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Abstracts of Presentations

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On the Cover (clockwise from top left):

Interactions between the tumor microenvironment and cancer cells drives disease progression and therapeutic recalcitrance. Shown here are basal cell carcinoma associated fibroblasts (CAFs) expressing the activation markers alpha-smooth muscle actin (αSMA - green) and fibroblast activation protein (FAP - red). Nuclei are stained with DAPI (blue). Activated CAFs, such as these, are hypothesized to be one of the primary culprits within the tumor microenvironment that communicate with cancer cells to promote disease aggressiveness. Our laboratory is focused on "reprogramming" these interactions by modulating CAFs from an activated to a quiescent phenotype, as reported by a loss of expression of these activation markers. We hypothesize that chemical modulation of the tumor microenvironment affords enhanced therapeutic susceptibility, and is a promising approach to improve the clinical utility of a wide variety chemo- and radiotherapies. (Courtesy of: Sriram Anbil, HHMI Medical Fellow, Harvard Medical School. Mentors: Tayyaba Hasan, PhD, Harvard Medical School and Massachusetts General Hospital and Edward Maytin, MD, PhD, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University)

Mice vaccinated with Herpes Simplex Virus type-2 (HSV-2) strains lacking glycoprotein D (gD) gain sterilizing and protective immunity against both HSV-1 and HSV-2. In the absence of gD, virions are unable to enter wild-type cells and must be grown in complementing cultures to remain infectious. Infecting these cultures with HSV-2 ΔgD leads to the formation of large syncitia characteristic of HSV. In this image, complementing cells were infected with an HSV-2 ΔgD strain in which gD was replaced with a robust rfp (HSV-2 ΔgD::rfp). The fluorescent marker allows us to visualize the immune response to HSV-2 ΔgD::rfp, further exploring its potential as a therapeutic agent and a vaccine vector for pathogens like influenza and HIV. (Courtesy of: Joseph Dardick, HHMI Medical Fellow, Albert Einstein College of Medicine. Mentor: William Jacobs, Jr., PhD, Howard Hughes Medical Institute and Albert Einstein College of Medicine)

Neural stem cells (NSCs) persist in the adult mammalian brain. NSCs capable of generating adult-born neurons are located in two distinct regions in the adult mammalian brain: the hippocampus and the subventricular zone (SVZ). These complex microenvironments are responsible for supporting neuroplasticity in the hippocampal and olfactory cortices, respectively, through continuous postnatal neurogenesis. When cultured, NSCs undergo a glia-to-neuron transition when provided with factors mimicking specific environmental cues of neuronal differentiation; a process termed neuropoiesis. Shown here are fully differentiated interneurons derived from NSCs harvested from the SVZ niche of adult nestin-CreERTm; rosa26r-tdT omato mice following tamoxifen injection for NSC lineage-tracing (tdTomato protein shown in red). When fully differentiated, these interneurons express the neuron-specific cytoskeletal protein MAP2 (shown here in green) and the inhibitory neuronal marker GAD67 (shown here in cyan). (Courtesy of: Gabriel Neves, HHMI Medical Fellow, Duke University School of Medicine. Mentor: Chay T. Kuo, MD, PhD, Duke University School of Medicine)

Novel optical techniques are under development, which permit the visualization and characterization of infiltrating tumor cells in unprocessed tissue during surgery, with the aim of guiding the resection of invasive malignancies. Stimulated Raman scattering (SRS) microscopy is a label-free imaging technique which generates image contrast based on the different chemical species present within a sample. By imaging the vibrational modes of protein and lipid molecules, SRS permits real-time, high-resolution imaging of cytoarchitecture in fresh tissue without dyes or stains. Further, by imaging the vibrational modes of small molecule metabolites, SRS is capable of performing real-time metabolomic profiling at the cellular level. Here we see an intraoperative SRS image of resected meningioma tissue, depicting the vibrational modes of protein (blue) and lipid (red). The collagen deposition (dark blue fibers), hypercellularity (crowded, pale blue nuclei), and whorled tissue architecture typical of this malignancy are all clearly visible. (Courtesy of: HHMI Medical Fellow Spencer Lewis, University of Michigan Medical School. Mentor: Daniel Orringer, MD, University of Michigan Medical School)
Host lymphodepletion enhances the frequency, persistence, and antitumor efficacy of loco-regionally delivered tumor-specific CAR T-cells against glioblastoma

Melissa Abel, HHMI Medical Fellow, Duke University School of Medicine

Mentors: John Sampson, MD, PhD and Luis Sanchez-Perez, PhD, Duke University School of Medicine

Glioblastoma (GBM) is the most common malignant primary brain tumor and is uniformly lethal. Conventional treatment is limited in part by a lack of specificity leading to damage of healthy brain tissue. The adoptive transfer of T-cells genetically engineered to express chimeric antigen receptors (CARs) represents a promising strategy to safely and efficiently target tumor cells within the central nervous system. CARs are artificial transmembrane receptors that couple the antigen-binding regions of an antibody with intracellular signaling components of a T-cell receptor. We have developed human and murine CARs that specifically recognize epidermal growth factor receptor (EGFR), a commonly amplified gene among GBMs, as well as EGFRvIII, a tumor-specific variant expressed in GBMs and other neoplasms. Previous studies from our lab have evaluated loco-regional delivery of EGFRvIII-specific CAR T-cells as a way to bypass variability in T-cell migration and enhance CAR T-cell survival and proliferation. CARs prolonged survival at modest doses, but were still hampered by poor persistence and displayed markers of T-cell exhaustion. In order to improve efficacy, we evaluated the effects of lymphodepletion prior to intra-cranial (IC) EGFRvIII CAR T-cell administration. These studies resulted in 90-100% long-term cures, while immune reconstitution impaired therapeutic efficacy. Additionally, host lymphodepletion enhanced the persistence of CARs in the brain over time and led to a significant decrease in CAR expression of T-cell exhaustion markers. The benefits associated with host lymphodepletion for adoptive T-cell immunotherapy are thought to include the improved functionality and the homeostatic-driven proliferation of exogenously delivered tumor-specific T-cells. We investigated two mechanisms that could be driving this result: (1) depletion of endogenous immune cells including regulatory T-cells (Tregs), and (2) a peripheral and/or local surge of IL-7 and IL-15 gamma chain cytokines. We have measured Treg counts in peripheral blood of lymphopenic mice and confirmed a drastically reduced number of Tregs compared to their lymphopre-plate counterparts. To determine whether Treg depletion is required for the intratumoral persistence and efficacy of IC-delivered CAR T-cells, we established a DEREG mouse model that allows for selective depletion of Tregs. Early studies show that temporary depletion of Tregs at the time of CAR infusion has no effect on treatment efficacy. Continued work will investigate whether sustained Treg depletion throughout the duration of tumor-killing activity will alter CAR T-cell efficacy. Ongoing studies include isolating the effects of homeostatic cytokines on CAR T-cell activity using transgenic IL-7 and IL-15 knockout mice. Upcoming studies will involve using our EGFR-specific CAR model to examine how lymphopenia might affect the efficacy and toxicity of targeting a tumor-associated antigen in GBM. Our results will help to clarify the mechanisms by which lymphopenia enhances CAR T-cell efficacy, and have potential to change approaches in the treatment of GBM.

Tmprss3 gene expression and gene therapy in a mouse model of human deafness

Hena Ahmed, HHMI Medical Fellow, Harvard Medical School

Mentor: Jeffrey Holt, PhD, Harvard Medical School and Boston Children’s Hospital

The inner ear is a complex structure that allows us to perceive sound. It is composed of the organ of Corti, spiral ganglion cells, the inner and outer spiral sulcus, and interdental cells, and (2) lack of Tmprss3 results in structural degeneration of hair cells and auditory impairment relative to wild-type mouse controls. I anticipate that hearing loss can be moderately recovered using gene therapy to drive Tmprss3 expression. If successful, our data will establish a foundation for development of gene therapy to treat DFNB8/10 in humans.
Stromal reprogramming with differentiation-promoting agents overcomes pancreatic ductal adenocarcinoma resistance to photodynamic therapy

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Mentors: Tayyaba Hasan, PhD, Harvard Medical School and Massachusetts General Hospital and Edward Maytin, MD, PhD, Cleveland Clinic Lerner Research Institute

Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease that is associated with the poorest of prognoses. The dismal outcomes associated with this condition are due in part to the highly active cancer-associated stroma that surrounds and communicates with cancer cells. Cancer-associated fibroblasts constitute a significant proportion of the cancer-associated stroma, and play a major role in the signaling interactions that increase tumor aggressiveness and resistance to therapy. Our project is focused on “reprogramming” cancer-stromal interactions to facilitate increased tumor susceptibility to standard-of-care chemotherapies and to the clinically promising light-based modality photodynamic therapy (PDT). Here, we test the efficiency of FDA-approved differentiation-promoting agents such as vitamin D (calcipotriol), all-trans retinoic acid (ATRA), and 5-fluorouracil (5-FU) to reprogram cancer-stromal interactions in vitro, and probe whether stromal reprogramming improves the cytotoxic efficacy of PDT in three-dimensional (3D) co-culture models comprising PDAC cells and cancer-associated fibroblasts (CAFs). We show that calcipotriol and 5-fluorouracil reduce the expression of α-smooth muscle actin (αSMA), a marker of stromal activation, in basal cell carcinoma-associated fibroblasts (BCAF). In addition, calcipotriol and 5-FU abrogate BCAF and pancreatic cancer-associated fibroblast (PCAF) induced changes in PDAC cell metabolic activity. Together, these results indicate that vitamin D and 5-FU may reprogram CAF-PDAC interactions. We probe whether PDAC resistance to PDT is overcome with this approach, and observe that BCAF and PCAF 3D co-cultures exhibit heightened PDT susceptibility following vitamin D pretreatment, suggesting that stromal reprogramming may be a viable strategy to overcome the profound therapeutic recalcitrance seen in clinical PDAC. In vivo studies are underway to confirm these findings, with the aim of improving clinical PDAC outcomes and augmenting ongoing clinical trials.

Subcortical cholinergic arousal nuclei during seizures: in vivo whole-cell recordings in a rat model

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Mentor: Hal Blumenfeld, MD, PhD, Yale School of Medicine

The mechanism of how seizures impair consciousness is not fully understood, but recent evidence suggests that ictal suppression of subcortical cholinergic arousal circuits, such as the nucleus basalis (NB) and pedunculopontine tegmental nucleus (PPTg), may play a role. Functional imaging and extracellular techniques show ictal suppression of activity in the NB and PPTg in a rat model of limbic seizures, but the postsynaptic signaling associated with this depressed firing rate is not known. The purpose of this investigation is to elucidate postsynaptic changes in neurons of the subcortical cholinergic arousal system during seizures.

Whole-cell recording (WCR) from neurons in vivo is a powerful technique for characterizing postsynaptic neuronal signaling from intact physiologic circuits. Whole cell recording is commonly used to access cortical neurons in vivo, but the literature reporting whole-cell recording from deep brain structures in vivo is sparse and in vivo WCR in the PPTg has not been previously reported. In this study, whole cell recordings were obtained in neurons of the PPTg, 6–8 mm deep, measured from the cortex, in the brain of head-fixed, anesthetized rats during limbic seizures.

One aspect limiting the depth of WCR is reproducible creation of long, narrow glass pipettes of sufficiently low resistance. A second obstacle to deep WCR is maintaining a viable pipette tip that can form a seal deeper than 1 GΩ with the target neuronal cell membrane (i.e., gigaseal) after passing through large amounts of tissue. The former problem was addressed using a 2.5 x 4.5 mm filament in a model P-1000 micropipette puller (Sutter Instruments) and creating a multi-line pulling program with a single high-velocity line followed by 3–4 low-velocity lines to produce micropipettes with a 10-mm taper and 4–6 MΩ resistance. To maintain a viable tip during descent through over 6–7 mm of tissue, the first pipette was lowered to the target region and left in place for 1 hour to allow the brain to form a narrow, low-resistance canal through which subsequent pipettes may pass. In addition, intrapipette positive pressure was maintained at 500 mbar throughout the descent, and dropped to <30 mbar at the target region. In this way, brain architecture was minimally perturbed and pipettes could access the PPTg.

Preliminary results of WCR in the PPTg during seizures show hyperpolarization of membrane potential associated with decreased firing in a subset of neurons histologically confirmed to be cholinergic. These data...
suggest cholinergic arousal systems are inhibited during hippocampal seizures, which may contribute to loss of consciousness. Ultimately these studies are aimed at identifying the synaptic mechanisms of depressed subcortical arousal during seizures, which may lead to new treatments aimed at preventing these changes and improving ictal and postictal cognition.

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The effect(s) of programmed death ligands 1 and 2 on the treatment of acute myeloid leukemia with anti-CD33 chimeric antigen receptor-engineered T lymphocytes

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Mentor: Terrence Geiger, MD, PhD, St. Jude Children’s Research Hospital

T lymphocytes engineered to express a chimeric antigen receptor (CAR) have shown profound clinical responses in relapsed/refractory B cell malignancies including leukemia, multiple myeloma, and lymphoma. This success of re-targeted T cells has yet to be extended to other hematologic malignancies. To this end, we have developed an immunotherapeutic approach to treat acute myeloid leukemia (AML) using CART T cells re-directed against the myeloid-specific antigen CD33 (CART-33). CART-33 cells are potent and specific in eliminating AML cells in vitro and in vivo. Despite this, CART-33 cells have shown inadequate in vivo expansion and persistence in NOD-SCID IL2rγ (−/−) (NSG) AML xenograft models. To address this, we assessed the impact of AML-expressed programmed death ligands 1 and 2 (PD-L1/L2) on CART-33 activity. PD-L1 inhibits T cell functions upon binding PD-1, an inhibitory receptor expressed on T cells that have been activated. Less is known about PD-L2’s effects. Interferon-gamma (IFN-γ), a primary effector cytokine secreted by CD4+ and CD8+ effector T cells, is a known inducer of PD-L1 on AML blasts. Using human AML cell lines U937, OCI-AML3, CMK, MV4-11, and a panel of primary human AML samples, we show that co-culturing AML with CART-33 cells, IFN-γ, TNF-α, or activated CART-33 supernatant can induce upregulation of both PD-L1 and PD-L2 on AML. Antibody-mediated blockade of cytokines in AML cultures mixed with activated CART-33 supernatant revealed that upregulation of PD-L1/L2 is dependent on CART-33 IFN-γ and TNF-α, and independent of other T cell-secreted effector cytokines. Additionally, IFN-γ and TNF-α synergize strongly in upregulating PD-ligands on AML. The kinetics and induction of PD-L2 are distinct from those of PD-L1. Although PD-L1 is well documented to suppress T cell function via ligation of T cell-expressed PD-1, the induction of PD-L1/L2 on AML had no effect on the cytolytic activity of CART-33 cells against AML in short-term (<48 h) cultures. Paradoxically, 24-hour pre-treatment of AML with either IFN-γ or CART-33 supernatant increased AML susceptibility to killing by CART-33 cells despite their elevated expression of PD-L1/L2. Moreover, addition of PD-1 blocking antibody reduced the frequency of CART-33 cells undergoing apoptosis in co-cultures with AML but had no effect on CART-33 cytolytic activity. Our results demonstrate how re-targeted T lymphocytes regulate expression of the immune-inhibitory PD-ligands PD-L1 and PD-L2 on AML and argue that AML-expressed PD-ligands affect the long-term sustainability, but not short-term cytolytic activity, of adoptively transferred CAR T cells in the treatment of AML.

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Evaluating organic fluorophore phototoxicity: quantification of phototoxic potential and effects

Thomas C. Binns, HHMI Medical Fellow, University of Arkansas for Medical Sciences College of Medicine (Janelia Research Campus)

Mentors: Luke Lavis, PhD and Zhe J. Liu, PhD, Janelia Research Campus

Visualization of the spatiotemporal relationships underlying biological processes is becoming increasingly vital to our understanding of cellular physiology and pathology. Recent advances in imaging technologies have expanded the utility of live-cell microscopy, allowing the visualization of subcellular processes with high spatiotemporal resolution. Phototoxicity, light-mediated damage produced through the absorption of light by endogenous and exogenous chromophores, becomes more limiting to such techniques as excitation wavelength and intensity are increased. Previous reports have identified various phototoxic effects exerted by fluorophores utilized in live-cell microscopy, including the generation of reactive oxygen species, induction of apoptosis, and decrease of mitotic activity; however, a comparison of commonly used fluorophores and excitation wavelengths has yet to be reported due to the lack of a standardized method of evaluation. This study describes the development of an assay designed to compare the phototoxic potential of various small molecule fluorescent dyes starting with the current line of Janelia Fluor dyes. Results obtained through this assay, in combination with those of functional and endpoint analyses, will inform both dye utilization and dye development aimed at mitigating imaging-induced phototoxicity, ensuring that biological systems being studied are not influenced by the tools used to study them.
Targeting the bromodomain and extraterminal domain family of proteins in Merkel cell carcinoma

Jae Eun Choi, HHMI Medical Fellow, University of California, San Diego, School of Medicine (University of Michigan)

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

Merkel cell carcinoma (MCC) is a neuroendocrine cutaneous malignancy that is lethal in its metastatic form. The basic helix-loop-helix transcription factor atonal homolog-1 (ATOH1) has been shown to be essential for normal development of Merkel cells and implicated in the pathogenesis of MCC. The bromodomain and extraterminal domain (BET) family of proteins, such as BRD4, mediate the transcription of genes by binding to acetylated lysine residues on histones and have promise as therapeutic targets in several cancer types. Here we investigate the therapeutic potential of BET protein in MCC using novel BET inhibitors and degraders.

To investigate the role of BRD4 in MCC, we first screened 17 MCC cell lines for their response to two different classes of BET inhibitors, ZBC-11 and ZBC-246. ZBC-11 is a small molecule inhibitor that binds to BRD4 and displaces it from chromatin. On the other hand, ZBC-246 is a fusion of ZBC-11 and thalidomide, which induces proteasomal degradation of BRD4 via the recruitment of ubiquitin complex. Sensitivity to these BET inhibitors was assessed by performing cell viability assays following 5 days of treatment. MCC cell lines were found to be very sensitive to BET inhibition, with median IC50 values at nanomolar concentrations. Since ZBC-246 leads to a complete loss of BRD4 protein in comparison to partial inhibitory activity of ZBC-11, MCC cell lines demonstrated greater sensitivity to ZBC-246 (IC50 60 pM to 73 nM) when compared to ZBC-11 (IC50 15 nM to >6 μM).

In order to identify the gene targets of BRD4 in MCC cells, we performed gene expression analysis on MCC-47 cells treated with both BET inhibitor (ZBC-11) and BET degrader (ZBC-246) at 3 and 24 hours. Microarray analysis demonstrated downregulation of ATOH1 and its downstream target SOX2. To validate this observation, we performed quantitative polymerase chain reaction (qPCR) and Western blotting for ATOH1 following 3- and 24-hour treatment with ZBC-11 and ZBC-246. There was significant downregulation of ATOH1 mRNA and protein at both time points.

Based on this observation, we hypothesized that the suppression of cell growth demonstrated by BRD4 inhibition occurred via suppression of ATOH1. To test our hypothesis, we investigated the role of ATOH1 knockdown on the proliferative capacity of MCC-47 cells. To our surprise, siRNA-mediated knockdown of ATOH1 had no effect on the viability of the MCC-47 cell line, suggesting that the effect of BET inhibitors on MCC cells is independent of ATOH1. As a future direction, we are mining the gene expression data to nominate BRD4-regulated genes that may have a vital role in MCC cell survival.

Taken together, our results demonstrate that MCC cell are very sensitive to compounds targeting the BET family of proteins and provide a compelling rationale for clinical evaluation of BET inhibitors and degraders in MCC.

Leishmania-specific skin-resident CD4+ T cells are formed from recently activated effector T cells

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Mentor: Phillip Scott, PhD, University of Pennsylvania School of Veterinary Medicine

Tissue-resident memory T (Trm) cells are critical components of protective immunity against a variety of pathogens. The majority of studies have focused on Trm cells at the site of infection or immunization, where inflammation promotes T cell recruitment. In contrast, few studies have focused on how Trm cells gain access to non-inflamed sites, an important issue for designing vaccines to target these cells. In mice that have resolved a primary infection with Leishmania, skin-resident memory CD4+ T cells have recently been shown to provide protection against challenge at sites distant from the initial infection site. This provides a model to determine when and how CD4+ T cells enter non-inflamed skin. We found that while Leishmania-specific CD4+ T cells enter the site of infection within a few hours, T cells were not found in non-inflamed skin distant from the primary infection site until 2 weeks after infection, and continued to enter the non-inflamed skin for at least 5 weeks. However, using parabiosis of naive mice and immune mice that had resolved infection, we found that Leishmania-specific CD4+ T cells present in the immune partner could not enter the non-inflamed skin of the naive partner. In contrast, upon re-challenge of the parabionts, these CD4+ T cells re-gained the ability to enter the non-inflamed skin of the naive parabiont. To understand what allows entry of CD4+ T cells into non-inflamed skin sites, we examined P- and E- selectin ligand (P&ESL) expression, and found that the CD4+ T cells capable of entering non-inflamed skin sites expressed high levels of P&ESL. To further characterize these cells, we examined their proliferation, and found that the cells entering non-inflamed skin sites have recently proliferated, suggesting that only activated effector T cells gain entry to non-inflamed skin. Combined, these data demonstrate that recently activated effector CD4+ T cells, but not memory CD4+T
cells, are capable of entering non-inflamed skin sites, and suggest that P&ESL expression plays a role in this process. Future studies will examine whether P&ESL expression is required for entry of CD4+ T cells into non-inflamed skin sites, identify what other factors are involved, and determine what promotes the retention of Trm cells in the skin. This work will be a critical contribution to the development of vaccines targeting the generation, migration, and retention of pathogen-specific resident memory T cells to relevant tissue sites.

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Application of isotopic tracing analysis reveals heterogeneous patterns of intratumoral dihydrotestosterone biosynthesis from adrenal androgens in localized prostate cancer

Charles Dai, HHMI Medical Fellow, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University

Mentor: Nima Sharifi, MD, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University

Androgen deprivation therapy (ADT) by medical or surgical castration is a mainstay of treatment for advanced prostate cancer. ADT disrupts the critical androgen signaling axis required for prostate cancer progression by depleting gonadal testosterone, the canonical precursor to the potent androgen dihydrotestosterone (DHT). While initially effective, ADT is invariably followed by recurrence of castration-resistant prostate cancer (CRPC). A major mechanism of CRPC is the intratumoral biosynthesis of DHT from the adrenal androgen dehydroepiandrosterone (DHEA), via three critical enzymatic steps (DHEA → androstenedione → 5α-androstanedione → DHT), designated as the "5α-androstanedione (5α-dione) pathway." We sought to determine the accessibility of the 5α-dione pathway in ADT-naïve, localized prostate cancers to better understand the transition of early-stage disease to CRPC and the potential underlying genotypic drivers of this metabolic phenotype.

The feasibility of an ex vivo culture system was first established using primary tissue from men undergoing prostatectomies for ADT-naïve, localized prostate cancer (n = 16). Selective isotopic tracing analysis was employed to measure the efficiency in conversion of isotope-labeled androstanedione (AD) into downstream steroid metabolites including DHT. Patient primary tissue samples, four different prostate cancer cell lines, and xenograft-derived tumors were incubated in media spiked with isotope-labeled AD over 48 hours, with and without equimolar unlabeled testosterone co-substrate to recapitulate a eugonadal versus androgen-deprived setting. Metabolites in media were captured by liquid chromatography and quantified via a radioactivity detector for tritium-labeled compounds or tandem mass spectrometry for 13C-labeled compounds (LC-MS/MS).

Tracing studies demonstrated that AD is readily converted to 5α-dione and ultimately to DHT in the majority of patient samples, although considerable variation was observed in the degree of metabolic utilization. Accessibility of the 5α-dione pathway was unaffected by the presence of testosterone co-substrate. Distinct metabolic phenotypes of robust versus limited metabolizers of AD were also noted across tested cell lines. To explore potential genotypic drivers of these differential phenotypes, publicly available cancer genomic data were queried for selective enrichment of genomic aberrations in genes expressing known steroidogenic isoenzymes involved in androgen metabolism. Expression of genes of interest was confirmed in cell lines in vitro by real-time PCR and Western blotting and then functionally tested through transient overexpression assays, siRNA-mediated gene silencing, and CRISPR/Cas9-mediated gene editing.

Our studies suggest that adrenal androgens may contribute to the biosynthesis of DHT in both early-stage, ADT-naive prostate cancer and CRPC via the 5α-dione pathway, but metabolic heterogeneity exists in the efficiency of substrate utilization. Active work is focused on the role of copy number alteration among genes in the 17β-hydroxysteroid dehydrogenase enzyme family toward determination of these phenotypes.

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Turning out the lights: RFP-tagged herpes simplex viruses (HSV) as novel tools for studying antibody dependent cell cytotoxicity (ADCC)

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

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

In collaboration, the Jacobs and Herold laboratories generated an attenuated herpes simplex virus type 2 (HSV-2) strain deleted in glycoprotein D (HSV-2 ΔgD) and demonstrated that it triggers an FcγRIV-activating response that elicits both antibody-dependent cell-mediated cytotoxicity and phagocytosis (ADCC and ADCP, respectively). The antibodies rapidly clear virus and prevent the establishment of latency in mice and guinea pigs following challenge with several clinical isolates of both HSV-1 and HSV-2. Quantifying ADCC and defining the responsible effector cells is technically challenging. Antibody-dependent cell-mediated cytotoxicity
(ADCC) has typically been measured by chromium release assays, which are cumbersome and require the use of a Cr\(^{51}\) radioisotope. Newer methods do away with the radioactive reagent, but can be inflexible, indirect, and expensive. To address these problems, we introduced \(rfp\) into HSV-2 (G) \(\Delta gD\) under control of an EF1\(_{\alpha}\) promoter. This reporter virus allows us to quantify the kinetics of ADCC and ADCP by separating infected from uninfected cells. The addition of cell membrane and cytosol markers then allow for direct quantification of the proportion of cells that undergo apoptosis due to effector function. By adding effector cells alongside immune sera or monoclonal antibodies from vaccinated mice, we are able to measure ADCC/ADCP activity as reductions in the viable \(rfp\) expressing cell population over time. Viability is measured using the membrane and cytosol markers. Moreover, since gD is required for viral entry, HSV-2 (G) \(\Delta gD::rfp\) has the additional utility of not spreading from cell to cell. As a result, it does not cause widespread cell death characteristic of HSV infection in wild-type cells. We hypothesize that this system will allow us to very finely evaluate the efficacy of different antibodies in mediating ADCC and ADCP function. Moreover, we propose that this assay can be used as a model to screen effector cell mutants and cells from different species to determine the genes and cytokines essential for ADCC function in HSV infection.

Preliminary experiments show that cells infected with 1 multiplicity of infection (MOI) of HSV-2 (G) \(\Delta gD::rfp\) start expressing the fluorescent marker around 4.5 hours post-infection. Expression increases up to 24 hours, at which point most cells undergo apoptosis due to the high MOI. Prior to infection, we stain non-adherent HEK cells with PKH67, a green fluorescent cell membrane marker, and tag-it violet cell cytosol markers. These target cells are then infected with 1 MOI of HSV-2 (G) \(\Delta gD::rfp\). At 1 hour post-infection, we incubate the target cells in pooled serum from HSV-2 \(\Delta gD\) vaccinated animals and then add these target cells to cultures of activated effector cells (such as splenocytes or bone marrow-derived macrophages [BMDMs], NK cells, or neutrophils). ADCC activity is then quantified with cytometric cell sorting. The proportion of \(rfp\) expressing cells that are double positive for the membrane and cytosol markers will decrease over time as infected cells are killed.

Preliminary experiments show that serum from HSV-2 \(\Delta gD\) vaccinated guinea pigs causes BMDMs to kill approximately 20–40% more of infected cells compared to serum from control vaccinated animals (a two to four-fold increase).

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The SRCAP complex subunit YL1 as a novel epigenetic target in melanoma

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Mentor: Emily Bernstein, PhD, Icahn School of Medicine at Mount Sinai

Metastatic melanoma remains therapeutically challenging, often resistant or refractory to current targeted and immune therapies. Targeting epigenetic regulators represents an advantageous strategy, as it most centrally affects aberrant transcriptional programs that promote or sustain the malignant phenotype. Such regulators, known as histone variants, contain sequence and structural variants of canonical histones and replace their conventional counterparts in chromatin through deposition by histone chaperone complexes. We previously found that H2A.Z, an H2A variant, is overexpressed in melanoma, with high expression correlating with decreased patient survival. The H2A.Z.2 isoform, in particular, promotes melanoma cell proliferation through increased transcription of E2F gene targets. We additionally showed that H2A.Z-specific chaperone complex SNF2-related CBP activator protein (SRCAP), which functions to replace nucleosomal H2A with H2A.Z, interacts with H2A.Z-containing nucleosomes in melanoma cells. Thus, we hypothesize that SRCAP complex is crucial to the epigenetic machinery controlling transcription in melanoma and its inhibition presents a novel strategy in melanoma treatment.

Using a short hairpin RNA-based loss-of-function mini-screen of the SRCAP complex members in melanoma cell lines, we found that knockdown of the subunit YL1 revealed significant inhibition of melanoma cell proliferation. Further, using gene expression and patient outcome data from The Cancer Genome Atlas (TCGA), YL1 was found to be overexpressed in 22% of cutaneous melanoma patients, and increased expression correlated with decreased patient survival. Mining of available datasets and immunoblot characterization of benign and malignant tissue corroborated increased levels of YL1 protein and mRNA in melanoma compared to melanocytes and benign nevi. Importantly, functional assays of YL1 knockdown in BRAF- and NRAS-mutant melanoma cell lines revealed decreased H2A.Z levels in chromatin, and cell cycle arrest followed by apoptosis.

Our findings suggest YL1 is aberrantly upregulated in melanoma and is crucial to maintain oncogenic proliferation and progression, likely through its role in H2A.Z-associated transcriptional mechanisms. Ongoing RNA and chromatin immunoprecipitation (ChIP) sequencing approaches will further reveal the biological role of YL1 in H2A.Z-mediated gene regulation and H2A.Z genomic occupancy in melanoma cells and its potential as a novel target in future melanoma pharmacotherapeutics.
**Strategies to characterize the subcellular localization of neuropilin-2 and its secreted ligand semaphorin-3F in the restriction of cortical neuron dendritic spine density and distribution**

**Teresa Easwaran**, HHMI Medical Fellow, Indiana University School of Medicine (Johns Hopkins University School of Medicine)

**Mentor:** Alex Kolodkin, PhD, Howard Hughes Medical Institute and Johns Hopkins University School of Medicine

Dendritic spines are small protrusions along the dendrite shaft that are the major location for excitatory synaptic input, and they undergo dynamic regulation following changes in activity and experience. It is critical to maintain excitatory and inhibitory balance for a coordinated and functional nervous system; disruption of this balance is seen in various neurological disease, such as epilepsy and autism. The semaphorins play critical roles in neural circuit assembly, dendritic spine formation, and neuronal morphogenesis. We have demonstrated that a secreted guidance cue, semaphorin 3F (Sema3F), is a negative regulator of dendritic spine number and synapse formation in the postnatal nervous system. Previous work demonstrates that loss of Sema3F, or its co-receptors neuropilin-2 (Npn-2) or plexin A3 (PlexA3), leads to an increase in spine density in layer V cortical pyramidal neurons and also in dentate gyrus granule cells. Further, *Sema3F*−/− and *Npn-2*−/− null mutant mice undergo spontaneous seizures, suggesting an imbalance in excitation and inhibition. It remains unclear what the specific cellular sources are that secrete Sema3F to restrict spine density and synapse formation. Therefore, we have developed a genetic strategy to conditionally knock out Sema3F from selective neuron populations and then assess spine morphology and distribution when Sema3F is removed from these specific cell types. To characterize dendritic morphology and spine distribution in these genotypes, we are labeling layer V pyramidal neurons using a g-deleted rabies virus expressing GFP so that we can achieve sparse labeling restricted to only layer V neurons, allowing for robust spine analysis and quantitation. We plan to determine which cells secrete Sema3F to restrain spine formation and distribution using this approach. Many questions also still remain regarding the expression, subcellular localization, and trafficking of the Npn-2. To address these issues, we have employed CRISPR/Cas9 technology to create an HA epitope-tagged *Npn-2* knock-in mouse. This knock-in mouse allows us to robustly assess endogenous Npn-2 cell surface distribution and also how it responds to Sema3F and interacts with the PlexA3 signaling receptor. This will provide understanding of how classical guidance molecules allow for proper cortical circuit assembly, with implications for understanding how dynamic regulation of synaptic morphology and function is maintained to influence synaptic plasticity and scaling in response to changes in neuronal activity.

**Identifying the NAD⁺ consuming enzyme promoting pathological axonal death**

**Kow Essuman**, HHMI Medical Fellow, Washington University in St. Louis School of Medicine

**Mentor:** Jeffrey Milbrandt, MD, PhD, Washington University in St. Louis School of Medicine

Axon degeneration is a form of programmed subcellular death resulting in the destruction of axons in injury and disease states. Injury-induced axon degeneration is thought to be independent of classic cell death pathways, as genetic and pharmacologic inhibition of these pathways do not significantly delay or prevent degeneration. Sterile alpha and TIR motif-containing-1 (SARM1) was recently reported to be an essential mediator of an injury-induced axon death pathway, though the mechanism behind its action remains to be solved. Here, we report that SARM1 mediates axon degeneration locally, by a catastrophic depletion of nicotinamide adenine dinucleotide (NAD⁺). Using a pharmacologically controlled dimerization system, we showed that activation of SARM1 signaling via dimerization of its TIR domain is sufficient to induce axon degeneration. This axonal demise is caused by a catastrophic loss of NAD⁺ in axons and can be inhibited by supplementation with the NAD⁺ precursor nicotinamide riboside (NR). The NAD⁺ consuming enzyme underlying SARM1-dependent axonal death has also remained unknown, although NADases like PARP1 and CD38 have been previously eliminated as candidates. Through a series of experiments, we have now identified the axonal NADase responsible for axon degeneration. This newly identified enzyme provides a novel therapeutic target against the many neurological diseases characterized by axonal degeneration, including peripheral neuropathy, traumatic brain injury, and neurodegenerative diseases.
Stromal interaction molecule 1 (STIM1) is required for a normal contractile phenotype in the uterus and is associated with obesity

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Mentors: Paul Rosenberg, MD and Chad Grotegut, MD, Duke University School of Medicine

Appropriate regulation of uterine contractility is necessary for successful parturition; inadequate uterine contraction is associated with prolonged labor, need for induction/augmentation, and cesarean delivery. Maternal obesity (BMI ≥ 30) increases the risk of obstetric complications secondary to poor uterine contractility. The mechanisms by which obesity affects myometrial contractility are unknown. Stromal interaction molecule 1 (STIM1) is a single-pass transmembrane protein that functions as a calcium (Ca\(^{2+}\)) sensor by activating store-operated Ca\(^{2+}\) (SOC) channels. STIM1 is required for maintaining cellular Ca\(^{2+}\) homeostasis and has been implicated in both excitation-contraction (EC) coupling and metabolic regulation of skeletal muscle, suggesting a possible link between myocyte contraction and obesity. To date, the role of STIM1 in uterine smooth muscle remains unknown. Herein, we characterize the role of STIM1 in the normal myometrium and begin to examine whether STIM1 function is altered in obesity.

STIM1-LacZ reporter mice underwent timed matings; uterine horns were isolated for histological and biochemical analysis. To assess contractility, uterine horns were harvested from STIM1-deficient (STIM1\(^{-/-}\); STIM1\(^{-/-}\)) mice, and myometrial strips were suspended in a tissue organ bath and stimulated with increasing doses of oxytocin. Parallel experiments were performed in both non-pregnant (NP) and term-pregnant (TNL) wild-type (WT) C57Bl/6 mice; muscle strips were pre-treated with a SOC channel inhibitor or vehicle control prior to stimulation with oxytocin. Human myometrial expression was measured in uterine biopsy samples obtained from term-pregnant women undergoing cesarean delivery at Duke University Medical Center. Primary cell lines derived from STIM1-deficient mouse models were used to assess cytosolic and mitochondrial Ca\(^{2+}\) dynamics and cellular metabolism. Finally, STIM1 polymorphisms were genotyped in skeletal muscle samples collected as part of the STRRIDE exercise intervention trial.

STIM1 is expressed in both the murine and human myometrium; STIM1 expression is upregulated in pregnancy and remains elevated throughout gestation and immediately postpartum. In myometrial strips isolated from NP and TNL mouse models, STIM1 is required for synchronous and sustained uterine contraction. Both STIM1 deficiency and SOC channel inhibition result in decreased contractile force, reduced contraction frequency, and diminished basal tone in response to myogenic (spontaneous) and oxytocin-induced stimulation. Preliminary data in both WT and STIM1-deficient mouse models suggests STIM1 expression is altered in obesity and that STIM1 deficiency results in reduced mitochondrial Ca\(^{2+}\) stores, leading to a Ca\(^{2+}\)-related block in oxidative metabolism. Last, initial genotyping experiments suggest STIM1 polymorphisms are associated with elevated BMI in adults.

We have demonstrated that STIM1 is required for a normal contractile phenotype in the myometrium. Our preliminary results suggest STIM1 as a potential link between obesity and myometrial contractility. Further studies are required to investigate this relationship, as understanding the effect of obesity on uterine contractility would lead to improved maternal and perinatal outcomes.

Real-time decoding of unconstrained upper limb movements using surface electrocorticography signals

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

Motor deficit is among the most debilitating aspects of injury to the central nervous system. Despite ongoing progress in brain-machine-interface development and in the functional electrical stimulation of muscles and nerves, very little is understood currently about how neural signals in the brain may be used to potentially control movement in one’s own unconstrained paralyzed limb. Here, we examined how neural activity in ventral premotor cortex (vPMC) of a rhesus macaque represents information about both planned and performed reach-return limb movements in free-space. We recorded from high-density electrode arrays and used real-time motion tracking techniques to correlate spatial-temporal changes in neural activity with arm movements made toward objects in space at millisecond precision. We find that many sites in the vPMC encode reaching movement and that neural activity from even a small number of electrodes within the vPMC can be used to accurately predict reach-return movement onset and left-right direction. Moreover, whereas higher gamma frequency field activity was more informative about movement direction during performance, mid-band beta and low gamma activity was more predictive of movement prior to onset. We speculate these dual signals may be used to optimize both planning and execution of movement during natural reaching, with prospective relevance to the future development of neural prosthetics aimed at restoring motor control over one’s own paralyzed limb.
Reshaping the immune microenvironment in metastatic prostate cancer with a TLR-4 agonist-conjugated anti-CDCP1 antibody and a CD47 antagonist

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Mentors: Irving Weissman, MD and Michael Longaker, MD, MBA, Stanford University School of Medicine

Prostate cancer is the second leading cause of cancer death in men in the United States. Severe morbidity and fatal outcome of the disease are directly linked to cancer metastasis. Recent discovery of the role of the immune system in clearance of metastatic prostate cancer cells (mPCs) has spurred interest in therapies that empower immune effectors against cancer cells. Antibodies are a class of drugs that can be designed to specifically target cancer cells and flag them to be killed by the immune system.

By comparing microarray gene expression between normal prostate, localized prostate cancer, metastatic prostate cancer, and a human metastatic prostate cancer cell line (PC-3), we identified a unique surface marker on mPCs, CUB domain-containing protein-1 (CDCP1), and developed a mouse IgG1 anti-human CDCP1 antibody (ACA). Initially, ACA treatment of luciferase-GFP+ PC-3 cells incubated with mouse macrophages did not increase phagocytosis over IgG1 isotype control, suggesting PC-3 cells possess mechanisms to evade antibody-mediated phagocytosis. We found that PC-3 cells highly express CD47, an anti-phagocytic signal and ligand to the SIRP-α molecule on macrophages. Although a CD47 antagonist, GV3, doubled phagocytosis compared to ACA treatment of luciferase-GFP+ PC-3 cells by FACS (P < 0.05), but an increased proportion of M1 (6.03% vs. 0.68%; P < 0.05) and decreased proportion of M2 macrophages (16.5% vs. 66.3; P < 0.05) in TCACA+GV3-treated versus isotype control conditions. In conclusion, we have shown that the combination of an ODN agonist-conjugated antibody targeting CDCP1 and the CD47 blocker GV3 allows for a specific and effective mechanism of immune-mediated mPC killing.

Tissue-specific intron retention is a new mechanism for FLNA mutation

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Mentor: Christopher A. Walsh, MD, PhD, Howard Hughes Medical Institute, Harvard Medical School and Boston Children’s Hospital

Periventricular nodular heterotopia (PVNH) is a rare neuronal migration disorder characterized by uncalcified nodules of neurons ectopically situated along the surface of the lateral ventricles. Loss of function of the human filamin A (FLNA) locus on Xq28 causes the most commonly inherited form of PVNH, predominantly affecting heterozygous females, while exhibiting prenatal or neonatal lethality in most males with the hemizygous variant.

Previous studies have shown that the probability of identifying a pathogenic FLNA variant in an individual with classic bilateral PVNH was 49%, leaving a high incidence of negative molecular genetic testing in individuals, particularly males. The absence of classical loss-of-function FLNA mutations in nearly half of patients with PVNH and the limited number of additional contributory genes suggest that novel mutational mechanisms impacting FLNA may account for many of the apparently FLNA-negative clinical testing results.

Deep sequencing of individuals with PVNH revealed several candidate mutations impacting conserved amino acids, intronic splice sites, and regulatory regions within FLNA. Most notably, a single base substitution was identified in intron 14, which is known to exhibit tissue-specific retention resulting in the premature termination of the protein. The T>C substitution was confirmed by custom in vitro assays to result in the loss of normal splicing, causing complete retention of the intron and premature protein truncation. These data suggest a novel mechanism where FLNA levels are partially regulated through the inclusion of introns, which in turn dysregulate the normal patterns of retention by intronic splicing mutations in individuals with PVNH, resulting in tissue-specific intronic retention, a reduction of protein and loss of FLNA. Together, these findings suggest that enhanced screening of FLNA through the inclusion of deep intronic and regulatory positions in individuals with typical and atypical PVNH could improve the diagnostic rate of clinical FLNA testing in individuals with PVNH.
Androgen receptor signaling regulates a feed-forward mechanism of androgen synthesis through transcriptional upregulation of HSD3B1

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Mentor: Nima Sharifi, MD, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University

Prostate cancer is the second leading cause of cancer related deaths in US men. Androgen deprivation therapy (ADT), by either surgical or pharmacological means, is a mainstay of treatment for metastatic disease; however, ADT invariably fails and results in the formation of castration-resistant prostate cancer (CRPC). CRPC is driven, to some extent, by the synthesis of intratumoral androgens and subsequent stimulation of androgen receptor (AR). 3β hydroxysteroid dehydrogenase/Δ5→Δ4 isomerase (3βHSD1) is the rate-limiting step of androgen biosynthesis, making it an ideal target for therapy in CRPC. Currently, little is known regarding the transcriptional regulation of HSD3B1, which is the coding gene for 3βHSD1. The aim of this study was to elucidate the transcriptional machinery responsible for regulation of HSD3B1, uncovering potential drug targets to prolong the life of patients with CRPC. Initial observations utilizing quantitative PCR in LNCaP and LAPC4 prostate cancer cell models demonstrated that transcription of HSD3B1 increased upon androgen stimulation for approximately seven to nine days. This is unique, as canonical androgen response is typically seen within 24–72 hours. Western blotting confirmed that increases in transcription correlated with increases in 3βHSD1 protein. Interestingly, this response also varies between cell models, with CWR22Rv1 and VCaP cell lines demonstrating induction as early as 24 hours and 72 hours, respectively.

LAPC4 cells treated with the synthetic androgen R1881 for 10 days were then treated with actinomycin D, an inhibitor of transcription. Actinomycin D treatment did not alter the half-life of HSD3B1 mRNA, which suggests that increases in mRNA seen with prolonged androgen exposure are the result of transcriptional upregulation rather than a change in mRNA stability. AR-stimulated increases in HSD3B1 transcript and 3BHSD1 protein were accompanied by an increase in enzyme activity, as assessed by metabolic flux from [3H]-DHEA to androstenedione and downstream steroids. Taken together, these data demonstrate a role for AR stimulation in HSD3B1 expression. To identify potential AR response elements in the HSD3B1 gene, we queried Cistrome Research Finder, a public database of ChIP-seq data. We limited the search to those experiments targeting AR in the presence of androgen stimulation, and returned multiple potential binding sites both upstream and downstream of the transcription start site that are currently being explored. Overall, the current data suggest a role for AR signaling in the regulation of HSD3B1 expression, and future studies will work to elucidate whether this is a direct or indirect action of AR and the cofactors required for upregulation of HSD3B1. This will reveal potential therapeutic targets to help extend life for patients with CRPC.

Elucidating the role of oxidized macrophage inhibitory factor (oxMIF) in glioblastoma

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

Glioblastoma (GBM), a WHO grade IV glioma, is a highly aggressive primary brain cancer, with an average life expectancy of under two years from time of diagnosis in part due to its characteristic invasiveness and resistance to therapies. Our lab has identified alterations in secretion of macrophage inhibitory factor (MIF)—a pleiotropic cytokine secreted by most cell types with known immunomodulatory and inflammatory properties—as one of the mechanisms of resistance of GBM to bevacizumab, an anti-VEGF monoclonal antibody (Oncogene, in press). MIF has been associated with GBM invasiveness and proliferation in cultured cells, and elevated MIF expression has been associated with worse clinical outcomes. Our studies have demonstrated that MIF drives a pro-inflammatory shift in the GBM microenvironment, and that inhibition of MIF signaling in GBM leads to a more invasive phenotype. Tumor cells are known to produce a build-up of reactive oxygen species (ROS) that may lead to increased production of oxidized MIF (oxMIF), an immunologically distinct redox-dependent conformational isoform of MIF. We believe this can explain the incongruent pro-tumoral vs. anti-tumoral effects reported in our study and others. We have found that inhibition of oxMIF via a monoclonal antibody specifically targeting oxMIF decreases GBM cell invasion in Matrigel invasion assays in cell culture. We are analyzing cell culture and in vivo models to fully define the effect of oxMIF inhibition on GBM proliferation, invasion, and modulation of the tumor microenvironment. We believe that elucidating further understanding of the different roles of MIF vs. oxMIF in GBM phenotype may open the doors to new therapeutic options.
Depression and other stress-related mood disorders are associated with cognitive dysfunctions, including deficits in reward processing and behavioral flexibility. The prefrontal cortex (PFC), which mediates higher-order cognitive abilities, is particularly vulnerable to stress-induced impairments in both structure and function. However, it is not well understood how chronic stress affects PFC activity at the level of individual neurons and local circuits, or how changes in PFC activity might correlate with behavioral deficits.

To characterize the effects of chronic stress on reward-directed behavior and PFC activity, we performed a longitudinal behavioral and imaging study in mice subjected to chronic social defeat stress (a well-established model of depressive behaviors in rodents). Adult male mice were trained on an operant sucrose preference task involving varying concentrations of sucrose and water rewards to assess adaptability to changing reward values; task performance was measured repeatedly before, during, and after 10 consecutive days of social defeat. In a subset of these mice, two-photon calcium imaging of pyramidal neuron activity in the M2 secondary motor region of medial PFC (mPFC) was recorded while animals were concurrently engaged in the sucrose preference task.

Chronic social defeat stress caused a significant decrease in sucrose preference, nearly abolishing preference for sucrose over water by the end of the stress period. Stressed mice also exhibited a significant increase in total acquisition of both sucrose and water rewards. Notably, chronic stress had a major effect on reward acquisition strategy; while control and pre-stress mice consistently demonstrated reward-directed behavior (in which actions are continually modified in response to changes in outcome value), mice subjected to chronic stress shifted toward a much more repetitive, reward-insensitive pattern of activity over the course of the stress period. Collection and analysis of calcium imaging data in conjunction with the above behavioral data are ongoing.

Our preliminary results suggest that chronic stress impairs reward-directed activity by shifting overall action strategy from a flexible, reward-sensitive approach to a more habitual, reward-insensitive pattern of behavior. Ongoing analysis of imaging data will provide further insight into the contribution of mPFC to reward-directed behaviors and the effect of chronic stress on mPFC neural dynamics.
ALK4 loss drives epithelial-mesenchymal transition to promote the malignant phenotype in pancreatic ductal adenocarcinoma

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Mentor: Gerard Blob, MD, PhD, Duke University School of Medicine

Activin receptor-like kinase 4 (ALK4) is a type I transforming growth factor-β (TGF-β) superfamily receptor that mediates signaling from several TGF-β superfamily ligands. ALK4 loss-of-function mutation in PDAC has been associated with an aggressive disease phenotype and poor survival. Here, we explore the mechanisms of ALK4 action in PDAC. Using shRNA-mediated silencing of ALK4 in PDAC cells (Panc-1), we demonstrated that decreased ALK4 expression induces epithelial-mesenchymal transition (EMT) and promotes migration. Specifically, ALK4-silenced cells had decreased expression of epithelial markers E-cadherin and β-catenin, with upregulation of mesenchymal markers N-cadherin and vimentin. In vivo, orthotopic implantation of luciferase expression shALK4-silenced HPNE cells resulted in significantly smaller primary tumor burden relative to shNTC HPNE implanted mice (0.5 g vs 1.5 g, P < 0.01). However, we observed a 3-fold increase in the incidence of mesenteric lymph node and liver metastasis in the shALK4 HPNE mice compared to shNTC. Histologic staining of harvested tissue revealed the upregulation of epithelial marker ZO-1 in shNTC HPNE tumors and higher mesenchymal marker vimentin expression in shALK4 HPNE tumors, suggesting that loss of ALK4 expression promoted EMT in vivo.

Mechanistically, ALK4-silenced Panc-1 cells demonstrated increased expression of integrins αvβ5 and α5β1, but no increase in receptor tyrosine kinase receptors, including EGFR, VEGF, or FGFR. PDAC-associated desmoplasia provides an extracellular matrix rich in integrin ligands that can activate integrin signaling via focal adhesion kinases and their recruitment of non-receptor tyrosine kinases such as c-Src. Integrins promote a variety of adhesion-dependent effects on tumor progression, including survival, proliferation, invasion, and chemotherapeutic resistance. Currently, CRISPR/Cas9 ALK4 silenced PDAC cell lines are being generated to elucidate the role of integrins as mediators of tumor progression in ALK4 mutant PDAC. Using these cell lines, we will further investigate the activation of MAP kinase and PI3/Akt pathways by fibronectin, an integrin αvβ5 and α5β1 ligand, and any effects it may have on resistance to chemotherapy with gemcitabine.

These data suggest that ALK4 silencing facilitates the development of highly metastatic tumors, albeit with small tumor burden. Further studies directed toward the observed upregulation of integrin receptors will provide deeper insight into the role of ALK4 in PDAC tumor progression.

AP1 is required for Notch1 activation by histone deacetylase inhibitors in neuroendocrine cancers

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Mentor: Herbert Chen, MD, University of Alabama School of Medicine

Notch signaling is minimally active in neuroendocrine (NE) cancer cells, and the induction of Notch isoforms at the transcriptional level alters the malignant NE phenotype, suppresses cancer cell proliferation, and leads to apoptosis. While histone deacetylase inhibitors (HDACi) induce Notch1 and suppress NE cancer growth, the molecular mechanism underlying this interplay has not been determined.

NE cancer cell lines BON, H727, and MZ-CRC-1 were treated with known HDACi: Thalidomide (TDP-A) and valproic acid (VPA), and Notch1 mRNA expression was measured with RT-PCR. Truncated genomic fragments of the Notch1 promoter region fused with a luciferase reporter were used to identify the HDACi-associated transcription factor (TF) binding sites. The key regulatory TF was identified with the electrophoretic mobility shift assay (EMSA). The effect of HDACi on Notch1 level was determined before and after silencing the potential TF by siRNA transfection. In addition, the plasmid of the potential TF was transiently transfected to evaluate its effect on Notch1 induction.

TDP-A and VPA induced Notch1 mRNA in a dose-dependent manner in NE cancer cell lines in nanomolar and millimolar concentrations, respectively. A functional DNA motif at -75 to -60 from the Notch1 start codon for the HDACi-dependent Notch1 induction was identified. The mutation of this core sequence containing the potential TFBSs failed to induce luciferase activity despite HDACi treatment. EMSA showed the greatest gel shift with AP1 in nuclear extracts of NE cancer cells after HDACi treatment. siRNA transfection achieved a greater than 4-fold decrease in AP1 transcript level, which significantly attenuated the effect of TDP-A and VPA on Notch1 induction. Interestingly, AP1 transfection did not alter Notch1 levels, which suggest that the presence of AP1 is necessary but insufficient for HDACi activation of Notch1.

We identified that AP1 is the transcription factor that binds to a specific transcription-binding site within the Notch1 promoter region that triggers Notch1 transcription. Elucidating the HDACi activation mechanism may lead to the development of novel therapeutic options for patients with NE cancers and facilitate the identification of clinical responders and prevent adverse effects.
Modulation of TGFβ in cardiac neural crest-derived cells titrates aneurysm severity in Marfan syndrome mice

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Mentor: Harry Dietz, MD, Howard Hughes Medical Institute and Johns Hopkins University School of Medicine

Aneurysm of the proximal aorta is the leading cause of mortality in Marfan syndrome (MFS), a disorder caused by mutations in the gene (FBN1) encoding the extracellular matrix protein fibrillin-1. Fibrillin-1 interacts with latent TGFβ, and tissues (including the aortic wall) from MFS patients and mice show an unambiguous signature for elevated TGFβ activity. Furthermore, antagonism of TGFβ attenuates aneurysm severity in MFS mice. Shprintzen-Goldberg Syndrome (SGS) and Loeys-Dietz Syndrome (LDS) are closely related disorders that show substantial phenotypic overlap with MFS, including aortic root aneurysm. SGS is caused by loss-of-function (LOF) mutations in SKI, encoding a prototypical TGFβ repressor that recruits negative and displaces positive regulators of transcription to TGFβ target genes. Paradoxically, LDS is caused by LOF mutations in FBN1, encoding the extracellular matrix protein fibrillin-1. Fibrillin interacts with latent TGFβ, and tissues (including the aortic wall) from LDS patients and mice show an unambiguous signature for elevated TGFβ activity. We hypothesize that excessive TGFβ signaling in CNC-derived cells will drive TGFβ activity in opposite directions, aortic tissue from both disorders shows the same unambiguous signature for high TGFβ signaling as seen in MFS. These findings have generated ambiguity about the precise role of TGFβ in aneurysm progression. We propose a reconciling model drawing upon the observation that aneurysms in all three disorders arise at sites where vascular smooth muscle cells (VSMCs) of differing embryonic origin overlap. We hypothesize that neighboring VSMC lineages respond differentially to a deficiency of TGFβ signaling, and loss of signaling in one lineage causes compensatory secretion of TGFβ ligand that overstimulates its less vulnerable neighbor.

In agreement with this model, we observed that LDS second heart field (SHF)-derived VSMCs in the aortic root show diminished TGFβ signaling and amplified levels of TGFβ expression, while adjacent cardiac neural crest (CNC)-derived VSMCs remain signaling competent. We engineered MFS mice to overexpress a transgenic SKI allele in specific lineages of VSMC during particular time periods, permitting fine control over suppression of TGFβ target gene activity. We also developed a conditional SKI knockout mouse, permitting a targeted increase of TGFβ target gene expression in specific cells. In preliminary studies, MFS mice that overexpress SKI in CNC-derived cells (predicted to decrease TGFβ activity) display decreased aneurysm growth compared to MFS littermates. Additionally, MFS mice that are SKI-null in CNC-derived cells (predicted to increase TGFβ activity) display greater aneurysm growth than MFS mice with functional SKI alleles. As an independent and parallel test of hypothesis, we are developing a transgenic mouse allele that expresses a constitutively active form of TGFβRI in a spatially and temporally controllable manner. We hypothesize that excessive TGFβ signaling in CNC-derived cells will prove sufficient to cause aneurysm, and that supplementary TGFβ signaling in SHF-derived cells can protect against aneurysm in TGFβ vascularopathies.
Our findings also provide evidence that some cases of MRKH may represent a blended phenotype resulting from pathogenic variation at more than one locus. In particular, we have identified probands with rare variants at two loci: one associated with a skeletal phenotype, and one associated with urogenital development. For example, one patient harbors a heterozygous variant in PLEKHM1 (p.E942*), implicated in autosomal recessive osteopetrosis (OMIM #611497), and a heterozygous predicted damaging variant in TRX18 (p.F554S), implicated in autosomal dominant congenital anomalies of the kidneys and urinary tract (OMIM #143400). We suggest that each of these variants contributes to development of the blended phenotype, which includes anomalies of urogenital development and thoracocervical osseous malformations.

In summary, we present WES analysis of a MRKH cohort that identifies likely pathogenic variants in genes known or suspected to play key roles in genitourinary development based on known developmental pathways and protein-protein interactions, as well as genes for which limited functional data are available. We additionally provide evidence that, in some individuals, MRKH may result from a blending of two distinct phenotypes in a single individual.

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The effects of cell wall deficiency (CWD) forms of Mycobacterium tuberculosis on energy metabolism

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Mentor: Adrie Steyn, PhD, Africa Health Research Institute

*Mycobacterium tuberculosis* (Mtb) is now ranked alongside HIV as the leading cause of death among infectious diseases. *Mtb* is an obligate aerobe that requires the use of flexible energy metabolism pathways to grow and survive. Recently bedaquiline, clofazimine, and Q203 have been used to study energy metabolism in active whole cells of *Mtb*. However, another form of *Mtb* exists with minimal metabolic activity, referred to as cell wall-deficient (CWD) *Mtb*, which was first described in 1883 in infected tissues and granulomas. It has been postulated that CWD *Mtb* can persist in a dormant state for many years inside the host cell, even though it is resistant to frontline anti-TB drugs. Ultimately, when the host immune system is suppressed, CWD *Mtb* reactivates and converts back to pathogenic *Mtb*. We believe that examining CWD *Mtb* susceptibility to energy metabolism-targeting compounds is imperative to our understanding of *Mtb* physiology, and complete sterilization of *Mtb* infection.

To study the role of the cell wall in oxidative phosphorylation and drug susceptibility, we generated CWD mycobacteria (spheroplasts) in vitro using glycine and lysozyme-based approaches. Spheroplasts were isolated from whole cells of *Mtb* using flow cytometry. We microscopically confirmed spheroplast generation with scanning and transmission electron microscopy.

We generated the bioenergetic profiles of spheroplasts using extracellular flux technology (Agilent Seahorse XF96), and measured energy metabolism in the presence of different energy metabolism-targeting compounds and different carbon sources. Using 13C stable isotopes, we did carbon tracing of the metabolites in energy pathways such as glycolysis, tricarboxylic acid cycle, and the pentose phosphate pathway.

Our XF96 results indicated that CWD forms of *Mtb* show reduced bioenergetic capacity, as is evident by a reduced oxygen consumption rate (OCR). Furthermore, we were able to demonstrate that bedaquiline, which targets ATP synthase, had no effect on cellular respiration of CWD *Mtb*. However, exposure of CWD *Mtb* to the uncoupler CCCP rapidly increased OCR, demonstrating a functional electron transport chain. We conclude that understanding *Mtb*’s ability to regulate bioenergetic pathways in CWD forms and their ability to laterally persist in host cells may lead to novel therapeutic interventions that target these processes. By applying cutting-edge bioenergetic technologies to study CWD *Mtb*, we hope to make innovative contributions toward better understanding *Mtb* physiology and pathogenicity.

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Dynamics of escape after a single infusion of a broadly neutralizing anti-HIV-1 antibody in human subjects

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Mentor: Michel Nussenzweig, MD, PhD, Howard Hughes Medical Institute and Rockefeller University

Sequencing the HIV-1 envelope gene is challenging due to the large diversity of the virus, even within a single infected person. The high turnover and mutation rates result in “quasispecies.” Recently, monoclonal antibodies against HIV-1 have been isolated that are able to neutralize a large diversity of HIV-1 viruses. These broadly neutralizing antibodies (bNabs) offer a potential treatment for HIV-infected individuals. Analysis of the existing and emerging quasispecies within subjects treated with bNabs against HIV-1 is important for understanding how the antibody affects the host viral population. In a phase I clinical trial, a single dose of monoclonal antibody 10-1074 was infused in 19 HIV-1-infected individuals. Single genome amplification (SGA) was used to sequence HIV-1 env genes...
isolated from plasma on the day of infusion and several weeks after infusion. Sequence analysis revealed single point mutations most commonly at the N332/S334 potentially glycosylated site at the base of the V3 loop, where 10-1074 is known to bind. At week 4 after infusion, the majority of amplified sequences were mutated, with phylogenetic analysis revealing a polyphyletic emergence of resistance. Pseudovirus generation and testing through TZM.bl assay confirmed that these mutations conferred resistance to 10-1074. The same pseudoviruses were also tested against the CD4-binding-site antibodies VRC01 and 3BNC117 and found to be sensitive. At week 20 after infusion, by which point the serum concentration of 10-1074 was well below neutralization level, the unmutated wild-type virus reemerged as the predominant quasispecies, suggesting a potential fitness cost of the mutated virus. Deeper sequencing through a Primer ID-based approach did not reveal preexisting resistant minority variants. Together these data show that HIV-1 resistance emerges rapidly to bNAb 10-1074 through single point mutations; however, these mutations may have a fitness cost given the reemergence of the wild-type virus following clearance of the bNAb. HIV-1 treatment with bNAbs will require a combination of antibodies targeting different epitopes.

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ATR kinase inhibition attenuates expression of PD-L1 on murine cancer cells post-ionizing radiation treatment

Pooja Karukonda, HHMI Medical Fellow, University of Pittsburgh School of Medicine

Mentor: Christopher Bakkenist, PhD, University of Pittsburgh School of Medicine

Ataxia telangiectasia and Rad3-related (ATR) is a DNA damage-signaling kinase, inhibition of which leads to profound radio sensitization of cancer cells, reportedly via cell cycle checkpoint blockade. We propose a novel mechanism for ATR inhibitor (ATRi)-induced radio sensitization of cancer cells: blockade of immune checkpoint signaling via decreased cell surface expression of programmed death-ligand 1 (PD-L1), an inducer of T cell exhaustion.

Using flow cytometry, we show an increase in PD-L1 surface protein expression on two immunogenic murine colon cancer cell lines 24 hours after treatment with 6 Gy of ionizing radiation (IR), a sub-lethal dose. Importantly, we show significant attenuation of this increase with addition of ATRi. PD-L1 expression has been shown to increase on apoptotic cells; thus, we also evaluated expression after 2 Gy, a non-lethal dose, which resulted in similar findings. Additionally, we show with annexin-V staining that cell death is not significantly different between IR and IR + ATRi treatment arms after 24 hours. Taken together, these data suggest that signaling mechanisms outside of apoptotic pathways are relevant in our model of ATR-dependent PD-L1 expression.

We proceed to show, via the creation of p53-knockout (KO) cell lines, that the ATR-dependent increase in PD-L1 expression is not dependent on p53, a key downstream effector of ATR kinase. Using real-time polymerase chain reaction (RT-PCR), we have identified interferon regulatory factor-1 (IRF-1), an immune-modulatory transcription factor, as a potential downstream target, as its expression increases after IR, and is subsequently inhibited with ATRi after 2 Gy. We show, however, that ATRi leads to a substantial increase in PD-L1 mRNA levels after 6 Gy, which leads us to believe that there are separate mechanisms in play after different doses of IR, and that after 6 Gy, PD-L1 expression may not be regulated at the transcriptional level.

Future experiments aim to flesh out the mechanistic details behind the immunoregulatory effects of ATRi, a potentially powerful cancer therapeutic when used in combination with IR. We will additionally perform functional studies, including co-culture of cancer cells with T cells to investigate the overall effects of ATRi on T cell exhaustion, as well as in vivo mouse experiments to look at the direct effects of ATRi on tumor burden and tumor-infiltrating lymphocytes.

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Establishing a multichannel dorsal root ganglion neural interface for restoring sensation and reducing phantom limb pain after amputation

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Mentors: Lee Fisher, PhD and Elizabeth Tyler-Kabara, MD, PhD, University of Pittsburgh School of Medicine

Recent evidence suggests that electrical stimulation of peripheral nerves can evoke focal sensations that emanate from the missing limb in amputees. Further evidence suggests that restoring physiologic sensations is associated with long-term mitigation of phantom limb pain (PLP). This study aims to characterize the normal patterns of activity in primary afferents (PAs), and to use those patterns in future studies to guide the programming of stimulation parameters for a neuroprosthesis to restore naturalistic sensation and reduce PLP in people with amputation.
We initially characterized PA responses of 125 recorded neurons to standing bidirectional (anterior vs. posterior) platform perturbation tasks using penetrating microelectrode arrays (MEAs) implanted in the lumbar dorsal root ganglia (L6-L7) of 5 cats. We observed a subset of PAs that resembled primary muscle spindles, as evidenced by units that rapidly increased their firing rates in response to unique perturbation directions that were also strongly correlated with joint angular velocity. As assessed using a linear regression model, the firing rate of most PAs was weakly correlated with limb kinematics and ground reaction forces. However, as multiple predictors were combined in the regression model, the ability to predict firing rate was significantly improved, suggesting that PAs encode multiple limb-state variables simultaneously.

In preliminary clinical trials, we assessed the ability to evoke focal sensations and mitigate PLP using electrical stimulation of the cervical roots (C6-C8). Via a percutaneous approach, the dorsal roots and rootlets were targeted with 3 spinal cord stimulator leads using a lateral percutaneous approach in 2 subjects with transhumeral amputations. Subjects reported various evoked sensations, such as paresthesias, pressure, and movement immediately in response to stimulation. We demonstrated that non-naturalistic sensations were associated with an acute increase in PLP intensity, as measured via a visual analog scale, which was further modulated by the stimulus amplitude. Subjects characterized sub-chronic PLP utilizing the McGill Pain Questionnaire (78-point scale) and Trinity Amputation and Prosthesis Experience Scales at baseline, weekly, and 1 month following explantation. Both subjects experienced a clinically significant reduction (>5 points) in PLP from baseline at 1-month follow-up, although the frequency of episodes was not affected. This study suggests that restoring sensory feedback from the amputated limb may be associated with increased PLP in the immediate post-stimulation phase with long-term coupling to PLP reduction. Subsequent experimentation will evaluate the effect of naturalistic stimulation paradigms on sensory percepts and PLP mitigation.

**A translational rodent model of hepatocellular carcinoma for in vivo studies of cancer dormancy and metabolism**

**Ryan Kiefer, HHMI-SIRF Medical Fellow, Perelman School of Medicine at the University of Pennsylvania**

**Mentor:** Terence Gade, MD, PhD, Perelman School of Medicine at the University of Pennsylvania

Despite evidence that novel targeted therapies have led to improved tumor response rates for hepatocellular carcinoma (HCC), the rate of cure remains low owing to recurrent disease, which can result from dormant malignant cells that persist without detection by conventional imaging methods. How dormant cancer cells survive in a growth-restricted environment is poorly understood but may offer insight into improved methods of detection and treatment. HCC cell survival following transarterial embolization (TAE) provides an ideal method through which to study tumor dormancy, as sustained ischemia has been shown to activate the metabolic stress response and induce quiescence. Using an oral diethylnitrosamine (DEN)-induced HCC model in male Wistar rats, we analyzed characteristics to optimize the autochthonous cancer model and described a novel endovascular approach that mimics clinical treatment that is known to induce dormancy. We found that rats with greater body weight (>400 g) at the start of the DEN diet had significantly longer survival (mean 108 vs. 88 days; \( P < 0.0001 \)) without a significant increase in tumor latency (\( P = 0.310 \)). Transarterial embolization via the right common femoral artery was described and found to be successful for the induction of ischemia. Embolized tumors were fluorescently stained with bromodeoxyuridine (BrdU), pimonidazole, and DAPI to confirm cellular hypoxia and the arrest of proliferation as evidence of dormancy. With this in vivo model of cancer dormancy, quantitative metabolic flux analysis will be performed using mass spectrometry and dynamic nuclear polarization-carbon-13-nuclear magnetic resonance spectroscopy. The detection of altered metabolism may uncover new imaging probes for the improved detection of dormant cancer as well as novel targeted therapies.
Determining neuronal elements of IOP regulation using mice

Alexander Kokini, HHMI Medical Fellow, Indiana University School of Medicine (Jackson Laboratory)

**Mentor:** Simon John, PhD, Howard Hughes Medical Institute and Jackson Laboratory

The nervous system is important in controlling intraocular pressure (IOP); however, the precise mechanisms of neural control need further evaluation. The mouse enables functional experiments, and so we are characterizing limbal innervation in mice. To understand neuronal functions regulating IOP, we are using modern molecular techniques and fluorescent reporter mice to map limbal neurons and determine pressure-dependent neuronal activity. We used a whole-mount procedure of the anterior mouse eye to study the innervation of the entire limbus in 3D. The Prox1-GFP mouse strain was used to label Schlemm’s canal (SC). The Thy1-YFP strain was used to identify sensory neurons. Antibodies to detect sympathetic neurons (tyrosine hydroxylase), parasympathetic neurons (vesicular acetylcholine transporter, choline transporter), nitricergic neurons (neuronal nitric oxide synthase), and axons (neurofilament) have been used. 3D limbal images generated using confocal microscopy and Imaris were used to map and identify the number and type of neurons and nerve termini in SC and trabecular meshwork (TM). Neuronal activity in response to pressure elevation was assessed using fluorescent neuronal activation sensors (GCaMP). To do this, eyes were cannulated and held at pressures of 13 and 33 mmHg for 30 minutes, followed by fixation, immunolabeling, and confocal microscopy. We have identified sympathetic nerve terminals and possibly non-nociceptive sensory terminals terminating in SC and TM. The TH-labeled termini have varicosities and the YFP-labeled termini appear as bouton-like structures. Using GCaMP calcium sensor mice, we have identified neurons that are activated in the limbus in response to elevated pressure. These neurons are present in the SC and associated TM region. We are constructing a detailed 3-dimensional (3D) map of the neuronal innervation of the AQH drainage structures to facilitate understanding of the neural control of IOP. Both SC and TM are highly innervated with activation of a subset of neurons in response to experimental elevation of IOP.

Altered glymphatic 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, DMS, 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. Recently, however, a broader model has emerged from the discovery that 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 the impairment of the astrocytic aquaporin 4-dependent paravascular clearance pathways of the glymphatic system. We theorized that impaired glymphatic 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 track the flow patterns of tracers infused into the cerebrospinal fluid of mice that received either the spared nerve injury (SNI) of peripheral neuropathic pain or sham injury. The rate of clearance 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]), and microgliosis (CD68), were then quantified by multi-channel fluorescence imaging of ex vivo brain and spinal cord slices. In the SNI model, we found that neuropathic pain behavior, including thermal hyperalgesia and mechanical allodynia, peaked at 3 days following SNI. We found that glymphatic clearance of tracers was significantly impaired in mice that received SNI, but not sham-injured mice. Glymphatic impairment was 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 glymphatic function in SNI vs. sham-injured mice in vivo, using serial contrast-enhanced computed tomography. Our results suggest that suppression of glymphatic function may contribute to the onset and maintenance of chronic neuropathic pain.
Effects of polymorphic \textit{Alu} repetitive elements on the human transcriptome and disease risk: \textit{Alu}-mediated disruption and introduction of mRNA splice sites and functional consequences

\textbf{Maria Kryatova}, HHMI Medical Fellow, Johns Hopkins University School of Medicine

Mentor: Kathleen Burns, MD, PhD, Johns Hopkins University School of Medicine

Polymorphic \textit{Alu} repetitive elements are a common source of structural variation among humans, accounting for \( \sim 17\% \) of structural variants in the human genome. While \textit{Alu} insertions have been previously implicated in causing rare monogenic diseases, it remains unclear if common \textit{Alu} polymorphisms affect inherited risk for common diseases. We hypothesize that some \textit{Alu} variants alter disease risk by modifying mRNA transcript structure, and focus on two mechanisms by which \textit{Alu} elements affect mRNA splicing. \textit{Alu} elements may either disrupt existing splice sites and cause exon skipping or introduce new splice sites leading to exonization of \textit{Alu} sequence.

We have curated a list of 115 intronic polymorphic \textit{Alu} elements within 100 base pairs of alternatively used exons and are performing minigene splicing assays with subsequent RT-PCR analysis to identify \textit{Alu} variants that cause exon skipping. To focus on \textit{Alu} candidates most likely to affect disease risk, we prioritize polymorphic \textit{Alu} elements in linkage disequilibrium with genome-wide association study (GWAS) signals. Of nine \textit{Alu} variants assayed in the minigene splicing assay thus far, four cause skipping of an adjacent exon (25–97 base pairs away). In one case the exon is always skipped in the presence of the \textit{Alu} element, and in three cases the amount of the shorter elements remain to be evaluated at the endogenous locus using CRISPR/Cas9 genome editing.

Detection of non-polymorphic reference genome \textit{Alu} elements. \textit{Alu} exonization events detected by the pipeline will be validated by RT-PCR. As with exon skipping events described above, \textit{Alu} variants of greatest clinical interest will be prioritized for further investigation using genome editing.

Are there sex-specific differences in arteriovenous fistula maturation?

\textbf{Tambudzai Kudze}, HHMI Medical Fellow, Yale School of Medicine

Mentor: Alan Dardik, MD, PhD, Yale School of Medicine

Hemodialysis is the most common method of renal replacement in patients with end-stage renal disease. The arteriovenous fistula (AVF) is the preferred method of dialysis access but must adapt, e.g., dilate and thicken, to the higher pressure and flow arterial environment. Although women frequently require dialysis, women have lower rates of AVF maturation compared to men (38% vs. 60%), preventing optimal AVF use.

Using a novel mouse model that recapitulates human AVF maturation, we hypothesize that there is a difference in AVF patency and maturation between male and female mice.

A 25-gauge needle was used to create fistulae between the abdominal inferior vena cava (IVC) and aorta in male and female C57BL/6 mice (9–10 wk). Doppler ultrasound was used to monitor aortic and IVC diameters and flow velocity at days 0, 3, 7, 14, and 21; shear stress was calculated. AVFs were examined at day 21, and computer morphometry was used to measure AVF wall thickness.

Male mice weighed more than female mice preoperatively and at day 21 (25.4 ± 0.5 g vs. 18.3 ± 0.3 g; \( P < 0.05; n = 15 \)); male mice also had larger suprarenal aortic diameter (1.23 ± 0.05 mm vs 1.06 ± 0.02 mm; \( P = 0.002 \)) but smaller suprarenal IVC diameter (1.53 ± 0.06 mm vs 1.73 ± 0.06 mm; \( P = 0.02 \)) at baseline. After AVF creation, there was increased suprarenal IVC dilation in male mice (53 ± 13% vs 25 ± 5%, \( n = 5-8 \); \( P = 0.04 \)) at day 7; at day 21, there was a trend toward increased IVC dilation in male mice (infrarenal, 86 ± 23% vs. 63 ± 10%, \( P = 0.22 \); suprarenal, 61 ± 17% vs. 42 ± 6%, \( P = 0.23 \)). Velocity in the suprarenal IVC was also increased in male mice, both at baseline (40 ± 6 mm/s vs. 25 ± 2 mm/s; \( P = 0.012 \); \( n = 15 \)) and at day 7 (79 ± 16 mm/s vs. 44 ± 6 mm/s; \( P = 0.04 \); \( n = 5-8 \)). Similarly, shear stress was increased in male mice at baseline (15 ± 2 vs. 8 ± 1 dynes/cm²; \( P = 0.002 \); \( n = 11-15 \)) and day 7 (30 ± 8 vs. 18 ± 5 dynes/cm²; \( P = 0.12 \); \( n = 5-8 \)). At day 21, AVF neointimal thickness was similar ( \( P = 0.9 \)).

In a mouse AVF model that recapitulates human AVF maturation, preliminary data suggests lower magnitudes of laminar shear stress and increased neointimal thickening in female mice. These findings may suggest...
Unique growth characteristics of E. coli via the NtrB/NtrC two-component system in vitro vs. in vivo—a signal transduction pathway associated with dysbiosis in IBD

Chiraag Kulkarni, HHMI Medical Fellow, Northwestern University, The Feinberg School of Medicine (Perelman School of Medicine at the University of Pennsylvania)

Mentor: Gary Wu, MD, Perelman School of Medicine at the University of Pennsylvania

Crohn’s disease is associated with bacterial dysbiosis consisting of reduced bacterial diversity and outgrowth of Proteobacteria, notably Enterobacteriaceae. However, a definitive mechanism for the development of dysbiosis is not known. An analysis of the gut microbiome in pediatric Crohn’s disease revealed a genomic signature for nitrogen metabolism associated with dysbiosis. Specifically, a bacterial two-component system responsible for sensing and responding to environmental nitrogen sources, NtrB/NtrC, was associated with dysbiosis, an increase in fecal amino acids, and the abundance of E. coli. Herein, we examined the effect of ntrC on growth characteristics of E. coli under different environmental conditions in vitro as well as its effects on intestinal colonization in vivo.

ΔntrC and wild-type MP1, a murine commensal strain of E. coli, were cultured under aerobic and anaerobic conditions using lysogeny broth (LB) and M9 minimal media. C57BL/6 mice were pretreated with antibiotics and polyethylene glycol (PEG) per established protocols and subsequently gavaged with a mixture of ΔntrC and wild-type MP1 that have tetacycline-induced fluorescent protein tags. Stool serial dilutions were performed and colony counts obtained weekly for calculation of competitive indices.

When cultured aerobically, ΔntrC MP1 exhibited both a growth delay and reduction in growth yield that were more pronounced in LB medium. Anaerobically, ΔntrC showed a growth delay but reached a similar OD in stationary phase compared to wild-type MP1 in both M9 and LB media. Complementation of the ntrC deletion in trans restored wild-type growth patterns. Additionally, ΔntrC MP1 showed a modest cell aggregation phenotype during late log and early stationary phase growth, specifically under aerobic conditions. Surprisingly, despite a clear growth defect under all conditions measured in vitro, oral co-inoculation of ΔntrC MP1 with wild-type MP1 in mice resulted in higher engraftment of ΔntrC MP1 compared to wild-type MP1 (~10^9 colony forming units [cfu] versus 10^7 cfu) in the gastrointestinal tract. This 2-log competitive advantage for ΔntrC MP1 over wild-type MP1 was sustained for a period of at least 1 month (P < 0.01).

Despite the clear growth disadvantage of ΔntrC MP1 under all culture conditions examined in vitro, ΔntrC MP1 led to an engraftment advantage over wild-type MP1 in vivo, demonstrating that the murine gut provides a unique environment for E. coli favoring the absence of this nitrogen signaling pathway. This ntrC-dependent response in vivo is unique among the 33 two-component signaling systems in the MP1 E. coli genome. One possible explanation under current investigation is that the cell aggregation phenotype of ΔntrC MP1 observed in vitro provides a colonization advantage in vivo. This observation may have particular relevance to IBD, where E. coli is an important component of the dysbiotic microbiota.

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Unique growth characteristics of E. coli via the NtrB/NtrC two-component system in vitro vs. in vivo—a signal transduction pathway associated with dysbiosis in IBD

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Directing B and T cell differentiation from induced pluripotent stem cell-derived hemogenic endothelium

Jerry Lee, HHMI Medical Fellow, Duke University School of Medicine (Harvard Medical School)

Mentor: George Daley, MD, PhD, Harvard Medical School and Boston Children’s Hospital

Autologous cell therapy for the treatment of hematological malignancies and primary immunodeficiencies has been limited by the inability to yield functional human hematopoietic stem cells (HSCs) from induced pluripotent stem cells (iPSCs) in vitro. Although previous approaches have produced immortalized cells with HSC-like morphology and cell surface markers, they have failed to demonstrate murine engraftment in secondary recipients—the gold standard for demonstrating HSC self-renewal and differentiation. Our lab has recently uncovered five transcription factors (TFs) necessary for differentiating iPSCs into hemogenic endothelial cells (HEs), the fetal precursors to HSCs, and confirmed hematopoiesis with multi-lineage secondary engraftment. Building on this discovery and focusing on therapeutic applications, we now set out to characterize and enhance the capacity of HEs to produce more terminally differentiated lymphocytes, for the ultimate aim of B and T cell replacement in immunocompromised hosts.

Following embryoid body formation, human iPSCs derived from bone marrow mesenchymal stem cells were transduced with polycistronic lentiviral vectors (RUNX1-ERG, LCOR-HOXA9-HOXA10) on day 3 of
endothelial-hematopoietic transition (EHT) induction. A candidate screen of 17 cytokines, epigenetic modifiers, and small molecules aimed at inducing HE proliferation was conducted during EHT induction in a RUNX1c gene reporter assay. Flow cytometry of HEs cocultured with MS5 or OP9-DL1 stromal cells was performed weekly for 6 weeks after initiating lymphoid differentiation.

In the candidate screen, IFN-γ enhanced RUNX1c reporter 2.4-fold during EHT, which was confirmed by flow cytometry showing increased hematopoietic progenitor cells (CD34+CD45+). Several compounds were suppressive to EHT, including IFN-α, which led to loss-of-function EHT enhancement when co-introduced with IFN-γ. After 1 week of lymphoid directed differentiation, the cell population was characterized by the predominance of myeloid progenitors (CD38+CD45+CD45RA−), with roughly 5% of progenitors characterized as common lymphoid progenitors (CD10+CD34+CD45RA−). At 3 weeks, CD19+IgM+ B cell progenitors as well as mature IgM+ B cells emerged, which were comparable to human cord blood (CB) controls; CD3+CD4+CDB8+ T cells were also identified at this time.

We determined that transient exposure to IFN-γ greatly improved the EHT of HEs, which may bolster the typically low engraftment efficiency of iPSC-derived HSCs. In our lymphoid differentiation assay, HEs were comparable to CB in the formation of mature and immature B/T cells. This finding suggests that our approach to creating engraftable HSCs holds significant potential for modeling hematopoietic disease, both in animal models and as a "disease-on-a-dish," and for developing therapeutic strategies in genetic blood disorders.

Reducing T cell-mediated cardiac injury through TLR9 signaling with CpG oligodeoxynucleotides

Erik Levinsohn, HHMI Medical Fellow, Yale School of Medicine (Harvard Medical School)

Mentor: Andrew Lichtman, MD, PhD, Harvard Medical School and Brigham and Women’s Hospital

T cell-mediated damage in the heart may occur following various stimuli, including viral infection, autoimmune provocation, and immunostimulatory cancer therapy. The PD-1/PD-L1 pathway prevents or limits T cell-mediated damage to the heart, as shown by mouse models in the Lichtman laboratory, and recently by the increased risk of myocarditis in cancer patients treated with anti-PD-1 antibodies. CpG oligodeoxynucleotides (ODN) are known to induce the expression of type I interferons (IFN) by acting as a ligand for endosomal Toll-like receptor 9 (TLR 9), and type I IFN have been shown to induce the immune checkpoint marker programmed death-ligand 1 (PD-L1) in the heart. However, the therapeutic potential of CpG ODN in T cell-mediated cardiac disease has not been established. We evaluated the immunomodulatory properties of CpG ODN in a variety of murine models of myocarditis. Furthermore, as there is little data regarding the expression of PD-L1 in the human heart, we investigated PD-L1 expression in both diseased and healthy human tissue.

Two models of autoimmunity were used to induce myocarditis in mice. CD4+ T cell-dependent disease was induced with two injections of of myosin-heavy chain (MyHC) peptide with complete Freund’s adjuvant into BALB/c mice. CD8+ cytotoxic T lymphocyte (CTL)-mediated disease was induced by adoptively transferring T cell receptor (TCR) transgenic CTL (OT-I) cells specific for ovalbumin peptide into mice expressing ovalbumin only in the heart under the MyHC promoter (cMy-mOva mice). The expression of PD-L1 as influenced by CpG ODN was evaluated by immunohistochemistry and qRT-PCR in BALB/c mice treated with one intraperitoneal injection of 50 μg CpG ODN or PBS 24 hours prior to sacrifice. Histological sections of normal and myocarditic human hearts were analyzed for expression of PD-L1 and class II MHC, a marker of interferon activity, by immunohistochemistry.

Relative to PBS-injected mice, CpG ODN-injected mice demonstrated a 3.2-fold increase in PD-L1 mRNA expression in myocardial tissue. Immunohistochemistry performed on CpG ODN-treated mice demonstrated increased PD-L1 protein expression, in an endothelial pattern, compared to PBS-treated mice. We have treated cMy-mOva mice with CpG ODN during OT-I-mediated myocarditis to assess the effects of induced myocardial PD-L1 on disease. Immunohistochemistry of the myocarditic human heart demonstrates focal expression of PD-L1, on both myocytes and endothelial cells, and increased endothelial class II MHC, accompanying T cell infiltration. CpG ODN markedly induces expression of PD-L1 in murine myocardium, and PD-L1 upregulation in human hearts occurs in the setting of T cell infiltration, as it does in mice. Therapeutic induction of myocardial PD-L1 may represent a therapeutic option for preventing or treating T cell-dependent cardiac disease. Further experimentation to assess the utility of such treatment in preventing disease progression is in progress for various murine models.
Virtual histopathology and immunohistochemistry with stimulated Raman scattering microscopy

Spencer Lewis, HHMI Medical Fellow, University of Michigan Medical School

Mentor: Daniel Orringer, MD, University of Michigan Medical School

Differentiating tumor-infiltrated tissue from surrounding normal brain during surgery is extremely challenging. Because the surgeon cannot visualize the tumor margin, peripheral tumor infiltration cannot be accurately assessed, leading to suboptimal resection. This difficulty contributes to high postoperative morbidity and mortality, as well as decreasing median time to local tumor recurrence. Improving intraoperative brain tumor detection is an important step toward improved outcomes in brain tumor surgery.

Stimulated Raman scattering (SRS) microscopy is a label-free optical technique that uses the intrinsic chemical composition of fresh tissue to generate image contrast. We have previously demonstrated exceptional structural correlation between SRS and conventional H&E microscopy, allowing SRS images to yield reliable diagnoses of human glioma tissue. Quantitative SRS has facilitated automatic tumor detection, leveraging the distributions of axons and nuclei to segment tumor infiltration with extremely high accuracy. Until now, however, it has not been possible to execute SRS in a clinical environment. Further, despite the potential for chemically specific SRS imaging, only structural features have been used to make diagnoses with SRS.

Here, we report the first execution of SRS microscopy in a clinical setting, using a portable, fiber-laser-based SRS microscope to image unprocessed specimens from 93 neurosurgical patients. SRS had near-perfect (k > 0.89) concordance with standard methods in a simulation on intraoperative pathologic consultation. We also present a multi-layer perceptron-based on image attribute quantification capable of predicting tumor diagnosis with 86.6% accuracy.

We further investigated how we could leverage the chemical specificity of SRS to image mutational status at the cellular level. Isocitrate dehydrogenase 1 (IDH-1) mutations are the most common mutations in low grade gliomas. These mutations result in a predictable set of metabolomic alterations, allowing SRS images to yield reliable diagnoses of human glioma tissue. Quantitative SRS has facilitated automatic tumor detection, leveraging the distributions of axons and nuclei to segment tumor infiltration with extremely high accuracy. Until now, however, it has not been possible to execute SRS in a clinical environment. Further, despite the potential for chemically specific SRS imaging, only structural features have been used to make diagnoses with SRS.

Here, we report the first execution of SRS microscopy in a clinical setting, using a portable, fiber-laser-based SRS microscope to image unprocessed specimens from 93 neurosurgical patients. SRS had near-perfect (k > 0.89) concordance with standard methods in a simulation on intraoperative pathologic consultation. We also present a multi-layer perceptron-based on image attribute quantification capable of predicting tumor diagnosis with 86.6% accuracy.

Using two isogenic lines of normal human astrocytes differing by the most common oncogenic point mutation in the IDH-1 gene (R132H), we have profiled the Raman spectral changes associated with this mutation, as well as their metabolomic correlates. Further, we have demonstrated that these changes can be induced in wild-type cells by exposing them to cell-permeable 2-HG, and that exposure of IDH-1-mutant cells to a small-molecule inhibitor of the mutant form of IDH-1 reverses these changes.

Because SRS amplifies the Raman signal with no resonant background, we believe this spectral difference can be leveraged to rapidly generate high resolution hyperspectral images of live wild-type and R132H cells in vitro. Importantly, this would represent a unique visualization mutational status without tags or immunohistochemical stains.

Pro-inflammatory M1 macrophages promote osteogenesis by mesenchymal stem cells via the COX-2-prostaglandin E2 pathway

Laura Lu, HHMI Medical Fellow, Stanford University School of Medicine

Mentors: Stuart Goodman, MD, PhD and Michael Longaker, MD, MBA, Stanford University School of Medicine

Bone fractures are among the most common orthopedic problems that affect individuals of all ages. Acutely after injury, activated macrophages dynamically contribute to and regulate an acute inflammatory response. As a result of this inflammatory milieu, different macrophage phenotypes arise: undifferentiated M0, pro-inflammatory M1, and anti-inflammatory M2. These macrophages work in concert with other cells at the injury site, including mesenchymal stem cells (MSCs), to modulate bone healing. The dynamics of macrophage-MSC interaction and their subsequent effects are not well understood.

In this study, we co-cultured undifferentiated M0 and polarized macrophages (M1 and M2) with primary murine MSCs to determine the cross-talk between polarized macrophages and MSCs and their effects on osteogenesis. At two weeks of culture, MSCs co-cultured with M1 macrophages had reduced alkaline phosphatase (ALP) activity compared to MSCs grown alone and the M0-MSC and M2-MSC groups. However, after four weeks of co-culture, MSCs grown in the presence of macrophages, especially M1 macrophages, had enhanced bone matrix mineralization compared to MSCs grown alone. Despite low ALP activity early in co-culture, the M1-MSC culture produced the most bone.

Bone formation was found to be closely associated with prostaglandin E2 (PGE2) secretion early in osteogenesis (two weeks). To determine the necessity of PGE2 in bone formation, cultures were treated with celecoxib, a cyclooxygenase-2 (COX-2)-selective inhibitor, for one week. This treatment resulted in significantly reduced bone mineralization in all co-cultures but most dramatically in the M1-MSC group. This result confirmed the
importance of COX-2 activity and PGE2 in macrophage-mediated enhancement of MSC osteogenesis. The presence of macrophages also reduced the expression of osteoprotegerin (OPG), the decoy RANKL receptor. This finding suggests that macrophages negatively regulate OPG secretion and thus may alter osteoclast activity in addition to enhancing bone formation.

Taken together, our study shows that an initial pro-inflammatory phase modulated by M1 macrophages promotes osteogenesis in MSCs via the COX-2-PGE2 pathway. Gaining a better understanding of the complex interactions between macrophages and MSCs in regulating inflammation provides opportunities to better understand and optimize immune modulation of bone healing and other regenerative processes in the presence of inflammation. As the role of COX-2 and PGE2 extends beyond musculoskeletal diseases into cancer biology, endocrinology, and cardiovascular health, it is imperative to gain a granular understanding of the nuances of COX-2 and PGE2 activity in all systems of the body. Moreover, this study provides a biological explanation for the adverse effects of non-steroidal anti-inflammatory drugs (NSAIDs) in the setting of inflammation and bone injury.

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HIF1α, B cells, and pancreatic cancer

Richard Maduka, HHMI Medical Fellow, Perelman School of Medicine at the University of Pennsylvania

Mentor: M. Celeste Simon, PhD, Perelman School of Medicine at the University of Pennsylvania

Pancreatic ductal adenocarcinoma (PDAC) is one of the leading causes of mortality related to cancer in the world. PDAC tumors, characterized by an extensive desmoplastic stromal response and hypovascularity, are likely impacted by significant oxygen scarcity during both initiation and progression. We have demonstrated, using a well-defined autochthonous KrasG12D-driven murine model, that hypoxia and stabilization of hypoxia-inducible factor 1 alpha (HIF1α), a principal mediator of hypoxic adaptation, emerge early during the preinvasive stages of PDAC. Previous data show that pancreas-specific deletion of the Hif1α gene actually accelerates early oncogenesis (PanIN 1-3) through increased recruitment of B-lymphocytes to the mouse pancreatic epithelium, dependent on increased expression of the B cell chemokine CXCL13. In order to further explore the role of HIF in later stages of PDAC, where hypoxia is likely to be more severe, we generated lentivirus with short hairpin RNAs designed to deplete both HIF1α and related HIF2α in a series of three mouse PDAC cell lines. Using knockdown variants of these PDAC cells, we performed subcutaneous injections into wild-type C57BL/6 mice. Rate of growth, size, and weight of the subcutaneous tumors were followed for three weeks and subsequently harvested for RNA and immunohistochemistry analysis. Gene expression analysis reveals adequate HIF1α and HIF2α ablation. In-vitro proliferation assessment reveals no significant difference in growth in the HIF1α- and HIF2α-knockdown cell lines when compared to controls. However, preliminary subcutaneous tumor analysis reveals subdued growth in HIF1α-knockdown tumors in comparison to control, illustrating the significant effect the tumor’s microenvironment has on PDAC growth.

Further subcutaneous tumor injections will be completed using the remaining PDAC cells, and tumor analysis will be performed with immunohistochemistry (IHC) for differences in cellular proliferation (Ki67), vascular angiogenesis (CD31), and apoptosis (cleaved caspase 3). Leukocyte recruitment will be assessed through both IHC (CD45) and flow cytometry (CD19+, CD43+, IgM+, CD5+) analysis. Finally, selected PDAC cells will be orthotopically transplanted into wild-type C57BL/6 mouse pancreata to further assess the effects of HIF on late-stage PDAC oncogenesis.
and IL-2 in the presence of b9-23 peptide will result in the induction of islet β cell specific T-regs which subsequently will prevent or reverse the onset of T1D.

To test this hypothesis, we used a model of T1D, in which non-obese diabetic (NOD) female mice spontaneously develop autoimmune diabetes at approximately 12 weeks. We have demonstrated that the subcutaneous injection of the microparticle cocktail increases the percentage of T-regs in the draining lymph node of the NOD mice. To determine the specificity of our induced T-regs, we will employ the b9-23 MHC-tetramer (a complex of MHC II molecules loaded with b9-23 peptide such that it will only bind to T-cell receptors that react to that peptide). We have optimized the staining protocol of the b9-23 tetramer and are currently characterizing the specificity the induced T-regs. Our initial studies in attempting to treat recent-onset diabetes have demonstrated an inability to reverse recent-onset hyperglycemia for longer than a few days. We suspect that this lack of sustained response is due to the rapid progression of disease outpacing the time needed to induce T-regs. Currently, we are exploring the use of a short course of immunosuppression to allow our system to induce significant numbers of T-regs without continued progression of disease. Additionally, we are also determining whether our system can prevent the onset of diabetes when administered in prediabetic 8-week-old NOD mice.

Mutation annotation files, HLA types, and clinical annotations were obtained from published data. We used standardized pipelines for somatic variant calling, mutational signature deconvolution, and neoantigen prediction. Patients were stratified into clinical benefit (CB) and no clinical benefit (NCB) groups using three previously published response metrics based on objective tumor regression (RECIST) and durations of progression-free survival (PFS) and overall survival after starting immune checkpoint therapy.

Nonsynonymous mutational burden was significantly higher in CB vs. NCB using all three response categorizations. To assess the impact of mutational processes contributing to overall mutational burden, we used a non-negative matrix factorization framework to infer mutational activity in tumors from six signatures previously seen in melanoma: aging (S1), T>C substitutions (S5), ultraviolet light (S7), mismatch repair (S6), alkylating agents (S11), and T>G substitutions (S17). Across all samples, the proportion of mutations in S7 or S11 was positively correlated with mutational burden (Spearman’s rho = 0.66), while S5 and S1 were anti-correlated (rho = -0.62). Additionally, in a multivariate logistic model, S7 activity and S11 activity were each independent predictors of CB adjusting for mutational load ($P < 0.05$), with the sum of S7 and S11 activity being a strong predictor ($P < 0.001$). Of the patients with low mutational burden (less than median) with CB, 79% had a majority of mutations in S7 or S11, compared to only 51% of NCB ($P < 0.01$; Pearson’s chi-squared). Neoantigen burden was strongly correlated with mutational burden, and did not improve the ability to predict CB. In examining mutations in specific genes, more than 500 genes were mutated significantly more frequently in either CB or NCB ($P < 0.05$, Fisher’s exact). Restricting analysis to recurrently mutated genes in cancer and correcting for patient mutational burden, nonsynonymous mutations in ACSL3 and MET and truncating alterations in ARID2 were significantly enriched in CB.

In this meta-analysis of 220 patients, harmonized clinical and whole exome analysis confirmed that mutational burden correlates with clinical response to anti-PD-1 and anti-CTLA-4 therapies in metastatic melanoma, with mutational signatures and alterations in specific genes potentially providing additional predictive power.
Performance requirements for a scanning tethered capsule endoscope

Mark Michael, HHMI Medical Fellow, Baylor College of Medicine
(Rice University)

Mentor: Rebecca Richards-Kortum, PhD, Rice University

Over 400,000 people die from esophageal cancer annually. These deaths are largely a result of the late onset of symptoms and the lack of low-cost early detection technologies.

To address this need, we are developing a novel, tethered capsule endoscope that images the entire mucosa at 10-μm resolution using narrow-band imaging (NBI) to detect abnormal vasculature indicative of early neoplasia. The capsule (13 × 26 mm) is small enough to be swallowed without sedation. Here, we describe laboratory validation studies to assess the feasibility of the design.

The system has two components: a reusable, tethered capsule that is swallowed to collect images of the mucosa and supporting hardware to manipulate and display the collected data on a tablet computer. A 3D printed capsule was designed with an axial hole matching the cross-sectional shape of the twisted wire of a gastric cytology brush, which served as the tether. The tether causes the capsule to rotate as it descends through the esophagus during peristalsis so that it captures high-resolution images from the entire esophagus. A theoretical analysis was performed to determine the sensor parameters needed to achieve the desired spatial resolution. Results were experimentally validated in a laboratory setting.

The passive mechanical guidance system allowed controlled rotation of the capsule during descent. Theoretical calculations and laboratory measurements indicate that the entire esophagus can be imaged with a spatial resolution of 10 μm if the capsule descends over 60 seconds and incorporates a sensor with a minimum frame rate of 3.6 frames per second and maximum exposure time of 0.8 ms. We designed an optical system that incorporates candidate sensors meeting these requirements and that fit within the spatial constraints of the capsule.

The tethered capsule system incorporating consumer-grade image sensors is designed to acquire high-definition images to enable visualization of capillary loops; this is a significant advantage compared to current, untethered “PillCam” systems.

Molecular mechanisms of SMARCB1 recurrent somatic missense mutations in meningioma tumorigenesis

Julio Montejo, HHMI Medical Fellow, Yale School of Medicine

Mentor: Murat Günel, MD, Yale School of Medicine

Meningiomas are the most frequent primary intracranial tumors. Although the majority are histopathologically classified as “benign,” all can damage precious brain tissue. Despite standard of care, malignant meningiomas have a dismal median overall survival of less than 3 years, necessitating the development of novel therapies.

Inactivating mutations in the NF2 tumor suppressor gene, located on chromosome 22, and/or chromosome 22 loss account for 40–60% of sporadic meningiomas. However, these driver mutations are thought to be associated with tumor initiation rather than malignant progression. Within non-NF2 meningiomas, next-generation sequencing recently revealed driver mutations in TRAF7, KLF4, AKT1, SMO, and POLR2A. Moreover, co-occurring with NF2 mutations and/or chromosome 22 loss, recurrent somatic missense mutations in the SMARCB1 tumor suppressor gene have been identified in sporadic meningiomas, interestingly, with a predilection for malignancy.

SMARCB1, located on chromosome 22, encodes a core subunit of the SWI/SNF chromatin-remodeling complex involved in epigenetic regulation. Functionally, the SMARCB1 protein is implicated in the recruitment of the SWI/SNF complex to specific target genes where chromatin modifications such as histone 3, lysine 27 (H3K27) acetylation, a marker of enhanced transcription, mediate gene expression. However, the precise genomic architecture and molecular mechanisms of SMARCB1-driven meningioma remain unknown, limiting avenues for the development of novel therapies.

In this study, we hypothesized that recurrent somatic missense mutations in SMARCB1 may confer the SWI/SNF complex with a neomorphic function in its repertoire of target genes where aberrant H3K27 acetylation may contribute to meningioma tumorigenesis.

To identify a meningioma cohort enriched in SMARCB1 mutations, targeted screening via molecular inversion probes (MIPs) sequencing of NF2, TRAF7, KLF4, AKT1, SMO, POLR2A, and SMARCB1, in series with chromosome 22 quantitative real-time PCR (q-PCR), was performed. To further characterize the genomic landscape of meningiomas, mutation-unknown samples via targeted screening were analyzed via whole exome sequencing. All candidate variants were confirmed via Sanger sequencing. Additionally, to identify potential genetic and phenotypic relationships, correlation between genetic and clinicopathological variables was performed.

To investigate chromatin status in an unbiased genome-wide manner, chromatin immunoprecipitation (ChIP) against H3K27 acetylation coupled
with next-generation sequencing (ChIP-sequencing) was utilized. Moreover, to quantify gene expression in an unbiased transcriptome-wide manner, RNA-sequencing was utilized. Epigenetic enrichment via ChIP-sequencing and gene expression levels via RNA-sequencing were computed for (1) normal adult meningeal tissue, (2) non-SMARCBI-mutant/NF2-mutant/Chr22-loss meningiomas, and (3) SMARCBI-mutant/NF2-mutant/Chr22-loss menigioma.

Our experiments are undergoing analysis. To identify potentially pathogenic target genes, all genes showing differential ChIP binding will be correlated with gene expression values to enrich for genes showing differential regulation, which may be associated with SMARCBI-driven menigioma tumorigenesis in the background of NF2 mutations and/or chromosome 22 loss. Identification of these target genes could open avenues for novel therapies.

PHIAL (Precision Heuristics for Interpreting the Alteration Landscape) was originally developed as a method of clinically interpreting cancer genomic data. The first iteration of PHIAL (PHIAL1) interpreted somatic whole exome sequence data using a co-developed database of tumor alterations relevant for genomics-driven therapy (TARGET). While PHIAL1 was successfully able to provide rapid clinical interpretation of genomic data, it was limited to first-order genomic relationships, was unable to rank multiple putatively actionable variants, did not make observations based on somatic-germline interactions, and could not leverage transcriptome data. Additionally, the initial version of the TARGET database (TARGET1) only referenced gene-level alterations to actions, did not record the relative predictive implications or cancer specificities of alteration-action relationships, and had no mechanism for recording relevant literature citations.

Both PHIAL1 and TARGET1 underwent several concurrent revisions to improve their ability to provide accurate clinical annotations. The second version of PHIAL (PHIAL2) was revised to utilize the presence of single nucleotide variations, insertions/deletions, somatic copy number alterations, fusions, and global features (such as mutational burden) together to predict actionability. Updates to the TARGET database (TARGET2) enabled greater specificity in alteration specification (including specification of the alteration and alteration type), added systems for measuring the predictive implication and cancer context of an annotation, and allowed the addition of citations to annotations. Additionally, a web-based system was built to allow other researchers convenient access to TARGET2 and to solicit future additions.

We applied both PHIAL1/TARGET1 and PHIAL2/TARGET2 to a 255-patient cohort with both whole exome and transcriptome sequencing data (146 castration-resistant prostate cancer and 109 metastatic melanoma samples). PHIAL1 identified 1,342 putatively clinically actionable/biologically relevant events across the cohort, with a median of 3 events per patient; 95% of patients had at least one putative event. PHIAL2 identified 2,508 putative events, with a median of 6 events per patient; 98.5% of patients harbored at least one event. Of these events, 8.12% were associated with an FDA-approved therapy and 2.09% with a clinical trial. PHIAL2 identified events in 9 patient samples that PHIAL1 associated with no events.

Our revisions to PHIAL and TARGET allowed for the identification of a larger number of putatively actionable/biologically relevant events, and for more precise assessment of the predictive implication of each suggested clinical action. These improvements may increase the clinical utility of whole exome/transcriptome sequencing by providing additional context and annotations for these data.

**Next-generation clinical interpretation algorithms for precision cancer medicine**

**Nathanael Moore**, HHMI Medical Fellow, Indiana University School of Medicine (Harvard Medical School)

**Mentor:** Eliezer Van Allen, MD, Harvard Medical School and Dana-Farber Cancer Institute

PHIAL (Precision Heuristics for Interpreting the Alteration Landscape) was originally developed as a method of clinically interpreting cancer genomic data. The first iteration of PHIAL (PHIAL1) interpreted somatic whole exome sequence data using a co-developed database of tumor alterations relevant for genomics-driven therapy (TARGET). While PHIAL1 was successfully able to provide rapid clinical interpretation of genomic data, it was limited to first-order genomic relationships, was unable to rank multiple putatively actionable variants, did not make observations based on somatic-germline interactions, and could not leverage transcriptome data. Additionally, the initial version of the TARGET database (TARGET1) only referenced gene-level alterations to actions, did not record the relative predictive implications or cancer specificities of alteration-action relationships, and had no mechanism for recording relevant literature citations.

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**FGF1 suppresses lipolysis to maintain glucose homeostasis in type 2 diabetes**

**Christopher Moutos**, HHMI Medical Fellow, University of Arkansas for Medical Sciences College of Medicine (Salk Institute for Biological Studies)

**Mentor:** Ronald Evans, PhD, Howard Hughes Medical Institute and Salk Institute for Biological Studies

Current pharmacological management of type 2 diabetes mellitus (T2DM) begins with metformin to improve insulin sensitivity. As the disease progresses, however, several classes of insulinotropic compounds, such as sulfonylureas, are required to achieve glycemic control, with insulin as a final option when glycemic control cannot otherwise be achieved. We previously established fibroblast growth factor 1 (FGF1) as an essential factor in adipose remodeling in response to nutrient status. Through injection of this peptide, we uncovered FGF1 as a potent anti-diabetic compound that normalizes blood glucose levels within hours across a range of diabetic models. Chronic injection of FGF1 achieved sustained glucose
lowering and insulin sensitization. These effects were in part mediated through the anti-lipolytic actions of the FGF1-FGF receptor 1 (FGFR1) signaling cascade in the adipose tissue. Importantly, suppression of adipose tissue lipolysis and free fatty acid release by FGF1-FGFR1 acted synergistically with insulin. Though FGF1 works with insulin to suppress lipolysis, it enacts transcriptomic changes separate from insulin. This allows FGF1 to lead to persistent glucose lowering in insulin-resistant mice. A single injection of recombinant FGF1 decreased serum free fatty acids, adipose lipolysis, hepatic acetyl-CoA, and gluconeogenesis, in addition to normalization of blood glucose levels. Continued work on this project will be focused on further understanding the mechanism of FGF1 action, as well as exploring compounds that may synergize with FGF1.

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In situ signal characterization of dermal sensory interfaces

Andrej Nedic, HHMI Medical Fellow, Wayne State University School of Medicine (University of Michigan Medical School)

Mentors: Melanie Urbanchek, MS, PhD and Paul Cederna, MD, University of Michigan Medical School

Dermal sensory interfaces (DSIs) consist of residual sensory peripheral nerves implanted into de-epithelialized skin grafts placed subcutaneously. DSIs may transduce electrical pulse signals to compound sensory nerve action potentials (CSNAPs) to provide sensation from prostheses. Our purpose is to characterize in situ DSI signal transduction evoked while varying key electrical stimulation parameters of: (1) amplitude, (2) frequency, and (3) pulse width.

Rats were grouped by surgery: innervated skin (IS, n = five), control nerve (CN, n = five), transected nerve (TN, n = five), or DSI (n = ten; historical data). Each DSI was constructed by encasing a transected sural nerve with a de-epithelialized skin graft. Electrical stimulation was applied distally to: the skin at the ankle (IS), the sural nerve (CN), the transected sural nerve (TN), and the skin graft (DSI). Peak-to-peak voltage (Vpp) and percent of stimuli transduced (sensitivity of signal) were recorded at stimulation current amplitudes (threshold [T], T + 50, T + 100, T + 150 μA), frequencies (2, 20, 50, 100 Hz), and pulse widths (30, 50, 100, 500 μsec). Evoked nerve responses were recorded proximally at the sural nerve.

As expected, threshold current required to elicit CSNAPs was significantly higher for both IS (450.0 ± 70.3 μA) and DSI (465 ± 109.9 μA) groups compared to CN (31.0 ± 28.3 μA) and TN (22.0 ± 17.9 μA) (P < 0.05). In the DSI group, increasing stimulation amplitude from threshold to threshold + 150 μA resulted in a linear increase in Vpp (11.4 ± 10.6 to 43.1 ± 24.2 μV; r² = 0.97), importantly, with no significant difference from IS (10.8 ± 2.0 to 17.6 ± 5.9 μV). Both increases were smaller than CN (28.8 ± 5.9 to 146.8 ± 85.6 μV) and TN (63.3 ± 34.5 to 304.7 ± 32.2 μV) (P < 0.05). There were no significant differences in Vpp when varying stimulation frequency for all groups. There were no significant differences in Vpp in CN and TN when varying pulse widths. For IS, CSNAPs were not transduced below 100 μsec pulse width. Electrical stimulation applied to DSIs reliably elicited CSNAPs with 96% or higher signal sensitivity at all tested parameters.

Electrical stimulation applied to DSIs produced CSNAPs similar to normal innervated skin, with better transduction sensitivity. Findings from this study suggest that patterned electrical stimulation is successfully transduced across DSIs in a similar fashion to native skin.

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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

Postnatally born neurons hold exciting promise for the treatment of brain injuries. Ideally, replacement neurons should reestablish the connections lost by injured neurons in a way that approximates the brain’s function to pre-injury levels. Thus, understanding the mechanisms that govern integration of immature neurons into functional circuits is of great importance. Neurogenesis in the adult rodent subventricular zone (SVZ) is a robust experimental model for investigating and understanding the integration capacities of newborn neurons. Using this system, we show that SVZ neurons have reduced integration capacities when ectopically transplanted into cortical circuitry versus the olfactory bulb circuitry—their normal target. Interestingly, this behavior was reversed when transplanting undifferentiated neurons into the cortex, suggesting that perhaps SVZ neurons at earlier developmental stages may still retain the necessary cellular plasticity to integrate into different contexts. To provide a source of neurons capable of adult cortical integration, we used Fezf2, a master gene for glutamatergic projection neurons, to alter SVZ neuronal fate. Lentiviral-mediated delivery of Fezf2 to the postnatal neural stem cells and neuronal progenitors led to ectopic Fezf2 expression in differentiated neurons. Presently, we are finalizing characterization of the Fezf2+ neurons,
as well as performing live-imaging and electrophysiological experiments on Fasl2+ neurons transplanted into cortical and olfactory bulb circuits to characterize their integration properties; these data that will be presented at the ASCI-AAP Joint Meeting. We are optimistic that these results will serve as proof of principle that newborn neurons can be re-engineered to integrating into distinct neuronal circuits.

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Structural analysis of GPCR-transducer complexes using cryo-electron microscopy

Anthony Nguyen, HHMI Medical Fellow, Tufts University School of Medicine (Duke University Medical Center)
Mentors: Robert Lefkowitz, MD, Duke University Medical Center and and Georgios Skiniotis, PhD, University of Michigan

G protein-coupled receptors (GPCRs) are cell-surface receptors involved in the signaling and regulation of many physiological processes. They bind an array of ligands, from small molecules to polypeptides, which act to stabilize active receptor conformations. In its active forms, the receptor interacts with G protein, thus initiating signaling cascades that eventually lead to functional outcomes. To prevent overstimulation, G protein-coupled receptor kinases (GRKs) phosphorylate the activated GPCR, which facilitates binding of the adapter protein β-arrestin (βarr) to the receptor. βarr recruitment to the GPCR has two important consequences: (1) desensitization of G protein signaling, since βarr binds to similar region of the G protein, thereby sterically blocking its binding to the GPCR; and (2) internalization of the GPCR-βarr complex, facilitated by βarr interacting with endocytic proteins such as clathrin and AP2. βarrs also serve as signaling molecules, capable of inciting molecular pathways that are distinct from those associated with G protein signaling, which ultimately leads to unique physiological consequences.

In contrast to this classical understanding of GPCR function, we have recently discovered that some GPCRs in fact are capable of forming mega-complexes, whereby one GPCR actively engages with both a G protein and βarr at the same time. This discovery is supported by our previous negative stain electron microscopy work, which directly observed a conformation of βarr that only engages the phosphorylated receptor tail, leaving the G protein binding interface on the intracellular transmembrane receptor core open. These mega-complexes have been shown to mediate sustained G protein signaling, as the receptor is internalized into intracellular compartments such as endosomes.

In the last few years, our lab has successfully purified a number of GPCR-transducers complexes, including a GPCR-βarr complex stabilized by an antigen binding antibody fragment (Fab) as well as a GPCR–G protein-βarr mega-complex stabilized by both a Fab and a nanobody. Furthermore, we have characterized the overall architecture of these complexes using negative stain electron microscopy. Currently, we are conducting preliminary trials in order to screen for optimal constructs and freezing conditions for high-resolution cryo-electron microscopy (cryo-EM) studies. Structural elucidation of these GPCR-transducer complexes using cryo-EM can provide crucial mechanistic insight into GPCR signaling, as well as pave the way for the creation of pharmaceutical compounds that might modulate pathogenic signaling pathways more specifically.

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FOXG1 overexpression and epigenetic landscape in a human iPSC-derived forebrain organoid model of severe, macrocephalic autism spectrum disorder

Neal Nolan, HHMI Medical Fellow, Yale School of Medicine
Mentor: Flora Vaccarino, MD, Yale School of Medicine

Autism spectrum disorder (ASD) is a highly heritable developmental disorder that in some cases is associated with early embryonic cortical overgrowth, macrocephaly, and GABAergic neuronal and synaptic imbalance. FOXG1, a transcription factor involved in early forebrain patterning, has been implicated in contributing to ASD phenotype in a subset of severely affected macrocephalic patients. Previous findings show that FOXG1 is particularly upregulated as well as co-expressed with other ASD-associated genes in these ASD patients’ forebrain organoids. These organoids are a human induced pluripotent stem cell (hiPSC)-derived three-dimensional model system for early telencephalic development. In these patients, increased FOXG1 activity may explain some features of ASD pathophysiology, for example, FOXG1’s known associations with cortical growth and GABAergic predominance. Our previous publication supports this connection, showing that FOXG1 knockdown by RNA interference (RNAi) in these ASD-derived organoids normalized several ASD-associated gene network and neurophenotypic changes.
However, to what extent FOXG1 upregulation specifically contributes to ASD, and the details of the FOXG1 regulatory network in health and disease, remain unclear. Additionally, the epigenetic landscape of ASD is not well understood, particularly with regard to FOXG1. Our previous ASD-derived organoids showed increased FOXG1 expression but without mutations in the FOXG1 sequence or upstream promoter regions, suggesting that epigenetic or distal regulatory changes might contribute to these patients’ ASD phenotype.

This project explored these uncertainties surrounding FOXG1 by two avenues. First, by using a doxycycline-inducible FOXG1 overexpression system in organoids derived from ASD patients’ fathers, who serve here as unaffected controls, we used several metrics, including RNA-sequencing (RNA-seq) and immunocytochemistry, to examine the extent to which FOXG1 overexpression alone recapitulates the ASD transcriptomic and neurophenotypic changes seen in organoids derived from their autistic sons. Second, by performing chromatin immunoprecipitation sequencing (ChIP-seq) (markers H3k4me3, H3k27ac, H3k27me3) on organoids derived from ASD patients and their unaffected fathers at time points corresponding with early forebrain development, we probed further into the dynamic developmental transcriptomic and epigenetic landscape of our ASD cohort; for example, we aim to investigate potential epigenetic causes for the previously unexplained overexpression of ASD-associated genes such as FOXG1.

FOXG1 over-expression, RNA-seq, and ChIP-seq experiments are ongoing in several clonal lines derived from multiple families. FOXG1 over-expression experiments, in particular, have already yielded unexpected and interesting results suggestive of the nuanced FOXG1 regulatory environment in pluripotency and neuronal differentiation. This set of experiments using hiPSC-derived forebrain organoids should better illustrate the role FOXG1 overexpression plays in creating the ASD phenotype seen in our patient cohort, as well as illustrate the network of transcriptional and epigenetic changes associated with ASD.

51 Platelet-mediated protection against alveolar injury through the release of thrombospondin-1 during Pseudomonas aeruginosa acute intrapulmonary infection

Tolani Olonisakin, HHMI Medical Fellow, University of Pittsburgh School of Medicine

Mentor: Janet Lee, MD, University of Pittsburgh School of Medicine

Pseudomonas aeruginosa (PA) acute lung infection is a major cause of opportunistic infection, and Pseudomonal sepsis is independently associated with increased mortality. Secretion of exoproteases such as Pseudomonas elastase (LasB), a metalloendoprotease that degrades tissue elastin and collagen, enhances PA pathogenicity, but host-derived factors that disarm pathogen-encoded proteases are not fully known. Thrombospondin-1 (TSP-1), a multifunctional extracellular matrix glycoprotein involved in cell-cell and cell-matrix interactions, is secreted by activated platelets during inflammation and inhibits neutrophil granule serine proteases. Moreover, TSP-1 upregulates the release of PA elastase LasB and that platelets provide protection against pathogen-triggered proteolysis through the release of TSP-1 during acute PA-induced injury. Utilizing natively thrombocytopenic mice deficient in the thrombopoietin receptor Mpl (Mpl–/–), we demonstrate that Mpl–/– mice are prone to severe alveolar injury and succumb to early mortality upon acute P. aeruginosa intrapulmonary infection. However, acute Klebsiella pneumoniae intrapulmonary infection fails to elicit alveolar injury or enhanced mortality in Mpl–/– mice compared to WT controls, highlighting a specific host-pathogen interaction. Furthermore, PA-secreted bacterial exoprotease(s) mediate the alveolar injury, as cell-free filtered supernatant obtained from P. aeruginosa reproduces the phenotype observed with whole live bacteria in Mpl–/– mice. We further show that cell-free filtered supernatant obtained from PA mutant containing a transposon insertion in LasB gene (lasB) lacks exoprotease activity and induces significantly less alveolar injury in Mpl–/– mice compared to parent PA strain. In vitro, Pseudomonas elastase activity is inhibited by rhTSP-1 monomer and purified platelet-derived TSP-1 in a dose-dependent manner. Collectively, our findings suggest that thrombocytopenia enhances lethality of P. aeruginosa-induced alveolar injury and propose a role for platelet-mediated protection against P. aeruginosa beyond hemostasis, in part, through the release of TSP-1.
The role of glial unfolded protein response in diet-induced obesity

Rebekah Packer Zahedi, HHMI-BWF Medical Fellow, University of Georgia College of Veterinary Medicine (University of California, Berkeley)

Mentor: Andrew Dillin, PhD, Howard Hughes Medical Institute and University of California, Berkeley

When faced with a stressor such as infection, overnutrition, or hypoxia, cellular resources become taxed and proteins can become misfolded, unfolded, and aggregated. The unfolded protein response of the endoplasmic reticulum (UPR\textsuperscript{ER}) helps maintain organismal proteostasis through signaling pathways that decrease protein translation and increase protein folding capacity. The UPR\textsuperscript{ER} is attenuated as an organism ages, and dysfunction of this system has been associated with numerous neurodegenerative and age-related diseases. One transcription factor from these pathways, X-box binding protein (XBP-1s), exerts significant control over the UPR\textsuperscript{ER}. Constitutive neuronal expression of xbp-1s in Caenorhabditis elegans compensates for systemic age-dependent loss of the UPR\textsuperscript{ER} through a cell non-autonomous mechanism and significantly increases worm longevity. Furthermore, glial-specific constitutive expression of xbp-1s in C. elegans also results in the activation of the UPR\textsuperscript{ER} in distal tissues and extends lifespan beyond the benefit conferred by neuronal-specific xbp-1s expression. The UPR\textsuperscript{ER} cell non-autonomous mechanism was recently found to be conserved in mammals, with neuronal xbp-1s expression shown to protect against diet-induced obesity and increase hepatic insulin sensitivity in mice.

We hypothesized that expression of xbp-1s in glia of mice would similarly induce the UPR\textsuperscript{ER} in distal tissues and prevent diet-induced obesity. To test this, we utilized an inducible Cre/lox site-specific recombination system to constitutively express xbp-1s in astrocytes in mice. To evaluate UPR\textsuperscript{ER} up-regulation, brains and livers were assessed using immunohistochemistry, quantitative polymerase chain reaction (qPCR), and Western blots. Age-matched cohorts of XBP1-s Cre/lox mice were then placed on a high-fat diet to characterize metabolic function and subsequent development of obesity. The results from this study will hopefully uncover new mechanisms of metabolic regulation and protein homeostasis that could serve as targets of therapy for human metabolic and age-related diseases.

Role of BBS Proteins in Fear Learning

Thomas Pak, HHMI Medical Fellow, University of Iowa Roy J. and Lucille A. Carver College of Medicine

Mentor: Val Sheffield, MD, PhD, University of Iowa Roy J. and Lucille A. Carver College of Medicine

Cilia are essential to life. Cilia are involved in vision, kidney function, mucin clearance in lungs, and brain development and function. Primary cilia are located on neurons, and are involved in neuronal signaling. This implicates a role of cilia in behavior. Mice, without primary cilia in the brain, have altered behavior. Bardet-Biedl syndrome (BBS) is a pleiotropic, autosomal recessive ciliopathy. We hypothesize that BBS proteins play a crucial role in anxiety processing and response. To study this, we tested fear conditioning (FC) in a homozygous knockin mouse model (BBS1\textsuperscript{M390R/M390R}) of the most common BBS mutation (BBS1\textsuperscript{M390R}) in humans. We discovered that BBS1\textsuperscript{M390R/M390R} mice have both impaired cue and context FC ($P < 0.01$). This fear conditioning dysfunction is recapitulated when the Bbs8 (gene is knocked out postnatally. To rule out confounding variables on the FC test, we performed control studies. These studies show that BBS1\textsuperscript{M390R/M390R} mice have normal motor function, including normal cerebellar function (rotarod performance test) and normal hearing (auditory brainstem response) compared to control mice.

To determine the mechanism for the FC defect, we investigated the electrophysiology of the hippocampus and amygdala. Whole-cell patch recordings of pyramidal neurons of the lateral amygdala show that the frequency and amplitude of mEPSCs (miniature excitatory postsynaptic currents) are smaller in BBS1\textsuperscript{M390R/M390R} mice compared to control mice ($P < 0.01$), whereas field recordings show that long-term potentiation of the hippocampus is not different between BBS1\textsuperscript{M390R/M390R} mice and controls. Furthermore, the paired pulse ratio (PPR) is larger in the hippocampus and amygdala of BBS mutant mice compared to the PPR in control mice ($P < 0.05$). The electrophysiology data point to a vesicle release defect. However, the BBS mouse ultrastructure morphology (synaptic density, vesicle density, vesicle docking density, vesicle size, and mitochondrial density) is normal compared to control mice.

Western blotting of synaptosomes shows that BBS proteins are located in the synapse. In addition, Western blot data show that the GSK/AKT signaling pathway in fear conditioning is affected in BBS mutant mice. These findings could explain our results that chronic lithium chloride treatment rescue context-dependent fear conditioning in BBS mutant mice. These findings provide a new understanding of the molecular mechanisms underlying anxiety disorders, and uncover a novel role for ciliary proteins in neural signaling.
**A genome-wide CRISPR/Cas9 screen for host factors that mediate cellular invasion of Chlamydia trachomatis**

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* is a Gram-negative bacterium that is the most common cause of sexually transmitted disease in the US and infectious blindness worldwide. An intracellular obligate parasite, *Chlamydia trachomatis* invades epithelial cells and replicates within a membrane-bound vacuole known as an inclusion body. To comprehensively identify the host factors that mediate *Chlamydia* invasion, we undertook a genome-wide CRISPR/Cas9-based screen in cultured human epithelial cells (HT29). We engineered a transgenic *C. trachomatis* serovar L2 strain expressing the mCherry fluorescent protein and infected a pooled mutant library of HT29 cells at high multiplicity of infection, leading to a very high invasion rate (~90%). Fluorescence-activated cell sorting (FACS) was then used to enrich for cells that did not express an intracellular mCherry signal, which reflects a sub-pool of mutant cells resistant to invasion. The nature of the genetic perturbations in the resistant cells was identified by next-generation sequencing of the chromosomally integrated guide RNAs. Having acquired preliminary hits from the screen, efforts are now underway to validate and characterize the function of these genes during *C. trachomatis* infection, which is of global urgency.

**Transcriptomic characterization of ductular and stellate cells in a murine liver injury model at single-cell resolution**

Fatemeh Parvin-Nejad, HHMI Medical Fellow, Icahn School of Medicine at Mount Sinai

Mentor: Scott Friedman, MD, Icahn School of Medicine at Mount Sinai

End-stage liver disease from hepatic fibrosis contributes significantly to US and worldwide morbidity and mortality, yet treatment remains limited. Severe liver disease is accompanied by ductular reaction (DR), comprised of expanded ductular cells (putative liver progenitor cells [LPCs]), hepatic stellate cells (HSCs), and fibrosis. DR correlates with severity of disease and fibrosis; however, its underlying mechanisms are poorly characterized. It is also unclear whether HSCs, the main fibrogenic cells in liver, contribute to regeneration by activating ductular cells, or if ductular cells activate HSCs to enhance fibrogenesis. Characterizing the cellular contributions to fibrogenesis may provide new targets for antifibrotic therapies. The aim of our study was to identify and characterize ductular cells and HSCs using single-cell transcriptomics. To address this aim, our laboratory has generated a hepatic autophagy-deficient mouse line (Alb-Cre:Arg7fl/fl mice) that develops hepatomegaly, spontaneous fibrosis, and marked expansion of LPCs, allowing us to explore their fibrogenic potential as well as their contribution to hepatic regeneration following injury. To visualize fibrogenic cells in this model, Alb-Cre:Arg7fl/fl mice, a liver-specific knockout of autophagy-related protein 7 (Atg7, a key component of the autophagy pathway), were crossed with Tg(Col1-GFP) mice (expressing GFP driven by the collagen 1a1 promoter) to identify collagen I-transcribing cells. Livers from these reporter mice were analyzed by immunofluorescence for markers of LPCs (CD133, EpCAM) or HSCs (desmin) and correlated with sites of GFP expression. In addition, LPCs were isolated using in situ perfusion and magnetic cell sorting to enrich for EpCAM-expressing cells, which were analyzed by single cell sequencing using the Fluidigm C1 platform. Single-cell sequencing data were analyzed via t-distributed stochastic neighbor embedding (t-SNE) to define clusters of genetically similar cells. Lists of differentially regulated genes among these clusters were reviewed to further characterize clusters and to identify genes of interest. Double immunofluorescence revealed close proximity between fibrotic HSCs (GFP+, desmin+) and LPCs (CD133+, CK7+), as well as scattered co-localization of GFP and CD133 expression in regions displaying a ductular reaction, suggesting cross-talk of LPCs with HSCs. Single-cell sequencing revealed five clusters of cells:
three clusters corresponded to resident hepatic cell populations: hepatocytes (Alb⁺, CRP⁺, ApoB⁺), HSCs (Col1a1⁺, Lrat⁺, Vimentin⁺), and immune cells (IL-1R2⁺, CD103⁺, Ccl5⁺); two clusters displayed upregulated expression of progenitor markers including EpCAM and Sox9; one progenitor-like cluster was specific for upregulation of LPC markers (Sox9, EpCAM), associated with downregulation of the HSC markers Lrat and vimentin, whereas the final cluster expressed a diverse genetic signature, including Sox9, as well as other HSC and endothelial cell markers. These data indicate a diversity of phenotypes within the LPC compartment, highlighting the need to functionally define LPC sub-populations and characterize their contributions to fibrogenesis and repair.

We next used RaPID-MS to identify that RC3H1 binds the SM1v1 motif and that increased RC3H1 leads to decay of RNAs bearing the SM1v1 motif. Given that RC3H1 upregulation correlates with decreased breast cancer survival, our findings suggest RC3H1 overexpression may perturb post-transcriptional regulation of RNAs bearing SM1v1-like motifs, altering disease course specifically through this mechanism. Given that RNA viruses co-opt host proteins at their untranslated regions (UTRs), we next applied RaPID-MS to identify the Zika virus (ZIKV) host interactome. We found striking enrichment of cell cycle regulatory proteins, paralleling ZIKV-infected neural progenitor cell (NPC) gene perturbations, and of QKI, a RNA binding protein highly expressed in NPCs. QKI depletion decreased ZIKV viral RNA levels by 90%, suggesting QKI is critical for ZIKV replication. As QKI loss mimics Zika congenital syndrome, this RNA-protein interaction may underlie the overlapping phenotypes.

Finally, we applied RaPID-MS to identify APOL4 as a highly specific protein interactor with wild-type corneodesmosin (CDSN) transcripts in differentiating keratinocytes, but not with transcripts bearing a mutant allele found in psoriasis. We newly describe the strong induction of APOL4 in terminal keratinocyte differentiation, and propose APOL4 as a novel RBP that regulates CDSN transcripts in differentiating KCS and whose function may be altered in psoriasis. Loss of APOL4 markedly reduced corneodesmosin RNA and protein levels, suggesting a regulatory relationship that may offer insight into the coordinate regulation of terminal differentiation with lipid barrier formation.

Here we describe the development and broad applications of RaPID, a modular system to identify and validate novel RNA-protein interactions across a range of human disease in living cells. This efficient, cost-effective approach powerfully expands the scope, feasibility, and depth of vicinal proteomic discoveries.

Characterizing the vicinal RNA-protein interactome across human disease

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Mentor: Paul Khavari, MD, PhD, Stanford University School of Medicine

Physical interactions between specific RNAs and proteins play critical roles in diverse cellular functions, and their perturbations contribute broadly to human disease. Here we develop a novel vicinal proteomic approach to study RNA-protein interactions in living cells and characterize its applications in genetic disease, cancer, virology, and skin biology. We use a promiscuous biotin ligase (BirA*) to biotin-tag proteins bound to RNA motifs followed by streptavidin pull-down and Western blot (WB) or mass spectrometry (MS) to identify the proteins, terming our method RaPID, for RNA-protein interaction detection.

We first evaluated iron metabolism regulation by iron responsive element (IRE) RNA motif binding by IRE binding proteins (IREBP). The IRE L-ferritin (FTL) transcript is mutated in hereditary hyperferritinemia-cataract syndrome (HHCS). RaPID-WB with wild-type FTL IRE motif showed significant IREB2 enrichment, and RaPID-MS identified IREB2 as the top IRE binding partner. HHCS FTL IRE disease-associated point mutation-containing transcripts from patients bearing the London, Paris, and Verona mutations demonstrated decreased IREB2 binding compared to wild-type, offering evidence for the hypothesis that HHCS severity is attributable to impaired IRE-IREBP interactions.

We next used RaPID-MS to identify that RC3H1 binds the SM1v1 motif and that increased RC3H1 leads to decay of RNAs bearing the SM1v1 motif. Given that RC3H1 upregulation correlates with decreased breast cancer survival, our findings suggest RC3H1 overexpression may perturb post-transcriptional regulation of RNAs bearing SM1v1-like motifs, altering disease course specifically through this mechanism. Given that RNA viruses co-opt host proteins at their untranslated regions (UTRs), we next applied RaPID-MS to identify the Zika virus (ZIKV) host interactome. We found striking enrichment of cell cycle regulatory proteins, paralleling ZIKV-infected neural progenitor cell (NPC) gene perturbations, and of QKI, a RNA binding protein highly expressed in NPCs. QKI depletion decreased ZIKV viral RNA levels by 90%, suggesting QKI is critical for ZIKV replication. As QKI loss mimics Zika congenital syndrome, this RNA-protein interaction may underlie the overlapping phenotypes.

Finally, we applied RaPID-MS to identify APOL4 as a highly specific protein interactor with wild-type corneodesmosin (CDSN) transcripts in differentiating keratinocytes, but not with transcripts bearing a mutant allele found in psoriasis. We newly describe the strong induction of APOL4 in terminal keratinocyte differentiation, and propose APOL4 as a novel RBP that regulates CDSN transcripts in differentiating KCS and whose function may be altered in psoriasis. Loss of APOL4 markedly reduced corneodesmosin RNA and protein levels, suggesting a regulatory relationship that may offer insight into the coordinate regulation of terminal differentiation with lipid barrier formation.

Here we describe the development and broad applications of RaPID, a modular system to identify and validate novel RNA-protein interactions across a range of human disease in living cells. This efficient, cost-effective approach powerfully expands the scope, feasibility, and depth of vicinal proteomic discoveries.
Immunotherapy is emerging as a powerful paradigm in cancer treatment. Recent studies have also indicated that the presence of immune cells within the tumor microenvironment plays a critical role in prognosis and survival. MicroRNAs (miRNAs), a class of small non-coding RNA molecules that post-transcriptionally regulate gene expression, have been reported to mediate immune cell recruitment and activation in the tumor microenvironment and directly target cancer-related immune pathways. This study aims to combine mRNA and miRNAs in order to determine the fractions and types of immune cells within a specific tumor and use these to predict patient survival in breast invasive carcinoma and ovarian serous cystadenocarcinoma. Preprocessed gene expression array, RNA-seq, miRNA-seq, and survival data from The Cancer Genome Atlas (TCGA) were downloaded from the UCSC Xena Browser. Using regression-based computational methods, we constructed, for the first time, immune cell signatures based on miRNA expression from breast and ovarian cancer datasets. We evaluated these using statistical analysis and by comparing them to known miRNA signatures for a subset of the cells. Next we applied CIBERSORT, an analytical tool developed by Newman et al., to quantify the relative levels of immune cell subsets in the tumor samples using both CIBERSORT’s own immune cell mRNA expression signature matrix and our constructed immune cell miRNA expression signature matrix. The immune cell subsets most related to survival were identified using univariate Cox analysis. In the breast cancer data, applying both the mRNA expression and miRNA expression signatures yielded the most significantly prognostic immune cell population compared to using either signature matrix alone; M2 macrophages emerged as the most significantly prognostic cell type with an adverse relationship to survival in the breast cancer data ($P = 2.74e^{-7}$). Moreover, using the combination of the mRNA and miRNA expression signatures generated the greatest number of significantly prognostic immune cell types in the ovarian cancer data with neutrophils being the most correlated with poor survival ($P = 0.0009$). These results suggest that our integrated mRNA and immune cell signatures could be used to better delineate prognostic immune cell subsets within cancers, while further investigation may reveal important regulatory relationships between the predictive miRNA and immune cell associations found within our signature matrix.
T Lymphocytes and Type 1 Diabetes: Receptor for Advanced Glycation Endproducts Signaling

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Mentor: Kevan Herold, MD, Yale School of Medicine

The role of the receptor for advanced glycation endproducts (RAGE) in adaptive immunity is not understood. Patients with type 1 diabetes (T1D) express increased levels of intracellular RAGE in T cells, compared to healthy controls, as do euglycemic at-risk relatives who progress to disease. We studied gene expression by nanostring in T cells from type 1 diabetics and found differences in pathways utilized by RAGE+ versus RAGE- T cells including those affecting apoptosis, tumor necrosis factors/nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB)/B cell lymphoma-2 proteins, interferon-γ/interleukin-12-induced interferon-γ production, and platelet derived growth factor signaling via signal transducer and activator of transcription and NF-κB proteins.

RAGE is expressed intracellularly by Jurkat cells and therefore we used these cells to study RAGE signaling in T cells. We silenced intracellular RAGE with pooled siRNAs causing a 75% reduction in RAGE by flow cytometry fluorescein isothiocyanate mean fluorescence index. Using Jurkat RAGE knockdowns, we found that RAGE siRNA interferes with T cell receptor (TCR) signaling. Western blots of Jurkats with knocked-down RAGE express decreased baseline levels and signal amplitude of phosphorylated zeta-chain-associated protein kinase 70 at the beginning of the TCR cascade. RAGE siRNA decreases the signal amplitude of phosphorylated extracellular signal-regulated kinases 1/2 (Erk 1/2) downstream of the TCR on western blots. The addition of high mobility group box 1 protein (HMGB1), a known RAGE ligand, increases the amplitude of phosphorylated Erk 1/2 signal in submaximally stimulated Jurkats transfectected with negative control siRNA but not in Jurkats transfectected with pooled RAGE siRNAs.

RAGE+ cells may require the receptor for maximal signal through the TCR and RAGE activation through HMGB1 might further augment the signaling cascade. We postulate that this mechanism permits survival and proliferation of potentially autoreactive T cells in type 1 diabetes.

The role of postsynaptic cell-adhesion molecules in the trafficking of AMPA-type glutamate receptors

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Mentor: Thomas Südhof, MD, Howard Hughes Medical Institute and Stanford University School of Medicine

Long-term potentiation (LTP), an activity-dependent, long-lasting change in synaptic strength, is a critical component of synaptic plasticity underlying learning and memory and has been implicated in various neuropsychiatric disorders, including depression and addiction. The mechanism of LTP is dependent on the trafficking of AMPA receptors (AMPARs) to the post-synaptic membrane.

The present project is guided by the unexpected role by which cell adhesion proteins mediate trans-synaptic signaling. Specifically, the project focuses on post-synaptic neurologin 1, an isoform found in excitatory synapses, binding pre-synaptic neurexin. This heterophilic interaction is present in mature and developing synapses, and its absence has been shown to impair LTP.

Here, we generated various neurologin constructs where different functional domains of neurologin 1 are mutated. These constructs were then used to determine which neurologin domains are necessary for AMPAR exocytosis so that the precise mechanism by which neurologin are required for LTP can be elucidated. Methodically, dissociated mouse hippocampal cultures where all four neurologin isoforms have been conditionally knocked out were infected by viruses carrying the different neurologin 1 constructs. Subsequently, the ability of the neurologin 1 constructs to mediate LTP was monitored by inducing chemical LTP in these neurons and measuring the exocytosis of AMPARs using immunocytochemistry.

Our data suggest that chemical LTP is impaired upon deletion of neurologin 1, can be rescued solely by the extracellular domain of neurologin 1, is impaired when neurexin binding is inhibited, and does not require dimerization. Ongoing work includes investigating novel binding partners to the neurologin-neurexin complex.
Modeling fetal hemoglobin reactivation in sickle cell anemia induced pluripotent stem cells

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

Mentor: George Daley, MD, PhD, Harvard Medical School and Boston Children’s Hospital

Human induced pluripotent stem cells (hiPSCs) hold promise for both disease modeling and the development of novel therapeutic treatments for sickle cell anemia (SCA). Such models are practical systems to screen new drug therapies and to examine the effects of gene editing. In our study, we aim to employ hiPSCs to test the therapeutic hypothesis that genetic manipulation of BCL11A, a master regulator of fetal hemoglobin (HbF) expression, will ameliorate sickling. hiPSCs can theoretically produce all cell types, including erythroid cells. However, in vitro modeling of SCA with reprogrammed cells has been limited by their inability to differentiate into β-globin-expressing, enucleated erythroid cells. Here, we propose strategies to produce both in vitro and in vivo models of SCA using these cell types. We derived hiPSCs from sickle cell patients with hemoglobin SS disease seen at our hematology clinic at Boston Children’s Hospital. CRISPR/Cas9-mediated repair of the sickle mutation served as a key control. Using a cocktail of transcription factors promoting self-renewal and multi-potentiality expressed under the control of a doxycycline-regulated promoter (Erg, HoxA9, RORa, Sox, Myb), we began generating conditionally immortalized hematopoietic cell lines that serve as a renewable source of robust cell types. We differentiated from these lines was assessed following the transfusion of these cells into immune-radiated mice, with the goal of producing a humanized mouse model of SCA. Concurrently, we further assessed the efficacy of different in vitro differentiation protocols, laying the groundwork needed to test and evaluate gene knockdown of BCL11A and the CRISPR/Cas9 gene-editing of the erythroid-specific enhancer of BCL11A to reactivate HbF. The generation of hiPSC-SCA models will be critical in broadening the current understanding of the molecular mechanisms of this disease, the development of improved pharmacological treatments, and a future of autologous cell therapy for the cure of SCA.
and compare our graph-based results against a parallel assessment using conventional tools.

While our group’s immediate clinical focus is on orofacial cleft genomics, the technology we are developing has fundamental importance to genomics and will have broad application across many diseases, including other types of structural birth defects, other inherited disorders, and cancer.

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TPOT-MDR: toward automated machine learning pipeline design for GWAS analysis

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

Mentor: Jason Moore, PhD, Perelman School of Medicine at the University of Pennsylvania

Machine learning has been gaining larger traction in efforts toward meeting the demand for the analysis and predicative modeling of big data applications in the biomedical sciences. Effective utilization of machine learning, however, requires domain expertise (i.e., feature engineering, model-selection, hyper-parameter tuning, model validation, etc.), all of which can prove to be a high barrier of entry. Therefore, the existence of off-the-shell tools that make machine learning more accessible can prove to be beneficial. To this end, we have been developing an open-source tree-based approach toward automated machine learning pipeline design, TPOT-MDR, for bioinformatics using evolutionary computation (EC). EC is a biological evolution-inspired approach to optimization that has demonstrated its ability to find solutions to a wide variety of tasks, in which the evolutionary algorithms have outperformed manually constructed solutions.

TPOT-MDR is an extension of Tree-Based Pipeline Optimization Tool (TPOT), which is a Python tool that uses genetic programming (an implementation of EC) to automatically create and optimize machine learning pipelines for general data science. TPOT implements many popular techniques in machine learning; however, analysis of biomedical data sets benefits greatly from the addition and use of domain-specific knowledge and algorithms. As such, TPOT-MDR extends TPOT for use in bioinformatics with biological filter algorithms, multi-factor dimensionality reduction, and an expert knowledge source operator. The expert knowledge source operator allows TPOT-MDR to utilize and leverage any available results from previous data analysis on a given biomedical data set as a feature subset selector in the pre-processing step of the pipeline assembly.

Analysis of TPOT-MDR was performed on 16 different simulated data sets and a real-world data set. TPOT-MDR with and without expert knowledge sources (obtained from 4 biological filters—ReliefF, SURF, multiSURF, SURFstar) were compared against logistic regression and eXtreme Gradient Boosting (XGBoost), both which enjoy much success and popularity in data science. TPOT-MDR with the expert knowledge sources performed the best across all simulated and real-world data sets. Our results indicate that TPOT-MDR can automatically incorporate external expert knowledge sources in constructing and optimizing machine learning pipelines for biomedical data science.

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Identifying drivers of chemoresistance in group 3 medulloblastoma through a CRISPR/Cas9 genome-wide activation screen

Anne Song, HHMI Medical Fellow, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University

Mentor: Jeremy Rich, MD, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University

Medulloblastoma (MB) is the most common malignant brain tumor in children, accounting for nearly 20% of all pediatric brain cancers. Based on transcriptional profiling studies, MB has been characterized into four molecular subgroups—WNT, SHH, group 3, and group 4—that have distinct genetics, patient demographics, histology, and clinical outcomes. Group 3 MB commonly develops resistance and becomes refractory to standard therapy. As these mechanisms of resistance remain poorly understood, we are using the CRISPR/Cas9-based synergistic activation mediator (SAM) system to conduct a genome-wide, gain-of-function, positive selection screen to identify the specific drivers of chemoresistance in group 3 MB. We cloned and sequenced a library consisting of 70,290 single-guide RNAs (sgRNAs) targeting each of the 23,430 coding isoforms from the human RefSeq database. We have transduced this library into a group 3 MB patient-derived model stably expressing the other SAM components (dCas9, VP64, MS2, p65, and HSF1) and then selected with standard-of-care chemotherapy conditions: (1) cisplatin; (2) vincristine; (3) 4-hydroperoxycyclophosphamide; and (4) combination of cisplatin, vincristine, and 4-hydroperoxycyclophosphamide. Modulators of chemotherapy sensitivity will be prioritized by sgRNAs that have activated genes conferring chemoresistance, and we will perform next-generation sequencing to identify
Impact of inhibitory luminopsins in a mouse tetanus toxin model of spontaneous and recurrent epileptiform discharges

Matthew Stern, HHMI-CURE Medical Fellow, Emory University School of Medicine
Mentor: Robert Gross, MD, PhD, Emory University School of Medicine

The current worldwide burden of epilepsy is estimated at over 50 million, with about one third of patients refractory to medication. Although treatment options are available, open resection, gamma knife radiosurgery, and radiofrequency ablation are destructive and permanent, while responsive neurostimulation and deep brain stimulation are non-destructive and reversible options, each carries a risk for hardware malfunction and infection. Additionally, these methods are non-specific, impacting all tissue in the target region. Lumigenetics may provide a method for circumventing these limitations. Similar to traditional optogenetics, lumigenetics allows for cell type-specific neuromodulation. However, lumigenetic agents, luminopsins, obviate the need for implanted hardware by having a bioluminescent luciferase enzyme coupled directly to their opsin component. To assess the potential impact of lumigenetics in the context of epilepsy, an adeno-associated virus carrying an inhibitory luminopsin construct (iLMO2) that encodes halorhodopsin under a CAMKIIa promoter was injected intracranially into V1 in a mouse tetanus toxin model of spontaneous and recurrent epileptiform discharges. iLMO2 expression spanning V1 was verified by fluorescence microscopy (n = 2). Bioluminescent activity (n = 2) was characterized via transcranial bioluminescence imaging: iLMO2-expressing mice demonstrated greater than 4 hours of luminescence following intraperitoneal administration of e-CTZ (a synthetic analogue of the native coelenterazine substrate), with peak luminescence occurring at a 52.5 ± 0.5 minutes and luminescence greater than half of the maximum occurring from 18.5 ± 5.5 minutes to 111.5 ± 3.5 minutes (mean ± standard error). Epileptiform activity was measured through electroencephalography (EEG). A template matching algorithm that utilized a training set of manually identified epileptiform discharges was implemented across the EEG traces to identify similar discharges. Trace elements with convolution at least 4 standard deviations greater than baseline were isolated and individually verified. Preliminary data (n = 2) suggest that activation of iLMO2 through intraperitoneal injection of e-CTZ is able to decrease the occurrence of these discharges (20.75 ± 9.50 and 0.25 ± 0.50 discharges per hour before and after e-CTZ, respectively; mean ± standard error) as compared with vehicle (20.00 ± 3.00 and 3.50 ± 3.00 discharges per hour before and after vehicle, respectively; mean ± standard error).

It was noted that there was a propensity for the occurrence of these discharges during movement and that the mice exhibited less activity following CTZ or vehicle administration, which may account in part for the decrease observed in both experimental and control conditions. Thus, although potentially promising, the sample size will need to be increased, the impact of mouse activity further investigated, and the seizure model refined to produce more traditional ictal activity before more definitive conclusions can be drawn as to the potential of lumigenetics as a treatment for epilepsy.

Identifying small molecule epigenetic modifiers that impact the viability of patient tumor-derived primary glioblastoma cell lines

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Mentors: Albert Kim, MD, PhD and Joshua Rubin, MD, PhD, Washington University in St. Louis School of Medicine

Glioblastoma is an aggressive brain tumor with a median survival time of 15 months despite standard-of-care treatment that consists of maximal safe surgical resection, chemotherapy with DNA alkylating agent temozolomide (TMZ), and radiation therapy. Therapy resistance has been associated with a tumorigenic and self-renewing subpopulation of cells within the bulk tumor termed glioblastoma stem-like cells (GSCs). In addition to genetic and metabolic mechanisms, epigenetic processes are critical for maintenance of the GSC state. Thus, disrupting the epigenetic mechanism that governs the GSC state is a promising therapeutic approach. To this end, our laboratory first conducted a small molecule library screen of 85 epigenetic-modifying compounds to identify novel drugs that reduce the viability of patient tumor-derived, primary glioblastoma stem-like cell lines. To test these drugs in pooled primary cancer lines, a barcoding strategy was utilized by transducing three primary GSC lines with 30 unique base pair sequences paired with expression of either enhanced GFP, mCerulean,
or turbo RFP to generate stable lines expressing the fluorescent protein of interest. Preliminary results indicated that 1) qPCR with cell line DNA using primers specific for each barcode and 2) fluorescence intensity of expressed fluorescent proteins correlated well with cell number for each individual line. Using this system, we then screened for epigenetic-modifying drugs that decreased fluorescence intensity in pooled cell lines after administration of the drug library alone and in combination with TMZ for a period of 10 days. This screen yielded 21 drug hits that significantly reduced the viability of at least two of the three cell lines tested compared to the appropriate controls. Using the MTS colorimetric assay as an independent measure of cell number, we then selected eight of these drugs, whose effects on GSCs have not been previously described, for validation of the initial screen results. These drugs represented a number of the major classes of epigenetic modulators, such as DNA methyltransferase inhibitors, histone deacetylase inhibitors, histone methyltransferase inhibitors, and sirtuin inhibitors. These results 1) demonstrate the power of a bar-coded, pooled cell line approach that can be scaled up to screen multiple cell lines simultaneously in a time-, cost-, and labor-efficient fashion relative to non-pooled cell screens; and 2) reveal novel therapeutic agents that reduced GSC viability, with implications for GSC tumorigenicity and augmentation of the standard of care in glioblastoma patients.

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Epigenetic activation and cytoplasmic sensing of endogenous retroviral elements fuel pathologic immune signaling in mesenchymal subclones of small cell lung cancer

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Mentor: David Barbie, MD, Harvard Medical School and Dana-Farber Cancer Institute

Small cell lung cancer (SCLC) is an aggressive malignancy with significant intratumoral heterogeneity responsible for differential chemoresistance and metastatic capacity among tumor subclones. In particular, mesenchymal subclones in SCLC have been shown to harbor distinct cytokine profiles and activation of pathogenic innate immune signaling networks, including the TBK1 and STAT1 pathways. Recent work has suggested that exposure of these mesenchymal tumor cells to IFN-γ leads to production of double-stranded RNA (dsRNA) through transcription of endogenous retroviral (ERV) elements located in the 3’ UTR of IFN-γ-inducible genes in a sense and anti-sense direction. However, the mechanism of ERV de-repression or the consequences of this dsRNA production for pathologic innate immune signaling in mesenchymal subclones remains unclear. We began by comparing ERV expression patterns against CCLE and TCGA gene expression datasets across tumors and found strong correlation of ERV expression with targets of DNA methyltransferases (DNMTs) and the H3K27 histone methyltransferase EZH2. Treatment of the SCLC cell line H69 with the DNMT inhibitor decitabine and the EZH2 methyltransferase inhibitor GSK126 both independently resulted in significant increases in ERV gene expression. In particular, H69 cells treated with EZH2 inhibition adopted a mesenchymal morphology and an increased production of CXCL10, an innate immune cytokine strongly linked to tumorigenesis, pTBK1, a marker of pathogenic TBK1 innate immune signaling, and PD-L1, a T-cell immunosuppressive factor thought to help mediate immune evasion widely across tumors—suggesting EZH2i-induced de-repression of ERVs contributes to tumor propagation and immune evasion. To further characterize the role of dsRNA production in the activation of cytokine circuits in mesenchymal SCLC subclones, we show that treatment of the chemoresistant H69AR cell line with the synthetic dsRNA oligonucleotide poly(I:C) induces CXCL10 production, with this induction shown to be specifically through activation of IFN-β and largely independent of IFN-γ. Importantly, this CXCL10 upregulation is shown to be completely reversed with CRISPR-mediated deletion of the cytoplasmic dsRNA sensor MAVS in the H69AR cell line. In addition, CXCL10 production is partially inhibited by the dual TBK1/IKKε inhibitor MRT67307, and completely inhibited by the JAK1/2 inhibitor ruxolitinib. Taken together, these results support a mechanism in which H3K27 demethylation in an SCLC model leads to epigenetic de-repression of endogenous retroviral elements and production of dsRNA, whose sensing by MAVS fuels a pathologic inflammatory circuit marked by CXCL10, pTBK1, and PD-L1. Hence, these suggest that mesenchymal-associated alterations in histone demethylation lead to de-repression of ERVs and priming of immunogenicity in tumor subclones, identifying unique points of intervention which may have important consequences for immunotherapy in small cell lung cancer.
The impact on transcriptome diversity after oncogenic transformation of human mammary epithelial cells

Anthony Tran, HHMI Medical Fellow, Sidney Kimmel Medical College at Thomas Jefferson University (Harvard Medical School)

Mentor: Kornelia Polyak, MD, PhD, Harvard Medical School and Dana-Farber Cancer Institute

Breast cancer is a leading cause of cancer-related morbidity and mortality worldwide. In the USA, one of eight women will be diagnosed with breast cancer over the course of their lifetime. A major obstacle hindering the treatment of breast cancers is intratumor heterogeneity, since cytotoxic drug sensitivities among different clonal populations may vary. Previously, we have shown that cancer cells display an increased repertoire and abundance of all transcripts within a cell (termed transcriptome diversity) compared to normal cells. The driving mechanism(s) of cellular intratumor heterogeneity has yet to be fully elucidated. In this study, we utilize immortalized human mammary epithelial cells (MCF10A) transformed with an array of doxycycline-inducible lentiviral vectors encoding known genetic drivers of breast tumorigenesis (TP53 mutant, BRCA1 mutant, PIK3CA mutant, shPTEN, and c-MYC) alone and in combination to determine their impact on transcriptome diversity based on RNA-seq. Our strategy enables strict temporal control and reversibility of gene expression alterations via the doxycycline-inducible promoter. Transcriptome diversity will also be interrogated using single-cell RNA sequencing to determine how individual cellular transcriptional profiles contribute to the overall population-based transcriptional landscape. These results will be compared to patient tumor samples’ transcriptome diversity and correlated with their respective clinicopathologic characteristics. Advancing our understanding of cellular transcriptome diversity will enable new avenues of treatment for breast cancers resistant to current clinical interventions such as metastatic and triple-negative breast cancers, both of which are characterized by high transcriptome diversity.

The role of synaptic zinc in tinnitus-related hyperactivity

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Mentor: Thanos Tzounopoulos, PhD, University of Pittsburgh School of Medicine

Increased neuronal spiking (hyperactivity) in the dorsal cochlear nucleus (DCN), a part of the auditory brainstem, is linked with the induction of tinnitus (Li et al., 2013). This hyperactivity can result from increased excitatory synaptic transmission. Among modulators of excitatory DCN neurotransmission, zinc is a major modulator that inhibits NMDA and AMPA glutamate receptors (Anderson et al., 2015; Kalappa et al., 2015). We hypothesize that noise-induced loss of synaptic zinc in DCN synapses cause an increase in NMDA and AMPA receptors signaling, leading to neuronal hyperactivity and noise-induced tinnitus.

To assess noise-induced tinnitus in mice, we have been using the gap-prepulse inhibition of the acoustic startle reflex (GPIAS) paradigm, which is the most commonly used animal model of tinnitus (Li et al., 2013). This paradigm has been challenged recently due to observations inconsistent with the key assumption that the sound of tinnitus "fills in" the silent gap in background sounds (Campolo et al., 2013; Hickox and Liberman, 2014). Thus, we decided to further develop and employ an additional animal model of tinnitus through a modified version of the conditioned place preference test ("conditioned crossing" test, in our case) (Yang et al., 2011). This model entails the training of mice to associate the perception of a tone to crossing behavior (to cross from one room to the other room), which allows us to infer tinnitus perception from crossing behavior during silent periods.

In the conditioned crossing paradigm, mice are trained to cross from one room to the other room when they hear an external sound, whereas, silence is expected to not produce any crossing behavior. During training, whenever a pure tone is played, the mouse receives a mild footshock if it does not cross to the other room within ten seconds. When the success rate reaches a 70% threshold, mice are noise exposed to induce tinnitus. If the crossing rate during silence is significantly increased after noise exposure, this is considered behavioral evidence of tinnitus.

Over several different iterations of training with varying tone and silence conditions, our current most optimal iteration of training program averages 15.33 days until the mouse is trained. However, with this training program, only one of fifteen mice (6.67%) has yielded behavior consistent with tinnitus, with an increased crossing rate. Refinements to the training programs continue to be tested to better simulate tinnitus tone conditions without
compromising efficiency in generating mice with behavioral evidence of tinnitus. Once we establish this behavioral paradigm, we will perform ex vivo electrophysiological and imaging experiments in brain slices to investigate tinnitus-induced changes in zinc signaling. These experiments will determine whether impaired zinc transmission is correlated with tinnitus.

Understand the mechanisms of a neuron specific proteotoxic stress response

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Mentor: Steve Finkbeiner, MD, PhD, University of California, San Francisco, School of Medicine

Abnormal deposition of aggregated protein in neurons is a shared pathologic feature of the major neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, frontotemporal dementia, Huntington’s disease, and ALS. This fact suggests that there is a mismatch between the production and clearance of misfolded proteins in neurons. In an effort to understand whether and how neurons recognize and respond to misfolded protein, we found evidence that the heat shock response (HSR), one of the principal coping responses to misfolded protein, is significantly reduced in neurons, both in vitro and in vivo, compared with non-neuronal cells. Further studies by our lab suggested that heat shock factors (HSFs), the principal transcriptional activators of the HSR, may be processed differently by neurons and non-neuronal cells. We believe neuron-specific processing of HSFs may play a role in attenuating a canonical HSR in neurons and are currently studying potential mechanisms of neuron-specific HSF regulation. We also performed RNAseq to verify the absence of a canonical HSR and to probe for the existence of unique, neuron-specific responses to proteotoxic stress. Our analysis not only confirmed the absence of a canonical HSR in neurons but suggested a novel transcriptional response, which included upregulation of small nucleolar RNAs (snoRNAs). We are currently investigating the role of this transcriptional response, including snoRNA upregulation, as part of a novel, neuron-specific response to proteotoxic stress.

Tracking the frequency-power correlation of the subcallosal cingulate local field potential (SCC-LFP) during chronic SCC deep brain stimulation (DBS) for major depressive disorder (MDD)

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SCC-DBS is an emerging intervention for treatment-resistant MDD, with recent innovations to improve targeting by multimodal imaging-guidance. However, subsequent to implantation, no biomarkers exist to guide initial stimulation parameters or adjustments over the course of therapy, leaving such decisions to time-consuming trial-and-error approaches. Utilizing SCC-LFP data collected from the DBS electrode over a course of therapeutic SCC-DBS, we present results of a longitudinal assessment of the SCC as the MDD patient recovers from illness. Of several candidate biometrics, our results highlight fluctuations in the correlation between LFP power and frequency (hereafter defined as the 1/f slope)—previously assessed as a dynamic brain activity biometric relevant for tracking aging and disease state—which suggests DBS increases the randomness of SCC activity in SCC-DBS treatment responders.

MDD patients (n = 6) were implanted with an investigational stimulation/sensing system (Activa PC+S) with institutional informed consent and regulatory approval. SCC-LFP recordings with stimulation off were collected from a 1-month-post-implantation resting phase, weekly during a 6-month treatment phase, and from a 1-week single-blinded discontinuation experiment. 1/f slope was quantified on Welch periodograms of 15-second SCC-LFP epochs using simple linear regression. To validate the 1/f slope measure, we compared recordings between known waking and sleeping hours to attempt a reproduction of previously reported circadian 1/f slope transitions. Comparative statistics include a general linear model (GLM) to compare 1/f slopes across treatment phases, and a paired t test for sleep-wake comparisons. 5 of 6 patients who responded to SCC-DBS treatment by 6 months were included in this analysis.

As reported in previous human and animal studies, we observe a significant increase in the 1/f slope magnitude during sleeping hours (P < 0.05). As supported by the GLM (P < 0.05), the 1/f slope in the left electrode undergoes a decrease in magnitude with stimulation and a mild increase during the 1-week discontinuation experiment, notably not reaching the pre-treatment baseline, but returning to the pre-discontinuation slope levels with resumed stimulation. This study demonstrates through circadian 1/f changes the sensitivity of the SCC-LFP to detect large shifts in
SCC neurophysiological activity. 1/f changes across treatment phase, while less pronounced, and observed in a small sample size, suggest that SCC-DBS stably, and potentially therapeutically, increases the randomness of SCC neural activity. The observed time course provides a potential biomarker of clinical response over the course of DBS therapy.

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IL-33 producers modulate interactions between sensory neurons and regulatory T cells in skeletal muscle

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Mentor: Diane Mathis, PhD, Harvard Medical School

A distinct population of regulatory T cells (Tregs) that accumulates in injured skeletal muscle helps control inflammation and enhances muscle regeneration. These muscle Tregs express high levels of ST2, which encodes the receptor for the alarmin IL-33. The signaling of IL-33 through ST2 on Tregs has been shown to promote Treg proliferation and to promote muscle repair. The primary IL-33 producers in skeletal muscle are a subset of fibro-adipogenic progenitors (FAPs) within the stromal compartment, expressing the markers Sca-1 and PDGFRα. Using immunostaining of muscle whole-mounts, we observed that IL-33-producing FAPs are frequently associated with peripheral nerve bundles as well as small-diameter sensory fibers. In addition, low-input RNA-seq analysis revealed that both components (CALCRL and Ramp1) of the receptor for the pain-related neuropeptide calcitonin gene-related peptide (CGRP) are expressed in FAPs. These observations prompted us to further investigate potential neural-immune system cross-talk, specifically between sensory neurons and muscle Tregs, mediated by IL-33-producing FAPs. We observed that uninjured TRPV1-Cre/DTA mice have fewer muscle FAPs compared to wild-type littermates, while no difference was seen in IL-33 expression. The total percentage of IL-33+ cells was therefore lower in TRPV1-depleted mice due to the decrease in the FAP population. This suggests that TRPV1+ neurons may have some function in supporting the development or maintenance of the muscle FAP population. The fraction of Tregs in the CD4+ compartment was also reduced in TRPV1-depleted muscle. Single-cell RNA-seq analysis further revealed that CALCRL expression was highest in muscle FAP populations expressing the highest levels of IL-33. Intraperitoneal injection of CGRP resulted in a rapid decrease in IL-33 protein within muscle FAPs according to flow cytometric analysis, which was not directly correlated with a decrease in IL-33 mRNA, suggesting that CGRP signaling may trigger the release of IL-33. To determine whether the physiologic effect of CGRP release by nociceptors is mediated by the CGRP receptor on muscle FAPs, we performed intramuscular injections of capsaicin, which activates pain-sensing TRPV1+ neurons and stimulates CGRP release from TRPV1+ sensory afferent fibers. Capsaicin injection resulted in a significant decrease in IL-33 protein within FAPs by flow cytometry, consistent with the effect seen with CGRP injection. When the CGRP receptor antagonist peptide CGRP8-37 was injected prior to capsaicin, no significant difference in IL-33 expression was observed. Furthermore, repeated intraperitoneal injections of CGRP expanded the Treg population in uninjured muscle. Together, these results suggest that activation of nociceptors in skeletal muscle may mediate IL-33 release via CGRP signaling, promoting muscle Treg expansion. Furthering understanding of the interplay between stromal, immune, and neural components of the muscle ecosystem could reveal new targets for improving regeneration and limiting destructive fibrosis in diseases such as Duchenne muscular dystrophy and age-related sarcopenia.

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Reciprocal roles for mRNA binding protein IMP1 in exosome secretion and colorectal cancer cell tumor growth

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Mentor: Anil Rustgi, MD, Perelman School of Medicine at the University of Pennsylvania

Colorectal cancer (CRC) remains the third leading cause of cancer-related death in the United States. Mutations in oncogenes or tumor suppressor genes, such as APC, BRAF, KRAS, SMAD4, and p53, are known mediators of CRC initiation and progression, but less is known about mechanisms of CRC metastasis. The 5-year survival of patients with CRC is <15%, underscoring the importance of studies elucidating new molecular mechanisms contributing to CRC development or progression. Metastasis occurs when one or more cells with tumor-initiating potential dislodge from the primary tumor site and migrate via the blood or lymphatic system to a secondary site, where they proliferate to form additional lesions. In CRC, the liver is often the site of metastasis. Understanding the mechanisms that confer metastatic potential, migration, invasion, and proliferation is paramount to reducing and treating metastatic disease. Recent work shows that mRNA binding proteins play a role in CRC pathogenesis.
The insulin-like growth factor 2 (IGF2) mRNA binding protein 1 (IMP1) is highly expressed in CRC patient tumors and correlates with a worse prognosis. IMP1 is critical during development and is overexpressed in cancers, including colorectal cancer. IMP1 overexpression promotes xenograft tumor growth and dissemination into the blood. Exosomes are secreted vesicles that carry intracellular mRNA, miRNA, and/or protein cargo. Exosomes have been implicated in tumor metastasis, namely the preparation of a “pre-metastatic niche,” but the mechanisms governing exosome generation and cargo are not fully characterized. We sought to study roles for IMP1 in CRC metastasis and exosome biology.

We generated SW480 and HT-29 colorectal cancer cell lines that express different IMP1 levels. Cells were cultured in media containing exosome-depleted FBS. Exosomes were isolated from cultured medium by ultracentrifugation and quantified by nanoparticle-tracking analysis. Tumor formation was assessed using xenograft models.

Electron microscopy demonstrates that IMP1 expression increases cellular multi-vesicular bodies, which house exosomes prior to secretion. Exosome production is directly related to IMP1 protein levels, with cells expressing the highest levels of IMP1 protein secreting the highest number of exosomes. Remarkably, cell lines that produce fewer exosomes form larger subcutaneous xenograft tumors, implicating a possible switch between cell division/proliferation and exosome production in vivo. Additionally, an IMP1-overexpressing cell line that produces more exosomes also results in a higher metastatic tumor burden. Our findings indicate that IMP1 plays a novel role in exosome generation, which impacts tumor growth and may be critical to metastasis.

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**Dynamics of human IgE+ B cell differentiation**

**Chung-an Wu, HHMI Medical Fellow, University of California, San Francisco, School of Medicine**

**Mentor:** Christopher Allen, PhD, University of California, San Francisco, School of Medicine

Allergic diseases affect over 400 million individuals worldwide and represent a major burden on the quality of life of those afflicted. IgE is a central molecular mediator of the type I allergic hypersensitivity reaction and a critical point that may be targeted therapeutically, but how the IgE antibody response is regulated under physiological conditions is poorly understood. Prior studies in mice showed that IgE+ B cells exhibited a greater propensity for differentiation into antibody-secreting plasma cells and participated minimally in germinal centers, which are important for the generation of higher-affinity antibody and humoral memory. This suggests a physiological limiter on long-lasting, high-affinity IgE responses; however, whether these properties of IgE+ B cells in mice extend to humans is yet unclear. Here we used a sensitive flow cytometric approach to characterize the differentiation of human IgE+ B cells generated from blood and tonsil lymphocytes in an anti-CD40/IL-4-driven in vitro assay. We observed that IgE+ B cells had a stronger tendency to differentiate into plasma cells when compared with IgG+ B cells, consistent with existing reports in murine models. To better define the molecular signals guiding IgE antibody production, we also examined the effects of IL-21, which has previously been reported to amplify human IgE responses in vitro. We found that increased IgE production in the presence of IL-21 was largely due to enhanced B cell proliferation and plasma cell differentiation. In contrast, effects on IgE class switch recombination depended on other signals received by the cell, such as strength of CD40 stimulation or presence of B cell receptor ligation. Our results highlight key similarities and differences in the behavior of human IgE+ B cells relative to more established murine models, and our specific focus on cellular analysis of the IgE response provides new insights that may be relevant to human disease.

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**Mutation in an autism susceptibility gene, ANK2, leads to loss of L1CAM binding in long axon tracts**

**Danwei Wu, HHMI Medical Fellow, University of Central Florida College of Medicine (Duke University Medical Center)**

**Mentor:** Vann Bennett, MD, PhD, Howard Hughes Medical Institute and Duke University Medical Center

Multiple genome-wide studies have identified ANK2 as a high-probability gene associated with high functioning autism. ANK2 encodes two major ankyrin-B isoforms, a 220-kD isoform that is widely expressed and a 440-kD isoform that is expressed only in unmyelinated axons in the developing and adult nervous system. De novo frameshift and nonsense human mutations in ANK2 lead to either loss of both 440-kD ankyrin-B and 220-kD ankyrin-B or loss of only 440-kD ankyrin-B, suggesting that 440-kD ankyrin-B is the key autism target. Here, we investigate the role of 440-kD ankyrin-B in the nervous system using two models: (1) mice with loxP sites flanking the giant exon of 440-kD ankyrin-B crossed with mice expressing Nestin-Cre recombinase and (2) mice generated harboring a frameshift mutation (fs2580a) in the giant exon of ANK2. Both mouse lines are viable and fertile. Homozygous mice bearing fs2580a mutation have complete loss of 440-kD ankyrin-B, and heterozygotes exhibit a 50% reduction in expression. In wild-type mice, 440-kD ankyrin-B is found only in neurons and not in supporting glial cells, with highest expression in the neonatal period. 440-kD and 220-kD ankyrin-B are co-expressed in long axon tracts with L1CAM, a cell adhesion molecule linked to a neurodevelopmental disorder in humans characterized by abnormal axonal pathfinding in the corpus callosum and corticospinal tracts. Using the proximity ligation assay to detect association of L1CAM with ankyrin-B, we found a strong proximity signal of L1CAM and ankyrin-B in the long axon tracts of wild-type mice. In contrast, a proximity signal was barely detectable in mice with Y1228H mutation in L1CAM, which eliminates ankyrin binding and causes abnormal long-range axonal targeting. Strikingly, mice lacking only 440-kD ankyrin-B expression but retaining 220-kD ankyrin-B exhibited nearly complete loss of L1CAM proximity signal in long axon tracts, including corpus callosum, internal capsule, and fibrae. We conclude that 440-kD ankyrin-B is the major ankyrin-B isoform interacting with L1CAM in axons. These findings suggest a functional connection between 440-kD ankyrin-B and L1CAM, a protein known to function in axonal pathfinding, which could provide a molecular mechanism for aberrant neural wiring associated with autism.
Exploring the role of p63 in cancer

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Mentor: Paul Khavari, MD, PhD, Stanford University School of Medicine

p63 is a member of the p53 family (p53, p63, and p73) of tumor suppressor proteins. All three proteins share similar structures, containing a highly conserved set of functional domains and undergoing splicing to generate various isoforms. The most prominent member of the family, p53, is well known for its function in cell cycle control, DNA repair, apoptosis, and other tumor-suppressive roles in a vast number of cancers. However, the contribution of additional p53 family members to tumor suppressor function is not well understood. p63 has a well-established role as a master regulator of epidermal homeostasis, where it is required for progenitor function as well as terminal differentiation. Unlike p53, mutation of p63 is not observed in most cancers, suggesting it may have pleiotropic roles in a cancer setting. This may be in part due to expression of different isoforms in various tissues. Recent studies have shown that the ratio of two major p63 isoforms, one containing the amino-terminal transactivation domain (TAp63) and an alternatively spliced isoform without this domain (ΔNp63), contributes to tumor-suppressive function, with the TAp63 isoform having been shown to possess tumor suppressor activity. Interestingly, the ΔNp63 isoform is frequently expressed and/or amplified in squamous cell carcinomas, suggesting a potential oncogenic role in cancer. To better understand how the function of p63 is altered in the cancer setting, we turned to proteomics to investigate the p63 interactome in cancer cell lines. We first inspected the expression level of ΔNp63 at the mRNA and protein level across a variety of epithelial cancer cell lines using qPCR and Western blot analysis. We found high expression of ΔNp63 primarily in squamous cell carcinoma cell lines. In addition, we assessed the p63 dependency of these cancer cell lines using proliferation assays after siRNA-mediated knockdown of ΔNp63. We identified a number of p63-dependent cancer cell lines originating from epidermal, head and neck, and cervical squamous cell carcinomas. We then sought to characterize the p63 protein interactome in these cell lines using a method known as BioID. To achieve this, we introduced a fusion protein of p63 with BirA*, a promiscuous biotin ligase that upon addition of excess biotin to media attaches biotin to proximally interacting proteins. Using streptavidin immunoprecipitation followed by mass spectrometry, we have identified hundreds of ΔNp63-interacting proteins. Candidate interactors will be placed in a CRISPR screen for proliferation to determine the functional role of these proteins in cancer. Ultimately, this study will help us better understand the role of p63 in cancer and identify novel targets for therapy.

A three-component strategy to study neural control of feeding behaviors at the midbrain/hindbrain boundary in mice

Hui Yang, HHMI Medical Fellow, Albert Einstein College of Medicine (Janelia Research Campus)

Mentor: Scott Sternson, PhD, Janelia Research Campus

Neural circuits that have evolved to motivate food procurement during scarcity may contribute to excess caloric intake, obesity, and other metabolic diseases. Recently, intensive efforts to understand circuits that control feeding behavior in mice have pointed to an important role for axon projections from the paraventricular hypothalamic nucleus (PVH) to a poorly defined area including the ventrolateral periaqueductal gray (vPAG), dorsal raphe (DR), or parabrachial nucleus (PBN). We used muscimol nanoinjections to precisely identify the location of these appetite control neurons at this midbrain/hindbrain boundary. With a more precise localization of this appetite control region, we have set out to investigate the neural processes by which these neurons participate in appetite control. For this, we have been developing a three-component strategy to identify novel neuron types involved in feeding behavior. First, we will use single cell RNA sequencing (RNA-seq) to obtain gene expression markers of neurons in this appetite-controlling region, and define neuronal types based on these molecular markers. Second, neuronal activity within this region during the behavior of interest will be monitored by Ca2+ imaging. Third, after in vivo imaging, we will post hoc validate the molecular markers selected from single cell RNA-seq, and correspond Ca2+ dynamics with the molecular identities by multiplexed fluorescent in situ hybridization (fISH). Ultimately, these molecular markers can be used to develop strategies to manipulate the activity of these neurons. This approach may facilitate uncovering the role of neuronal types in a previously poorly understood brain region, and parallelize investigations of in vivo neuronal dynamics of all molecularly defined cell types in a brain region.
**Exercise blunts tumor growth via differential immune system modulation**

**Xiaojie Zhang,** HHMI Medical Fellow, Duke University School of Medicine  
**Mentor:** Mark Dewhirst, DVM, PhD, Duke University School of Medicine

Exercise as a mode of therapy has long been used in managing illnesses from diabetes to coronary artery disease. However, defining the role of exercise in cancer therapy has been limited. In our murine models of orthotopically implanted breast tumor 4T1-luc cells, exercise slowed tumor growth rates, reduced metastasis, and improved survival. We have shown that exercise reduces tumor hypoxia and improves tumor perfusion. These effects have shed light on the mechanisms by which exercise augments tumor killing by chemotherapy and radiation therapy (RT). As an adjunct to RT, exercise delayed tumor growth by 30% compared to irradiated controls. Tumors from exercising mice that reached endpoint (1,500 mm$^3$) exhibited 50% reduction in hypoxic area compared to sedentary mice. Furthermore, exercise led to greater CD31 colocalization to desmin, a marker of vascular maturity, as well as upregulated expression of angiogenic factors like VEGF.

Exercise modulation of tumor hypoxic environment gives rise to the deeper question of the role of the immune system in exercise-impacted tumorigenesis. It has been shown that immune cells are mobilized by exercise, but their activity is inversely related to hypoxic stress. Since exercise improves oxygenation, the present study aims to investigate the novel, *in vivo* effects of voluntary wheel running on the immune system in syngeneic BALB/c mice with mammary pad implants of 4T1-luc tumors. We hypothesize that exercise slows tumor growth via altered cytokine release and differential immune cell recruitment to the tumor site. Our preliminary results reveal that exercise increased tumor infiltration by cytotoxic T cells: via flow cytometry, we detected a two-fold infiltration by CD8$^+$ cytotoxic T cells in the tumors of exercise mice compared to sedentary mice. This differential recruitment of immune cells combined with less hypoxia may contribute to greater immune surveillance. We have also found exercise reduced pro-inflammatory cytokines such as leptin by 20% compared with sedentary control mice. Moreover, when the ratio of the anti-inflammatory cytokine adiponectin to leptin is examined, exercise increased this ratio by 50%.

We will further investigate whether exercise-induced tumor immune response is transferable from immunocompetent exercised vs. sedentary donor mice using an adoptive transfer method. We are currently establishing a radiation dose that is sufficient to deplete lymphocytes in recipient mice, without inducing whole body toxicity. This dose will be used for our adoptive transfer study. If the immune effects are transferable, tumor-bearing recipients of lymphocytes from exercise donor mice would demonstrate delayed tumor growth compared to recipients of lymphocytes from sedentary donor mice. Furthermore, we could see enhanced anti-tumor effects when lymphocytes are transferred from exercising donors to exercising recipients. This study will investigate novel mechanisms underlying how a highly accessible mode of therapy can affect tumor microenvironment and, ultimately, disease progression.