these observations, and on the fact that Thpok is expressed in effector and memory CD4+ but not CD8+ T-cells, we hypothesized that Thpok expression could be responsible for the reduced half-life of the CD4+ memory pool, relative to its CD8+ counterparts. Here, combining well-defined infectious models and specific deletion of Thpok in CD4+ T-cells, we demonstrate that Thpok is not needed for the initial expansion of antigen-specific CD4+ T-cells. However, contrary to our expectations, we show that Thpok is necessary for memory CD4+ T-cells maintenance and functions. In fact, Thpok-deletion, at the time of the activation, results in a decrease of the memory CD4+ T-cells pool after primary infection. Moreover, the remaining cells have a reduced ability to proliferate and produce cytokines upon recall showing that Thpok also affects the function of memory T-cells. Strikingly, as a result of dysfunctional memory CD4+ T-cell response, Thpok-deficient animals are not able, compare to control animals, to clear the pathogen after viral rechallenge. Last, inducible-deletion models together with large-scale gene expression analyses demonstrate that Thpok limits the proliferation and terminal differentiation of effectors CD4+ T-cells while it promotes memory differentiation. These experiments demonstrate that Thpok, by imprinting a memory program in effectors T-cells, is a crucial transcription factor involved in the differentiation and function of memory CD4+ T-cells. Current research using single-cell gene analyses aims to identify the mechanism by which Thpok promotes the emergence of memory precursors at the effector phase.

National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS)

Shreya Bhattacharya

Postdoctoral Fellow

Immunology - General

Epithelial DLX3-dependent transcriptome regulates keratinocyte-leukocyte interaction to maintain skin immune homeostasis.

Immune response is a critical defense mechanism retained by coordinated release of pro- and anti-inflammatory cytokines by leukocytes. Extensive crosstalk between leukocytes and keratinocytes is required for maintenance of skin immune homeostasis and to prevent chronic inflammation. Chronic inflammatory skin disorders such as psoriasis and atopic dermatitis are associated with deregulated immune reaction, abnormal epidermal proliferation and differentiation. Dlx3, a homeobox transcription factor, is an essential regulator of epidermal differentiation. Its expression is down-regulated in psoriatic human skin and epidermal deletion of Dlx3 in murine skin induces an IL-17 linked psoriatic-like inflammation. Here we sought to identify the triggering signals from Dlx3-null keratinocytes responsible for the psoriatic-like inflammation and to comprehend the reciprocal signaling crosstalk between epidermis and dermis that maintain immune homeostasis. We performed acute deletion of Dlx3 in adult epidermal keratinocytes using a tamoxifen-inducible K14cre-ERT system (K14creERT;Dlx3f/f). FACS analysis was performed to characterize the inflammatory cells that sequentially infiltrate the skin at early (3day) and late stages (1 week and 2 week) after Dlx3 deletion. We also analyzed the signaling molecules and inflammatory cytokines that are differentially regulated in skin of K14creERT;Dlx3f/f using RNA-seq profiling. We observed dermal accumulations of macrophages and neutrophils within 3 days of tamoxifen-induced Dlx3 ablation, with enhanced infiltration starting one week after deletion. We also detected increased numbers of CD3+ T-cells in the dermis, specifically IL-17 secreting $\gamma\delta$ T-cells, after one and two week of Dlx3 deletion. RNA-seq analysis of early (3 day) and late (1 and 2 week) time points in Dlx3-ablated skin showed that Sonic hedgehog and Wnt/ β -catenin signaling pathways are altered initially, followed by cell cycle regulation impairment and subsequent production of pro-inflammatory cytokines IL-36, IL-6 and IL-12. Furthermore, two weeks after Dlx3 deletion there was a down-regulation of the anti-inflammatory cytokine IL-10 and up-regulation of pro-inflammatory IL-17 cytokine. Therefore we demonstrate that Dlx3 regulates skin immune homeostasis by modulating the balance of pro- and anti-inflammatory cytokines in the course of keratinocytes and leukocytes interplay.

National Institute of Allergy and Infectious Diseases (NIAID)

Ines Martin-Martin

Visiting Fellow

Immunology - Infectious Disease

Investigating the role of a salivary hyaluronidase from the sand fly Lutzomyia longipalpis as a vaccine candidate against leishmaniasis

Leishmaniasis is a parasitic vector-borne disease which affects two million people worldwide each year. There are evidences of acquirement of protective immunity after contact with the parasite, which

supports that vaccines against leishmaniasis may be possible. However, up to now there is no approved vaccine in humans. *Leishmania* spp. parasites are transmitted to the vertebrate host through the bite of infected sand flies. Insect salivary components are injected at the bite site facilitating blood meal acquisition and have been shown to enhance pathogen transmission. Therefore, identifying salivary proteins of different leishmaniasis vectors has become a major task in the field of anti-*Leishmania* vaccine development. We have investigated the physiological role of a salivary protein from the sand fly *Lutzomyia longipalpis* (hyaluronidase: LuloHYA) in blood-feeding and establishment of parasite infection in a mouse model. LuloHYA is an enzyme that degrades hyaluronic acid, a constituent of the extracellular matrix of vertebrates. It has been referred as a spreading factor since it increases tissue permeability for other salivary components that serve as antihaemostatic, vasodilatory or anti-inflammatory agents. LuloHYA was expressed in mammalian cells and biochemically characterized showing highest activity at pH 6 and low ion strength (50 nM NaCl). Rabbit-raised anti-LuloHYA antibodies completely abrogated hyaluronidase activity in vitro. Moreover, in vivo experiments demonstrated that blocking LuloHYA with specific antibodies interferes with sand fly blood-feeding. Concretely, only 21% of the sand flies attempted to feed on mice that had circulating anti-LuloHYA antibodies succeed in ingesting blood compared to 53% success in the control group (reduction of 60.38%, $p < 0.0001$). Vaccination studies were conducted with C57BL/6 strain and a homozygous mutant mice strain (B6.129S2-Ighmtm1Cgn/J) which lacks mature B cells. C57BL/6 animals immunized with LuloHYA controlled *Leishmania* major lesion development through the 8 weeks of measurements. This protective immunity was abrogated when B6.129S2-Ighmtm1Cgn/J mice were used for vaccination suggesting that anti-LuloHYA antibodies might play a role for protection against the parasite. This work highlights the relevance of vector salivary components in blood feeding and parasite transmission and further suggests the inclusion of this protein as a component for an anti-*Leishmania* vaccine.

National Human Genome Research Institute (NHGRI)

Tuoqi Wu

Visiting Fellow

Immunology - Infectious Disease

The transcription factor TCF1 is required for anti-viral CD4 and CD8 T cell responses during chronic viral infection

Effective T cell responses result in clearance of pathogens and generation of protective T cell memory. However, T cell exhaustion, characterized by poor effector function, reduced proliferative capacity, and loss of memory, is frequently observed during chronic viral infections, such as HIV. How virus-specific T cells persist in the presence of immunosuppression caused by chronic infections is still unclear. Data indicate that T follicular helper (T_{fh}) cells, which provide help to antibody producing B cells, dominate antiviral CD4 responses during chronic viremia and are instrumental for keeping chronic viral infections in check. I recently found that TCF1, a transcription factor that forms a negative feedback loop with IL2 and Blimp1, is required for antiviral T_{fh} responses during acute viral infection (Wu et al, Cell Reports, 2015). To evaluate whether TCF1 is important for responses to chronic viral infection, we challenged mice with lymphocytic choriomeningitis virus (LCMV) clone 13, which causes chronic viremia in mice. Similar to our previous observations in acute infection, we found that virus-specific CD4 T cells could be segregated into CXCR5+TCF1+Blimp1- T_{fh} cells and CXCR5-TCF1-Blimp1+ Th1 cells after LCMV clone 13

infection. Early after infection with clone 13, T-cell-specific TCF1 deficiency (cKO) led to a reduction in Tfh but not overall CD4 T cell responses. However, antiviral CD4 T cell responses were not maintained without TCF1, leading to a significant loss of virus-specific cKO CD4 T cells as viremia progressed. Similarly, antiviral CD8 T cell responses in TCF1 cKO mice were moderately reduced early after infection, but declined sharply over time. To evaluate whether these defects were cell-intrinsic, we made mixed bone marrow chimeras with WT and cKO donors and followed their antiviral responses. Our data indicate that both virus-specific CD4 and CD8 T cells intrinsically required TCF1 to persist during chronic viral infection. Intriguingly, virus-specific CD8 T cells could be separated into TCF1+Blimp1-Tim3- and TCF1-Blimp1+Tim3+ populations, and the Tim3- (TCF1+) cells persisted better than their Tim3+ counterparts during chronic infection. Moreover, TCF1 deficiency reduced the Tim3- population, while over-expression of TCF1 expanded it, indicating that TCF1 is crucial for these long-lasting Tim3- virus-specific CD8 T cells. Thus, TCF1 is critical to sustain both viral-specific CD4 and CD8 T cell responses to chronic viremia.

National Institute of Neurological Disorders and Stroke (NINDS)

Elliott Moseman

Postdoctoral Fellow

Immunology - Infectious Disease

Visualization of T cell behavior within the virally infected brain reveals indirect T cell mediated neuronal protection

The olfactory epithelium is a specialized structure containing layers of olfactory sensory neurons (OSNs). Because OSNs require contact with the outside environment in order to perform their chemosensory duties, they are acutely vulnerable to infection. In addition, because OSNs directly link the central nervous system (CNS) to the outside environment, they potentially undermine the primary protective mechanisms the CNS has evolved to protect itself, namely the blood-brain and blood-cerebrospinal fluid barriers. Neurotropic viruses, such as vesicular stomatitis virus (VSV), can readily infect OSNs. Little is known, however, about how local innate and adaptive immune responses prevent viral spread from the OSNs into the CNS. Following intranasal VSV infection, we observed immune responses in the olfactory epithelium where the OSN cell bodies reside, and in the olfactory bulb (OB), where their axons terminate in the glomeruli. After intranasal VSV infection both CD4+ and CD8+ T cell depleted animals are vulnerable to fatal VSV, associated with heightened viral escape from the glomeruli into distal brain regions. We sought to understand the T cells play in preventing viral escape beyond OSNs in the glomerular layer. By developing a technique to image the olfactory bulb in vivo via two-photon microscopy, we were able to visualize immune cell behavior within infected animals. VSV-specific CD8+ T cells move rapidly through infected axon tracks, yet T cell motility analysis revealed a decrease in viral-specific T cell velocity (indicative of antigen recognition) in the presence of infected OSNs. Studies of bone marrow chimeric animals indicated that hematopoietic antigen presentation is sufficient to prevent fatal neuroinvasion, as well as elicit T cell calcium signaling within the OB in vivo. In order to understand what protective signals T cells provide the brain, we generated a VSV expressing Cre recombinase. VSV-Cre infection of floxed IFNAR mice and floxed IFNgR revealed that continued IFNAR signaling is critical, whereas IFNgR expression is not required to clear VSV infected cells. In addition, IFNg and perforin-deficient mice are similarly resistant to VSV infection. Collectively, these data indicate that

antiviral T cells can control virus in infected neurons (likely in a noncytopathic manner) through interactions with hematopoietic cells.

National Institute of Allergy and Infectious Diseases (NIAID)

Lydia Roberts

Postdoctoral Fellow

Immunology - Infectious Disease

Antigen-specific, poly-functional CD4+ T cells are required for vaccine-mediated protection in tularemia

The virulent intracellular pathogen *Francisella tularensis* subsp. *tularensis* (Ftt) causes an acute, lethal disease called tularemia. Following inhalation of less than 50 organisms, bacteria replicate exponentially over the first 3 days of infection and kill the host within 4-5 days. This rapid course of infection is due to Ftt's ability to evade and suppress host innate immunity. Given the swift nature of disease progression, survival is dependent on the presence of an effective adaptive immune response. However, the nature of such a response has not been elucidated. Thus, we designed in vitro and in vivo models to characterize the protective immune responses with the goal of applying these features to novel vaccines. Utilizing unlicensed vaccine strains with varying efficacy, we found that vaccinated mice lacking CD4+ T cells succumbed rapidly to Ftt challenge, surviving only 1 day longer than naïve animals. In contrast, animals depleted of CD8+ T cells survived as long as 11 days after Ftt infection. These data indicated that vaccine-mediated protection requires a pool of CD4+ T cells capable of immediately controlling Ftt replication. Controlling Ftt replication in macrophages requires both IFN- γ and TNF- α . Indeed, effective vaccination correlated with the presence of poly-functional CD4+ T cells producing IFN- γ , TNF- α , and IL-2 and these purified pulmonary and splenic CD4+ T cells controlled Ftt replication in vitro. To follow and eventually manipulate the antigen-specific response, we generated vaccine and Ftt strains expressing the well-characterized epitope gp61 from LCMV. Immune animals had persistent numbers of gp61-specific CD4+ T cells in their lymph nodes and spleens, whereas vaccinated, but non-immune animals did not. Therefore, we hypothesized the expansion of high avidity, antigen specific CD4+ T cells would convert a poorly efficacious vaccine to one that engenders protection. As predicted, inclusion of the gp61 epitope in both the vaccinating strain and virulent Ftt challenge strain converted a vaccine that failed evoke adequate T cell responses to one that was 100% protective. Together our work has revealed successful vaccines directed against an aggressive, highly pathogenic organism requires a large pool of high avidity, poly-functional CD4+ T cells. Moreover, the elucidation of *Francisella* epitopes that elicit high-avidity CD4+ T cell responses, specifically in humans, will be required for successful vaccine development.

National Institute of Allergy and Infectious Diseases (NIAID)

Arielle Glatman Zaretsky

Postdoctoral Fellow

Immunology - Innate and Cell-mediated Host Defenses

Cutaneous IgA production contributes to microbial regulation

The microbiota is a critical regulator of the host immune system. In healthy individuals, the immune system maintains these organisms as nonpathogenic symbionts. However, microbiota-colonized sites are also barriers, with constant pathogen exposure, thereby requiring a balance between tolerance and protection. IgA is the major antibody class found at mucosal surfaces colonized by the microbiota, making it the most prevalent isotype in the body. In the gut, IgA differentially coats bacteria, providing an immunological distinction between pathogenic and commensal species. The skin is also a barrier and extensive commensal niche. Our laboratory has shown that skin-resident microbes promote immunity to pathogens via a commensal-specific skin-resident T cell population that enhances the immunological barrier. Although IgA is present in the skin, and IgA overproduction can lead to skin pathologies, little is known about its function or regulation at this site. We hypothesize that IgA contributes to the regulation of the microbiota of the skin, ultimately shaping immunity and control of pathogens. Initial studies reveal low levels of IgA⁺ B cells at distinct sites in the skin of naïve mice, as in human skin. Notably, commensal populations on mouse or human skin vary significantly depending on the area sampled. Thus, the observation that IgA⁺ B cells are only present at defined sites suggests that IgA production may shape local commensal populations or be uniquely elicited at certain sites. Microbial comparisons reveal that IgA-deficient mice have a skin microbiota similar to WT controls. However, when these mice are associated with a new commensal, *Staphylococcus epidermidis*, the lack of IgA is associated with a divergence in the cutaneous microbiota. Notably, upon *S. epidermidis* colonization, control mice exhibit an outgrowth of microbes from the class Clostridia, while IgA-deficient animals have an outgrowth of Bacilli, which includes *S. epidermidis*. These findings suggest that skin IgA can contribute to control of the microbiota after exposure to a new skin microbe and may limit over-colonization of defined microbial species. Further studies are underway to determine how IgA controls commensal colonization in the context of inflammation or infection. This will provide a new understanding of the humoral component of the skin-specific interplay between the microbiota and the host immune system, which could translate to improved treatment for skin diseases.

National Institute of Environmental Health Sciences (NIEHS)

Seddon Thomas

Postdoctoral Fellow Postdoctoral Fellow

Immunology - Innate and Cell-mediated Host Defenses

MYD88-dependent dendritic and epithelial cell crosstalk in the lung orchestrates immune responses to inhaled allergens

Allergic asthma is an inflammatory disease of the airway stemming from inappropriate immune responses to inhaled environmental allergens. Although asthma was previously regarded as a single disease, it is now seen as a heterogeneous set of diseases. Some forms of asthma are predominantly eosinophilic and steroid-responsive, whereas others are neutrophilic and steroid-resistant. Thus, there is an urgent need for new therapies that target specific types of asthma. MYD88, the adaptor molecule for TLR and IL-1 family member signaling, is required for allergic sensitization through the airway. It has been proposed that airway epithelial cells (AECs) communicate with lung dendritic cells (DCs), but the molecular signals involved remain poorly understood. To address these questions, we used mice bearing a 'floxed' version of the *Myd88* gene to generate animals lacking MYD88 in either AECs (AEC-KO), or in CD11c⁺ DCs (DC-KO). Following allergic sensitization and challenge, AEC-KO mice had significant

reductions in eosinophils compared to WT mice, whereas DC-KO mice had marked reductions in airway neutrophils. To better understand how MYD88 signaling in AECs and DCs affect immune responses in the airway, we analyzed RNA from AEC-KO and DC-KO mice post-sensitization. MYD88 signaling in AECs triggered immune response gene activation as early as 2 hours following sensitization, whereas CD11c-dependent MYD88 signaling directed delayed responses. Analysis of RNA from purified AECs and DCs revealed MYD88-dependent transcriptional crosstalk from AECs to DCs. Next, we employed ATAC-seq to study how cell-intrinsic and AEC-dependent MYD88 signaling affects chromatin structure in DCs. We identified a large number of loci in DCs where open chromatin near transcription start sites (TSS) was dependent on MYD88 signaling. In comparing WT and DC-KO DCs, a number of WT loci appeared “poised” for activation with open chromatin at both baseline and following sensitization as compared to DC-KO, while increases in WT gene transcription were detected only after allergic sensitization. A separate set of loci were identified in which open chromatin at TSS in DCs was dependent on MYD88 signaling in AECs. Thus, MYD88 signaling in AECs and DCs controls chromatin structure and gene transcription in distinct sets of genes, and is associated with specific forms of inflammation of the airway.

National Institute of Allergy and Infectious Diseases (NIAID)

Oliver Voss

Postdoctoral Fellow Postdoctoral Fellow

Immunology - Innate and Cell-mediated Host Defenses

LPS-recognition by CD300b regulates both TLR4/MyD88- and TLR4/TRIF-mediated signaling to exacerbate septic shock.

LPS, present in gram-negative bacteria membranes, causes strong immune responses following detection by TLR4 on immune cells. Excess immune cell activation resulting from severe infection initiates a pro-inflammatory cytokine storm that leads to a more severe immunopathology, like septic shock and, subsequently, death. Recently, the myeloid-specific Cd300b receptor was implicated in regulating the immune response to bacterial infection by an unknown mechanism. Here, we identified LPS as a ligand for Cd300b and found that wild-type (WT) mice, unlike Cd300b^{-/-} mice, were highly susceptible to endotoxemia and septic peritonitis. WT but not Cd300b^{-/-} mice demonstrate increased serum levels of pro-inflammatory cytokines (e.g. TNF α) and a reduced level of the anti-inflammatory cytokine, IL-10. Neutralization of IL-10 in Cd300b^{-/-} mice diminishes their survival advantage over WT animals. In vivo depletion and adoptive transfer studies identify CD300b-expressing macrophages (M ϕ) as the key cell type augmenting septic shock, suggesting that Cd300b amplifies the TLR4-LPS induced immune response thereby causing lethal inflammation. Indeed, CD300b and its adaptor, DAP12, associated with TLR4/CD14 upon LPS binding, promoting MyD88/TIRAP dissociation from the complex and the recruitment and activation of Syk and PI3K. This results in the activation of AKT, which subsequently leads to a reduced production of the IL-10 by M ϕ , via a PI3K/AKT-dependent inhibition of the MEK1/2-ERK1/2-NF κ B signaling pathway. In addition, CD300b also enhances TLR4/CD14-TRIF-IRF3 signaling responses, resulting in elevated IFN- β levels. In sum, these findings describe a previously unidentified LPS-induced signaling complex consisting of CD300b/DAP12/TLR4/CD14/Syk/PI3K that effectively amplifies TLR4-mediated inflammation. Furthermore, our data change the paradigm of how

LPS mediates TLR4 signaling in myeloid cells and identify potential targets for future clinical intervention.

National Institute of Allergy and Infectious Diseases (NIAID)

Seong-Ji Han

Research Fellow

Immunology - Innate and Cell-mediated Host Defenses

Immunity in the mesenteric adipose tissue

Each organ is surrounded by adipose tissue that serves as a scaffold for the lymphatic network that allows immune communication between tissues and lymphoid sites. However, to what extent the adipose tissue represents an immune compartment and/or directly contributes to immune protection remains unknown. The gut is a major barrier site, which in addition to its protective function against pathogens, is constantly exposed to commensal bacteria. The gut has developed a range of strategies to limit microbial exposure, including antimicrobial peptides, a mucus layer, an epithelial layer and various innate immune cells such as a specialized population of antigen presenting cells. Collectively, these different layers of defense have been referred to as the mucosal firewall. We hypothesized that the mesenteric adipose tissue (MAT) that connects the gut to the mesenteric lymph nodes directly contributes to protective immunity. We previously showed that infection with *Yersinia pseudotuberculosis* leads to a massive accumulation of innate immune cells and a permanent remodeling of the MAT. Our results also reveal that remodeling of the MAT can occur after most gastrointestinal infections. Analysis of the MAT under steady-state conditions revealed an accumulation of T cell subsets organized in fat associated lymphoid clusters (FALCs). Transcriptional analysis of MAT DCs showed a unique immunological and lineage signature compared to gut resident DCs. Deletion of defined DC lineages was associated with specific impairment in either Th2 or Th1-Th17 cell accumulation in the MAT. Further characterization of the MAT revealed that the majority of T cells express memory T cell markers. Analysis of surgically conjoined mice demonstrated that the majority of MAT-resident T cells are indeed resident memory T cells. Resident memory T cells are long-lived tissue-resident cells specific for a previously encountered pathogen. Infection with *Yersinia* showed a profound accumulation of *Yersinia*-specific memory T cells within the MAT. Collectively, these results suggest that the MAT provides a reservoir of rapid responders for subsequent intestinal infections. Our current work further assesses the contribution of T cells residing in the FALCs to long-term protective immunity against gastrointestinal infection. Together, our results support the adipose tissue as a previously unappreciated immune compartment and indicate that the MAT is an integral component of the mucosal firewall.

National Institute of Neurological Disorders and Stroke (NINDS)

Rejane Rua

Postdoctoral Fellow

Immunology - Innate and Cell-mediated Host Defenses

Alternatively activated brain-resident macrophages acquire and retain inflammatory properties

following CNS infection while interacting with effector and memory T cells

The meningeal and perivascular spaces of the central nervous system (CNS) are inhabited by specialized macrophages, but their homeostatic status and role in orchestrating the immune response against invading pathogens is not well understood. Examination of the naive brain by two-photon microscopy revealed that meningeal and perivascular macrophages are highly dynamic and constantly survey their immediate surroundings similar to microglia. Under steady state conditions, we uncovered that they are maintained in an alternatively activated state, which likely facilitates brain homeostasis. Interestingly, during the development of fatal meningitis induced by lymphocytic choriomeningitis virus, these cells were directly engaged by infiltrating virus-specific CD8⁺ T cells following acquisition of viral antigen and conversion into an inflammatory phenotype. Mechanistically, microarray analyses revealed that this transition relied on innate cytokine signaling and occurred in the absence of infiltrating inflammatory cells. Using a sub-lethal model of viral meningitis, we observed that despite elimination of previously infected cells, CNS macrophages remained activated for weeks after viral clearance, which was dependent on IFN- γ signaling and associated with tissue-resident memory T cell interactions. Collectively, these data indicate that brain-resident macrophages are highly plastic cells that can quickly participate in the antiviral defense against an invading pathogen and can also become imprinted with a prolonged activation program. The inflammatory properties and localization of these cells may explain why most CNS immune responses first develop in the meningeal and perivascular spaces.

National Institute of Allergy and Infectious Diseases (NIAID)

Michelle Sallin

Postdoctoral Fellow

Immunology - Lymphocyte Development and Activation

T-bet and IFN- γ have opposing effects on the generation of lung-homing Mycobacterium tuberculosis specific CD4 T cells

Approximately a third of the world's population is infected with Mycobacterium tuberculosis (Mtb), and each year there are ~1.5 million deaths due to tuberculosis. The only vaccine available, Bacillus Calmette-Guérin (BCG), provides limited efficacy in young children but no protection in adults, where most of the disease transmission occurs. The development of novel TB vaccination strategies would have dramatic global public health benefits, but have been hindered due to the incomplete understanding of the mechanisms of protective immunity against Mtb infection. It is clear that the Th1 response is required for host survival in Mtb infection. Utilizing an in vivo labeling technique we have previously shown that Mtb-specific CD4 T cells can be subdivided into parenchymal homing protective cells (T-bet^{int}/IFN γ ^{int}/CXCR3^{high}) and non-protective cells that accumulate in the lung vasculature (T-bet^{high}/IFN γ ^{int}/KLRG1^{high}/CXCR3^{high}). Based on the two subsets differential expression of T-bet and IFN γ , we examined the relative contribution of T-bet, and IFN γ in their differentiation. We find that Mtb infected T-bet^{-/-} mice display a robust Mtb-specific CD4 T cell response in the lung parenchyma, but a loss of the non-protective CD4 T cell subset in the lung vasculature. Furthermore, naïve polyclonal T-bet^{-/-} CD4 T cells adoptively transferred into infected WT mice showed similar loss of intravascular CD4 T cells, indicating the role for T-bet in the differentiation of intravascular CD4 T cells is T cell intrinsic. As expected we find that T-bet^{-/-} Mtb-specific CD4 T cells are deficient in IFN γ expression and produce IL-17A. Using T-bet^{-/-} mice that report T-bet

promoter activity with ZsGreen expression, we find that all of the ROR γ ⁺ Mtb-specific CD4 T cells are ZsGreen⁺, indicating that would be Th1 cells divert into the Th17 lineage. In contrast to T-bet^{-/-} mice, which showed a large increase in parenchymal T cells, IFN γ ^{-/-} mice showed a dramatic accumulation of terminally differentiated KLRG1^{high}/CX3CR1^{high} CD4 T cells in the lung vasculature. These data indicate T-bet is dispensable for CD4 T cell migration into the lungs of Mtb infected mice and promotes the generation of more terminally differentiated CD4 T cells which cannot enter the lung. At the same time, T-bet induces IFN γ expression, which provides a negative feedback signal preventing the accumulation of these non-protective CD4 T cells.

National Institute of Allergy and Infectious Diseases (NIAID)

Oliver Harrison

Visiting Fellow

Immunology - Lymphocyte Development and Activation

Local Foxp3⁺ Treg cells maintain lineage integrity of commensal-specific T cells in the skin.

The barrier surfaces of the body, including the skin, are colonized by unique communities of commensal micro-organisms, consisting of fungi, viruses and bacteria (microbiota). We recently demonstrated that exposure to skin commensal species drives unique cutaneous immune responses, which are key to local immune homeostasis and function. However, as inflammatory disorders of barrier tissues are likely the result of dysregulated responses to commensal microbes, it is key to understand how regulatory immune mechanisms control host-microbiota interactions. A key immune regulatory axis is Foxp3⁺ T regulatory (Treg) cells, a CD4⁺ T cell subset critical for prevention of pathological immune activation against self- and foreign antigens. We sought to determine the role of Foxp3⁺ Treg cells in commensal-specific T cell responses. To this end we generated a mouse model whereby Foxp3⁺ Treg cell function is selectively perturbed within the skin. Skin Foxp3⁺ Treg cells express uniquely high levels of transcription factor Gata3, and targeted deletion of Gata3 in Foxp3⁺ Treg cells resulted in selective impairment of Foxp3⁺ Treg cells within the skin. Loss of dermal Foxp3⁺ Treg cell function resulted in the aberrant accumulation of Th2 cells, eosinophils and basophils resulting in severe dermatitis. We assessed the role of local Foxp3⁺ Treg function in control of commensal specific T cell responses. Colonization of mice with commensal *S. epidermidis* leads to the recruitment of skin-resident IL-17A-producing CD8⁺ T cells in the absence of overt inflammation. By contrast, *S. epidermidis* colonization of mice with impaired dermal Foxp3⁺ Treg cells resulted in accumulation of IL-5 and IL-13-producing CD8⁺ T cells, which was not evident in control mice. Strikingly, aberrant production of IL-5 and IL-13 by dermal T cells is only observed in patients with severe skin disorders, such as atopic dermatitis. Importantly, these data demonstrate that local Foxp3⁺ Treg cells are key in maintaining the lineage integrity of commensal specific T cells within the skin. As such, these data suggest that perturbed regulatory responses to commensals may underlie clinical disorders of the skin. Investigating the mechanisms underlying maintenance and regulation of lineage-specification of commensal-specific T cells will aid our understanding of how to treat chronic inflammatory skin disorders.

National Human Genome Research Institute (NHGRI)

Bonnie Huang

Postdoctoral Fellow

Immunology - Lymphocyte Development and Activation

Using CRISPR-based mutagenesis to discover novel genes regulating T follicular helper cell development and function

T follicular helper (Tfh) cells are specialized CD4 helper T cells that signal B cells to produce high affinity antibodies and to become memory B cells. These processes are crucial for long-term protective immunity, and dysregulation of Tfh cells has been found in many human autoimmune diseases as well as chronic viral infections. Identifying the genes governing Tfh differentiation and function could provide targets for improving therapies, but the differentiation of T cells into Tfh cells instead of alternative specialized T cell subsets requires a large number of signaling cues, which are only partially understood. To discover novel Tfh-regulating genes, we sought to develop a functional genetic system to knock out genes directly in primary mouse T cells, and then measure the ability of these T cells to become Tfh cells in vivo. CRISPR is a powerful and versatile method for knocking out a gene of interest, by editing the gene sequence to introduce a premature stop codon and leading to complete loss of functional protein expression. To test whether CRISPR can knockout genes in T cells, we optimized a retroviral vector encoding Cas9 nuclease and a guide RNA sequence. We then transduced mouse T cells with this construct containing a guide RNA sequence against the gene Tcf7, which encodes Tcf1, a transcription factor that is important for Tfh differentiation. We observed up to 50% Tcf1-negative cells among transduced cells. PCR analysis of the genomic DNA from transduced Tcf1+ versus Tcf1- cells showed that only the latter had mutations in the targeted Tcf7 gene region, confirming that Tcf1 protein loss was specifically due to CRISPR activity. After control or Tcf7-edited T cells were adoptively transferred into wild-type mice and challenged with viral infection, the Tcf7-edited T cells were significantly impaired in Tfh differentiation compared to control T cells. These data validated that CRISPR-mediated gene knockout in primary T cells effectively modulates Tfh differentiation in vivo, and allows us to begin evaluating novel candidates from gene expression studies comparing Tfh to non-Tfh T cells in this viral infection model. Given the importance of cell-cell contact during Tfh help of B cells, we plan to screen adhesion and cytoskeleton genes selectively expressed by Tfh cells, in order to accelerate the search for novel targets that specifically modulate Tfh-B cell interactions in vivo without disrupting the function of other T helper cell subsets.

National Institute of Allergy and Infectious Diseases (NIAID)

Munir Akkaya

Visiting Fellow

Immunology - Lymphocyte Development and Activation

Toll-like receptor 9 signaling antagonizes the B cell receptor-dependent ability of B cells to process and present antigen to helper T cells.

B cells express both an adaptive antigen-specific B cell receptor (BCR) and innate Toll-like receptors (TLRs) allowing the functional outcome of the B cell's engagement with antigen to be modulated in response to pathogen-derived TLR ligands. In T cell-dependent antibody (Ab) responses, the BCR both signals for B cell proliferation and differentiation and also internalizes bound antigen for processing and

presentation to helper T cells (TH cells). Key events in T cell-dependent Ab responses in vivo are dependent on B cell presentation to TH cells. Ab responses are initiated in secondary lymphoid organs by the interaction of antigen-primed TH cells with activated antigen-specific B cells through MHC-class II peptide complexes presented on the B cell surface. Dependent, in part, on the quality of the B cell- TH cells interaction, B cells either enter germinal centers (GCs) or differentiate into either short-lived plasma cells (PCs) or GC-independent memory B cells (MBCs). Within the GC, B cells proliferate, somatically hypermutate and subsequently undergo antigen-dependent affinity selection. Selection is dependent on the ability of B cells to capture, process and present antigen to TH cells an event that ultimately results in the differentiation of GC B cells to long-lived MBCs and PCs. Here, using transgenic and knock out mouse models, I show that although the TLR9 ligand, CpG, does not affect early antigen-driven BCR signaling, CpG alters the outcome of BCR signaling, resulting in a unique transcriptional profile, enhanced proliferation and differentiation to PCs. Remarkably, CpG dramatically limits the ability of B cells to process and present antigen to TH cells. In the presence of CpG, BCR-induced upregulation of the expression of CD86 and MHC class II is antagonized and antigen internalized by the BCR is not properly trafficked to antigen processing compartments resulting in reduced numbers of peptide-MHC class II complexes on the B cell surface. Indeed, CpG treated antigen-specific B cells show a reduced ability to maintain contact with antigen-specific T cells and to activate antigen-specific helper T cell proliferation in vitro. These results indicate that TLR9 activation of B cells in T cell-dependent Ab responses would drive B cell toward proliferation and antibody secretion and away from events that are highly dependent on the ability of B cells to present antigen to helper T cells and produce long-lived MBCs and PCs.

National Institute of Environmental Health Sciences (NIEHS)

Derek Cain

Postdoctoral Fellow Postdoctoral Fellow

Immunology - Lymphocyte Development and Activation

Intrinsic Glucocorticoid Receptor Signaling Regulates B cell Recirculation through Bone Marrow by Modulation of CXCR4

Glucocorticoids exert potent regulatory effects on immune responses. However, the ubiquitous expression of glucocorticoid receptors (GRs), combined with the pleiotropic downstream effects of GR signaling, have obscured the characterization of glucocorticoid actions on individual cell types in vivo. Considerable effort has been dedicated to unraveling the direct effects of glucocorticoids on T cells, macrophages, and dendritic cells, yet little is known of intrinsic GR signaling on B cell biology. B cells express GRs throughout development and are sensitive to glucocorticoid-induced death. In mouse models of glucocorticoid perturbation, we found that glucocorticoid-mediated changes in B-cell populations could be explained not only by altered cell survival, but also by redirected tissue migration. To determine the intrinsic effects of GR signaling on B cells, we generated and characterized a novel B-cell specific GR knockout (B cell-GRKO) mouse. B-cell development was normal in B cell-GRKO mice, as were B-cell numbers in secondary lymphoid tissues. However, mature B cells were abnormally distributed, with reduced numbers of mature B cells in bone marrow and more in the circulation. Gene expression analyses revealed the chemokine receptor CXCR4 as a target of GR signaling in B cells, and GR-deficient B cells were impaired in migratory responses to the CXCR4 ligand CXCL12 ex vivo. GR-

deficient B cells were also less competent than control B cells in homing to bone marrow in vivo, whereas their migration to secondary lymphoid tissues was normal. Moreover, in B cell-GRKO mice, circadian fluctuations in circulating B cell counts were abolished, indicating that B-cell exchange between bone marrow and blood is coupled to diurnal patterns of glucocorticoid secretion. B-cell specific deletion of GR did not affect humoral responses to immunizations with T-dependent or T-independent (Type I) antigens; however, antigen-specific responses to a T-independent (Type II) antigen were significantly impaired. We conclude that glucocorticoids promote the trafficking of recirculating B cells through bone marrow via GR-mediated regulation of CXCR4. In contrast to the general view that glucocorticoids suppress adaptive immunity, our findings reveal a crucial role for endogenous glucocorticoids in promoting “innate” humoral responses by maintaining a pool of B cells in bone marrow capable of responding to multivalent antigens.

National Institute of Environmental Health Sciences (NIEHS)

YuanYuan Li

Research Fellow

Research Fellow
Informatics/Computational Biology

A comprehensive genomic pan-cancer analysis using The Cancer Genome Atlas gene expression data

The Cancer Genome Atlas (TCGA) has made available comprehensive molecular profiles including gene expression for many human tumor types and provided a great opportunity to use those data to identify features that can classify tumor types. Because gender differences in cancer susceptibility are one of the most consistent findings in cancer epidemiology, knowing whether the distinguishing features differ between males and females for the same tumor types might enhance their utility as biomarkers. Our goals are: to identify a set of genes whose expression levels can classify pan-cancer tumor types when gender is ignored; and to identify analogous sets of genes in sex non-specific tumors from men and from women separately. We analyzed RNA-seq data for 9096 TCGA tumor samples from 31 types using the GA/KNN algorithm we developed. We randomly assigned half of all samples into a training set and half into a test set, proportionally allocating samples from each tumor type and used the training set to obtain 2000 near-optimal classifiers (each set consisting of 20 genes) from repeated runs. We subsequently applied each resultant near-optimal classifier to the test set and compared the predicted class with the true class to calculate training and testing performances. To see if sex non-specific tumors (not related to reproductive organs) differ between males and females, we repeated the analysis for 24 tumor types separately for males and for females. In all analyses, we could accurately classify ~89% training and ~88% testing samples. The top 20 genes that separated the 31 tumor types were TCF21, TBX5, EMX20S, EMX2, PA2G4P4, HNF1B, NACA2, SFTPA1, ATP5EP2, PTTG3P, FTHL3, ANXA2P3, GATA3, NAPSA, SFTA3, HSPB1P1, HOXA9, IGBP1P1, RPL19P12, and SFTPB. Of the 100 top-ranked genes, 74 were common between males and females. A few genes showed considerable difference in ranking between genders. For example, BNC1 ranked 36 in males but 461 in females. Literature showed that BNC1 was hypermethylated in female compared to male hepatocellular carcinoma. It remains unclear if the top genes played any role in differential cancer susceptibility between genders. In conclusion, we were able to accurately classify ~88% of the tumor samples in all analyses. The performances are remarkable given the number of the tumor types (24 and 31) involved. We were also identified a few genes that might play a role in gender differences among sex non-specific tumors.

National Library of Medicine (NLM)

Chung-Chi Huang

Visiting Fellow

Informatics/Computational Biology

Discovering Biomedical Semantic Relations in PubMed Queries for Database Curation and Literature Retrieval

Identifying relevant papers from the literature is a common task in biocuration. Existing biomedical literature search systems typically rely on matching user keywords. Semantic search, on the other hand, seeks to improve search accuracy by understanding the entities and contextual relations in user keywords. With past research mostly focusing on semantically identifying biological entities (e.g. chemicals, diseases, and genes), in this work, we aim to discover biomedical semantic relations in PubMed queries in an automated and unsupervised fashion. Specifically, we focus on identifying semantically similar contextual information (or context patterns) that PubMed users use to represent semantic relations between entities such as "CHEMICAL-1 compared to CHEMICAL-2" and "CHEMICAL-1 versus CHEMICAL-2," and "CHEMICAL induced DISEASE" and "DISEASE due to CHEMICAL." With the advances in automatic named entity recognition, we first tag bio-entities in PubMed queries and then use tagged entities as knowledge to distinguish pattern meanings. More specifically, we estimate patterns' semantic similarity by their participating entities. To avoid data sparseness and specificity issues, entities are further projected to latent topics via latent semantic analysis (LSA) and semantically similar contextual patterns or semantic relations are mined based on their LSA topic distributions. To the best of our knowledge, little work like ours mines similar patterns without the help of manual data and human intervention (thus unsupervised approach). Our two separate evaluations of chemical-chemical (CC) and chemical-disease (CD) relations show that the proposed approach significantly outperforms a baseline, which measures pattern semantic similarity simply by participating entities. When compared against the ground truth, our approach achieves nDCG scores nearly as high as 0.9 and 0.85 respectively for the CC and CD task where nDCG is a standard measure of ranking quality. These results suggest that our approach can effectively identify and return related semantic patterns in a ranked order. To assess the potential utility of our similar patterns in semantic search, we perform a pilot study on 12 frequently sought semantic relations in PubMed involving 49 CC and CD patterns and observe improved PubMed search results based on post-hoc evaluation, implying our patterns, complementary to MeSH, benefit PubMed literature search. Further investigation in larger tests is warranted.

National Cancer Institute - Division of Cancer Epidemiology and Genetics (NCI-DCEG)

Xing Hua

Postdoctoral Fellow

Informatics/Computational Biology

MEGSA: A powerful and flexible framework for analyzing mutual exclusivity of tumor mutations

Cancers, driven by somatic mutations, cause over eight million deaths worldwide each year. Recent technical advances in next generation sequencing and bioinformatic analyses have greatly advanced the

characterization of tumor genomes. The central challenge in tumor sequencing studies is to identify driver genes and pathways, investigate their functional relationships and nominate drug targets. The efficiency of these analyses, particularly for infrequently mutated genes, is compromised when patients carry different combinations of driver mutations. Mutual exclusivity analysis helps address these challenges. To identify mutually exclusive gene sets (MEGS), we developed a powerful and flexible analytic framework based on a likelihood ratio test and a model selection procedure. Extensive simulations demonstrated that our method substantially improved existing methods for controlling type-I error rate, the statistical power and the capability of identifying the exact MEGS, particularly for highly imbalanced MEGS. We have implemented our algorithms in a publically available, platform-independent package MEGSA. MEGSA can be used for de novo discovery, pathway-guided searches or for expanding established small MEGS and thus is expected to help “understand” the big data produced from large-scale tumor sequencing studies. MEGSA has been extensively used in our division to analyze sequencing data of different cancer types, including lung adenocarcinoma and gastric cancer. We applied MEGSA to the whole exome sequencing data for fourteen cancer types from The Cancer Genome Atlas (TCGA). We identified novel MEGS in multiple cancer types. For acute myeloid leukemia, we identified a novel MEGS with five genes (FLT3, IDH2, NRAS, KIT and TP53) and a MEGS (NPM1, TP53 and RUX1) whose mutation status was strongly associated with survival ($P=6.7 \times 10^{-4}$). For breast cancer, we identified a significant MEGS consisting of TP53 and four infrequently mutated genes (ARID1A, AKT1, MED23 and TBL1XR1), providing support for their role as cancer drivers. These results demonstrated the usefulness of MEGSA for identifying mutually exclusive patterns and for nominating driver genes.

National Library of Medicine (NLM)

The Phuong Dao

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Elucidating Sequence-Structure Binding Motifs by Uncovering Selection Trends in HT-SELEX Experiments

Aptamers, short synthetic RNA/DNA molecules binding specific targets with high affinity and specificity, are utilized in an increasing spectrum of bio-medical applications. Aptamers are identified in vitro via the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) protocol. SELEX selects binders through an iterative process that, starting from a pool of random ssDNA/RNA sequences, amplifies target-affine species through a series of selection cycles. HT-SELEX, which combines SELEX with high throughput sequencing, is capable of generating over half a billion data points, challenging computational scientists with the task of identifying aptamer properties such as sequence-structure motifs that determine binding. None of currently available motif finding approaches possess the scalability required for HT-SELEX data, and taking advantage of important properties of the experimental procedure. We present AptaTRACE, a novel approach for the identification of sequence-structure binding motifs for massive amount of sequence data produced by HT-SELEX experiment. Our approach leverages the experimental design of the SELEX protocol and identifies sequence-structure motifs that show a signature of selection towards a preferred structure. In the initial pool, secondary structural contexts i.e. tendency of residing in a hairpin, bulge loop, inner loop, multiple loop, dangling end or being paired of each k-mer are distributed according to a background distribution. For sequence motifs involved in binding, in later selection cycles, this distribution shifts towards the structural context

avored by the binding interaction with the target site. Utilizing a relative entropy based scoring function, AptaTRACE is able to identify the motifs that converge to a specific structural context throughout the selection cycles of HT-SELEX experiments. We show our results of applying AptaTRACE to simulated data and high-throughput in vitro selection of 9 rounds of a cell-SELEX experiment. While most of motif finding methods can not handle the massive amount of cell-SELEX data, AptaTRACE takes several hours to finish. AptaTRACE also outperforms others in terms of sensitivity since it can uncover the motifs even when they are present only in a small fraction of the pool. Moreover, our method can also help to reduce the number of selection cycles required to produce aptamers with the desired properties, thus reducing cost and time of this rather expensive procedure.

National Human Genome Research Institute (NHGRI)

Stephen Bond

Postdoctoral Fellow

Informatics/Computational Biology

Recursive Dynamic Markov Clustering for Automated Orthogroup Classification

Inferred orthology (i.e., homology via speciation) among genes is commonly used to predict gene product function. Orthology is also a key consideration when classifying genes coherently and consistently across taxa, but the granularity of many popular prediction tools is too coarse to properly resolve clusters of orthologs (i.e., orthogroups) within specific gene families. As a result, final classification is often at the discretion of individual curators manually inspecting gene trees. Here, we present a new effort to fully automate the classification of orthogroups from a set of homologous sequences. For this study, we have extended a popular method for identifying natural clusters in all-by-all similarity graphs, called Markov clustering (MCL). Current MCL-based ortholog clustering tools rely on the BLASTP local alignment algorithm to create similarity metrics between sequences, but BLASTP discards information when sequences are very similar or very dissimilar. We have found that this limits the ability of MCL to resolve orthogroups within a gene family. However, we know that the sequences under study are homologous, so we switched to global alignment methods (e.g., Needleman-Wunsch) that generate more information-rich metrics. Another key weakness with current MCL-based approaches is a dependence on user-specified parameters that alter the size and composition of the final groupings. It is impossible to know the correct parameters for a dataset beforehand, so default values are conventionally used. We have shown this to be highly sub-optimal, so we implemented a novel scoring system for dynamic optimization of MCL parameters. A third significant weakness with MCL is an assumption of homogeneous rates of evolution among groups. This is generally an unrealistic assumption and we have now shown that recursively decomposing predicted orthogroups with further rounds of dynamic MCL, using our custom scoring system to identify subgroups, dramatically improves the final orthogroup prediction. In place of conventional experimental controls, new phylogenetic methods are validated on a combination of simulated and pre-curated datasets. Here, we have included simulated data from a range of evolutionary models and selected sample protein families with distinct biochemical properties. These controls illustrate how our new method, named Recursive Dynamic MCL, substantially improves the resolving power and accuracy of orthogroup clustering with MCL.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Vijay Walia

Research Fellow

Intracellular Trafficking

LPA-PI3K-Akt Signaling Network Regulates Primary Cilium Assembly by stabilizing Rab11-WDR44 interaction

A growing list of genetic diseases is associated with defects in primary cilium assembly and signaling. G0/G1-arrest, deprivation of serum factors or cell-confluency stimulates primary cilia assembly in many mammalian cells. However, the mechanism by which serum factor(s) block ciliogenesis is not known beyond the obvious links to cell cycle control. We previously showed that Rabin8, a Rab8 GEF, gets recruited to vesicular Rab11 membranes within minutes of serum removal to activate Rab8 for ciliary membrane growth in RPE-1 cells, marking the earliest observable step in ciliogenesis. To understand how serum signaling factors regulate this process, we screened individual growth factors and discovered that lysophosphatidic acid (LPA) inhibits Rabin8 trafficking and ciliogenesis. Furthermore, we show that G-protein coupled LPA receptor 1 (LPAR1), but not LPAR2-5, activation by LPA inhibits ciliogenesis. A chemical inhibitor screen of pathways downstream of LPAR1 identified PI3K/Akt as a negative regulator of ciliogenesis initiation. To determine if this pathway is a global regulator of ciliogenesis, we tested 57 adherent cell lines from NCI-60 panel. Among 23 of 57 (40%) cell lines that ciliate, 61% were responsive to LPAR1/PI3K/Akt inhibition. We theorized that Rabin8 interaction with Rab11-membrane vesicles is regulated directly by Akt phosphorylation of Rab11 binding protein (Rab11 effector). Bioinformatics and proteomics analysis suggested that Rab11-effector proteins are Akt substrate and could function in a Rab11-effector switch with Rabin8 to initiate ciliogenesis. To test this theory, we depleted these proteins and analyzed Rabin8 pre-ciliary trafficking in non-ciliating conditions (+ serum). We observed a significant increase in Rabin8 trafficking following depletion of WDR44/Rabphilin-11. WDR44 was the first reported Rab11 interacting protein but its Rab11 associated function is not known. We discovered that Akt phosphorylates WDR44 at Serine-342 in the Rab11 binding domain, and a phospho-inactive protein showed reduced binding to Rab11. Expression of the phospho-active WDR44 S342D mutant, but not S342A, strongly blocked ciliogenesis in RPE-1 cells and zebrafish embryos. Together these findings uncover a novel growth factor signaling pathway mediated by PI3K/Akt kinase and its novel downstream substrate, WDR44, in the regulation of ciliogenesis initiation via modulating Rab11-Rabin8 interaction and pre-ciliary vesicle trafficking to the centrosome.

National Institute of Child Health and Human Development (NICHD)

Maria Bagh

Research Fellow

Intracellular Trafficking

Unraveling lysosomal acidification defect in a lysosomal storage disorder model

Abstract removed at request of the author

National Institute of Environmental Health Sciences (NIEHS)

Shuang Tang

Visiting Fellow

Metabolomics/Proteomics

Metabolic and epigenetic regulation of mouse embryonic stem cell maintenance and embryogenesis by SIRT1

Abstract removed at request of author

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Christopher Rice

Visiting Fellow

Metabolomics/Proteomics

Immature C-kit+ neutrophils possess greater mitochondrial function which supports their suppressive function and hinders the anti-tumor immune response

Neutrophils are generally considered to be a homogenous population, however in tumor bearing mice neutrophils are heterogeneous and include an immature subset termed granulocytic- myeloid derived suppressor cells (g-MDSC), which play a major role in the tumor progression and the establishment of the metastatic niche. In this role they are commonly thought to work by suppressing T-cell proliferation and function through the generation of reactive oxygen species (ROS). The stem cell factor (SCF) receptor c-kit has previously been identified as a marker for immature neutrophil populations in cancer. Furthermore SCF/c-kit signaling at the tumor site has previously been implicated in the generation of a suppressive MDSC phenotype. Mouse bone marrow contains a significant subset of immature neutrophils that express c-kit. Our metabolic analysis show that c-kit expression by neutrophils correlates with a greater spare respiratory capacity, higher OCR/ECAR ratios, increased mitochondrial mass and increased mitochondrial DNA. Accordingly we find that c-kit+ neutrophils also possess increased expression of the master regulator of mitochondrial biogenesis, PGC1a, consistent with a known role for SCF/c-kit signaling in the generation of mitochondria. We find that in healthy mice, peripheral c-kit+ neutrophil represent a marginal population, however in a murine model of breast cancer this population is greatly expanded at the tumor site. We hypothesize that in the bone marrow this maintenance of mitochondria promotes longevity prior to maturation and release to the periphery. Whereas in the tumor site c-kit+ neutrophils utilize their mitochondria to produce ROS from a more diverse set of fuels, thus allowing them to suppress T-cell function in the glucose depleted tumor micro-environment (TME). Maintenance of mitochondrial mass and function in neutrophils at the tumor site may therefore promote their T-cell inhibiting properties as well as increasing their lifespan. This study supports recent findings that demonstrate tumor-associated MDSC are more oxidative than their circulating counterparts. Furthermore our studies show modulation of SCF/c-kit signaling may modulate neutrophil metabolism during cancer and could therefore pose an attractive approach for therapeutic intervention where significant neutrophil populations are seen as a poor prognostic marker.

National Institute of Allergy and Infectious Diseases (NIAID)

Casey Daniels

Postdoctoral Fellow Postdoctoral Fellow

Metabolomics/Proteomics

ADP-ribosylation in the innate immune response

ADP-ribosylation is a post-translational modification (PTM) important for DNA repair and inflammatory signaling. While inhibition of poly(ADP-ribose) polymerases (PARPs) – the enzymes responsible for ADP-ribosylation – is known to protect animal models from sepsis, the molecular mechanisms behind this response are unknown. In order to investigate the role of this PTM in the innate immune response, we have characterized ADP-ribosylation in macrophages, both before and after activation by lipopolysaccharide (LPS) – a molecule found on gram negative bacteria. The macrophage ADP-ribosylated proteome changes in response to LPS treatment, which we have shown by both Western blot and mass spectrometry; this dataset represents the first draft of the macrophage ADP-ribosylated proteome. The mass spectrometry results were acquired through application of our recently described method wherein ADP-ribose is cleaved down to its phosphoribose attachment site and then phosphoenriched – a pipeline which delivers both the phosphoproteome and the ADP-ribosylated proteome. Furthermore, we have shown by both fluorescence microscopy and Cytometric Bead Array technology that NFkB and the cytokines associated with its activation are altered in response to PARP inhibition, an effect which is dramatized by macrophage activation with LPS. Finally, we have shown that TLR4 is ADP-ribosylated on its TIR domain, an otherwise unknown modification on an important regulatory domain for all TLR signaling. The presence of this modification site in light of the importance of TLR4 signaling in sepsis pathogenesis, and the protective effect of PARP inhibition against sepsis, suggests that ADP-ribosylation may be a major player in TLR4 signaling. Our dataset represents the first proteome-wide assessment of both human and mouse macrophage ADP-ribosylation, and implicates PARP biology in the early innate immune response.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Arti Tripathi

Postdoctoral Fellow Postdoctoral Fellow

Microbiology and Antimicrobials

Structural basis of anti-adaptor mediated regulation of proteolysis

Regulated proteolysis of proteins in all organisms plays a very crucial role during growth, development, and stress. RpoS is an RNA polymerase sigma factor, required for activation of stress specific genes in multiple bacteria. The energy-dependent ClpXP proteolysis machinery, in collaboration with RssB, an adaptor, tightly regulates the amount of RpoS. Various stress-specific anti-adaptor proteins regulate the RssB-mediated proteolysis of RpoS. Each anti-adaptor protein controls the activity of RssB through direct binding. Here we report structural and functional characterization of IraP, an anti-adaptor induced in response to phosphate starvation. The crystal structure of IraP at 2.35-Å resolution was determined. The structure shows IraP is a dimer in which the N-terminal halves form a parallel helix-helix pair, similar to a leucine zipper. No electron density was observed beyond Gln42 of chain A and beyond Met40 of chain

B, indicating that the C-terminal halves are disordered. By structure-guided mutational studies, we identify two groups of residues in IraP; one is important for direct interaction with RssB, whereas the other is required for IraP dimerization. Mutations in the first region, leading to loss of IraP interaction with the adaptor, decreased the ability of IraP to stabilize RpoS. Loss of dimerization had modest effects on IraP activity, suggesting that dimerization is not essential for IraP activity. Mutations of residues in the C-terminal halves did not have much effect on binding of IraP with the adaptor, but did affect IraP dimerization, indicating that the flexible C-terminal halves help in maintaining the overall structure of IraP. Our data is consistent with a model in which IraP stabilizes RpoS by direct binding to RssB and hence sequestering RssB from the RssB-RpoS complex. Our study helps in understanding the stress response in microorganisms and suggests the possibility of targeting pathogenic bacteria by designing a factor/or molecule that disturbs regulated proteolysis and reduces the fitness of pathogens.

National Institute of Child Health and Human Development (NICHD)

Eric Cheng

Postdoctoral Fellow Postdoctoral Fellow

Microbiology and Antimicrobials

High throughput screen for inhibitors of the Legionella Type IV secretion system, a conserved virulence system

The recent emergence of drug-resistant pathogens calls for the development of novel therapeutic approaches and more efficient drug candidates. Additionally, current antibiotic treatments have been shown to impact the composition and homeostasis of the human microbiome, arguing for the need of new drugs that specifically target pathogenic bacteria but not commensal bacteria. Type IV secretion systems (T4SS) are sophisticated translocation machines that are used by bacterial pathogens to deliver effector proteins into host cells. These effector proteins rewire host cell signaling cascades to facilitate growth and survival of the pathogen. In an effort to find compounds that target and inhibit the Legionella T4SS, I adapted a fluorescence resonance energy transfer (FRET)-based reporter assay to measure bacterial T4SS activity in a multiplex plate format. I challenged macrophages with Legionella that expressed an effector protein fused to beta-lactamase, an enzyme that cleaves beta-lactam rings. A FRET substrate that consists of two fluorescent moieties separated by a beta-lactam ring will be cleaved within macrophages only when the reporter-effector fusion protein is translocated into the host cytosol. Cleavage of this ring resulted in a fluorescent emission change from green to blue and can be used as a readout for T4SS activity. Using this fluorescence-based assay, I screened over 18,000 compounds and identified ~600 that inhibit a green to blue emission shift, indicative of reduced T4SS activity. A more stringent validation screen confirmed ~20% (100 compounds) of the candidate compounds, and I am currently using an alternative reporter assay to validate these results and to further eliminate false positives. The remaining compounds will be selected for further study. Bacterial growth assays and cytotoxicity assays will be performed to eliminate compounds that have a general effect on cell viability from those candidates that specifically target the bacterium's T4SS. Ultimately, I will test each validated compound for its potency in blocking intracellular replication of Legionella pneumophila as well as related pathogens that also employ a T4SS during infection. The compounds identified here could eventually lead to new antimicrobials that selectively target pathogenic bacteria but not commensals when treating bacterial infections.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Francois De Mets

Doctoral Candidate

Microbiology and Antimicrobials

A processed Small non-Coding RNA regulates Acetate Metabolism in Coordination with the Metabolic State of the TCA Cycle

A plethora of bacteria use small non-coding RNAs (sRNAs) and the RNA chaperone Hfq to affect mRNA stability and translation. The Hfq chaperone is crucial for the stability of these sRNAs and facilitates sRNA-mRNA pairing. Most of the well-characterized Hfq-associated sRNAs in bacteria originate from transcripts expressed from conserved intergenic regions. However, recent RNA-seq profiling revealed that 3' regions of some mRNAs contain features similar to sRNAs, including being highly enriched by Hfq co-immunoprecipitation. We demonstrate that SdhX, a sRNA previously called RybD, is generated by 3'-end processing of the mRNA for *sdhCDAB-sucABCD*, encoding enzymes of the tricarboxylic acid cycle (TCA) in *Escherichia coli*. We found that both endoribonuclease RNase E and the Hfq chaperone are required to mediate an accurate cleavage of SdhX from the *sucD* 3' end. SdhX levels are regulated both by the *sdh* promoter (transcriptional regulation) and by sRNAs that post-transcriptionally affect the *sdh-suc* mRNA, such as Spot42, therefore tying SdhX abundance to expression of genes of the TCA cycle. Based on how sRNAs interact with the Hfq ring-shaped homo-hexamer protein, we recently categorized small RNAs in two classes. Class I sRNAs depend on proximal and rim Hfq faces for stability while Class II sRNAs depend on proximal and distal Hfq faces. SdhX is a typical Class II sRNA, losing its stability in Q8A proximal and Y25D distal Hfq mutants. SdhX sRNA is critical for acetyl-phosphate accumulation by negative regulation of *ackA*, encoding the enzyme that degrades acetyl-phosphate. Indeed, acetyl-phosphate levels become undetectable in a *sdhX* mutant. Acetyl-phosphate is a high-energy donor that acts as a global signal regulator in many cellular processes such as stress resistance or biogenesis of flagella and colanic acid (capsule). *pta*, a gene downstream from *ackA*, encodes the enzyme that synthesizes acetyl-phosphate, but is not affected by SdhX. We found that a promoter for *pta* allows this discoordinate regulation of these two neighboring genes. These results suggest that SdhX acts as a sensor of the TCA metabolic state, indicating a powerful mechanism for bacteria to link TCA cycle with acetate metabolism in central carbon metabolism. Current efforts are being focused on understanding the physiological role(s) of SdhX-mediated regulation of acetyl-phosphate levels in bacteria.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Su Chung

Postdoctoral Fellow

Molecular Biology - Eukaryotic

IL-13 is a critical mediator and therapeutic target in radiation lung injury

Background: Lung fibrosis is a late tissue injury that often develops after thoracic radiotherapy. In several inflammatory disease models, IL-13, a type 2 cytokine, has been shown to be a critical mediator of fibrosis. We sought to determine the importance of IL-13 and type 2 driven inflammation in radiation

lung fibrosis using a well characterized murine model. Methods: Eight to ten-week old female IL-13 deficient mice and C57BL/6Ncr wild-type mice were exposed to 5 daily fractions of 6Gy thoracic irradiation or no irradiation. In a separate study, thoracic irradiated C57BL/6Ncr mice were given anti-IL-13 IgG antibody (0.5mg per animal) or isotype control via IP injections on a weekly basis for eight weeks starting at three weeks after irradiation. Cohorts of mice were used for survival analysis and for bronchoalveolar lavage (BAL) fluid and lung tissue collection. BAL fluid and lung tissue were used to assess collagen accumulation and macrophage infiltration and polarization. Expression of pro-fibrotic cytokines and other pro-fibrotic factors such as matrix metalloproteinases and their inhibitors were also examined in lung tissues. Results: Thoracic irradiated C57BL/6Ncr wild-type mice developed lung fibrosis by 16 weeks after irradiation. At this time point, lung tissues from these mice had increased collagen deposition and hydroxyproline content with accumulation of alternatively activated (M2) macrophages. BAL fluid from irradiated mice had increased soluble collagen, protein, and cellularity mainly due to increase in macrophage population. IL-13, an inducer of M2 macrophage polarization, was also significantly elevated in irradiated lungs whereas IL-4 levels remained unchanged. Plasma from irradiated mice also displayed a transient increase in saturation of soluble IL13Ra2, a decoy receptor, with IL-13. Moreover, irradiated IL-13 deficient mice had prolonged survival and decreased lung fibrosis compared to irradiated wild-type mice, with decreased makers of Th2 driven inflammation and fibrosis. Similarly, C57BL/6Ncr mice treated with anti-IL-13 IgG antibody during peak IL13Ra2 saturation had less fibrosis and reduced markers of Th2 driven inflammation compared to mice treated with isotype control. Conclusion: Altogether, our data suggest that IL-13 is a potent driver of radiation induced lung fibrosis. Therapeutic inhibition of IL-13 is a promising strategy to prevent the development of lung fibrosis after thoracic irradiation.

National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS)

Jianliang Xu

Postdoctoral Fellow Postdoctoral Fellow

Molecular Biology - Eukaryotic

Watching single molecules at work in living B cells

Recent advances in high-resolution optical imaging make it possible to observe single molecules 'on the job' in living cells to answer key questions about the kinetic behavior of transcription factors (TFs), such as residence time, target-searching or interactions. Here we describe an approach to apply this novel method to study B cell biology. Live cell single molecule imaging requires gene fusion with a tag that binds a fluorescently labeled ligand. To achieve this, we used CRISPR/Cas9 to knock HaloTag into TFs of interest in ES cells. With JF549-conjugated HaloTag ligand, single molecules were visualized and residence times were gleaned from tracking single molecules across many frames. For a set of TFs we detected both fast and slow component, in good agreement with published data, validating our single molecule imaging system. To study the dynamic of TFs during B cell activation, we generated mice with HaloTag fused to endogenous JUND or CTCF protein. Surprisingly, even though CTCF is more a structural protein in defining topological associated domains of chromatin, we found the residence time of both JUND and CTCF were decreased to half in activated B cells compare to naive B cells. In contrast, the fast diffusion coefficient of both proteins significantly increased after cell activation, indicating that TFs become more "active" after cell activation. Indeed, 3D single molecule tracking revealed that both

molecules spent fewer trials on binding to non-specific sites, and overall searching time they spent before binding to their specific binding sites significantly decreased after B cell activation. Besides of transcriptional changes, activated B cells undergo somatic hypermutation (SHM) and class switch recombination (CSR) at Igh gene, which are mediated by activation induced cytidine deaminase (AID). To see the dynamic of CSR, we labeled switch mu (Sm) and alpha (Sa) regions with mCherry and GFP protein respectively. Ongoing experiments of imaging Sm and Sa in real time during cell activation are likely to add a new dimension to our understanding of immunoglobulin gene diversification and AID targeting.

National Institute of Allergy and Infectious Diseases (NIAID)

ABHISHEKA BANSAL

Postdoctoral Fellow

Molecular Biology - Eukaryotic

Plasmodium falciparum calcium dependent protein kinase 2 (PfCDPK2) is essential for exflagellation of male gametocytes

Plasmodium falciparum causes the most severe form of malaria and is responsible for a million deaths annually in the developing world. The emergence of resistance against currently used artemisinin based combination therapy (ACTs) in Southeast Asia is a cause of concern since ACTs are the front-line drugs for treating infected patients. Hence, there is an urgent need to find and validate new drug targets. Kinases have been extensively used as drug targets for treatment of various human ailments including cancer but have only recently gained entrance in the malaria drug development programs. Calcium dependent protein kinases (CDPKs) have been shown to play critical roles at various stages of the parasite life-cycle and importantly, are absent from the human host. These features mark them as attractive drug targets. Interestingly, one of the family members of PfCDPKs, CDPK2 is present only in *P. falciparum* and does not have a homologous protein in other *Plasmodium* species as evident from a manual blast search across all the *Plasmodium* genomes available. Earlier attempts to knock-out CDPK2 in the blood stages of *P. falciparum* were not successful, suggesting either the gene is essential for asexual proliferation or technical difficulties. However, with the advent of new gene editing technology, CRISPR-CAS9, we successfully knocked-out CDPK2 from the blood stages. The growth rate of the CDPK2 KO parasite clones, 1 and 13 was found similar to the wild type parasites, confirming CDPK2 function is redundant for the asexual proliferation of the parasite. However, there was a >90% reduction in male gametocyte exflagellation in the two CDPK2 KO clones, 1 and 13 compared to the wild type parasite, as evident by in vitro assay for exflagellation. Additionally, no oocysts were detected in the mosquitoes infected with the two CDPK2KO clones, 1 and 13 while the wild type parasite was able to infect mosquitoes. Complementation of the KO parasite with a functional copy of CDPK2 gene is in progress and will help in further validating the phenotype. We propose CDPK2 as a new candidate for drug discovery efforts that will be useful in blocking transmission of the parasite.

National Heart, Lung, and Blood Institute (NHLBI)

Julia Liu

Postdoctoral FellowPostdoctoral Fellow

Molecular Biology - Eukaryotic

MICU1 serves as a molecular gatekeeper to prevent in vivo mitochondrial calcium overload

Calcium entry into the mitochondria is critical for cellular homeostasis and is thought to modulate bioenergetic capacity as well as contribute to cell death. The biophysical properties of the mitochondrial calcium uniporter, the inner mitochondrial membrane channel by which mitochondria take up calcium, have been extensively studied for decades, but only recently have several of the component proteins of the uniporter complex been identified. MICU1 is a subunit of the uniporter complex that in cells has been shown to act as a “gatekeeper” by preventing calcium entry at low, resting cytosolic calcium levels. Notably, human patients with loss-of-function mutations in MICU1 develop severe neurological symptoms, along with a proximal myopathy. Here, we describe a mouse model of MICU1 deficiency. Consistent with cellular studies, mitochondria isolated from MICU1^{-/-} mice demonstrate altered calcium handling with increased uptake rates at low cytosolic calcium concentrations and reduced uptake rates at high calcium concentrations. In mice, the absence of MICU1 expression results in significant perinatal mortality, with less than one in six MICU1^{-/-} animals surviving into adulthood. Similar to afflicted patients, surviving MICU1^{-/-} mice exhibit marked ataxia and muscle weakness. These animals also exhibit increased levels of mitochondrial calcium, altered mitochondrial morphology, elevated tissue lactate levels, reduced ATP, and increased levels of mitochondrial reactive oxygen species. To confirm the role of calcium overload in these phenotypes, we generated additional mice with a targeted deletion in EMRE, a uniporter component essential for calcium uptake. Remarkably, the absence of one allele of EMRE rescued the marked perinatal mortality observed in MICU1^{-/-} mice. Furthermore, EMRE haploinsufficiency restored gatekeeping function to MICU1^{-/-} mitochondria, lowered mitochondrial calcium levels, and significantly improved the biochemical, neurological and myopathic features observed in MICU1^{-/-} mice. These results demonstrate that the primary function of MICU1 is to prevent in vivo mitochondrial calcium overload. They further suggest that manipulating calcium uniporter activity might provide a strategy to treat patients lacking MICU1, as well as for the growing number of other conditions characterized by mitochondrial calcium overload.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Kumari Kavita

Visiting FellowVisiting Fellow

Molecular Biology - Prokaryotic

Untangling the role of the C-terminus of Hfq in sRNA regulation

The bacterial Hfq is an RNA-binding chaperone that stabilizes small noncoding RNAs (sRNA) and facilitates riboregulation by base pairing of the sRNA with target mRNAs. These target mRNAs encode proteins mostly involved in stress responses and virulence. The homohexameric Hfq has a conserved N-terminal core, which is sufficient to bind RNA and promote pairing to targets, and a less conserved C-terminus. The long C-terminal extension, present in gamma and beta-proteobacteria, is not found in some other bacteria. Previous reports provided conflicting assessments of the necessity for the role of the C-terminus, possibly reflecting the different approaches and assays used by different groups. The

maintainance of the C-terminus tail during the course of evolution argues that it endows a beneficial function, which is not known yet. We constructed strains of *E. coli* in which Hfq deleted for the C-terminus is expressed from the chromosomal locus (single copy), allowing in vivo comparisons with the wild-type Hfq, expressed under similar conditions and at physiological levels. Recently, our group has shown that sRNAs fall into two classes, based on how they bind to Hfq. Class I binds the proximal surface and rim of Hfq, while Class II binds the proximal and distal faces. We compared Class I and Class II sRNA accumulation by northern blot analysis in wild-type Hfq and C-terminal deleted Hfq (hfq65) strains. Class II (ChiX, MgrR, CyaR and McaS) but not most Class I sRNAs (Spot42, GcvB and ArcZ) had reduced accumulation in the hfq65 strain compared to wild-type. One tested Class II sRNA (ChiX) was also defective for function in the hfq65 host. However, when repression by ChiX was measured in multicopy the defect in function for hfq65 was suppressed which may explain why some previous studies did not see any defect in function with hfq65. The reduced accumulation may reflect defects in initial binding to Hfq or effects on pairing and turnover of the sRNA. We tested both and found that the C-terminus did not affect intrinsic stability or binding of sRNA to Hfq. Rather, in the absence of the Hfq C-terminus, Class II sRNAs are more rapidly released and degraded after pairing with sRNAs. These results, coupled with in vitro studies of truncated Hfq carried out by our collaborators (Andrew Santiago-Frangos and Sarah Woodson, JHU) are consistent with a model in which the Hfq C-terminus helps to protect the protein from non-specific nucleic acid binding.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Gilad Kaplan

Postdoctoral Fellow

Molecular Biology - Prokaryotic

Engineering next generation anti-CD25 immunotoxins with low non-specific toxicity and immunogenicity

CD25 is expressed on regulatory T cells as well as on multiple hematological malignancies. LMB2 is an anti-CD25 recombinant immunotoxin composed of an Fv that binds to CD25 and a 38kDa fragment of *Pseudomonas* exotoxin A (PE38), which contains a processing domain and an ADP-ribosylation domain. In clinical trials, LMB2 has produced complete remissions in patients with Adult T cell leukemia and Hairy Cell Leukemia. Because LMB2 contains a bacterial toxin, it can only be given for 1 treatment cycle to patients with normal immune function before neutralizing antibodies form. This does not occur in many leukemia patients, because their immune system is suppressed and antibody formation greatly delayed. Our goal was to use rational protein design to develop a second generation LMB2 that is much less immunogenic so that it can be used in patients with normal immune function, and is also better tolerated by humans and can therefore be given at higher doses. Both immunogenicity and non-specific toxicity were decreased by deleting unnecessary regions of the toxin processing domain. For this we designed mutant immunotoxins combining truncations of different lengths and in different areas of the processing domain with stabilizing disulfide bonds and point mutations. These rationally designed mutants were produced and screened for cytotoxic activity. The most active mutant was one in which the processing domain was completely replaced by a critical furin protease cleavage sequence constrained by an engineered disulfide bond. The immunogenicity of this immunotoxin was then further reduced by introducing 6 point mutations into the remaining ADP-ribosylation domain that disrupt T and B cell epitopes. The final molecule, Anti-CD25-PE24-DM19, has a 90% decrease in immunogenicity.

Although it exhibits a 10-fold decrease in cytotoxic activity on CD25 expressing cells compared with LMB2 it is still very active (IC50s of 1.2pM and 0.11pM respectively), and can be given to mice at 100-fold higher doses than LMB2 (maximal tolerated dose of 24 and 0.3 mg/kg respectively) leading to a greatly enhanced therapeutic window. We conclude that because our engineered anti-CD25-PE24-DM19 immunotoxin exhibits high cytotoxic activity in the pM range, low non-specific toxicity in mice and low immunogenicity it is an excellent candidate for targeting regulatory T cells and CD25 expressing malignancies in humans.

National Cancer Institute - Division of Cancer Epidemiology and Genetics (NCI-DCEG)

Guoqin Yu

Research Fellow

Molecular Biology - Prokaryotic

Helicobacter pylori dominates the stomach microbiota of gastric cancer patients

Objective: *Helicobacter pylori* (Hp) is the primary cause of gastric cancer but we have little understanding of its relative abundance in the stomach compared to other taxa, especially at the time of gastric cancer diagnosis. New methods for characterizing bacterial communities may bring additional insight into the gastric microbiota. Design: A total of 80 pairs (non-malignant and tumor) of gastric tissue samples from China and 80 pairs from Mexico were sampled from gastric cancer cases. We characterized the bacterial taxonomic profiles by sequencing the 16S rRNA gene V3-V4 region and the functional profiles by prediction with PICRUSt. Results: Based on non-malignant tissues, Hp was the most abundant member of gastric microbiota with average relative abundance of 51% in Chinese and 24% in Mexico. The relative abundance of Hp exceeded 50% in 53% Chinese and 28% of Mexican cases. Other than *Helicobacter*, gastric microbiota of both Chinese and Mexican cases was dominated by oral-associated bacteria genera including *Streptococcus*, *Prevotella*, and *Haemophilus*. After *Helicobacter* reads were removed, the taxonomic profiles of gastric microbiota resembled oral microbiota, the predicted functional profiles, however, did not resemble the body sites characterized by the Human Microbiome Project. Gastric microbiota composition did not differ by Hp colonization status or stomach anatomic site, but did differ between non-malignant and tumor tissue in either Chinese or Mexican samples. Conclusions: We reported for the first time that Hp is the dominant bacteria in gastric microbiota of gastric cancer patients rather than diminished or depleted as traditionally believed. We also showed that the bacterial composition and structure of gastric microbiota resembled oral microbiota when *Helicobacter* reads were removed. Our findings provided important insight on gastric microbiota composition of gastric cancer cases, and may have important clinical implications.

National Institute on Drug Abuse (NIDA)

Brandon Warren

Postdoctoral Fellow

Neuropharmacology and Neurochemistry

Distinct neuronal ensembles in ventral medial prefrontal cortex mediate self-administration and

extinction of food self-administration in rats.

Operant learning involves learned associations between specific behaviors and unconditioned rewards. For example, rats can learn to perform an operant response (lever press) to receive rewarding food pellets. If food reward delivery is terminated, the learned response undergoes operant extinction. These reward-associated and extinction-associated memories in operant learning are thought to be distinct from each other. Since memories are thought to be stored within specific patterns of sparsely distributed neurons called 'neuronal ensembles', reward-associated memories and extinction-associated memories are also believed to be encoded by distinct neuronal ensembles. To test this hypothesis, we first trained animals to lever press for palatable food pellets in 7 daily training sessions. Rats were then subjected to 0, 2, or 7 daily extinction sessions. This enabled us to assess neuronal activity at three different points during extinction learning. During extinction, pressing the active lever (previously paired with delivery of a palatable food pellet) did not result in delivery of reward. Then, rats in each group were exposed to a 15 min test (non-reinforced) or were left in their homecage, as a control. We then assessed Fos immunoreactivity in the infralimbic and prelimbic cortices. Because Fos is associated with neuronal activity, Fos immunoreactivity allowed us to visualize specific neurons and neuronal ensembles activated by the recall of extinction memories. Here, we found maximal Fos immunoreactivity in the infralimbic cortex after 2 extinction sessions, suggesting that neuronal ensembles within the infralimbic cortex may encode extinction memory. To test this hypothesis, we used the newly developed Daun02 inactivation procedure to selectively inactivate neuronal ensembles associated with extinction or self-administration recall. Daun02 is converted to daunomycin only in activated neurons of transgenic cFos-lacZ rats. Because daunomycin produces apoptosis, injecting Daun02 into the infralimbic cortex in cFos-lacZ rats will selectively lesion Fos-expressing neurons. Using this method, we show that selective inactivation of extinction ensembles impaired extinction recall, while selective inactivation of acquisition ensembles disturbed acquisition recall. This provides the first evidence that neuronal ensembles encoding extinction and training memories are not only distinct, but can intermingle in the same brain area.

National Institute on Drug Abuse (NIDA)

Jordi Bonaventura

Postdoctoral Fellow

Neuropharmacology and Neurochemistry

Differential modulatory role of dopamine D4 receptor polymorphic variants on methamphetamine-induced dopamine release

The human dopamine (DA) D4 receptor gene contains a large number of polymorphisms in its coding sequence. The most extensive is found in the region coding the third intracellular loop, where a 48-bp sequence is repeated from 2 to 11 times. The two most common variants contain 4 repeats (D4.4) and 7 repeats (D4.7). D4.7 has been consistently associated with low constraint, constituting a risk factor for ADHD and substance use disorder, but little is known about the underlying mechanisms. Nevertheless it is generally assumed that D4.7 is "less functional" than D4.4 receptor. D4 receptors are highly expressed by pyramidal cortico-striatal glutamatergic neurons of the prefrontal cortex (PFC), including their striatal terminals. Cortico-striatal neurons –esp. those connecting the medial PFC and the Nucleus Accumbens (NAc) shell- play an important role processing reward-associated stimuli. Cortico-striatal

neurotransmission is also involved in the acute effects of methamphetamine (METH). Apart from producing a large increase in DA release, METH also increases the extracellular concentration of glutamate (GLU) in the NAc by not yet known mechanisms. To bring new clues into those mechanisms we used knock-in mice carrying a 7-repeat third intracellular loop identical to that found in the human D4.7. Using our recently introduced optogenetic-microdilysis technique, we measured in vivo GLU release in the NAc upon local and selective optogenetic stimulation of PFC nerve terminals. We found lower levels of optogenetically induced glutamate release in D4.7 animals compared with WT littermates. Systemic administration of METH but not cocaine produced a significantly lower increase in NAc DA in D4.7 knock-in mice compared to their WT littermates. METH also increased NAc GLU levels, but the effect was significantly lower in D4.7 than WT mice, mimicking the results obtained with optogenetic stimulation. The same differences were also found with locomotor activity, where METH but not cocaine produced significantly lower effect in D4.7 than WT mice. Our results indicate that this differential effect on both NAc DA and GLU depends on the increased inhibitory control of NAc GLU release by D4.7. The results predict D4 receptor polymorphic-dependent differential addictive and neurotoxic properties of METH.

National Institute of Child Health and Human Development (NICHD)

Pushpanathan Muthuirulan

Visiting Fellow

Neuroscience - Cellular and Molecular

Mapping Neurotransmitter Receptors to Active Synaptic Circuits by Fluorescence Complementation

Many neuropsychiatric and neurodegenerative disorders, ranging from mental retardation to Alzheimer's disease, are accompanied by altered synaptic circuits in the brain. Mapping the synaptic circuits could provide insight into the computation processes that underlie brain functions and pathophysiology of neurological diseases. The overwhelming complexity of neural circuits poses a great challenge to map its connection patterns and synaptic components. The recently developed GRASP (GFP reconstitution across synaptic partners) technique, based on functional complementation between two non-fluorescent GFP fragments has proven useful for mapping synaptic circuits but can't identify active synapses or synaptic components, such as neurotransmitter receptors. Here, we developed a novel receptor-based GRASP method to map neurotransmitter receptors to active synapses. In this method, we engineered different neurotransmitter receptors of interest with small split-GFP moiety (spGFP11), which reconstitutes with the large split-GFP moiety (spGFP1-10) tethered on synaptic vesicles (syn-spGFP1-10) to form functional GFP in active synapses. By a combination of molecular, histology and genetic approaches, we applied this strategy to map *Drosophila* motion detection circuits. Our previous studies revealed that motion-sensitive direction-selective T4 and T5 neurons expressed distinct combinations of nicotinic and muscarinic cholinergic receptors to receive synaptic inputs from multiple cholinergic transmedulla (Tm) neurons. To map the cholinergic receptors to specific synapses, we engineered spGFP11-tagged nicotinic (nAChR- $\beta 3$) and muscarinic (mAChR-B) receptors based on rational design. These spGFP11-tagged receptors, when expressed in the T4 and T5 neurons, specifically targeted to dendritic terminals. Furthermore, we additionally expressed in different Tm neurons syn-spGFP1-10, which reconstituted GFP fluorescence in vivo with spGFP11-tagged cholinergic receptors on T4 and T5. Based on these experiments, we suggest that T5 uses muscarinic receptors to receive Tm9 inputs and T4 uses

nicotinic receptors to receive Mi1 inputs. This method allows for the first time retrospective labelling of active synapses based on their usage of specific neurotransmitter-receptors. With the advance of the CRISPR/Cas9 genome editing technique, we envision this method could be applied effectively to map neurotransmitter receptors or other synaptic components to neural circuits of essentially any animal models.

National Institute on Drug Abuse (NIDA)

Lindsay De Biase

Postdoctoral Fellow

Neuroscience - Cellular and Molecular

Next generation RNA sequencing reveals similarities and differences in the functional state of basal ganglia microglia

Microglia possess motile processes and phagocytose synapses during circuit maturation and neurodegeneration. Microglia also release inflammatory and trophic factors that influence synaptic signaling and neuronal activity. We recently showed that microglia in the basal ganglia (BG) of young adult mice differ dramatically in their density, morphology, and electrophysiological properties, suggesting that microglial impact on surrounding neurons may vary considerably. To define the magnitude and nature of variation in BG microglial phenotype, we used RNA sequencing (RNAseq) to analyze the transcriptome of BG microglia. Published RNAseq studies used microglia isolated from whole brain, whole cortex, or whole spinal cord to define how microglia differ from other CNS cells. We developed a novel RNAseq protocol to analyze microglia from individual BG nuclei of individual mice. This protocol does not require pooling tissue across animals or enzymatic digestion at elevated temperatures, better preserving native gene expression of these dynamic cells. Analysis of cortex (Ctx), nucleus accumbens (NAc), ventral tegmental area (VTA), and substantia nigra (SN) microglia revealed robust expression of microglial-specific genes and no expression of neuronal, astrocyte, and oligodendrocyte lineage genes. RT-PCR analysis of gene expression showed strong agreement with expression levels measured by RNAseq ($R^2 = 0.85$, $P = 0.0001$, $N = 17$ genes). Comparison across brain regions indicated that NAc and Ctx microglia show the greatest degree of similarity (84.3% overlap in expressed genes), while VTA microglia were the most distinct (56.4 -60.0% overlap with Ctx, NAc, and SN). Functional annotation revealed that genes involved in process motility and immune function are well conserved across BG and Ctx microglia. Instead, genes involved in mitochondrial function, lysosome function, and homeostasis of reactive oxygen species were differentially expressed. Confocal imaging and immunostaining for CD68 indicated that microglial lysosome content varied significantly across BG nuclei, supporting the conclusion that lysosome function, and, possibly, phagocytotic activity vary. This is the first quantitative assessment of variation in BG microglial phenotype and the first RNAseq study of microglia from individual brain regions. These data provide a critical foundation for defining microglial contributions to BG circuit function during health and neurodegenerative or addictive processes.

National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)

Ramón Piñol

Postdoctoral Fellow Postdoctoral Fellow

Neuroscience - Cellular and Molecular

Differential Regulation of Energy Homeostasis by Hypothalamic Bombesin-Like Receptor-3 Populations

Obesity affects over one third of adults in the United States and is caused by disrupted energy homeostasis: energy intake exceeds expenditure. Mice lacking the orphan G protein-coupled receptor bombesin-like receptor subtype-3 (Brs3) are obese, with reduced energy expenditure and increased food intake. Concomitantly, Brs3 agonists both stimulate brown adipose tissue (BAT) thermogenesis through sympathetic activation and suppress food intake. Brs3 is expressed in several hypothalamic regions, including the paraventricular nucleus of the hypothalamus (PVH) and dorsomedial hypothalamus (DMH), two regions pivotal in energy homeostasis. The goal of this work is to elucidate the circuitry by which Brs3 regulates energy metabolism. We measured the effect of localized injections of Brs3 agonist MK-5046 on BAT temperature in anesthetized mice. Nucleus-targeted MK-5046 injections in the DMH, but not in the PVH, raised BAT temperature. Vehicle had no effect in either nucleus. To assess the effect of acute activation of specific Brs3 subpopulations in freely moving, conscious mice we used chemogenetics. We virally expressed the excitatory designer receptor exclusively activated by designer drugs (DREADD) hM3Dq in Brs3-Cre mice. Chemogenetic activation of DMH-Brs3 neurons increased total energy expenditure and body temperature, without having an effect on physical activity or food intake. In contrast, after Brs3-Cre-dependent expression of hM3Dq in the PVH, chemogenetic activation of PVH-Brs3 neurons robustly suppressed food intake and increased body temperature only modestly. Vehicle had no effect. We used an optogenetic approach to corroborate these findings and untangle the underlying circuitry. In the DMH of Brs3-Cre mice we expressed the light-activated excitatory ion channel ChR2 and an optic fiber was implanted over the DMH. Optogenetic activation of DMH-Brs3 neurons increased body temperature. To identify the projections by which DMH-Brs3 neurons drive thermogenesis, we also implanted an optic fiber over axonal projections of DMH-Brs3 neurons in brainstem rostral raphe pallidus (RPa). DMH-Brs3 → RPa pathway stimulation increased body temperature. Thus, different hypothalamic Brs3 neurons control energy homeostasis by increasing energy expenditure and BAT temperature, and by suppressing food intake. Specifically, PVH-Brs3 neurons can robustly suppress food intake and DMH-Brs3 neurons can drive BAT-mediated thermogenesis through projections to RPa.

National Institute on Aging (NIA)

Yuyoung Joo

Postdoctoral Fellow Postdoctoral Fellow

Neuroscience - Cellular and Molecular

Top3b-null Mice Show Defective Neurogenesis, Synaptic Plasticity and Increased Anxiety

Topoisomerase 3b (Top3b), the first RNA topoisomerase, interacts with FMRP, the disease gene product in fragile X mental retardation syndrome. Increasing evidence suggests that Top3b regulates RNA metabolism and promotes synapse formation. A recent study also shows that individuals carrying deletion of Top3b gene are at increased risk of developing schizophrenia and intellectual disability. However, the functional role and pathologic mechanism of Top3b in mental disorders are unclear. Here

we show that Top3b-deficient mice have increased anxiety and intensified fear conditioned memory compared with wild type mice in several behavioral tests. In addition, Top3b-deficient mice display enlarged ventricles, a phenotype commonly observed in schizophrenia patients. These enlarged ventricles could be due to reduced proliferation and differentiation of adult neural stem cells in subventricular zone (SVZ) and hippocampus of Top3b-deficient mice. Furthermore, we show that two forms of protein synthesis-dependent synaptic plasticity, long-term depression (LTD) and long-term potentiation (LTP) that involve activation of metabotropic glutamate receptors (mGluRs), are impaired in the hippocampus of Top3b-deficient mice. Mechanistically, Top3b binds to a group of mRNAs, which are crucial for adult neurogenesis and newly developed tissue structure; and may regulate an emotional condition including anxiety level and fear memory. Our data demonstrate that Top3b is required for adult neural genesis and synaptic plasticity, and provides a mechanism for how its mutation can lead to neurodevelopmental disorders.

National Institute of Mental Health (NIMH)

Amicia Elliott

Postdoctoral Fellow

Neuroscience - Cellular and Molecular

Probing the cellular and network-level activity in a Drosophila neural circuit using light-sheet microscopy

Sequences of coordinated motor activity, which are critical for everyday function, are produced by poorly understood central neural circuits. The complexity and scale of the circuitry involved in this process makes it very difficult to study in rodent animal models or humans. However, the neural circuits of the fruitfly drive similar complex behaviors, and are small enough to investigate via emerging methods in microscopy. A critical behavioral sequence for the fruitfly, called ecdysis, is a hormonally-initiated neural program that drives the flies to molt at each developmental stage. Ecdysis consists of three precisely timed morphological, physiological, and behavioral events. The neural circuit controlling the ecdysis program consists of approximately 300 peptidergic neurons that express the Ecdysis Triggering Hormone receptor (ETHR) and are activated by peripheral release of Ecdysis Triggering Hormone (ETH). The activation of these neurons leads directly to three phases of associated motor neuron activity, but a cellular-level understanding of how this is accomplished remains elusive. To address this question, we have built a light-sheet microscope that is capable of imaging the whole ecdysis neural circuit rapidly at high resolution. We are using fluorescent biosensors for Ca²⁺ to monitor the neural activity in ETHR-expressing neurons or motor neurons of excised brains in response to ETH. Existing data indicate that specific subpopulations of these neurons are required for each behavioral phase of ecdysis. Consistent with this, single-cell activity imaging on the light-sheet microscope confirms that individual neurons respond to ETH with varying degrees of lag and with distinct activity profiles. Collectively, these data will be used to generate a predictive model of the circuit underlying the ecdysis sequence. Addition of a second color channel, which will allow us to measure Ca²⁺ activity from two different populations of neurons at once, is currently in progress.

National Institute of Child Health and Human Development (NICHD)

Daniela Calvigioni

Doctoral Candidate

Neuroscience - General

Functional differentiation of cholecystinin-containing interneurons during brain development

Interneurons represent one of the most diverse cell populations of the brain. The complex architecture of the GABAergic system is essential during brain development to orchestrate migration, synapse differentiation and network activity. Therefore understanding the physiology of prenatal interneurons is crucial, particularly since dysfunction of interneuron during development is associated with psychiatric diseases. Cholecystinin(CCK) expression characterizes one of the most abundant GABAergic subpopulation, involved in multiple circuits such as food intake, anxiety and fear. Although extensively studied postnatally, the notorious difficulty to localize CCK protein has limited prenatal characterization. Here, we took advantage of a new mouse line encoding a CCK-driven RFP to study the spatio/temporal dynamics of CCK expression, neuronal identity and positioning through high-resolution neuroanatomy involving tissue clearing and lightsheet whole brain imaging. We then generated a dual reporter mouse co-expressing CCK-DsRed and Gad1-GFP to specifically interrogate the CCK-interneurons in vivo. Sequential lightsheet imaging at discrete developmental stages timed the appearance of CCK-interneurons to Embryonic day 12.5(E12.5) in the CGE migrating along the cortical plate and reaching the hippocampus by E14.5. Furthermore we identified a subclass of interneurons co-expressing CCK/CR/CB1R as early as E12.5 that accumulated in SR/SLM of the hippocampus by E14.5. Electrophysiology is a preferred method to test cell state-transitions, including gradual developmental maturation. Considering that embryonic brain contains morphologically immature neurons whose somata are packed in a relatively small volume, visual discrimination of interneurons subtypes for electrophysiological characterization is not possible without fluorescence tagging. Therefore, we took advantage of the CCK-DsRed mouse line for comparative analysis by electrophysiology throughout embryonic development. Whole-cell patch-clamp recordings of DS-Red expressing interneurons revealed the first Na⁺ and K⁺ channel activity by E12.5, and adult-like excitability by E18.5. In addition recorded interneurons were filled with LuciferYellow to analyze morphological development and gap junction coupling. In sum, our study defines prenatal molecular and electrophysiological features of CCK-interneurons at physiological levels, laying the foundation for an interneuron-specific analysis in pathological conditions

National Institute of Child Health and Human Development (NICHD)

Wei-Chia Tseng

Postdoctoral Fellow

Neuroscience - General

Establishment and characterization of Niemann-Pick Disease Type C zebrafish models

Niemann-Pick Disease Type C (NPC), is a rare autosomal-recessive lysosomal storage disease, estimated to affect 1 in 100,000 individuals. NPC symptoms and severity vary broadly becoming evident in early childhood. Common symptoms include neonatal jaundice, hepatosplenomegaly, ataxia, tremor, seizures, and learning difficulties. At cellular level, these symptoms are associated with excessive accumulation of unesterified cholesterol and glycolipids in late endosomes and lysosomes as well as

dysregulation of cholesterol homeostasis in NPC patients. In 95% of cases, NPC is caused by mutations in *npc1* gene. NPC1 is a transmembrane protein found to interact with NPC2 in late endosomes and lysosomes, and functions in the endocytic cholesterol trafficking pathway. Although mouse and feline models of NPC have been established and studied, mechanisms of NPC progression are still not fully understood. In this study, we aim to establish NPC models using zebrafish to complement current studies in mammalian models. Zebrafish have the advantage as a model system to perform many in vivo experiments with large numbers such as rescue and live imaging that are more difficult in mammals. Here we have generated a zebrafish *npc1* null mutant by CRISPR/Cas9-mediated gene targeting. Zygotic *npc1* mutant zebrafish exhibit no overt phenotype and are viable during the embryonic and larval stage. However, only a small fraction of *npc1* mutant zebrafish survives to adulthood. These surviving fish have significant growth retardation and developing motor and balance defects shortly before they die around 3-month post-fertilization. Approximately 50% of *npc1* mutant zebrafish develop more significant pathological defects and die during the juvenile stage. Our preliminary data also indicates that zygotic *npc1* mutant zebrafish develop an acute liver defect at 7dpf and die soon after a short-term starvation. These combined phenotypes mimic both the severe neonatal lethal presentation of NPC as well as the classical neurological presentation of the disorder found in NPC1 patients. We are currently analyzing the pathological defects and mechanisms in *npc1* mutant zebrafish. Ultimately, we aim to establish a platform using zebrafish models that could potentially be utilized to develop therapeutic strategies for treating NPC patients.

National Institute on Drug Abuse (NIDA)

Daniele Caprioli

Postdoctoral Fellow

Neuroscience - General

Role of the dorsal striatum in regulating incubation of methamphetamine craving after voluntary abstinence

Methamphetamine (Meth) addiction is characterized by high relapse rates that are often precipitated by exposure to craving-provoking drug-associated cues. Animal models of relapse show that cue-induced drug seeking progressively increases during extended forced abstinence periods after self-administration of addictive drugs, including Meth. This phenomenon, now termed 'incubation of drug craving', was recently observed in inpatient Meth users. In rodent studies of incubation of drug craving, drug seeking is assessed after experimenter-imposed forced abstinence. In contrast, in humans, abstinence is often voluntary due to the availability of alternative rewards. This aspect of human addiction (voluntary abstinence) is not captured by current animal models of drug craving and relapse. We recently established a choice-based rat model of relapse in which incubation of Meth craving is observed after prolonged voluntary abstinence. The model includes 4 phases: palatable food self-administration (SA), Meth SA, voluntary abstinence, and assessment of cue-induced Meth seeking during early (day 1) or late (day 21) abstinence. Voluntary abstinence is achieved via a discrete choice procedure (20 daily mutually exclusive choices) in which food-sated rats strongly prefers palatable food pellets over Meth. Here we studied the role of the dorsal striatum (DS; medial, M or lateral, L), a brain area previously implicated in cue- and context-induced drug relapse, in this rat model of relapse. Using RNAscope® to determine striatal sub-region and cell-type specificity of the activated neurons, we

measured the expression of the neuronal activity marker Fos in DS neurons expressing dopamine D1 and D2 receptors (Drd1 and Drd2) of the direct (striatonigral) and indirect (striatopallidal) pathways. We found that incubation of Meth craving after voluntary abstinence is associated with increased expression of the neuronal activity marker Fos in DMS neurons expressing Drd1 and Drd2 receptors with higher co-localization of Fos with Drd1. In contrast, incubation of Meth craving was not associated with increased Fos expression in DLS. We then found that both Drd1 and Drd2 in DMS play a causal role in incubation of Meth craving: injections of the selective dopamine D1 and D2 antagonists SCH39166 and raclopride, respectively, into DMS decreased this incubation. These data provide the first mechanistic account of a clinically-relevant novel animal model of drug relapse.

National Institute on Aging (NIA)

Qu Tian

Research Fellow

Neuroscience - General

Amyloid burden, a hallmark of Alzheimer's disease, links to mobility decline in normal aging

Older individuals tend to slow down in moving and thinking. We often perceive it as a sign of aging. We rarely think this might be a sign of Alzheimer's disease (AD). It was only recently that an increasing line of research demonstrated motor slowing strongly predicted risks for cognitive impairment and AD. With powerful neuroimaging tools, great progress has been made to unveil the neural control of mobility, which may underlie the intriguing connection between poor mobility and future high AD risk. Prior findings show that brain atrophy and white matter lesions are associated with poor mobility. Most of previous evidence, however, is neurovascular origin. Amyloid deposits, one primary neuropathological hallmark of AD, are known to be associated with subsequent memory loss. Whether amyloid burden plays a role in mobility decline, independent of memory loss, is unknown. Understanding this relationship is paramount as motor slowing may occur years prior to cognitive symptoms of AD. Using data from the Baltimore Longitudinal Study of Aging, we identified 59 cognitive normal older participants who had amyloid imaging in 2006-14, and repeated measures of upper- (mean tapping time) and lower-extremity (gait speed, 400m walk time, Health ABC Physical Performance Battery (HABCPPB) score, total standing balance time) motor function at and subsequent to amyloid imaging during a mean follow-up of 4.7 years. Linear mixed effects models were used to examine the relationship of baseline amyloid burden with motor changes. Models were adjusted for age, sex, body mass index, cardiovascular risk score, APOE e4 genotype, and concurrent memory decline. We found higher mean cortical amyloid burden was associated with steeper declines in gait speed, HABCPPB score, and 400m performance (all $p < 0.05$). Regional analysis revealed that the relationship was localized in motor planning-related early deposition regions, including putamen, dorsolateral prefrontal cortex, lateral temporal lobe, and precuneus, and not late deposition areas of primary motor cortex or the hippocampus. Results remained robust with adjustment. There were no significant associations with change in mean tapping time. These findings show for the first time that amyloid burden is a risk factor for mobility decline among cognitive normal older adults, independent of memory loss. Older adults who appear normal with progressive mobility decline may need to be screened for amyloid accumulation, a hallmark of AD.

National Institute of Child Health and Human Development (NICHD)

Jason Wester

Postdoctoral Fellow

Neuroscience - General

Interneurons Differentially Contribute to Spontaneous Network Activity in the Developing Hippocampus Dependent on Their Embryonic Lineage

During neural development, circuits throughout the brain spontaneously generate rhythmic bouts of network activity, which promotes and guides synapse formation. In the rodent hippocampus, this activity is observed as giant depolarizing potentials (GDPs) during the first postnatal week. Immature GABAergic interneurons importantly contribute to GDP generation, due to the depolarizing (excitatory) actions of GABA early in development. While they are highly diverse, cortical interneurons can be segregated into two distinct groups based on their embryonic lineage from either the medial or caudal ganglionic eminences (MGE and CGE). There is evidence suggesting CGE-derived interneurons are important for GDP generation; however, their contribution relative to those from the MGE has never been directly tested. Here, we used the light-activated proton pump archaerhodopsin to optogenetically inhibit either MGE- or CGE-derived interneurons during spontaneous GDPs in neonatal mouse hippocampal slices. Furthermore, we were able to focus the excitation light such that we could inhibit interneurons in a region-specific manner. In region CA1, where interneurons are the primary source of circuit excitation due to very sparse connectivity between pyramidal cells, we found that MGE-derived interneurons strongly and preferentially contributed to GDP generation: inhibiting these cells nearly abolished GDPs, while their excitation could reliably trigger GDPs. In contrast, inhibiting CGE-derived interneurons only weakly suppressed GDPs, while exciting them rarely produced GDPs. To explain the circuit mechanisms of these findings, we performed dual whole-cell patch recordings between interneurons and neighboring pyramidal cells in neonatal CA1. MGE interneurons formed synaptic connections to and from neighboring pyramidal cells at a much higher rate than those from the CGE. Furthermore, MGE interneurons demonstrated mature synaptic connections with high vesicle release probability. Post-hoc morphological analysis revealed their axons often targeted the perisomatic region of pyramidal cells. Finally, we found that inhibiting MGE interneurons in CA1 suppressed GDPs in neighboring region CA3 and vice versa; conversely, they could also trigger GDPs in CA1 that propagated to CA3 and vice versa. Our data demonstrate that MGE-derived interneurons are integrated into the local circuit early in development and play a key role in both generating and coordinating GDPs across the hippocampus.

National Institute of Mental Health (NIMH)

Benjamin Suarez-Jimenez

Doctoral Candidate

Neuroscience - Integrative, Functional, and Cognitive

Neural correlates of location-specific threat learning in humans

Learning about dangers in our environment is a vital adaptive behavior, and many have studied the

association of environmental cues with danger or safety. However, the outcome associated with a specific environmental cue can depend on where it is encountered, and relatively little is known about the neural mechanisms behind location-specific fear learning within a single environment. Through a series of experiments I developed a novel virtual reality task comprising safe and dangerous zones within a single environment. Healthy volunteers explored this environment while 'picking flowers', which they were told might contain bees. On contacting a flower, participants were frozen for a short period and, if 'stung,' received a mild electric shock at the end of this period. Participants had the opportunity to learn that bees only inhabited flowers in one (dangerous) half of the environment. Participants are able to discriminate zones that predict safety and threat within a single environment, with galvanic skin responses and subjective reports increasing as they approached and picked flowers in the dangerous half of the environment. Using functional magnetic resonance imaging, I found posterior medial temporal lobe structures (parahippocampus, posterior hippocampus) to be involved in memory for object locations. In contrast, anterior hippocampus, amygdala and ventromedial prefrontal cortex showed greater activity when approaching flowers, but this activity did not differentiate between safe and dangerous zones. However, once participants reached a flower in the dangerous zone, increased activity was seen in areas associated with imminent threat, such as the midbrain/periaqueductal gray, dorsal anterior cingulate and insula cortices. These results are the first to reveal mechanisms of location-specific fear learning in humans, in the absence of obvious boundaries delineating safety and danger zones. In future, I hope the new paradigm will be used to understand the overgeneralization of fear in anxiety disorders and post-traumatic stress disorder.

National Institute of Mental Health (NIMH)

Angela Ianni

Doctoral Candidate

Neuroscience - Integrative, Functional, and Cognitive

Dopaminergic Correlates of Behavioral Flexibility in Foraging Decision-Making in Humans

Foraging is experimentally defined as a reward-guided behavior that involves deciding whether to engage with the current environment or leave and search elsewhere. Foraging is crucial for survival, and abnormalities have been found in addiction, Parkinson's disease, and schizophrenia, all associated with dysfunction of the dopamine system. Although previous studies have identified an important role of the anterior cingulate cortex and of neuromodulators such as dopamine and norepinephrine in foraging behavior, it is unclear how regional variation in neuromodulator synthesis and receptors impact behavior in humans. In 41 healthy adults, we used PET imaging to directly measure three dopamine-related parameters: presynaptic synthesis capacity (with [18F]-DOPA) and D1 ([11C]NNC112) and D2-3 ([18F]Fallypride) receptor binding potential (BPnd). In these same individuals, we measured foraging behavior in four different reward environments using a computer-based foraging task. We tested for correlations between PET measures and adaptive foraging behavior (change in patch-leaving threshold between the two environments with maximally different average reward rates) using statistical thresholds of $p < 0.05$ (hypothesis-driven basal ganglia ROIs) and $p < 0.005$ (whole-brain), uncorrected. We found that adaptive foraging behavior was positively correlated with FDOPA uptake rate in extrastriatal regions including the anterior cingulate cortex ($r=0.50$, $p=0.00073$) and posterior midbrain ($r=0.47$, $p=0.0015$), D1 BPnd in the ventral striatum ($r=0.43$, $p=0.0071$), and D2-3 BPnd in the

dopaminergic midbrain ($r=0.41$, $p=0.033$). These data support the computational model proposed by Humphries et. al. that striatal D1 receptor activation is a key contributor to the tradeoff between exploitation and exploration and suggests that dopamine D1 receptors may be important for tracking changes in the mean reward rate and thus adjusting behavior (patch exit threshold) when moving from one environment to another. Furthermore, we provided evidence that monoaminergic synthesis capacity in the anterior cingulate cortex, a key area in the foraging network, is implicated in adaptive foraging behavior. These data provide direct insights into the roles of dopamine synthesis capacity and receptor availability in frontostriatal and midbrain circuitry during adaptive foraging behavior in humans.

National Institute of Mental Health (NIMH)

Michal Ramot

Visiting Fellow

Neuroscience - Integrative, Functional, and Cognitive

Direct modulation of aberrant network connectivity in Autistic Spectrum Disorder through real time NeuroFeedback

Numerous studies have documented aberrant patterns of brain functional connectivity in patients with Autistic Spectrum Disorders (ASD). Specifically, some cortical areas have been shown to be significantly under-connected in patients with ASD compared to typically developing (TD) control subjects, in a manner that is correlated with symptom severity. Traditional training techniques in ASD are limited and often do not generalize well beyond the training paradigm, and do not address the aberrant network structure. Recent studies have shown that real time neurofeedback can be used to train participants to modulate network activations, making it a promising candidate for a variety of clinical applications. However, specific, long-ranging, neurofeedback induced connectivity changes are yet to be shown. In this study we used previously collected data on large groups of ASD and TD participants to identify two target brain regions that showed the greatest under-connectivity in ASD while also being physically distant from each other and belonging to separate networks (in the superior temporal sulcus and in somatosensory cortex), and a third control region which was chosen to be uncorrelated to those regions in TD participants (in Inferior Parietal Lobule). We then used a novel method we developed for use with real time fMRI neurofeedback, allowing us to feedback correlations between regions, rather than just activation levels. Over a period of four days, participants with ASD were trained to increase the correlation between the two target regions, while simultaneously decoupling the target and control regions,. This training regime induced a large and significant change between the first and last day in the correlations between these three regions, with the two target regions showing increased coupling with training, whereas target-control pairs showed decreased coupling. Furthermore, whole brain analysis revealed that these changes were specific to the areas being trained. Moreover, preliminary results show a link between changes in behavior, and changes in the resting state connectivity following training. A control group of TD participants were trained on a different network configuration, to rule out any changes resulting from unforeseen task elements that were not the feedback itself. Together, these results suggest that neurofeedback can be used to directly alter clinically relevant, complex network connectivity patterns.

National Institute on Drug Abuse (NIDA)

Brooke Schmeichel

Postdoctoral Fellow Postdoctoral Fellow

Neuroscience - Integrative, Functional, and Cognitive

Hypocretin Neurotransmission within the Central Amygdala Mediates Escalated Cocaine Self-Administration and Stress-induced Reinstatement in Rats

Cocaine abuse affects nearly 1.7 million individuals per year and is characterized by patterns of excessive drug taking and preoccupation with obtaining cocaine. Experimental animals exhibit similar compulsive drug-seeking behaviors when exposed to extended access to cocaine self-administration. Compulsive behaviors in animals include excessive drug intake, elevated progressive ratio breakpoints, and reinstatement of cocaine-seeking following abstinence. Compulsive cocaine taking, in part, occurs through neuroplasticity of stress systems that mediate negative emotional states contributing to drug abuse. Brain stress systems play a significant role in the reinstatement of drug-seeking following extinction, a model of relapse. The hypocretin (HCRT) neuropeptide system has been implicated in stress processes, drug taking and reinstatement of drug-seeking. Evidence suggests HCRT may drive drug-seeking through activation of brain regions implicated in stress system dysfunction, including the central amygdala (CeA). The role of HCRT in compulsive behaviors associated with addiction has yet to be fully elucidated and is a potential novel target for treatment medications. The current studies characterized the role of HCRT within the CeA in the mediation of compulsive drug taking using behavioral and electrophysiological techniques in an animal model of cocaine addiction. Systemic and intra-CeA injections of the HCRT-receptor 1 antagonist, SB334867 (SB), were administered to rats allowed short (1h) or extended (6h) access to cocaine self-administration and tested for fixed and progressive ratio responding and stress-induced reinstatement of drug-seeking. Using electrophysiological techniques, we investigated GABA transmission in the CeA and sensitivity of GABAergic synapses to modulation of HCRT in short or extended cocaine-access rats. We found systemic administration of SB (0-30 mg/kg) dose-dependently decreased cocaine intake in extended, but not short, cocaine-access rats. Intra-CeA microinjections of SB also significantly reduced both cocaine intake and stress-induced reinstatement of cocaine-seeking in extended cocaine-access rats. Finally, electrophysiological data indicated enhanced GABA transmission within the CeA in extended cocaine-access rats, which was blocked with SB. These findings show a role for HCRT in the neuroplasticity associated with cocaine addiction, highlighting a significant new pharmacological target in the treatment of drug addiction.

National Institute on Drug Abuse (NIDA)

David Barker

Postdoctoral Fellow Postdoctoral Fellow

Neuroscience - Integrative, Functional, and Cognitive

Glutamatergic neurons of the Lateral Preoptic Neurons Synapse onto Ventral Tegmental Area GABAergic neurons, activation of this pathway provokes aversion

The Lateral Preoptic Area (LPO) serves as an important relay between neurons in forebrain limbic

structures and neurons in the ventral tegmental area (VTA), an area critically involved in addiction. Growing evidence has revealed that the VTA is far more complex than originally imagined, containing a mixture of dopamine neurons, glutamate neurons, GABA neurons, and even neurons capable of transmitting two or more of these neurotransmitters. Thus, while it has been known that LPO neurons target the VTA, and that this pathway plays a role in motivated behavior and drug addiction, it is unclear which cell types in the VTA are targeted by neurons in the LPO. To determine the VTA cell type that establishes synaptic contact with inputs from the LPO we injected into the VTA of three separate groups of mice expressing cre-recombinase in GABA, Glutamate, or Dopamine neurons (VGAT::Cre, VGLuT2::Cre and TH::Cre) a set of viral vectors that allow for cell-type specific retrograde tracing. This approach allowed us to retrogradely label and quantify LPO neurons that specifically synapse only on dopamine, glutamate or GABA neurons in the VTA. Surprisingly, we found that within the VTA, LPO neurons mainly synapse on GABAergic neurons, a few on glutamatergic and rarely on dopaminergic neurons. By a combination of retrograde tracing and in situ hybridization, we found that that a large number of LPO neurons targeting the VTA are glutamatergic. Thus we conclude that LPO glutamatergic neurons establish synapses with VTA GABAergic neurons. We next used an optogenetic approach to selectively express the light activated channel Channelrhodopsin2 (ChR2) in LPO glutamatergic neurons by injecting a viral vector encoding a Cre-dependent ChR2 into the LPO of VGLuT2::Cre mice. We found that stimulation of LPO glutamatergic terminals in the VTA resulted in escape behaviors and the development of a conditioned place aversion. We conclude that LPO glutamatergic neurons predominantly synapse on VTA GABA neurons, and that the activation of this excitatory projection from the LPO to the VTA signals aversion. This discovery opens a new avenue for investigating the role of this projection in drug abuse and motivated behavior, behaviors that have been associated with projections from the LPO to VTA.

National Institute of Environmental Health Sciences (NIEHS)

Shannon Farris

Postdoctoral Fellow

Neuroscience - Neurodegeneration and Neurological disorders

Transcriptome profiling in hippocampal dendrites

Several lines of evidence suggest that experience-dependent translation of mRNA in dendrites is required for the persistence of experience-dependent changes in the brain. This spatial restriction of mRNA to subcellular domains enables local regulation of proteins in a synapse-specific manner. It is currently unknown whether different cell types express different complements of dendritic RNAs to regulate specific forms of synaptic plasticity. Synapses in hippocampal area CA2 differ from those in neighboring CA1 in that they do not undergo typical long-term potentiation, a process that requires local protein synthesis. In fact, we found that even the maintenance of baseline synaptic transmission in CA2 may require dendritic protein synthesis, as translation inhibitors led to a decrease in excitatory synaptic responses in CA2, but not in CA1. These data suggest that local protein synthesis may play a critical role in gating synaptic plasticity in CA2 dendrites. To identify the RNA transcripts in CA2 and CA1 dendrites, we used laser-capture microdissection on hippocampal sections from a mouse line that expresses green fluorescent protein in CA2. RNA was extracted from CA2 and CA1 cell bodies and dendrites (N=3 adult male mice) and hybridized to transcriptome microarrays or used to generate cDNA libraries for RNAseq.

We identified 751 genes that were higher in CA2 cell bodies and 400 genes higher in CA2 dendrites compared to CA1, indicating that CA2 neurons express distinct complements of RNAs. Further, the RNAseq data correlated well with the array data ($R^2=0.79$, Pearson's correlation). Using NextBio gene ontology software to identify canonical pathways, we found that compared to CA1, CA2 cell bodies and dendrites were enriched for genes regulating lipid synthesis and metabolism. However, MAPK and calcium signaling pathways were enriched in CA1 dendrites, consistent with the high levels of plasticity there. Single molecule fluorescent in situ hybridization was used to confirm dendritic localization of CA2 mRNAs, including *Pcp4*, *Rgs14* and *Adcy1*, all of which have been proposed to regulate synaptic plasticity. This data set will allow us to identify alternative splice variants in CA2 transcripts that may mediate RNA stability and translation in dendrites. Identifying molecules in CA2 dendrites may lead to novel therapeutic targets for disorders such as autism, where dendritic protein synthesis has gone awry.

National Institute of Environmental Health Sciences (NIEHS)

Georgia Alexander

Research Fellow

Neuroscience - Neurodegeneration and Neurological disorders

Chemo-Genetic Activation of Hippocampal Area CA2 Neurons Increases Gamma Oscillations in Hippocampus and Prefrontal Cortex

Networks of neurons communicate through oscillations, which reflect synchronized action potentials of individual neurons. Communication between neuronal networks is the basis of cognition and is required for many behaviors. Gamma oscillations (30-120Hz) occur in the hippocampus, coordinating the activation of local neuronal networks and propagating to distant networks with which they communicate. Gamma oscillations have attracted considerable attention in schizophrenia research because individuals with the disease display abnormal gamma oscillations, as measured in hippocampus and prefrontal cortex (PFC). Although relatively understudied, the CA2 field of the hippocampus is becoming increasingly recognized as a socio-cognitive hub for processing memories with socially relevant information. Because impaired social cognition is a common symptom associated with schizophrenia, and because CA2 is unique within the hippocampus in its susceptibility to loss of a type of neuron that contributes to gamma oscillations in schizophrenia, abnormal gamma activity in CA2 emerges as a candidate mechanism for linking the known cellular and network pathologies with the sociocognitive impairments observed in individuals with schizophrenia. To address the relationship between CA2 neuronal activity and gamma oscillations in hippocampus and PFC, we infused viruses coding for a cre-dependent excitatory DREADD (Designer Receptors Exclusively Activated by Designer Drugs) into hippocampi of mice that express cre recombinase selectively in CA2 pyramidal cells. We then implanted electrode arrays to simultaneously monitor activity of hippocampal and PFC neurons before and following administration of the DREADD ligand, Clozapine-N-oxide (CNO), while animals freely explored an open field. We found that CNO increased firing of CA2 pyramidal neurons and dose-dependently increased gamma oscillations in both the hippocampus and PFC, but not in areas outside of PFC. At the level of the healthy brain, these findings demonstrate that activation of CA2 neurons is sufficient to induce gamma oscillations within the hippocampus as well as the PFC, suggesting that the CA2 circuitry plays into distributed neuronal networks. In the diseased brain, these findings support the idea that the loss of neurons from CA2 may underlie the abnormal gamma oscillations in patients with

schizophrenia and provide a mechanistic link to the sociocognitive impairments observed in these patients.

National Institute of Environmental Health Sciences (NIEHS)

Sheng Song

Visiting Fellow

Neuroscience - Neurodegeneration and Neurological disorders

NADPH oxidase-mediated mitochondrial dysfunction contributes to high vulnerability of locus coeruleus noradrenergic neurons in response to inflammation

Clinical evidence revealed that the loss of locus coeruleus noradrenergic (LC/NE) neurons is greater and occurs earlier than dopaminergic neurons in the substantia nigra (SN/DA) in Parkinson's disease (PD) patients. However, the mechanism remains unclear. The purpose of this study were: 1) to develop a rodent PD model displaying a temporal pattern of neurodegeneration that ascends caudo-rostrally from the lower brainstem to front cortex by a systemic injection of endotoxin, LPS; and 2) to elucidate mechanisms underlying the extreme vulnerability of LC/NE neurons during low-grade, chronic neuroinflammation. We found that LPS-injected mice displayed an ascending pattern of neurodegeneration similar to what found in PD brains: neuron loss started in LC/NE, followed by SN/DA, and finally in cortical and hippocampal regions. Mechanistic studies revealed that LC/NE neurons of normal mice exhibited higher degree of mitochondrial oxidative stress than neurons in other brain regions including SN/DA neurons, which was further elevated by LPS-induced neuroinflammation. Further studies showed that the activation of neuronal NADPH oxidase (NOX2) correlated with the degree of mitochondrial oxidative stress and subsequent dysfunction. We found that LPS treatment caused greater superoxide production and damage of mitochondria in LC/NE neurons than that of SN/DA neurons. These in vivo findings were further confirmed by in vitro studies showing that LPS activated neuronal NOX2, increased production of intracellular superoxide, suppressed ATP production and collapsed mitochondrial membrane potential in cultured neurons. Moreover, pharmacological inhibition or genetic ablation of NOX2 greatly attenuated LPS-induced mitochondrial dysfunction and afforded LC/NE neurons protection. Finally, this neuronal NOX2-mediated mitochondria dysfunction-based vulnerability of LC/NE neurons was further verified in a "two-hit" PD model generated by a single systemic LPS injection in transgenic mice over-expressing human A53T mutant alpha-synuclein. In summary, our findings pointed to neuronal NOX2 activation and a resulting mitochondrial dysfunction as critical factors contributing to the extreme vulnerability of LC/NE neurons in PD.

National Institute on Aging (NIA)

Roger Mullins

Postdoctoral Fellow

Neuroscience - Neurodegeneration and Neurological disorders

Low glucose utilization and high lactate production in the Alzheimer's disease brain

Two defining pathological characteristics of Alzheimer's Disease (AD) in the brain are the presence of

amyloid-beta plaques and severe abnormalities in glucose metabolism. In particular, the posterior cingulate/precuneus region is strongly affected by early amyloid-beta deposition and decreased glucose utilization associated with AD. In addition, brain regions with prominent amyloid-beta deposition also have a higher metabolic reliance on glycolysis, which results in production of lactate. We hypothesize that AD patients show decreased glucose metabolism and increased lactate production, resulting in higher concentrations of glucose and lactate in areas that are heavily affected by AD pathology. This study used a sample of 45 cognitively normal (CN) younger controls (42 ± 9 years old), 8 CN older controls (69 ± 10 years old), and 29 patients with biomarker-supported (low CSF amyloid-beta-42, high tau and/or p-181-tau) high-probability early AD (74 ± 9 years old). Glucose and lactate levels were obtained using Magnetic resonance spectroscopy (MRS), specifically a 2D junctional Point-Resolved Spectroscopy (jPRESS) sequence. Raw data from the 2D jPRESS sequence was analyzed using the ProFit tools in MATLAB to assess metabolite concentrations within a $25 \times 18 \times 20$ mm posterior cingulate/precuneus voxel. We found that diagnostic group had a significant effect on both glucose and lactate (normalized to creatine), with AD patients having higher glucose ($p < 0.0001$) and lactate concentrations ($p < 0.001$) than the CN participants. We propose that glucose and lactate levels in the posterior cingulate/precuneus region can serve as novel and potent biomarkers for understanding the abnormal brain metabolism associated with AD.

National Institute of Child Health and Human Development (NICHD)

Alejandro Alvarez-Prats

Visiting Fellow

Neuroscience - Neurodegeneration and Neurological disorders

DISSECTING THE ROLE OF A LIPID KINASE IN PERIPHERAL NERVE MYELINATION

Abstract removed at request of the author

National Institute of Neurological Disorders and Stroke (NINDS)

Rahilla Tarfa

Doctoral Candidate

Neurotransmission and Ion Channels

Comparison of high-frequency firing among dopamine neurons grouped according to axonal projection pattern.

Midbrain dopamine neurons are involved in a diverse number of brain circuits including reward, motivation, and movement, with individual dopamine neurons sending axonal projections to discrete brain nuclei. In addition to anatomical diversity, dopamine neurons differ substantially in their function and excitability. Specifically, dopamine neurons that project to the cortex (mesocortical) fire action potentials at substantially higher rates than those that project to the nucleus accumbens (mesoaccumbal), but the precise mechanism is unknown. Therefore, we analyzed ionic currents that enable high-frequency action potential firing in dopamine neurons grouped according to their axonal projection patterns. To do this, we retrogradely labeled dopamine neurons using fluorescent tracers and

recorded their electrical activity using whole-cell patch clamp techniques. Mesocortical neurons are unusual as they lack dopamine (D2) autoreceptors. The tonic release of dopamine during high-frequency firing is thought to inhibit spiking of mesoaccumbal neurons, while firing in mesocortical neurons is thought to be largely unaffected by D2 activation. To test the influence of auto-inhibition of D2-receptors, we recorded firing in the presence of D2-autoreceptor blockers. We observed no significant change in the maximal firing rates of both subpopulations. This suggests that differences in D2-autoreceptor activation cannot explain the discrepancy in firing frequency among the dopamine neuron subpopulations. But, why do mesocortical neurons fire at higher rates? Our results indicate that mesocortical neurons have the narrowest action potential widths. The amount of calcium entry during an action potential contributes to its width. Past studies have shown that calcium and its dependent currents are most prevalent in mesoaccumbal neurons. We tested for differences in the calcium-dependent potassium (SK) current and found that they are present in high densities in mesoaccumbal neurons, and limit their high frequency firing. Overall, the difference in dopamine subpopulation firing had been based on the incorrect assumption that D2 autoinhibition was the mechanism underlying the difference in firing. Instead, we show that differences in intrinsic currents, like SK, contribute to their heterogeneity, and provide alternative targets that can be used in treating dopamine subpopulation specific diseases like addiction and parkinson's.

National Institute on Alcohol Abuse and Alcoholism (NIAAA)

Kari Johnson

Postdoctoral Fellow

Neurotransmission and Ion Channels

Input-specific modulation of striatal glutamatergic neurotransmission by metabotropic glutamate receptors

The striatum is a part of the brain that plays important roles in movement, action selection, and habit formation. Chronic exposure to drugs of abuse alters striatal function and biases action selection to promote drug-seeking behavior. Understanding how striatal function is regulated will provide critical opportunities to pharmacologically modify drug-induced adaptations in striatal circuits and related behaviors. Striatal neurons are controlled by glutamatergic inputs from several brain regions, and the strength of these inputs can be modulated by activation of presynaptic G protein-coupled receptors (GPCRs). Previous efforts to evaluate GPCR-mediated modulation of striatal glutamatergic transmission have employed techniques that cannot distinguish the contributions of different inputs to the striatum; particularly, little is known about modulation of thalamostriatal circuitry. Inputs from individual cortical and thalamic regions exert differential control over striatum-dependent behaviors, so it is critical to understand how discrete circuits are modulated to identify novel therapeutic strategies that are targeted to the circuit-level causes of pathological behavior. To study modulation of striatal circuits by metabotropic glutamate receptors (mGlu2 and mGlu3), we used viral and transgenic strategies to express Channelrhodopsin-2 (ChR2) in cortical or thalamic projections to the mouse striatum. We performed ex vivo whole-cell patch clamp recordings of optically-evoked excitatory postsynaptic currents (oEPSCs) in striatal neurons and evaluated the effects of drugs targeting mGlu2/3 on corticostriatal and thalamostriatal transmission. The mGlu2/3 receptor agonist LY379268 produced a robust depression of thalamostriatal oEPSC amplitude. The effect of LY379268 on thalamostriatal

transmission was mimicked by an mGlu2-selective agonist and was not blocked by an antagonist of mGlu3, suggesting a major, previously-unrecognized role for mGlu2 in the modulation of thalamostriatal transmission. Interestingly, inhibition of corticostriatal transmission by mGlu2 was less pronounced. Finally, inhibition of EPSCs by LY379268 was disrupted by a two week alcohol vapor exposure procedure. Because drugs that enhance mGlu2 function are currently in clinical development for alcohol and drug use, these timely studies will provide critical information about the effects of mGlu2 activation on striatal function and how mGlu2 activation could correct drug-related behaviors.

National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)

Sarah Morgan

Postdoctoral Fellow

Pharmacology and Toxicology/Environmental Health

TSH and IGF1 Receptor Crosstalk Upregulates Thyroid-Specific Genes in Primary Cultures of Human Thyrocytes

Major regulation of thyroid gland function is mediated by thyrotropin (TSH) activating the TSH receptor (TSHR), a G protein-coupled receptor, on thyrocytes. TSHR signals via cAMP and beta-arrestin1 (bARR1). TSH stimulates upregulation of thyroid-specific genes including thyroglobulin (TG), thyroperoxidase (TPO), sodium-iodide symporter (NIS), and type II iodothyronine deiodinase (DIO2), that are involved in thyroid hormone synthesis. Evidence suggests that the insulin-like growth factor 1 (IGF1) receptor (IGF1R) may play a role in regulating the effects of TSHR stimulation. We sought to examine the potential role of TSHR/IGF1R crosstalk in primary cultures of human thyrocytes. TSH/IGF1 cotreatment elicited additive effects on TG, TPO, and DIO2 mRNA levels but synergistic effects on NIS mRNA. Similarly, TSH and IGF1 had additive effects on TG protein secretion (1.61±0.15 times TSH alone) yet synergistically induced NIS protein expression (11.9±4.5 times 1 mU/mL TSH alone). The IGF1R tyrosine kinase inhibitor linsitinib dose-dependently inhibited TSH-stimulated upregulation of NIS (up to 94±3%) but not TG. The effects of TSH alone were not inhibited by antibodies that block binding to the IGF1R and, therefore, were mediated by TSHR/IGF1R crosstalk initiated by binding to TSHR. The cooperative effects of TSH and IGF1 were not mediated by cAMP. Knockdown of bARR1 had no effect on TG expression via TSH alone or with IGF1, nor did it affect TSH-mediated NIS expression. However, bARR1 knockdown abolished the synergistic effect of TSH and IGF1 on NIS. Because bARR1 can signal via ERK1/2 and Akt, we examined the effects of ERK (U0126) or Akt (MK-2206) inhibition on the induction of these genes. Neither inhibitor had a significant effect on TG expression; however, both inhibitors reduced NIS stimulation by TSH (U0126 by 79±9%, MK2206 by 63±8%) and blocked the synergistic effect of IGF1 (by 64±8% and 75±5%, respectively). We conclude that crosstalk between TSHR and IGF1R plays an important role in thyroid cell function. IGF1 enhances the TSH-mediated effect on all genes examined, but significantly increases the response to TSH with regard to NIS expression that appears to occur via bARR1 signaling, including ERK and Akt activation. Fully understanding the nature of this crosstalk, and particularly the mechanisms of NIS regulation, has clinical implications for the treatment of thyroid diseases including thyroid cancer.

National Institute on Drug Abuse (NIDA)

Brendan Tunstall

Postdoctoral Fellow

Pharmacology and Toxicology/Environmental Health

Oxytocin Blocks Compulsive-like Alcohol Drinking

From today's global population, an estimated 440 million people will die or become disabled due to alcohol. Two main sources of motivation drive alcohol drinking: alcohol's rewarding and stress-relieving effects. We hypothesized that brain signaling by the neuropeptide oxytocin, known to be involved in both stress and reward function, contributes to compulsive alcohol drinking. To our knowledge, there are currently no studies comparing the effect of oxytocin on alcohol drinking in alcohol-dependent vs. nondependent rodents or humans. We used a preclinical model of alcohol dependence that reliably produces somatic and motivational signs of dependence to test our hypothesis. Wistar rats were trained to lever press for access to alcohol and then either made alcohol dependent (via repeated cycles of alcohol vapor exposure) or exposed to air to provide a nondependent control group. Oxytocin (0-1 mg/kg) was administered systemically (intraperitoneal route) and intranasally, a novel route of peptide administration, to facilitate brain penetrance and avoid peripherally mediated side-effects. The results indicate that alcohol-dependent rats developed escalated alcohol consumption and alcohol seeking behavior (responding when response requirement is increased) compared to nondependent controls. Both systemic and intranasal oxytocin blocked compulsive-like alcohol consumption and seeking in dependent rats, at doses which did not disrupt alcohol drinking in nondependent rats. This suggests that oxytocin can selectively block the compulsive-like alcohol motivated behaviors which emerge in alcohol dependence. Additional experiments controlling for side-effects of oxytocin indicated that systemic oxytocin reduced spontaneous locomotion (open field assay) as well as the consumption of sweet (non-caloric saccharin) and caloric (non-sweet maltodextrin) fluids, whereas intranasal oxytocin treatment had no effect in these measures. Collectively, these findings suggest that oxytocin can reduce alcohol drinking through a direct action on motivation for alcohol's pharmacological effect (rather than gustatory/caloric properties, or motivation in general) and that intranasal administration is more effective in this regard. This is important, as intranasal oxytocin represents a viable, novel approach to treating alcohol use disorders. A clinical trial informed by the present data is underway, testing the efficacy of intranasal oxytocin in humans with alcohol use disorders.

NIH Clinical Center (CC)

Kristina Brooks

Postdoctoral Fellow

Pharmacology and Toxicology/Environmental Health

Cobicistat Increases the Effects of Dabigatran on Thrombin Time in Healthy Volunteers

Background: Dabigatran etexilate (DE) is an oral direct thrombin inhibitor and substrate of intestinal Permeability-glycoprotein (P-gp) and renal multidrug and toxin extrusion-1 (MATE-1) transporters. Cobicistat (COBI) is a pharmacokinetic (PK) enhancer and inhibitor of P-gp and renal MATE-1 transporters, which may result in increased DE exposure when coadministered. Using thrombin time (TT), we sought to characterize the pharmacodynamic (PD) effects of COBI on DE, and if this potential interaction may be mitigated by separated administration. Methods: This was a single-center, open-

label, fixed sequence, intra-subject study conducted in healthy volunteers. The study was comprised of 3 phases: (1) DE 150 mg x1 alone (day 0), (2) DE 150 mg x1 two hours prior to COBI 150 mg (day 19±1), and (3) DE 150 mg x1 with COBI 150 mg (day 26±1). Subjects underwent a 5-day washout period following phase 1, followed by COBI 150 mg once daily throughout Phases 2 and 3 (days 5 - 26±1). Blood was collected at 0, 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 hours post-dose on DE dosing days. TT was determined using STA®-Thrombin reagent (Diagnostica Stago, Asnières-sur-Seine, France). Noncompartmental methods were used to derive DE PD parameters (TT area-under-the-effect-curve [AUEC] and thrombin time at 24 hours [TT-last]). Geometric mean ratios (GMR) with 90% confidence intervals [CI] were compared between phases and p-values were calculated using a two-tailed paired t-test. Results: A total of 18 subjects were enrolled, with 16 completing the study and 2 without phase 3 data due to lack of study compliance. There was a 30% and 33% increase in AUEC GMR between Phase 2 and Phase 1 (p below 0.0001, 90% CI [1.21, 1.39]), and Phase 3 and Phase 1 (p below 0.0001, 90% CI 1.22-1.44), respectively. Significant increases in TT-last GMR of 46% and 51% were also observed between Phase 2 and Phase 1 (p below 0.0001, 90% CI [1.30, 1.61]) and Phase 3 and Phase 1 (p below 0.001, 90% CI [1.24, 1.78]), respectively. No significant differences were noted between Phase 3 and Phase 2 in GMR of AUEC (p=0.551, 90% CI [0.95, 1.09]) or TT-last (p=0.595, 90% CI [0.89, 1.17]). Conclusions: COBI coadministration resulted in significant increases in TT AUEC and TT-last vs. DE alone. These effects were preserved despite separating COBI and DE administration by 2 hours, supporting the putative mechanisms of P-gp and MATE-1 inhibition by COBI. Further analyses of changes in DE PK-PD relationships are warranted.

National Institute on Alcohol Abuse and Alcoholism (NIAAA)

Yong He

Doctoral Candidate

Pharmacology and Toxicology/Environmental Health

Hepatic mitochondrial DNA-TLR9-microRNA-223 forms a negative feedback loop to control acetaminophen-induced acute liver injury and inflammation

Acetaminophen (APAP) overdose is one of leading causes of acute liver failure worldwide, which is accompanied with significant neutrophil infiltration in the liver; however, the mechanisms underlying hepatic neutrophil infiltration and neutrophilic inflammation remain obscure. In this study, we found microRNA-223 (miR-223), one of the most abundant miRNA in neutrophils that acts as a fine-tuner of the generation and function of neutrophils, was found to be significantly elevated in the liver and serum after APAP injection in mice. The function of miR-223 in APAP-induced liver inflammation was investigated in miR-223 knockout (KO) mice. Compared with wild-type (WT) mice, miR-223KO mice were more susceptible to APAP-induced liver injury, as indicated by higher levels of serum alanine transaminase (ALT), aspartate transaminase (AST), and liver necrosis with or without overnight-fasting. In addition, miR-223KO mice showed greater degree of neutrophil and macrophage infiltration in the liver after APAP injection than WT mice. Accordingly, inflammatory response and oxidative damage manifested by 4-Hydroxynonenal (4-HNE) and N-nitrotyrosine expression were more pronounced in the APAP-treated miR-223KO mice compared with WT mice. Intriguingly, TLR9 ligand or free DNA released from necrotic hepatocytes increased miR-223 expression in neutrophils in vitro and in vivo through a Toll-like receptor 9 (TLR9)/NF- κ B dependent mechanism. In addition, neutrophils lacking of miR-223

were more sensitive to TLR9 ligand-induced production of proinflammatory cytokines in vitro. Mechanistically, miR-223 was found to control APAP-induced liver injury and inflammatory response by regulating IKK α expression. Taken together, DNA released from necrotic cells exacerbates APAP-induced hepatotoxicity by activating TLR9-mediated inflammation, meanwhile, DNA-TLR9-miR-223 forms a negative feedback loop to control liver injury and inflammation. These findings strongly suggest that miR-223 is a key mediator controlling the acute neutrophilic response and may be used as a therapeutic target for the treatment of drug-induced liver injury.

National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)

Xinran Ma

Visiting Fellow

Physiology

Forkhead box A3 mediates glucocorticoid receptor function in adipose tissue

Glucocorticoids (GCs) are a class of steroid hormones that bind to GC receptor (GR) and exert broad physiological functions and have been widely used in the clinic for the treatment of inflammatory diseases. However, long-term GC treatment has been associated with numerous adverse effects on metabolism, such as central obesity, increased fat mass, muscle wasting, hepatic steatosis, hyperlipidemia and insulin resistance but the mechanism is still largely unknown. Forkhead-box (Fox) proteins are a large family of transcription factors that bind DNA through a conserved winged-helix binding motif and shown to be critically in aging, metabolism, and development. Among them, Foxa3 is a newly identified transcriptional factor that promotes the selective expansion of epididymal fat and suppresses energy expenditure in a nutritional- and age-dependent manner. Meanwhile, Foxa3 levels are elevated in adipose tissues in response to high fat diet regimen and aging; however the mechanism that controls Foxa3 levels in fat is not clear. Given the established roles of GCs in increasing central obesity and in reducing energy consumption, we examine the possible link between GCs and Foxa3. In silico analysis predicts a GR response element at Foxa3 promoter and molecular analysis validates that with GC treatment, GR binds to the Foxa3 promoter and activates Foxa3 transcriptionally. In addition, gene array analysis comparing cells with or without Foxa3 deficiency upon GC treatment shows that Foxa3 is required for the induction of GR target gene programs in both preadipocytes and adipocytes. Mechanistically, Chromatin-IP results show that Foxa3 facilitates the binding of the GR to its target gene promoters via chromatin remodeling events. Finally, physiological study of the chronic GC treatment in WT and Foxa3 global knockout mice reveals that Foxa3 ablation specifically attenuates GC signaling in fat depots and protects mice against fat accretion without affecting other pathological side effects in liver, muscle and spleen. In conclusion, we demonstrate that Foxa3 is a direct gene target of GR in fat. Once induced, Foxa3 acts as a “pioneer factor” to open chromatin structure for GR binding to more gene targets and mediates GC-induced gene programs in adipose tissues, which proposes Foxa3 as a potential clinical target for the attenuation of side effects of GC treatment in fat depots.

National Heart, Lung, and Blood Institute (NHLBI)

Xiangbo Ruan

Visiting Fellow

Physiology

Regulation of Gluconeogenesis by Long non-coding RNAs in Human Primary Hepatocytes

Long non-coding RNAs (lncRNAs) are RNA transcripts longer than 200 bp and without coding potential. They are widely transcribed from mammalian genomes while their physiological functions are largely unknown. We have recently demonstrated that lncRNAs play important roles in regulating glucose and lipid homeostasis in mice. However, since most lncRNAs are primate- or human-specific, the importance of lncRNAs to human metabolism remains to be determined. Hepatic gluconeogenesis is a major pathway that maintains plasma glucose levels during fasting and is also a key contributor to hyperglycemia in diabetes. In light of the potent effects of lncRNAs on energy metabolism in mice, we hypothesize that, similar to human protein coding genes, human lncRNAs regulated by fasting signaling might play important role in human gluconeogenesis. Thus, we first systemically screened for lncRNAs that are induced by glucagon, a hormone inducing glucose production from liver during fasting, in cultured primary human hepatocytes. Totally, we found 302 lncRNAs that were significantly upregulated by glucagon treatment. The regulations of top 10 most increased lncRNAs could be faithfully verified by real time PCR and 8 of them were also upregulated by glucagon in human hepatocytes isolated from a second donor. We next tested the function of these glucagon-induced lncRNAs in gluconeogenesis by depleting these lncRNAs with siRNAs and performing glucose production assay in primary human hepatocytes. We found loss-of-function of one of them, we termed lncGIRG (glucagon-induced repressor of gluconeogenesis), resulted in increased glucose production. Finally, to explore the mechanism of action of lncGIRG, we performed genome-wide gene expression analyses in lncGIRG-depleted human hepatocytes and identified increased expression of an array of genes in gluconeogenic pathway including glucose-6-phosphatase, catalytic subunit (G6PC) and phosphoenolpyruvate carboxykinase 1 (PCK1). These results suggest that lncGIRG might serve as a negative regulator of gluconeogenesis by suppressing gluconeogenic gene expression in human hepatocytes. Taken together, our study supports that lncRNAs could have important functional roles in human metabolism, and lncGIRG might serve as a negative regulatory mechanism of hepatic gluconeogenesis in human. lncRNAs such as lncGIRG could potentially constitute important therapeutic targets for managing hyperglycemia in patients with diabetes.

National Institute of Environmental Health Sciences (NIEHS)

Matthew Schellenberg

Visiting Fellow

Protein Structure/Structural Biology

SUMO Recognition Mediated by a Novel "Split-SIM" Class of SUMO-binding Motifs

Akin to Ubiquitination, post-translational modification of proteins with Small Ubiquitin-like Modifier (SUMO) plays important roles in many cellular pathways. Protein SUMOylation is recognized by "reader" proteins, which bind SUMO using a SUMO Interacting Motif (SIM) that contributes a fifth beta-strand to the SUMO beta sheet. A SIM consensus sequence is typically found in flexible and solvent accessible regions of proteins, however the semi-conserved nature of this motif obfuscates clear prediction of SIM

based on primary amino acid sequence, such that even in cases with clear biochemical evidence for SUMO binding a SIM sequence may not be apparent. To understand how such proteins may bind to SUMO, we targeted SUMO protein complexes with interesting SUMO-binding proteins for X-ray crystallography trials. Topoisomerase II (Topo II) relieves topological strain in double-stranded DNA, and is SUMOylated in response to chemotherapeutic drugs that target Topo II. Tyrosyl-DNA Phosphodiesterase 2 (Tdp2) binds to the SUMO2 isoform and is a key effector of cellular resistance to Topo II drugs. We found the catalytic domain of Tdp2 contains the SUMO2 binding site, however this globular domain lacks a canonical SIM sequence. We solved two crystal forms of Tdp2 bound to SUMO2. These share a common Tdp2-SUMO2 architecture, which we confirmed exists in solution using Small Angle X-ray Scattering (SAXS). Interestingly, Tdp2 makes use of amino acids distal in primary sequence to form a “split-SIM” by transitioning an alpha-helix near the N-terminus of Tdp2 to a beta-strand, along with extension of a C-terminal beta-strand to occupy the SIM binding site in SUMO2. Additional surface loops of the globular Tdp2 catalytic domain expand this interface. We determined the K_d for this complex is 0.88 micromolar using NMR, which places Tdp2 amongst the strongest SUMO binding proteins. We also designed mutations in the Tdp2 split-SIM and verified that they disrupt SUMO interaction both in vitro and in vivo. Our work shows that a SIM-like sequence can be composed of residues distal in primary sequence. This sets a precedent: that SIM-like SUMO binding can be effected by short protuberances from a globular domain. From our Tdp2-SUMO2 structure, we propose short consensus sequences that could be capable of forming a split-SIM in globular domains, and this will aid the field in identification of SUMO binding-sites in the future.

National Heart, Lung, and Blood Institute (NHLBI)

Madeleine Davison

Visiting Fellow

Protein Structure/Structural Biology

A novel HIV-1 inhibitor blocks ubiquitin recognition by Tsg101

Proton pump inhibitors (PPIs) are traditionally used to treat gastrointestinal bleeding, stomach ulcers, and acid reflux. Recently, high-throughput small-molecule screening also identified these compounds as potent inhibitors of HIV-1, the causative agent of AIDS. The inhibitors cause viral particles to become tethered to the surface of an infected cell, preventing their release to infect other cells. Tsg101, the human protein that was the target of the small-molecule screening, is known to be hijacked by HIV-1 Gag, and would normally recruit the cellular machinery that pinches off and releases the budding HIV-1 particle. In order to develop improved versions of the drugs tailored to the treatment of HIV-1, and to further understand the mechanism of inhibition, we determined the high-resolution structure of Tsg101 with one of the inhibitors identified in the screen. We located the binding site of tenatoprazole by following changes in the NMR spectrum of Tsg101 upon addition of the compound. However, the compound did not bind Tsg101 near to the binding site of HIV-1 Gag; instead, tenatoprazole formed a covalent interaction with a cysteine residue near to the ubiquitin recognition site of Tsg101, as confirmed by mass spectrometry. The fact that the drug targeted virus particle release is interesting, since the interaction did not interfere with the binding of Gag, but instead lowered the ubiquitin-Tsg101 binding affinity, indicating a central role of the latter in HIV-1 particle production. However, the covalent nature of the tenatoprazole-Tsg101 interaction complicated further structural studies since many NMR

experiments rely on a non-covalent interaction between protein and ligand. To solve this problem, we labeled the protein with a carbon isotope and used NMR filtering experiments to preferentially observe intermolecular Tsg101-tenatoprazole resonances while simultaneously removing intra-protein resonances. In this way, the orientation of the drug in the binding site could be determined for use in future computer-aided design of an improved inhibitor. In summary, the knowledge gained has revealed a new target for the inhibition of HIV-1 particle production, namely ubiquitin-Tsg101 interaction. Moreover, the high-resolution structure of the Tsg101-tenatoprazole complex has provided us with the necessary tools to improve the binding characteristics of this novel class of HIV-1 inhibitors.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Xiang Chen

Research Fellow

Protein Structure/Structural Biology

Structures of Rpn1 complexed with ubiquitin and K48 diubiquitin define Rpn1 as a novel proteasome ubiquitin receptor

Proteolysis by the proteasome is essential for diverse cellular events including orderly cell cycle progression, activation of transcription factors, and protein quality control. Misregulation of the ubiquitin-proteasome system (UPS) is associated with a broad range of human diseases, such as cancer, developmental disorders, neurodegenerative diseases, immune disorders, and microbial infections. Substrates for the proteasome are first ubiquitinated by an enzymatic cascade, allowing for their recognition by ubiquitin receptors that are intrinsic to the proteasome. Two proteasome subunits have been defined as ubiquitin receptors, Rpn10 and Rpn13. We have identified a third ubiquitin receptor in the proteasome, the 110-kDa proteasome component Rpn1. We find that a site within the Rpn1 toroid recognizes ubiquitin as well as ubiquitin-like (UBL) domains of proteins that shuttle substrates to proteasomes. We name this ubiquitin-binding region of Rpn1 T1 (Toroid 1). We use nuclear magnetic resonance (NMR) to solve the structures of the Rpn1 T1 alone and complexed with monoubiquitin or K48-linked diubiquitin. The Rpn1 T1 site consists of seven helices that form 3.5 helix-turn-helix hairpins (PC repeats). The structures of Rpn1 T1 with monoubiquitin or K48-linked diubiquitin show three neighboring outer helices of the T1 site engaging two ubiquitins. The two binding sites dictate a preference for K48- and K6-linked ubiquitin chains that can be exploited to place substrates at the ATPase ring, where they are unfolded and passaged into the proteasome proteolytic chamber. These structures also illustrate a new path on proteasome for binding ubiquitinated substrates that is on the same side of the proteasome as Rpn10 and Rpn13. We also find that proximal to the T1 site is a second UBL-binding site (T2) in the Rpn1 toroid that assists in ubiquitin chain disassembly, by binding the UBL of deubiquitinating enzyme Ubp6. Thus a two-site recognition domain intrinsic to the proteasome uses homologous ubiquitin/UBL-class ligands to assemble substrates, shuttling factors, and a deubiquitinating enzyme. Altogether this work provides fundamental information on how cells target proteins for proteolysis by the proteasome.

National Institute on Drug Abuse (NIDA)

Landing Moran

Postdoctoral FellowPostdoctoral Fellow

Psychiatry

Aripiprazole for cocaine abstinence: a randomized controlled trial with ecological momentary assessment

A major problem in treating addiction is the likelihood of relapse: it can occur after seemingly successful abstinence, with an array of precipitants that often include drug-associated environmental cues. In the rat reinstatement model of relapse, resumption of extinguished cocaine-seeking by cues and small priming doses of cocaine was reduced by aripiprazole, a partial agonist at dopamine D2 receptors with affinity for several other monoamine receptors. The clinical implication of these findings is that aripiprazole might prevent relapse in addicted cocaine users who have become abstinent. To date, studies on aripiprazole have shown mixed results regarding effects on stimulant use. However, the participants in these studies were currently misusing stimulants and not seeking treatment; relapse prevention was not an outcome measure. We designed a clinical trial to test the hypothesis that aripiprazole would prevent or delay cocaine relapse in abstinent former users of cocaine. We also hypothesized that aripiprazole would reduce cocaine craving in daily life, as assessed by ecological momentary assessment (EMA), in which participants carried PDAs to report their activities and moods in real time. We employed a randomized double-blind clinical trial for patients in an outpatient methadone-maintenance clinic with two treatment groups: aripiprazole (15 mg oral daily; n=9) and placebo (n=9). To establish abstinence prior to aripiprazole induction, contingent vouchers were given for each cocaine-negative urine specimen during the first 12 weeks. Participants who were abstinent from cocaine during weeks 11 and 12 were randomized to receive aripiprazole or placebo in weeks 13 through 27. Only 45% (18/40) of the enrolled participants met abstinence criteria for randomization. Aripiprazole appeared to increase the time to lapse (one cocaine-positive urine sample) and relapse (two consecutive cocaine-positive urine samples), but there was no significant difference between treatment groups in these measures or in the proportion of cocaine-negative urines. EMA reports of cocaine craving were greater in participants who received aripiprazole (3.01% vs. 0.93%, $F=18.28$, $p<0.001$). Although further assessment with a larger sample is needed to determine aripiprazole's potential utility as a treatment for cocaine addiction, the use of EMA provided novel information on craving in real time in the participant's normal environment, following aripiprazole treatment.

National Institute of Neurological Disorders and Stroke (NINDS)

Carine Maurer

Clinical FellowClinical Fellow

Psychiatry

Neural correlates of impaired self-agency in functional movement disorders

Background: Patients with functional movement disorders (FMD), a type of conversion disorder, represent one of the more common disorders referred to the modern neurology clinic. Despite its prevalence, the mechanisms underlying FMD remain poorly understood. Impairment in self-agency, the sense that one is controlling one's own actions, is a characteristic feature of FMD, with patients reporting lack of voluntary control over their abnormal movements despite physiologic evidence

demonstrating that the movements use voluntary motor pathways. According to the influential comparator model, the sense of self-agency relies upon the right temporo-parietal junction (TPJ), which plays the critical role of “mismatch detector”, comparing internal predictions of movement with feedback from actual external events. To investigate the neural mechanisms contributing to impaired self-agency in FMD patients, we explored group differences in resting state functional connectivity from the right TPJ between FMD patients and age- and sex-matched healthy controls (HCs). Methods: We obtained resting-state fMRI on 35 patients with clinically definite FMD and 35 HCs. Between-group differences in functional connectivity from the right TPJ were assessed using the AFNI tool 3dGroupInstaCorr. Scores on the Beck Depression Inventory, Childhood Trauma Questionnaire, and Hamilton Anxiety and Depression Rating Scales were included as covariates. Significant between-group differences were determined by Monte-Carlo simulation resulting in a cluster-level significance threshold of $p < 0.05$. Results: Compared to HCs, patients with FMD showed decreased functional connectivity between the right TPJ and bilateral sensorimotor regions, including the right sensorimotor cortex, bilateral cerebellum, bilateral supplementary motor area, and right insula. These findings were independent of depression, anxiety and childhood trauma scores. Conclusions: The decreased functional connectivity between the right TPJ and bilateral sensorimotor regions observed in patients with FMD supports a model whereby impaired motor feed-forward and sensory feedback from areas of sensorimotor integration to the right TPJ contributes to patients’ impaired sense of self-agency. Our study, the first resting-state fMRI analysis in the literature to date on FMD patients, has important implications with respect to how neurologists, psychiatrists, and patients conceptualize this debilitating disorder.

National Institute on Drug Abuse (NIDA)

William Kowalczyk

Postdoctoral Fellow

Psychiatry

Ninety minutes into the future: predicting momentary craving and mood from several hours of GPS tracks

Some behaviors (e.g. manic episodes, suicide) are better to prevent than to have to treat in their aftermath. In our specialty, addiction treatment, we want to prevent relapse, which is recognized as problem separate from persistent ongoing drug use. Prior attempts to predict imminent lapse have usually focused on time scales of weeks or months, which is problematic when attempting to predict a behavior that occurs in a moment. Our goal is to predict behavior in real time, to open up a world of live, just-in-time, mHealth interventions for drug lapses and other behaviors. The present study attempts to take a step towards that goal by making ambulatory predictions of craving and mood 90 minutes into the future. In two separate pools of polydrug-using, methadone-maintained outpatients (pilot study, $n = 27$; main study, $n = 81$), we collected time-stamped GPS data and ratings of mood, stress, and craving over 16 weeks at randomly prompted times via ecological momentary assessment (EMA). We mapped participants’ GPS tracks for the 6.5 hours before each EMA entry, tying the GPS tracks to an independently obtained observer rating of visible neighborhood disorder (NifETy). We dropped the 90 minutes of NifETy data immediately preceding an EMA response and used the remaining 5 hours to train randomForest machine-learning models to predict stress, mood, heroin and cocaine craving. A second

model adding demographic factors as predictors was run on the main study. The models predicted mood, drug craving, and stress 90 minutes into the future in both the pilot and main study. In the main study, using only GPS data, agreement was generally at a moderate level (agreement statistics, kappa: Stress .38-.45, Cocaine Craving .36-.55, Heroin Craving .52-.64; r: Positive Mood .71-.78, negative mood .52-.63) Adding person-level predictors increased the accuracy of the predictions to generally substantial levels (agreement statistics, kappa: Stress .43-.52, Cocaine Craving .40-.62, Heroin Craving .61-.69; r: Positive Mood .80-.83, negative mood .59-.68). Our goal is to harness advances in environmental data to reduce the burdens of behavioral disorders. We succeeded in an automated prediction of a patient's future state using GPS data; using a model that could ultimately run on a smartphone. This represents the first step towards the goal of a just-in-time mHealth intervention to predict drug lapses. The step represented here may be extended to other behavioral disorders.

National Institute of Mental Health (NIMH)

David Pagliaccio

Postdoctoral Fellow

Psychiatry

Behavioral and Neural Sustained Attention Deficits in Bipolar Disorder and Familial Risk for Bipolar

Bipolar disorder (BP) is among the most disabling and heritable psychiatric disorders but its genetic and environmental causes remain largely unknown. Recent neuroimaging work has aimed to identify endophenotypes for disorders like BP to help bridge the gap between presentation of a disorder and its genetic underpinnings. Endophenotypes are reliable and heritable traits that are present in both individuals with the disorder (actively or in remission) and those with a family history of the disorder. Increased reaction time variability, which is thought to index deficits in sustained attention, including attentional lapses, is a promising behavioral endophenotype for BP. This is the first study to identify neural mechanisms mediating sustained attention deficits in individuals with, or at risk for BP. We used a sophisticated fMRI analytic approach to quantify precisely trial-wise associations between reaction time and brain activity. The current study examined 106 unrelated individuals (8-25 years old) who completed an fMRI sustained attention task: 24 with BP, 29 at-risk based on a first-degree relative with BP, and 53 low-risk, unaffected individuals. Relative to low-risk individuals, those with BP or at-risk for BP exhibited increased reaction time variability ($F(2,102)=4.26, p=.02, \eta^2=0.08$) – replicating prior studies implicating reaction time variability as a behavioral endophenotypes for BP. Further, we identified blunted relationships between trial-wise variation in reaction time and brain activity in the inferior and middle frontal gyri, precuneus, cingulate cortex, caudate, and postcentral gyrus (all regions: $p < .001, \eta^2 > 0.06$) in at-risk and BP individuals, compared to healthy low-risk individuals. This blunting partially mediated group differences in reaction time variability ($\beta=0.010, 95\%$ Confidence Interval=[0.002-0.020], Sobel $Z=2.08, p=.038$). Importantly, in this study of BP, the use of a novel analytic approach that linked behavior and brain activity directly was integral to our findings, since robust group differences were not found with traditional fMRI analyses. In sum, in unaffected but at-risk individuals and in euthymic BP patients, we found blunting in key frontal, cingulate, and striatal areas that mediate the rapid implementation of attentional control - our findings thus meet several criteria for an endophenotype. Elucidating such neural endophenotypes can facilitate novel approaches to BP prediction, diagnosis, and prevention.

National Institute on Alcohol Abuse and Alcoholism (NIAAA)

Erica Grodin

Doctoral Candidate

Radiology/Imaging/PET and Neuroimaging

Decreased Subcortical Volumes in Alcohol Dependent Individuals: Effect of Polysubstance Use Disorder

Chronic alcohol use has been shown to have widespread effects on brain morphometry. Both cortical and subcortical regions have been shown to be impacted by heavy alcohol use. Furthermore, alcohol dependent individuals are often diagnosed with comorbid substance use disorders. Alterations in brain morphometry may be different in individuals that are dependent on alcohol alone and individuals dependent on alcohol and other substances. However, most research on alcohol dependence and brain morphometry has excluded individuals with comorbid substance use. The few studies that have investigated alcohol and comorbid substance dependence have only included participants with alcohol and comorbid stimulant dependence. Here, we compared subcortical brain volume in individuals with alcohol dependence only (ADO, n = 37), individuals with polysubstance use disorder (PS, n = 37), and healthy control participants (HC, n = 37). PS were all diagnosed with alcohol dependence and at least two additional substance use disorders. We focused on subcortical structures as they are implicated in domains disrupted by addiction, including reward (nucleus accumbens, amygdala), cognition (hippocampus), habit formation (caudate, putamen, globus pallidus), and compulsive behavior (thalamus). Participants underwent a structural MR scan and a model-based segmentation tool was used to measure the volume of 14 subcortical regions. Compared to HC, ADO had smaller volume in the bilateral hippocampus, right nucleus accumbens, and right thalamus. PS only had volume reductions in the bilateral thalamus compared to HC. PS had a larger right caudate compared to ADO. Regional subcortical volume was negatively associated with drinking measures only in the ADO group. The thalamus was the only subcortical region with significant volume reduction in both alcohol dependent groups. The thalamus is implicated in compulsive behaviors, and it serves as an important relay station from the striatum to the orbitofrontal cortex. Dysfunction in the striato-thalamo-orbitofrontal circuit has been hypothesized to result in compulsive behavior and increased drug-seeking motivation seen in addiction. This study confirms the association between alcohol dependence and reductions in subcortical brain volume. The structural volume and association findings underscore the importance of considering comorbid substance use in alcohol dependent populations.

National Institute on Alcohol Abuse and Alcoholism (NIAAA)

Ehsan Shokri Kojori

Visiting Fellow

Radiology/Imaging/PET and Neuroimaging

COMET: Connectivity-Metabolism Associations Characterized by Cost and Reactivity Indices

Regional dynamics in the associations between neuronal demand and metabolic supply remain largely underexplored. Here we propose a new framework to characterize different associations in brain regions between metabolic supply and neuronal demand. Specifically, in a two dimensional map of

demand and supply, we define a reactivity axis along which the positive end indicates high supply associated with high demand and the negative end indicates low supply associated with low demand. Perpendicular to reactivity axis, we define a cost axis along which the positive end indicates high supply associated with low demand and the negative end indicates low supply associated with high demand. We used resting-state local functional connectivity density (lFCD, indexing neuronal demand) and PET-FDG (indexing glucose metabolism) to assess regional differences in cost and reactivity measures. Each participant (n = 24; mean age = 32.5 years; 12 males), underwent a PET-18FDG scan. The data were transformed into metabolic images, and normalized in SPM8. Participants underwent 5-min resting-state scan (TR = 1.6 s) following each PET-18FDG scan. fMRI data were realigned, normalized, low-pass filtered (< 0.1 Hz), and scrubbed. The lFCD for each voxel was defined as the size of local cluster of correlated voxels. Reactivity and cost were computed by projecting lFCD and FDG values along the corresponding axes. Occipital and parietal cortices, middle and inferior frontal gyri, superior temporal gyrus had high reactivity, whereas cerebellum, limbic regions, superior temporal gyrus, pons, and putamen had low reactivity. High cost regions were middle and inferior frontal gyri, orbitofrontal cortex, occipital and parietal cortices, superior temporal gyrus, and putamen, whereas cerebellum, limbic regions, pons, and thalamus had low cost. Superior and inferior frontal gyrus, superior temporal gyrus, insula, thalamus, limbic regions, midbrain, precuneus and cuneus, had higher cost than reactivity, whereas cerebellum, middle temporal gyrus, lingual gyrus, inferior parietal lobule, and cingulate gyrus had higher reactivity than cost. We showed that the associations between glucose metabolism and slow neuronal oscillations are markedly different among brain regions. We conclude that categorizing brain regions based on having high metabolic cost vs. being proportionally reactive to neuronal demand is an effective approach to study demand-supply regimen of the human brain.

NIH Clinical Center (CC)

Xiaosong Wang

Visiting Fellow

Radiology/Imaging/PET and Neuroimaging

Unsupervised Category Discovery via Looped Deep Pseudo-Task Optimization Using a Large Scale Radiology Image Database

Obtaining semantic labels on a large scale radiology image database (215,786 key images from 61,845 unique patients) is a prerequisite yet bottleneck to train highly effective deep Convolutional Neural Network (CNN) models for image recognition. Nevertheless, conventional means of collecting image labels (e.g. Google image search followed by crowd-sourcing) are not applicable due to 1) the formidable difficulties of medical annotation tasks for clinically untrained annotators, 2) unavailability of a high quality or large capacity medical image search engine. On the other hand, even for well-trained radiologists, this type of “assigning labels to images” task is not aligned with their regular diagnostic routine work so that drastic inter-observer variations or inconsistency may be demonstrated. In this project, we proposed a Looped Deep Pseudo-task Optimization (LDPO) procedure for automatic category discovery (auto-annotation) of visually coherent and clinically semantic (concept) clusters. Our system can be initialized by domain-specific (CNN trained on radiology images and text report derived labels) or generic (ImageNet based) CNN models. Afterwards, a sequence of pseudo-tasks are exploited by the looped deep image feature clustering (to refine image labels) and deep CNN

training/classification using new labels (to obtain more task representative deep features). Our method is conceptually simple and based on the convergence of better labels leading to better trained CNN models which consequently feed more effective deep image features to facilitate more meaningful clustering/labels. Finally, 270 categories of images resulted from the AlexNet-FC7-Topic setting was preferred to other model-encoding settings in the subjective evaluation by two board-certified radiologists. It achieves 81.09% for Top-1 classification accuracy and 94.12% for Top-5 accuracy, which is significantly better than the most related previous work. In addition, LDPO is also evaluated on the Texture-25 dataset as an unsupervised texture classification problem. Using the same clustering method of k-means, the purity or accuracy measurements improve from 53.9% (0-th) to 66.1% at the 6-th iteration, indicating that LDPO indeed learns better deep image features and labels in the looped process. A US patent has been filed based on the proposed work and it has also been submitted to a top-tier computer vision conference for publication.

National Institute on Drug Abuse (NIDA)

Sufang Li

Postdoctoral Fellow/Postdoctoral Fellow

Radiology/Imaging/PET and Neuroimaging

Trait impulsivity is associated with different intrinsic functional connectivity with ventral striatum in smokers and nonsmokers

Ventral striatum (VS) is the key structure implicated in impulsivity. It modulated impulsive behaviors by interacting with other brain regions. However, little is known about how it interacts with other brain regions underlies impulsivity in addiction. Resting state functional connectivity (rsFC) provided us a novel method to explore the intrinsic functional connectivity of VS circuits, its association with impulsivity and potential alteration in smokers versus nonsmokers. 60 smokers and 60 matched nonsmokers participated in the resting state fMRI scan and their impulsivity was assessed by using Barrat Impulsive Scale. Voxel-wise rsFC between bilateral VS and all other brain regions were computed for each subject. A voxel-wise analysis of covariance was then conducted to identify the effect of smoking status, trait impulsivity and their interaction. Significant interactions were found in the dorsal anterior cingulate cortex (dACC) and bilateral amygdala. Specifically, significant positive correlation between impulsivity and rsFC of VS-amygdala circuit was found in smokers while not in nonsmokers; significant positive correlation between impulsivity and rsFC of VS-dACC circuit was found in nonsmokers but not in smokers. Main effect of smoking status was located in the left amygdala and bilateral ventral striatum, and smokers have increased VS-amygdala connectivity and decreased striatal connectivity than nonsmokers. Main effect of impulsivity was located in the left ventral striatum and right anterior insula. To further explore the role of these resting state functional connectivity in impulsivity, we also employed two tasks: Go/NoGo task activates the dACC and assesses neural computation during response inhibition and emotional memory task activates amygdala and assesses emotional response. VS-amygdala rsFC positively correlated with the amygdala activation during negative emotional response versus positive; and VS-dACC rsFC also positively correlated with the dACC activation during false inhibition (loss impulse control). Additionally, VS-dACC rsFC negatively predict nicotine dependence severity (FTND) and VS-amygdala rsFC positively predict anxiety in smokers. These results provided new evidence for the theory that the role of frontal-striatal circuit involved in impulse

control, and striatal-limbic circuit involved in impulse drive. It demonstrates an important linkage of mesocorticolimbic circuits with underlying impulsive behaviors in addiction.

NIH Clinical Center (CC)

Mingchen Gao

Postdoctoral Fellow

Radiology/Imaging/PET and Neuroimaging

Multi-label Deep Convolutional Neural Networks for Holistic Interstitial Lung Disease Detection

Holistically detecting interstitial lung diseases (ILD) using single CT images is a challenging but also important medical imaging problem. The difficulties lie on several aspects, which include the tremendous amount of variation in disease appearance, location, and configuration and also the expense required to obtain delicate pixel-level ILD annotations of large datasets for training. Finally, there are usually multiple diseases coexisting on the same patient, even on single CT slices. Beyond the basic approaches from most of the previous work, focusing on predicting a single ILD label to manually defined region of interest, we propose a multi-label deep regression model for holistic images. An end-to-end convolutional neural network (CNN) network is trained for multi-label image regression. The deep CNN regression model, which is inspired by the cortex of the brain, learns the deep image features and the final predictions to multi labels simultaneously. While CNNs are powerful tools, their feature learning strategy is not invariant to the spatial locations. To accommodate the large spatial variations of the ILD locations, the learned CNN features at different network depths are spatially aggregated and encoded through Fisher Vector (FV) method to turn them into location-invariant representations. The unordered features are then trained using a multi-variate linear regressor to regress the numbers of ILD pixels or binary labels. The proposed algorithms are validated on a publicly available dataset of 533 patients, called Lung Tissue Research Consortium (LTRC) dataset, using five-fold cross-validation. Four most typical ILDs are investigated here, Ground Glass, Reticular, Honeycomb and Emphysema. There are 18883 slices in total for training and testing. There are 3368, 1606, 1247 and 2639 positive slices for each disease, respectively. In total there are 11677 healthy CT images, 5675 images with one disease, 1410 images with two diseases, 119 images with three diseases, and 2 images with four diseases. We achieved high area-under-curve (AUC) scores of 0.982, 0.972, 0.893 and 0.993 for Ground Glass, Reticular, Honeycomb and Emphysema, respectively. As such, our work represents an important step forward in providing clinically effective ILD detection.

National Institute of Environmental Health Sciences (NIEHS)

Jonathan Busada

Research Fellow

Signal Transduction - General

Glucocorticoids are indispensable for normal gastric function in female mice but are dispensable in males.

Digesting food and protecting the body from ingested pathogens are some of the primary roles of the

stomach. Normal stomach function is dependent on glucocorticoids (GCs) secreted from the adrenal cortex, which stimulate acid production by gastric parietal cells and suppress inflammation of the gastric epithelium caused by exposure to pathogens and environmental toxins. Long-term GC deficiency, such as Addison's disease, causes reduced acid production and gastric inflammation. If untreated, chronic gastric inflammation in Addison's patients is correlated with gastric cancer. Despite the crucial relationship between GCs and the stomach, few studies have examined the requirements of GCs in maintaining normal gastric function. In this study, we adrenalectomized (ADX) male and female mice and examined their stomachs 2 months post surgery to investigate the consequences of chronic GC deficiency. The gastric glands of ADX female mice were grossly abnormal with hyperplasia of mucous neck cells and had prominent reduction of parietal cells and chief cells relative to sham controls. In addition, there was neutrophil infiltration into the lamia propria underlying the gastric glands. Surprisingly, the stomachs of ADX male mice were relatively normal. Next, we performed QRT-PCR analysis for a panel of gastric cancer markers. All of the cancer markers were significantly increased in ADX female stomachs but were only slightly increased in ADX male stomachs. To fully characterize differences in the transcriptome between male and female stomachs, we performed RNA sequencing on ADX mice treated for 3 hours with the synthetic GC dexamethasone. RNA sequencing revealed that 1,560 genes were uniquely regulated by GCs in males, 1,887 in females, and 2,243 genes were regulated in both male and female stomachs. Finally, we castrated and ovariectomized (OVX) male and female mice respectively and euthanized them 2 months post-surgery. Stomachs from castrated mice were histologically indistinguishable from ADX female stomachs, but stomachs from OVX mice appeared normal. Taken together, our data reveals that GCs are indispensable for normal gastric function in females but that testosterone compensates for GC deficiency in males. These results may explain why women are more susceptible than men to gastro-intestinal inflammation and autoimmune diseases.

National Institute of Environmental Health Sciences (NIEHS)

Hoai Nghia Nguyen

Visiting Fellow

Signal Transduction - General

The significance of a rare signaling paradigm: the mutually competing activities of IP7K, a bifunctional kinase/phosphatase

The metabolism of cellular signals exhibits common paradigms; evolution retains and adapts good ideas. One recurring example is the 'futile' cycle: interconversion of functionally-different forms of a molecule. For signaling purposes, the distinct enzymes catalyzing these competing events are generally spatially separated and individually regulated. We now describe rare outliers: two human PPIP5K isoforms with phosphatase and kinase domains in a single protein, which control cell-signaling by IP7 and IP8, two inositol pyrophosphates (PP-IP). We characterized PPIP5Ks in vitro using recombinant enzymes. The purification is technically demanding; the proteins are huge (130-160 kDa) and contain oxygen-sensitive iron. We used an anaerobic chamber to purify FLAG-tagged PPIP5Ks expressed in HEK cells; enzyme integrity was validated by SDS-PAGE. We found PPIP5Ks phosphorylate IP7 to IP8, and dephosphorylate IP8 back to IP7. To eliminate mutual interference between these opposing reactions, kinase activities were assayed with mutants rendered phosphatase-dead by a single-site mutation; conversely, phosphatase activities were assayed with kinase-dead mutants. At physiological [ATP] (3mM) and [IP7]

(1 μ M), the kinases operate at 4 nmol/mg/min (near V_{max}). Rates of IP8 dephosphorylation were similar, although influenced by substrate supply (at 0.1 and 1 μ M IP8, $v = 1$ and 6 nmol/mg/min, respectively). Similarity in rates of competing reactions is known to maximize a futile cycle's regulatory capacity. Upon reducing [ATP] from 3 to 1 mM, phosphatase activity of PPIP5K2 increased 50%, while kinase activity decreased 40%. We propose signaling sensitivity is enhanced by enforced co-localization of the kinase and phosphatase activities for their reciprocal regulation by ATP. Of further importance to the signaling field: this is the first description of a molecular mechanism to modulate IP7/IP8 turnover. Intriguingly, in prior reports, low [ATP], or high [IP7], have been separately linked to impaired DNA repair. So we adjusted the poise of the IP7/IP8 cycle in HCT116 cells by over-expressing PPIP5K that was either phosphatase-dead (low IP7/IP8 ratio) or kinase-dead (high IP7/IP8 ratio). Cells with a high IP7/IP8 ratio were more sensitive to etoposide-induced DNA damage (assayed by confocal imaging of pH2A.X histone phosphorylation). To summarize: [ATP], [PP-IPs], and DNA repair, are interconnected through an uncommon paradigm; a futile cycle catalyzed by a single protein.

National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)

Sai Prasad Pydi

Visiting Fellow

Signal Transduction - General

Mice lacking β -arrestin-2 in adipocytes show reduced adiposity, improved glucose tolerance, and enhanced insulin sensitivity

Background: In obese individuals, excess fat accumulates in adipose tissue (AT), leading to altered AT metabolism and increased insulin resistance, a key feature of Type 2 diabetes (T2D). Therefore, it is of great importance to understand the signaling pathways that regulate AT function. Activation of certain G protein coupled receptors (GPCRs) in AT has been reported to have beneficial effects on whole body glucose homeostasis. GPCR signaling is modulated by proteins of the β -arrestin family (barr1 and barr2), which can terminate GPCR signaling and/or mediate GPCR-independent signaling. Since recent studies have revealed that barr1 and barr2 regulate many important physiological functions, the in vivo roles of β -arrestins have become the focus of intense research. The role of barr1 and barr2 in adipocyte function remains unexplored. Objective: Deciphering the role of barr2 in adipocyte function, obesity and whole body glucose homeostasis. Methods: To assess the role of barr2 in adipocytes, we used Cre-lox technology to generate mutant mice that lacked barr2 selectively in adipocytes (adipo-barr2 KO mice). These mutant mice, together with their control littermates, were subjected to a series of metabolic tests. Moreover, we used various molecular and biochemical techniques to probe the molecular mechanism through which barr2 modulates adipocyte function. Results: Interestingly, adipo-barr2 KO mice maintained on a high-fat diet showed significantly reduced body weight, improved glucose tolerance, enhanced insulin sensitivity and reduced adiposity. RNA-seq analysis revealed increased expression of thermogenic genes, beige fat markers and mitochondrial marker genes in white adipose tissue (WAT) isolated from the mutant mice. Mature adipocytes from adipo-barr2 KO mice showed greatly increased lipolytic responses after treatment with β -adrenergic receptor (β -AR) agonists, including CL316243 (β 3-AR specific) and isoproterenol (non selective β -AR agonist). This effect was associated with a significant increase in mitochondrial respiration (oxygen consumption rate), as demonstrated by the use of Seahorse technology. Conclusion: Our data support the novel concept that

barr2 deficiency promotes beiging of WAT and improves whole body glucose metabolism, most likely by promoting signaling through adipocyte β -ARs. These findings suggest that pharmacological inhibition of barr2 signaling in adipose tissue may prove clinically useful for the treatment of T2D.

National Eye Institute (NEI)

Min Jae Song

Research Fellow

Stem Cells - General

3D bioprinting and iPS cells help generate patient-specific ocular tissue to model and treat age related macular degeneration

Pathological angiogenesis of capillaries located in the back of the eye (choroid) leads to an eye disease “wet” age-related macular degeneration (wet-AMD, one of the leading causes of blindness among elderly. In wet-AMD, choroidal capillaries grow and leak into the eye by breaching through the outer blood-retina-barrier that is formed by the tight junctions of the retinal pigment epithelium (RPE) cell layer located adjacent to these capillaries. Antibodies against Vascular Endothelial Growth Factor (VEGF) provide a temporary treatment by stopping capillary growth but do not cure the underlying disease. This is because there is no good model to identify mechanism of disease initiation. We have combined bioprinting and tissue engineering with induced pluripotent stem (iPS) cell technology to develop a 3D in vitro model of wet-AMD. Using a collagen-based gel for encapsulation of patient-specific iPS cell-derived endothelial cells, choroidal fibroblasts, and pericytes, we successfully bioprinted a microvascular network on one side of a ten micron thick biodegradable scaffold. On the other side of the scaffold, we grow a RPE monolayer differentiated from the same patient’s iPS cells. The scaffold serves as a transient support for RPE and choroid to secrete extracellular matrix and forms a membrane similar to Bruch’s membrane in the back of the eye. This 3D tissue shows electrical properties that are reminiscent of the outer blood-retina-barrier of the eye. Furthermore, similar to VEGF induced vascular growth in wet-AMD, the in vitro microvascular network also responds to VEGF. The use of patient-specific iPS cells allows us to dissect genetic pathways associated with wet-AMD initiation. In cancer field, drugs that inhibit transcription factor STAT3 induced VEGF secretion are currently being tested in phase 2 & 3 clinical trials. STAT3 is activated in inflamed RPE cells. We have obtained iPS cells from patients with STAT3 mutations and with STAT3 overexpression. It is expected that STAT3 mutant RPE & endothelial cells will be defective in inducing wet-AMD like processes in vitro, whereas STAT3 overexpressed cells will overtly induce wet-AMD in vitro. STAT3 inhibitory drugs will be tested as potential anti-AMD drugs. In conclusion, this work provides a platform to discover disease inducing pathways and the possibility of identifying potential therapeutic drugs for wet-AMD.

National Institute of Child Health and Human Development (NICHD)

Solji Park

Visiting Fellow

Stem Cells - General

EGG FROM OVARIAN STEM CELLS DEVELOPS INTO EMBRYO

The dogma in reproductive biology is that women have a finite ovarian reserve, which means the number of oocyte decrease gradually until the depletion at menopause. This is in contrast to men who possess germ line stem cells that create sperms over the lifespan. However, it has been questioned recently by the discovery of cells in the ovarian cortex capable of mitosis and meiosis in mouse, rat and human. In this study we found that these ovarian-derived stem cells (OSCs) are also present in non-human primates and they can develop into oocytes with fertilization potential. The ovarian cortex was digested with collagenase IV and DNase followed by FACS with the DDX4 antibody. The cells were expanded in culture, transfected with a GFP lentivirus, and 1.28-2.1 million cells were transplanted into the remaining ovary of the rhesus monkey. Following transplantation, gonadotropins were used for ovarian hyperstimulation to isolate oocytes. We confirmed that oocytes were transplant derivation by fluorescence, PCR and nested PCR. True oocyte phenotype with appearance of healthy zona pellucida and polar body was observed as well as the expression of oocyte specific genes. Two out of 68 oocytes obtained by follicular aspiration were confirmed OSCs origin, whereas 32 out of 71 from microdissection appear to arrest in the ovarian tissue after transplantation. Further studies are needed to elucidate the determinants and timeline of follicular development of OSC derived oocytes after transplantation. Collected MII oocytes were fertilized via intracytoplasmic sperm injection and embryo development potential assessed. A mature oocyte originating from OSCs developed into 64-cells stage embryo. This is the first fertilized embryo derived from stem cells to achieve fertilization in primates. This finding suggests that ovarian stem cell transplantation open the door to novel fertility preservation strategies for women with both age-related and premature ovarian insufficiency.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Yixing Han

Postdoctoral Fellow

Stem Cells - General

Lsh/HELLS regulates self-renewal/proliferation of NSPCs associated with control of Bmp4 and Cdkn1a expression

The cerebral cortex develops from multipotent neural stem cells that begin as neuroepithelial cells in the ventricular zone and expand into the intermediate neural progenitors in the subventricular zone. Epigenetic regulation plays a pivotal role in the maintenance of cell identity as well as the step-wise guidance towards cellular differentiation. Understanding molecular mechanisms and identifying key chromatin factors that regulate neural stem/progenitor cells (NSPCs) maintenance and neurogenesis is critical for the development of novel therapies of neural pathologies. Lsh is crucial for normal development as Lsh-deficient mice show multiple developmental defects. Lsh/HELLS genetic mutation causes ICF syndrome, which is a human developmental disorder with abnormal motor neuron development and evidence of mental retardation. At molecular level, Lsh deletion leads to genome-wide DNA hypomethylation. To clarify the role of Lsh in early neurogenesis, we performed the following experiments in mouse embryonic NSPCs and brain tissue: 1) Cell-based analysis and IHC; 2) Image-based time-lapse immunofluorescence and FACS; 3) RNA-seq for proliferating NSPCs and RT-qPCR for proliferation and differentiation NSPCs; 4) ChIP-qPCR and NoME-seq assay; and 5) Rescues assay for proliferating NSPCs. We found that Lsh deletion results in a profound decrease in NSPCs proliferation

and a compromised ability of self-renewal in vivo. In addition, NSPCs are of the ability to differentiate into main neural lineages but with a delayed program in the absence of Lsh. RNA-seq analysis identified that Lsh deletion resulted in robust decrease of the stem cell modulator Bmp4 and increase of the cell cycle regulator Cdkn1a expression. Epigenetic level assays suggest that altered chromatin states at specific enhancers of Bmp4 and Cdkn1a, which consistent with the deregulation of gene expression and reduced proliferation of Lsh^{-/-} NSPCs, indicating a novel role for the chromatin remodeler Lsh in neurologic function. Growth repression of Lsh^{-/-} NSPCs can be restored by adding exogenous Bmp4, suggesting a novel Bmp4 controlled pathway as potential therapeutic target in the ICF syndrome. In summary, we demonstrate functional requirements for Lsh in proliferation of NSPCs. Our results provide a fresh view of molecular signaling mechanism that Lsh regulates the chromatin status on specific enhancers of Bmp4 and Cdkn1a to coordinate NSPCs self-renewal, which may has therapeutic potentiality.

National Institute of Environmental Health Sciences (NIEHS)

RAJNEESH PATHANIA

Research Fellow

Stem Cells and Cancer

A role for ISL1 as a mammary tumor suppressor

Abstract removed at request of the author

National Institute on Aging (NIA)

Krisztina Marosi

Postdoctoral Fellow

Stress, Aging, and Oxidative Stress/Free Radical Research

Metabolic fuel switch from glucose to ketones regulates SIRT3 in the brain

SIRT3 is a member of the Sirtuin family of NAD⁺-dependent deacetylases and plays a critical role in metabolic regulation. Previously we found that neurons lacking the mitochondrial deacetylase SIRT3 are more vulnerable to dysfunction and degeneration in mouse models of epilepsy and Huntington's disease. We also showed that exercise and synaptic activity induce hippocampal SIRT3 expression to modulate mitochondrial protein acetylation and bolster neuronal resistance to oxidative stress and apoptosis. In our current study we investigated how physiological and metabolic changes resulting from dietary interventions and exercise affect SIRT3 levels and activity in the brain. We found that alternate day fasting and exercise promote a metabolic fuel switch involving elevated ketone levels and reduced blood glucose levels in C57BL/6J mice. Elevated plasma ketone levels were correlated with enhanced SIRT3 protein levels in the brain of the mice that were subjected to alternate day fasting and exercise compared to ad libitum fed sedentary controls. We then determined whether the ketone 3 β -hydroxybutyrate (3OHB) regulates SIRT3 expression in cultured neurons. 3OHB increased SIRT3 protein levels and activity in a low glucose condition, but not in a high glucose condition indicating that changes in substrate utilization regulate SIRT3. We showed that 3OHB induces ATP and NAD⁺ production by bypassing glycolysis and promoting mitochondrial respiration in the neurons, which may play a role in the modulation of SIRT3 activity. We are currently determining whether 3OHB and SIRT3 mediate

neuroprotective effects of fasting by suppressing ketone production using lentivirus-mediated expression of shRNA targeting an enzyme critical for 3OHB production in the liver.

National Heart, Lung, and Blood Institute (NHLBI)

Javier Traba

Visiting Fellow

Stress, Aging, and Oxidative Stress/Free Radical Research

Fasting regulates NLRP3 inflammasome activation in humans by modulating mitochondrial integrity

Fasting confers beneficial effects against inflammation-linked diseases, including vascular disease and diabetes. Activation of the Nod-like receptor family protein 3 (NLRP3) inflammasome, an intracellular innate immune surveillance complex involved in the cleavage and release of inflammatory cytokines, is implicated in this pathophysiology. This inflammasome includes cytosolic proteins NLRP3, the adaptor protein ASC and procaspase-1, which assemble on mitochondria, where the extrusion of mitochondrial content (reactive oxygen species [ROS], oxidized mitochondrial DNA or cardiolipin) mediates its activation. It has been proposed that members of the Sirtuin family of protein deacetylases are involved in mitochondrial homeostasis and are activated by caloric restriction. Thus, we hypothesized that fasting blunts the NLRP3 inflammasome via Sirtuin-mediated augmentation of mitochondrial integrity. To test this we performed a clinical study of 19 healthy volunteers. Each subject underwent a 24-hour fast and then was fed a fixed-calorie meal. Blood was drawn during the fasted and fed states and analyzed for NLRP3 inflammasome activation in leukocytes. Individuals showed less NLRP3 inflammasome activation, in parallel with signatures consistent with activation of the mitochondrial-enriched Sirtuin deacetylase SIRT3, in the fasted state compared with refeed conditions. In a human macrophage line, depletion of SIRT3 increased NLRP3 inflammasome activation in association with excessive mitochondrial ROS production. Furthermore, pharmacologic SIRT3 activation using the agonist Nicotinamide Riboside blunted NLRP3 activity in parallel with enhanced mitochondrial function in cultured cells and in primary leukocytes. We further characterized the role of SIRT3 by studying a downstream effector Superoxide Dismutase 2 (SOD2), an enzyme that controls mitochondrial ROS, a known inflammasome activator. We found that a constitutively active SOD2 mutant strongly inhibited the NLRP3 inflammasome, whereas an inactive mutant did not. SOD2-mediated inhibition of ROS accumulation prevents the localization and assembly of the NLRP3 complex on mitochondria. Together, our data indicate that nutrient levels regulate the inflammasome, in part through SIRT3-SOD2-mediated mitochondrial homeostatic control. Moreover, these results suggest that deacetylase-dependent inflammasome attenuation may be amenable to targeting in human disease.

National Institute on Aging (NIA)

Alberto Diaz-Ruiz

Postdoctoral Fellow

Stress, Aging, and Oxidative Stress/Free Radical Research

Increased non-mitochondrial respiration as a potential mechanism for lifespan extension in mice.

Mitochondrion constitutes the primary source of energy and power in mammalian cells. Strikingly, mild inhibition of mitochondrial respiration extends lifespan in yeast, *C. elegans*, *Drosophila* and mice. The molecular pathways beneath this process include changes in metabolic homeostasis and/or activation of mitochondrial retrograde signaling, a pathway of communication from mitochondria to the nucleus. In the event of mitochondrial dysfunction, oxygen consumption occurs at the cell surface through the trans-plasma membrane electron transport (PMET) complex to meet the energy demands. This non-mitochondrial PMET lowers intracellular oxidative stress, replenishes cellular NAD⁺ levels, and maintains ATP production through glycolysis in mitochondria-deficient cells. It is unclear whether PMET up-regulation mimics the beneficial effects of reduced mitochondrial respiration on healthspan and lifespan. To this end, we generated a mouse model that overexpresses two main components of the PMET, NQO1 and CYB5R3, in a C57Bl/6 background (RedTG). RedTG mice showed extended lifespan compared to WT littermates along with greater physical performance as an indicator of improved healthspan. RedTG mice preferentially used carbohydrates as a source of energy consistent with enhanced glycolysis. Thus, overexpression of NQO1 and CYB5R3 coupled with enhanced PMET-mediated cell surface respiration may contribute to extended longevity and healthspan, presumably via reduction in mitochondrial respiration and activation of retrograde signaling.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Natalia von Muhlinen

Visiting Fellow

Stress, Aging, and Oxidative Stress/Free Radical Research

delta133p53 isoform as a novel therapeutic target for premature aging and aging-related diseases

Progeroid syndromes are disorders with symptoms that resemble premature aging, among which Hutchinson-Gilford Progeria Syndrome (HGPS), a rare but fatal childhood disorder, is the most severe. HGPS is caused by a point mutation in the LMNA gene that generates the toxic form of pre-lamin A 'progerin', whose accumulation causes genomic instability, DNA damage and the early onset of senescence (irreversible cellular growth arrest), which all together contribute to the premature aging symptoms in HGPS patients. However, potential therapeutic targets to inhibit senescence and ameliorate DNA damage in HGPS remain unknown. Here, we showed that the N-terminally truncated p53 isoform delta133p53, an endogenous regulator of senescence in normal cells, is diminished in nearly-senescent patient-derived HGPS fibroblasts. Importantly, knockdown of delta133p53 accelerated the onset of senescence in HGPS cells, highlighting the functional importance of this isoform in proliferation of HGPS cells. In contrast, restoring delta133p53 expression rescued senescence and extended the replicative lifespan of HGPS fibroblasts. Our mechanistic studies showed that delta133p53 inhibited senescence-associated p53-target genes including p21 and mir-34a. Furthermore, delta133p53 expression decreased spontaneous DNA double strand breaks, probably by directly transactivating RAD51, a DNA repair factor essential for homologous recombination. Moreover, expression of human delta133p53 in otherwise nearly-senescent mouse embryonic fibroblasts derived from a progeria mouse model delayed the onset of senescence and extended their replicative lifespan. Our findings strongly suggest that delta133p53 is a novel therapeutic target that by delaying the onset of senescence and ameliorating DNA damage may extend the lifespan of HGPS children in vivo. We are currently generating a novel humanized mouse expressing delta133p53 isoform as 'proof-of-principle' that drugs enhancing

human delta133p53 expression may have therapeutic value to treat HGPS patients in vivo; we are also conducting high-throughput screenings of candidate compounds to identify novel modulators of delta133p53 expression for potential future clinical applications. These studies will be invaluable towards future therapeutic strategies to inhibit or delay the progression of aging-related diseases, such as the progeroid disorder Werner Syndrome as well as in neurodegenerative disorders such as Alzheimer's disease.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Yinmeng Yang

Doctoral Candidate

Tumor Biology and Metastasis

Efficacy of anti-CD19 chimeric antigen receptor T cell therapy against acute lymphoblastic leukemia is diminished in the presence of TCR antigen

Tremendous progress has been achieved employing immunotherapy for B cell acute lymphoblastic leukemia (ALL), which is the leading cause of death in children from cancer. Recent trials using chimeric antigen receptor T cells (CAR) targeting the B cell restricted antigen CD19 that utilize the autologous transfer of patients T cells, have demonstrated remarkable remission rates of 80% against relapsed or refractory ALL. The feasibility of therapy depends on the ability of the isolated autologous T cells to expand, which in this heavily pre-treated patient population is not always possible. In these instances, allogeneic donors may be an alternative source to produce CAR, however there is little known about the effectiveness of allogeneic CAR (alloCAR). These alloCAR would have specificity for two antigens, one being the CAR target and the other derived from their endogenous T cell receptor (TCR) that could have reactivity against alloantigens, so activation of the endogenous TCR would potentially impact the efficacy of the alloCAR against tumor. To address this, we established a syngeneic murine model to evaluate the biology of alloCAR. By giving gender mismatched CAR cells derived from a female mouse to treat a male mouse, we found significantly lower survival due to poor leukemia clearance compared to gender matched CAR treatment of female mice. We then controlled for both CAR and TCR specificity using an HY antigen TCR transgenic system, and found that concurrent CAR and TCR stimulation induced an increase in T cell exhaustion markers PD-1, Tim3, and Lag3, 6X reduction in CAR numbers, and 5X increase in apoptotic CAR cells in the bone marrow compared to CAR stimulation alone. Interestingly, the negative effects of the active TCR was predominantly in CD8 T cells, typically considered the primary effector cells, and not in CD4 T cells that are by dogma non-cytolytic. Interestingly, CD4 CAR acquire cytolytic capabilities through CAR activation. The use of a CD4 only CAR product in the presence of TCR and CAR antigens led to increased expansion, and better persistence than observed with CD8 CAR and led to leukemia clearance. In addition, CD4 CAR T cells produced higher levels of T cell growth factor IL2, IFN γ , and TNF α compared to CD8 T cells. These findings suggest that CD4 CAR may be better when using allogeneic donor cells for immunotherapy. Future work will seek to examine the downstream pathways in TCR vs. CAR and effects of TCR elimination.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Tara Gelb

Postdoctoral FellowPostdoctoral Fellow

Tumor Biology and Metastasis

Exploring the treatment and biology of Merkel cell carcinoma using high throughput chemical screening

Abstract removed at request of author

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Laura Vera Ramirez

Visiting FellowVisiting Fellow

Tumor Biology and Metastasis

Disseminated breast cancer cells require autophagy to enter cellular dormancy and survive long periods of growth-arrested state

Late metastatic recurrence, even decades after the apparently successful removal and treatment of the primary tumor, is a significant cause of breast cancer (BC) mortality which suggests that disseminated cancer cells are able to survive in a quiescent state, known as cell dormancy, until conditions trigger a dormant-to-proliferative metastatic switch. Autophagy has been shown to contribute to both cancer suppression and progression depending on the context and stage of the disease in which autophagy is activated. While autophagy can protect against the consequences of genomic instability and an inflammatory microenvironment, it can also provide an important protective mechanism for transformed cells to survive metabolic stress. Here, we show that disseminated BC cells require the activation of autophagy to enter dormancy and survive the stressful conditions encountered once they reach the metastatic target organ. To do this, we have used both in vitro and in vivo models consisting on 3-dimensional cultures and a preclinical model of fibrosis at the lung metastatic site, respectively. Our in vitro data using cellular markers for autophagy and a Tandem fluorescent-tagged LC3 (mCherry-GFP-LC3) reporter for the autophagic flux reveal that dormant BC cells activate autophagy earlier than those induced to proliferate in Collagen-1-enriched microenvironments. Furthermore, the inhibition of the autophagic flux by treating dormant cells with Chloroquine (CQ) induces cell death in practically the entire quiescent cell population. To study the effects of the inhibition of the autophagic flux in vivo, nude mice induced to develop lung fibrosis were injected with our dormant BC cells. Alterations of the microenvironment, such as fibrosis, have been shown to promote the dormant-to-proliferative switch in this model system. We next treated these mice with CQ and observed that both the total tumor burden and percentage of proliferative lung lesions significantly decrease in the treated group when compared with the non-treated group, suggesting that the inhibition of autophagy suppresses survival of dormant cells and their proliferative response to external stimuli. This study is of translational relevance since CQ is a FDA approved drug that could be added to the adjuvant therapy of BC patients to prevent long-term recurrences.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Khadijah Mitchell

Postdoctoral Fellow

Tumor Biology and Metastasis

UNRAVELING THE AFRICAN AMERICAN LUNG CANCER TRANSCRIPTOME

African Americans (AA) have the highest lung cancer incidence and mortality compared with any other population. These racial disparities are not due to smoking alone. In fact, AA smoke less than European Americans (EA), and AA never smokers have higher rates of lung cancer when compared with EA, suggesting biological determinants. No studies to date have compared the molecular biology of lung cancer in AA and EA. Comparative transcriptomic profiling of lung cancer in AA and EA was performed using tissues from the NCI-MD Study (n=33 AA, 33 EA) and Affymetrix gene expression arrays. There were 1044 and 1796 differentially expressed genes between tumor and normal tissues in AA and EA, respectively. GSEA and Enrichment Map analyses revealed unique pathways and distinct biologically relevant themes: Lung tumors from AA were enriched in stem cell and invasion pathways, while tumors from EA were enriched in cell cycle, mitosis, and proliferation pathways. After comparing AA and EA gene expression signatures with drug response profiles using Connectivity Mapping, AA and EA were predicted to have varying sensitivities to chemotherapeutic agents. Interestingly, AA were predicted to be resistant to irinotecan (a cell cycle inhibitor currently in clinical trials for lung cancer) while EA were sensitive to the same drug. Next, we identified novel AA-specific driver genes that could play a role in racial disparities. We identified 40 genes differentially expressed in AA tissues only. Four of these “population-specific” genes, all of which had lower expression in AA compared with EA, were found on 17q21.31. Interestingly, this region is a known area of genetic evolution—duplication of this region is observed in Europeans but not in Africans, thus in line with our gene expression observations in AA. To determine if this region was potentially associated with lung cancer in AA, we asked whether expression of these genes were altered in AA lung cancer. Indeed, we observed that two of the genes, ARL17 and KANSL1, were increased in tumor tissue. Moreover, these genes were also associated with poor survival, but only in AA, suggesting that this region of genetic heterogeneity contributes to a distinct aggressive phenotype in AA. Overall, our study reports clear tumor biology differences, predicted differential drug response, and novel AA-specific driver genes in lung cancer between AA and EA. These data can inform precision medicine approaches for AA lung cancer patients.

National Cancer Institute - Center for Cancer Research (NCI-CCR)

Ngoc-Han Ha

Research Fellow

Tumor Biology and Metastasis

Polymorphisms in the promoter of the circadian rhythm gene Arntl2 affect metastatic susceptibility in breast cancer

Breast cancer mortality is primarily due to metastatic lesions rather than primary tumors, yet relatively little is understood regarding the mechanisms of metastatic breast cancer, making it difficult to identify patients who are at risk for metastatic disease. Our hypothesis suggests that inherited germline mutations contribute to metastatic disease and that these single nucleotide polymorphisms (SNPs) could be used to predict outcome in breast cancer patients. To investigate the effect of inherited SNPs on

metastasis, we used a mouse genetics approach comparing strains with high (FVB) and low (MOLF) metastatic phenotypes and identified *Arntl2*, a circadian rhythm transcription factor, as a gene whose differential expression predicted outcome in breast cancer patients. To identify SNP differences in *Arntl2* between MOLF and FVB, we performed whole genome sequencing of MOLF and compared it to the FVB genome. Overlapping the data with DNase hypersensitivity sites, which mark regulatory regions in the genome, revealed 13 SNPs in the predicted promoter of *Arntl2*. In order to test the causative role of the SNPs on *Arntl2* expression in vivo, metastatic cell lines were engineered using the CRISPR-Cas9 approach to specifically replace the FVB *Arntl2* promoter with that of MOLF. In agreement with our hypothesis, substituting in the MOLF promoter reduced *Arntl2* transcript levels and subsequently decreased lung metastases in orthotopic implantation assays. In vitro pulldown experiments with strain-specific promoter probes revealed potential differential binding of chromatin modifier proteins, further demonstrating the significance of the SNPs in regulating *Arntl2* transcription. Finally, analysis of SNPs associated with *Arntl2* expression in a cohort of Chinese breast cancer patients revealed significant correlation of *Arntl2* expression with overall survival, validating this gene as a marker in humans. Since *Arntl2* is a transcription factor, current studies are focused on identifying *Arntl2*-regulated genes to investigate downstream pathways involved in metastasis. This study has important implications regarding the role of circadian rhythm in cancer progression and provides a potential mechanism to explain the increased risk of breast cancers in nightshift workers. Furthermore, this provides the first evidence that transcriptional control elements can be engineered using CRISPR-Cas9 to establish the causative role of SNPs in inherited susceptibility to cancer metastasis.

National Institute on Aging (NIA)

Douglas Dluzen

Postdoctoral Fellow

Vascular Disease and Biology

Racial differences in microRNA and gene expression in hypertensive women

Hypertension affects one in three adults in the U.S. and is a major risk factor for cardiovascular diseases (CVDs). African American (AA) women have the highest prevalence of hypertension in the U.S., nearly one in two, but are the least studied population. Identifying novel mechanisms influencing hypertension disparities, especially in AA women, may help elucidate additional targets for personalized treatment. We hypothesized that differential gene expression influences the disproportionate prevalence of hypertension among AA females. We performed microarray profiling of peripheral blood mononuclear cells from AA or white, normotensive or hypertensive females and found that thousands of mRNAs were significantly- and differentially-expressed by both race and hypertension. A majority of genes in hypertension-related pathways, e.g. renin-angiotensin signaling, were significantly elevated in AA hypertensives compared with white hypertensives. Significant increases in gene expression in AA hypertensives was also observed in pathways novel to essential hypertension, including regulation of the actin cytoskeleton and focal adhesion signaling. In order to identify a mechanism contributing to these differences, we profiled microRNAs (miRNAs) in an expanded cohort. miRNAs negatively regulate target mRNA expression levels and play a critical role in many CVDs. We observed over 30 miRNAs significantly- and differentially-expressed in our cohort. We used in silico miRNA target prediction algorithms, coupled with gene pathway analysis, to identify miRNA/mRNA pairs that are differentially-

and reciprocally-expressed in our cohort and involved with pathways identified in our microarray screens. Several identified miRNA/mRNA pairs, including miR-4717/PLD1, miR-4709/PLD1, miR-4763/APOL3, and miR-20a/MCL1 exhibit significant differential-expression in AA female hypertensives. Expression of each miRNA/mRNA pair was validated using RT-qPCR and miRNA functionality for each target was confirmed at both the mRNA and protein level using human aortic endothelial cells. We have identified novel miRNAs and mRNA targets differentially-expressed by race that may influence hypertension etiology in AA women. This approach highlights the need to study at-risk populations in order to find additional targets that could be relevant for the prevention and treatment of hypertension in a more personalized manner, with the ultimate goal of eliminating hypertension-related disparities.

National Heart, Lung, and Blood Institute (NHLBI)

Scott Gordon

Postdoctoral Fellow

Vascular Disease and Biology

A High Density Lipoprotein Proteome Index Correlates with Cardiovascular Disease Severity in Humans

Cardiovascular disease (CVD) has been the leading cause of death in developed countries for over a century. In the clinic, doctors evaluate a patient's risk for developing CVD by measuring the cholesterol content of lipoproteins. High density lipoprotein associated cholesterol (HDL-C), commonly referred to as the "good cholesterol," has been the traditional biomarker for estimating cardiovascular protection derived from HDL particles in the blood. However, several recent studies have indicated that HDL-C may not represent the best indicator of the protective capacity of HDL. Recent proteomics studies have identified over 90 different proteins consistently found to associate with HDL and ongoing studies are unveiling interesting roles for many of these proteins in HDL function, but for the majority of these proteins, the functional importance of their association with HDL has not yet been determined. In this study, we tested the hypothesis that the protein composition of HDL is associated with CVD severity in human subjects. A total of 101 subjects were selected and grouped based on disease severity as determined by CT-angiography: normal (0% stenosis; n = 31), mild (< 30% stenosis; n = 31), moderate (30 – 49% stenosis; n = 30) and severe (>70% stenosis; n = 9). HDL was separated from plasma by size-exclusion chromatography and HDL bound proteins were analyzed by electrospray ionization tandem mass spectrometry. A spectral index value (derived from normalized spectrum counts and the number of subjects in which the peptide was detected) was used to evaluate changes in protein abundance between disease groups. We then developed a novel metric for the analysis of HDL proteome data, which takes into account all identified proteins as a single proteome index that represents the deviation of the whole proteome in disease compared to the healthy controls. This new metric, the HDL Proteome Index, displays an impressive correlation with CVD severity as measured by coronary artery calcification ($r^2 = 0.9524$; $p < 0.001$) and plaque volume ($r^2 = 0.9318$; $p < 0.01$), even after correction for other known risk factors. These findings establish the HDL proteome as a significant factor in the development of CVD and indicate its potential for further development as a biomarker of cardiovascular risk with even greater predictive power than the current clinical measure of HDL-C.

National Cancer Institute - Division of Cancer Epidemiology and Genetics (NCI-DCEG)

Payal Khincha

Clinical Fellow

Vascular Disease and Biology

Pulmonary Arteriovenous Malformations: an Uncharacterized Phenotype of Dyskeratosis Congenita

Background: Dyskeratosis congenita (DC) is a cancer-prone inherited bone marrow failure (BMF)

syndrome caused by germline mutations in telomere biology genes. The triad of reticular skin

pigmentation, dysplastic nails and oral leukoplakia is diagnostic. Many patients may present with other

manifestations; all are at risk of other medical problems including pulmonary fibrosis (PF) and liver

disease. Pulmonary arterio-venous malformations (PAVMs) have been reported in a limited number of

DC patients, often in relation to hepatopulmonary syndrome (HPS). However, the underlying biology of

PAVMs in DC is unclear. PAVMs can lead to clinically significant right-to-left shunting resulting in

decreased oxygenation and respiratory insufficiency. Objective: To characterize PAVMs as a phenotype

of DC. Design/method: In this IRB-approved multi-institutional case series we evaluated patients of any

age, race and gender diagnosed with DC and PAVMs concurrently or at separate times. Data were

obtained by retrospective review of medical records by the primary institution and maintained at NCI.

Results: We report thirteen unrelated patients with DC and PAVMs. Nine (70%) had no evidence of liver

disease or portal hypertension (and hence no HPS) at PAVM diagnosis. The median age at DC diagnosis

was 13 years (range 1-27) and 15 years (range 3-32) for PAVM. The genetic cause of DC varied (one

DKC1, five TINF2, two TERT, two RTEL1, one PARN, two unknown). Ten patients (77%) underwent

hematopoietic cell transplant (HCT) for BMF or myelodysplasia. Two patients had PAVMs diagnosed

prior to DC diagnosis, and five (38%) were prior to HCT. Diffusion capacity for carbon monoxide (DLCO)

was consistently decreased out of proportion to other pulmonary function tests (PFTs), including in

patients with PF (12-55% of predicted). Bubble echocardiography with agitated saline was indicative of

PAVMs in all but one patient. The majority of PAVMs in DC were multiple and microscopic, only one

patient could undergo PAVM embolization. Conclusion: This case series establishes PAVMs as an

important pulmonary phenotype of DC that can occur independently of HPS. Clinicians should be vigilant

of respiratory symptoms and abnormal PFTs, particularly decreased DLCO, not explained by other

pulmonary pathology. Early detection of PAVMs is key to timely symptomatic management. Further

research is needed to determine the underlying pathophysiology and optimal therapeutic options for

patient management.

National Institute of Allergy and Infectious Diseases (NIAID)

Seong-In Hyun

Doctoral Candidate

Virology - DNA

Vaccinia virus protein I2 is required for a critical stage in virus morphogenesis

Poxviruses are large enveloped double-stranded DNA viruses that replicate in the cytoplasm of host cells

and are responsible for diseases of humans and other animals. Vaccinia virus (VACV), the most

extensively studied member in the family, was used as the vaccine to prevent smallpox and is now being

used as a vector to produce vaccines against other pathogens and cancer. VACV encodes approximately

200 proteins, of which 100 are conserved in all members of the vertebrate subfamily of poxviruses and

have roles in gene expression, DNA replication, morphogenesis and cell entry. A previous report suggested that the I2 protein, encoded by one of the highly conserved genes, has an essential role in virus entry. However, this conclusion was based on repression of I2 synthesis, rather than deletion of the gene. To construct a deletion mutant, I first made a cell line that constitutively expressed the I2 protein. Next, the I2 gene was deleted from the VACV genome by homologous recombination in the complementing I2-cell line, which was also used for propagation of the mutant virus. The I2-deletion mutant was unable to replicate in control cells demonstrating that the protein has an essential role in VACV replication. Transmission electron microscopy revealed a striking defect in virus morphogenesis. During normal VACV morphogenesis, spherical immature particles shed the viral D13 scaffold protein and assume the brick shape of mature infectious particles. However, this transition did not occur in cells infected with the I2-deletion mutant and dense spherical particles accumulated. Furthermore, Western blotting and immuno-electron microscopy showed that the scaffold protein was retained on the defective particles. In addition, the membrane proteins comprising the entry/fusion complex were greatly diminished in amount on these particles although most other proteins were present at normal levels. Based on these data, I propose that the primary role of the I2 protein is to participate in the removal of the scaffold protein from immature virus particles, which is necessary for subsequent steps in morphogenesis including the incorporation of the entry/fusion proteins into the viral membrane.

National Institute of Allergy and Infectious Diseases (NIAID)

Samuel {prter

Doctoral Candidate

Virology - DNA

Optimizing Papillomavirus Infection of Primary Human Keratinocytes

Human Papillomavirus (HPV) is the most common sexually transmitted disease in the United States. As the etiologic agent of greater than 99% of cervical cancers, and a growing number of anogenital, and head and neck cancers, HPV remains a major worldwide health burden. HPV infects dividing keratinocytes in the basal levels of a stratified epithelium. The viral genome then undergoes an initial, short burst of replication, then maintains a low copy number to avoid immune system detection. Once the host keratinocytes begin to differentiate, the genome is amplified to a very high copy number. As the keratinocytes become terminally differentiated, the production of capsid proteins L1 and L2 is triggered and the genome is packaged and released from desquamating epithelial cells in the stratum corneum. Because of this unique life cycle, cell culture production of native HPV virions for use in infection studies is challenging. Organotypic 3D rafts of HPV-infected keratinocytes can be used to produce viruses but viral yields are low, and only wild type viruses that can complete the viral life cycle will be packaged. Therefore, we have optimized the production of recombinant HPV virions (quasiviruses) in 293TT cells. In this system, recircularized viral genome is cotransfected with a plasmid that expresses the capsid proteins L1/L2 and the resulting virus is purifying by ultracentrifugation on an Optiprep gradient. We have optimized the purity of the virus stock by inducing replication of the viral DNA in 293TT cells, and by enriching for genome containing virions. With the optimized quasivirus infection model, the observed infectivity in primary foreskin keratinocytes improved 10-fold. We are also able to package a series of mutated genomes and genomes that contain selectable markers in their late region. HPV infection of primary keratinocytes has not been well studied because of the difficulties in

producing virus particles, and inefficient infection of these cells. Therefore we have screened many primary keratinocyte strains to improve the sensitivity of the assay. Using a technique established in our laboratory, we can conditionally immortalize these cells. We are now using CRISPR-Cas9 technology to inactivate cellular factors that will likely restrict viral infection. This will allow us to study the initiation and establishment of HPV infection in the host keratinocyte.

National Institute of Allergy and Infectious Diseases (NIAID)

Dennise de Jesús Díaz

Postdoctoral Fellow

Virology - RNA and Retroviruses

Reconstructing the human gastrointestinal tract in vitro: a new model system for the study of noroviruses and other enteric pathogens

Noroviruses (NoV) cause acute gastrointestinal disease worldwide, with diarrhea and vomiting that can lead to life-threatening dehydration. Although millions of cases occur annually in the U.S. alone, no effective antiviral drugs or vaccines are available. The absence of a permissive cell culture system for the study of NoV infectivity in vitro has been a major research obstacle in the development of control strategies. Studies have shown that NoV disease can be recapitulated in human volunteers challenged orally with stool samples containing the virus, but animal models do not present the same pathophysiology of vomiting and diarrhea when challenged. Therefore, we hypothesized that a human-like gut environment may be necessary for successful viral replication in vitro. In order to mimic the human gastrointestinal (GI) tract, we established small intestinal organoids (SIO) by the sequential differentiation of human pluripotent stem cells into definitive endoderm followed by hindgut endoderm. Comparative analysis of SIOs to human intestinal biopsies by immunohistochemistry demonstrated that these structures were morphologically authentic and expressed Cdx2, Muc2, ChgA and villin, markers for intestinal epithelium, goblet, enteroendocrine and enterocytes cells, respectively. However, although this system resembled the human GI tract structurally and norovirus particles bound to the SIOs as measured by RT-qPCR of viral RNA, the intestinal epithelial layer alone was not sufficient to support viral replication. Because the human GI tract is a complex and dynamic environment characterized by a mixture of immune components, bacteria and its metabolites, all of which could influence NoV infections, we initiated studies to identify the environmental features of the gut associated with norovirus infection in ill patients. First, we are analyzing the gut microbiome of patients during an acute NoV episode and after recovery in context of the metabolites and factors that might facilitate norovirus infection. Second, we are screening molecules and peptides present in the intestinal tract as additives to the SIOs during norovirus infection experiments, and we have already generated promising results. By combining these two systematic approaches we expect to successfully establish a cell culture system for human NoV. This system would advance the field by allowing study of NoV infectivity and pathogenesis, critical areas of understanding in vaccine and drug design.

National Institute of Allergy and Infectious Diseases (NIAID)

Masfique Mehedi

Research Fellow

Virology - RNA and Retroviruses

Virus-Induced Cellular Filopodia are a Previously Unrecognized Mechanism for Human Respiratory Syncytial Virus Spread

Human respiratory syncytial virus (RSV) is the leading cause of severe lower respiratory disease in young children worldwide, causing over 60,000 hospitalizations annually in the US alone. Currently, there is no vaccine or specific antiviral therapy available. To identify host factors involved in RSV infection, we performed high-throughput human genome-wide siRNA screening on RSV-infected A549 cells, a human lung epithelial cell line. We identified actin-related protein 2 (ARP2) as one of the host factors involved in the RSV life cycle. ARP2 is part of the ARP2/3 complex, which regulates actin polymerization and affects cellular shape, structure, and motility. ARP2 knockdown in A549 cells did not reduce RSV entry or gene expression at early time points after infection; however, virus budding, release of infectious virions, and syncytia formation were reduced at late time points. This suggested that ARP2 plays a role in viral spread. Staining of A549 cells for filamentous actin, microtubulin, and RSV fusion (F) protein revealed that infection induced extensive formation of finger-like actin protrusions that were mostly microtubulin-deficient, identifying them as filopodia. Using stimulated emission depletion (STED) microscopy, we found that the filopodia appeared to shuttle RSV particles to nearby uninfected cells. ARP2-knockdown reduced RSV-induced filopodia formation and virus spread, confirming its role on cell-to-cell spread. By examining the formation of filopodia during infection with two related viruses, human parainfluenza virus type 3 (PIV3) and human metapneumovirus (MPV), we found that filopodia-mediated spread was most extensive in RSV infected cells. Filopodia-mediated RSV spread also seemed to be specific to human lung epithelial cells, as it was reduced or absent during RSV infection in monkey kidney cells. Transient expression of the RSV F protein in A549 cells or infection with chimeric bovine/human PIV3 virus expressing RSV F protein induced actin protrusions, suggesting a new role for the RSV F protein in the viral spread. Our results show that RSV-induced filopodia represent a previously-unrecognized mechanism for cell-to-cell spread. This provides a new target for the development of RSV antivirals.

National Institute of Allergy and Infectious Diseases (NIAID)

Jonathan Dougherty

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Virology - RNA and Retroviruses

Human Myxovirus resistance protein B (MxB) restricts Ebola virus replication

Abstract removed at request of author

National Heart, Lung, and Blood Institute (NHLBI)

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Virology - RNA and Retroviruses

Intercellular transmission of enteroviral populations with vesicles

During cell-to-cell transmission, viruses are largely thought to behave as discrete infectious units. Upending this view, we recently discovered that enteroviruses could travel between cells, not only as independent viral particles but also as clusters of viral particles. We reported that members of the enterovirus family of positive-strand RNA viruses, including poliovirus, coxsackievirus, and rhinovirus, all are released non-lytically from cells in culture within large vesicles that contained up to several hundred viral particles. We discovered that the vesicle membrane surrounding the enterovirus particles was enriched in phosphatidylserine (PS) lipids. We show that vesicular PS lipids are co-factors to the relevant enterovirus receptors in mediating subsequent infectivity and transmission, in particular to primary human macrophages. These vesicles then facilitated virus spread to other susceptible cells by collectively transferring multiple viral genomes into the cytoplasm. We found that the enterovirus containing extracellular vesicles were highly infectious. Surprisingly, we observed significantly higher infection efficiencies when cells were infected with viral particles within vesicles as opposed to equivalent titers of free viral particles. Additionally, viral particles within vesicles are capable of suppressing the production of antiviral mRNAs of primary human macrophages rather than free single viral particles. This study reveals a novel mode of viral transmission, where enteroviral genomes are transmitted from cell-to-cell en bloc in membrane-bound PS vesicles instead of as single independent genomes. This has implications for facilitating genetic cooperativity among viral quasispecies, enhancing viral replication as well as regulating innate immune responses.