Medical Research Fellows Program

Abstracts

April 20–22, 2018
On the Cover (clockwise from top left):
Melanin transfer from epidermal melanocytes to neighboring keratinocytes underlies skin pigmentation and protection from damage by ultraviolet (UV) radiation. This symbiotic system is known the epidermal-melanin unit. However, the exact molecular mechanism of melanin transfer within an epidermal-melanin unit has not been fully clarified. Therefore, experimentally tractable models of melanocyte–keratinocyte communication remain an important part of understanding disorders of pigmentation. When co-cultured primary human melanocytes and keratinocytes spontaneously form an epidermal-melanin units making it an ideal model to study melanin transfer and analyze the effect of specific genes or defined factors such as UV radiation. Shown here is a 2D maximum intensity Z-projection of primary melanocytes and keratinocytes forming an epidermal-melanin unit after two days of co-culture. Expression of HMB-45 and MART-1 in melanocytes are shown in green and expression of cytokeratin 5 in basal keratinocytes are shown in red.
(Courtesy of: Brandon M. Law, HHMI Medical Fellow, Harvard Medical School. Mentors: R. Rox Anderson, MD, Massachusetts General Hospital and David E. Fisher, MD, PhD, Massachusetts General Hospital)

Glioblastoma is an aggressive brain cancer that prolifically invades local tissue. The mechanisms responsible for this invasion are poorly understood and studies directed at the tumor cells in isolation fail to model cancer for what it really is: a complex organ with interacting cellular and extracellular elements. Therefore, there is growing interest in studying the stroma around cancer to better understand the forces that drive invasion. Site-directed biopsies taken from the subventricular zone, a region known to harbor particularly aggressive glioblastoma, have demonstrated increased expression of EDA-Fibronectin, a component of the extracellular matrix thought to promote tumor invasion. Shown here is an immunofluorescent image from a glioblastoma biopsy from the subventricular zone demonstrating EDA-fibronectin (Green), total fibronectin (Red) and DAPI (blue).
(Courtesy of: Jonathan W. Rick, HHMI Medical Fellow, University of California, San Francisco. Mentor: Manish K. Aghi, MD, PhD, University of California, San Francisco)

eIF4e is an integral part of the eukaryotic translation initiation factor 4F (eIF4E) complex, which initiates translation of target mRNA transcripts, via the binding of eIF4E to the 5′-prime methyl-7 guanosine (m7G) cap of mRNA transcripts. eIF4E modulates the translation and thus expression of a subset of mRNAs, many of which are implicated in proliferation, survival, and oncogenesis. Additionally, eIF4e has been shown to be overexpressed in some 30% of malignancies. The anti-viral drug, ribavirin, has been demonstrated to have anti-neoplastic effects through the modulation of eIF4E, including via the nuclear to cytoplasmic relocalization of eIF4E. Pictured here is the nuclear and cytoplasmic localization of atypical teratoid rhabdoid tumor (AT/RT) cells treated with ribavirin, and costained for eIF4E (GFP, green) and DNA (DAPI, blue). These studies allow us to better understand ribavirin's mechanistic effects in AT/RT.
(Courtesy of: Joshua Casaos, HHMI Medical Fellow, Johns Hopkins University School of Medicine. Mentor: Henry Brem, MD, Johns Hopkins Hospital)

Many cancers are known to be highly aneuploid but experimentally derived aneuploid cells have a distinct growth disadvantage and can produce inflammatory cytokines. To address how aneuploidy affects the immune system's control of cancerous or pre-cancerous cells, aneuploid cells can be created from immortalized or transformed cell lines by inducing chromosome missegregation. In this image, mitosis of a melanoma cell treated with a small molecule inhibitor is visualized with stains for tubulin (anti-DM1-alpha, red) and DNA (DAPI, blue). Comparing the immune response to these cells and their euploid counterparts in vivo and in vitro can help uncover mechanisms the body uses to prevent tumor growth as well as those that cancer cells use to evade the immune system.
(Courtesy of: Emily MacDuffie, HHMI Medical Fellow, Massachusetts Institute of Technology. Mentor: Angelika Amon, PhD, Howard Hughes Medical Institute and Massachusetts Institute of Technology)
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Characterization of External Globus Pallidus Neurons from the Dbx1 Lineage

Zachary Abecassis, HHMI-PF Medical Fellow, Northwestern University, The Feinberg School of Medicine

Mentor: Chi-Yung Savio Chan, PhD, Northwestern University, The Feinberg School of Medicine

Note: Abstract numbers based on poster presentation numbers at AAP-ASCI-APSA Joint Meeting

Parkinson’s Disease (PD) is a prevalent neurodegenerative disease that affects the central nervous system. Early stages of the disease include symptoms of resting tremor, muscle rigidity, bradykinesia, and gait instability, that often leads to other complications such as dementia or depression. Dysfunction within the basal ganglia, particularly the external globus pallidus (GPe), has been implicated in the motor manifestations of the disease. The GPe most notably projects to other areas within the basal ganglia circuit, primarily the dorsal striatum and subthalamic nucleus (STN). Its communication with other areas of the brain, such as the cortex and thalamus, remains largely unknown. We recently identified a subtype of GPe neurons that originate from progenitor cells expressing the protein-coding gene developing brain homeobox 1 (Dbx1+) during embryologic development. Contrary to the majority of the GPe that originates from the medial and lateral ganglionic eminences, these neurons originate from the preoptic area. The objective of this study was to comprehensively profile these neurons to determine if they possess any unique qualities as compared to the known subtypes of GPe neurons, i.e. parvalbumin (PV) and Npas1 transcription factor expressing neurons. By crossing a Dbx1-Cre transgenic mouse with a floxed-stop tdTomato (Ai14) mouse, we have been able to identify neurons originating from the Dbx1 lineage (Dbx1+) with tdTomato fluorescence. Immunohistochemistry was performed for known GPe proteins and intrinsic cell properties were captured using cell-attached and whole-cell patch-clamp recordings. Finally, retrograde tracers (fluorogold and cholera toxin subunit B) were injected into various brain regions including the subthalamic nucleus, cortex, and parafascicular nucleus of the thalamus (PF), in order to evaluate potential projection sites of Dbx1+ GPe neurons. Immunohistochemistry revealed that Dbx1+ GPe neurons comprise 10 percent of GPe neurons. These neurons were heavily PV+ (~62%) with some expressing Npas1 (~10%). Contrary to two-thirds of Npas1 neurons, the Npas1+ Dbx1+ neurons did not express Foxp2, a subset of Npas1 neurons known to project exclusively to the striatum. Electrophysiological analysis revealed that Dbx1+ GPe neurons appear to match the results seen in the immunohistochemistry, representing a subset of both PV+ and Npas1+ neurons. Retrograde tracing revealed projections to both the STN and PF. Electrophysiology and immunohistochemistry reveal that GPe neurons of Dbx1 lineage appear to be representative of the greater neuronal population of the GPe. However, the projection to the PF represents a novel projection site of these neurons, not seen by any other biomarkers within the GPe. Therefore, the unique origin of these neurons presents an opportunity to leverage this transgenic mouse to further explore this anatomical projection.

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Sulfasalazine as a Treatment for Acquired Epilepsy

Oscar Alcoreza, HHMI-CURE Medical Fellow, Virginia Tech Carilion School of Medicine (Virginia Tech Carilion Research Institute)

Mentor: Harald Sontheimer, PhD, Virginia Tech Carilion Research Institute

Epilepsy affects approximately 2.2 million Americans, with 150,000 new cases being diagnosed each year. Despite some success managing epilepsy, current therapeutics offer no benefit to 1-in-3 patients. Previous studies from our lab revealed that primary brain tumors release glutamate and induce electroencephalographic (EEG)-confirmed behavioral seizures in adult mice implanted with human-derived glioma cells. These studies identified increased expression of system xc (SXC), a cysteine/glutamate exchanger, on glioma cells as a major contributor to elevated glutamate levels in tumor-implanted mice. Inhibition of SXC via sulfasalazine (SAS), an FDA-approved anti-inflammatory drug, decreased glutamate release and EEG-confirmed behavioral seizures.

SXC is normally expressed on glial cells and gliosis is a prominent feature of many forms of epilepsy. Additionally, recent studies revealed that astroglial dysfunction, leading to pathological changes in the extracellular environment and neuronal metabolism, may play a critical role in the initiation of seizures and development of epilepsy. We therefore hypothesize that gliosis may cause an increase in SXC expression leading to enhanced glutamate release in acquired epilepsies and that treatment with SAS can decrease seizure occurrence in mouse models of epilepsy. To test this, we used the kainic acid (KA)-induced model of acquired epilepsy, which present with gliosis, to characterize changes in the expression of SXC. Our preliminary data reveal that animals treated with KA showed increase protein expression of SXC in the hippocampus and that treatment with SAS decreases the expression of SXC. Using the beta-1 integrin knockout mouse model,
which is characterized by widespread chronic astrogliosis and spontaneous seizures, our preliminary results suggest that treatment with SAS decreases EEG-confirmed behavioral seizures.

These results suggest that SXC may play a role in the pathogenesis of acquired epilepsies and that further studies on SXC and the effects of SAS is warranted as a potential novel therapeutic modality. As most treatments for epilepsy involves modifying neuronal excitatory or inhibitory mechanisms, SXC provides an unexplored glial target for decreasing glutamate release in acquired epilepsies.

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The Congenital Heart Disease Candidate Gene Myelin Regulatory Factor Plays an Unexpected Role in Left Right Patterning

Sarah Amalraj, HHMI Medical Fellow, Yale School of Medicine

Mentor: Mustafa Khokha, MD, Yale School of Medicine

Congenital heart disease (CHD) is the most common major birth defect, affecting nearly 3% of children. Although the causes of CHD are not well understood, abnormalities in left-right (LR) patterning (known as Heterotaxy (Htx)) are associated with severe forms of CHD. A recent analysis of Htx/CHD patients identified numerous candidate genes, including the gene myelin regulatory factor (MYRF). The protein MYRF is known to be a transcription factor for the generation of myelin in the central nervous system during development; however, its role in LR patterning and heart development is undefined. Here, we show that depletion of myrf using CRISPR based gene modification in Xenopus tropicalis causes midline heart looping defects phenocopying our patient. We then analyzed LR patterning markers pitx2 and coco and found abnormal bilateral expression of pitx2, but normal expression of coco in myrf depleted embryos. Additionally, we depleted myrf in one cell of a two-cell embryo and found that left-sided injected embryos resulted in heart looping defects, abnormal bilateral pitx2 expression, and normal coco expression. Conversely, right-sided injected embryos had no LR patterning defects. Together, our data suggests MYRF plays a role in LR patterning, possibly by acting as a midline protein regulating transcription of nodal gene xnr1, as nodal signaling occurs between coco and pitx2 expression in the LR patterning cascade. We conclude that patient driven gene discovery from patients with congenital heart disease can provide new insights into the molecular mechanisms that drive cardiac patterning and LR axis formation.

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IL-33-T Regulatory Cell Axis Triggers Development of a Cancer-Promoting Immune Environment in Chronic Inflammation

Amir Ameri, HHMI Medical Fellow, Harvard Medical School

Mentors: Shawn Demehri, MD, PhD, Massachusetts General Hospital

Chronic inflammation is a well-characterized driver of cancer in the skin and other epithelial organs; however, the mechanism underlying the development of cancer-promoting chronic inflammation is unknown. We previously showed chronic allergic contact dermatitis (ACD) is a type 2 inflammatory disease and potent inducer of squamous cell carcinoma in mice and humans. In contrast, acute ACD, a common skin inflammatory condition, is marked by type 1 inflammation, including T helper 1, cytotoxic T and NK cells, which inhibit cancer development. The opposite effects of chronic versus acute ACD on cancer provide a unique paradigm to investigate how cancer-promoting chronic inflammation develops. To determine the mechanism underlying the transition from acute to chronic ACD, we examined the epithelium-derived cytokines, IL-33, TSLP, and IL-25 that are master drivers of type 2 inflammation in barrier organs. IL-33 expression markedly increased during the transition from acute to chronic ACD, initiating tumor-promoting, type 2 inflammation in chronic ACD. Mice lacking IL-33 or IL-33 receptor (ST2) were protected from ACD-induced skin cancer compared to wild-type controls and IL-33 was required for the progression of inflammatory bowel disease-induced colorectal cancer. Notably, IL-33’s direct effect on T regulatory cells was required for the development of a cancer-promoting immune environment in the skin and colon. Our findings elucidate a novel mechanism underlying the formation of a tumor-initiating immune environment in chronic inflammatory diseases and yield novel targets for cancer treatment and prevention in chronic inflammatory contexts.
Recent studies have demonstrated that therapies targeting neoantigens can elicit effective anti-tumor immunity. These antigens arise from somatic tumor mutations, and are attractive targets for immunotherapy given their exquisite tumor specificity and exemption from central tolerance. Personalized neoantigen vaccines have been shown to be feasible, safe and immunogenic in highly mutated cancers, such as melanoma, but have not been tested in tumors with lower mutation rate.

A Phase I/Ib trial was conducted of personal neoantigen vaccines for glioblastoma (GBM), a tumor with ten-fold lower mutation burden than melanoma. Following standard of care surgery and radiation, eight patients received vaccine consisting of up to 20 synthetic long peptides (median 12, range 7–20) that encode predicted neoepitopes, and poly-ICLC adjuvant. Changes in the tumor microenvironment were assessed in post-vaccination surgical resection specimens from five patients. Vaccination induced de novo circulating polyfunctional neoantigen-specific CD4+ and CD8+ T cell responses, and increased tumor-infiltrating lymphocytes (TILs) in two patients who did not receive dexamethasone during vaccine priming. In these patients, infiltrating CD8+ T cells increased significantly from two patients who did not receive dexamethasone during vaccine priming.

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In contrast, all three patients who received dexamethasone during vaccine priming failed to generate circulating immune responses, with no significant change in T cell infiltrate.

We hypothesized that post-vaccination TILs were specific for immunizing neoantigens. Single cell T cell receptor (TCR) analysis of CD3+ TILs and peripheral T cells in vitro expanded against immunizing peptides was performed from one patient with circulating neoantigen-specific T cells. Four CD4+ and two CD8+ T cell clonotypes in peripheral blood were identical to TILs. In order to probe their specificity, we applied a pipeline developed in our lab to clone and express TCR sequences of immunizing neoantigens. Single cell T cell receptor (TCR) analysis of CD3+ TILs and peripheral T cells in vitro expanded against immunizing peptides was performed from one patient with circulating neoantigen-specific T cells. Four CD4+ and two CD8+ T cell clonotypes in peripheral blood were identical to TILs. In order to probe their specificity, we applied a pipeline developed in our lab to clone and express TCR sequences of immunizing neoantigens.

We identified a TCR shared in CD4+ T cells in peripheral blood and TILs specific for ARH-GAP35, a neoantigen targeted by vaccination, and capable of discriminating between the mutant and wildtype peptide. The intratumoral TCR repertoire was probed further using an algorithm called Grouping of Lymphocyte Interactions by Paratope Hotspots (GLIPH), to cluster TCRs by similarity. Representative TCRs from each cluster are being tested for neoantigen specificity and reactivity against autologous tumor.

Our observations demonstrate that neoantigen vaccines favorably alter the immune milieu of GBM, and that neoepitope-specific T cells can traffic from the periphery into intracranial tumors. These results are particularly promising in a tumor with relatively low mutation burden and low immune infiltrates at baseline. However, there remain significant immunosuppressive factors to overcome for neoantigen-specific T cells to generate clinically significant anti-tumor activity. We anticipate that combination therapy with checkpoint blockade may be more effective in augmenting anti-tumor immunity and improving outcomes to therapy.

Circulating Exosomal MicroRNA as a Non-Invasive Biomarker for Pediatric Medulloblastoma

Sydney Ariagno, HHMI Medical Fellow, Weill Cornell Medical College

Mentors: David Lyden, MD, PhD and Praveen Raju, MD, PhD, Weill Cornell Medicine and Memorial Sloan Kettering Cancer Center

Medulloblastoma (MB) is the most common malignant pediatric brain tumor, and over one-third of children with this cerebellar tumor die within five years of diagnosis. Clinicians are currently limited in their abilities to diagnose this tumor without surgical biopsy and identify metastatic or recurrent disease at early stages. The development of a noninvasive biomarker could help address these problems. Exosomes are microvesicles that are secreted from tumors constitutively and contain cell-type specific proteins and genetic material, including microRNAs (miRs). In addition, exosomes are easily isolated from plasma and provide an unexplored reserve for noninvasive biomarkers.

Using a sporadic Sonic Hedgehog (SHH)-driven mouse model of MB, we assessed exosomal profiles from tumor tissue explant cultures and plasma at early and late tumor stages. Exosomes from cerebellar tissue explants and plasma were similarly collected from age-matched control mice. Tissue explants were cultured for 24 hours in exosome-depleted media. Conditioned media from explants and plasma samples then underwent differential ultracentrifugation to isolate exosomes, and exosomal protein amount was quantified. Tissue- and plasma-derived exosomes then underwent miR
sequencing. Exosomal protein amount and miR expression were compared between early stage preneoplastic and late tumor stages, as well as between tissue and plasma samples at each stage.

We found that exosomal protein abundance from late stage tumor tissue was significantly higher than early stage preneoplastic tissue (0.246 vs. 0.114 ug/mg; ***p<0.0001) after normalization for explant brain weight. Additionally, exosomal protein amount from late stage tumor tissue was significantly higher than that of late stage control cerebellum (0.246 vs. 0.126 ug/mg; ***p<0.0001). A similar trend was seen with exosomal plasma samples, although did not reach statistical significance (0.04 vs. 0.0207 ug/mg, p=.232; 0.039 vs. 0.025 ug/mg, p = .136). With regards to tumor exosomal content, miR sequencing revealed the presence of the miR-17~92 cluster which is specifically associated with the human SHH-driven MB subtype. qPCR validation of miR expression in circulating exosomes confirmed that miR-17, 19b, 20a, and 92 (all members of the 17~92 cluster) were approximately twice as prevalent in exosomes from tumor mice as compared with control mice.

In conclusion, our data suggests that tumor exosomes may be a potential noninvasive biomarker for monitoring disease status in children with MB. Tumor tissue explant and plasma exosomal protein amount in our SHH-MB mouse model correlates with tumor stage. Exosomal miR sequencing shows upregulation of components of the miR-17~92 cluster that are specific for the SHH MB subgroup. Additional studies will be needed to validate these preclinical findings in human MB tissue and plasma samples.

The DYRK1A Pathway Illustrates a Paradoxical Program in Normal Versus Malignant Lymphopoiesis

Rahul Bhansali, HHMI Medical Fellow, University of Illinois College of Medicine (Northwestern University, The Feinberg School of Medicine)

Mentor: John Crispino, PhD, Northwestern University

Given the critical role of DYRK1A in lymphopoiesis, we aimed to seek whether DYRK1A is a suitable target in ALL. We first generated murine models of ALL in pan-hematopoietic Dyrk1a conditional knock-out mice. Excision of Dyrk1a in leukemic mice increased survival after transplant nearly threefold. Next, in order to determine viability of targeting DYRK1A, we treated ALL cell lines with EHT1610, a potent and selective DYRK1A-specific inhibitor. EHT1610 sensitivity was observed in ALL cell lines harboring various oncogenic mutations, including those associated with both non-DS and DS-ALL. We also found that 50% (9/18) of primary ALL samples were sensitive to EHT1610 treatment in vitro, with 75% (6/8) of the DS-ALL samples displaying sensitivity. Of note, the observed induction of cycling also conferred increased sensitivity to traditional cytotoxic drugs, such as cytarabine. Furthermore, we saw a marked decrease in leukemic burden in ALL-PDX models after treatment with EHT1610, indicating that DYRK1A is a target in ALL.

Lastly, we performed global and directed phosphoproteomic studies to determine DYRK1A substrates in pre-B cells. Loss of DYRK1A activity in pre-B cells leads to changes in phosphorylation of substrates that regulate cell cycle, splicing, and RNA metabolism. One of the identified targets is serine 329 of FOXO1, a pro-apoptotic transcription factor that is critical in B lymphopoiesis and negatively regulated by PI3K/Akt signaling. Using a green fluorescent protein-tagged FOXO1, we found that treatment of large pre-B cells with EHT1610 shifted FOXO1 localization from the cytoplasm to the nucleus, where it is active despite intact PI3K/Akt signaling. Moreover, treatment of pre-B cells with EHT1610 and AS1842856, an inhibitor of activated FOXO1, rescued the G2-M blockade. Paradoxically, despite its tumor suppressor role in normal lymphocytes, ALL cell lines are very sensitive to FOXO1 inhibition, suggesting a unique requirement for its role in the survival of ALL cells. In all, these studies indicate that DYRK1A is an effective target in ALL through a loss of proliferative capacity, sensitization to commonly used cytotoxic drugs by induction of cycling, and dysregulation of substrates which are key to lymphocyte survival.
Characterization of Novel Photosensitizers based upon the Janelia Fluor Rhodamine Scaffold

Thomas Binns, HHMI Medical Fellow, University of Arkansas for Medical Sciences College of Medicine (Howard Hughes Medical Institute, Janelia Research Campus)

Mentors: Luke Lavis, PhD and James Liu, PhD, Howard Hughes Medical Institute, Janelia Research Campus

Photosensitizing agents are excited by electromagnetic radiation, typically in the form of visible or ultraviolet light, and subsequently generate free radicals via the transfer of energy to adjacent molecules. Photosensitizers see broad use in the basic sciences through techniques such as targeted cell ablation and chromophore-assisted light inactivation of specific proteins as well as in the clinic through applications including photodynamic therapy for the treatment of cancers and inactivation of pathogenic microbes. This study describes the characterization of photosensitizing agents based upon HaloTag ligand variants of the Janelia Fluor scaffold using two approaches—halogenation and substitution of the central xanthene oxygen with a sulfur. The resulting candidate molecules are compared for singlet oxygen generation potency, HaloTag binding kinetics, and photosensitization availability due to the lactone-zwitterionic equilibrium typical of rhodamine dyes. Experiments in their use for the manipulation of biological systems are currently underway. This study provides the characterization of several new photosensitizing agents for use in the biomedical sciences and compares two canonical approaches to photosensitizer development, highlighting potential pitfalls of each in the context of compatibility with self-labeling enzyme tagging systems such as HaloTag.

Dynamics of Dexmedetomidine-induced Loss and Return of Consciousness Across Primate Neocortex

Jessica Briscoe, HHMI Medical Fellow, Geisinger Commonwealth School of Medicine (Harvard Medical School)

Mentors: Emery Brown, MD, PhD and Emad Eskandar, MD, Massachusetts General Hospital and Harvard Medical School

Consciousness is the experience of perceiving the outside, as well as our internal worlds; by synthesizing multiple sensory inputs, developing emotions, or forming and reflecting on opinions. However, we have yet to understand how the components of the brain interact to mediate consciousness and arousal. Physicians and scientists have manipulated arousal and consciousness for decades, using anesthesia for purposes of surgery and procedures without the knowledge of how it affects the interchange of brain networks. The body’s natural neural oscillations that create varying arousal and unconsciousness states during sleep has been shown to be physiologically distinct from the neural oscillation patterns seen during anesthesia-induced arousal and unconsciousness. This distinction may reveal more information about anesthesia’s effects, as well as the phenomenon of changes in arousal states. More importantly, electrophysiological studies conducted in rodents, non-human primates and humans are largely conducted under anesthesia without a basic understanding of how anesthetic agents interfere with baseline brain functional synchrony and neural oscillations. We hypothesize anesthetic-induced altered states of arousal are generated by highly structured and distinctive oscillations, causing characteristic spatial and temporal neural patterns along the neocortex, thalamus and brainstem.

We have investigated how the anesthetic dexmedetomidine effects neural oscillations during loss of consciousness (LOC) and return of consciousness (ROC) in the primate neocortex.

We conducted microelectrode array intracortical recordings in the somatosensory (SI) and frontal ventral premotor (PMv) networks in primates. Following a behavioral task, to determine alertness and performance measures, dexmedetomidine infusion is administered through a vascular port. Alertness and trial-by-trial performance was then used to analyze the primates behavior during LOC, ROC, and return of pre-anesthetic performance (ROPAP) using the state-space model.

We first analyzed the change in spectrograms in SI and PMv. During wakefulness, beta oscillations dominated in SI and PMv, while the onset of LOC was identified by a brief increase of alpha power oscillations more obvious in SI than PMv. Throughout anesthesia, slow-delta oscillations appeared, while ROC was associated with a return of alpha oscillation dominance and decrease of slow-delta waves. As the animal recovered,
alpha waves appeared to increase its frequency toward the beta range; however, ROPAP did not achieve pre-anesthetic beta oscillation frequency and power. Single neuron spike responses were clearly different in S1 and PMv. The PMv spike firing rate increased upon LOC while S1 neurons decreased; a response that continued through recovery.

These findings suggest that dexmedetomidine-induced LOC and ROC are both associated with an increase of alpha oscillations in S1 and PM. The slow-delta oscillations are dominant while the animals are unconscious. Spike firing response appears to be region specific, but clearly associated with consciousness changes. Further investigation is underway to identify mechanisms of cortical, thalamic, and brainstem responses under dexmedetomidine anesthesia.

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Enteroendocrine Cell Activity Modulates Vagal Response in vivo

Kelly Buchanan, HHMI Medical Fellow, Duke University School of Medicine
Mentor: Diego Bohórquez, PhD, Duke University

Overeating is linked to an altered perception of the reward value of nutrients. But how this signal is transduced from the gut to the brain remains unknown. Like taste receptor cells in the tongue, the gastrointestinal tract also has cells that sense nutrients. These are called enteroendocrine cells (EECs). EECs are known to respond to luminal stimuli and release hormones that act on vagal nerve fibers. Recently, we discovered that EECs can also form synaptic links with vagal nodose neurons. Here, we sought to establish if EECs directly transduce stimuli onto vagal nerve fibers. We combined whole nerve electrophysiology of the cervical vagus nerve and EEC optogenetics to dissect the functional connectivity of this gut-brain neuroepithelial circuit. Our results show the following:

First—sugars delivered intra-luminally stimulate vagal firing rate. Sucrose, D-glucose, the non-nutritive sweetener sucralose, and the non-metabolized sugar methyl α-D-glucopyranoside, but not fructose, cause a rapid and significant increase of vagal firing rate when delivered intra-luminally in mice. The response is not observed when the stimuli is applied intraperitoneally or directly onto the cervical vagus. The effects on cervical vagal firing rate are abolished after a subdiaphragmatic vagotomy, indicating that an intact vagus is necessary for the response.

Second—EECs transduce the stimuli of both nutritive and non-nutritive sugars onto the vagus nerve. Using a mouse in which EECs express the excitatory channelrhodopsin 2, we found that an intraluminal stimulus with 473nm laser rapidly and significantly increases vagal firing rate. This increase is comparable to the sucrose response. Then, using a mouse in which EECs express the inhibitory halorhodopsin, we found that both sucrose and sucralose vagal responses are attenuated when EECs are stimulated with a 532nm laser light.

Third—EEC transduction of a sucrose stimulus is dependent upon the sodium glucose transporter SGLT1. We found that the sucrose but not sucralose stimulus is abolished when the solution contains the SGLT1 receptor blocker phloridzin.

These data suggest that EECs modulate vagal activity within seconds in response to D-glucose and that the response is dependent upon the sodium glucose transporter SGLT1. This neuroepithelial circuit represents a therapeutic target to alter the transduction of caloric reward from gut to brain and to modulate appetite.

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Ribavirin as a Potential Therapeutic for Atypical Teratoid/Rhabdoid Tumors

Joshua Casaos, HHMI Medical Fellow, Johns Hopkins University School of Medicine
Mentor: Henry Brem, MD, Johns Hopkins University School of Medicine

Atypical teratoid/rhabdoid tumors (AT/RT) are highly aggressive pediatric brain tumors with no current standard of care. A recent genetic analysis reported AT/RT to have high expression of enhancer of zeste homolog 2 (EZH2), a methyltransferase known to have oncogenic properties in many cancers. Our laboratory previously demonstrated that the anti-viral drug ribavirin, approved by the FDA for treatment of hepatitis C, inhibited glioma cell growth in vitro and in vivo, potentially through modulation of EZH2. Based on these findings and the lack of a pre-clinical model of ribavirin in AT/RT, we investigated the effect of ribavirin on AT/RT in vitro and in vivo. Three different human AT/RT cell lines (BT12, BT16, and BT37) were selected for investigation. Cell proliferation was assessed via cell counting. Cell cycle and cell death processes were quantified using flow cytometry. Tumor migration, invasion, and adhesion capacities were assessed via scratch wound, Boyden chamber, and adhesion assays, respectively. Ribavirin’s mechanism of action in
AT/RT was studied using Western blots for several known ribavirin targets. Finally, we tested ribavirin efficacy in vivo using an orthotopic xenograft AT/RT model in athymic mice.

We provide evidence that ribavirin significantly impairs AT/RT cell growth, increases cell cycle arrest, and induces cell death, potentially through modulation of the EZH2 and/or eIF4E pathways. We also demonstrate that ribavirin significantly impairs AT/RT cell migration, invasion, and adhesion. Most importantly, we show that ribavirin significantly improves the survival of mice orthotopically implanted with BT12 cells. Ribavirin-treated animals exhibited a significantly increased median survival (56 days) compared to controls (37 days) (p<0.0001).

Our work establishes that ribavirin is effective against AT/RT in vitro and in vivo. Given the lack of standard therapy for AT/RT, these findings fill an area of unmet need and could represent a new therapeutic option for children with this deadly disease.

**Glucose Transporter GLUT3: A Novel Molecular Target in Bevacizumab-Resistant Glioblastoma**

**Ankush Chandra**, HHMI Medical Fellow, Wayne State University School of Medicine (University of California, San Francisco, School of Medicine)

**Mentor**: Manish Aghi, MD PhD, University of California, San Francisco

In spite of optimal treatments and recent advances in our understanding of its biology, glioblastoma (GBM) remains the most lethal primary adult brain tumor, with a poor median survival of less than 15 months from diagnosis. While phase II clinical trials showed encouraging results, randomized phase III clinical trials failed to show efficacy of VEGF neutralizing antibody bevacizumab, which devascularizes the tumor, as most tumors progress during treatment and develop a much more aggressive and invasive molecular phenotype associated with resistance. Our lab has identified and found overexpression of the glucose transporter GLUT3 to be a hallmark of resistance to anti-angiogenic therapy in GBM causing a metabolic re-circuitry within the tumor. In our studies, we have also shown GLUT3 overexpression to be associated with increased survival and proliferation of bevacizumab-resistant GBM cells, both, in vitro and in vivo. We have also found that GLUT3 inhibition using an indirect inhibitor decreased survival of bevacizumab-resistant GBM cells in culture, suggesting GLUT3 as a potential targetable biomarker of resistance. We believe that direct molecular inhibition of GLUT3 will reverse the aggressive biology of bevacizumab-resistant GBM and overcome resistance to anti-angiogenic therapies. Using six novel direct GLUT3 inhibitors synthesized and provided by our collaborators, we found that all six inhibitors inhibited bevacizumab-resistant GBM proliferation with three of the six inhibitors inhibiting proliferation by 45%. We are analyzing the effects of these inhibitors on bevacizumab-resistant GBM cell biochemistry, morphology, invasiveness, and growth in culture and in vivo to fully define the effect of direct GLUT3 inhibition on bevacizumab-resistant GBM. We believe that elucidating the biological actions of direct GLUT3 inhibitors will define targeting GLUT3 in bevacizumab-resistant GBM as an effective strategy to overcome GBM resistance to bevacizumab and unraveling the full therapeutic potential of anti-angiogenic agents.

**NGF signaling in stress fracture healing**

**Leslie Chang**, HHMI Medical Fellow, University of California, San Diego, School of Medicine (Johns Hopkins University School of Medicine)

**Mentors**: Thomas Clemens, PhD and Aaron James, MD, PhD, Johns Hopkins University

In contrast to the large body of literature on the role of peripheral nerves in other tissues, few studies have investigated the function of peripheral nerves in the skeleton. Developing peripheral tissues dictate innervation by secretion of specific neurotrophins, which promote neuronal survival by activation of tyrosine kinase receptors. The prototypic target tissue-derived neurotrophin is nerve growth factor (NGF). Importantly, the vast majority of nerves in mature bone are thinly myelinated or unmyelinated sensory neurons, which express the NGF high affinity receptor TrkA. As sensory nerves have also been shown to be important mediators of early response to mechanical loading; we sought to develop a better understanding of the expression of the neurotrophin NGF and subsequent innervation of bone in a clinically relevant stress fracture model.

In the present study, 18 week old male Thy1-YFP and NGF-eGFP reporter mice underwent stress fracture injury, using a previously validated cyclic axial compression of the right forelimb. Bone repair was assessed using routine histology as well as immunohistological analysis to assess for inflammatory response (CD45), and vascular proliferation (CD31). In Thy1-YFP reporter samples, immunohistochemistry was performed for the sympathetic marker TH (Tyrosine Hydroxylase) and sensory nerve marker CGRP (Calcitonin gene-related peptide).

Stress fracture induces early and robust NGF reporter activity within the injured bone. At 1 and 3 days post-injury, increased NGF reporter activity
was observed among the resident periosteal stromal cells as well as inflammatory cells adjacent to the fracture site. CD45 immunohistochemical staining highlighted a dual source of NGF within the early fracture period among both stromal cells and inflammatory cells. Next, neuronal sprouting and ingrowth were examined within the same model using Thy1-YFP reporter animals (a pan-neuronal reporter). In the uninjured periosteum, YFP+ nerve fibers are of thin caliber and indiscerete. Early timepoints post fracture demonstrated an increased density of YFP+ nerve fibers was observed adjacent to the fracture site. The bulk of YFP+ nerves surrounding the fracture site were found to be CGRP+ sensory nerves, while a small minority were found to be TH+ sympathetic fibers.

Additionally, as neural density increased, a trend toward increased vascularity of the fracture site was also observed.

Our data documents a timeline of NGF expression in an appendicular stress fracture model within the early stromal/inflammatory milieu of the soft callus. This robust expansion of an NGF+ domain within early bone healing corresponds to later neural sprouting and ingrowth of predominantly CGRP+ sensory fibers into and around the bone repair site. Moreover, sensory nerve ingrowth seems to be coordinated with vascular ingrowth. These new findings suggest a previously undescribed role for sensory nerves in the trophic support for fracture repair.

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Using Artificial Intelligence to identify Non-Invasive Imaging Biomarkers in Lung Cancer—a Deep Learning Radiomic Approach

Tafadzwa Chaunzwa, HHMI Medical Fellow, Yale School of Medicine (Harvard Medical School)

Mentor: Hugo Aerts, PhD, Dana-Farber Cancer Institute, Brigham and Women’s Hospital and Harvard Medical School

Lung cancer is the leading cause of cancer death and morbidity. The high degree of intra- and inter-tumor heterogeneity presents a challenge in treatment. However, early-stage disease, specifically, Stage I Non-Small Cell Lung Cancer (NSCLC), is amenable to curative resection. Poor surgical candidates may benefit from Stereotactic Body Radiation Therapy (SBRT). Many prognostic molecular and genomic biomarkers have been identified in lung cancer, and there is a growing body of evidence suggesting radiomic phenotypes (drawn from medical images) can augment prognostic power, when used in combination with both clinical features and tumor genomic profiles. Recent advances in computation and convolutional neural networks (CNNs) have also opened new avenues to tumor patch analysis and feature extraction from different imaging modalities.

In this study we set out to develop a deep learning model that can act as a non-invasive prognostic biomarker in patients with Stage I NSCLC treated with surgery. In contrast to “conventional” radiomics, which relies on engineered quantitative imaging features (e.g. Shape Compactness, wavelet Grey Level Nonuniformity HLH), deep learning based image analysis algorithms are able to automatically find optimal rules and parameters from data. Our model would be able to assign patients to short term or long term survival groups, based on computed tomography (CT) characteristics.

Initial data mining yielded a discovery cohort of 300 patients who underwent surgery for Stage I NSCLC at Massachusetts General Hospital (MGH) between 2004 and 2010. Pre-surgical CT studies with or without contrast were retrieved for ~300 patients. Image pre-processing included manual tumor identification and segmentation, with seed-point placement to predefine a 50 voxel cubical region of interest (ROI) that contains tumor, and creation of isotropic voxel dimensions in CT data with spatial interpolation. Further data curation yielded 233 volumetric and 2-dimensional imaging samples to feed into our neural networks. A 2-dimensional VGG-16 CNN modeled after the mammalian visual cortex was used to perform visual recognition and data analysis. The model was trained on 186 labeled CT scans, matched to one of two groups based on 2 year survival. Data containing variations of scanners and imaging protocols were used in training to create a model that is robust for the variations.

Our model learned to classify patients with long term vs short term survival in a test set of 47 patients, with 83% accuracy. These preliminary findings suggest that Artificial Intelligence-enhanced radiomic feature extraction and predictive modeling can improve the clinician’s ability to assess the benefits of treatment in patients with early-stage NSCLC. An even larger dataset of ~1300 patients with Stage II and Stage III lung cancer, treated at MGH, is also being curated and added to separate analyses. In addition, pre-trained 3-dimensional models will also be employed.
Targeting the Vitamin B6 Pathway as a Novel Therapeutic Strategy for Cutaneous T-cell Lymphoma

Cynthia Chen, HHMI Medical Fellow, Columbia University College of Physicians and Surgeons

Mentor: Larisa Geskin, MD, Columbia University Medical Center

Cutaneous T-cell lymphoma (CTCL) is a malignancy of predominantly skin-homing CD4+ lymphocytes. Patients with advanced stages of CTCL have a high mortality rate, and even those with more indolent disease experience severely diminished quality of life due to recurrent skin infections, pain, and pruritus. Currently, there are few specific therapeutic targets known in CTCL, highlighting the need to identify not only key mediators in disease pathogenesis, but also druggable ones. One aspect of CTCL cells that has not been well-studied thus far is their dependence on unique metabolic pathways to support dysregulated growth. Here, we identify the vitamin B6 pathway as a novel potential target for the treatment of CTCL. Using an sgRNA-mediated knockout platform, we found that loss of pyridoxal kinase (PDXK), an enzyme that converts vitamin B6 to its active form, pyridoxal phosphate (PLP), resulted in reduced proliferation of the CTCL cell lines MyLa and PB2B. Consistently, administration of the FDA-approved drug isoniazid, commonly used to treat tuberculosis and known to block the vitamin B6 pathway, recapitulated this proliferation defect in vitro. Furthermore, cells cultured with isoniazid demonstrated higher levels of apoptosis and delayed cell cycle progression. Future studies will assess the feasibility of repurposing isoniazid as a new treatment for CTCL treatment by testing the drug’s effects on primary samples from patients with CTCL.

Pan-Cancer Akt Pathway Synthetic Essential Long Intergenic Non-Coding RNA LINC00499

Jasper Chen, HHMI Medical Fellow, University of Texas Medical School at Houston (The University of Texas-MD Anderson Cancer Center)

Mentor: Ronald DePinho, MD, The University of Texas-MD Anderson Cancer Center

The Akt pathway is one of the most commonly activated in oncogenesis. To identify gene targets that could lead to the development of therapies across multiple cancer types, we applied the principle of synthetic essentiality, a recently described framework for identifying potential synthetic lethal interactions by screening for genes that are consistently retained in the context of a tumor suppressor deletion or an oncogene amplification, on a pan-cancer scale to analyze 11,025 cases encompassing 32 cancer types from the Cancer Genome Atlas for genes that possess loss-of-function mutations that are mutually exclusive of loss-of-function in PTEN, gain-of-function in EGFR, PIK3CA, AKT1, AKT2, AKT3, or high phospho-Akt (Ser473) by RPPA. Based on these criteria, 1,010 cases were classified as Akt pathway gain-of-function and 10,015 cases were classified as wild-type. Our screening yielded a lincRNA, LINC00499, that had zero loss-of-function mutations in the Akt gain-of-function cohort and 40 loss-of-function mutations in the wild-type cohort. LINC00499 was overexpressed in PTEN-deficient SF763 glioblastoma and DU145 prostate cancer cell lines, suggesting increased dependency on this lincRNA during the PTEN-deficient state. Thus, we hypothesized that PTEN deletion disinhibits the Akt pathway, thereby inhibiting FoxO, which disinhibits LINC00499 expression. Three conserved FoxO binding sites were found using FIMO to search for the consensus binding motif between -8 kb from the transcription start site to +5 kb from the transcription end site of LINC00499 in aligned sequences of human, mouse, and at least one of cow, rabbit, sheep, pig, dog, cat, or horse with p-value < 0.0001. BLAST against Human RefSeqGene sequences determined putative binding elements in two splice variants of LINC00499. Depletion of LINC00499 by CRISPR-mediated deletion of the binding element for each splice variant yielded a reduction in cell proliferation and survival in PTEN-deficient SF763 GBM cells. The splice variant of LINC00499 with the greater difference in phenotype between PTEN-KO and WT was further analyzed by chromatin isolation by RNA purification in order to validate the 166 potential gene targets identified by BLAST. The binding sites varied from as short as 18 bp in length with 100% sequence identity to the lincRNA binding element to as long as 120 bp with 72% identity. The ErbB signaling pathway was found to be highly represented in this list by KEGG pathway enrichment.
Mature adipocytes impair the antimicrobial function of reactive dermal adipogenesis: an explanation for impaired cutaneous defense in obesity

Stella X. Chen, HHMI Medical Fellow, University of California, San Diego, School of Medicine

Mentor: Richard Gallo, MD, PhD, University of California, San Diego, School of Medicine

To defend against cutaneous infections, the skin must maintain both a physical and immunological barrier. Recently, our lab has shown that a layer of adipocytes in the dermis is necessary for antimicrobial defense by producing the cathelicidin antimicrobial peptide (Camp). However, obesity is paradoxically associated with increased cutaneous infection rates. In this study, we investigated how the antimicrobial-adipogenic response is impaired in obesity. Using mouse models of diet-induced obesity, we found that obese mice fed a high-fat diet lost dermal adipogenic-antimicrobial activity and had increased susceptibility to Staphylococcus aureus infection. In vitro studies of mouse primary dermal adipogenic fibroblasts showed that Camp expression was transiently produced in early adipogenesis but subsequently lost as adipocytes matured. Functional antimicrobial assays confirmed that newly differentiating, immature adipocytes led to a 97% (p<0.05) reduction in methicillin-resistant Camp growth, but this antimicrobial effect was lost with further adipogenesis. Surprisingly, we found that primary dermal fibroblasts co-cultured with mature adipocytes prior to fibroblast differentiation could no longer inhibit S. aureus growth during early adipogenesis. These fibroblasts expressed decreased Camp expression (p<0.01), increased mature adipocyte marker (Fabp4) expression (p<0.01), and increased collagen (Col1a1) expression (p<0.02). Together, these results suggest that undifferentiated adipogenic fibroblasts are an antimicrobial reserve that fight infection by rapidly producing Camp. Mature adipocytes not only lose antimicrobial function, but may indirectly deplete (through spontaneous differentiation) and directly impair (through fibrosis) the adipogenic stem cell pool, leading to impaired antimicrobial defense. These findings provide a possible explanation for impaired cutaneous defense in obesity.

Chromosome bridge resolution requires mechanical forces from actin-based contractility

Anna Cheng, HHMI Medical Fellow, USF Health Morsani College of Medicine (Harvard Medical School)

Mentor: David Pellman, MD, Howard Hughes Medical Institute, Dana-Farber Cancer Institute and Harvard Medical School

Chromosome bridges result from errors in cell division and form chromatin threads that connect daughter nuclei after division. These bridges eventually break (“resolve”) and the daughter cells inherit broken chromosome fragments. This is thought to initiate a major pathway for oncogene amplification and tumor genome evolution called the “breakage-fusion-bridge” (BFB) cycle. However, we still lack a complete understanding of the mechanism(s) causing chromosome bridges to break in the first place. Here we present new evidence that bridge breakage requires actin-dependent contractile forces. As daughter cells connected by a bridge move away from each other, the bridge is typically stretched over long distances before breakage. Using fibronectin micropatterns to limit daughter cell separation, we were able to block bridge resolution, with over 90% of bridges still intact as the daughter cells entered the next mitosis. In cells not constrained by micropatterns, bridge resolution was similarly blocked by timed addition of inhibitors of actin contractility. We propose that mechanical forces from actin-based contractility play a central role in bridge resolution.

We are also studying the genomic consequences of bridge breakage. BFB cycles have been observed in association with another form of localized mutagenesis called chromothripsis. It has also been shown that individual cells experiencing telomere dysfunction can grow into clonal populations with chromothripsis. These findings suggest a mechanistic link between bridge breakage and chromothripsis, but the details of this relationship are unclear. To address this question, we are using our “Look-seq” approach, which combines long-term imaging with single-cell sequencing. We will discuss whether bridge breakage occurs directly via chromothripsis, or if the relationship is indirect, with chromothripsis occurring as a downstream consequence perhaps through formation of micronuclei in subsequent cell cycles.
Loss of function of the hepatic fatty acid translocase receptor CD36 is insufficient to protect from liver steatosis in a murine model of parenteral nutrition-induced liver injury

Bennet Cho, HHMI Medical Fellow, Harvard Medical School

Mentor: Mark Puder, MD, PhD, Boston Children’s Hospital

Children with intestinal failure (IF) due to insufficient bowel length or loss of function require intravenous parenteral nutrition (PN). However, long-term PN administration can lead to IF-associated liver disease (IFALD), characterized by cholestasis and hepatic inflammation. IFALD can progress to end-stage liver disease requiring liver transplantation. Although there is no current Food and Drug Administration (FDA)-approved treatment, replacing the standard soybean oil-based lipid emulsion in PN with a fish oil-based lipid emulsion (FOLE) has been found to reverse IFALD. Our laboratory has demonstrated that FOLE protects from hepatosteatosis in a murine model of PN-induced liver injury, a process that is dependent on G-protein coupled receptor 120 (GPR120). Peroxisome proliferator-activated receptor γ (PPARγ) expression, a transcription factor associated with hepatic lipogenesis and lipid droplet deposition, is normalized by FOLE treatment. Among transcriptional targets of PPARγ, CD36 expression is increased in this model of liver injury and is normalized by FOLE treatment in a GPR120-dependent fashion. Fatty acid translocase receptor CD36 is a free fatty acid and lipoprotein transporter receptor associated with increased hepatic fatty acid uptake and triglyceride storage. The goal of this study was to determine if loss of function of CD36 is sufficient to protect from PN-induced liver injury.

Cd36 knockout (KO) C57BL/6 male mice and wild type (WT) littermates were fed ad libitum chow or PN diet for 19 days. Livers, spleens, and kidneys were weighed and stained for hematoxylin and eosin (H&E) histologic analysis. Total RNA was extracted from liver and subjected to quantitative real-time polymerase chain reaction (qPCR) for measurement of hepatic regulators of lipid metabolism, including acetyl-CoA carboxylase 2 (ACC2) and PPARα.

H&E staining revealed marked steatosis in livers from both WT and KO mice fed PN, whereas WT and KO mice fed regular chow revealed histologically normal livers. qPCR data demonstrated increased expression of ACC2 and PPARα in PN-fed CD36 KO mice compared to chow-fed WT and KO mice.

The results of this study demonstrate that loss of function of CD36 by itself is not sufficient to prevent development of hepatosteatosis in a murine model of PN-induced liver injury. Increased ACC2 and PPARα expression in CD36 KO mice suggests compensatory lipid accumulation via parallel pathways in the absence of CD36 function. Further studies investigating these alternate pathways may elucidate the pathologic mechanisms involved in the development of IFALD.

Oncogenic potential of non-canonical PIK3CA mutations and implications for molecular targeting strategies in head and neck cancer

Janice Cho, HHMI Medical Fellow, Wake Forest School of Medicine of Wake Forest Baptist Medical Center (University of California, San Francisco, School of Medicine)

Mentor: Jennifer Grandis, MD, University of California, San Francisco

Despite the recent FDA approval of PD1 inhibitors for head and neck squamous cell carcinoma (HNSCC), about 50% of patients succumb to their disease and predictive biomarkers to guide therapy are lacking. PIK3CA, the most commonly mutated oncogene in HNSCC, encodes a phosphoinositide-3-kinase (PI3K) isoform, which triggers downstream pathways involved in cell growth and survival. According to The Cancer Genome Atlas Network (TCGA), approximately 63% of PIK3CA mutation clusters at three canonical, “hotspot” mutation sites (E542K, E545K, H1047R) while 37% of PIK3CA-mutated HNSCC are non-canonical, rare mutations. Although there is considerable evidence illustrating the oncogenic effect of canonical, “hotspot” PIK3CA mutations, the role of non-canonical, rare mutations in HNSCC tumorigenesis is unclear. Additionally, preliminary data from our lab demonstrate that PIK3CA mutations may lead to tumorigenesis via hyperactivation of the PI3K/COX2/PGE2 signaling pathway, and that combined therapeutic targeting of PI3K and COX2 may be a viable therapeutic strategy for PIK3CA-altered tumors. Through a genomics-based approach to treatment selection, this project aims to uncover the role of PIK3CA non-canonical mutations in conferring oncogenic driver function with the goal of identifying a potentially viable therapeutic strategy for subsets of patients who possess PIK3CA-mutated tumors. To do this, I am engineering a panel of non-canonical PIK3CA mutation variants in a representative HNSCC cell line sensitive to serum deprivation. These mutants will then be functionally assessed for activating potential through proliferation in the absence of serum and probed for PI3K/COX2/PGE2 signaling activation via immunoblot analysis. Subsequently, I will determine whether PIK3CA non-canonical mutations exhibiting “driver” tumor growth augment response to conventional or molecular targeting strategies used in the treatment of HNSCC. If successful, this project will guide therapy for patients possessing PIK3CA-mutated tumors.
Immune Profiling of Rejection Biomarkers in HIV-Positive Transplant Recipients

Simon Chu, HHMI Medical Fellow, University of California, San Francisco, School of Medicine

Mentors: Qi Zhi Tang, PhD and Peter Stock, MD, PhD, University of California, San Francisco

HIV+ solid-organ transplant recipients are predisposed to a three times higher rate of rejection episodes when compared to HIV- recipients, but immunological correlates of rejection in this population have not previously been identified. Here we describe our investigation of immunologic phenotype and gene expression profiling to identify functional differences between Rejectors (Rej) and Non-Rejectors (NR).

Donor and recipient peripheral blood mononuclear cells (PBMCs) were collected prior to transplant. Rej were selected based on biopsy-proven acute cellular rejection. Kidney transplant recipients were stratified by Rej (n=28) versus NR (n=56), as compared to matched HIV- kidney transplant recipients, HIV+ non-transplant controls and HIV-, ESRD-healthy control subjects (n=25 per group). These patients were profiled using flow cytometric panels to characterize cellular subsets, activation status, and Treg phenotype. Groups were compared for variance using the Kruskal-Wallis test, with pairwise comparison performed between groups by Dunn’s post-test. For gene expression analysis, pre-transplant HIV+ liver recipient PBMCs from Rej (n=2) and NR (n=2) were co-cultured in mixed lymphocyte reaction (MLR) in vitro with either CD40L-stimulated donor or 3rd party B cells. Donor B cells were removed by immunodepletion and recipient cells were analyzed using a custom NanoString panel. Raw counts were normalized and p-values were adjusted using the Benjamini-Hochberg procedure.

HIV+ Rej were found to have markers of increased pre-transplant immune activation as compared to NR, with a bias toward activation of the innate immune system. They exhibited a significantly altered monocyte phenotype, including decreased HLA-DR expression on CD14+CD16+ intermediate monocytes (p=0.0042). Moreover, Rej have increased B cell activation by HLA-DR expression (p=0.016) and less activated Tregs by decreased percentage of CD39+ Tregs (p=0.0271). The frequency of Tregs did not differ between the two groups. After alloantigen stimulation, Rej showed increased gene expression of T-cell activation markers, CD28 and ICOS (p=0.0242, 0.0334). Interestingly, NR displayed upregulation of regulatory ligands in the leukocyte immunoglobulin-like receptor family (LILR), including LILRB4, LILRB1, and LILRA1 (p=0.0384, 0.017, 0.0317). Differential gene expression between Rej and NR was preserved irrespective of stimulus by either donor or 3rd party.

Overall, our results suggest that increased rates of rejection in HIV+ kidney and liver transplants correlate with pre-transplant, recipient-specific immune dysfunction. Concordance in gene expression profile following stimulation with donor or 3rd party suggests that differential gene expression is an intrinsic, recipient-driven propensity to immune activation in Rej and immune regulation in NR.

Quantitative Analysis of RPE Morphology after Gene Therapy Rescue in a Mouse Model of Retinitis Pigmentosa

Michelle Chung, HHMI-FFB Medical Fellow, Indiana University School of Medicine (Harvard Medical School)

Mentors: Connie Cepko, PhD, Howard Hughes Medical Institute and Harvard Medical School and David Wu, MD, PhD, Harvard Medical School and Massachusetts Eye and Ear Infirmary

Retinitis pigmentosa (RP) is a disease in which a mutation in >70 different loci leads to a conserved pattern of clinical symptoms and cell death in the eye. Photoreceptors, the cell types that capture and process light, are the cells primarily affected by the genetic lesion. Rod photoreceptors, which we use for dim light vision, die first, followed by cones, the type that we use for our daylight and color vision. Retinal pigmented epithelial cells (RPE) provide support for rods and cones, and they are also affected in RP.

We recently showed that an adeno-associated viral (AAV) vector overexpressing Nrf2, a transcription factor that fights oxidation and inflammation, rescues cones and vision in an RP mouse model (rd1). We have more recently found that it also rescues the RPE. My project concerns quantifying the degree of RPE rescue so that we can better score this phenotype and test Nrf2 relative to other RP treatments that we are developing.

Eyes from rd1 mice received a subretinal injection of an AAV encoding for Nrf2 driven by an RPE-specific promoter (BEST1) or an AAV encoding GFP driven by the same promoter. The eyes were then flat-mounted and the RPE cytoskeletons imaged with phalloidin. The uniform hexagonal monolayer of the RPE became increasingly dysmorphic in untreated or control mice but remained regular in Nrf2 treated mice. Manual segmentation of RPE cells and quantification with Fiji found that the RPE of untreated or control mice had mean areas 12% larger than in treated mice. Control (AAV-GFP) rd1 RPE also had a mean aspect ratio 16% lower than that found in rd1 RPE from eyes treated with Nrf2. Because of the time-consuming nature of this analysis, we developed a customized eight-step
CellProfiler pipeline to automate segmentation. CellProfiler was less effective than manual segmentation for both mean cell area (ROC AUC 0.68 for manual vs 0.56 for CellProfiler) and aspect ratio mean (ROC AUC 0.96 for manual vs 0.77 for CellProfiler).

We were able to demonstrate a quantitative difference between rescued and non-rescued RPE utilizing parameters of cell area and cell aspect ratio. Aspect ratio mean was a better parameter for discriminating between treated and untreated RPE than mean cell area. Although manual segmentation of RPE offers more sensitivity, CellProfiler is able to detect differences in rescued vs non-rescued eyes. Further improvements to the CellProfiler pipeline may bring its performance closer to that of human segmentation.

Together, these results suggest that renalase and its serum binding protein may play an important role in the regulation of injury and recovery in pancreatitis. To better understand how, I am using immunoprecipitation assays to confirm the identity of the binding protein and investigate its interactions with renalase during periods of pancreatic injury and recovery. I am also working with human samples to confirm that the same RNLS binding protein(s) found in murine plasma is the same in humans.

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The Role of Renalase and its Potential Serum Binding Partner in Pancreatitis

Shang-Lin Chung, HHMI Medical Fellow, Yale School of Medicine

Mentor: Fred Gorelick, MD, Yale School of Medicine

Renalase (RNLS) is a plasma protein secreted by the kidney and other tissues that has been found to be protective in acute injury including models of ischemic injury to the kidney and heart. In addition, it has been shown that renalase appears to disappear from serum during acute kidney injury. Given its apparent cytoprotective properties and connection to other organ systems and disease, we investigate the relationship of renalase with pancreatitis. Using isolated murine pancreatic lobules, pretreatment with recombinant human renalase (rRNLS) blocked zymogen activity caused by both cerulein and carbachol, two agents that can cause pancreatitis in vivo. Cerulein-induced histological changes commonly seen in pancreatitis was also prevented with rRNLS pretreatment, and cerulein administration to mice with a genetic deletion of renalase resulted in more severe pancreatitis. Furthermore, serum endogenous renalase was found to be undetectable within an hour after the onset of experimental cerulein-induced murine pancreatitis; RNLS returned to levels higher than basal values once cerulein administration ceased. Similarly, in preliminary studies in human plasma from individuals with acute pancreatitis we observed significant decreases in plasma levels when compared to healthy controls. We found that renalase is transported in serum in a high molecular weight complex, and this complex may have a role in its apparent disappearance from serum during the onset of disease. Through mass spectrometry, we determined that putative murine RNLS binding protein candidates in murine models include murinoglobulin, pregnancy zone protein, C3, and C5, which are members of the alpha-2-macroglobulin domain containing gene family.

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Mice Lacking Type-1 Interferon Receptor Generate ADCK Antibodies in Response to ΔgD-2 Vaccination but have Defects in ADCK Mobilization

Joseph Dardick, HHMI Medical Fellow, Albert Einstein College of Medicine

Mentor: William Jacobs, PhD, Howard Hughes Medical Institute and Albert Einstein College of Medicine

ΔgD-2 is a live-attenuated Herpes Simplex Virus type-2 (HSV-2) strain genetically deleted for glycoprotein D (gD), one of the immunodominant antigens. gD is required for cell entry, limiting ΔgD-2 to a single round of replication in non-complementing cells. ΔgD-2 is a novel immunogen in that it elicits completely protective immunity against HSV-1 and HSV-2 through antibody-dependent cell-mediated cytotoxicity (ADCK). The mechanisms by which immunogens elicit ADCK activating antibodies are not well understood, but type-1 interferons (IFNα/β) have been shown to play a role activating humoral immunity and FcγR-mediated immune modulation.

To investigate the role of IFNα/β in generating an effective ADCK antibody response, we prime-boost vaccinated IFNα/β receptor knockout mice (IFNAR-/-) with a fluorescent ΔgD-2 variant and compared the resulting immunity to that of WT mice. Both strains of mice vaccinated with ΔgD-2 generated similar proportions of total activated and gB-specific CD8+ T cells. Additionally, ΔgD-2 vaccinated IFNAR-/- mice developed similar overall and isotype specific anti-HSV IgG titers as compared to WT mice. ADCK assays using bone-marrow derived macrophages (BMDMs) from WT mice showed that serum from vaccinated IFNAR-/- mice induces significantly more ADCK killing than serum from vaccinated WT mice (p<0.05). To determine whether the humoral immunity in IFNAR-/-...
mice initiated ADCK in vivo, serum from vaccinated IFNAR\(-/\) mice was passively transferred into naive WT and FcγRI\(\text{IV}\)-/- knockout mice. WT mice that received the vaccinated serum were partially protected from 10x LD\(_{90}\) challenge with HSV-2 but FcγRI\(\text{IV}\)-/- mice were not (p<0.05). However, in a simultaneous experiment, serum from vaccinated WT mice did not confer any protection to naive IFNAR\(-/\) mice, indicating difficulty in mobilizing an ADCK response (p<0.01). Surprisingly IFNAR\(-/\) mice that were prime-boost vaccinated with ΔgD-2 were still completely protected from morbidity and mortality following a 10x LD\(_{90}\) challenge with HSV-2 (p<0.0001). Subsequent quantification of viral copies in the dorsal root ganglia of the challenged mice found no difference in sterilization of infection between vaccinated WT and IFNAR\(-/\) mice. To determine whether the disruption in ADCK during the passive transfer experiments was due to defects in macrophage response, assays were performed using WT BMDMs in the presence of IFNAR blocking Abs. However, the addition of anti-IFNAR antibodies did not affect ADCK activity, indicating that IFNAR signaling is not required for macrophages to carry out ADCK. Together, our data shows that the production of ADCK activity, indicating that IFNAR signaling is not required for macrophages to carry out ADCK. Together, our data shows that the production of ADCK antibodies in response to ΔgD-2 is independent of IFNα/β, phages to carry out ADCK. Together, our data shows that the production of ADCK antibodies in response to ΔgD-2 is independent of IFNα/β, IFNAR-/- mice that were prime-boost vaccinated with ΔgD-2 were still completely protected from morbidity and mortality following a 10x LD\(_{90}\) challenge with HSV-2 (p<0.0001). Subsequent quantification of viral copies in the dorsal root ganglia of the challenged mice found no difference in sterilization of infection between vaccinated WT and IFNAR\(-/\) mice. To determine whether the disruption in ADCK during the passive transfer experiments was due to defects in macrophage response, assays were performed using WT BMDMs in the presence of IFNAR blocking Abs. However, the addition of anti-IFNAR antibodies did not affect ADCK activity, indicating that IFNAR signaling is not required for macrophages to carry out ADCK. Together, our data shows that the production of ADCK antibodies in response to ΔgD-2 is independent of IFNα/β, but IFNAR signaling is required for protection by passive transfer. These findings illustrate a novel drawback of the IFNAR\(-/\) model while highlighting the utility of ΔgD-2 as a vaccine vector for pathogens which are commonly studied in IFNAR\(-/\) mice.

Reciprocal Transplantation and ATAC-seq Profiling of Fibroblasts Reveal Scar-Forming Behavior is Cell Intrinsic

Heather desJardins-Park, HHMI Medical Fellow, Stanford University School of Medicine

Mentor: Michael Longaker, MD, MBA, Stanford University School of Medicine

Scars can be physically and psychologically devastating, and represent a major financial burden on the US healthcare system. Interestingly, mammalian fetuses heal scarlessly by regenerating native tissue. In mice, the transition from regenerative healing to scarring occurs between embryonic day (e)16.5 and e18.5. A lineage of fibroblasts, the cells known to produce scars, defined by embryonic expression of Engrailed 1 deposits all scar tissue in adult mice. However, these Engrailed 1-positive fibroblasts (EPFs) contribute to scarless healing before e16.5, then transition into a scarring phenotype as development progresses. Therefore, we hypothesized that EPFs accumulate epigenetic changes over time that result in the shift from scarless to scarring phenotype, and that EPF healing phenotype is thus cell intrinsic rather than dependent on cell microenvironment.

Fibroblasts were isolated from En1\(^{\text{Cre;R26mTmG}}\) mice using fluorescence-activated cell sorting (FACS) at gestational ages e10.5, e16.5, e18.5 and postnatal day (p)1 and p30. Reciprocal transplantation experiments were performed to analyze EPF behavior in vivo, by injecting EPFs from e16.5 or p1 En1\(^{\text{Cre;R26mTmG}}\) mice into the dorsum of C57BL/6j mice at p1 or e16.5, respectively. Epigenetic analysis was performed using the Assay for Transposase Accessible Chromatin with high-throughput sequencing (ATAC-seq).

EPFs at e16.5 transplanted into a scarring microenvironment (p1) exhibit non-scarring morphology and colocalize with type I collagen in only 2.13% of cells, whereas EPFs at p1 transplanted into a scarless microenvironment (e16.5) exhibit scarring morphology and colocalize with type I collagen in 24.18% of cells. ATAC-seq profiling reveals e10.5 fibroblasts are of a single lineage; they were thus excluded from analysis. Time course analysis of e16.5-p30 EPFs and Engrailed 1-negative fibroblasts (ENFs) demonstrates reliable biological reproducibility. Principal component analysis indicates that while EPFs and ENFs are similar at e16.5, they eventually diverge as distinct populations, with the greatest differences observed at p30. Examining the EPF lineage specifically, the most epigenetic changes occur between e16.5 and e18.5, with fewer epigenetic changes occurring postnatally between p1 and p30 (significant peaks = 124 vs. 20). Upon analyzing the genes for a-smooth muscle actin and vimentin, two genes implicated in fibrosis whose expression is thought to be characteristic of all fibroblasts, we found that their genetic accessibility significantly differs between EPFs and ENFs.

Our data strongly suggest that fibroblast phenotype is linked to transcriptional regulation. Additionally, fibroblasts, while they may be capable of activation, behave in a stereotyped manner not dependent on cell microenvironment. Directed interventions on EPFs may thus enable scarless healing in adult animals. In future experiments, we hope to identify targets for genetic perturbation using CRISPR-Cas9. By corroborating our ATAC-seq data with functional assays such as Western blots and RNA analysis, we will pinpoint genes regulating fibroblast behavior to ultimately identify novel therapeutic options for human patients.
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The Role of Spinal Meningeal Lymphatic Vessels in Recovery from Spinal Cord Injury

Michael Dong, HHMI Medical Fellow, University of Virginia School of Medicine
Mentor: Jonathan Kipnis, PhD, University of Virginia

Spinal cord injury is a debilitating disease involving damage from initial injury and secondary degeneration with recruitment of immune cells to facilitate repair. However, the trafficking of immune cells between the peripheral immune system and the spinal cord is poorly understood. In addition, the controversy over the beneficial or harmful aspects of the inflammatory response in spinal cord injury has led to greater discussion over immunotherapy strategies.

The spinal meningeal lymphatic network has recently been characterized and provides an unexplored avenue for communication between the nervous system and immune system. The role of spinal meningeal lymphatics has never been studied in the context of spinal cord injury. We demonstrate marked alterations in lymphatic vessels in the spinal cord dural meningeal compartment as a result of spinal cord injury. We have assessed their morphological and functional changes and the role of major lymphangiogenesis-promoting factors in meningeal lymphatic vessel changes associated with spinal cord injury. We hypothesize that spinal meningeal lymphatic vessels respond to injury and communicate with the peripheral immune system to activate the adaptive immune response. Further work is required to establish the causal link between meningeal lymphatic trafficking of immune cells and recovery in spinal cord injury. We aim to set the foundation for spinal meningeal lymphatic modification as a therapeutic option in spinal cord injury.

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Translocation of a Pathobiont Induces Lymphocyte Migration to Internal Organs and Systemic Autoimmunity

Rebecca Fine, HHMI Medical Fellow, Yale School of Medicine
Mentor: Martin Kriegel, MD PhD, Yale School of Medicine

The mechanisms and sites of host-microbiota interactions in autoimmunity are largely unknown. We hypothesized that certain commensals traverse the gut barrier in autoimmune-prone hosts and interact with immune cells in non-gut organs to promote systemic autoimmunity. During this process, gut-imprinted lymphocytes are expected to migrate to sites of pathobiont colonization. To test these hypotheses, we used the lupus-prone (NZWxBXSB)F1 mouse model which carries a duplication of toll-like receptor 7 (TLR7) on the Y chromosome.

We first determined the importance of the microbiota by modulating the commensal community with single antibiotics. Treatment with oral vancomycin reduced activated CD44+ T cells, Th17 cells, T follicular helper cells, anti-β2GPI, -RNA IgG, -dsDNA autoantibodies, and protected mice from autoimmune deaths. Culture of small intestinal segments, mesenteric lymph nodes (MLN), livers, and spleens revealed progressive translocation of the gram-positive commensal Enterococcus gallinarum that was suppressed by vancomycin. Intramuscular vaccination of (NZWxBXSB)F1 males with E. gallinarum, but not other commensals, prevented translocation, autoantibody production, and autoimmune-related deaths.

To elucidate the contribution of host genetics to E. gallinarum-driven lupus, we tested whether internal organs of female (NZWxBXSB)F1 mice, which do not carry the TLR7 duplication and develop milder disease than males, are colonized by translocated E. gallinarum. E. gallinarum translocated to MLN in females only after first depleting the niche with broad spectrum antibiotics. To determine the role of E. gallinarum in the development of lupus in the absence of genetic predisposition, we next studied whether dense monoclonization of non-autoimmune-prone C57BL/6 germ-free mice with E. gallinarum could induce signs of systemic autoimmunity. In this gnotobiotic setting, E. gallinarum was able to translocate, and induce Th17 cells and serum autoantibodies. Aged monocolonized C57BL/6 mice had also greater levels of Peyer’s patch and splenic CD4+ T cells than germ-free controls. Gut-homing α4β7+ CD4+ T cells were significantly increased in spleens, suggesting that lymphocytes previously imprinted in the gut follow E. gallinarum to internal organs. Furthermore, in (NZWxBXSB)F1 males, circulating CD3+CD4+ cells expressing α4β7+ or CCR9 increased with age and
were reduced with oral antibiotics. Lastly, *E. gallinarum* was detected in human livers of patients with autoimmune hepatitis and systemic lupus erythematosus but not non-autoimmune cirrhotic livers.

Together, these data support that a gut commensal translocates spontaneously to initiate autoimmunity in genetically prone hosts by inducing Th17 responses, autoantibodies, and homing of gut-imprinted lymphocytes to internal organs colonized by the pathobiont. Vaccination against this pathobiont prevented translocation and autoimmune deaths. Because the same species was detected in human livers from autoimmune patients, similar processes may occur in humans. Overall, these discoveries represent a new paradigm of host-microbiota interactions in autoimmunity and reveal a novel treatment target aimed at the microbiota.

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**Pro-efferocytic Nanotherapy for Atherosclerosis**

**Alyssa Flores**, HHMI Medical Fellow, Geisel School of Medicine at Dartmouth (Stanford University School of Medicine)

**Mentor:** Nicholas Leeper, MD, Stanford University School of Medicine

Atherosclerotic cardiovascular disease continues to be the leading cause of death worldwide. It is now known that defective clearance of apoptotic tissue (efferocytosis) is a key driver of atherosclerotic plaque progression. We previously demonstrated that blocking the “don’t eat me” signal CD47 can reverse this defect and prevent plaque expansion, however this antibody-based therapy also caused off-target clearance of red blood cells.

To avoid this toxicity, we are evaluating single-walled carbon nanotubes (SWNTs) as a novel platform that may be able to deliver CD47 inhibitors specifically to the atherosclerotic plaque, without any off target toxicity.

Small-molecule inhibitors of CD47 signaling were evaluated in vitro in RAW264.7 macrophages to verify disruption of tyrosine phosphatase SHP1, the common downstream signaling pathway of CD47. SWNTs were prepared by loading carbon nanotubes with polyethylene glycol (PEG) for solubilization, Cy5.5 fluorophore for detection by fluorescence-activated cell sorting (FACS), and pro-efferocytic therapies (small-molecule inhibitors of CD47-SHP1 signaling). SWNTs were then evaluated for their ability to be taken up by monocytes/macrophages and promote efferocytosis in vitro by FACS. To study SWNT accumulation in the arterial plaque and other systemic tissues, samples of atherosclerotic aortae, blood, spleen, liver, lung, and heart were dissociated and analyzed 24h, 48h, and seven days after tail vein injection of SWNTs into atheroprone mice (apolipoprotein-E deficient, apoE -/- mice).

Immunofluorescence staining confirmed that small-molecule SHP1 inhibitors suppress phosphorylation of SHP1 in macrophages. Dye-conjugated (naked) SWNTs rapidly and preferentially accumulated in mouse RAW264.7 and human THP1 macrophages in vitro (>80%), compared to other immune cells and vascular smooth muscle cells. In vivo pharmacokinetic studies showed enhanced uptake of SWNTs into intraplaque macrophages of apoE -/- mice over time. Following seven days in circulation, total SWNT detection decreased in mouse blood, liver, lung, spleen, and heart, indicating minimal systemic accumulation. Standard in vitro efferocytosis assays demonstrated that SHP1-loaded SWNTs effectively promote clearance of apoptotic cells (4.3-fold), compared to naked SWNTs and SHP1 inhibitors alone. This effect was similar to efferocytosis rates induced by anti-CD47 antibodies in vitro.

Future studies will evaluate pro-efferocytic SWNTs for their ability to stably deliver to macrophages and enhance lesional phagocytosis in mouse models of atherosclerosis, without causing anemia. If effective, SWNTs may form the basis of a new “Trojan horse” platform for delivery of pro-efferocytic therapies to prevent atherosclerosis.

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**Elucidating Endocrine Functions of Peripheral Neuromedin U in Regulating Metabolism**

**Mollie Friedlander**, HHMI Medical Fellow, Stanford University School of Medicine

**Mentor:** Seung Kim, MD, PhD, Stanford University School of Medicine

Metabolic diseases are polyhormonal disorders, and a subset of the hormones that regulate these diseases remain uncharacterized. Previous research has focused on understanding hormones that regulate insulin secretion in the fed state, but it is apparent that sustained fasting also alters the dynamics of insulin regulation, resulting in diminished insulin output, glucose intolerance, and “starvation diabetes.” While the role of incretins in promoting insulin secretion during feeding is well-documented, a counter-regulatory hormone that decreases insulin output (a “decretin”) during fasting has not yet been identified in mammals. Recently, our group characterized an index decretin hormone in *Drosophila* and identified its mammalian orthologue, Neuromedin U (NMU).

NMU encodes a secreted peptide that is expressed in the hypothalamic “feeding center” and gastrointestinal cells. Both NMU deficiency and excess have been linked to altered glucose metabolism in mammals. Mice expressing...
a truncated form of NMU demonstrate increased adiposity and hyperinsulinemia, and a familial variant of NMU encoding a missense substitution (R165W) cosegregates with early-onset obesity and hypertriglyceridemia in humans. Conversely, ubiquitous overexpression of NMU in mouse results in hypoinsulinemia and reduced body weight, and our group previously reported ectopic NMU expression from enteroendocrine cells of the gastrointestinal tract during fasting in patients with pancreatic ductal adenocarcinoma, a disease associated with pancreaticogenic diabetes.

While prior research has investigated the role of NMU in central regulation of satiety, our studies aimed to characterize the function of peripheral NMU in regulating insulin secretion and metabolic homeostasis. Specifically, we (1) profiled NMU-expressing enteroendocrine cell gene expression by RNA-Seq, (2) evaluated the effects of NMU and peripheral NMU receptor (NMUR1) signaling on insulin secretion from pancreatic islets, and (3) generated transgenic models to explore the role of NMU misexpression on insulin secretion from wildtype islets, this effect was lost in mice lacking NMUR1. To investigate the role of peripheral NMU in vivo, we developed a conditional knockout allele and an inducible NMU misexpression allele in mice.

Collectively, our results support a model in which NMU secreted from enteroendocrine cells of the gastrointestinal tract during fasting interacts with pancreatic islets to suppress insulin secretion in an NMUR1-dependent manner. Furthermore, we present mouse models to investigate the link between NMU misexpression and altered metabolism in human disease. These studies address essential questions about links connecting peripheral NMU function and insulin biology, and a better understanding of the role of decretins in insulin regulation may transform current therapeutic approaches to treating metabolic diseases.

**Exome Sequencing Uncovers the Molecular Pathogenesis of Vein of Galen Cerebral Arterio-Venous Malformations**

**Jonathan Gaillard**, HHMI Medical Fellow, Yale School of Medicine

**Mentor:** Kristopher Kahle, MD, PhD, Yale School of Medicine

Vein of Galen malformations (VOGMs) are particularly severe arterio-venous malformations of the developing brain. If untreated, VOGMs cause high-output cardiac failure, hydrocephalus, brain hemorrhage, and death. Our limited knowledge of the molecular genetics of VOGMs has hindered the development of novel therapies. We hypothesized that the apparent sporadic occurrence of VOGM may frequently be attributable to damaging de novo mutation events or incomplete penetrance of rare transmitted variants. Unbiased whole-exome sequencing (WES) can overcome these barriers for gene discovery.

Using a dynamic and innovative HIC/IRB-approved social media campaign and teaming with multiple domestic and international collaborators, we recruited the largest VOGM cohort to date in less than 1.5 years. Germline DNA was isolated from 50 unrelated probands harboring radiographically-confirmed VOGMs. Both parents were available for 48/50 probands. WES was performed on all participating individuals (n=148). Variants in both cohorts were called using the Genome Analysis Toolkit (GATK) Haplotype Caller and annotated for allele frequencies in Exome Aggregation Consortium (ExAC), 1000 Genomes, Kaviar, and Exome Variant Server (EVS). De novo mutations were identified using TrioDeNovo and the impact of missense mutations was inferred using MetaSVM and a custom bioinformatics pipeline. Transfections of constructs harboring VOGM mutations in HEK293 cells, followed by immunoprecipitation and immunoblotting, were performed to determine impact on biochemical function. Further functional validation in vivo was performed in *Xenopus tropicalis* using CRISPR/Cas9 gene editing.

Gene burden analysis revealed exome-wide significant enrichment of rare transmitted damaging mutations in *ephrin type-B receptor* 4 (*EPHB4*: n=4; \(p = 2.34 \times 10^{-7}, 80.52\)-fold enrichment), a key regulator of arterio-venous differentiation. Additional damaging rare inherited mutations were identified in the upstream EPHB4 ligand ephrinB2 (*EFNB2*); the downstream EPHB4 effector Ras GTPase-activating protein 1 (*RASA1*), mutated in CM-AVM type 1; and the associated EphrinB2-EphB4 regulator *activin A receptor like* type 1 (*ACVRL1*), mutated in Hereditary Haemorrhagic Telangiectasia. Two novel damaging mutations were identified in the *ACVRL1* paralog *activin A receptor type* 1 (*ACVRI1*), not previously implicated in human disease. Together, mutations in EPHB4 signaling components accounted for 9/50 (18%) of cases. VOGM-associated *EPHB4*
mutants, relative to wild type, are associated with significant impairment of RAS/MAPK/ERK1/2 and PI3K/AKT/mTOR signaling in mammalian cells. \textit{X. tropicalis} embryos injected with CRISPR/Cas9 targeted against \textit{RASA1} or \textit{EPHB4} harbored severe neural tube defects compared to controls, and decreased and irregular expression of \textit{msr} and \textit{prox1}, demonstrating widespread impairment of vascular and lymphatic development.

This work demonstrates the power of social media to recruit actionable genetic cohorts for rare diseases in unprecedented time, and has uncovered the critical genetic determinants and molecular mechanisms of VOGM. These results have potential diagnostic screening implications for family members, and identify specific genes and pathways for the development of targeted therapeutics.

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**Investigating Key Residues of PCSK9 Processing and Modulators of PCSK9-Mediated Degradation**

**Adri Galvan,** HHMI Medical Fellow, Penn State Milton S. Hershey Medical Center College of Medicine (University of California, San Francisco, School of Medicine)

**Mentor:** Kevan Shokat, PhD, Howard Hughes Medical Institute and University of California, San Francisco

Proprotein convertase subtilisin/kexin type 9 (PCSK9) binds the LDL receptor (LDL-R) and induces lysosomal-mediated degradation, increasing LDL cholesterol (LDL-C), and, consequently, cardiovascular risk. Thus, PCSK9 has emerged as a prime therapeutic target against heart disease. Self-proteolysis of PCSK9 produces a mature, yet catalytically inactive protein which is shuttled extracellularly to bind and chaperone LDL-R for degradation. We previously identified PCSK9 processing as occurring via two independent mechanisms: proteolysis and proteolysis— independent secretion. We then developed parallel high-throughput luminescence assays capable of assessing each step. These assays allowed us to analyze key residues for both events using saturation mutagenesis libraries determining residues P6 and P4 through P1’ of the cleavage sequence to be highly specific for maintenance of function. We also evaluated single nucleotide polymorphism (SNP) effects on each mechanism of PCSK9 processing and found a high percentage of the SNPs analyzed to alter proteolysis. Based on our results, we examined potential structural implications of the SNPs, defined proteolysis to be the rate limiting step of PCSK9 processing, and identified a region of the protein involved in allosteric regulation of PCSK9 proteolysis.

While PCSK9 processing involves these two steps for production, little is known about mediators of PCSK9 and LDL-R binding. Cell-based studies have shown LDL-C inhibits PCSK9-mediated degradation, though the mechanism of its effect is unclear. Recently, heparan sulfate proteoglycans (HSPG) were suggested to act as co-receptors on the hepatocyte facilitating the LDL-R-PCSK9 interaction. By adapting our high-throughput assay, we investigated the relationship of these two effectors on PCSK9 uptake to elucidate the mechanism of LDL-C’s inhibitor effect on PCSK9. We also compared the inhibitory effects of LDL-C variants, such as oxidized LDL-C and lipoprotein(a), on PCSK9-mediated degradation. We found the inhibition of PCSK9 uptake to be additive with the introduction of exogenous heparin, competing for HSPG chaperone binding, and LDL-C. However, maximal inhibition of PCSK9 uptake could be achieved with heparin alone indicating related pathways which supports previous data demonstrating a similar N-terminal PCSK9 binding region for both mediators. Our studies provide further insight into PCSK9 biochemical processing and modulators of PCSK9 metabolism, with implications for finding new targets to lower LDL cholesterol and prevent atherosclerosis.

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**Investigation of Changes in Extracellular Vesicles from Glioblastoma Cells treated with Gene-Mediated Cytotoxic Immunotherapy**

**Alexandra Giantini Larsen,** HHMI Medical Fellow, Harvard Medical School

**Mentors:** Ennio Chiocca, MD, PhD and Sean Lawler, PhD, Brigham and Women’s Hospital and Harvard Medical School

Glioblastoma (GBM) is a devastating malignant brain cancer whose progression is driven by a subset of GBM stem-like cells (GSCs). In order to improve survival, novel therapeutics using gene-mediated cytotoxic immunotherapy (GMCI) are being studied. GMCI inserts the gene for a therapeutic enzyme or protein into cells using an engineered virus. One use of GMCI to target GBM involves intratumoral injection of a non-replicating adenovirus (AdV) that expresses the herpes simplex virus thymidine kinase (HSV-tk) gene. An anti-herpetic produrg that requires activation by HSV-tk is administered and the activated toxic nucleotide causes cell death that promotes an immune response. GMCI is now in clinical trials for patients with GBM. However, the identification of biomarkers that measure tumor responsiveness to the treatment is still needed. Extracellular vesicles (EVs) are important mediators of intercellular communication, and can transmit biological information. Tumor-derived EVs
are found in several biological fluids, including CSF and blood, and represent an excellent source of biomarkers. The goal of this study was to identify changes in EVs secreted by GBM cells treated with GMCI compared to control cells.

To determine the time of maximum expression of the HSV-tk gene and optimal time for exosome collection, the expression of green fluorescent protein (GFP) was monitored after cells were infected with an AdV-expressing the GFP gene. Maximal expression occurred at 24 hours. Viral infection of cells with an AdV-expressing the HSV-tk gene (AdV-tk) was confirmed by treatment of cells with increasing concentrations of the anti-herpetic prodrug ganciclovir (GCV). GCV showed strong cytotoxicity against the GSCs with an IC50 of 0.1 uM at 96 hours. Differences in EV size were detected between control cells and virally infected cells using the NanoSight at 24 and 48 hours after infection. The concentration of EVs in virally infected cells was 100-fold less than control cells at 24 and 48 hours. However, control EVs showed a main population of EVs around 100 nm, while virally infected cells secreted two different populations of EVs around 85 and 140 nm. At 48h after infection, the size of the main population of EVs remained consistent while the virally infected cells secreted EVs shifted more to the larger population.

Our results suggest that AdV-tk treatment of glioblastoma cells may alter EV quantity and size. Ongoing studies are being performed to further characterize these EVs.

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AIRE in Pregnancy: Autoimmune Regulator Gene May Support Maternal-Fetal Tolerance via Deletion of Reactive Maternal T cells

Eva Gillis-Buck, HHMI Medical Fellow, University of California, San Francisco, School of Medicine

Mentors: Tippi MacKenzie, MD and Mark Anderson, MD, PhD, University of California, San Francisco

Complications of pregnancy, such as recurrent miscarriage, may represent a failure of maternal-fetal immune tolerance. The autoimmune regulator gene (AIRE) is a crucial component of tolerance to self-antigens, but its relevance for maternal-fetal tolerance has not yet been explored. AIRE contributes to both central and peripheral tolerance via the presentation of tissue specific antigens (TSAs), leading to the clonal deletion of self-reactive T cells. These TSAs include placental antigens, which we call “distant self” antigens, since they are encoded in the maternal genome, but have not been expressed since the mother was herself an embryo with a placenta. We hypothesize that AIRE-driven maternal tolerance of “distant self” placental TSAs are critical for successful embryo implantation and development. To test this, we first investigated AIRE expression in pregnant and nonpregnant female mouse tissues. Using qPCR, RNAscope, and immunohistochemistry, we found AIRE-expressing cells in the uterine draining lymph nodes. To test the functional role of AIRE during pregnancy, we next used an AIRE-diphtheria toxin receptor (DTR) transgenic mouse model to ablate AIRE-expressing cells during the first nine days of an alloge neic pregnancy. We found significantly smaller litter sizes (p<0.0001) and smaller embryos (p=0.005) in a subset of AIRE-DTR pregnancies (30% of N=10), compared to wildtype (WT) pregnancies given DT (N=11). Flow cytometry of maternal tissues showed AIRE-DTR pregnancies had more CD4+ T cells (p=0.003) and fewer FoxP3+ Tregs (p=0.003) in the uterus, and more CD25+CD4+ T cells (p=0.019) in peripheral lymph nodes. AIRE-DTR mice were less likely to be pregnant after a confirmed plug (rate of pregnancy on embryonic day 9.5: 67% AIRE-DTR vs 92% WT), suggesting decreased fertility or early pregnancy loss. Thus, although AIRE expression may decrease during pregnancy, AIRE-expressing cells may still play an important role in maternal-fetal tolerance by deleting reactive maternal T cells and promoting maternal Treg differentiation. Future work will use RNA-sequencing to compare transcription profiles of thymic and peripheral AIRE-expressing cells in pregnant versus nonpregnant female mice, with the goal of identifying candidate placental TSAs that may be responsible for maternal T cell reactivity towards the placenta and developing embryo.
Examining the postmitotic roles of STAG2 mutations in solid tumors

Mary Guan, HHMI Medical Fellow, University of Michigan Medical School

Mentor: Arul Chinnaiyan, MD, PhD, Howard Hughes Medical Institute and University of Michigan

Mutations in the cohesin complex are associated with cancer, namely Ewing’s Sarcoma (EWS) and bladder cancer (BC). The STAG2 subunit of cohesin is essential for its loading onto chromatin and proper function of the complex. EWS and BC patients who carry a STAG2 mutation show decreased survival. How STAG2 functions as a tumor suppressor is not fully understood. Some studies show that STAG2 mutations cause aneuploidy in cancers; while other data suggest that STAG2 mutations result in altered gene expression. We hypothesize that in EWS, where the EWSR1-FLI1 fusion is pathognomonic, the genetic epistasis of EWSR1-FLI1 and STAG2 is due to the suppressive role of STAG2 on EWSR1-FLI1.

In this study, we used co-immunoprecipitation (co-IP) to examine a ternary interaction between EWSR1-FLI1 and STAG2. We explored the phenotypic effect of STAG2 knockdown in vitro in HT1376 BC cells and A673 EWS cells via siRNA knockdown and proliferation curves. Finally, we determined the effect of STAG2 knockdown on expression of EWSR1-FLI1 and its downstream targets using RT-PCR.

We did not detect any physical interaction between STAG2 and EWSR1-FLI1 in A673 EWS cells via co-IP with STAG2 or EWS-FLI1. Phenotypically, in vitro proliferation was not affected upon STAG2 knockdown in HT1376 BC cells. We also counted HT1376 cell at 7 days post siSTAG2 transfection. Consistently, no significant difference was observed from cell count between siSTAG2 and negative control (siNC). Utilizing CellTiter-Glo Luminescent Cell Viability Assay, we further observed from cell count between siSTAG2 and negative control (siNC). Phenotypically, proliferation was not affected upon STAG2 knockdown in HT1376 BC cells. We also counted HT1376 cell at 7 days post siSTAG2 transfection. Consistently, no significant difference was observed from cell count between siSTAG2 and negative control (siNC).

In summary, our results suggest STAG2 mediates expression of EWSR1-FLI1 and its targeted genes; however not through physical interaction. Instead, STAG2 could act on chromatin to regulate oncogenic transcriptional programs associated with EWSR1-FLI1. Currently there exists no medical therapy for EWS, as the only treatments are chemotherapy, radiation therapy, and surgery. If STAG2 regulates oncogenesis of EWS, this will shed new light on the treatment of EWS, by targeting STAG2 in a specific, non-invasive way.
and spleen from animals treated with isoform-specific anti-TGF-β therapy with and without RT is underway to understand the contribution of αTGF-β1 versus αTGF-β3 to anti-tumor efficacy.

TGF-β1 and TGF-β3 are expressed on numerous lymphoid and myeloid cells in B16 tumors and spleens. TGF-β isoform expression peaks 5 days post-RT. Anti-TGF-β therapy is effective in delaying tumor growth and may synergize with RT in certain cancers. This demonstrates rationale for the use of anti-TGF-β therapy to enhance the effectiveness of RT in cancer.

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Mouse Endothelial Cell Transcriptomes in Healthy and Diseased States

Jennifer Hu, HHMI-ASHG Medical Fellow, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University (Harvard Medical School)

Mentor: Matthew Warman, MD, Howard Hughes Medical Institute, Boston Children’s Hospital and Harvard Medical School

Endothelial cells line the interiors of blood and lymphatic vessels. Depending on the vessel type (e.g., capillary, vein, artery, sinusoid), they have diverse morphologies and functions. Somatic mutations have been identified in endothelial cells isolated from sporadically occurring vascular malformations; these include: PIK3CA mutations in lymphatic and venous malformations and GNAQ mutations in congenital hemangiomas. How a mutation in an endothelial cell gives rise to a specific type of vascular malformation is unknown.

We hypothesize that a somatic mutation’s effect depends upon the transcriptome of the endothelial cell in which the mutation arose. For instance, baseline transcriptional differences between sinusoidal and arterial endothelial cells account for the ability of a somatic GNAQ mutation to produce a hepatic hemangioma in the former and no malformation in the latter. To begin testing this hypothesis, we are performing RNA sequencing on mouse endothelial cells freshly recovered from different vascular compartments.

Thus far, we have immuno-affinity purified endothelial cells from collagenase digested lung, liver, aorta, inferior vena cava/jugular veins, and uterus samples from adult C57BL/6J mice using anti-CD31 antibodies. Our highest yield came from the liver (~1 million cells) and our lowest yield came from the aorta (~13,000 cells). Extracted RNA was used to create RNA-seq libraries which were characterized using an Illumina MiSeq. As expected, known endothelial cell transcripts (e.g., Vwf) were present in every sample. However, our ability to detect differences in the transcriptomes of the different endothelial cell populations was confounded by contaminating neighboring cells. For example, albumin transcripts (A Bh) were enriched in endothelial cells isolated from liver and surfactant associated protein transcripts (Sftp b) were enriched in endothelial cells isolated from lung.

We are currently developing more stringent enrichment methods to recover endothelial cells, applying computational methods to eliminate contaminating transcripts from endothelial cell datasets, and performing single cell RNA sequencing to precisely define different endothelial cell transcriptomes. Once this work is complete, we will begin determining endothelial cell transcriptomes in mice with a conditional Pik3ca p.H1047R allele that has been associated with venous and lymphatic malformations or a GNAQ p.Q209L allele that has hepatic malformations. Finally, endothelial cells from different vascular compartments of wild-type and conditional mutant mice will be co-cultured with wild-type cells to determine how mutant endothelial cells affect the behavior of non-mutant neighbors.

Completion of these experiments will elucidate the transcriptional profiles of endothelial cells from different vascular sites in the absence or presence of a malformation-causing somatic mutation. It will also identify cell autonomous changes in gene expression that can recruit non-mutant cells into the malformation process. This knowledge could lead to therapies that halt the development or progression of a vascular malformation, or promote the malformation’s regression.

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Neutrophil Extracellular Traps Propagate Post-Traumatic Heterotopic Ossification

Charles Hwang, HHMI Medical Fellow, University of Michigan Medical School

Mentors: Stephen Weiss, MD and Benjamin Levi, MD, University of Michigan

Neutrophil extracellular traps (NETs), a component of the host defense mechanism, have been implicated in pro-inflammatory conditions such as arthritis and autoimmune disorders. Heterotopic ossification (HO) is a pathology of impaired musculoskeletal wound healing characterized by ectopic osseous lesions that cause joint immobility, disfigurement, and pain. This acute-on-chronic inflammatory process has both clinically and experimentally exhibited an association between mechanical strain and increased local inflammation. We show that mechanically disrupted NETs independently induce NETosis, thereby augmenting inflammation in response to injury, and causing wound pathology. Furthermore, interrupting signaling downstream to nascent NETs attenuated ectopic formations.
Wild-type mice underwent hindlimb Achilles tenotomy to generate trauma-induced HO. Mice were allowed to ambulate with the injured hindlimb either mobile or cast-immobilized. Mice were treated with vehicle control, i.e. DNase-I to chemically disrupt NETs, or ODN-2088 (TLR 7/8/9 inhibitor) to inhibit DNA receptor activity. Inflammation was assessed by flow cytometry to quantify infiltrate at 48 hours and cartilage was assessed histologically (Safranin O) at three weeks. For in vitro studies, neutrophils were isolated from wild-type mice and treated with phorbol-12-myristate 13-acetate (PMA) to induce NETosis. NETs were mechanically disrupted using proven protocols or left intact. Neutrophils treated with ODN-2088 were exposed to either disrupted or intact NETs followed by quantification of "secondary" NETosis using anti-citH3/DAPI staining. For μCT analysis, mice received 150ng/g i.p. injections daily with ectopic lesions quantified at nine weeks. All significant results were calculated at p<0.05.

Immobile hindlimbs reduced normalized neutrophil (1.0 v. 0.27) and macrophage (1.0 v. 0.26) counts compared with mobile hindlimbs 48 hours after injury corroborated by histology showing increased citH3 immunostaining in the mobile hindlimb. Chemical disruption of NETs through DNase-I also significantly increased normalized neutrophil (1.0 v. 6.39) and macrophage (1.0 v. 3.0) counts in the immobile hindlimb at 48 hours; however, no differences were observed in the mobile hindlimb (n=5/group). Treatment with DNase-I to chemically disrupt NETs also led to the presence of ectopic cartilage. TLR interruption via ODN-2088 significantly reduced inflammation in the mobile hindlimbs. In vitro studies confirmed that mechanically disrupted NETs induce robust secondary NETosis compared with intact NETs (46.6/hpf v. 20.2/hpf). Treatment of secondary neutrophils with ODN-2088 reduced secondary NETosis (92.7/hpf v. 0.90/hpf) validating the role TLRs serve as major receptors for NET-induced NETosis. Post-traumatic treatment with ODN-2088 successfully reduced the CT volume of ectopic bone (5.36 v. 9.52mm3), confirming the signaling and recruiting role of NETosis in HO.

These results strongly suggest mechanical or chemical disruption of NETs augments joint inflammation and endochondral ectopic bone formation by inducing further NETosis among neutrophils. TLR 7/8/9 inhibition via ODN-2088 prevents this secondary NETosis and downstream development of ectopic lesions, suggestive of a valuable target in preventing NETotic pathologies and dysregulated inflammation.
These data demonstrate the superior reperfusion efficacy of a long-pulse (1600 cycles) vs. short-pulse (35 cycles) US delivery system for the treatment of MVO. This in vivo observation aligns with our previous in vitro findings showing that longer pulse length is associated with greater reperfusion efficacy. Results obtained from this study should inform clinical translation and optimization of sonoreperfusion of MVO.

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Deciphering the functional heterogeneity of the human macrophage response to Mycobacterium tuberculosis infection

Christopher Itoh, HHMI Medical Fellow, University of California, Davis, School of Medicine (Harvard University School of Public Health)

Mentor: Sarah Fortune, MD, Harvard University School of Public Health

Mycobacterium tuberculosis (Mtbd), the causative agent of tuberculosis, is the leading cause of infectious death in the world. Macrophages are critical to both the progression and control of Mtbd infection, providing a protected niche for the bacterium in some cases but clearing the bacterium in others. We understand little about how differences in macrophage antimicrobial capacity are established and maintained. Canonically, M1 macrophages are thought to be antimicrobial and M2 macrophages are immunoregulatory. However, several lines of evidence now indicate that there is extensive transcriptional and functional heterogeneity within macrophage populations that is not captured by these classifiers (Murray, 2017). To define the dynamics of macrophage memory and plasticity, we developed HERMES (High-dimensional Evaluation of Response to Macrophage Environmental Stimuli), an in vitro platform to characterize macrophage responses and assess their phenotypic stability. This assay captures both static and dynamic features of macrophage state, including surface marker expression, cytokine production, and antimicrobial capacity. HERMES involves a tiered checkerboard assay in which we polarized macrophages with a primary differentiation stimuli, M-CSF or GM-CSF, modulated their state with LPS, IFN-γ, IL-4, IL-6 or IL-10, and measured their response to different TLR stimuli. Macrophages were indistinguishable by canonical surface markers; however, by using a strategy that is both combinatorial and state specific, we uncovered routes of macrophage state modulation that are both terminal and plastic as indicated through their cytokine release profiles. Furthermore, we observed that polarized macrophages had differential capacity to control Mtbd growth indicating that macrophage antimicrobial programs were also differentially engaged. In conclusion, macrophage exposure and stimulation history give rise to combinatorial plasticity, as well as differential response in Mtbd control. HERMES allows us to investigate the complexity of macrophage plasticity and help define strategies capable of activating a pathway for the most effective macrophage state to control disease.

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Elucidation of an Exquisite Synthetic Lethal Interaction Between ATR Inhibitors and Alkylating Agents in MGMT-Methylated Glioma Cells

Christopher Jackson, HHMI Medical Fellow, Columbia University College of Physicians and Surgeons (Yale School of Medicine)

Mentor: Ranjit Bindra, MD, PhD, Yale School of Medicine

Glioblastoma is the most common primary brain tumor in adults. The current standard of care for this condition consists of surgery with maximal resection followed by concurrent temozolomide (TMZ) and radiation therapy. TMZ belongs to a class of alkylating agents which are thought to exert their main cytotoxic effects on cells via methylation of DNA bases, including guanine bases at the O6 position (O6meG). O6-methylguanine-DNA methyltransferase (MGMT) is a suicide enzyme that repairs TMZ-induced DNA damage by removing these alkyl adducts. The promoter region of MGMT contains CpG island regions that are methylated in approximately 45% of glioblastoma cases. Patients with methylation of the MGMT promoter respond more readily to TMZ and survive approximately 9 months longer than patients with an unmethylated MGMT promoter. Despite this prognostic benefit, these patients still have a median survival of only 21.7 months. Ample opportunity still exists to better understand the relationship between MGMT and TMZ and to introduce treatments that further extend survival in these patients.

Here, we report exquisite synergistic interactions between TMZ and inhibitors of a key DNA damage response protein, ataxia telangiectasia and rad3-related protein (ATR). Preliminary data suggest a mechanism of action which involves acute replication stress leading to fork collapse, and consequent induction of DNA double-strand breaks. TMZ induces activation of ATR specifically in MGMT-methylated glioma cells, and inhibition of ATR signaling sensitizes the glioma cells to TMZ. The magnitude of TMZ sensitization by ATR inhibition is marked—over 100-fold—and entirely dependent on MGMT status. These data lay the foundation for future clinical trials testing this combination specifically in MGMT-methylated glioma, and they challenge previously described mechanisms of action proposed for the differential sensitivity of TMZ in MGMT-methylated tumors.
The Role of Teneurins in Neurodevelopmental Disorders

Hudin Jackson, HHMI Medical Fellow, University of Connecticut Health Center, School of Medicine (Baylor College of Medicine)

Mentor: Hugo Bellen, DVM, PhD, Howard Hughes Medical Institute and Baylor College of Medicine

Neurodevelopmental disorders (NDD) encompass conditions in which development of the central nervous system is perturbed and manifest as neuropsychiatric problems or impaired motor function, learning, language, or non-verbal communication. The underlying etiologies for these disorders are highly complex, but increasing evidence has shown that different rare and common genetic alterations can contribute to similar disease mechanisms underlying shared clinical phenotypes. This suggests that determining the genetic basis and pathogenic mechanisms of rare monogenic disorders has the potential to provide critical insights for many highly prevalent NDD. Therefore, we developed a large-scale collaborative effort to identify individuals with either rare conditions or rare genetic etiologies and to determine the functional consequences of the human gene alterations in a high-throughput Drosophila system.

Through multi-institutional collaborations, several individuals with neurodevelopmental disorders were identified with candidate pathogenic variants in vertebrate teneurin genes. These individuals exhibit a range of neurological deficits that were not previously shown to be associated with this gene family. The evolutionarily conserved teneurins encode transmembrane proteins involved in a number of processes integral to the proper development of the nervous system, including neuronal outgrowth, synaptic organization, and synaptic transmission. Previous studies of the fly teneurins, ten-a and ten-m, demonstrated that knockdown of ten-m and ten-a causes synaptic loss, impaired apposition of pre- and post-synaptic partners and defective synaptic transmission. Teneurin knockdown in flies results in locomotor deficits, marked by poor crawling. Studies in vertebrates demonstrated that TENM4 knockout mice display impaired odor discrimination and TENM4 knockout mice exhibit tremors. TENM4 knockdown also causes hypomyelination of small axons and inhibition of oligodendrocyte differentiation within the spinal cord of TENM4 mutant mice.

In order to determine the pathogenic nature of the rare human variants, we employed a genetic system where the endogenous locus for the fly homolog is disrupted resulting in a loss of function allele that simultaneously expresses the yeast transactivator gene (GAL4) under the same spatiotemporal regulation of the endogenous locus. To study the human teneurins, we generated a novel ten-a-T2A-GAL4 allele and obtained the ten-m Gal4 allele (w^{118};PBac(IT.GAL4)Ten-m^0257-G4/TM6B, Tb^1). The ten-a-T2A-GAL4 and ten-m-Gal4 alleles are used in conjunction with transgenic fly alleles expressing wildtype and variant fly and human teneurin cDNAs (hcDNA) under the regulation of Upstream Activating Sequences (UAS). The UAS-GAL4 system allows us to perform a high-throughput in vivo analysis of the consequences of the human gene variants on nervous system development and function. These findings have the potential to elucidate the role of teneurins in human NDD and identify shared pathogenic mechanisms that may provide insight into novel therapeutic avenues.

Oocytes and Embryos from Mice of Advanced Maternal Age Show Decreased Mitochondrial Mass but No Changes in Imprinted Methylation

Audrey Kindsfather, HHMI Medical Fellow, University of Pittsburgh School of Medicine

Mentor: Mellissa Mann, PhD, University of Pittsburgh School of Medicine

Over the last several decades, the average age of first-time mothers has risen steadily. Advanced maternal age, defined in humans as above 35 years old, is known to increase the risk of spontaneous abortion, stillbirth, preterm birth, aneuploidy, and other chromosomal abnormalities and birth defects. As a woman ages, molecular changes occur in her oocytes that can affect the ability of the oocytes to be fertilized and the viability of the embryo. These changes include oxidative stress, which is known to damage mitochondria. In addition to other cellular processes, mitochondria likely play a role in regulating epigenetic mechanisms, such as genomic imprinting. Genomic imprinting is an epigenetic phenomenon that restricts expression to predominantly one parental allele through various mechanisms including cytosine methylation. Mitochondria provide both ATP and methyl groups used for imprinting acquisition in germ cells and imprinted methylation maintenance in preimplantation embryos as the rest of the genome is demethylated. The aim of this project is to determine whether maternal age affects mitochondrial activity and methylation of the imprinted genes Sorpu, H19, and Kcnq1ot1 in mouse oocytes and preimplantation embryos.

Female C57BL/6 CAST7 mice from very young (<2 months), young (2-5 months), middle (6-10 months), and advanced (>10 months) age groups were mated with C57BL/6 males. Morula and blastocysts were collected at day E3.5 by flushing the uterus and oviducts with M2 medium. Oocytes were collected by treating the ovaries with collagenase and trypsin/EDTA. All samples were stained with Mitotracker Red to visualize...
active mitochondria, Mitotracker Green to visualize total mitochondria, and Hoechst to visualize nucleic acids, and imaged with confocal microscopy. Total and active mitochondrial levels were quantified by the green and red intensity, respectively. In oocytes, total and active mitochondrial patterning was characterized as perinuclear, peripheral, homogenous, or aggregated. Individual oocytes, morula, and blastocysts were subjected to bisulfite mutagenesis and assessed for Snrpn, H19, and Kcnq1ot1 imprinted methylation levels.

Our data show that there was a significant decrease in active mitochondrial mass in both oocytes and blastocysts with increasing maternal age. While the mitochondria in oocytes from young and middle-aged mothers was usually arranged in perinuclear and homogenous patterns, oocytes from mothers of advanced age show peripheral and aggregated patterns more frequently. However, our data to date have not shown any difference between imprinted methylation levels of Snrpn, H19, and Kcnq1ot1 in oocytes and blastocysts between mice of any of the age groups. Future studies will determine the consequences of the observed mitochondrial dysfunction, as well as examine how assisted reproductive technologies such as superovulation and embryo culture affect mitochondrial activity and imprinted methylation in oocytes and embryos from mice of advanced maternal age.

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Altered cerebrospinal fluid flow patterns in a mouse model of chronic neuropathic pain
Benjamin Kress, HHMI Medical Fellow, State University of New York Upstate Medical University (University of Rochester School of Medicine and Dentistry)
Mentor: Maiken Nedergaard, MD, DMSc, University of Rochester School of Medicine and Dentistry

Altered neuronal connectivity and hyperexcitability of nociceptive neurons is generally believed to represent the causal substrate of persistent neuropathic pain. It is now accepted that, in addition, a deviation from the normal supportive functions of glial cells, in particular astrocytes and microglia, contributes to both the initial rewiring of spinal pain pathways, as well as the maintenance of the hypersensitivity believed to underlie the persistent component of neuropathic pain. To date, the exact impairment of the supportive functions of glial cells that contributes to the establishment and maintenance of neuropathic pain remains unknown. Here we tested the hypothesis that peripheral nerve injury triggers functional and structural changes in astrocytes that results in pathologically significant alterations in cerebrospinal fluid (CSF) flow patterns. We theorized that impaired clearance of interstitial fluid and solutes may lead to the accumulation of excitatory or pro-inflammatory mediators (e.g., cytokines, ATP, or glutamate), which could subsequently contribute to maladaptive synaptic plasticity and the sensitization of nociceptive neurons. To test this hypothesis, we used fluorescence imaging to monitor flow patterns of tracers infused into the CSF of awake mice that had (i) received either the spared nerve injury (SNI) model of peripheral neuropathic pain or (ii) sham injury. The accumulation of tracers from the brain and spinal cord parenchyma, as well as the expression of markers of astrogliosis (glial fibrillary acidic protein [GFAP] and aquaporin 4 [AQP4]), or microgliosis (CD68), were then quantified after multi-channel fluorescence imaging of ex vivo brain and spinal cord slices. In the SNI model, neuropathic pain behavior, including thermal hyperalgesia and mechanical allodynia, peaked at 3 days following SNI. Accumulation of tracers within the parenchyma was markedly altered between mice that received SNI vs. sham-injury. Altered CSF flow patterns were detected as early as 3 days following SNI, and positively correlated with both pain behavior and astrogliosis, determined by increased expression of GFAP/AQP4. The early administration of the analgesics pregabalin and clonidine, but not morphine, prevented the suppression of glymphatic clearance. We are currently evaluating CSF flow in awake nerve injured vs. sham-injured mice in vivo, using serial contrast-enhanced computed tomography. Our results suggest altered glial cell function and CSF flow patterns may contribute to the onset and maintenance of chronic neuropathic pain.

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Lysosomal Calcium Stores Mediate Ultraviolet Light Responsiveness in Human Melanocytes
Brandon Law, HHMI Medical Fellow, Harvard Medical School
Mentors: Richard Anderson, MD and David Fisher, MD, PhD, Massachusetts General Hospital and Harvard Medical School

Mutations in genes encoding lysosome-related organelles are implicated in impaired pigmentation, but the exact mechanism of this relationship remains poorly understood. Pigment production by human epidermal melanocytes occurs in response to ultraviolet (UV) light. In this study, we hypothesize that lysosomes in melanocytes play a critical role in mediating UV response. To query this hypothesis, we use live-cell imaging to probe for UV light-evoked response in cultured primary human melanocytes. We show that UV light evokes rapid lysosomal calcium release. Pharmacologic pathway modulation indicates that this calcium release is GPCR-mediated and requires the presence of a retinaldehyde chromophore,
suggesting that a phototransduction cascade mediates this UV-response rather than a nonspecific cell damage pathway. Action spectrum studies show that calcium response robustly corresponds to the wavelength sensitivities of known UV-specific photoreceptors. UV-induced lysosomal calcium release is attenuated by pharmacologic blockade of lysosomal acidification or depletion of lysosomal calcium stores, further implicating the role of lysosomal ion homeostasis in UV response. Additional studies using CRISPR/Cas9 to genetically edit candidate lysosomal ionic transporter genes in primary human melanocytes derived from individuals with light, medium, and dark skin tones are ongoing. Taken together, these findings suggest a general paradigm that lysosomes serve as critical signaling organelles for UV response and provide additional mechanistic evidence for the emerging role of lysosome-related organelles in diseases of pigmentation.

Fibrolamellar Hepatocellular Carcinoma (FL-HCC) is a rare and lethal liver cancer that primarily affects adolescents and young adults. Surgical resection is the mainstay of treatment; however, due to the non-specific nature of the disease symptoms (e.g., weight loss, abdominal pain), FL-HCC is often diagnosed at a fairly late stage, at which point the tumor has often metastasized and surgery is no longer an option. There is a lack of systemic therapies for the disease, and the mortality rate is high with a five-year survival of only 30-40%.

Previously, our lab discovered a recurrent genetic mutation found in all FL-HCC tumor samples sequenced. The mutation is an in-frame deletion of approximately 400 kilobases on one copy of chromosome 19. This deletion results in a fusion gene that contains the first exon from DNAJB1, which encodes for a member of the heat shock protein family, and exons 2-10 from PRKACA, which encodes for the catalytic subunit α of Protein Kinase A (PKAc). This chimeric gene leads to a chimeric mRNA, and ultimately, a chimeric protein that retains the full catalytic activity compared to the native kinase. More recently, we have found that creating the chimera is hydrodynamically transfected into the liver of mice, the mice also develop the tumor. When the same chimera is expressed with a point mutation to kill the kinase activity, no tumors are formed. Together these data implicate the kinase activity of the DNAJB1-PRKACA as the oncogenic driver for this cancer.

Here we initiate the discovery and development of a novel drug to target the kinase activity of the chimeric protein via a high-throughput drug screen. The chimeric protein was produced in BL21-CodonPlus (DE3)-RIL E. coli cells. The protein was purified in a single affinity step using a 20-residue peptide derived from Protein Kinase Inhibitor, a naturally occurring inhibitor of PKAc. This allowed for the purification of the chimera without the use of an affinity tag. The pure protein was found to have comparable activity to the native kinase, and was inhibited by H89, a known potent inhibitor of PKAc.

The kinase activity screen was optimized and had a Z-factor of 0.85 with H89 as the positive control. The screen included close to 400,000 compounds. The positive threshold was set to 15% normalized percentage inhibition with a z-score ≥3.5. There were 82 compounds that met this cut-off. Secondary screens of these “hits” are in process, and these will ultimately be tested in cells and mouse models of FL-HCC.
our results showed distinct populations defined by high and low ribosomal protein (RP) expression. Interestingly, this translational signature correlated with worse prognosis of patients from whom the CTCs were acquired. We hypothesize that the dramatic shift in cell phenotype that occurs as tumor cells undergo metastasis likely requires equally dynamic changes in protein synthesis and translational machinery. To better understand the dynamics of ribosomal protein regulation, we fused three ribosomal protein gene promoters (RPS6, RPL10a, RPL12) to a destabilized green fluorescent protein (GFP) gene, allowing quantification of GFP fluorescence to serve as a dynamic readout of RP expression. We stably infected both a breast cancer CTC line and an immortalized breast epithelial cell line to study temporal changes in ribosomal protein expression at both a single cell and bulk population level. We are currently investigating the regulation of the protein biosynthetic pathway and its potential role in mediating CTC survival. Ultimately, this study aims to characterize the regulation of translational machinery and its contribution to CTC biology, expanding our understanding of metastasis and identifying potential novel targets for inhibition.

Peripheral blood was collected from patients undergoing resection of lung metastatic brain tumors (n = 27). Immunosuppressive monocytes (CD45+ CD11b+ CD163+/- PD-L1+/-) and myeloid-derived suppressor cells (MDSCs) (CD11b+ CD33+ HLA-DRlo PD-L1+/-) were quantified through flow cytometry. Tumor tissue was obtained from resection of brain metastases and used to generate cell cultures (n = 6) from which tumor conditioned media (TCM) was collected. TCM was analyzed for immunosuppressive cytokines, including interleukin-6 (IL-6), by enzyme-linked immunosorbent assay (ELISA). Naive monocytes were stimulated with TCM for 24 hours in the presence of antibodies targeting the IL-6 receptor (tocilizumab), IL-6 (siltuximab), or IgG control.

Patients with brain metastatic lung carcinoma demonstrated increased peripheral monocyte PD-L1 compared to healthy controls (p<0.05), with an average 2.5-fold increase. All patients exhibited an increased abundance of MDSCs (p<0.05), with an average 14.9-fold increase. Elevated plasma IL-6 was associated with increased peripheral myeloid PD-L1 in patients with brain metastatic lung carcinoma (p<0.05). TCM stimulated monocytes expressed increased PD-L1 compared to unstimulated controls (17.7 vs. 7.4% PD-L1 positive, p<0.001). Correlation of TCM IL-6 levels with PD-L1 expression in TCM stimulated monocytes demonstrated a dose-dependent relationship (R² = 0.87, p<0.01). In addition, treatment with anti-IL-6 and anti-IL-6 receptor antibodies inhibited the increase in monocyte PD-L1.

Patients with lung cancer and brain metastases exhibit signs of peripheral immunosuppression, including increased PD-L1+ monocytes and MDSCs. IL-6 was found to stimulate induction of immunosuppressive myeloid cells. Monitoring of these immunosuppressive factors in peripheral blood may be used as a biomarker to predict patients at greater risk for brain metastases and suggest a new target for therapeutic intervention.
An Immunogenomics Approach to Neoantigen Identification in Preclinical Models of Glioblastoma

Connor Liu, HHMI Medical Fellow, Washington University in St. Louis School of Medicine
Mentors: Gavin Dunn, MD, PhD and Robert Schreiber, PhD, Washington University in St. Louis School of Medicine

Glioblastoma (GBM) remains the most common and lethal malignancy of the central nervous system (CNS) in adults. The past decade of GBM research has seen limited progress in improving patient survival beyond the current standard-of-care surgical resection followed by radiation and temozolomide chemotherapy. However, recent success in the use of immunotherapy to treat other solid and blood malignancies, in addition to experimental evidence challenging the notion of the CNS as an immunologically privileged environment, has generated new interest in using immune-based treatments for GBM patients. In particular, ‘cancer immunogenomics’ describes an approach to immunotherapy design aimed at leveraging the tumor-specific mutant peptides presented on a patient’s individual human leukocyte antigen (HLA) molecules, as therapeutic targets. These tumor specific mutant peptides, called neoantigens, have come under intense focus as the immunodominant targets mediating anti-tumor responses to immunotherapy.

Most recently, using DNA whole exome sequencing, RNA-sequencing, and computational algorithms to prioritize neoantigens predicted to bind major histocompatibility complexes (MHC), our group isolated neoantigen-specific CD8+ T-cells from tumor infiltrating lymphocytes (TIL) in two checkpoint sensitive mouse glioma models, GL261 and SMA560. To assess the anti-glioma immune response following neoantigen vaccination, here we demonstrate that peptide vaccination with a mutant Imp3 neoantigen confers a significant survival advantage in mice bearing intracranial GL261. To our understanding, these findings represent the first documentation of a personalized ‘mutation-to-vaccine’ pipeline in a preclinical GBM model and provides a framework for studying the cellular basis of neoantigen-dependent responses to immunotherapy. Additionally, we show that the CT2A syngeneic mouse glioma model recapitulates the highly aggressive and checkpoint blockade resistant phenotype seen in the human GBM setting. Applying DNA whole exome sequencing we identified 2,392 nonsynonymous mutations in the CT2A tumor genome, of which 802 were also expressed in RNA-sequences. We used the open-source, in silico neoantigen prediction software pVAC-seq to identify top-ranking H-2kb and H-2Db restricted CT2A candidate neoantigens for synthesis and immunogenicity screening by IFNγ ELISPOT. Thus, by determining the immunogenicities of high priority neoantigens in CT2A, we extend our neoantigen discovery pipeline to a third and highly immunosuppressive glioma model. In conjunction with our work in GL261 and SMA560, characterization of the CT2A neoantigen landscape will help establish a GBM immunotherapy model that captures clinically relevant biology and that will provide a platform to further study combination immunotherapies in GBM.

Defining the role of the immune system in aneuploid cell clearance

Emily MacDuffie, HHMI Medical Fellow, The Warren Alpert Medical School of Brown University (Massachusetts Institute of Technology)
Mentor: Angelika Weis-Amon, PhD, Howard Hughes Medical Institute and Massachusetts Institute of Technology

Aneuploidy, the loss or gain of one or more chromosomes, is a hallmark of oncogenic transformation. Although aneuploidy is rare in normal tissues (0-4% of cells), it is estimated to be present in 90% of solid cancers. Despite its pervasiveness in cancerous tissue, experimentally-derived aneuploid cells have a significant proliferative disadvantage compared to euploid cells both in vitro and in vivo. To assess the effects of aneuploidy on cellular fitness and tumorigenesis, mouse models were developed that lack a functional spindle assembly checkpoint (SAC). Without a functioning checkpoint, cells initiate anaphase before correct kinetochore attachments are made, resulting in aneuploid daughter cells. Mouse models of chromosomal instability (CIN) due to disruption of the SAC show high levels of aneuploidy in tissues that are formed during embryogenesis such as brain. However, tissues that regenerate during adulthood, such as skin, intestine, and peripheral blood harbor very low levels of aneuploidy. It is unknown how aneuploid cells are selected against in these tissues. We tested the possibility that aneuploid cells are cleared via apoptosis. We quantified cleaved caspase 3, a marker of apoptosis, in the intestine of CIN mouse models but detected no increase in apoptotic cells compared to control mice, suggesting that other mechanisms may be responsible for aneuploid cell elimination. We hypothesized that aneuploid cells exhibit immunogenic stress responses that are recognized by the immune system and result in their ultimate removal.

To test the above hypothesis, we optimized a protocol to generate arrested aneuploid cells with complex karyotypes from RPE1 cells. Broad protein profiling of secreted cytokines in the cellular supernatant revealed increased levels GM-CSF, IL-6, and CCL2, inflammatory cytokines known to attract cells involved in both innate and adaptive immunity. Co-cultures of aneuploid RPE-1 cells and natural killer (NK) cells have
previously demonstrated NK-mediated cell killing. Continuing investigations are exploring the interactions between aneuploid cells and phagocytic cell types including macrophages and dendritic cells.

To determine if immune mediated clearance of aneuploid cells occurs in vivo, tissue inflammation was assessed in two mouse models expressing mutated components of the SAC. Intestine, skin, kidney, liver, and brain tissue were stained with immune cell markers and the number of immune cells per area was quantified. Preliminary experiments suggest that macrophages are more abundant in the regenerating tissues of CIN models but that the number of CD4+ helper T cells does not differ between CIN models and controls. Ongoing experiments are evaluating quantity of NK cells, CD8+ cytotoxic T cells, and dendritic cells in CIN tissues.

Understanding immune system recognition of these non-cancerous aneuploid cells is the first step towards the development of therapies that can augment the immune system’s response to cancerous aneuploid cells.

We screened for NRX-I in 744 women ages 65 and older participating in an ongoing, prospective cohort study in Oregon (Women Engaged in Advancing Health Research [WEAR] study). This approach was used to enhance the efficiency of identifying a subpopulation of women harboring impactful, high-risk mutations. We examined the HUMARA assay results and any association with previous health history using a cross-sectional study design. Analysis of variance and logistic regression analyses were used to examine the relationship between HUMARA assay results and baseline CVD risk, controlling for age and race.

Women displaying highly skewed NRX-I were 2.5 times as likely to report a history of diabetes controlled with insulin injection (p-value 0.078, 95% CI: 0.76, 8.99) compared to women without NRX-I. They were 1.4 times as likely to report hypercholesterolemia (p-value: 0.39, 95% CI: 1.00, 1.97) and 1.5 times as likely to report us of cholesterol lowering medication (p-value: 0.01, 95% CI: 1.08, 2.24). Women with NRX-I were 2.54 times as likely to report a history of myocardial infarction (p-value: 0.077, 95% CI: 0.761, 8.98). Women with NRX-I were 1.48 times more likely to report daily aspirin use, (p-value=0.016, 95%CI: 1.05, 2.0) than women without NRX-I. Women with and without NXR-I were not found to differ with respect to race, age, body mass index, smoking history, hypertension, family history of CVD, or previous history of transient ischemic attack and stroke.

Prediction of major adverse cardiac events is based the presence of traditional risk factors including high blood pressure, high cholesterol, uncontrolled diabetes, smoking, and family history. Yet there is significant residual risk; many will still die from CVD without these risk factors. NRX-I, in addition to enriching for mutations known to confer CVD and HM risk, may be a marker for additional and unique health risk. An association between NRX-I and DM as well as hypercholesterolemia, supports the current biological hypothesis that clonal hematopoiesis, perhaps irrespective of the cause or underlying driver mutation, is a driver of CVD.

### Evaluation of Non-Random X-inactivation in Blood Cells as a Marker for Cardiovascular Disease and Adverse Health Risk

**Emily Marre**, HHMI Medical Fellow, Chicago Medical School at Rosalind Franklin University of Medicine and Science (Oregon Health & Science University School of Medicine)

**Mentors:** Brian Druker, MD, Howard Hughes Medical Institute and Oregon Health & Science University and Kim-Hien Dao, DO, PhD, Oregon Health & Science University

Age-related clonal hematopoiesis (CH) is a common condition that is associated with an increased risk of hematologic malignancies (HM) and cardiovascular disease (CVD). The majority of candidate driver mutations occur in epigenetic regulatory genes such as ASXL1, DNMT3A, and TET2 genes. However, a significant proportion of older people harbor clonal hematopoiesis without candidate driver mutations. In older women, clonal hematopoiesis can be detected by the human androgen receptor A gene (HUMARA) assay regardless of the presence or absence of candidate driver mutation(s). The HUMARA assay evaluates non-random X inactivation (NRX-I) as a marker for clonal hematopoiesis. The purpose of this study is to evaluate the association between NRX-I and cardiovascular risk factors and other health correlates.
Atypical Xist RNA Localization to the Inactive X in a Female-biased Murine Model of Systemic Lupus Erythematosus

Anna Martin, HHMI-BWF Medical Fellow, University of Pennsylvania School of Veterinary Medicine

Mentors: Michael Atchison, PhD and Montserrat Anguera, PhD, University of Pennsylvania School of Veterinary Medicine

Systemic lupus erythematosus (SLE) is a severe autoimmune disease that affects women at a rate nine times higher than men. The genetic basis for this bias is the X-chromosome, where the greatest concentration of immunity related genes on any chromosome can be found. Females have two X chromosomes (XX), and through a process, known as X-chromosome inactivation (XCI), silence one of their X-chromosomes randomly to have a similar level of X-linked gene expression as males (XY). In XCI, XIST RNA, a long non-coding RNA, is expressed from the future inactive X (Xi) and is bound to it in cis by the transcription factor YY1. As XIST RNA coats the Xi, it recruits heterochromatin modifiers to condense and silence it. Previous research has shown that human SLE patient B cells exhibit altered localization of XIST RNA, which indicates that they have partial reactivation of the Xi. To explore this relationship, we worked with NZB/W F1 mice, which are a well-characterized murine model of SLE-like disease that also displays a female bias. The hypothesis of our study is that the Xi in splenic B cells of NZB/W F1 mice during disease progression will exhibit compromised silencing due to reduced epigenetic modifications, resulting in increased expression of X-linked genes. Preliminary results indicate that the protein and mRNA levels of YY1, which is required for XIST RNA localization to the Xi, is reduced across all stages of disease in female NZB/W F1 compared to age-matched wild type (WT) mice. Using XIST RNA FISH, we have observed that the activated B cells of late stage disease NZB/W F1 mice have decreased localization of XIST RNA to the Xi when compared to WT, suggestive of compromised silencing of the Xi. In addition, we observed that the expression of Tlr7 and Cxcr3, two important immunity-related X-linked genes, is increased in diseased NZB/W F1 when compared to age-matched WT. We conclude that there is evidence of perturbations with XCI in the female-biased lupus mouse model that could explain how X-linked genes become abnormally overexpressed in B cells during SLE.

The Role of ARID1A in the Pathogenesis of Nonalcoholic Fatty Liver Disease

Austin Moore, HHMI Medical Fellow, University of Texas Southwestern Medical School

Mentor: Hao Zhu, MD, University of Texas Southwestern Medical School

Nonalcoholic fatty liver disease (NAFLD) is a rapidly growing cause of chronic liver damage, cirrhosis, and hepatocellular carcinoma both in the United States and worldwide. The role of epigenetic regulation in the pathogenesis of fatty liver disease is unclear. ARID1A, a DNA-binding component of the SWI/SNF ATP-dependent chromatin remodeling complex, is frequently mutated in HCC and contributes to nucleosome repositioning and access by transcriptional regulators. We hypothesized that chromatin remodeling is important for the pathogenesis of fatty liver disease. We analyzed previously existing human gene expression datasets and found that ARID1A expression is suppressed in the liver tissues of patients with nonalcoholic steatohepatitis (NASH), a more progressive form of NAFLD that involves liver inflammation and damage.

The functional impact of this suppression was examined in mice with Cre-mediated liver-specific deletion of Arid1a (Arid1a LKO), which develop age-dependent fatty liver disease as measured via the NAFLD activity score (NAS). These Arid1a LKO mice exhibit a phenotype marked by increased liver weight, elevated cholesterol, and increased markers of liver damage. They are also more susceptible to high fat diet induced liver steatosis and inflammation. RNA-sequencing analysis of Arid1a-deficient liver tissue revealed upregulation of lipogenesis genes, such as Srebf1 and Fasn, and downregulation of fatty acid oxidation genes, including Acoxl. Chromatin immunoprecipitation sequencing (ChIP-seq) studies corroborated these results, demonstrating direct binding of Arid1a to the promoters of differentially regulated genes involved in lipid handling and fatty acid metabolism.

To better study the later stages of fatty liver disease progression, we combined adenoviral Cas9-mediated Pten deletion with our Arid1a LKO model, which synergistically drove fatty liver development. Inhibition of lipogenesis using CAT-2003, a potent SREBP inhibitor, showed improvements in markers of fatty liver disease progression in this Arid1a/Pten double knockout model. Collectively, our data show that ARID1A plays a role in the epigenetic regulation of hepatic lipid homeostasis, and its suppression contributes to fatty liver pathogenesis.
Investigating Context-Dependent Integration Potential of Postnatally Born Interneurons

**Gabriel Neves, HHMI Medical Fellow, Duke University School of Medicine**

**Mentor:** Chay Kuo, MD, PhD, Duke University School of Medicine

Understanding the principles guiding neuron integration into neural circuits have important implications for functional recovery post-injury. Firing action potentials (AP) is a central characteristic of all neurons, quintessential for circuit-level computation. Despite our mechanistic understanding of how APs are generated, how newborn adult neurons develop abilities to fire APs remains unknown. This is likely a key step to successfully transplant neurons as a therapy. The Ankyrin proteins organize voltage-gated ion channels necessary for AP generation in neurons. Their roles have not been studied during adult born interneuron integration. Using adult neurogenesis in the rodent subventricular zone (SVZ) as a model for understanding neuronal integration into functional circuitry, we combined innovations in mouse genetics, microscopy, and electrophysiology to characterize Ank3 expression and function during olfactory bulb (OB) interneurons maturation. To determine developmental Ank3 expression by OB interneurons, we generated a novel DCX-CreER<sup>tm</sup> transgenic driver line that specifically targets newborn neurons migrating from the adult SVZ to the OB enabling us to age these interneurons. Using this strategy, we were able to characterize Ank3’s developmental expression in OB interneurons finding Ank3 upregulating during the initial phase of neuronal integration. Electrophysiological recordings showed that Ank3 upregulation paralleled the interneurons’ ability to fire APs. Using the DCX-CreER<sup>tm</sup> transgenic driver crossed to a <sup>lox</sup> allele, tamoxifen-induced Ank3 deletion resulted in decreased dendritic complexity, as well as reduced AP firing frequencies and amplitudes. Strikingly, 4 weeks following tamoxifen-induction, Ank3-deletion resulted in significantly fewer integrated interneurons compared to control littermates, revealing Ank3 as a critical regulator of neuronal integration into adult neural circuits. Together, our data demonstrated that Ank3 upregulation during interneuron integration in the adult brain is required for proper AP generation, promoting interneuron survival.

Nanomechanics of a Tip-Link Protein from the Cochlea

**Aaron Oswald, HHMI Medical Fellow, Weill Cornell Medical College (Rockefeller University)**

**Mentor:** A. James Hudspeth, MD, PhD, Howard Hughes Medical Institute and Rockefeller University

Sound transduction occurs in cochlear hair cells when deflection of hair bundles opens mechanically sensitive ion channels. Each hair bundle comprises 100–200 stiff, cylindrical projections called stereocilia, whose distal ends are connected by tip links, filamentous biopolymers consisting of dimers of protocadherin 15 (PCDH15) and cadherin 23 (CDH23). Numerous pathogenic mutations affecting both proteins are associated with Usher Syndrome and nonsyndromic deafness in humans. The sensitivity and dynamic range of mammalian hearing require a certain amount of elasticity in a hypothetical mechanical element, the “gating spring” that transmits tension to the transduction channels. Although tip links are candidates to be gating springs, molecular-dynamics simulations of fragments of PCDH15 and CDH23 suggest that tip links are far too stiff for this purpose. Even though the mechanical properties of full-length tip-link proteins and their dimers remain unknown, other structures are therefore believed to dominate gating-spring elasticity.

We hypothesize that the mechanical properties of tip links play a key role in the elasticity of gating springs, and that some of the pathogenic mutations in tip-link proteins perturb tip-link elasticity. To test these hypotheses, we characterize the mechanics of individual PCDH15 molecules by confining the proteins between a glass substrate and a polystyrene bead 1 µm in diameter. By holding the bead in an optical trap, we subject the protein to tensions as great as 40 pN. Tethered by PCDH15, the bead explores a dynamic range of mammalian hearing requiring a certain amount of elasticity. In a hypothetical mechanical element, the “gating spring” that transmits tension to the transduction channels. Although tip links are candidates to be gating springs, molecular-dynamics simulations of fragments of PCDH15 and CDH23 suggest that tip links are far too stiff for this purpose. Even though the mechanical properties of full-length tip-link proteins and their dimers remain unknown, other structures are therefore believed to dominate gating-spring elasticity.

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A FACS-based CRISPR screen for host determinants of Chlamydia trachomatis invasion reveals the multiple roles of COPI during infection.

Joseph Park, HHMI Medical Fellow, Boston University School of Medicine (Harvard Medical School)

Mentor: Matthew Waldor, MD, PhD, Howard Hughes Medical Institute and Harvard Medical School

Chlamydia trachomatis, an obligate intracellular bacterium, is a major cause of human genital and ocular infections worldwide. The pathogen is not easily genetically tractable and the advent of CRISPR editing in human cells enables a new approach for investigating how this pathogen interacts with host. Using a fluorescence-activation cell sorting (FACS) approach, we undertook an unbiased genome-wide screen in a CRISPR-edited genome-wide library of HT29 human epithelial cells that were infected with fluorescently labelled C. trachomatis. Enrichment of mutants that were resistant to invasion and deep sequencing of their sgRNAs led to identification of multiple hits in the COPI vesicular trafficking pathway. In siRNA-transfected or temperature-sensitive mammalian cell lines, COPI depletion led to a significant reduction in bacterial attachment. This was caused by a decrease in cell surface heparan sulfate, the receptor for C. trachomatis binding. Acute inactivation of COPI trafficking via golgicide A treatment, which spared heparan sulfate, unmasked a defect in pathogen Type III secretion and consequent internalization. Furthermore, internalized bacteria were closely associated with COPI-labelled structures in microscopy. Together, these findings reveal the diverse roles of COPI during the early steps of C. trachomatis invasion into host cells and demonstrate the utility of FACS-based CRISPR screening in studying the mechanisms of entry of intracellular pathogens.

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Delineating the mechanisms underlying oncogenic transcription in ALKF1174L/MYCN neuroblastoma

Monica Pomaville, HHMI Medical Fellow, Michigan State University College of Human Medicine (Harvard Medical School)

Mentor: Rani George, MD, PhD, Harvard Medical School

Neuroblastoma, a malignancy of the developing sympathetic nervous system, is the most common extracranial solid tumor in children and has a survival rate of <50% in high-risk patients. One of the most influential determinants of poor prognosis in children with neuroblastoma is amplification of the transcription factor MYCN, found in approximately 20% of tumors. Because small-molecule inhibition of MYCN has not been clinically achieved, recent investigations have focused on the identification of critical targets that modulate MYCN. In a disease with few recurrent somatic mutations, a gain-of-function mutation, a phenylalanine to leucine substitution at codon 1174, exists in a gene encoding the cell surface receptor tyrosine kinase ALK. Interestingly, the ALKF1174L mutant is preferentially associated with MYCN amplification and accounts for a subset of patients with a particularly poor clinical outcome. In vivo studies have shown that concomitant expression of MYCN and ALKF1174L in neural crest cells leads to development of neuroblastoma with earlier onset, higher penetrance, and enhanced lethality compared to tumors in mice with isolated MYCN amplification. Mice bearing tumors with MYCN amplification and ALKF1174L show a distinct transcriptional profile compared to those with isolated MYCN amplification, suggesting a positive cooperative effect between the two. However, we do not have a clear understanding of the contribution of ALKF1174L in altering gene expression.

The goal of this study is to understand the mechanism(s) underlying the ability of ALKF1174L to affect oncogenic transcription of MYCN. To investigate this, I performed shRNA-mediated knockdown of ALK in cell lines expressing ALKF1174L or wild-type (WT) ALK together with and without amplified MYCN. Depletion of oncogenic ALKF1174L resulted in a decrease in MYCN protein levels, while knockdown of WT ALK showed no discernible change in MYCN protein expression, supporting a role for the mutant receptor but not its WT counterpart, in regulating the expression of oncogenic MYCN. By examining the genome-wide changes caused by inducible overexpression and deletion of ALKF1174L in isogenic cell line pairs with and without MYCN amplification, we hope to better characterize this oncogenic relationship and potentially identify therapeutically targetable nodes in high-risk neuroblastoma.
Small Molecule Allosteric Modulators of the β2-Adrenergic Receptor

Paula Rambarat, HHMI Medical Fellow, Columbia University College of Physicians and Surgeons (Duke University School of Medicine)

Mentor: Robert Lefkowitz, MD, Howard Hughes Medical Institute and Duke University Medical Center

The β-adrenergic receptors (βAR) are a subfamily of G-protein coupled receptors (GPCR) implicated in the pathophysiology of cardiovascular and pulmonary diseases. Drugs that bind to the highly conserved, orthosteric hormone binding site of the βARs are a mainstay of treatment for conditions like asthma and heart failure. However, the use of such orthosteric drugs is often limited by off-target side effects. For instance, antagonists for the cardiac-predominant β1AR are often contraindicated in patients with asthma, as binding to β2ARs in the lung causes undesirable bronchoconstriction. Likewise, the use of β2AR agonists in asthma is limited by adverse effects like tachycardia. Targeting allosteric sites, which are topographically and evolutionarily distinct from the orthosteric one, has potential to yield more highly specific, efficacious medications. Allosteric drugs exert their effects by modulating the activity of orthosteric ligands. Negative allosteric modulators (NAMs) diminish the activity of orthosteric agonists, whereas positive allosteric modulators (PAMs) enhance agonist function.

Here, we present our isolation and characterization of the first PAM specific for the β2AR, Compound-6, and compare its properties with our recently reported β2AR NAM, Compound-15. These compounds were discovered through affinity-based, in vitro screening of highly diverse DNA-encoded small molecule libraries (DELs) against the purified human-β2AR. Of note, compound-15 was isolated by screening DELs against the unliganded β2AR, whereas compound-6 was obtained by screening against β2AR bound to a high-affinity agonist. Both compounds have low micromolar affinity for the β2AR and display diametrically opposed allosteric activity in radioligand binding studies. As expected for a NAM, compound-15 decreases β2AR affinity for orthosteric agonists, whereas the PAM, compound-6, enhances agonist affinity for the receptor. Both compounds also show unbiased allosteric modulation of β2AR-mediated signaling in cellular assays measuring cAMP production and β-arrestin recruitment downstream of the activated receptor. Compound-15 decreases the responsiveness, and therefore efficacy, of orthosteric agonists, while compound-6 increases agonist activity at the β2AR. Importantly, both modulators are highly specific for the β2AR, as their ability to modulate agonist function at the closely related β1AR is greatly diminished. Interestingly, compound-15 binds to the canonical, intracellular transducer binding site of the β2AR thereby sterically blocking transducer function, whereas compound-6 potentiates transducer function, suggesting a different binding site on the receptor. Additionally, using structurally modified analogs of each compound, we define the respective chemical groups that are key to their biological activity.

Overall, our studies outline a generally applicable, proof-of-concept strategy to isolate conformational-specific, small molecule GPCR ligands with unique functional profiles such as compound-6 and 15. It will be of interest to test these compounds in animal disease models to further determine their potential utility as therapeutics. In addition, comparative analyses of their atomic level interactions with the β2AR will offer further mechanistic insights into GPCR allostery.

Germline BARD1 Mutations Predispose to Neuroblastoma Through Defective DNA Double-Strand Break Repair

Michael Randall, HHMI Medical Fellow, Perelman School of Medicine at the University of Pennsylvania

Mentor: John Maris, MD, Perelman School of Medicine at the University of Pennsylvania and Children’s Hospital of Philadelphia

Neuroblastoma, an embryonal malignancy of the autonomic nervous system and the most common cancer diagnosed during the first year of life, is a complex genetic disease. While a genome-wide association study (GWAS) has identified several neuroblastoma predisposition loci, each individual SNP association has a relatively low effect size. By contrast, rare germline variants may have a larger effect size and contribute more significantly to malignant transformation. Our sequencing of neuroblastoma patients’ germline DNA has revealed significant enrichment for putative loss-of-function mutations in the BRCA1-associated RING domain 1 (BARD1) gene (7/766, 0.9%, p<0.001). Notably, no tumors showed loss of heterozygosity of the wild type (WT) allele. BARD1 heterodimerizes with BRCA1 and promotes its stability and subcellular localization; the BRCA1-BARD1 complex is essential for homology-directed repair (HDR) of DNA double-strand breaks (DSB). We hypothesized that germline BARD1 variants contribute to malignant transformation via dysregulation of HDR and ensuing genomic instability.

We first used a biochemical approach to evaluate the impact of BARD1 variants on the formation and function of the BARD1-BRCA1 heterodimer.
Five BARD1 germline variants (p.R112*, p.R150*, p.E287fs, p.Q564*, and p.R641*) were prioritized for study here. These variants were characterized for BARD1-BRCA1 heterodimerization after co-transfection with BRCA1 cDNA into HEK293T. Co-immunoprecipitation demonstrated that all but one truncated BARD1 protein (p.R112*) maintained the ability to bind BRCA1, though only p.E287fs stabilized BRCA1 comparably to WT BARD1, as measured by persistence following cycloheximide treatment. Immunofluorescence and immunoblotting after nuclear fractionation showed that three variants with truncations within or near the BRCA1-binding RING finger domain caused aberrant BRCA1 localization to the cytoplasm. We next introduced the E287fs variant into the IMR-5 neuroblastoma cell line as a monoallelic knock-in via CRISPR/Cas9 (IMR-5 BARD1 WT/E287fs). Successful knock-in was confirmed using Sanger sequencing. BARD1 WT/E287fs IMR-5 cells assembled significantly fewer RAD51 foci after UV irradiation than WT IMR-5 cells (38% vs. 12% of nuclei with ≥3 foci; p<0.001), suggesting reduced capacity for DNA DSB repair. Moreover, IMR-5 BARD1 WT/E287fs cells were six-fold more sensitive to poly (ADP-ribose) polymerase (PARP) inhibition with olaparib relative to WT IMR-5 cells (IC50 of 860 vs. 5400 nM). Taken together, these data suggest that BARD1 germline mutations predispose to neuroblastoma by disrupting the stability and localization of BRCA1. The resultant dysregulation of DNA DSB repair and increased sensitivity to PARP inhibition may provide a therapeutic opportunity. Efforts are ongoing to engineer additional heterozygous BARD1 variant knock-ins, and to further characterize these variants’ effects on BARD1-BRCA1 heterodimerization and DNA DSB repair.

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Immune Regulation of Cartilage Tumors

Spencer Richardson, HHMI Medical Fellow, University of Tennessee Health Science Center College of Medicine (St. Jude Children’s Research Hospital)

Mentor: Maureen McGargill, PhD, St. Jude Children’s Research Hospital

Osteochondromas are the most common type of benign bone tumor caused by neoplastic outgrowths of cartilage and bone. Although these tumors are benign, osteochondromas cause severe pain, deformity, and constriction of tendons and neurovascular structures. Individuals can develop multiple osteochondromas in a condition called multiple hereditary exostoses (MHE). MHE is linked to heterozygous mutations in the genes encoding exostosin glycosyltransferase 1 (EXT1) or 2 (EXT2). Although most MHE patients bear mutations in either EXT1 or EXT2, studies in both mice and humans indicate that heterozygous EXT mutations are not sufficient to cause disease. Mice with a heterozygous deletion of Ext1 model the loss-of-function EXT1 mutations observed in MHE patients, however these mice show very limited penetrance of osteochondromas. Thus, it is not understood how a loss of function of one EXT allele causes such severe disease. We previously demonstrated that the immune system impacts the formation and severity of osteochondromas in a mouse model of multiple osteochondromas caused by genetic deletion of Erk1 and Erk2 in CD4+ cells. This was surprising as no immune regulator of osteochondroma growth had been described. Thus, we hypothesize that EXT mutations render individuals susceptible to osteochondromas, but that additional “hits” mediated by inflammation and the immune system are necessary for tumor formation and growth.

To test whether activation of the immune system impacts the incidence and severity of osteochondromas, we stimulated the innate immune system in two different mouse models of multiple osteochondromas; heterozygous Ext1 (+/−) mice and mice deficient in Erk1 and Erk2 in CD4+ cells (Erk double knockout [DKOCD4]). The innate immune system was activated by giving the mice various adjuvants including polyinosinic-polycytidylic acid, complete Freund’s adjuvant, and bacterial lipopolysaccharide at weekly intervals starting at three weeks of age. We found that the incidence of osteochondroma formation was accelerated after sequential treatment with multiple adjuvants. Histological analysis of the affected bones demonstrated an increase in severity of osteochondromas in mice treated with adjuvants compared to littermate controls. These data indicate that stimulation of the innate immune system can impact osteochondroma growth. Importantly, we show that Ext1 +/- mice develop larger and more numerous tumors following innate stimulation, which supports our hypothesis that the immune system can serve as an additional “hit” resulting in tumors. The immune system may also contribute to the wide spectrum and severity of tumors observed in MHE patients. These data reveal a novel role for the immune system in development of osteochondromas.
Characterizing the Role of Fibronectin Splice Variants in Glioblastoma

Jonathan Rick, HHMI Medical Fellow, University of California, San Francisco, School of Medicine

Mentor: Manish Aghi, MD PhD, University of California, San Francisco

Glioblastoma (GBM) is an aggressive primary brain cancer with a dismal overall survival of under two years from diagnosis. A defining feature of these tumors is their invasiveness, which enables escape from surgical resection and drives inevitable recurrence, with over 90% of recurrences occurring within 2 cm from the original tumor. Work to identify mediators of invasion and target them has thus far yielded little progress. This knowledge gap could be explained by the fact that studies focused on cancer cells themselves are unable to capture GBM for what is really is, an organ with complex dynamic interplay between tumor cells and their microenvironment. Consequently, we are interested in studying the milieu surrounding GBM tumors to identify factors that permit invasion. One suspect is EDA expressing fibronectin, a splice variant that has been associated with other malignancies. In other cancers, EDA is thought to play a role in invasion and in making the tumor microenvironment more susceptible to growth. In work completed under the fellowship to date, I have demonstrated that EDA fibronectin is expressed in GBM and have also shown that this expression is particularly elevated in periventricular tumor locations. In areas rich with EDA expression, this protein appears to form scaffolds that may serve a role for angiogenesis, haptotaxis and/or macrophage polarization. We have also identified that cancer associated fibroblasts (CAFs) in GBM preferentially accumulate in periventricular tumor locations, where they are the cellular source of EDA fibronectin. Ongoing work seeks to elucidate the roles that EDA fibronectin plays in pro-tumoral invasiveness and macrophage polarization and, in so doing, identify a potential therapeutic target.

Characterization of the Inflammatory Infiltrate in Primed Mycobacterial Uveitis

Kevin Rolnick, HHMI-FFB Medical Fellow, University of Wisconsin School of Medicine and Public Health (University of Washington School of Medicine)

Mentor: Russell Van Gelder, MD, PhD, University of Washington School of Medicine

Uveitis is the fifth leading cause of blindness in the U.S., however our understanding of its immunologic pathophysiology is only nascent. To develop novel treatments for this set of diseases and to understand the ramifications of uveitis associated with viral vector mediated gene therapy for retinal degeneration, a more robust understanding of the inflammatory sequence in uveitis is required. Both innate and adaptive immunity are implicated in uveitis. Recently, an animal model incorporating innate and adaptive immunity, primed mycobacterial uveitis (PMU), was developed. This study aims to describe in detail the immune responses associated with PMU. Further, we aim to determine if aqueous samples represent the inflammatory response that occurs throughout the entire eye in PMU, as this could potentially serve as a diagnostic approach for uveitis.

To generate uveitis in C57BL/6J mice, mycobacterial H37Ra antigen was delivered subcutaneously one week prior to unilateral intravitreal injection of the same antigen (n=5). Inflammation was confirmed via vitreoretinal and anterior chamber optical coherence tomography at day 1 post-intravitreal injection (D1). At D1, cell suspensions were generated from inflamed eye aqueous (‘Ia’ sample), inflamed eye vitreous and choroid (‘Ivc’ sample), as well as from fellow eye aqueous, vitreous, and choroid (‘FE’ sample). Flow cytometry was performed to identify ocular cells and leukocytes as well as lymphocytic and granulocytic lineages.

Isolated cells were first gated to identify CD45+ leukocytes. The vast majority of CD45+ cells in Ia as well as Ivc were granulocytic (CD3-, CD19-, NK1.1+) with neutrophils (Ly6g+) representing >90% of these cells in Ia and Ivc. Of CD45+/CD3+ cells, a minority were CD8+ (17.2%, 16.4%, and 22.4% in Ia, Ivc, and FE, respectively). A larger proportion of CD3+ T cells and CD19+ B cells were observed in Ivc than Ia (CD3+: 0.72% in Ia, 1.50% in Ivc; CD19+: 0% in Ia, 0.27% in Ivc). A significant population of plasmacytoid dendritic cells (CD45+/CD11chigh/CD11blow) was found in both Ia and Ivc (0.25% and 1.81% of CD45+/CD3+/CD19+/NK1.1+ cells, respectively).

These findings illustrate the complex manifestations of intravitreal mycobacterial exposure. Both the anterior and posterior eye experience a granulocyte-predominant response at D1 following H37ra exposure. Further, the inflammatory infiltrate in the anterior chamber is distinct from that...
In this study, we aim to utilize human induced pluripotent stem cells (hiPSCs) to model sickle cell anemia (SCA) in vitro. This approach allows us to mimic the disease in a controlled environment without the limitations associated with animal models. We have generated hiPSCs from sickle cell patients with hemoglobin SS disease seen at our hematology clinic at Boston Children’s Hospital. Using a cocktail of transcription factors that promote self-renewal and multipotency expressed under the control of a doxycycline-regulated promoter (ERG, HOXA9, RORA, SOX4, MYB), we generated conditionally immortalized hematopoietic progenitors that serve as a renewable source of robust erythroid cells in vitro. Erythroid progenitors differentiated from these lines underwent globin-switching once transfused into immunodeficient mice, with a 27% induction of beta-globin expression. The generation of hiPSC-derived enucleated, beta-globin-expressing red blood cells (iRBCs) from sickle cell patients with beta-thalassemia major could benefit from autologous, engineered red blood cells. Here, we describe preliminary iRBC production from hiPSCs and the development of novel therapeutic treatments for sickle cell anemia (SCA). hiPSCs can theoretically produce all cell types including induced red blood cells (iRBCs). Sickle cell patients could benefit from autologous, engineered red blood cells as these patients have rare blood types, are frequently allo-sensitized to blood products, and are at risk of iron overload from recurrent transfusions. However, in vitro modeling of SCA as well as iRBC production from hiPSCs has been hampered by their inability to differentiate into terminally-mature, enucleated, beta-globin-expressing red blood cells. Here, we describe strategies to improve in vitro production of iRBCs. We generated hiPSCs from sickle cell patients with hemoglobin SS disease seen at our hematology clinic at Boston Children’s Hospital. Using a cocktail of transcription factors that promote self-renewal and multipotency expressed under the control of a doxycycline-regulated promoter (ERG, HOXA9, RORA, SOX4, MYB), we generated conditionally immortalized hematopoietic progenitors that serve as a renewable source of robust erythroid cells in vitro. Erythroid progenitors differentiated from these lines underwent globin-switching once transfused into immunodeficient mice, with a 27% induction of beta-globin expression. An in vitro protocol incorporating human plasma can be used to produce 30-40% beta-globin-expressing cells. 10-50% of generated iRBCs are also enucleated. Preliminary iRBC analysis reveals nearly 36% of the enucleated population to be RNA negative erythrocytes and 64% RNA positive reticulocytes. With an expandable source of erythroid progenitors capable of producing mature red cells, we hope to assess the feasibility of this platform for autologous cell therapies. In future studies, we anticipate the improvement of the sickling model via a robust induction of beta-globin expression. The generation of hiPSC-derived SCA models will be critical in broadening the current understanding of the molecular mechanisms of this disease, and the development of improved pharmacological treatments for the treatment of SCA.

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**Enucleation in Induced Red Blood Cells: A Platform for Autologous Cell Therapy and In Vitro Modeling of Sickle Cell Anemia**

**Tolulope Rosanwo, HHMI Medical Fellow, Case Western Reserve University School of Medicine (Harvard Medical School)**

**Mentor:** George Daley, MD, PhD, Boston Children’s Hospital, Dana Farber Cancer Institute and Harvard Medical School

Human induced pluripotent stem cells (hiPSCs) hold tremendous promise for disease modeling and the development of novel therapeutic treatments for sickle cell anemia (SCA). hiPSCs can theoretically produce all cell types including induced red blood cells (iRBCs). Sickle cell patients could benefit from autologous, engineered red blood cells as these patients have rare blood types, are frequently allo-sensitized to blood products, and are at risk of iron overload from recurrent transfusions. However, in vitro modeling of SCA as well as iRBC production from hiPSCs has been hampered by their inability to differentiate into terminally-mature, enucleated, beta-globin-expressing red blood cells. Here, we describe strategies to improve in vitro production of iRBCs. We generated hiPSCs from sickle cell patients with hemoglobin SS disease seen at our hematology clinic at Boston Children’s Hospital. Using a cocktail of transcription factors that promote self-renewal and multipotency expressed under the control of a doxycycline-regulated promoter (ERG, HOXA9, RORA, SOX4, MYB), we generated conditionally immortalized hematopoietic progenitors that serve as a renewable source of robust erythroid cells in vitro. Erythroid progenitors differentiated from these lines underwent globin-switching once transfused into immunodeficient mice, with a 27% induction of beta-globin expression. An in vitro protocol incorporating human plasma can be used to produce 30-40% beta-globin-expressing cells. 10-50% of generated iRBCs are also enucleated. Preliminary iRBC analysis reveals nearly 36% of the enucleated population to be RNA negative erythrocytes and 64% RNA positive reticulocytes. With an expandable source of erythroid progenitors capable of producing mature red cells, we hope to assess the feasibility of this platform for autologous cell therapies. In future studies, we anticipate the improvement of the sickling model via a robust induction of beta-globin expression. The generation of hiPSC-derived SCA models will be critical in broadening the current understanding of the molecular mechanisms of this disease, and the development of improved pharmacological treatments for the treatment of SCA.

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**Understanding the Molecular Function of RBFOX and Determining the Role in Congenital Heart Disease**

**Harsh Shah, HHMI Medical Fellow, Mayo Medical School (Harvard Medical School)**

**Mentor:** Caroline Burns, PhD, Massachusetts General Hospital and Harvard Medical School

Congenital heart disease (CHD) accounts for nearly one-third of all major congenital anomalies and affects approximately 40,000 births per year in the United States. Although critical breakthroughs in cardiovascular diagnostics and surgery have led to increased survival rates in newborns with CHD, the etiologies of most CHDs remain largely unknown. Progress on this front has been recently achieved with the advent of whole exome sequencing where both inherited and de novo mutations were identified in newborns that segregate with CHDs. Although these mutations are likely to be causative for disease, this hypothesis has yet to be tested. Moreover, because the majority of loci discovered have not been previously linked to cardiovascular development, their mechanisms of action are elusive. One locus identified by the whole exome approach encodes RBFOX2, an evolutionarily conserved RNA binding protein of unknown function. During my internship in the Burns Lab at Massachusetts General Hospital/Harvard Medical School, I will uncover the molecular and cellular mechanisms underlying the cardiovascular phenotypes I observe in a new genetic zebrafish model of RbFox-mediated CHD.

The zebrafish genome contains three RBFOX paralogs including RBFOX1, RBFOX1-like, and RBFOX2. Using whole mount in situ hybridization, we only observed RBFOX1-like and RBFOX2 transcripts in the zebrafish heart. Based on their expression patterns, we generated new zebrafish strains that harbor small deletions in RBFOX1-like and RBFOX2 by CRISPR-Cas9 genome editing to learn whether they play a required role in heart development or function. Compared to single mutants that develop grossly normal hearts during embryogenesis, double mutant embryos show severe cardiac edema indicative of cardiovascular abnormalities.
Heartbeat monitoring revealed a significantly decreased rate of ventricular. Moreover, sarcomere structure appears highly disorganized in RBFOX double mutant ventricular cardiomyocytes. Together, these data demonstrate that RBFOX is required for normal heart function during embryogenesis.

In the coming months, I plan to more deeply characterize the RBFOX cardiac phenotype. Specifically, I will assess heart function by analyzing fractional shortening, calcium transients, and action potential duration and heart structure by counting cardiomyocyte numbers and by measuring cardiomyocyte cell size with the goal of uncovering the primary cellular mechanism underlying the observed cardiac failure. Concurrently, I plan to compare the transcriptomes of control to double mutant embryos by deep sequencing analysis to gain insight into potential molecular mechanisms underlying the heart failure phenotype. Ultimately, my research will demonstrate that mutations in RBFOX are causative for cardiovascular phenotypes in zebrafish and uncover the mechanisms by which RBFOX regulates heart development and function.


Poster Abstracts

Creation of a Robust Genetically Engineered Mouse Model of IDH-mutant Glioma

Diana Shi, HHMI Medical Fellow, Harvard Medical School

Mentor: William Kaelin, M.D., Howard Hughes Medical Institute, Dana-Farber Cancer Institute and Harvard Medical School

Glioblastoma (GBM) is the most common and aggressive form of brain tumor. Secondary GBMs are a subset of GBMs that progress from a low-grade or anaplastic astrocytoma, and display a high prevalence (70-90%) of mutations in the gene isocitrate dehydrogenase (IDH) 1. The ability to study IDH1 mutations in vivo has been hampered, however, by a lack of faithful mouse models. The goal of this project is to create a genetically engineered mouse model (GEMM) of IDH1-mutant glioma by introducing the most common glioma-associated IDH1 mutation (encoding the IDH1R132H oncoprotein) into mouse brain cells in vivo concomitantly with other driver mutations that frequently co-occur in IDH1-mutant gliomas.

Data from low-grade glioma patients compiled by The Cancer Genome Atlas (TCGA) show that IDH1 mutations often co-occur with activating PIK3R1 or PIK3CA mutations, together with loss of function mutations in TP53 and ATRX. We first investigated the cooperativity between IDH1 and PIK3R1 mutations in a human astrocytic cell line immortalized via ectopic expression of telomerase (hTERT), which phenocopies inactivating mutations in ATRX, as well as E6 and E7, which inactivate p53 and Rb, respectively. Lines expressing either IDH1 or PIK3R1 mutants exhibited significantly increased colony size and number in soft agar assays compared to the empty vector-expressing line, and these phenotypes were enhanced further in cells expressing both mutants together. Furthermore, intracranial injection of these cell into immunodeficient mice demonstrated significantly increased tumor incidence and aggressiveness in mice injected with cells expressing both oncogenes relative to either oncogene alone.

We used this knowledge to develop our strategy for generating an IDH1-mutant GEMM. We constructed an adeno-associated virus (AAV) encoding Cre-recombinase and sgRNAs targeting murine isoforms of ATRX, TP53, and RB1. This virus was intracranially injected into four different engineered mouse strains (designated: (1) LSL-Cas9; (2) LSL-Cas9; LSL-PIK3CAH1047R; (3) LSL-Cas9; LSL-IDH1R132H; (4) LSL-Cas9; LSL-ATRXH132H; LSL-PIK3CAH1047R), where the indicated genes were placed downstream of a loxP-stop-loxP (LSL) cassette. These mice have been monitored for tumor initiation with serial MRIs. One of the first mice injected developed a needle track osteosarcoma, and validated the in vivo efficacy of our sgRNAs and Cre activation efficiency. We then re-engineered our AAV so that Cre was controlled by a tissue-specific glial fibrillary acidic protein (GFAP) promoter and injected it into the four engineered mouse strains. At 3 months post-injection, 3 out of 10 LSL-Cas9; LSL-IDH1R132H; LSL-PIK3CAH1047R mice demonstrated intraparenchymal enhancement on MRI suspicious of developing GBMs. Furthermore, 0 out of 8 double-mutant mice (LSL-Cas9; LSL-PIK3CAH1047R) showed evidence of tumor formation on MRI at this same time point, suggesting that mutant IDH1 is acting as a driver in our model. We anticipate that our IDH1-mutant mice will develop tumors that recapitulate the histopathological, metabolic, and epigenetic changes seen in human IDH1-mutant gliomas.
Lymphatic Bile Acids Play an Important Role in Modulating Enhanced Insulin Sensitivity Following Roux-En-Y Gastric Bypass

Ornella Simo, HHMI Medical Fellow, Meharry Medical College (Vanderbilt University School of Medicine)

Mentor: Naji Abumrad, MD, Vanderbilt University School of Medicine

Bariatric surgery is the most effective and durable treatment for obesity as well as Type 2 diabetes, though the mechanisms underlying its effectiveness remain to be fully elucidated. Roux en Y Gastric Bypass (RYGB) is the most effective and most widely used bariatric procedure. We previously demonstrated (Nat. Comm, 2015) that bile diversion to the ileum (GB-IL) has identical metabolic effects to RYGB in rodent models of obesity. We showed that that the metabolic improvements, particularly the enhanced insulin sensitivity, are associated with circulating bile acids (BAs). In this study we hypothesize that BAs increase incretin responses. We fed C57BL6 mice a high fat diet for 12 weeks, following which the mice were subjected to either bile diversion from the gall bladder to the ileum (GB-IL), or RYGB vs. sham (controls); additionally, each mouse was fitted with a chronic cannula into the mesenteric lymphatics. Four weeks following surgery, and after an overnight fast, lymph samples were collected on hourly basis before (2 h) and after (4 h) nutrient mixed meal bolus (Ensure®). GB-IL and RYGB mice had significant weight loss and reduced food intake during the first week postop. At 4 weeks post-surgery, body fat composition was significantly lower in RYGB and GB-IL and had significantly lower blood glucose levels. Lymph content of triglycerides and phospholipids were remarkably higher in RYGB and GB-IL mice. Plasma BA levels in RYGB and GB-IL were 2-fold and 10-fold higher following surgery, and after an overnight fast, lymph samples were collected on hourly basis before (2 h) and after (4 h) nutrient mixed meal bolus (Ensure®). GB-IL and RYGB mice had significant weight loss and reduced food intake during the first week postop. At 4 weeks post-surgery, body fat composition was significantly lower in RYGB and GB-IL and had significantly lower blood glucose levels. Lymph content of triglycerides and phospholipids were remarkably higher in RYGB and GB-IL mice. Plasma GLP-1 levels were undetectable in both groups, but lymphatic incretin levels (GLP-1 and GIP) were significantly higher in GB-IL and RYGB mice. However, only GB-IL mice had significantly higher lymphatic levels of C-peptide compared to other study groups. These results suggest a differential effect of BAs on pancreatic insulin and C-peptide secretion, which may be responsible for a significant component of the improved insulin sensitivity following bariatric surgery.

Weakly and semi-supervised deep learning for immunohistochemistry feature representation in tissue microarrays with generative adversarial networks

Andrew Sohn, HHMI Medical Fellow, Jefferson Medical College of Thomas Jefferson University (Perelman School of Medicine at the University of Pennsylvania)

Mentors: Jason Moore, PhD and Michael Feldman, MD, PhD, Perelman School of Medicine at the University of Pennsylvania

Histopathology is still considered to be the gold standard in the diagnoses of many cancers. Recently, there have been two major developments that are poised to change the face of pathology at the center of clinical diagnosis: (1) digital pathology, and (2) molecular profiling technologies. The emergence of digital pathology allows for the introduction of automated image analysis to pathology, which can greatly aid a pathologist’s workflow and offer new quantitative tools that are otherwise laboriously prohibitive. Molecular technologies offer complementary information probed at a resolution that is not accessible by tissue morphology and phenotype analyses. While we are presently in the era of ‘omics’, histological images still provide important features that are unavailable from molecular profiling/omics data, such as the spatial context of the tumor microenvironment.

The 50,000-foot view of our work is an attempt to extract phenotypic features from tissue microarrays (TMAs) and correlate with genetic features. To extract phenotypic features, we use techniques from semi-supervised deep learning, and more specifically, generative adversarial networks (GANs). Very briefly GANs are a class of generative models (as opposed to discriminative models) that can perform representation learning on unlabeled data by imitating a real data distribution via transforming noise variables into synthetic data within target domain. Using GANs, we perform data augmentation and image-to-image translation/image style transfer (among IHC stains) as proxies for representation learning as well as disentangled representation learning. Once a robust deep learning model has been trained, we will search for a common feature space for the phenotypic and genetic features, followed by clustering analyses to observe whether any phenotypic and genetic features correlate with one another.

We perform our analyses on a dataset of BRCA1 and BRCA2 germline mutation-associated breast and ovarian tumors. The dataset consists of TMAs with twenty different immunohistochemistry stains (stromal and immune) and genetic profiles generated from exome sequencing. This dataset has been generated and compiled from patients seen at the Hospital of the University of Pennsylvania.
Evaluation and Prevention of Heart Disease Using Three Fatty Acids from Postprandial Pythons

Kelsey Spaur, HHMI Medical Fellow, University of Colorado Denver School of Medicine (University of Colorado at Boulder)

Mentor: Leslie Leinwand, PhD, University of Colorado at Boulder

Extreme environments drive species to develop novel adaptations, which have been a source of scientific innovation for years. For the Burmese python, its environmental pressure is infrequent feeding. Following a meal weighing up to its body weight, multiple organs undergo significant growth to accommodate a 40-fold increase in metabolic rate. Regarding the heart, the Leinwand lab showed that this hypertrophy is physiologic rather than pathologic by several criteria including a signature of gene expression changes that were distinct from those of pathological hypertrophic growth. Most notable of the genes activated by serum from post-fed pythons was the water and glycerol transporter aquaporin 7 (AQP7). From post-fed python plasma, they identified a combination of three fatty acids (FAs) that replicated cardiac hypertrophy when administered subcutaneously to pythons and mice and when applied to cultured neonatal rat ventricular myocytes (NRVMs). The FAs were: 40 mM myristic (C14:0), 100 mM palmitic (C16:0), and 7.5 mM palmitoleic (C16:1).

In this study, we evaluated the capacity for the FAs to prevent or reduce negative cardiac changes due to pathologic stimuli. This hypothesis was first tested using FAs dissolved in dimethyl sulfoxide (DMSO) and NRVMs. Administering FAs to NRVMs prior to the pathologic hypertrophic stimulus, phenylephrine (PE), blunted the hypertrophic effect of PE compared to DMSO controls after 48 hours of total treatment. We also applied FAs to NRVMs following PE for 48 hours and still saw a significant increase in AQP7 expression relative to DMSO controls. This induction of AQP7 and the reduction of pathologic hypertrophy in the presence of a pathologic stimulus suggests that the FAs can exert a dominant effect. These in-vitro findings led us to investigate in-vivo. Due to variability seen using DMSO-FAs mini-pumps, we investigated oral gavage as an alternate route of administration. Several recent papers have shown efficacy of FAs via oral gavage for other purposes. The FAs were suspended in corn oil and administered to two cohorts of mice for four weeks. The groups were either six or ten weeks of age at the beginning of the experiment. We did not see cardiac hypertrophy in either group, but the six week group did show significant up-regulation of α-MyHC, which is an indication of a physiologic effect. Although oral gavage does not cause a robust enough response to be utilized for future experiments, this finding indicates there was some effect. We recently conjugated the FAs to bovine serum albumin (BSA). Our next step is to test BSA-FAs on NRVMs to ensure they induce the hypertrophy we have seen with DMSO-FAs. If successful, we will perform mini-pump experiments in mice. Our ultimate goal is to administer FAs in-vivo prior to or following a pathologic stimulus such as PE or trans-aortic constriction.

HLA-E-Expressing “Universal” Pluripotent Stem Cells as a Source of Retinal Pigment Epithelium to Treat Age-Related Macular Degeneration

Marta Stevanovic, HHMI-FFB Medical Fellow, Emory University School of Medicine (Keck School of Medicine of the University of Southern California)

Mentors: Mark Humayun, MD, PhD, University of Southern California Roski Eye Institute and Dennis Clegg, PhD, University of California Santa Barbara

Identifying a suitable stem cell source for retinal pigmented epithelium (RPE) has been a challenge in treating dry age-related macular degeneration (AMD). Using human embryonic stem cells (hESC), which express polymorphic human leukocyte antigens (HLA), may cause immune rejection. Creating induced pluripotent stem cells from a patient’s own tissue is costly and time-consuming. A solution may be to use “universal stem cells” (Ucells), which do not express polymorphic HLA proteins. We differentiated Ucells into RPE and tested the phenotype and function of RPE derived from U3 (lack HLA class I expression), U37 (lack HLA class I and II expression), and the controls U1 and H9 in vitro. We will inject U1-, U3-, and U37-RPE cell suspensions into the subretinal space of pigmented athymic nude-RCS rats with RPE dysfunction to test the ability of Ucell-RPE to rescue photoreceptor function in vivo.

Quantitative PCR (Q-PCR) was used to analyze RPE gene expression and test for genes of contaminating cell types. Morphology and epithelial polarity were assessed using light microscopy and immunohistochemistry. Differentiation efficiency was examined by flow cytometry using the Pmel17 antibody, which labels pigmented cells. Loss of Oct4, a pluripotency marker, was also determined by flow cytometry. Ucell-RPE function was analyzed by measuring secretion of the growth factor PEDF using ELISA assays and also by measuring the ability of Ucell-RPE to carry out phagocytosis of bovine photoreceptor outer segments. For all in vitro studies, hESC-derived
(H9) RPE was a positive control. For the in vivo study, immunocytochemistry of retinal tissue will be performed 1 and 3 months after injections. Phagocytic capability of the Ucell-RPE will be examined by staining tissue sections for rhodopsin and quantifying phagosomes. For all in vivo experiments, U1-RPE will be used as a positive control and sham surgery as a negative control.

Ucell and H9-RPE showed similar expression of RPE-specific genes, including visual cycle genes (RPE65, CRLBP), RPE membrane channel and transporter genes (BEST1), and pigment and melanin biosynthesis genes (TYRP1, tyrosinase, and MITF). The Pmel17 gene was expressed in 86.12±0.9248 % of U1-, 84.65±1.392 % of U3-, and 88.67±1.506 % of H9-RPE. There was no significant difference between U3- and U1-RPE (Mann-Whitney test, two-tailed, p=0.4) or U3- and H9-RPE (Mann-Whitney test, two-tailed, p=0.1) Pmel17 expression. Ucell-RPE morphology and phagocytosis ability were also similar to those of H9-RPE.

Our results in vitro show that Ucell-RPE is similar in form and function to H9-RPE (in vivo results pending). RPE from Ucells is a novel approach to bypass immune-mediated graft rejection without using immunosuppression. Using Ucells as progenitors may be also be applicable to transplantation of other cell types.

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Exploring Substrate Promiscuity towards a Novel Method of Sortase-Mediated Bioorthogonal Protein Labeling

Erica Storm, HHMI Medical Fellow, Stanford University School of Medicine

Mentor: Jennifer Cochran, PhD, Stanford University

Sortases are bacterial transpeptidases with wide applications for post-translational covalent modification of proteins. Sortase A is highly specific for its LPXTG peptide recognition motif which it incorporates to any substrate with a terminal oligoglycine moiety. Recent engineering of sortase A has improved its catalytic activity, but its utility for in vivo labeling remains limited by the lack of cell permeable and commercial available oligoglycine peptide probes. Thus, there remains a need for a versatile bioorthogonal method of site-specific protein labeling in living cells.

We present a two-step, sortase-based approach to in vivo protein labeling using an intermediate azide tag. First, we demonstrated that an engineered sortase A variant (termed 7M) exhibits promiscuous activity with an array of primary amines including 3-azido-1-propanamine (Azp) and propargylglycine, with incorporation rates of up to 80-100%. By using sortase A 7M to enzymatically label proteins with Azp, we showed that fluorescent probes could then be added through click chemistry via the azide tag. We have demonstrated this strategy both in vitro and in vivo using an Escherichia coli expression system. Application of 25 mM Azp to E. coli cultures coexpressing sortase A 7M and an LPTEG-tagged GFP variant, followed by incubation with a Cy3-dibenzocyclooctyne (DBCO) modified dye yielded complete conversion to the labeled protein conjugates.

To demonstrate the versatility of our sortase bioconjugation system, we sought to optimize the sortase labeling reaction in mammalian cells. Sortase A 7M was successfully expressed at low levels in adherent HEK293 cells, but not in suspension HEK293 cultures. Conversely, suspension HEK cultures displayed 80-fold lower sensitivity to Azp concentrations than adherent HEK cells. Thus, engineering of sortase A for selective Azp activity is essential not only to expand the toolbox of sortase variants but to reduce concentrations of Azp substrate to sustainable levels for mammalian culture.

Towards this end, a yeast-display library of 160,000 sortase A 7M variants was generated through saturation mutagenesis of four residues within the predicted oligoglycine pocket of sortase A. In order to quantify the bioconjugation reaction of yeast-displayed sortase variants, we developed a simplified dual-protein expression vector, termed pCL. Our construct utilizes both termini of the a-agglutinin mating protein (Aga2p) subunit to facilitate internal conjugation of the LPETG recognition motif by co-displayed sortase A. Induction of the pCL yeast library in the presence of Azp was followed by copper-free click reaction with biotin-DBCO and conjugation detected by staining with PE-labeled avidin. Fluorescence activated cell sorting (FACS) will be used to select enzyme variants for sequencing and additional kinetic characterization. Identifying sortase variants with improved Azp functionality will further demonstrate the versatility of our pCL display system and facilitate bioorthogonal site-specific protein labeling in mammalian cells.
Decreased expression of TCF12 transcription factor in glioblastoma stem-like cells results in an increased mesenchymal subtype signature.

Rukayat Taiwo, HHMI Medical Fellow, Washington University in St. Louis School of Medicine

Mentor: Albert Kim, MD, PhD, Washington University in St. Louis School of Medicine

The basic helix-loop-helix (bHLH) transcription factor, TCF12, has been implicated in precursor cell proliferation during embryonic neurogenesis and continues to be expressed postnatally in mitotically active regions of the brain. TCF12 has been shown to be preferentially expressed in glioblastoma stem-like cells (GSCs), a key subpopulation of cancer cells thought to underlie tumor therapy resistance and recurrence. But the exact role of TCF12 in gliomagenesis has remained unclear since it has been shown to be both a proto-oncogene and tumor suppressor depending on the cellular context. We therefore investigated the potential role of TCF12 in regulating the biology of GSCs. Using microarray analysis of 24 patient-derived GSCs, we find that TCF12 is highly expressed in proneural GSCs relative to normal human astrocytes, and differentiation of proneural GSCs in culture markedly downregulated TCF12 mRNA. To test the role of TCF12 in GSCs, loss-of-function approaches using both RNA interference and CRISPR-Cas9-mediated knockout demonstrated reduced self-renewal capacity, a key measure of stem-like cell identity, relative to control infection. Conversely, over-expression of TCF12 using a lentiviral approach increased GSC self-renewal capacity. Mechanistically, a mini-screen of candidates known to regulate GSC self-renewal revealed that knock-down of TCF12 led to a decrease in the expression of the bHLH transcription factor, OLIG2, helix-loop-helix protein, ID1. By contrast, over-expression of TCF12 led to increased expression of these markers. Furthermore, bioinformatic analysis of the genomic regions upstream of OLIG2 and ID1 transcription start sites revealed the presence of evolutionarily conserved TCF12 binding sites, implicating these stem-identity genes as potential transcriptional targets of TCF12. Taken together, these findings suggest that TCF12 controls the stem-like identity of proneural GSCs by stimulating expression of transcriptional regulators OLIG2 and ID1. Finally, using OLIG2 and CD44 as markers of proneural and mesenchymal identity respectively, knock down of TCF12 demonstrated decreased OLIG2 expression with a concomitant increase in CD44 expression. These findings suggest that TCF12 is necessary for the maintenance of a proneural stem identity and loss of TCF12 may lead to a switch from proneural to mesenchymal subtype.

Modelling Inflammation in Heterotopic Ossification Using Human Induced-Pluripotent Stem Cells

Amy Ton, HHMI Medical Fellow, University of California, San Francisco, School of Medicine

Mentor: Edward Hsiao, MD, PhD, University of California, San Francisco, School of Medicine

Heterotopic ossification (HO) is a debilitating process where bone forms abnormally in soft tissue. This can occur in response to trauma or injury and complicates over 40% of hip replacement surgeries. However, what factors predispose HO formation are largely unknown. To address this, we established an iPS cell model of fibrodysplasia ossificans progressiva (FOP), a congenital disease of HO caused by a gain-of-function mutation in the ACVR1 receptor of the BMP pathway. Given that FOP patients develop HO in response to trauma, sepsis, or immunizations, we sought to understand how the classical ACVR1 R206H mutation may affect acute inflammation. Macrophages and endothelial cells are found in nascent HO lesions and both cell types play critical roles in inflammation of numerous tissues. Our prior clinical studies showed that FOP patients are in a pro-inflammatory state with increased populations of pro-inflammatory monocytes and elevated circulating inflammatory cytokines. In addition, FOP iPS cell-derived endothelial cells (iEC) can secrete increased inflammatory factors when co-cultured with mesenchymal stem cells. Since HO lesions show a massive infiltration of immune cells by histology, we hypothesized that the FOP endothelial cells induce increased chemotaxis of monocytes in response to injury. Because primary tissue specimens are not possible to obtain from FOP patients due to the risk of inducing more HO, we generated macrophages from our iPS cell lines (iMac) using a new highly efficient and reproducible differentiation protocol. iMacs express macrophage lineage markers of CD14 and CD11b, with FOP iMacs having larger populations of CD206-expressing cells than that of CD163. We are currently co-culturing our iMacs with iECs in a transwell model to assess macrophage migration and chemotaxis. These data suggest that FOP iMacs have different immune responses caused by abnormal BMP signaling that may affect cell-cell interactions. This immune activation may predispose FOP patients to the development of HO, and may also be a key regulator of bone formation in non-genetic forms of HO.
Biomimicry of the Breast Cancer Microenvironment in a 3D, Patient-Derived, Tissue Engineered Model

Yoshiko Toyoda, HHMI Medical Fellow, Weill Cornell Medical College

Mentor: Jason Spector, MD, Weill Cornell Medical College

Obesity is a known risk factor for the development and prognosis of breast cancer. Obesity is associated with less response to breast cancer therapy and more aggressive disease. Adipocytes have been identified as a source of exogenous lipids in many cancer cell types, including breast, and provide energy to fuel malignant survival and growth in breast cancer. Autologous fat transfer is an increasingly ubiquitous procedure for breast reconstruction after mastectomy, but the oncologic safety is largely unknown. There is a compelling need to better understand the biology behind the obesity-breast cancer link. We have developed a 3D, patient-derived tissue engineered platform to directly assess the metabolic interactions among cells of the breast cancer tumor microenvironment.

Breast tissue was enzymatically digested to retrieve adipocytes. Polydimethylsiloxane wells were filled with type I collagen encapsulated with stromal cells and adipocytes labeled with the fluorescent lipid dye borondipyrromethene (BODIPY) 493/503 and RFP-labeled MDA-MB-231 breast cancer cells on the surface. Cultures of MDA-MB-231 in non-adipocyte containing collagen matrices as well as adipocyte containing constructs without breast cancer cells served as controls. After 1, 2, 3, and 7 days, constructs were fixed then immunohistochemically stained with Hoechst nuclear stain and Cytokeratin 19. Lipid transfer and breast cancer cell invasion into the collagen bulk were analyzed using laser scanning confocal microscopy and images were processed with Imaris.

The 3D collagen culture platform demonstrated BODIPY-stained mature adipocytes neighbored by stromal cells, akin to the native architecture in human breast tissue. At the interface of the cancer cells with the biomimetic stroma, lipid transfer was observed—breast cancer cells which were close in proximity to the lipid-filled adipocytes demonstrated small, green fluorescent lipid droplets in the cytoplasm. Breast cancer cell invasion into the collagen bulk was assessed via confocal Z stacks on Imaris.

We have established a novel 3D tissue engineered platform to study breast cancer microenvironment, including metabolic interactions between primary human breast adipocytes and breast cancer cells. Transfer of fluorescently-labeled lipids directly from adipocytes to breast cancer cells may induce aberrant metabolism to fuel malignant growth and adaptive survival which may have implications in breast cancer prognosis in patients of different obesity as well as in the setting of autologous fat transfer after oncologic resection.

The Transcriptional Response of Endothelial Cells to Statin Treatment in vivo

Katherine Turnbull, HHMI-BWF Medical Fellow, Iowa State University College of Veterinary Medicine (University of Michigan Medical School)

Mentor: David Ginsburg, MD, Howard Hughes Medical Institute and University of Michigan Medical School

Statins inhibit hydroxymethylglutaryl-CoA reductase, an enzyme in the cholesterol synthesis pathway, and are widely prescribed for cardiovascular risk reduction. In addition to lowering plasma cholesterol levels, statins have also been proposed to further reduce cardiovascular risk via other pleiotropic anti-inflammatory and pro-angiogenic effects, potentially mediated through the endothelium, though the underlying molecular mechanisms are still largely unknown. Analysis of endothelial cells (ECs) in vivo has been limited due to their extensive heterogeneity across vascular beds as well as their interspersed anatomical distribution. In addition, isolation of ECs from complex tissues and/or expansion in vitro results in rapid phenotypic drift and changes in gene expression programs.

To characterize changes in the EC transcriptome induced by statin treatment, we applied the Translating Ribosome Affinity Purification (TRAP) technique, tagging the ribosomal protein RPL22 with hemagglutinin (HA) in all ECs in the mouse in vivo by crossing a conditional Rpl22-HA allele with a Tck-Cre recombinase transgene. Mice are perfused with cycloheximide at the time of sacrifice, freezing actively translating mRNA within the ribosomal complex. EC ribosomes are then selectively isolated from tissue lysates by immunoselection for the HA tag and the associated mRNA is then purified from this complex. Multiple tissues (brain, heart, kidney, liver, lung and skeletal muscle) were subjected to high throughput RNA sequencing (RNASeq) and EC-enriched transcripts were identified by comparison of the HA-selected mRNA to total tissue mRNA. To study the effects of statin treatment on the endothelium, 3 male mice were treated orally with 1mg/kg body weight atorvastatin daily for one week, and 3 untreated males were used as controls.

RNASeq analysis of control tissues identified a set of pan-EC enriched genes in all tissues evaluated, including the known EC markers Tek, Cdh5, Flt1, and Nos3. Additionally, each tissue exhibited a set of vascular bed-specific EC transcripts, which was most prominent in brain. Gene ontology analysis of identified EC markers showed enrichment for vascular-related biologic processes, including ‘vascular development’, ‘blood vessel morphogenesis’, and ‘angiogenesis’. Though analysis of the statin treated samples is still in progress, preliminary unsupervised hierarchical clustering analysis of the brain samples showed high similarities between transcriptional profiles for the statin-treated
Towards a single-neuronal basis of social reciprocity within the macaque anterior cingulate cortex

Amy Wang, HHMI Medical Fellow, Harvard Medical School

Mentor: Ziv Williams, MD, Massachusetts General Hospital and Harvard Medical School

Social dysfunction is a core component of many psychiatric disorders, but its single-neuronal and causal underpinnings remain largely unknown. Reciprocity, a central feature of social interaction, allows individuals in a group to forge alliances towards augmenting individual and mutual fitness. Here, we studied the neuronal correlates of group interaction by obtaining multiple-neuronal recordings in the anterior cingulate cortex (ACC) of rhesus macaques as they performed a structured social task.

We devised a three-agent social task in which three macaques interacted with each other over multiple rounds. The task required the monkeys to sit around a rotary table apparatus; in each trial, one individual would offer a food reward to one of the other two. Throughout sessions, individuals could reciprocate past rewards that had been delivered to them. Based on this design, we could associate core computations associated with interactive behavior: the animal’s own decisions, the decisions of others, their social identities, and past interactions. During task performance, we recorded neuronal activity from their ACC using micro-electrode arrays.

The monkeys showed strategic preferences for other individuals, and preferred to reward those who reciprocated. Engaging in this social strategy increased the amount of reward received by a given animal, enhancing individual fitness. Maintaining a mental representation of specific preferred individuals is a prerequisite for acting out strategic social preferences. We discovered a sub-population of neurons encoding such a signal: these neurons tracked the reward received by other group members and displayed differential activity in response to different individuals.

These findings demonstrate a novel sub-population of neurons in the primate ACC that encode information about particular individuals, forming the necessary basis for social reciprocity. These results lay the groundwork for identifying specific, neurobiologically-guided targets for treatment of social behavioral disorders.

Hypoxia-induced Translational Profiles of Embryonic and Adult-Derived Macrophages: Implications in Cardiac Injury and Repair Responses to Ischemia

Nicholas Wilcox, HHMI Medical Fellow, Yale School of Medicine

Mentor: Jeffrey Bender, MD, Yale School of Medicine

Myocardial infarction (MI) remains one of the leading causes of mortality. Healing after MI depends on tight regulation of the inflammatory response because cardiomyocytes have a limited ability to regenerate following injury and consequent cell loss. Macrophages are important in both injury and repair responses to MI because they clear necrotic cardiomyocytes, promote angiogenesis and produce cytokines, chemokines and growth factors. The mammalian heart contains at least 2 macrophage subsets. Yolk sac-derived macrophages (YSDMs) establish themselves during embryonic development and undergo self-renewal. In contrast, bone marrow-derived macrophages (BMDMs) primarily originate from circulating monocytes recruited to the heart following an acute perturbation.

Evidence suggests that YSDMs and BMDMs have disparate roles in tissue repair. Data describing differences between these macrophage subsets has been generated largely by total RNA analyses, which do not necessarily correlate with functional gene expression due to dynamic post-transcriptional regulation. For example, the RNA-binding protein HuR promotes active translation by binding to the three prime untranslated region (3’-UTR) of mRNA, while miRNAs cause mRNA degradation or prevent active translation. We hypothesize that following hypoxia in vitro, which assimilates ischemia in vivo, YSDMs are anti-inflammatory and promote favorable cardiac repair and remodeling, while BMDMs are pro-inflammatory and hinder tissue repair. To test this, we are employing translating ribosome affinity purification (TRAP), a novel translational profiling approach, which couples with a transgenic murine model enables the isolation of cell-specific, polyosomal mRNA.

We have determined, by quantitative polymerase chain reaction (qPCR), the optimal duration (16 to 20 hours) of hypoxic incubation of murine BMDMs following seven-day culture for induction of an mRNA subset. This includes vascular endothelial growth factor (Vegf), matrix metalloproteinase 9 (Mmp9) and glucose transporter 1 (Glut1). Gene ontology analysis identified a subset of putative hypoxia-inducible mRNA targets of HuR and miRNA regulation, including endoplasmic (Hsp90b1), a molecular chaperone regulating secretory protein processing including toll-like receptors and integrins. Relevantly, Hsp90b1 is necessary for tumor-associated macrophages to drive inflammation. qPCR analysis of...
polysomal RNA from BMDMs showed enrichment of select transcripts following hypoxia, including Hsp90b1, Vegf and Mmp9. Finally, Mmp9 was enriched in polysomal RNA relative to non-polysomal RNA after hypoxia.

We have employed TRAP to isolate sufficient yields of polysomal RNA from murine BMDMs, derived from HuR wild type or knockout mice after hypoxia or normoxia, for RNA-seq analysis. We have demonstrated efficient HuR deletion in BMDMs from knockout mice by flow cytometry and western blot. RNA-seq will enable unbiased detection of novel or less abundant transcripts and elucidate HuR-mediated stabilization of polysomal RNA for targets of interest. Similar experiments will establish a comparative translational profile for YSDMs. Results would provide a molecular basis to explain the non-overlapping properties of these macrophage subsets and identify novel therapeutic targets.

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Using Artificial Antigen Presenting Cells to Enhance Antigen Presentation in the Treatment of Glioblastoma

Yuanxuan Xia, HHMI Medical Fellow, Johns Hopkins University School of Medicine
Mentor: Michael Lim, MD, Johns Hopkins University School of Medicine

Immunotherapy has become a promising tool in the oncologist’s armamentarium for treating cancer. Glioblastoma (GBM), however, exhibits an extremely immunosuppressive tumor microenvironment through multiple mechanisms, including upregulation of checkpoint molecules like PD-1, its ligand PD-L1, and the presence of suppressive myeloid cells such as macrophages and myeloid-derived suppressor cells. To counteract the immunosuppressive milieu in GBM, we have developed a biodegradable microparticle-based system of artificial antigen presenting cells (aAPCs) that can be placed locally within a tumor. As artificial constructs, aAPCs can perform the duties of native APCs without experiencing tumor-induced immunosuppression. Through a mixture of poly(beta-amino ester) (PBAE) and poly(lactic-co-glycolic acid) (PLGA), we generated biodegradable microparticle cores that can be loaded with any combination of immunostimulatory molecules. Normally, APCs activate the immune system by presenting a specific peptide (Signal 1) and a variety of co-stimulatory molecules (Signal 2) to activate T cells and drive an adaptive immune response against the peptide of interest. Using the well-characterized OT-1 OVA system, we loaded MHC I dimer expressing OVA chicken peptide (Signal 1) and co-stimulatory anti-CD28, anti-OX40, and anti-41BB molecules (Signal 2) onto our microparticle surface. To identify what combination of Signal 2 molecules best stimulates a cytotoxic immune response, we performed co-culture assays with OT-1 CD8 T cells and aAPCs with each permutation of co-stimulatory molecules. Surprisingly, aAPCs loaded with just MHC-OVA generated as great a proliferation response as those aAPCs loaded with both MHC-OVA and co-stimulatory molecules. This suggests that OVA system aAPCs bearing only Signal 1 are sufficient to stimulate a strong response in vitro. Next, to translate this OVA aAPC system into in vivo experiments for GBM therapy, we tested the efficacy of adoptive transfer of OT-1 T cells into mice implanted with OVA-expressing GL261 tumors. To test whether transfer of OT-1 T cells alone eradicates GL261 tumors, we treated mice with escalating doses of OT-1 T cells. Interestingly, no dose of OT-1 T cells significantly improved survival compared to controls. Currently, we are testing the in vivo efficacy of aAPCs in combination with PD-1 blockade in mice harboring OVA-expressing GL261 tumors.

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The effect of vitamin D on Th17-hyperresponsive Systemic Lupus Erythematosus

Erin Yamamoto, HHMI Medical Fellow, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University
Mentor: Trine Jorgensen, PhD and Xiaoxia Li, PhD, Lerner Research Institute, Cleveland Clinic Foundation

Recently, approximately 20% of Systemic Lupus Erythematosus patients of European decent were found to harbor a mutation in ACT1 (rs33980500) which leads to a non-functional ACT1 protein variant (ACT1 D10N). ACT1 is the key downstream signaling molecule for IL-17, a characteristic cytokine for Th17 cells. The Act1<sup>-/-</sup> mouse displays a lupus-like phenotype that is initiated by bacterial gut colonization and driven by Th17 cells and corresponding cytokines. Since vitamin D is a known suppressor of Th17 differentiation, and vitamin D deficiency is suspected to contribute to lupus pathology, we hypothesize that vitamin D supplementation will ameliorate the Act1<sup>-/-</sup> phenotype. Additionally, we propose that patients with the ACT1 D10N variant will display a Th17 hyperresponsive lupus similar that of the Act1<sup>-/-</sup> mouse. The Act1<sup>-/-</sup> Th17 cell populations and disease pathology were assessed after 8 weeks of either a vitamin D low (0 IU/g), normal (2 IU/g), or high (10 IU/g) diet. Compared to mice on a normal vitamin D diet, the spleen weights of mice in the low and high vitamin D groups were approximately...
Breast cancer cells exploit an orphan RNA to drive metastatic progression

Steven Zhang, HHMI Medical Fellow, Stanford University School of Medicine (University of California, San Francisco, School of Medicine)

Mentor: Hani Goodarzi, PhD, University of California, San Francisco

Cancer cells often hijack endogenous regulatory programs to achieve pathological gene expression landscapes. Notable examples of these strategies include somatic mutations, genetic amplifications and deletions, and epigenetic modifications. Additionally, post-transcriptional pathways have also emerged as major regulators of this transformation, including those involving miRNAs, RNA binding proteins, and tRNAs and tRNA fragments. However, a shared characteristic among these strategies is that they rely on existing pathways within the cell. Given that cancer can be viewed as an evolutionary process, there is a distinct possibility that cancer cells are capable of evolving or rewiring new regulatory pathways during disease progression. One possible source for novel pathways is non-coding RNAs, as these molecules can arise due to ectopic activation of RNA polymerases or nucleases while also possessing versatile regulatory capacity. We posited that non-coding RNAs arise in cancer cells as a consequence of tumorigenesis and provide a pool of cancer-specific molecules with regulatory potential. In this study, we performed a systematic search for the emergence of such neo-regulators of gene expression in breast cancer. We performed a systematic search for such cancer-specific RNA species by comparing the small RNA profiles of eight human breast cancer cell lines to human mammary epithelial cells. We identified a pool of small RNAs whose presence was further associated with breast cancer using data obtained from The Cancer Genome Atlas Data and the small RNA profiles of patient-derived xenograft samples. We subsequently discovered that a functional oncRNA termed T3p, originating from the 3’ end of TERC, acts as a broad regulator of gene expression and is a robust promoter of breast cancer metastasis. We found that T3p exerts its pro-metastatic effects by interacting with the RISC complex and increasing the expression of pro-metastatic genes NUPR1 and PANX2. Our findings raise the possibility that further examination of the cancer-specific RNA landscape and investigation into oncRNAs may yield novel strategies in developing diagnostic and therapeutic methods across many cancer types.

Strain-Dependent Activation and Inhibition of Human Immunodeficiency Virus-1 Entry by a PF-68742 Diastereomer

Connie Zhao, HHMI Medical Fellow, Harvard Medical School

Mentor: Joseph Sodroski, MD and Navid Madani, PhD, Dana-Farber Cancer Institute and Harvard Medical School

Human Immunodeficiency Virus-1 (HIV-1) entry into cells is mediated by the envelope (Env) trimer of gp120 and gp41 heterodimers. Sequential binding to the target cell receptors, CD4 and CCR5 or CXCR4, triggers the metastable Env to undergo entry-related conformational changes. PF-68742 was recently identified as a small molecule that inhibits infection of a subset of HIV-1 strains by interfering with an Env function other than receptor binding, with resistance determinants mapping to the gp41 disulfide loop (DSL) and the gp120 C5 region. We investigated the antiviral mechanism of PF-68742.
Recombinant luciferase-expressing HIV-1 pseudotyped by wild-type (WT) or mutant HIV-1 Envs was incubated with increasing concentrations of PF-68742 and/or other entry inhibitors and/or antibodies. The virus-inhibitor mixture was then added to CD4+ CCR5+, CD4+ CXCR4+, or CD4- CCR5+ target cells, and luciferase activity was measured 48 to 72 hours later.

Of the four PF-68742 diastereomers, only one, MF275, inhibited the infection of CD4+ CCR5+ cells by some HIV-1 strains. Unexpectedly, MF275 activated the infection of CD4- CCR5+ cells by several HIV-1 strains resistant to the compound's inhibitory effects in CD4+ CCR5+ target cells. In both cases, the strain susceptibility profiles were unique from those of other entry inhibitors. Sensitivity to other entry inhibitors in the presence of MF275 indicated that MF275-activated virus entry requires CCR5 binding as well as gp41 heptad repeat formation and exposure. Washout of MF275 prior to addition of the virus mixture to target cells was able to abrogate its inhibitory effect in CD4+ CCR5+ target cells but not its activating effect in CD4- CCR5+ target cells. In contrast to CD4-mimetic compounds, MF275 inhibitory and activating activity did not depend upon availability of the gp120 Phe43 cavity. Mutants with altered Env reactivity or State 1 preference (previously demonstrated to be resistant to CD4-mimetic compounds) remained susceptible to MF275. Conversely, changes in the gp41 DSL and gp120 C5 regions conferred resistance to MF275 but not CD4-mimetic compounds. The half-lives of the onset and decay of the MF275-activated state were different from those of CD4-mimetic compounds. Finally, while MF275 and CD4-mimetic compounds both enhanced susceptibility of some HIV-1 strains to the 17b and 19b antibodies against a CD4-induced (CD4i) epitope and the gp120 V3 loop, respectively, only MF275 enhanced susceptibility to the 4e10 antibody against the gp41 membrane-proximal external region (MPER).

MF275 apparently binds a site on the HIV-1 Env unique from the CD4 binding site and activates a conformational cascade that leads to virus entry. This pathway is parallel to but distinct from that triggered by CD4 and CD4-mimetic compounds. An understanding of the mechanisms of activity of MF275 should assist efforts to optimize its utility.

Chemotherapy augments recombinant oncolytic poliovirus efficacy for treatment of glioblastoma

Justin Zhuo, HHMI Medical Fellow, Duke University School of Medicine

Mentor: Matthias Gromeier, MD, Duke University School of Medicine

Glioblastoma (GBM), the most common primary malignant brain tumor in adults, is a highly lethal cancer with nearly all patients experiencing tumor recurrence after standard of care surgery, radiation, and chemotherapy. Thus, novel therapeutic strategies are urgently mandated. PVSRIPO is a highly-attenuated, recombinant polio:rhinovirus chimera that has demonstrated promising and robust clinical and radiographic responses in phase I clinical trials for recurrent GBM. PVSRIPO causes direct tumor cytotoxicity via binding to the CD155 poliovirus receptor expressed in GBM and virtually all solid neoplasms. Eliciting type I interferon signals in antigen presenting cells leads to robust innate and adaptive antitumor immunity. An unexpected discovery during the phase I trial was the achievement of complete and durable responses in patients who were treated with single-dose chemotherapy months after tumor progression following PVSRIPO infusion. This led to the initiation of an ongoing phase II trial comparing the efficacy of PVSRIPO and single-dose chemotherapy to PVSRIPO alone.

The goal of this study is to elucidate the therapeutic mechanisms of combined PVSRIPO and single-dose lymphodepletive chemotherapy using syngeneic mouse tumor models. We hypothesize that post-PVSRIPO lymphodepletive chemotherapy elicits an 'immunologic reset' that unmasks antitumor immune responses initially generated by PVSRIPO. To test this hypothesis, mice were implanted subcutaneously on the right flank with CT2A murine glioma cells transduced with human CD155. After one week, PVSRIPO was injected intratumorally, with intraperitoneal temozolomide administration occurring the following week. Tumor caliper measurement data and survival analyses demonstrate that combination treatment decreases tumor growth and increases overall survival compared to either treatment alone. Furthermore, early flow cytometry analyses of intratumoral lymphocyte populations suggest that chemotherapy administration following PVSRIPO treatment may induce memory T cell and regulatory T cell infiltration of the tumor. Ongoing experiments utilizing temozolomide-resistant cell lines will serve to better highlight the role of chemotherapy in restoring antitumor immunity by minimizing its ability to cause direct tumor cytotoxicity. This study has important implications not only for guiding future GBM treatments, but also for the potential inclusion of chemotherapy in other immunotherapeutic approaches.