Dishevelled modulates ciliogenesis through an interaction with Developmentally Regulated GTP binding protein 1 (Drg1)

Cilia are critical structures for proper embryonic development and maintaining homeostasis. Malformation or dysfunction of both non-motile and motile cilia causes inherited human disorders, ciliopathies, which includes a wide spectrum of manifestation such as retinal degeneration, renal disease, cerebral abnormalities, and obesity. Motile ciliopathies are characterized by dysfunction of tissues and organs harboring the motile cilia machinery for generating fluid flow or mucus clearance, resulting in defects of brain development and fertility, and chronic airway diseases. Although cutting-edge proteomic and genomic analysis and imaging technologies have uncovered numerous novel components of cilia, there are still significant gaps in our understanding of all the proteins involved in regulating and directing ciliogenesis. We identify Drg1 (Developmentally regulated GTP binding protein 1) as a novel binding partner of Dishevelled (Dvl), a known regulator of ciliogenesis. Using the Xenopus laevis embryo as a model system, we show that morpholino-mediated knockdown of Drg1 causes a reduction in the length and number of cilia in the gastrocoel roof plate (GRP) as well as in multiciliated cells (MCCs) of the embryonic epidermis. While expression of wild type Drg1 rescues these ciliogenesis defects in the morphant, a Drg1 mutant with a reduced ability to interact with Dvl fails to rescue the phenotype. In addition, Drg1 morphants display defective basal body migration and docking to the apical surface of MCCs, as well as abnormal rotational polarity of basal bodies. Moreover, apical actin and RhoA activity are also decreased in Drg1 morphants, suggesting a possible cause for the deficient basal body behavior. Lastly, Drg1 knockdown results in decreased protein complex formation between Dvl and Daam1, and between Daam1 and RhoA. These results further support the concept that the Drg1/Dvl interaction regulates the nucleation and stability of MCC apical actin. Thus, Drg1 is a newly identified partner of Dvl in regulating ciliogenesis.

Design and application of LED-activated, thermolabile lipid nanocarriers for delivery of polyphosphate cell-signal into cell cytoplasm

Inositol phosphates (IPs) offer the quintessential example of the versatility of phosphate arrays in signal transduction: combinatorial placement around the inositol ring of three (as in IP3) to eight (IP8)
phosphates. This large family of signals controls diverse aspects of cell biology. But, with the exception of IP3, IP actions are poorly understood at the molecular level. Quantitative mechanistic information is essential to explain rheostatic (dose/response) actions; IPs are rarely on/off switches. Unfortunately, the high polarity of phosphate prevents IPs from directly entering populations of cultured cells. Currently, manipulation of cellular IP levels “with little quantitative control” relies on genetic or pharmacological targeting of enzymes supervising IP turnover; off-target effects present an added complication. Here, we describe the first use of thermolabile lipid nanocarriers, to deliver into cells any IP, in a titratable manner. For this work, we obtained a fluorescently-tagged IP (FAM-IP7) to optimize procedures. We used a lipid-film hydration/extrusion method to prepare biomimetic liposomes that included dipalmitoylphosphatidylcholine (DPPC) plus cyanine dye (DiR). FAM-IP7 was encapsulated into the liposomes (with 33% efficiency) through a postformation sonication/freeze-thaw/extrusion protocol. Transmission electron microscopy showed the liposomes are unilamellar. Dynamic light scattering showed size uniformity (204 nm diameter; polydispersity = 0.21), ensuring consistency of liposome interactions with cells. Liposomes were added to HCT116 cells in 6-well plates for 6 hr. Confocal microscopy and flow cytometry indicated >90% cell accumulation of liposomes into endosomes; no FAM-IP7 release into cytosol was observed. Next, we used high-power LED illumination (λ = 730 nm; 0.5 W; 2 min) to induce photothermal conversion by the DiR. This nanoscale heating initiates gel-liquid transition of the liposomal membranes (Tm DPCC = 41.1 °C), promoting fusion with the surrounding endosomal membrane; >60% of FAM-IP7 escapes into the cytosol. Electrophoresis of cell extracts showed the FAM-IP7 remained intact. There was no effect of LED irradiation upon cell integrity markers. This all-purpose method applies to any IP, or metabolically-stable analog, and avoids complex synthetic chemistry; we are currently delivering into cells defined amounts of IP7 to quantitatively study its role in cancer metastasis.

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Biochemistry - General, Proteins, and Lipids

Adipocyte Gi signaling regulates whole-body glucose homeostasis and insulin sensitivity
Adipocyte dysfunction links obesity to insulin resistance and type 2 diabetes (T2D). Receptor-mediated activation of heterotrimeric G proteins plays a pivotal role in regulating adipocyte function. Little is known about the potential in vivo metabolic roles of Gi-type G proteins expressed by adipocytes, primarily due to the lack of suitable animal models. To address this issue, we generated adipocre-ROSA26PTX mice in which Gi signaling was selectively disrupted in adipocytes ('adipo-Gi KO mice'). Compared to control mice, adipo-Gi KO mice displayed increased lipolysis and decreased fat mass when maintained on regular chow or a high fat diet (HFD). Moreover, HFD adipo-Gi KO mice showed impaired glucose tolerance, reduced insulin sensitivity, and significantly increased plasma levels of several pro-inflammatory cytokines, as compared with control mice. In vivo 14C-2-deoxy-glucose uptake assays demonstrated that the lack of adipocyte Gi signaling led to significantly reduced insulin-induced glucose uptake in adipose and skeletal muscle tissues. Consistent with this observation, in vitro studies with primary adipocytes prepared from adipo-Gi KO mice indicated that the lack of Gi signaling led to impaired insulin signaling and reduced insulin-stimulated glucose uptake. In parallel, we used a chemo-
genetic approach to generate a mouse line selectively expressing a CNO-sensitive, Gi-coupled DREADD (designer receptor exclusively activated by a designer drug) in adipocytes (adipo-GiD mice). CNO-induced activation of adipocyte Gi- signaling in adipo-GiD mice significantly improved glucose tolerance and insulin sensitivity independent of the diet that the mice consumed (regular chow or HFD). In vivo 14C-2-deoxy-glucose uptake assays demonstrated that activation of adipocyte Gi signaling enhanced insulin-induced glucose uptake in adipose tissues. Taken together, our data clearly demonstrate that adipocyte Gi signaling functions as a key regulator of insulin signaling in adipocytes and is critical for the maintenance of glucose homeostasis. Our data suggest that agents able of enhancing Gi signaling in adipose tissue might prove useful as novel antidiabetic drugs by enhancing insulin action in fat and other peripheral tissues.

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Biochemistry - General, Proteins, and Lipids

Intestinal PPARA deficiency protects against obesity-related non-alcoholic fatty liver disease and non-alcoholic steatohepatitis

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disorder in developed countries. NAFLD triggers an increased risk of non-alcoholic steatohepatitis (NASH) and the end-stage liver diseases cirrhosis and hepatocellular cancer. Drug therapy targeting NAFLD/NASH is extremely limited. Peroxisome proliferator-activated receptor alpha (PPARA) is a nuclear receptor that regulates lipid homeostasis and inflammation. PPARA controls lipid transport and beta-oxidation in liver. In recent years, intestine has emerged as a central organ participating in the control of lipid homeostasis. Interestingly, PPARA expression in the intestine was significantly higher in obese mice compared to that of lean mice. However, to date, the role of intestinal PPARA in NAFLD/NASH is poorly understood. To address this issue, intestine-specific PPARA knockout (Ppara-dIE) mice were examined to clarify the role of intestinal PPARA in the development of NAFLD and NASH. Wild-type (WT) mice fed a 60% high-fat diet (HFD) had marked obesity and hepatic steatosis, while Ppara-dIE mice showed less body weight gain and lower hepatic steatosis. Using a high fat/cholesterol/fructose diet (HFCFD) that promotes human-like NASH pathologic features in mice, Ppara-dIE mice exhibited lower NASH activity score (NAS) and fibrosis score compared with WT mice. Consistent with a decreased NAS, hepatic genes related to inflammation and fibrosis were significantly down-regulated in HFCFD-fed Ppara-dIE mice. Mechanistically, intestinal PPARA depletion reversed nutrient-excess diet-induced chemokine C-C motif ligand 2 (CCL2) expression in the intestine, thus decreased the recruitment of pro-inflammatory macrophage via the CCL2-C chemokine receptor type 2 (CCR2) axis and the subsequent production and secretion of pro-inflammatory cytokines such as IL-1beta, TNFalpha and IL-6. Besides, intestinal cholesterol absorption and transport was reduced in Ppara-dIE mice via down-regulation of Niemann-Pick C1-Like 1 (NPC1L1) and scavenger receptor class B type 1 (SRB1), leading to lower cholesterol levels in both serum and liver of Ppara-dIE mice. These factors collectively contribute to the amelioration of obesity-related NAFLD/NASH in Ppara-dIE mice. In summary, this study revealed that intestinal PPARA deficiency protected against obesity-related NAFLD/NASH, and thus intestinal PPARA antagonism may be a druggable target for the treatment of NAFLD/NASH.
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Spatially-segmented single-cell transcriptomics by diffusional accessibility to a small-molecule dye

Single-cell RNA-sequencing (scRNA-seq) is useful for describing cell states, but identifying the spatial arrangement of these states in tissues remains challenging. We describe SEgmentation by Exogenous Perfusion (SEEP), a rapid, integrated, method for providing spatial-segmentation to scRNA-seq data. Tissues are divided into layers based on accessibility of a fluorescent dye allowing sorted cells to be characterized by transcriptomic and regional identity. We use SEEP to explore the transcriptional states of cells in direct contact with peritoneal fluid in high grade serous ovarian cancer. High throughput scRNA-seq is used to describe complex tissues by characterizing transcriptional states of individual cells. Defining a cell’s position, both in regard to tissue margins and its social context, is essential for understanding the intrinsic and extrinsic variables that effect the transcriptional identity of individual cells. Mainstream high-throughput scRNA-seq assays, however, decouple cells from their original locations within tissues. In situ hybridization readouts of gene expression and in situ sequencing preserve spatial information in tissues, but currently have a lower total read threshold than NextGen sequencing imposing a restriction on either cell throughput or transcriptional breadth. Combining the above methods or using regional barcodes to define 2D positions have spatially-reconstructed tissue regions but are seldom employed in unspecialized laboratories. We describe an integrated method for correlating radial-spatial cell positions with scRNA-seq data. Critically, our method can be performed with common biological laboratory equipment and adds negligible cost to a high-throughput scRNA-seq assay. By employing a basic stain-and-sort method using an off-the-shelf live-dead stain, we are able to correlate transcriptional profiles with radial-spatial positions in both radially symmetric tissues (e.g., spheroids, organoids, spherical tumor masses) and linear tissue samples (e.g., punch biopsies). SEEP uses an imaging-based calibration step to inform the parameters of a FACS and sequencing based measurement step.

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The Impact of Cell Death on Tumor Heterogeneity and Fitness 
The complexity of treating cancer is largely associated with the vast levels of genomic diversity of tumor cells, commonly known as tumor inter- and intra-heterogeneity. Cancer therapeutics have failed to successfully eliminate tumor cells with different genomic landscapes. This clonal diversity drives therapeutic relapse as each tumor cell has a unique gene signature that allows it either to respond or to resist treatment. This response also varies over time as treatment can eliminate certain populations of sensitive cells, creating a vacant niche in which the resistant cells that overcame the cellular stresses of cell death are able to dominate and expand into more diverse populations. This selected-for tumor heterogeneity has been linked to worse patient prognosis and a decrease in overall survival. Research in identifying the drivers and cellular pathways that promote diversity has been limited. Thus, we hypothesize that increased levels of cell death fosters a greater level of tumor diversity due to selective, intrinsic mechanisms of resistant clones to bypass apoptotic stimuli, dominate the tumor cell population, and create a more aggressive disease state. Utilizing single cell RNA transcriptomic data of brain, colorectal, breast, head and neck, melanoma, and liver cancers, we show that the levels of cell death, determined by the expression of 92 genes involved in apoptosis, within each tumor type is positively associated with cell population diversity as measured by the Shannon Entropy Index. Moreover, this apoptosis gene signature is significantly associated with worse overall survival of liver cancer patients using bulk transcriptome data. To directly test if induction of apoptosis results in increased cellular diversity, we established liver cancer cell lines in which key anti-apoptotic genes are conditionally deleted by CRISPR/Cas9 gene editing. We have successfully and selectively knocked out MCL-1 in a liver cancer cell line to control for various amounts of cell death. Moving forward, heterogeneity of tumor cells will be quantified when subjected to various amounts of cell death to determine the causes of cancer cell diversity. While it seems paradoxical to associate cell death with tumor heterogeneity, this phenomenon may in part explain the selective advantages and fitness cancer cells acquire post-treatment and emphasizes the need to address cancer cell clonal evolution and adaptations when designing therapeutic interventions.

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Carcinogenesis 
Host immune reactivity and history of virus exposures defines early onset of liver cancer 
Liver cancer is one of the most common and lethal type of malignancies. Its incidence has more than tripled in US since 1980 to an estimate of 42,030 new cases and 31,780 deaths in 2019. The poor prognosis is mainly due to most of patients are diagnosed at an advanced stage. It is challenging to
detect liver cancer at an early stage so potentially curative treatments can be implemented. Patients with history of viral infections such as hepatitis B and C viruses are with higher risk of liver cancer. However, not all hepatitis B/C viruses infected individuals develop cancer. It would be very helpful for early diagnosis if we can predict cancer from viral infection history. Currently, the effect of viral infection on host long term health and the mechanism by which virus causes cancer remains elusive, and the association and interplay between host immune reactivity to viral infection has not been explored. We hypothesized that viral infection can alter host immunity in more subtle ways, leaving an indelible footprint on the immune system, which may contribute to cancer development. In this study, we tested an emerging synthetic virome technique termed phage immunoprecipitation sequencing (PhIP-Seq), a high-throughput method to profile the history of viral exposures using patient’s serum samples. This method can detect footprint of more than 200 human viral species including over 1000 strains. We successfully obtained viral infection profiles in a cohort of 487 patients with liver cancer or with chronic liver diseases as well as 412 healthy volunteers. Using Xgboost algorithm, we identified a viral signature that can identify individuals with liver cancer with an accuracy of over 85%. In addition, this signature was validated with a prospective cohort of over 200 patients. With these exciting results, we are now expanding our study by profiling a cohort of 3,400 patients with liver cancer from the TIGER-LC consortium. Our results indicate that this unique viral signature may be useful for early detection of liver cancer. Understanding of host-virus interaction may also provide mechanistic insight into hepatocarcinogenesis.

Leila Toulabi
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Carcinogenesis

A novel tumor suppressive role of Dopamine receptor D1 in lung cancer

We studied inherited susceptibility to lung cancer among genes associated with nicotine addiction and found that a single nucleotide polymorphism (SNP) in the Dopamine Receptor D1 (DRD1) gene is protective against the risk of developing lung cancer. As we made our observation in both ever and never smokers, the finding led us to hypothesize that the dopamine pathway (DAP) plays a role in lung cancer independent of nicotine addiction and smoking. As the DAP has not been studied in lung before, we first verified that dopamine receptors, metabolizing and synthesizing enzymes and dopamine transporters are expressed in normal human lung tissue. Using samples from the NCI-MD case-control study we found that DRD1 expression is reduced in lung cancer, data that we further validated in The Cancer Genome Atlas (TCGA). Using two cohorts we showed that this downregulation of DRD1 expression is due to enhanced methylation of the DRD1 promoter in lung cancer tissues compared with adjacent normal tissues. Notably, high DRD1 expression and low DRD1 methylation are associated with good patient survival (P <0.01). These findings suggest that DRD1 has tumor suppressive-like functions in lung cancer. To investigate this, we generated overexpression and knockdown cell line models. The main phenotype that we observed was modulation of cell proliferation and was validated in an additional 2 cell lines. An analysis of global phosphorylation levels of up to 100 proteins in these cell line models suggested that DRD1 decreases EGFR signaling via MAPK. To validate this, we specifically examined EGF/MAPK and found that DRD1 decreases EGFR phosphorylation, p-MEK, p-ERK and cyclin
D1 in both overexpression and knockdown cell line models. We further observed that DRD1 inhibits EGFR signaling by reducing EGFR mRNA levels. Interestingly, DRD1 agonists and antagonists are used, for a range of physiological applications. To assess whether these observations could be pharmacologically relevant, we treated three lung cancer cell lines with both DRD1 agonists and antagonists. Only DRD1 agonists induced cell death, a mechanism we were able to show is due to downregulation of BCL-2 and BCL-X. Our novel findings show that DRD1 acts like a tumor suppressor in lung cancer. These findings could be translated to a novel therapeutic strategy since FDA-approved DRD1 agonists exist and could be used as combinatorial agents with other drugs to induce apoptosis in lung tumor cells.

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Cell Biology - Cell Adhesion and Motility

Focused Ion Beam Scanning Electron Microscopy of Escherichia coli uncovers the role of a bacterial histone-like protein in the fimbriae formation

Fimbriae, or pili, are proteinaceous appendages found on both commensal and pathogenic bacteria that facilitate attachment to host tissues. The formation of fimbriae is under environmental regulation through complex gene regulatory networks involving specific and global transcription regulators. The histone-like protein HU is a conserved chromosomal architectural protein of the bacterial kingdom. HU not only participates in the condensation and three-dimensional (3D) organization of the bacterial genomes, but it also regulates the expression of a large number of genes. HU is essential in many bacterial pathogens and has been used as a target to develop new antibiotics. Although HU is not essential in Escherichia coli, the absence of HU causes the formation of filamentous and anucleate cells due to the improper organization of the genome. Because of large-scale changes in gene expression, we hypothesized that the absence of HU results in other morphological changes in E. coli. To test our hypothesis, we performed high-resolution 3D imaging of E. coli using focused ion beam scanning electron microscopy (FIB-SEM). To preserve ultra-structures in a near-native state, we cryofixed bacteria by high-pressure freezing and carried out freeze-substitution to bring samples to ambient temperature for imaging. Strikingly, we observed the presence of fimbriae in the strain lacking HU but not in the wild-type. To further investigate if the formation of fimbriae in the absence of HU is due to up-regulation of fimbriae genes, we carried out global transcription analysis using DNA microarray. We indeed observed the up-regulation of both fimbriae structural genes and regulatory genes in the strain lacking HU demonstrating that HU controls the formation of fimbriae through the transcriptional regulation of fimbriae genes. Interestingly, we observed that HU inhibited flagellar genes. Flagella are another proteinaceous appendages that are larger than fimbriae and are required for bacterial motility. Based on these observations, we propose that HU plays a crucial role in the host colonization through the coordinated transcriptional regulation of flagellar and fimbriae genes. We are currently investigating the molecular mechanism of how HU regulates flagellar and fimbriae genes. Our findings have broader implications in the understanding of the colonization of animal hosts by commensal and pathogenic bacteria.
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Cell Biology - Cell Cycle and Division  

*Investigation of the roles of Drosophila DDX42 on ovarian growth and oocyte development.*

The ovary is extremely sensitive to environmental stresses and nutritional resources. Our lab is interested in understanding how metabolism and its dysregulation impact meiotic progression and growth during oogenesis. Target of Rapamycin Complex 1 (TORC1) is a master regulator of metabolism and promotes growth in response to multiple upstream inputs. Compromising TORC1 activity in the female germline leads to a reduction of ovarian growth and a block of oocyte development. Conversely, mutations in inhibitors of TORC1 result in premature ovarian failure in humans. Our lab completed a large-scale RNAi-based screen to identify genes that when silenced, suppress the ovarian phenotypes of mutants with low TORC1 activity. From this screen we identified a novel gene encoding for the putative DEAD box-containing RNA-binding helicase (DDX42). To confirm that DDX42 suppresses the growth deficit associated with low TORC1 activity we generated a null mutant of ddx42 by CRISPR-Cas9. Notably, the ddx42 mutants rescued the TORC1 mutant growth phenotype, thus confirming the results from our screen. ddx42 null homozygous mutant ovaries display the presence of multiple same-sized previtellogenic follicles further implying that DDX42 regulates TORC1 activity and growth during oogenesis. Since DEAD box proteins have been widely implicated in multiple stages of RNA processing and translation, we investigated the genome-wide gene expression changes induced by loss of ddx42 in the ovary. By RNA-seq and real-time quantitative PCR we found that dFOXO is the most significantly downregulated gene (more than 30 times) compared to control ovaries. dFOXO is the only Drosophila ortholog of the forkhead family of transcription factors which have important roles in the regulation of growth and the response to stress and is deleted in a wide array of cancers. Moreover, a deeper transcriptome analysis of the exon regions and splice junctions (JunctionSeq) revealed that almost 600 genes are differentially spliced (p<0.0001) in the absence of DDX42 in the ovary. Currently, we are using a GFP-tagged version of DDX42 to perform pull down experiments and identify partners of DDX42 and its target mRNAs. By this approach, we hope to gain a better understanding of how this novel interplay between DDX42, dFOXO and splicing contributes to the regulation of TORC1-dependent growth during oogenesis.

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**Prabhu Sankaralingam**  
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NIDDK  
Cell Biology - Cell Cycle and Division  

*In vitro Analysis suggests that ZYG-1 Phosphorylates SAS-5 to control Centriole Assembly Outcomes.*

Centrioles are microtubule-based organelles that direct bipolar spindle formation and serve as basal bodies for nucleating cilia. In order to function properly, centriole number must be tightly maintained, and in large part, this is accomplished through a precise duplication event that happens once each cell cycle. Underscoring the importance of maintaining proper centriole number, defects in centriole
duplication have been linked to cancer, growth retardation syndromes, and primary microcephaly. Pioneering studies in C. elegans have identified a conserved set of centriole assembly proteins (SPD-2, ZYG-1, SAS-5, SAS-6, and SAS-4), that function more or less the same across genera. Although general details of the assembly process have been worked out, significant gaps in our knowledge still exist. In particular, we do not understand how the master regulator of this process, the kinase ZYG-1/Plk-4, orchestrates the protein-protein interactions underlying centriole formation. A major hurdle to address this and other questions has been the inability to produce sufficient quantities of full-length and functional recombinant proteins. Using a novel approach, we have managed to express abundant levels of full-length centriole assembly factors in E. coli. Using these proteins, we have demonstrated that ZYG-1 binds to SAS-5 and phosphorylates it in vitro. Many of the phosphorylated residues are conserved, and interestingly four of these are located in a conserved motif that we have found mediates binding to both ZYG-1 and SAS-4. Further, our work indicates that the precise pattern of phosphorylation within this region dictates binding specificity; phosphorylation of two residues drives SAS-5 to exclusively bind SAS-4, presumably leading to production of a daughter centriole, while phosphorylation of all four residues excludes SAS-4 and drives SAS-5 to enter into an apparent dead-end complex with ZYG-1 to prevent further daughter centriole formation. We have begun validating our findings in vivo and have found that some of these phosphorylation events are important for proper centriole assembly. In summary, our work reveals a novel mechanism of regulation that determines the fate of early centriole assembly intermediates and thereby likely contributes to maintaining the proper number of centrioles.
that the E3 ubiquitin ligase function of nopo is required in the nucleus to prevent MB cell death. In addition to small MBs, nopo mutants have axon guidance defects including MB axons improperly crossing the midline and misguided axon tracts in the central brain. Mutants for bendless, which encodes an E2 that interacts with Nopo, have properly sized MBs that improperly cross the midline similar to nopo mutants. Thus, our working model is that nopo has multiple functions in neurogenesis: a DDR function in the nucleus during interphase, a centrosome-spindle cohesion function during mitosis, and a potential function with bendless in axon guidance. Our work reveals a novel mitotic role for a DDR gene in neurogenesis, suggesting a deeper link between the two pathways commonly mutated in microcephaly.

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Cell Biology - General

Adipocyte death preferentially induces liver injury and inflammation via the activation of CCR2+ macrophages and lipolysis

BACKGROUND AND AIM: Adipocyte death occurs under various physiopathological conditions, including obesity and alcohol drinking, and can trigger organ damage particularly in the liver. Although a link between adipocyte death and liver injury has been suggested by numerous studies, the underlying mechanisms by which adipocyte death trigger liver injury and other organs remain obscure. To explore these mechanisms, we developed a unique mice model of inducible adipocyte death by human CD59 (hCD59) on adipocytes (adipocyte-specific hCD59 transgenic mice). METHODS: The Cre-inducible human CD59 knockin mice were generated by crossing Cre-inducible floxed STOP-CD59 knock-in mice with adiponectin Cre transgenic mice. Acute adipocytes death was induced by Intermedilysin (ILY), which rapidly lyses hCD59 expressing cells exclusively by binding to the hCD59 but not mouse CD59. At the time point of sacrifice, blood serum, liver tissues and adipose tissues were collected and analyzed to define the role of adipocyte death in liver injury. RESULTS: Injection of ILY causes acute selective death of adipocyte death and elevation of serum free fatty acid (FFA) levels in ihCD59AdTG mice. ILY injection also resulted in the secondary damage to multiple organs with the strongest injury observed in the liver, with inflammation and hepatic macrophage activation. Mechanistically, acute adipocyte death elevated epinephrine and norepinephrine levels and activated lipolysis pathways in adipose tissue in a CCR2+ macrophage-dependent manner, which was followed by FFA release and lipotoxicity in the liver. Additionally, acute adipocyte death caused hepatic CCR2+ macrophage activation and infiltration, further exacerbating liver injury. DISCUSSION: Acute and selective adipocyte death can trigger adipose tissue macrophage infiltration and lipolysis and predominantly induces liver damage via the activation of CCR2+ macrophages, which is probably due to the superior sensitivity of hepatocytes to lipotoxicity and the abundance of macrophages in the liver. These findings should stimulate further studies of adipocyte death and lipolysis in NASH patients.
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*A 3D Bioprinted Human Neurovascular Unit as a tissue-in-a-well model for drug screening for Glioblastoma and neurological diseases*

Glioblastomas (GBM) are a fast-growing, aggressive type of central nervous system tumors that arise from astrocytes and form on the supportive tissue of the brain. In spite of all the efforts towards the understanding of the physiology of GBM tumors, the prognosis for patients with high-grade gliomas is generally poor, and as a result there is a tremendous medical need for more effective treatments.

Biofabricated 3D tissue models are being developed as physiologically-relevant assay platforms for drug testing. It is widely accepted that angiogenesis and vascular permeability play critical roles in the progress of GBM by increasing the local concentration of vascular endothelial growth factors (VEGFs). We therefore have developed a 3D neurovascular unit (NVU) containing human primary/pluripotent stem cell derived vascular endothelial cells, pericytes, and astrocytes using bioprinting/bioink technologies. This NVU provides a physiologically-relevant microenvironment for GBM growth and spread, and because it is developed in a multiwell plate platform, it is possible to use it for rapid drug screening. For the NVU tissue biofabrication, GFP expressing primary ECs, pericytes, and astrocytes are mixed with a fibrin-based hydrogel, called bioink, and are bioprinted onto the 24/96 well plates. Bioprinting was used to create a spatial design with an initial cell-free zone made with cell-free hydrogel within the NVU on the well to allow the quantitation of angiogenesis (vascular sprouting) and the vasculogenesis (tubulogenesis of ECs) in the tissue. Vasculogenesis and angiogenesis was observed within three days after the bioprinting. Increased number of new vessels and the vessels reaching into the center of the cell-free zone were quantitated using cell-based fluorescence imaging and an in-house developed MATLAB algorithm. The in vitro induced angiogenesis within the 3D NVU resembles in vivo microvascular characteristics. In conclusion, an innovative 3D bioprinting platform that enables rapid and robust production of 3D neurovascular unit tissues in multiwell plate format has been developed. This bioprinted 3D NVU is a powerful platform upon which to build increased cellular complexity by incorporating neuronal cells and GBM tumor cell and thus creating a more predictive assay platform for drug development for GBM and other neurological diseases.
Human, Murine, and In Vitro Studies Identify RORA as a Modulator of Hepatic Steatosis and Severity of Non-Alcoholic Fatty Liver Disease

Non-alcoholic fatty liver disease (NAFLD) is becoming the most common cause of chronic liver disease with limited treatment options. A single nucleotide polymorphism (SNP), rs339969 in the RAR-related Orphan Receptor Alpha (RORA) gene, is associated with plasma liver enzymes levels. Previous studies demonstrated a role for RORA in the regulation of lipid and energy metabolism. However, the role of RORA in the liver metabolism remains unexplored. The current study aims to uncover whether and how hepatic RORA affects NAFLD progression. In a cohort of 768 subjects with biopsy-proven NAFLD, SNPs within the RORA gene (rs339969, rs1657792, and rs8040067) are significantly associated with liver fat, histological liver injury, and/or plasma liver enzyme levels. The minor allele frequency of rs339969 is significantly different between NAFLD and healthy controls. Hepatic expression of RORA is increased 2.7-fold in patients with NAFLD compared with healthy controls. Further, the minor alleles of rs339969 and rs1657792 associate with increased RORA gene expression, while the minor allele in rs8040067 has an opposite effect, consistent with their different patterns of association with liver histology, indicating these variants may affect NAFLD progression by regulating RORA expression. In HepG2 cells, knocking-down the expression of RORA by siRNA stimulated lipid accumulation and increased the gene expression of fatty acid synthase (FASN), a key de novo lipogenesis (DNL) gene. Consistently, siRORA significantly increased DNL as determined by 14C-glucose incorporation assay. Mechanistically, immunofluorescence staining and qPCR demonstrated that siRORA increased nuclear translocation and activation of SREBP1, a key transcription factor regulating lipid synthesis, by inhibiting the expression of INSIG2 and thus augmenting proteolytic activation of SREBP1. Data obtained from primary human hepatocytes confirmed modulation of the INSIG2-SREBP1-FASN pathway by RORA. In vivo, liver-specific Rora knock-out mice (Rora-KO) mice on high-fat diet had hepatic steatosis, elevated serum liver enzymes levels, worse insulin resistance, and elevated hepatic expression of lipid synthesis-, fibrosis-, and inflammation-related genes, supporting an important role of Rora in driving hepatic steatosis and injury. In conclusion, our data suggest that RORA plays important roles in hepatic lipid metabolism and the pathogenesis of NAFLD by regulating DNL.

Towards autonomous Microscopy: Artificial intelligent microscopy screening (A.I.M.S.)
The identification of new pathways via genetic screens has been a central strategy in cell biology. Historically, this work was performed in yeast; however, for translational research in would be important to develop mammalian screening platforms. Traditionally, RNAi-based screens have been used to elucidate pathways in human cells. This approach has relied on two major strategies: arrayed screens, which have high specificity but require the production of each vector separately creating a
technical bottleneck, and pooled screens, where production is easier, but specificity and reproducibility suffer. To overcome these limitations, we have developed a novel screening approach termed Artificial-Intelligence Microscopy Screening (designated AIMS). The new platform "converts" single cells into separate wells by applying machine learning and deep learning algorithms to detect subcellular phenotypes. In brief, a genome-wide suppressor screen is performed on cells expressing dCas9 (CRISPR inhibition) by infecting a single chamber of cells with pooled guide-RNA (gRNA)-expressing lentivirus so that every cell will express a distinct gRNA. These cells are also stably-expressing a photo-activatable red fluorescent protein (pa-mCherry). Before the screen, an AI-model is trained on several examples of the desired phenotype. Pooled gRNA-infected cells are screened by microscopy and individual cells exhibiting the desired phenotype are identified, photoactivated, and isolated via flow cytometry. To define phenotype to genotype connections, the gRNA lentiviral integration site is sequenced in single cells from the selected sorted cells. Using AIMS, we were able to identify critical factors involved in selective organellar degradation pathways with high accuracy. Currently, AIMS screens aimed to identify genes involved in distinct subcellular scenarios, such as protein localization, organelle formation, and organelle positioning are in development. Our approach is not only a novel demonstration of how machine learning can be used to improve cell biology research and discovery, but, our new platform also enables phenotypic-based screening at the subcellular level, an approach which has only been available to big pharma or committed cores in the past. Additionally, this screening approach can be implemented for drug target exploration and may provide a new way to sort single cells from complex human samples, for example, T-cells for immunotherapy.

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Impaired lysosome transport to distal axons contributes to autophagic stress in the neurodegenerative lysosomal storage disorder Niemann-Pick Type C

Niemann-Pick Type C (NPC) is a neurodegenerative lysosomal storage disorder characterized by accumulation of multiple lipids in late endosomes and lysosomes. NPC patients typically present clinically with heterogenous systemic changes followed by progressive neurodegeneration that leads to premature death. An early pathologic feature of NPC is axonal dystrophy, which consists of bulbous swellings along axons that contain accumulated organelles associated with the autophagy-lysosomal pathway. Such changes occur before symptom-onset and degeneration in NPC mice and suggest that defects in axonal organelle transport contribute to early NPC pathology. However, the mechanisms underlying these pathologic changes remain obscure. Here, by culturing neurons in microfluidic devices that provide physical and fluidic separation of axons from neuronal cell bodies and dendrites, we demonstrate that mature lysosome delivery to distal axons is significantly reduced in cortical neurons from Npc1 null mice, resulting in fewer numbers of lysosomes in NPC distal axons. Fluorescence microscopy analysis of the cell body revealed increased integrated density of endolysosomal proteins Lamp1 and glucocerebrosidase in NPC relative to WT, while western blot showed no change in total levels of these proteins between the genotypes. These results indicate that reduced axonal lysosome density in NPC does not result from an overall reduction in neuronal lysosome levels but suggests a
change in lysosome distribution. Consistently, time-lapse imaging of live cortical neurons displayed reduced anterograde lysosome transport in NPC distal axons, reflecting a specific defect in lysosome delivery. To determine the potential impact of this defect on axonal health, we assessed autophagosome distribution and motility in WT and NPC axons. We found that decreased axonal lysosome density leads to increased axonal autophagic stress that occurs without changes to autophagosome transport in NPC axons. Interestingly, this reduction in axonal lysosome density could be overcome by elevated expression of Arl8b, a small GTPase that mediates kinesin-1-dependent lysosome transport to the cell periphery. Rescuing axonal lysosome density by Arl8b expression reduced autophagic stress in NPC axons. Collectively, these observations suggest that impaired lysosome transport to distal axons disrupts maturation and progression of the autophagy-lysosomal pathway and contributes to altered axonal homeostasis in NPC.

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Cell Biology - Intracellular Trafficking, Cytoskeleton, and Extracellular Matrix
OXYSTEROL-BINDING PROTEIN-RELATED PROTEIN 3 (ORP3) IS A PKC-REGULATED LIPID TRANSPORTER THAT WORKS AT CONTACT SITES BETWEEN THE PLASMA MEMBRANE AND ENDOPLASMIC RETICULUM
Cellular cholesterol metabolism is integral to the control of lipid homeostasis in all eukaryotic cells. In fact, several human diseases are caused by the improper synthesis or transport of cholesterol, including familial hypercholesterinemia and Smith-Lemli-Opitz syndrome as clinically-relevant examples, and understanding how lipids are distributed between organelles will help us to comprehend the
pathophysiology of these diseases. Recently, oxysterol-binding protein (OSBP)-related proteins (ORPs) have been shown to mediate the non-vesicular transfer of lipids between cellular membranes; including examples of cholesterol and phospholipid exchange between the plasma membrane (PM) and endoplasmic reticulum (ER). Interestingly, our recent work has identified ORP3 as a potential regulator of lipid homeostasis that associates with the PM after activation of protein kinase C (PKC). However, it remains unclear what lipids are specifically targeted for transport by ORP3 as well as how PM recognition is occurring. Previous studies have identified phosphoinositides (PPIs) as important regulators of ORP functions, so we chose to use GFP-tagged forms of ORP3 to investigate if the PPI composition was responsible for the interaction of ORP3 with the PM in transiently transfected HEK293 cells. Therefore, a chemically-inducible heterodimerization approach was used to selectively modify PPI levels by recruiting PPI phosphatases directed against either the 4- or 5-phosphates of phosphatidylinositol 4-phosphate (Pi4P) and/or 4,5-bisphosphate (Pi(4,5)P2) to the PM. PM attachment of ORP3 was monitored with TIRF microscopy, while changes in PPI lipids were measured using our recently developed BRET-based biosensors in live cells. Importantly, catalytically inactive phosphatases were used as control for each experiment. Our results show that Pi(4,5)P2 is necessary for PM association of ORP3 and Pi4P also plays a lesser, but significant role. The significant changes were assessed by ANOVA followed by Holm-Sidak test. We also found that ORP3 mediates the transfer Pi4P from the PM to the ER, consistent with the use of the Pi4P gradient between the PM and ER for the counter-transport of a secondary lipid out of the ER. Current experiments are aimed at explicitly identifying the lipid cargoes of ORP3 using lipidomic analysis of cells following inactivation of the endogenous protein and defining the relationship between ORP3 with other proteins that are localized to contact sites.

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Cell Biology - Intracellular Trafficking, Cytoskeleton, and Extracellular Matrix
*Malaria parasite protein trafficking and insertion at the host membrane tracked with a novel split reporter assay*
Several malaria parasite proteins are exposed on the host cell surface, mediating critical functions such as cytoadherence and nutrient acquisition. How these proteins traffic to and insert in the host membrane is unknown, hindering therapy development against these vulnerable targets. Mechanistic
insights have been limited by difficulties in parasite transfection and the lack of a host membrane insertion-specific reporter. We addressed these problems with CRISPR/Cas9 transfection of the human pathogen, Plasmodium falciparum, and a novel split NanoLuc reporter assay. CRISPR editing permitted insertion of a varying copy number of a small 11 aa NanoLuc bait fragment into an extracellular loop of CLAG3, a parasite protein linked to channel-mediated nutrient uptake from human plasma. Successful host membrane insertion was confirmed with 1, 2, 3, 5, and 7 copies of the bait using luminescence measurements after extracellular addition of recombinant LargeBiT, a protein that interacts with the bait to yield luciferase activity. Protease susceptibility, nutrient uptake assays, confocal imaging, and immunoblotting all supported this conclusion. Because our studies suggested a compromised signal and altered nutrient transport in parasites engineered with higher bait copy number, we then used bioluminescent microscopy to image individual infected erythrocytes. This approach determined that nearly all cells from the clone carrying a short single copy of the bait faithfully inserted the protein into their host cell; in contrast, < 1% of the cells that express the 7-copy bait correctly trafficked the chimeric protein, implicating previously unknown epigenetic control of protein trafficking in this clone. A separate transfection, using a truncated clag3 gene, revealed that a C-terminal transmembrane domain is also required for host membrane insertion of this parasite protein. Finally, we used kinetic studies over the parasiteâ€™s life cycle to track stage-specific CLAG3 membrane insertion. A miniaturized version of this kinetic assay is being optimized for high-throughput screens to find chemicals that block trafficking of parasite proteins to the cell surface. These studies define the constraints on parasite protein trafficking to the host membrane. They also provide insights into activation of nutrient channels at the RBC membrane and should guide development of novel therapies that interfere with host cell remodeling.

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**Alessandro Bonifazi**  
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Chemistry  
The significance of chirality in drug design and synthesis of bitopic ligands as D3R selective agonists
The development of selective dopamine receptor agonists is a subject of increasing interest due to the potential therapeutic applications in neurological disorders. Due to the large degree of homogeneity among the D2-like family of dopamine receptors, achieving ligands capable of discrimination among them remains a significant challenge. Previous work from our lab has shown the use of bitopic ligands to
be a powerful strategy in achieving increased D3R selectivity for antagonists. Inspired by the potential for chemical modification of the D3 preferential agonists (+)-PD128907 and PF592379, we sought to synthesize and test a variety of bitopic structures to further improve their D3R selectivity. When in a bitopic configuration, the (S,S) conformation of the PF592379 primary pharmacophore resulted in a privileged architecture with increased affinity and selectivity for the D3R orthosteric binding site. Driven by an earlier finding revealing the inclusion of a cyclopropyl moiety in the linker of the bitopic molecule may induce a structural orientation favorable for D3R selectivity and allosteric modulations, we proceeded to synthesize the bitopic compounds of the privileged (S,S)-PF592379 primary pharmacophore with a cyclopropyl ring in the linker. Incorporation of the ring in the linker and full resolution of the chiral centers present allowed us to analyze the effect of the stereochemistry of the linker on the final affinity and selectivity of the bitopic molecules synthesized. Binding studies were performed in presence of agonist radioligand, to specifically assess affinities for the receptors’ active states. Lead compound FOB02-04 (D2R Ki: 106 nM, D3R Ki: 2.84 nM, D4R Ki: 315 nM, D2R/D3R: 37.3, D4R/D3R: 111) and its most active enantiomer FOB02-04A (D2R Ki: 87.8 nM, D3R Ki: 1.85 nM, D4R Ki: 286 nM, D2R/D3R: 47.5, D4R/D3R: 155), may have the highest D3R to D2R selectivity reported for agonists, to date. The high structural complexity of these compounds may inspire future computational studies to better understand ligand-receptor interactions, as well as underscore potential biased agonism as a consequence of specific receptor conformations. Moreover, due to their high D3R selectivity and metabolic stability in mice liver microsomes, FOB02-04 and the eutomer FOB02-04A, may have the potential to become the main pharmacological reference tools for future D3R in vitro and/or in vivo studies.

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Chemistry

* Determination of E/Z-Conformation for 5-Methylene Substituted Hydantoins by EXSIDE NMR
The determination of stereochemistry within a compound is critical for the development of small molecule therapeutics as structure is highly involved in a drug’s activity. NMR spectroscopy is a primary method for the determination of molecular structure and configuration, especially when acquiring an x-ray crystal structure is hindered by the inability obtain to well-diffracting crystals. However, NMR can often provide data that are difficult to interpret or inaccessible due to molecular flexibility or complexities in 3D configuration. Moreover, NMR experiments to correctly identify stereochemistry (i.e., E/Z-conformation) of small molecules are often very time consuming. Thus, it is imperative to develop an NMR platform which can rapidly and easily identify stereoisomers of small molecules through easy-to-interpret NMR parameters; e.g., the chemical shift and long-range 1H, 13C coupling constants (3JCH), the latter of which can distinctly identify E/Z conformation. Here, we developed and demonstrated the viability of this approach for nearly 40 5-methylene substituted hydantoins utilizing a variation of the standard HSQC experiment called the Excitation-Sculptured Indirect-Detection Experiment (EXSIDE). After structural assignment for each compound by the straightforward measurement of chemical shifts (1H and 13C) and homonuclear coupling constants (3JHH) with a standard suite of 1D and 2D NMR experiments, EXSIDE was used to measure long-range
heteronuclear coupling constants (3JCH) to accurately identify the E/Z-conformation. Additionally, we implemented a rapid acquisition technique, non-uniform sampling (NUS), which reduced data acquisition time of the EXSIDE NMR experiment for a single compound from 12 hours to 2 hours. The development of analytical techniques to expeditiously acquire quantitative markers for structural deconvolution of small molecules is essential, especially in the design of therapeutic treatments. As such, our application of the EXSIDE NMR coupled with NUS provided an efficient method for the structural identification of 5-methylene substituted hydantoins where biological activity was imparted by the stereochemistry of the exocyclic double bond. The stereospecific assignment of these compounds enabled us to positively identify an active Z-stereoisomer that inhibited the Six1-Eya2 transcriptional protein-protein complex whose overexpression is implicated in diverse tumor types such as Wilms’ tumor, ovarian cancer, and breast cancer.

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Chemistry
LUMO stabilization leads to a new generation of red-shifted dyes
The minimal autofluorescence produced by the biological tissues in the near-IR (NIR) region makes it an important region of the electromagnetic spectrum to have fluorophores to operate in. The current set of NIR-emitting dyes, for example, cyanine-based fluorophores, suffer from inherently low photostability and poor physical properties. Various fluorescence microscopy techniques require bright NIR-emitting fluorophores with high chemical and photostability. Only a few fluorophores suitable for these applications absorb beyond 700 nm; a void that our project aims to address. Initial in-silico DFT analysis predicted a stabilization effect on the LUMO of the pyronin motif, when the C-10 position is replaced by electronegative functionalities, resulting in red-shifted dyes. For the first time, a correlation between the experimental absorbance maxima and the LUMO electronic Mulliken population at C-10 position (R2 = 0.96), was established. On the basis of our in-silico modeling, we synthesized a series of keto-rhodamine dyes that were obtained by replacing the ether linkage on a xanthene scaffold with a ketone-functionality. These keto-rhodamine dyes have dramatically red-shifted absorbance and emission maxima (abs. max. = ~851 nm and em. max. = ~905 nm) in comparison to their rhodamine counterparts (abs. max. = 498 nm, em. max. = 529 nm). This observation validated our DFT predictions. In an attempt to extrapolate this hypothesis to other dyes, we chose the widely used amino-coumarin dye. It was
observed that introducing a gem-difluoro ketone functionality into the core structure of the amino-coumarin scaffold to replace lactone motif (abs. max. = 377 nm, em. max. = 450 nm), resulted in a substantially red-shifted scaffold (abs. max. = 481 nm, em. max. = 579 nm). We also found that these difluoro coumarin dyes can be further bathochromic-shifted into NIR via modifications at the C-2 and C-3 positions. So far, the most red-shifted analog operates at abs. max. = 600 nm and em. max. = 720 nm. In conclusion, introducing electronegative functionalities leads to dramatically red-shifted dyes via LUMO-stabilization and will lead to the identification of new molecules for in vivo imaging applications.

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Chromatin and Chromosomes
The erythroid differentiation-associated gene HEMGN cooperates with SWI/SNF complex ATPase Brg1 and cohesion to regulate globin gene activation and hemoglobin switching

Considerable effort is devoted to exploring novel regulators involved in the switch from fetal to adult hemoglobin during development since reactivation of fetal hemoglobin in adult life significantly ameliorates the symptoms of Sickle Cell Anemia and Beta-thalassemia. We found that the erythroid differentiation-associated gene HEMGN (also called EDAG) is required for globin gene activation and globin switching during differentiation of human primary erythroid CD34+ cells. Knock down of HEMGN in CD34+ cells attenuated both fetal (gamma) and adult (beta) globin gene expression and delayed the hemoglobin switch since expression of the adult globin gene was almost eliminated. In accord, knock out of HEMGN in human K562 cells by CRISPR/CAS9 genome editing significantly impaired the transcription of embryonic and fetal globin genes detected by RNA-seq. Up-regulated genes upon HEMGN loss were associated with biological process of protein deacetylation and regulation of histone modification, while the down-regulated genes were highly enriched in the pathway of regulation of hematopoietic progenitor cell differentiation, hemoglobin complex and chromosome organization, suggesting a role for HEMGN in regulation of epigenetic status and chromosome architecture. ChIP-qPCR revealed that HEMGN mainly occupied the enhancer region in the beta-globin locus, including DNA Hypersensitive sites (HS1-HS4) in locus control region (LCR) and the newly identified gamma globin enhancer, BGLT3. In addition, HEMGN binds to HS5 which is occupied by CTCF and cohesion proteins. Mass Spectrometry identified novel co-factors of HEMGN including SWI/SNF remodeling complex (Brg1 and BAF47), cohesion complex (SMC1 and SMC3) and erythroid transcription factor LDB1. Interestingly, loss of HEMGN resulted in reduced occupancy of Brg1 in the LCR enhancer and gamma globin promoter, and correspondingly increased nucleosome density. Moreover, SMC3 occupancy in HS5 and 3â€™HS1 and LDB1 occupancy in LCR were significantly decreased upon HEMGN KO. Consistent with cohesin/SMC3 and LDB1 as major regulators of chromatin looping, KO of HEMGN disrupted HS5/gamma globin and LCR/ gamma globin looping, which is required for activation of globin genes. Our results reveal that the key function of HEMGN in globin gene activation is carried out through recruitment of nucleosome remodelers and looping modifiers to control LCR chromatin accessibility and chromatin organization in the beta-globin locus.
Niek Van Wietmarschen
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Chromatin and Chromosomes

TA dinucleotide repeats are sites of recurrent spontaneous replication fork stalling

Complete genome duplication is critical for maintaining genome integrity in dividing cells. However, replication forks are known to stall frequently during S-phase, possibly leading to stretches of non-replicated DNA. Under-replicated DNA interferes with chromosome segregation, leading to gain or loss of chromosome segments or entire chromosomes, and onco genesis. Replication intermediates at under-replicated DNA are normally cleaved by mitotic nucleases MUS81 and GEN1 to prevent chromosome mis-segregation, at the expense of generating DNA double strand breaks (DSBs) which are not repaired until after mitosis. Although spontaneous replication fork stalling is believed to occur multiple times in each replicating cell, it is currently not known if this occurs stochastically or at specific genomic loci. Here, we used END-seq to map sites of replication fork stalling genome-wide. END-seq can detect DSBs, but not stalled forks. To overcome this limitation, we treat isolated genomic DNA with purified GEN1 enzyme in vitro to cleave replication forks into detectable DSBs. We performed END-seq on resting murine B cells, which reside in G0/G1, and cycling B cells, ~70% of which are in S-phase. We detected thousands of recurring stalled forks throughout the genome in GEN1-pretreated cycling cells, but not in resting cells or without GEN1 treatment. These stall sites are highly reproducible and occur at stretches of TA dinucleotides, indicating these TA repeats impede replication fork progression. DSBs are produced at the same sites in vivo when cells are forced to prematurely enter mitosis, indicating the same stalled forks are bona fide targets of mitotic nucleases. We performed the same experiment in several human and murine cell lines, and detected spontaneous DNA breakage at TA repeats even in the absence of GEN1 pre-treatment. Breaks are not detected when S-phase cells are blocked from entering mitosis, but are reconstituted when DNA from these cells is pre-treated with GEN1. We conclude that replication fork frequently stall at TA repeats in S-phase, and these sites are cleaved during mitosis because replication cannot be completed during S-phase. Our data indicate that while fork stalling at TA repeats occur in all cell types, forks only break during mitosis in (pre)cancerous cells, potentially contributing to onco genesis. We are currently investigating the mechanism behind the observed breakage patterns and how breaks are repaired.

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Chromatin and Chromosomes

The accessibility of human centromeric chromatin is modulated by elastic CENP-A nucleosomes

At the heart of a mitotic chromosome sits the centromere, which is epigenetically defined by the histone H3 variant CENP-A/CENH3. CENP-A nucleosomes seeds the kinetochore, creating the physical interface between chromosomes and mitotic spindles. Despite lack of sequence conservation at the level of CENP-A protein or its associated centromeric DNA, CENP-A chromatin provides the epigenetic and structural
foundation of the centromere. This is made possible by the recruitment of a triad of inner kinetochore proteins: CENP-B, CENP-C, and CENP-N. In specific, CENP-C bound to CENP-A nucleosomes is essential for kinetochore formation. How the inner kinetochore complex structurally affects CENP-A chromatin in vivo remains an open question. Here, we combined in silico, in vitro, and in vivo methods to understand how CENP-A nucleosomes create a unique centromeric chromatin environment. Using all-atom molecular dynamic simulations, we found that CENP-A nucleosomes are highly distortable compared to H3 nucleosomes, but CENP-C fixes specific conformational states. We computationally and experimentally tested the relative effective elasticity of free CENP-A nucleosomes compared to CENP-C bound CENP-A nucleosomes. To our surprise, both in silico and in vitro methods show that CENP-A is far more elastic relative to H3 nucleosomes. Remarkably, upon CENP-C binding in vitro, CENP-A nucleosomes stiffen by three-fold, and subsequently cause three-dimensional clustering of either recombinant or native CENP-A chromatin fibers. In parallel, overexpression of CENP-C in vivo leads to overcompaction of centromeric chromatin, which is concomitant with a marked reduction in the levels of RNA polymerase 2 (RNAP2) occupancy at centromeres. The loss of RNAP2 occupancy correlated with reduced new CENP-A loading in early G1. Finally, we show that overexpressing CENP-C leads to extensive mitotic defects, which can be rescued by expressing mutants of CENP-A which are either unable to bind CENP-C, or which cause CENP-C to be sequestered away. Together, these data suggest a model in which the innate distortable structural properties of CENP-A nucleosome create a chromatin environment that is conducive of transcription, an essential event for new CENP-A loading, but which is repressed by kinetochore bound CENP-A chromatin. It stands to reason that in dividing cells, a balance between kinetochore bound and free CENP-A chromatin must exist to maintain centromere fidelity.

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Chromatin and Chromosomes  
INO80 Regulates Chromatin Landscape and Transcription to Maintain the Primed Pluripotent State  
Recent studies suggest that there exist different pluripotent states. Mouse embryonic stem cells cultured in 2i/LIF medium are in the naïve state and mimics the early epiblast cells. They can contribute to blastocyst chimeras and the germline when injected into pre-implantation embryos. In contrast, mouse post-implantation epiblast stem cells represent the primed state. They do not form blastocyst chimeras efficiently or germ cells. Like mouse cells, human naïve and primed stem cells have also been derived. In both species, cells in the naïve and primed state require different culture conditions and show distinct epigenome. However, how these states are maintained and how the dynamics between the two states is regulated epigenetically remain poorly defined. To address these questions, we systematically examined the chromatin accessibility by ATAC-seq in the naïve, transitional, and primed state. We found that a global change of chromatin accessibility during the transition from the naïve to the primed state and hypothesized that the changes may be regulated by chromatin remodeling complexes. Next, we examined the involvement of the four major ATP-dependent chromatin remodelers, including SWI/SNF, ISWI, CHD and INO80. We found the genomic occupancy of INO80 highly correlates with the chromatin accessibility in each state. Further, INO80 occupancy mirrors enhancer markers and is associated with genes that show state-specific expression. To test its function,
we examined the impact of Ino80 deletion on chromatin accessibility and gene expression in two states. In the primed state, Ino80 deletion led to cell differentiation and death, suggesting it is required for maintaining the primed state. Consistently, Ino80 deletion led to largely impaired chromatin accessibility and expression of genes near INO80-bound loci. Surprisingly, in the naïve state, Ino80 deletion had minimal effect on both cell growth, chromatin accessibility, and gene expression. Compared to the naïve state, we observed that the chromatin in the primed state is more compact with more prominent occupancy by repressive marks such as polycomb group proteins. Therefore, we propose that INO80-dependent chromatin remodeling is selectively required to facilitate gene expression in the primed state. Together, our study uncovers a unique chromatin state in the naïve pluripotent cells, and defines the role of INO80 in the dynamic regulation of chromatin and transcription in different cellular states.

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Chloride intracellular channel protein 4 (CLIC4) is a serological cancer biomarker released from tumor epithelial cells via extracellular vesicles and required for metastasis.

Introduction CLIC4 is a highly conserved metamorphic protein originally described as an ion channel. It translocates to the nucleus serving as an integral component of TGF-ß signaling. In multiple cancers, CLIC4 is a tumor suppressor, excluded from the nucleus and lost from the cytoplasm of progressing cancer cells. In contrast, CLIC4 is upregulated in the tumor stroma acting as a tumor promoter. CLIC4 lacks a secretory sequence, but recent reports indicate that CLIC4 is detected in the circulation of cancer patients serving as possible biomarker and has been detected in extracellular vesicles (EVs). Methods EVs from cell culture supernatants or biological fluids were isolated by differential centrifugation, following ultracentrifugation and Optiprep density gradients. EV size distribution and concentration were analyzed by NTA and TEM. The presence of prototypical markers and CLIC4 were analyzed by immunoblot and by tissue staining. Results CLIC4 was present in EVs released from primary normal and multiple breast tumor cell lines and increased in EVs from TGF-ß-induced myofibroblasts. In vivo, in two different orthotopic syngeneic mouse breast cancer models, CLIC4 levels in EVs isolated from plasma increased with tumor burden and lung metastatic load. Moreover, CLIC4 levels in EVs isolated from plasma of breast cancer patient was elevated when compared to healthy age and race matched controls. To dissect the contribution of stromal vs tumor epithelial compartments as the source of the CLIC4-high EVs, CLIC4 was either deleted in tumor cells lines by CRISPR/Cas9 or CLIC4 KO females were implanted CLIC4 WT tumor cells. CLIC4 is reduced in circulating EVs from CLIC4 KO tumor bearing mice when compared to WT and it is present in circulating EVs from CLIC4 KO females bearing WT tumors, indicating that the major contribution of CLIC4 into circulation is from tumor epithelium. Additionally, CLIC4 KO females display no difference in primary tumor size and a significant reduction in both size and number of lung metastases. Summary CLIC4 levels in EVs from biological fluids may have value as a cancer biomarker, in conjunction with other markers, to detect or analyze tumor progression or recurrence. The low lung metastasis frequency in CLIC4 KO females may due to a defect in lung tissue to support the formation of a metastatic niche mainly by poorly neutrophil recruitment, myofibroblast conversion, and probably neovasculature.
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**Voriconazole and cutaneous squamous cell carcinoma risk among lung transplant recipients**

**Background:** Solid organ transplant recipients have greatly elevated risk of cutaneous squamous cell carcinoma (SCC), an aggressive skin cancer. Voriconazole (vori) is used to prevent and treat invasive aspergillosis in lung recipients. It can cause phototoxicity and has been associated with increased SCC risk, but previous studies were modestly sized. **Methods:** In order to examine the association between vori exposure and SCC, we used the Scientific Registry of Transplant Recipients (SRTR), restricted to white lung recipients (2007-2016). Exposure to immunosuppressant and antifungal (vori, itraconazole [itra], posaconazole [posa], other) medications was ascertained from pharmacy claims linked to the SRTR. Follow-up began at transplantation and ended at the earliest of SCC, transplant failure/retransplant, death, loss to follow-up or 12/31/2016. Person-time was divided into 30-day intervals, with medication exposure assessed at the start of each interval. We excluded all persons missing >25% of immunosuppressant interval coverage. Intervals lacking an immunosuppressant or antifungal claim were assigned unknown exposure status, because that suggested incomplete pharmacy data. Antifungal use was treated as a time varying exposure. Cox models adjusted for sex, age, transplant reason and number, induction therapy, smoking history, total ambient ultraviolet exposure, and other antifungal use were used to assess the association between vori and SCC. **Results:** There were 9,738 lung recipients (median age 59 years, interquartile range [IQR] 48-65), of whom 59% were male. Median follow-up was 3.0 years (IQR 1.4-5.0), and recipients had immunosuppressant medication data for 75% (IQR 51%-90%) of 30-day intervals. There were 1,031 SCCs observed over 32,064 person-years (incidence 322 per 10,000 person-years). Overall, 41%, 26%, and 13% of recipients had a claim for vori, itra, and posa, respectively. Compared to individuals with no observed vori use, those with 1-3, 4-7, 8-15, and >15 months of vori experienced increasingly elevated SCC risk: adjusted hazard ratio (aHR) 1.1 (95%CI 0.9-1.3), 1.4 (1.2-1.7), 2.0 (1.7-2.5), and 3.0 (2.4-3.9) For each 30-day vori use, SCC risk increased 5% (aHR 1.05, 95%CI 1.04-1.06). There was no association between other antifungals and SCC. **Conclusions:** Vori may play a role in the etiology of skin cancer, and high-risk lung recipients (e.g., fair skin) should be counseled on potential adverse effects of vori before initiation.

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Clinical and Translational Research - General

**NAD+ supplementation prevents age-related hearing loss in mice**

**Age-related hearing loss (ARHL) is one of the most common disorder affecting elderly populations. The prevalence of hearing loss accelerates dramatically with age. Despite its high prevalence and cost, there are no interventions that prevent ARHL. Studies have revealed that defects in the cochlea are major**
contributors to ARHL. The cochlea is the auditory portion of the inner ear and it contains specialized cells including inner and outer hair cells. These cells have many mitochondria, are metabolically active, reflecting high energy demand for proper auditory function. Indeed, reduced mitochondrial function has been proposed as one of the major mechanisms underlying ARHL. We have reported that mitochondrial deficits can be reversed by supplementation with the essential metabolite NAD+. Given that hair cells are high-energy requiring cells, we hypothesize that NAD+ repletion may ameliorate the hearing decline in ARHL through enhancement of mitochondrial homeostasis. To address this hypothesis, we have examined hearing loss in aging mice by measuring electrical potential changes derived from the auditory brain stem (ABR). ABR is a technology to record brain wave activity and measure hearing thresholds in response to sound with different intensities (Decibel-dB) and frequencies (Hertz-Hz). We initially measured hearing status of young mice at 2 months of age (23 male and 22 female) and we observed that the hearing threshold for high-frequency sound (32 kHz) was 30.1 dB, which is considered in normal range for healthy hearing. Following initial ABR measurements, we randomly split the cohort into 2 groups for NAD+ supplementation for 6 months. We used Nicotinamide Riboside (NR) as the NAD+ precursor delivered in drinking water. At 8 months, the non-treated group significantly developed high-frequency hearing loss by 21.2 dB (p<0.0001; N=20), and hearing threshold increased by %70. Strikingly, NR-treated mice did not develop any significant hearing loss at high-frequencies (p=0.23, N=25) and hearing threshold increased only by 20%, suggesting that NAD+ intervention prevents high-frequency hearing loss in mice. Next, we will perform cochlear histology analysis to identify whether NAD+ augmentation prevents cochlear hair cell loss and improves mitochondrial quality in cochlear cells. Given that NR is a natural product with no known toxicities in mice, rats or people, it might be an effective intervention against hearing loss in aging populations.

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NCI-CCR
Clinical and Translational Research - General
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Adipokines and Gestational Diabetes- A Longitudinal Study to identify early biomarkers/predictors in a multi-racial pregnancy cohort
Gestational Diabetes (GDM) is a common pregnancy complication and has adverse health implications on both women and their offspring. Hyperglycemia in pregnancy has been regarded as a teratogen affecting fetal structure, function, and development starting in early pregnancy. GDM is typically
screened for in late pregnancy, leaving little time for intervention to be effective. Therefore, early prediction and intervention to prevent GDM is important. Several adipokines such as leptin and adiponectin are implicated in the development of GDM, but the etiological and clinical relevance of recently identified adipokines to GDM has been underexplored. Therefore, we prospectively investigated a panel of adipokines during early to mid-pregnancy in association with GDM risk and in prediction of GDM. Among the NICHD Fetal Growth Studies-Singleton cohort (2009-2013) of 2808 pregnant women, 107 GDM cases were identified and matched to 214 non-GDM controls on age, race, and gestational week (GW) at blood draw. Plasma leptin, soluble leptin receptor (sOB-R), free leptin, chemerin, fatty acid binding protein 4 (FABP4), retinol binding protein 4, adiponectin, omentin1, and vaspin were measured twice at GWs 10-14 and 15-26, before GDM diagnosis. Adjusting for maternal age, GW of blood collection, nulliparity, and family history of diabetes, conditional logistic analysis was performed to estimate adjusted odds ratios (aOR) for associations of adipokines with GDM. Receiver-operating-characteristic (ROC) curves with n-1 cross-validation assessed the predictive value of adipokines for GDM diagnosis. At GW 10-14, more than 12 weeks before GDM is typically screened for, adiponectin and sOB-R were significantly and inversely related to GDM risk. For example, across increasing quartiles of sOB-R the aOR of GDM were 1.00, 0.37, 0.28, and 0.24 (Ptrend=.0001). In contrast, leptin, free leptin, chemerin, and FABP4 were significantly and positively associated with GDM risk (all P for trend <.001). At GW 15-26, similar but slightly stronger associations of adipokine concentrations with GDM were observed. In addition, at GW 10-14, sOB-R significantly improved GDM prediction over conventional risk factors (age, GW, race, nulliparity, family history of diabetes, prepregnancy BMI) and glucose (P=0.02). A panel of adipokines may be implicated in the pathogenesis of GDM and some may improve the prediction of GDM in early pregnancy, more than 12 weeks before GDM is usually screened for.

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A Methylation Density Binary Classifier for Predicting and Optimizing the Performance of Methylation Biomarkers in Clinical Samples
Aberrant DNA methylation is a hallmark of carcinogenesis and commonly heralded as a promising cancer biomarker. A particularly attractive application for methylation biomarkers is use in liquid biopsies,
which represent minimally invasive assays to detect circulating tumor DNA (ctDNA) and associated epigenomic alterations when shed from cancerous tissues into the blood. However, there exist a number of technical hurdles involved in translating promising methylation biomarkers for use in liquid biopsies, including (i) the small proportion of plasma ctDNA relative to cell-free DNA (cfDNA) derived from healthy cells, (ii) heterogeneity of methylation at a given locus, (iii) age-associated accrual of methylation confounding marker selection, and (iv) differences in the yield of extracted cfDNA between samples. In the present work, we introduce a methylation density binary classifier aimed at maximizing the performance of methylation biomarkers in dilute, composite solutions (e.g., liquid biopsies) by leveraging statistical differences in the methylation density profile between sample cohorts.

Methylation density, or the proportion of methylated CpG-dinucleotides in a given genomic locus, takes advantage of coupled CpG methylation that can overcome background methylation due to technical or biological effects but is agnostic to changes to the methylation pattern and therefore also has the potential to achieve high sensitivity. With this classifier, we first demonstrate our ability to optimize classification of ovarian cancer tumors based on methylation density profiles derived from reduced representation bisulfite sequencing reads of ZNF154, a recurrently methylated locus in multiple cancer types. We then use in silico simulations to predict the performance of the classifier in liquid biopsies and validate these predictions using quasidigital melt curve analysis of cfDNA from 91 plasma samples from individuals with and without ovarian carcinoma. We find strong agreement between expected and observed classifier performance; and that implementation of this approach with ZNF154 outperforms the conventional protein biomarker, CA125, for detection of etiologically-diverse ovarian cancer types. Our results indicate that methylation density profiles can be exploited to predict and facilitate implementation of methylation biomarkers for clinical applications, and that ZNF154 methylation shows promise as a clinically-useful biomarker for ovarian and other cancer types.

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Experimental employment in opioid use disorder: improved stress, craving, and psychological flow during work in a therapeutic workplace

People with opioid use disorder (OUD) often also have employment problems, with only a minority of people in OUD treatment employed full-time. However, there may be important benefits of work for those who are employed. Using ecological momentary assessment (EMA), in which people respond to smartphone-delivered questions about their emotions and activities during daily life, our laboratory previously found that people in methadone treatment for OUD reported less stress and craving, and more happiness, when at work vs. elsewhere. Because those participants worked their own community jobs, it is unclear how much self-selection effects impacted the results. Therefore, we presently conducted EMA as people with heroin dependence (n = 161, 55.9% male) were randomized to work or not work for up to 110 days on standardized job activities (data entry skills training) in a contingency-management-based therapeutic workplace (TW). At intake, participants were randomized to immediate TW access (immediate group, IG), allowing them to work 4 h/day Mon.-Fri., or to a 4-week waitlist-delay condition (delayed group, DG), providing payment like IG but otherwise preventing TW access. Both
groups had thrice-weekly urinalysis; participants’ TW wages depended on verified drug abstinence. For all participants, EMA began 1 week after randomization. In mixed linear statistical models, participants reported less stress, less heroin craving, less cocaine craving, and more happiness at work vs. elsewhere, replicating the prior EMA findings. Participants also reported more psychological flow (ie, absorption, enjoyment, intrinsic motivation, challenge-skill matching) at work vs. elsewhere, extending those findings. For the randomization, DG reported more stress and craving throughout, with no significant interactions with TW access. Heroin- and cocaine-craving decreased over time for both groups. DG reported initially less happiness but ultimately more happiness than IG. Thus, we observed psychological benefits associated with work, even without participants’ self-selecting their own particular jobs. Notably, some of these effects are opposite those found in studies of the general population (eg, less happiness when working). Contrary to expectations, differences between DG and IG remained as DG worked, suggesting waiting itself may have affected participants. Together, these results clarify some of the ways that work, and work-based contingency management, could aid OUD recovery.

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Rapid Perceptual Processing in Detecting Prostate Cancer  
Radiologists often report having a sense or hunch that ‘something’ is present in an image, even before they locate a specific abnormality. Recent studies have confirmed that radiologists can identify whether an image is abnormal or normal better than chance in breast or lung images presented for half a second. To date, this perceptual gist processing has only been demonstrated in static two-dimensional (2D) images (e.g. mammograms). Standard practice in radiology is moving to three-dimensional (3D) volumetric modalities. In volumetric imaging, such as multiparametric MRI (mpMRI), used in prostate screening, a single case consists of a series of image slices through the body that are assembled into a virtual stack. Radiologists can acquire a 3D representation of organ structures by scrolling through stacks. Here, we developed a new behavioral task paradigm to investigate whether radiologists can extract perceptual gist from volumetric images. We tested 14 radiologists with prostate mpMRI experience on 56 cases, each comprising a stack of 26 T2-weighted prostate mpMRI slices. A trial consisted of a single movie of the stack. After each case, participants localized the cancerous lesion on a
prostate sector map, then indicated whether a cancerous lesion was presented, and gave a confidence rating. Presentation duration was varied between groups. Radiologists viewed cases presented at either 48 ms/slice (20.8 Hz, n = 5), 96 ms/slice (10.4 Hz, n = 5), or 144 ms/slice (6.9 Hz, n = 4). Lesion detection performance was well above chance in the 96 ms/slice and 144 ms/slice groups, but not in the 48 ms group [d’ mean(sd): 48 ms/slice 0.77(0.67); 96 ms/slice 0.71 (0.29); 144 ms/slice 0.14 (0.14)]. Radiologists could localize the lesion with 40% accuracy in the 48 ms/slice condition and with 48% accuracy for both 96 ms/slice & 144 ms/slice conditions. Our data indicate that radiologists do develop gist perception for 3D modalities. Surprisingly, slower presentation rates did not improve performance. There may be an optimal framerate for processing 3D information, depending on anatomical site and/or lesion conspicuity. Our findings offer meaningful and novel implications that can lead to improved computer aided technologies used to assist radiologists, as well as develop training protocols that leverage the ability to detect an abnormality in a brief glance to in order to improve cancer detection.

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Developmental Biology
Tissue-specific roles of thyroid hormone receptors ensures coordinated and complete metamorphosis in Xenopus tropicalis
Thyroid hormone (TH) is essential for the development throughout vertebrates including humans and amphibians. During the development of anuran amphibians, the tadpole undergoes metamorphosis to become a frog in a process that is totally controlled by TH, whose effects are mediated by thyroid hormone receptors (TRs). There are two types of TRs, TR-alpha and TR-beta in all vertebrates, and both are expressed during the postembryonic developmental period when plasma TH levels are high. In mammals, this corresponds to a period around birth when it is difficult to manipulate the uterus enclosed mammalian embryos. Thus, the functions of TR during this important developmental period remain largely unexplored. We use frog as a model of vertebrate development due the ability to manipulate the externally developing tadpoles, which offers a unique opportunity to study how the adult organs are developed in vertebrate. To determine TR function during vertebrate development, we knocked out TR-beta by using CRISPR/Cas9 technology in TR-alpha knockout background and generated TR double knockout [TRDKO] animals. TRDKO tadpoles showed the resistant to external TH treatment with no change in TH target genes expression. Developmentally, TRDKO tadpoles completed the limb
formation but failed to survive past stage 61, the climax of metamorphosis (similar to mammals around birth), with little tail resorption and gill repression taken place. In addition, the intestine, which undergoes remodeling into the adult organ in all vertebrates during postembryonic development, had abnormal morphology in the TRDKO tadpoles at the stage 61, with a multiple folded structure resembling adult intestine of wild type animals but few proliferating stem cells as observed in the wild type animals. RNA-seq analysis revealed that the extracellular matrix organization related genes were precociously up-regulated at the larval stage tadpoles in TRDKO tadpoles while genes related to stem cell, cell proliferation and apoptosis, which are known to be important for intestinal remodeling, were repressed in the TRDKO tadpoles during metamorphosis. Our data suggest that TRs are not required for the initiation of metamorphosis but is essential for the completion of metamorphosis. Furthermore, the differential effects of TR knockout on different organs/tissues indicate tissue specific roles for TR to control temporal coordination and progression of metamorphosis in various organs.

Marina Venero Galanternik
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Developmental Biology

Studying the origin and function of novel vascular-associated cells in the zebrafish meninges

The meninges is a complex vascularized connective tissue that surrounds the Central Nervous System protecting it from mechanical shock, supporting brain buoyancy and maintaining brain homeostasis. Despite its critical roles, the molecular identity, developmental origins, and functional properties of different meningeal cell types remain poorly characterized. We are using the powerful experimental tools available in the zebrafish to carry out a comprehensive anatomical, molecular, and genetic characterization of the meninges, its cellular constituents, and their roles in brain homeostasis. To examine the anatomical structure of the meninges and the morphology of its resident cell types, we are using histology, electron microscopy, and super-resolution confocal imaging. Our studies have shown that the meninges is a complex three-layered tissue containing a number of unusual cell types. Using single-cell RNAseq, we are profiling all meningeal cell types, and correlating our expression and anatomical data to define the morphological and molecular identities and interrelationships of the different meningeal cells. Our studies thus far have revealed a number of unusual cell populations in the meninges, including fluorescent Granular Perithelial cells (FGPs) a novel meningeal perivascular cell population. Although the morphology and scavenging behavior of FGPs is reminiscent of macrophages, these cells are molecularly closely related to lymphatic endothelial cells. Using zebrafish genetics we have identified two new mutants with FGP defects: y281, an ENU-induced mutant specifically deficient in FGPs, and mrc1a(sa21118), a nonsense mutant that develops excess FGPs with defective scavenging capabilities. We are using these mutants and methods for ablating FGPs to define the functional role of these cells in the brain. Besides FGPs, we have also identified a meningeal population of cells expressing high levels of ependymin, a meningeal cerebrospinal fluid glycoprotein. Ependymin-expressing cells (EPDs) reside in close association with meningeal blood vessels and FGPs, and our preliminary super-resolution imaging data suggests that EPDs and FGPs may help guide each others’ migration and patterning. Together, our ongoing studies using the powerful tools and
methods available in the fish are facilitating comprehensive understanding of meningeal development and function.

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*The Remain of the Male: Unexpected Contribution of the Male Tract Mesenchyme to the Female Reproductive Tract*

Formation of the female reproductive tract is the first critical step in determining women’s reproductive health. At early fetal stage, female embryos contain both primitive male and female reproductive tracts, which are surrounded by their own different mesenchyme. During sexual differentiation, female embryos eliminate the primitive male tracts, and only maintain the primitive female tracts, which further differentiates into the oviduct, uterus, cervix and upper part of the vagina. The proper differentiation and functions of these critical female reproductive organs require mesenchymal cells. The degeneration of the primitive male tract in the female embryo leads to the logical assumption that the mesenchyme surrounding the male tract is eliminated as well, and does not contribute to female reproductive tract formation. To test this assumption, we developed a tamoxifen-inducible lineage tracing mouse model, where we labeled the male tract mesenchyme and traced the fate of labeled cells and their progeny during development. We found that, instead of being eliminated, the male tract mesenchyme remained and differentiated into smooth muscle and fibroblasts in adult female reproductive tract organs. To understand how the mesenchyme derived from primitive male tract and female tract differs, we performed fluorescence-activated cell sorting of these two mesenchymal populations for transcriptomic analysis. We found that male tract mesenchyme-derived cells had higher expression of genes involved in extracellular matrix synthesis. Therefore, the mesenchyme surrounding the primitive male tract gives rise to a distinct mesenchymal population in female reproductive tract organs. We then investigated whether the male tract mesenchyme was sufficient for maintaining the female tract formation under the condition when the female tract mesenchyme is experimentally removed. We designed a Cre-mediated cell ablation model that removed cells from the female tract mesenchyme. When the female tract mesenchyme was ablated, the basic structure of female reproductive tract organs was partially maintained, indicating that the male tract mesenchyme can compensate for the loss of the female tract mesenchyme. Taken together, our study
unveils an unexpected contribution of mesenchymal progenitor cells surrounding primitive male tracts to the female reproductive tract formation. This discovery prompts a consideration of male cells in physiology and pathology of female reproduction.

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Developmental Biology
Title: Discovering Macrophage Role in Ocular Pathologies Using 3D Bioprinted RPE/Choroid
We previously developed a 3D-Bioprinted in-vitro outer blood retina barrier that contains an intact, confluent RPE monolayer and a choroid with a dense capillary network. In this model, we're able to study ocular pathologies such as choroidal neovascularization (CNV) and degenerative conditions in the back of the eye. The purpose of this study was to investigate the role of mature macrophages on choroid development and pathology. By adding these components, we aim to increase the accuracy of our in vitro models as well as explore the obfuscating role that these cells play in disease initiation and progression. A collagen-derived gel is used for encapsulation of endothelial cells, choroidal fibroblasts, and ocular pericytes for bioprinting with additional hydrogels to provide microenvironment conducive for microvascular network formation. We bioprinted a 3D engineered vascularized tissue with a defined geometry on the basal side of a degradable PLGA scaffolds. We then seeded IPSC-RPE on the apical side at 7 days post printing. Finally, primary M1, M2, and M1+M2 polarized macrophages (Dr. Anju Singh/NCATS/NIH) were added to the tissues at set concentrations at days 0, 7, and 21. A combination of confocal microscopy, quantitative western blotting, trans-epithelial resistance measurements, and flow cytometry were used to evaluate the health of these constructs. We observed M1 and M2 specific phenotypes at various time points, indicating a strong influence on vasculogenesis and angiogenesis in developing vasculature. M2 macrophages promoted tissue growth, and heavily favored angiogenesis at all time points. In contrast, M1 macrophages supported vasculogenesis only when added on day 0. When added to the tissue at later timepoints, M1 polarized macrophages curtailed angiogenesis and leading to vascular degeneration, and degradation of the retinal pigment epithelium. Conclusions: We have successfully created an in-vitro construct which allows for investigation of immune mediated development and pathologies of the eye. Future studies will attempt to expand on the mechanisms of macrophage subpopulations in the choroid and RPE, as well as their roles in ocular development and progression of pathological conditions.

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NIA
DNA-binding Proteins/Receptors and DNA Repair
Biological contribution of CSB-LEO1 cooperativity in RNA transcription and DNA repair
Cockayne syndrome (CS) is a premature aging disease characterized by photosensitivity, developmental
defects and neurodegeneration. Mutations in CSA, CSB, XPB, XPD, XPG, XPF-ERCC1 give rise to CS. To identify molecular pathways that engage the CS proteins towards understanding the disease etiology, we conducted yeast two-hybrid (Y2H) screens using full-length or C-terminal (Pro1010-Cys1493) CSB as bait. CSB is a DNA-dependent ATPase and chromatin remodeler, with demonstrated roles in transcription-coupled nucleotide excision repair, which resolves lesions that block RNA polymerase II. A total of 12 interactors were identified, but only LEO1 (Phe381-Ser568 region) appeared as a CSB binding partner in both screens. LEO1 is a member of the polymerase associated factor 1 complex (PAF1C) with roles in transcription elongation and chromatin modification. Thus, my hypothesis is that the LEO1-CSB interaction represents a novel link that coordinates RNA transcription with DNA repair. My work has revealed that recombinant LEO1 and CSB directly interact and exist in a common complex within human cells. UV irradiation promoted a coordinated CSB-LEO1 response to DNA damage. A dose-dependent study showed that the highest co-immunoprecipitation (co-IP) of CSB-LEO1 was at 10 J/m2 UV, while a time-dependent experiment found the greatest amount of complex formation at 1h post irradiation. Cell fractionation revealed that LEO1 and CSB get recruited to chromatin in response to UV damage, suggesting this collaboration may occur in the context of DNA repair. Consistently, the CSB-LEO1 interaction responded to a broad range of genotoxins, including the crosslinking agent cisplatin and the oxidizing agent menadione. shRNA-mediated LEO1 knockdown revealed that LEO1 deficiency does not obviously affect UV sensitivity, yet CSB recruitment to chromatin is reduced in the absence of the PAF1C complex member. Ongoing work includes investigating further for CS cellular phenotypes in LEO1-deficient cells or PAF1C defects in CS cells, and exploring the recruitment of LEO1-CSB to localized sites of DNA damage using laser irradiation and confocal microscopy. The ongoing effort to characterize this novel interaction for its biological function will shed important insight into the molecular processes that regulate the coordination between transcription and DNA repair and may have implications for age-related disease treatment.

Arindam Datta
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DNA-binding Proteins/Receptors and DNA Repair

Targeting BRCA2-linked cancers with a WRN helicase-specific inhibitor

Timely and accurate DNA replication is central to genome integrity. Smooth DNA synthesis, however, is often challenged by various genomic lesions leading to fork stalling. The tumor suppressor BRCA2 stabilizes stalled forks by protecting them from extensive nuclease-mediated degradation, thereby preserving genome stability. The WRN helicase-nuclease mutated in the premature aging disorder Werner syndrome also plays a role in stabilization of stalled forks, but its mechanism is not well understood. Here we employed a WRN helicase-specific small molecule inhibitor (NSC617145, designated WRNi) to investigate the role of WRN at stalled forks when BRCA2 function is lost. Results from single-molecule DNA fiber experiments demonstrated a significant (p=0.01) increase in replication fork asymmetry (RFA) in WRNi-treated DLD1/BRCA2-/- cells (% RFA=48.2±3.5) compared to isogenic DLD1/BRCA2 wild-type cells (% RFA=29.1±7.2), indicating replication fork stalling at multiple genomic loci. Importantly, we observed profound MRE11 nuclease-mediated degradation of nascent DNA strands in BRCA2-deficient cells upon WRNi treatment. Fork restart experiments further demonstrated that
stalled fork recovery is significantly perturbed in BRCA2-deficient cells under conditions of hydroxyurea-induced replication stress and pharmacological inhibition of WRN helicase activity. Consistent with the DNA fiber results, BRCA2 mutant cancer cells showed a marked 4-fold greater sensitivity to the WRNi compared to the BRCA2 wild-type cells accompanied by increased DNA damage accumulation and apoptosis. In vivo studies with a NOD/SCID mouse xenograft model demonstrated that DLD1/BRCA2−/− tumors displayed a statistically significant (p=0.04) three-fold greater tumor growth inhibition compared to DLD1/BRCA2 wild-type tumors. We further showed that WRNi treatment is synthetic lethal with the poly(ADP-ribose) polymerase (PARP) inhibitor olaparib in BRCA2-mutated ovarian cancer cells. This finding has important therapeutic implications as acquired resistance to PARP inhibitors is one of the major challenges in treating BRCA mutant ovarian cancers. Collectively, our study provides mechanistic insight into the interactive roles of WRN and BRCA2 to stabilize stressed replication forks and suggests that pharmacological inhibition of WRN helicase is a therapeutic strategy to combat BRCA2 mutant cancers.

Nicholas Ashton
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DNA-binding Proteins/Receptors and DNA Repair

_DNA polymerase iota is a novel substrate of the USP7 deubiquitinating enzyme_

Cancers develop due to DNA mutations that endow cells with a selective growth advantage, enabling them to evade cell death and proliferation limits, to metastasize throughout the body. DNA mutations occur because of intrinsic infidelities of the replication machinery, as well as the inaccurate replication or repair of damaged DNA. In many cancers, the rate of DNA mutation is accelerated by the mutation of DNA replication proteins. DNA polymerase iota (Pol iota) is a poorly understood DNA polymerase, which is frequently down-regulated in cancer. Other members of this family function in â€œtranslesion synthesisâ€™, a process used to bypass DNA damage lesions during replication. Unlike for related proteins, a defined role for Pol iota however remains elusive. To further understand the function of Pol iota, we sought to characterize its regulatory network. To do so, we immunoprecipitated Pol iota from human cell lines, and used mass spectrometry to identify co-eluting proteins. These analyses revealed a novel association with the USP7 deubiquitinating enzyme, a protein also known to regulate several other DNA replication proteins. As a small portion of Pol iota is known to be mono-ubiquitinated in cells, we thus questioned whether Pol iota might also be a USP7 substrate. To test this, we overexpressed wild type USP7, or a catalytically inactive mutant, in 293T cells and immunoblotted for Pol iota. Whilst we could readily detect mono-ubiquitination of Pol iota in non-transfected cells, this modification was completely suppressed in cells expressing wild type USP7. Furthermore, overexpression of catalytically inactive USP7 proved to have a dominant-negative effect, increasing Pol iota mono-ubiquitination tenfold. To confirm a direct role for USP7 in regulating Pol iota, we next sought to characterize and disrupt the USP7-Pol iota interaction. To narrow down the USP7 Pol iota-binding region, we firstly truncated USP7 and performed co-immunoprecipitation assays; these revealed a Pol iota-binding site within the N-terminal USP7 TRAF domain. As this domain primarily binds PxxS motifs â€œseven of which exist in Pol iota â€œwe then individually mutated each of these motifs within Pol iota. One of these mutations, S580A, not only disrupted binding to USP7, although also markedly increased the basal levels
of Pol iota mono-ubiquitination. These data thus support that USP7 is a novel, negative modulator of Pol iota mono-ubiquitination.

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DNA-binding Proteins/Receptors and DNA Repair
Crosstalk between DNA damage and mitochondrial responses after stress
DNA is constantly under endogenous and exogenous threats which could potentially induce damage and cause genomic instability. In response to DNA damage, there is a cascade of events that occur termed the DNA damage response. This includes efforts to initiate DNA repair, cell cycle arrest, or apoptosis. Many of these processes are well-characterized in the nucleus, however, much less is known about how mitochondria respond, even though mitochondria significantly contribute to cell fate decisions after damage. Here, we aimed to investigate how mitochondria respond to DNA damage induced by gamma irradiation (IR). Primary human and mouse embryonic fibroblast cells were treated with gamma irradiation at 2 Gy or 6 Gy and responses were compared to untreated cells. In both cell types we found: (1) mitochondrial content, ROS, membrane potential and calcium influx were increased; and, most interestingly, (2) the levels of mitophagy which is defined as the selective degradation of damaged mitochondria by autophagy were also dramatically increased. We then evaluated the mitochondrial IR-response in mice using an in vivo mitophagy reporter strain (mt-Keima mice). Here we observed a significant increase in mitophagy in Purkinje cells (cerebellum neurons important for motor learning and controlling), but not in the dentate gyrus, liver, or heart after IR. These results indicate that there are tissue-specific mitochondrial responses. We then further investigated which mitophagy pathway was involved in mitophagy induction after IR by knocking down different known proteins involved in mitophagy in the human embryonic fibroblasts and western blots were used to confirm knockdown. Among the candidates we have tested so far (Parkin, BNIP3 and Spata18), the most dramatic suppression of IR-induced mitophagy was after knockdown of SPATA18 which is a p53-inducible protein that helps eliminate oxidized mitochondrial proteins from mitochondria. In short, we found that mitophagy is induced following DNA damage by IR in vitro and in some tissues in vivo. Further, we have identified SPATA18 as one protein that regulates the induction of mitophagy in this process. We will continue to explore the significance of SPATA18 loss on DNA damage response and its mechanisms.

Matthew Taves
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Endocrinology
Thymic glucocorticoids specifically target antigen-signaled thymocytes to regulate selection
Glucocorticoids (GCs) are adrenal hormones that circulate in the blood to orchestrate systemic homeostasis, and GC dysregulation leads to metabolic, inflammatory, and psychiatric disorders. GCs can
also be synthesized by extra-adrenal tissues such as the thymus, intestine, and skin, and may function as local signals. It is unclear, however, if local GC synthesis occurs in vivo, is biologically relevant, and if GCs can be targeted to specific cells. We examined these questions in the thymus, where GCs antagonize T cell antigen receptor (TCR) signaling in immature T cells (thymocytes) and are critical for the generation of a competent TCR repertoire. First, to identify the source of thymic GCs, we used mice with thymic epithelial cell (TEC) or thymocyte deletion of the GC synthetic enzyme Cyp11b1 (Cyp11b1-foxn1 and Cyp11b1-lck, respectively). We found reduced expression of GC-dependent genes in Cyp11b1-foxn1 but not Cyp11b1-lck thymi, indicating that TECs, not thymocytes, synthesize GCs. This reduction was identical in Cyp11b1-/- mice, indicating that TECs are the main source of thymic GCs. Next, to test if TEC GCs alter the TCR repertoire, we activated wild-type, Cyp11b1-foxn1, and Cyp11b1-lck T cells with TCR crosslinking antibodies (a repertoire-independent stimulus) or allogeneic antigen-presenting cells (APCs, a repertoire-dependent stimulus). T cells proliferated similarly upon TCR crosslinking, indicating normal activation, but Cyp11b1-foxn1 T cells had reduced proliferative responses to allogeneic APCs, indicating a weak repertoire. Cyp11b1-foxn1 mice also had reduced T cell responses to viral infection. Thymic GCs, therefore, are required for a competent T cell repertoire. Finally, we aimed to identify the targets of TEC GCs. As current approaches cannot detect intra-tissue GCs with high resolution, we developed a technique using fluorescence detection of chromatin-associated GC receptors as cell-specific, quantitative biosensors of GC signaling. We found that antigen-signaled CD4+8+TCRhi thymocytes were uniquely exposed to elevated GC levels, and that this GC exposure was reduced in Cyp11b1-foxn1 mice. Together, these studies demonstrate extra-adrenal GC synthesis, its nonredundant role in thymocyte selection, and targeted TEC-thymocyte GC delivery. GCs therefore act as targeted, paracrine signals, as well as systemic signals. Manipulation of local synthesis may lead to GC treatments that avoid the severe side-effects of systemic GC administration.

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The transcriptional mediator JAZF1 plays a critical role in regulating metabolic syndrome and adipocyte differentiation

Obesity is a global epidemic associated with increased morbidity and mortality. Related to this epidemic is a substantial rise in obesity-related conditions, type II diabetes, cardiovascular disease and cancers. GWAS studies have indicated that single nucleotide polymorphisms in the Juxtaposed with Another Zinc Finger 1 (JAZF1) gene is highly associated with metabolic syndrome and type II diabetes. JAZF1 encodes a nuclear zinc-finger protein involved in the regulation of gene transcription, expressed in multiple metabolic tissues including adipose tissue and pancreatic islet cells. However, its regulatory function in metabolic and catabolic processes is still unknown. To further explore the role of JAZF1, we generated JAZF1 knockout mice (JAZF1-KO). We demonstrated that when fed a high fat diet (HFD), JAZF1-KO mice exhibit lower body-weight and fat-mass with reduced adipocyte size compared to controls. In addition, HFD JAZF1-KO mice exhibited normal blood glucose levels and maintained high insulin sensitivity, while obese WT-littermates developed severe hyperglycemia and developed insulin resistance. Furthermore, energy expenditure analysis of both O2 consumption and CO2 output revealed that HFD-treated JAZF1-
KO mice have increased energy expenditure. Our microarray and RNA-seq analysis in adipocytes and liver tissue of JAZF1 mice, showed upregulation of catabolic processes in several tissues of JAZF1-KO mice. The differential gene expression was confirmed by qPCR. Pathway analysis indicated that JAZF1 regulates genes involved in lipolysis, lipogenesis and adipocytes development. Interestingly, gene expression analysis showed that B3 adrenergic receptor (ADRB3) was greatly upregulated in white adipose tissue of HFD-treated KO-mice. ADRB3 plays a critical role in regulation of thermogenesis and lipolysis which is linked with obesity. Upregulation of ADRB3 in JAZF1-KO correlates with increased lipolysis. Our studies analyzing the role of JAZF1 during adipocyte differentiation using in-vitro culture system demonstrated that significant increase in JAZF1 and ADRB3 expression during adipocyte differentiation consistent with their role in the regulation of adipocyte functions. We identified a critical role for JAZF1 in the development of metabolic syndrome and in the regulation of energy homeostasis through its regulation of adipocyte development. Importantly, our findings provide a mechanism for the role for JAZF1 in regulating and the development of obesity.

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Endocrinology

WNK1 in the Uterus: A Previously Undescribed Role In Mediating Implantation

Infertility is a major health burden worldwide, and our lacking knowledge of the uterine function during pregnancy attributes to the high rate of unsuccessful reproductive treatments. It is therefore critical to understand the initiation and maintenance of pregnancy at the molecular level. We have previously shown that Epidermal Growth Factor Receptor (EGFR) is of vital importance in the endometrium during early pregnancy, and a subsequent proteome approach identified With No Lysine Kinase 1 (WNK1) as an EGFR downstream target in human endometrial stromal cells in vitro. Whilst well established as a kinase with ion homeostasis modulating functions in the renal epithelial cells, WNK1’s significance in uterine biology remains unknown. In this study, we examined the in vivo role of WNK1 in pregnancy using a uterine Wnk1 deletion mouse model, where Wnk1-floxed mice (WNK1f/f) were crossed to the PRCre mice to generate mice with specific Wnk1 deletion in the female reproductive tract (WNK1d/d). A 6-month breeding trial revealed that WNK1d/d mice were severely subfertile with >50% reduction in the number of litters produced and the average litter size compared to their WNK1f/f littermate. A closer examination during early and mid-pregnancy showed that subfertility was due to delayed implantation...
and increased embryo resorption. We then demonstrated that the delayed implantation is associated with aberrant signaling in the luminal epithelium during the window of implantation (WOI) - including retained Progesterone Receptor (PR) expression and failure of FOXO1 nuclear localization, both of which are prerequisite for successful implantation. A subsequent RNA-sequencing analysis identified elevated PI3K/AKT signaling during the WOI in the Wnk1d/d uterus, suggesting that the decreased nuclear FOXO1 may be attributed to its increased phosphorylation by AKT and hence the consequent cytoplasmic localization. Indeed, siRNA-mediated WNK1 inhibition in HEC1A cells increased the levels of phosphorylated (activated) AKT, and pharmacological inhibition of AKT was able to rescue WNK1 inhibition-induced FOXO1 phosphorylation and cytoplasmic retention. In summary, these results illustrate a novel and critical role of WNK1 in mediating the AKT-FOXO1 signaling axis during WOI, a disruption of which impaired the timing of implantation, leading to abnormal embryo development in utero and eventually resulting in increased resorption and subfertility.

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Modification of the association between daily aspirin use and colorectal cancer risk by other cancer risk factors

Purpose: Daily low-dose aspirin is recommended for certain individuals for prevention of cardiovascular disease and colorectal cancer (CRC). However, there are also known risks associated with aspirin use. Identifying finer subgroups of individuals most likely to benefit from aspirin for CRC prevention would help to tailor recommendations to maximize benefits and minimize risks. To aid in identifying these subgroups, we investigated whether the association between daily aspirin and CRC varies by other cancer risk factors, including age, body mass index (BMI), smoking status, physical activity, and family history of CRC. Methods: This study pooled 352,005 individuals from the NIH-AARP Diet and Health Study (1995-2011) and the control arm of the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (1993-2009), of whom 6902 developed CRC. Multivariable Cox proportional hazards regression was used to examine associations between daily aspirin use, reported at baseline, and CRC. Analyses were conducted overall and stratified by baseline age (50-59, 60-69, 70+ years), BMI (&lt;25, 25-29.9, 30+ kg/m2), smoking status (current, former, never), physical activity (&lt;1, 1-3, 4+ hours/week), and family history of CRC. Statistical interaction was tested via likelihood ratio tests. Results: Compared to non-users/non-daily users, individuals who used aspirin daily experienced a 15% reduction in CRC risk (hazard ratio [HR]: 0.85, 95% confidence interval [CI]: 0.80-0.89). Similar risk reductions were observed across strata of baseline age (p-interaction=0.76), smoking status (p-interaction=0.92), and physical activity (p-interaction=0.53). Risk reductions were also similar for individuals with a family history of CRC (HR: 0.81, 95% CI: 0.68-0.96) and without (HR: 0.86, 95% CI: 0.81-0.91, p-interaction=0.57). The risk reduction was strongest among individuals with BMI &lt;25 kg/m2 (HR: 0.78, 95% CI: 0.71-0.87) and attenuated with increasing BMI (BMI 25-29.9 kg/m2: HR: 0.86, 95% CI: 0.79-0.93; BMI 30+ kg/m2: HR: 0.90, 95% CI: 0.81-1.00; p-interaction=0.16). Conclusion: In accordance with prior studies, daily aspirin use significantly reduced CRC risk. Risk reductions were consistent across strata of other cancer risk factors, with the possible exception of BMI. These findings suggest that aspirin may be most efficacious
at preventing CRC among individuals with low or normal BMI, and that BMI may need to be considered in risk-benefit calculations for aspirin use.

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*Genome-wide association study reveals novel African ancestry-specific loci influencing hormones involved in appetite regulation*

African migrants to the US, Europe, and African Americans are disproportionally affected by obesity compared to their European ancestry counterparts in these environments. In Africa the rates of obesity have been increasing rapidly over the past two decades. Obesity results from an imbalance between energy expenditure and energy intake, the latter being driven by a complex system of appetite regulation. Evidence on the genetic basis of appetite regulation is scarce, in general, and lacking in African ancestry populations. Two main appetite regulators are leptin and ghrelin, where leptin suppresses food intake and ghrelin is involved in meal initiation. We aimed to detect genetic loci influencing leptin and ghrelin levels among continental Africans enrolled from Ghana, Kenya and Nigeria as part of the Africa America Diabetes Mellitus (AADM) Study. First, we performed genome-wide association studies for leptin and ghrelin in 3,777 individuals in analyses ran with and without adjustment for BMI. We identified 15 genome-wide significant variants (p<5x10-8) across five loci for leptin (LEP, SGPP1, and three intergenic). After adjustment for BMI, four variants in the LEP gene (rs28954105, rs17151919, rs28954096, rs76076185) which codes for leptin, remained strongly associated (rs28954105 p=1.4x10-9). These four SNPs are African ancestry-specific (i.e. monomorphic in other 1000 Genome project populations). Associated p-values for the intergenic loci and loci in SGPP1 were attenuated after adjustment for BMI, suggesting a mediating effect of adiposity. One intergenic locus (rs374806964) was only associated with leptin after BMI adjustment. Four loci were identified for ghrelin (USP4, SETD7 and two intergenic), which were all independent of BMI. Secondly, we performed replication in a sample of African Americans from the Howard University Family Study (HUFS; n=1,567). We replicated three of the 15 variants for leptin (at p<0.05/15), all three in the LEP gene. Of the two ghrelin loci that could be tested in HUFS, neither replicated. The findings of this first GWAS for leptin and ghrelin in African populations suggest that African ancestry-specific loci in the LEP gene may be contributing to variations in circulating leptin in Africans, independently of BMI. These findings into the genetic basis of appetite regulation in African ancestry populations promise to provide novel insights into the biology of weight gain and perhaps obesity prevention strategies.

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Persistent infection with oncogenic human papillomavirus (HPV) is a necessary cause of cervical cancer, with 70% of cervical cancers attributed to HPV16/18. The bivalent HPV vaccine (targeting HPV16/18) contains virus-like particles that display epitopes essential to eliciting an immune response and generating neutralizing antibodies. Earlier studies have documented the cross-protective effect of the bivalent HPV vaccine and, to a lesser extent, the quadrivalent HPV vaccine. However, the durability of such protection remains unknown. To evaluate the efficacy of the HPV vaccine to reduce HPV incidence and related neoplasia, the Costa Rica HPV Vaccine Trial (CVT) randomized 7466 women aged 18-25 to receive either 3 doses of the bivalent vaccine or an active placebo. Of note, a subset of women received only 1 or 2 doses of the HPV vaccine. Cervical samples were collected and tested for HPV infections annually for 4 years. At the end of the study, the vaccinated women and a newly enrolled unvaccinated control group were followed for an additional 7 years during the Long-Term Follow-Up (LTFU) with biennial collection and testing. We compared the rate, \( r(\text{vac}) \), of incident HPV infection in vaccinated women to the rate, \( r(\text{unv}) \), in unvaccinated women. We then estimated the average vaccine efficacy (VE=1- \( r(\text{vac})/r(\text{unv}) \)) against one-time detected HPV infections in women receiving 1, 2, or 3 doses of the vaccine and tested for a change in VE over time using General Estimating Equations. In women receiving 3 doses of the HPV vaccine, we observed significant cross-protection over 11 years against HPV31 (VE=56.1%, 95%CI:46.9-63.8%), HPV33 (VE=33.3%, 95%CI:10.5-50.3%), HPV35 (VE=22.2%, 95%CI:1.9-38.4%), HPV45 (VE=73.1%, 95%CI:64.4-79.6%), and HPV58 (VE=21.3%, 95%CI:6.3-33.8%). Moreover, there was no statistically significant decrease in VE over time (p-value for trend>0.05 for HPV31/33/45). As a benchmark, VE against HPV16/18 was 75.3% (95%CI:70.4-79.4%). In women receiving only 1 or 2 doses of the HPV vaccine, we observed similarly high VE against cross-protected types. Substantial cross-protection afforded by the bivalent vaccine against HPV31/45 and, to a lesser extent, HPV33, 35, and 58, was sustained and remained stable after 11 years post-vaccination, regardless of the number of vaccine dose received. Our study reinforces the notion that the bivalent vaccine is a competitive vaccine option that could further avert 9.6% of cervical cancers through cross-protection.

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Risk of therapy-related myelodysplastic syndrome/acute myeloid leukemia after childhood cancer: a population-based study

Background: Advances in therapy for childhood cancer have resulted in improved survival and a substantial increase in number of childhood cancer survivors in the United States. These changes have led to greater focus on the long-term health of childhood cancer survivors and risks for therapy-related adverse effects. Therapy-related myelodysplastic syndrome and acute myeloid leukemia (tMDS/AML) are rare yet serious complications of cytotoxic cancer therapies. The limited data available on tMDS/AML risk among childhood cancer survivors derive from case-control/cohort studies, conducted in patients treated in the 1980-1990s. Population-based data on tMDS/AML risk among childhood cancer survivors treated in recent years are lacking. Methods: Patients included 6-month survivors treated with chemotherapy for first primary non-myeloid childhood cancer (aged less than 20 years), as reported to US population-based cancer registries. We evaluated calendar year trends in tAML risk during 1975-2015 among 25,933 survivors in nine registries, then comprehensively investigated tMDS/AML risk among 36,975 survivors in 17 registries during 2000-2015, when MDS became reportable and the registry program expanded. Results: tAML risk within five years after chemotherapy for childhood cancer increased significantly during 1975-2015 [standardized incidence ratio [SIR] prior to 1990=40; 95% confidence interval [CI], 17-79; SIR 2000-2015=133; 95%CI, 98-177; P_{trend}<0.001]. Among survivors diagnosed during 2000-2015 (N=186 tMDS/AML cases), SIRs were significantly higher less than 5 versus 5 or more years following lymphoid (SIR less than 5=90; SIR 5 or more=29; P=less than 0.001) or solid cancer (SIR less than 5=153; SIR 5 or more=47; P=less than 0.001). tMDS/AML risk was elevated after chemotherapy for most first primary cancers investigated, with the highest SIR following sarcoma (SIR=204; 95%CI, 156-261). tMDS/AML was rare, with an excess absolute risk of 8.3 cases/10,000 person-years and cumulative-incidence at 10 years of less than 1%, except after sarcoma (1.4%; 95%CI, 1.1%-1.8%). Conclusion: Large-scale, population-based data demonstrate substantially elevated tMDS/AML risks after chemotherapy for a range of childhood cancers in the current treatment era, following a significant increase in tAML risk since 1975. Although absolute risks are low, continued refinements in childhood cancer treatments are needed to minimize life-threatening risk of tMDS/AML.

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The associations of maternal polycystic ovary syndrome and hirsutism with behavioral problems in offspring

Background: Behavioral problems in children are prevalent worldwide, yet much remains unknown about their etiology. Maternal polycystic ovary syndrome (PCOS) and hirsutism are marked by hyperandronemia which may influence fetal brain development. Previous studies have found a positive association between maternal PCOS and offspring attention deficit hyperactivity disorder (ADHD). The associations of maternal hirsutism with offspring behavioral problems are unknown. Objectives: We aimed to determine the associations between maternal PCOS and hirsutism with behavioral problems in offspring in Upstate KIDS, a birth cohort in upstate New York. Methods: Mothers reported a history of
PCOS or hirsutism diagnosis at 4 months postpartum. When children were 7 or 8 years old, mothers indicated any ADHD, anxiety, or conduct disorder diagnoses (n=1909). In addition, they rated their child’s behavior with the Strengths and Difficulties Questionnaire (SDQ) at 7 years (n=1386). We classified children with borderline total, emotional, peer relationship, conduct, hyperactivity, and prosocial problems using the SDQ scores. Adjusted risk ratios (aRR) and 95% confidence intervals (CI) were estimated using multivariable Poisson regression with inverse probability weights to account for non-response to follow-up and multiple imputation for missing exposure and covariates. Results: Prevalence of PCOS and hirsutism was 12% and 4%, respectively; 84% of women with hirsutism had PCOS. After adjustment for sociodemographic covariates, pre-pregnancy body mass index, and parental history of affective disorders, children born to mothers with PCOS had higher risk of anxiety (aRR 1.62; 95% CI 1.02-2.57) and borderline emotional problems (aRR 1.66; 1.18-2.33) compared with children born to mothers without PCOS. Hirsutism was related to higher risk of children’s ADHD (aRR 2.33; 1.28, 4.24) and conduct disorder (aRR 2.54; 1.18, 5.47) and borderline total, emotional, peer relationship, and conduct problems (aRRs 2.40 (1.33, 4.32), 2.61 (1.69, 4.05), 1.92 (1.16, 3.79), and 2.22 (1.30, 3.79), respectively). Conclusions: We report novel associations between maternal PCOS and offspring anxiety as well as maternal hirsutism and various offspring behavioral problems. Future studies are needed primarily to replicate these findings in other well-characterized prospective cohorts, and secondarily to explore the role of maternal androgens during pregnancy.

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Epidemiology/Biostatistics - Prognosis and Response Predictions

Pre-diagnostic blood levels of organochlorines and risk of non-Hodgkin lymphoma in three prospective cohorts in China and Singapore

Background: Organochlorines (OCs) are a structurally diverse group of chlorinated hydrocarbons and environmentally persistent pollutants that have been extensively used as pesticides in agricultural settings, and for industrial applications worldwide. Specific OCs have been associated with non-Hodgkin lymphoma (NHL) risk with varying degrees of evidence. These associations have not been evaluated in Asia, where the high exposure and historical environmental contamination levels of certain OC pesticides (e.g. dichlorodiphenyltrichloroethane (DDT) and ß-hexachlorocyclohexane (HCH)) differ from Western populations. Methods: We evaluated NHL risk in relation to pre-diagnostic blood levels of five OC pesticide metabolites in a case-control study of 167 NHL cases and 167 controls. The study was nested within three prospective cohorts in Shanghai and Singapore. Blood levels of OCs were measured using gas chromatography/high resolution mass spectrometry. Associations between lipid-adjusted OC levels and NHL risk were analyzed using conditional logistic regression. Results: Median blood levels of p,p’-dichlorodiphenyldichloroethylene (p,p’-DDE), the primary DDT metabolite, and ß-HCH were up to 12 and 65 times higher, respectively, in samples from the Asian cohorts compared to blood samples collected from several previous cohort studies in the United States and Europe. An increased risk of NHL was observed among those with higher ß-HCH levels (3rd vs. 1st tertile OR=1.8, 95%CI=1.0-3.2; ptrend =0.049) and this association was consistent across the three Asian cohorts and in men and women. The ß-HCH association was consistent among cases that were diagnosed less than 7 and more
than 7 years after blood collection and was strengthened when excluding those diagnosed within two years of blood collection (3rd vs. 1st tertile OR = 2.0, 95%CI =1.1-3.9; p\text{trend} = 0.03). No significant exposure-response associations were observed for other OCs, including for p,p’-DDE, and NHL risk. Conclusions: Our findings provide the first evidence suggesting an association between blood levels of β-HCH and NHL risk in Asians. This is a concern because substantial quantities of persistent and toxic residues of HCH have been discharged into the environment worldwide. Although there is limited evidence that DDT/p,p’-DDE is associated with NHL based on studies in Western populations, our findings for p,p’-DDE based on substantially higher blood levels among Asians do not support an association.

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Association of Urinary Levels of Bisphenols F and S Used as Bisphenol A Substitutes with Asthma Outcomes

Bisphenols are compounds with two hydroxyphenyl groups that are used in polycarbonate plastics and epoxy resins. Exposure to bisphenols occurs mainly through the ingestion of contaminated food or beverage as well as via skin and inhalation of house dust. Bisphenol A (BPA) is the most common bisphenol and was first tested as a synthetic estrogen before being used to manufacture plastics. Due to reported health concerns, the use of BPA has been restricted in several countries and has been gradually replaced by analogs such as bisphenol F (BPF) and bisphenol S (BPS) in products often advertised as “BPA freeâ€”. Despite a well-documented association of BPA with asthma and allergic diseases, the relationship between these structural analogs and asthma outcomes is unknown. Therefore, we examined the association of exposure to BPF, BPS, and BPA with asthma outcomes for the first time in a large, nationally representative sample of the U.S. population. We analyzed data from 3,694 adults and children who participated in the National Health and Nutrition Examination Survey (NHANES) from 2013 to 2016. Levels of BPF, BPS, and BPA were measured in spot urine samples and asthma outcomes were assessed using questionnaires. Logistic regression was used to determine the association of urinary bisphenol levels with asthma outcomes, adjusting for socio-demographic characteristics (age, gender, race/ethnicity, household income, and education level of the household reference person), known asthma risk factors (exposure to cigarette smoke, body mass index, and family history of asthma), kidney function, and urinary creatinine to account for dilution. Detectable levels of urinary BPF were positively associated with current asthma (odds ratio [OR]: 1.47, 95% confidence interval [CI]: 1.13-1.91), especially in adults (OR: 1.63, 95% CI: 1.21-2.19) and with asthma attacks in the past 12 months (OR: 1.46, 95% CI: 1.01-2.12). The association of log10-transformed urinary BPS with current asthma depended on gender (interaction P-value = 0.03) and a positive relationship was only found in men (OR: 1.71, 95% CI: 1.10-2.65). Our findings suggest that BPF and BPS are associated with asthma outcomes and may not be safe alternatives to BPA. This work has important public health relevance as BPF and BPS are replacing BPA in a variety of consumer products. These results should however be confirmed by future prospective studies with repeated measures of exposure to the BPA analogs.
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Epidemiology/Biostatistics - Prognosis and Response Predictions
Background: Despite substantial declines in cardiovascular disease (CVD) mortality during past decades, progress against CVD deaths in midlife has stagnated with rates increased in some US racial/ethnic groups. Identifications of characteristics of groups where rates and trends are particularly alarming are important for targeted public health interventions. Therefore, we conducted a detailed examination of the trends in premature (ages 25-64) mortality due to CVD from 2000 to 2015 by demographics and county-level factors, including education, rurality, and prevalence of smoking, obesity and diabetes.
Methods: We ascertained National Mortality data from the SEER dataset. We first estimated age-standardized premature mortality rates (ASR) and calculated the average annual percent changes (AAPC) in ASR by age, sex, race/ethnicity and county-level factors (in quintiles). We then conducted multivariate Quasi-Poisson regression, adjusting for age, to calculate the relative risks of CVD mortality across quintiles of each county-level factor. Results: From 2000 to 2015, 2.3 million CVD deaths occurred among 25-64-year-old individuals in the US. There were significant declines in CVD mortality for blacks, Latinos and Asians and Pacific Islanders (AAPC [range]: -1.7 to -3.2%) although blacks continued to have the highest CVD mortality rates. Mortality rates were second highest for American Indians/Alaskan Natives and increased significantly among 25-49-year-old individuals (AAPC, 2.1% in women; 1.3% in men). In whites mortality rates plateaued among 25-49-year-old white women (AAPC, 0.05%). We observed important declines in mortality rates for most major CVD subtypes, except for hypertensive heart disease which showed significant increases in mortality across most racial/ethnic groups, and endocarditis where rates increased in white individuals and American Indian/Alaska Native men. Counties with the highest prevalence of diabetes were at the most risk of CVD mortality. Conclusions: There have been substantial declines in premature CVD mortality in much of the US population. However, increases in CVD mortality before age 50 years among American Indians/Alaska Natives, flattening rates in whites, and overall increases in deaths from hypertensive disease suggest that targeted public health interventions are needed to prevent these premature deaths.

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Epidemiology/Biostatistics - Prognosis and Response Predictions
Aspirin use and survival from biliary tract cancer
Background: Biliary tract cancers are rare but lethal. Because median survival time is less than 12 months, there is a critical need for treatments that extend life. Aspirin inhibits tumor growth and metastases in vitro. Previous studies suggest that aspirin may improve cancer survival, although results
have been conflicting, and there is limited evaluation of the role of aspirin in biliary tract cancer survival. Objective: To determine whether postdiagnosis aspirin use confers overall survival benefit in patients with biliary tract cancer. Design, Setting, and Participants: Patients diagnosed with gallbladder, cholangiocarcinoma, ampulla of Vater, or mixed biliary tract cancer between 1990-2017 were selected from primary care records using the United Kingdom’s Clinical Practice Research Datalink. The associations between postdiagnosis aspirin use and overall survival were analyzed using time-dependent Cox proportional hazards regression adjusting for age, diagnosis year, sex, proxies of health status, comorbidities, and prior statin use. We also modeled this relationship using propensity scores with inverse probability treatment weights to mimic a randomized trial design. Exposures: Postdiagnosis prevalent and incident aspirin use was operationalized as "use" versus "non-use" and number of prescriptions in both the time-dependent and propensity score-adjusted models. Main Outcomes and Measures: Overall survival within each cancer site. Results: Of 2,934 patients with biliary tract cancers, there were 2,415 deaths (82%), with a median survival time of 5.8 (interquartile range: 2 – 15) months. Aspirin use was associated with decreased risk of death in gallbladder cancer (hazard ratio (HR): 0.63, 95% confidence interval (CI): 0.47, 0.83), cholangiocarcinoma (HR: 0.70, 95% CI: 0.58, 0.83), ampulla of Vater cancer (HR: 0.40, 95% CI: 0.23, 0.69), and mixed type (HR: 0.61, 95% CI: 0.44, 0.84) in the multivariable, time-varying adjusted analysis. The number of prescriptions was also associated with increased survival. When stratified by previous aspirin use, incident post-diagnosis aspirin users saw the greatest survival benefit. The propensity score-adjusted associations were similar to the results from the main analysis. Conclusions and Relevance: Aspirin use after biliary tract cancer diagnosis was associated with a lower risk of overall mortality. If confirmed in future studies, aspirin could be used to extend life in these patients.

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*Auxin inducible degron (AID) system to define direct chromatin regulatory functions of Bcl11b and BRG1 in mouse primary T cells*

Differentiation of naïve CD4 T cells into different T helper lineages is regulated by specific epigenetic and transcription factors that can initiate lineage-specific gene transcription. Defining genomic targets of these regulatory factors is critical to our understanding of the molecular mechanisms of differentiation. Although many studies have identified potential targets of transcription factors using traditional non-reversible gene deletion strategy, they require at least 2 to 3 days to abrogate the protein expression of genes of interest. This may lead to accumulation of indirect secondary effects and thus compromise our effort to identify direct targets of the epigenetic and transcription factors under study. To solve this problem, we have employed the plant auxin-inducible degron (AID) system to induce rapid and reversible degradation of target proteins. Here, we have generated mouse lines carrying the AID-tagged Bcl11b and Brg1 genes at their endogenous loci, respectively. BCL11B is a key transcription regulator essential for T cell differentiation, while BRG1 is an ATPase subunit of the chromatin-remodeling BAF complexes. Using primary CD4 T cells from these mice, we demonstrated that Auxin treatment of Tir1-expressing T cells efficiently induced depletion of BCL11B and BRG1 proteins within 3 hours. To understand their roles and molecular mechanisms during CD4+ T cell differentiation, we have analyzed genome-wide changes with RNA-seq, ChIC-seq and MNase-seq assays after the rapid depletion of BCL11B or BRG1 in auxin-treated Th2 cells. We have confirmed that auxin induced degradation of BCL11B induced depletion of its binding to chromatin genome wide. This was accompanied with loss of active histone marks, and nucleosome reorganization at the BCL11B binding sites, indicating that a rapid and direct role of BCL11B in modulating the states of chromatin. We demonstrate that the AID degron system is a powerful tool for studying the kinetics and function of these key transcription and epigenetic regulators in primary cells. Future studies will investigate the kinetics of transcription and epigenetic states after removing auxin from the cells to restore the expression of these critical factors. We believe this new strategy will bring us new insights into how these key transcription factors and chromatin remodelling factors regulate their direct targets to promote T cell differentiation and maintain the specific T cell identity.

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*Structural and functional characterization of maternally expressed gene 3 lncRNA*

Long non-coding RNAs (lncRNAs) have emerged as key players in gene regulation and cancer. Maternally expressed gene 3 (Meg3) lncRNA is a tumor suppressor expressed in various normal human tissues where it upregulates the transcription of p53-driven genes, leading to G2/M cell cycle arrest and apoptosis. However, its expression is low or undetectable in various cancerous tissues and ectopic expression of this RNA in these cells inhibits tumor growth. Meg3 RNA is also involved in epigenetic...
regulation of oncogenes via its interaction with polycomb repressive complex 2 (PRC2). Resolving the structure of Meg3 RNA and characterizing its interactions with cellular binding partners will deepen our understanding of tumorigenesis and provide a framework for RNA-based anti-cancer therapies. Herein, we determine the architectural landscape of Meg3 RNA, define the functionally important regions of this RNA and characterize its interactions with PRC2. We report the first experimentally derived secondary structure of the 1595-nt Meg3 lncRNA transcribed in-vivo in U87 glioblastoma cells using selective 2' hydroxyl acylation analyzed by primer extension and mutational profiling (SHAPE-MaP). Our results indicate that Meg3 RNA is intricately branched and contains five structurally stable regions. Employing a luciferase reporter assay to measure the activation of p53 promoter activity of various structure-guided deletion mutants of Meg3 RNA in U87 cells, we show that two out of the five structurally stable regions of the RNA are required for p53 promoter activation by the RNA. The nucleotide sequence of these two regions was found to be conserved throughout recent evolution. Using gel-based and SHAPE-MaP based foot-printing assays, we also identified a previously unreported 20-nt stem-loop high affinity PRC2 binding site on an in-vitro transcribed full-length Meg3 RNA. Deletion of this stem-loop led to about 80% abrogation of Meg3 mediated p53 driven transcriptional activation suggesting that the Meg3 mediated p53 promoter activation is achieved by epigenetic regulation. We are currently working on the delivery and sustained expression of the full-length and minimal fully-functional region of Meg3 RNA in cancer cells using an adeno-associated virus 2 to harness the therapeutic potential of Meg3 RNA.

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Epigenetic modules governing skin homeostasis  
Skin homeostasis is directed by spatiotemporal regulation of gene expression, where precise expression profiles determine each stage of keratinocyte terminal differentiation. The epidermal layer of skin is the outermost layer and comprises of two broadly classified sub-layers, proliferating basal and differentiated suprabasal layers. The poise between adequate proliferation and differentiation ensures proper barrier formation. Studies so far have reported epigenetic profiling of skin either from mixed cell populations or cultured cells, which undermines tissue heterogeneity and restricts identification of cell-specific changes in epigenetic and transcriptomic landscapes. We aim to identify the underlying epigenetic modules that drive epidermal equilibrium. We used epidermis of newborn (1-2days old) pups and obtained the basal and suprabasal cell populations via percoll gradient separation. Using a multi-omics approach, we performed ATAC-Seq (Assay for Transposase-Accessible Chromatin using sequencing) and RNA-Seq (n=3). Open chromatin loci was identified with the ENCODE (Encyclopedia of DNA Elements) pipeline, and RNA-Seq data was analyzed using Partek suite. ATAC-Seq peaks suggested moderately higher fractions of open chromatin in basal cells, although the enrichment in various genomic regions was comparable between the two cell types. Gene ontology analysis of RNA-seq data showed enrichment for terms encompassing keratinocyte and epithelial cell differentiation in suprabasal cells, whereas processes linked to cell cycle and chromosome organization were enriched in basal cells. We have previously shown homeobox transcription factor Dlx3 mediating skin homeostasis. ChIP
(Chromatin immunoprecipitation) sequencing of Dlx3 in differentiated suprabasal cells showed Dlx3 binding to putative promoter regions of Hox and keratin genes, the epidermal lineage determinants Grhl3 and Klf4, and epigenetic modulators such as Ezh2 and Stat3. These Dlx3 peaks coincide with H3K4me3 (promoter) and H3K27ac (enhancer) marks, implying Dlx3’s involvement in regulation of these genes. We are currently mapping genome-wide interaction maps with Hi-C and occupancy profiles of H3k4me1 and H3k27me3, regulators Ctcf, Klf4, Med1 and Pol II in these cells. Integration of this multi-dimensional data will enable mapping of cell-specific regulatory regions, identification of novel Dlx3 targets and tracing of higher order epigenetic modules that determine balance of skin homeostasis.

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Gene Expression
GLIS2 causes kidney fibrosis through transcriptional regulation of cell migration- and immune cell recruitment-related genes
GLI-Similar 2 (GLIS2) is a member of the GLIS subfamily of Krüppel-like zinc finger transcription factors. GLIS2 is expressed in several tissues, with highest expression in kidney. GLIS2 deficiency in humans and mice lead to the development of nephropathisis, a cystic kidney disease that is the most common genetic cause of renal failure in young adults. Using GLIS2 knockout mice (GLIS2-KO), we showed that this cystic renal disease is associated by cyst formation, renal atrophy, fibrosis and severe inflammation. This is accompanied with increased apoptosis and infiltration of inflammatory lymphocytes. To obtain insights into the physiological function of GLIS2 and the mechanism of development this cystic renal disease, we compared the gene expression profiles between kidneys from wild type and GLIS2-KO mice using RNA-Seq. Analysis of the RNA-Seq data showed that most of the differentially expressed genes were induced in GLIS2-KO kidneys. Many of these up-regulated genes were repressed in IMCD3 cells overexpressing GLIS2 suggesting that GLIS2 largely functions as a transcriptional repressor. This is consistent with our Mass Spec data showing that GLIS2 is associated with a co-repressor complex containing RCOR1, KDM1, CTBP1 and HDAC2. To identify genes that are directly regulated by GLIS2, we performed ChIP-Seq analysis using GLIS2-HA overexpressed IMCD3 cell line to identify DNA binding sites that GLIS2 directly binds. ChIP-Seq analysis showed about 23% of the GLIS2 binding site (GLISBS) were located in the proximal promoter region. To examine which of the differentially-expressed genes were directly regulated by GLIS2, the genes identified by ChIP-Seq were compared with those from RNA-Seq and the overlapping genes selected for further analysis. TGFB1, IRF1, JUNB, NFKB2, ITGAS, ITGB2, CXCL10, IFNGR1, AEBP1, EDN1, C3, S1PR2, LPAR1, FSTL1, FOSL1, HSPB1, EFEMP2, CRLF1, GLIPR2, and NFKBIE were among the genes directly regulated by GLIS2. Pathway analysis identified extracellular matrix, cell migration, adhesion and inflammation as major pathways regulated by GLIS2. Interestingly, GLIS2 binding sites are associated with a previously identified repressor region in the TGFB1 regulatory region, which is consistent with data showing that GLIS2 represses TGFB1 transcription. Our results suggest that GLIS2 is transcriptional repressor that is a critical regulator of cell migration, inflammation, and renal fibrosis.
**Varun Sood**  
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Gene Expression

*Identifying determinants of stochastic transcriptional bursting*

Stochastic bursting is a fundamental hallmark of transcription across species. Yet the molecular mechanisms that regulate *On* and *Off* times of transcriptional bursts are unknown. We have used high-throughput imaging to screen small molecule libraries of 510 inhibitors of chromatin modifying enzymes and kinases to identify factors that regulate transcriptional stochasticity in HBEC cells. The libraries were screened for their effect on nine genes which covered a wide range of expression levels. To determine bursting frequency, we imaged active transcription sites through nascent RNA FISH and used the average transcription sites/cells as proxy for transcriptional bursts. We hypothesized that factors that increase bursting will increase the number of transcription sites in random population samples. We validated this approach using the slow bursting gene Errfi1 which has a median off-time of 3h as determined by live cell observation using an MS-RNA tagging system. In a pilot screen, we identified four broad categories of inhibitors that increased the firing rate of Errfi1 by 2-4 fold: Inhibitors of histone deacetylases, demethylases, janus kinase and bromodomain containing proteins (BCP). Live cell measurements of Errfi1-MS2 showed that these inhibitors primarily decreased the median off- times by 20-50%, while the BCP and Janus kinase inhibitors increased the median on-time. Importantly, these changes increased Errfi1 mRNA levels by several folds. Extending our nascent RNA-FISH screen to the full set of target genes revealed differences in the sensitivity of various genes groups to inhibitors. Genes with long off-times and consequently low burst frequencies (Slc2A1, Rrpa3 and Sec16a) showed an increase in bursting with the same class of inhibitors as Errfi1, while genes with high burst frequencies (CANX, DNAJC5, RhoA and Rab7a) showed a decrease in bursting with the same inhibitors. An exception was Myc, which behaved like a high burster despite its low burst frequency. Our results provide the first insight into the molecular basis of transcriptional bursting. They suggest that regulation of bursting predominantly occurs by modulating the off-time of genes and that regulation of gene specific bursting is a multi-factorial process influenced by expression levels, chromatin context and signaling events. We will now dissect how chromatin states regulate on- and off-times by coupling chromatin assays with live-cell studies for the remaining nine genes.

**Seung Bum Park**  
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Gene Expression

*Generation of a Conditional CRISPR-Cas9 System to Activate Gene Editing and Minimize Off-Target Effects in Human Stem Cells*

CRISPR-Cas9 system has emerged as a powerful and efficient tool for genome editing. However, one of the important drawbacks of CRISPR-Cas9 system is the constitutive endonuclease activity when Cas9 endonuclease and its sgRNA are co-expressed. This constitutive endonuclease activity results in
undesirable off-target effects that hinders studies using CRISPR-Cas9 system such as understanding gene functions or its therapeutic use in humans. Here, we describe a novel method that allows temporal control of CRISPR-Cas9 activity by combining transcriptional regulation of Cas9 gene expression and protein stability control of Cas9 protein. To achieve this dual controls, we combine the doxycycline-inducible system for transcriptional regulation and FKBP12-derived destabilizing domain fused to Cas9 for protein stability regulation. We showed that Cas9 gene expression and its protein stability are tightly regulated by a doxycycline and a synthetic ligand (Shield1). We also confirmed that approximately 10% of Cas9 gene expression was observed when only one of the two controls was used. By combining two regulatable systems, we were able to markedly lower the baseline Cas9 gene expression and limit the exposure time of Cas9 endonucleases in the cell, resulting in little or no off-target effects. We assess knock-out efficiency of our system in human stem cells (hESC or hiPSC) by targeting several tumor suppressor genes such as p53, phosphatase and tensin homolog (PTEN), and adenomatous polyposis coli (APC). For in vivo application of our system, an inducible p53 gene knock-out SW iPSC clone was generated and engrafted subcutaneously into the athymic nude mice. Currently, we are improving Cas9 gene expression in vivo by replacing the promoter for Cas9 gene expression because we observed low level of Cas9 gene expression in vivo. We anticipate that our novel conditional CRISPR-Cas9 system will serve as a valuable tool for the systematic characterization and identification of genes for various pathological processes as well as paving the way to develop safer method for clinical use of CRISPR-Cas9 system in humans.

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Gene Expression
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Gene Expression
Single-molecule analysis reveals linked cycles of transcription factor binding and chromatin remodeling at specific gene promoter in Saccharomyces cerevisiae
Introduction: Transcription is a process by which DNA is copied into RNA by an enzyme RNA polymerase. This is the first stage of gene expression and impaired gene expression leads to many human diseases including cancers. Hence, it is essential to understand the molecular mechanism of transcription. It is known that transcription factors (TFs) bind to gene promoters transiently, generally in the range of seconds. Gene promoters are occupied by well positioned nucleosomes, hence chromatin remodeling is required to move or remove nucleosomes from the gene promoters to make them accessible for TF
binding. It is not known how the fast exchange of the TFs at gene promoters is molecularly linked with chromatin remodeling and transcription. Methods: We have developed a Single-Molecule Imaging method for quantifying transcription factor binding at specific gene promoters in the yeast S. cerevisiae. We took the advantage of the 10 copies of the CUP1 gene array naturally present in this yeast. This system allowed us to visualize live transcription site (TS) upon gene activation and we could measure the binding of the TFs (Ace1p) at the specific TS. We used Single-Molecule Fluorescence in Situ Hybridization (smFISH) to measure transcription output in terms of number of mRNA per cell. We applied bursting gene expression model to our SMT and smFISH data to extract burst dynamics of CUP1 transcription and certain transcription parameters (transcript production time and transcription initiation rate). Results: SMT of TF Ace1p at the CUP1 array showed that chromatin remodeler RSC speeds up the search process of the Ace1p for finding its target site. We demonstrated that RSC regulates transcription bursts of CUP1 only by modulating Ace1p binding, but it does not affect transcription initiation and elongation rates. SMT of RSC at the CUP1 array showed fast exchange of RSC, suggesting rapid remodeling events at the gene promoters. Therefore, transient binding of Ace1p and rapid bursts of transcription of CUP1 may be dependent on short repetitive cycles of nucleosome mobilization. This type of regulation reduces the transcriptional noise and ensures a homogeneous response of the cell population to heavy metal stress. Ongoing: We are investigating the Single-Molecule dynamics of histones and RNA PolII to understand how histones behave at the gene promoters upon transcription activation and how binding of a TF recruits RNA polII at specific gene promoter.

Stefan Barisic  
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Developing a Murine Model for the Cellular Therapy of Clear Cell Renal Cell Carcinoma  
Clear cell renal cell carcinoma (ccRCC) is the most common type of kidney cancer in humans. Despite significant advances in immunotherapy of metastatic ccRCC, the mortality remains high. Our group has developed a potentially curative approach for treating ccRCC by targeting human endogenous retrovirus type E (HERV-E)-derived HLA-A11 restricted antigen named CT-RCC-1. Importantly, this HERV-E is expressed exclusively in ccRCC, with no detectable expression in normal tissues. We have characterized a T-cell receptor (TCR) that recognizes CT-RCC-1 antigen in the context of HLA-A11. CD8+ T-cells transduced with a retroviral vector containing this TCR sequence (HERV-E T-cells) show specific recognition and exhibit potent cytotoxic effects against HLA-A11+ HERV-E expressing RCC cells in vitro. To further investigate the potency of HERV-E T-cells, we are developing a murine xeno-model for tracking their behavior and efficacy in vivo. RCC cells (HERV-E expressing and non-expressing) transduced with a retroviral luciferase vector were mixed with Matrigel at 1:1 ratio. A total of 2.5 x 106 Luc+ RCC cells were injected s.c. in the abdominal region of the immunodeficient NSG mice. When tumors reached palpable size, the animals were divided into three groups (6 mice per group) and received: a) HERV-E TCR-transduced T-cells, b) mock-transduced T-cells and c) no treatment. Treated mice received 1x107 T-cells and then IL-2 (200 000 IU, i.p.) daily for 3 days. Tumor engraftment was successful in all animals and tumor size was determined by imaging luciferase bioluminescence at the following timepoints: pre-treatment, 7, 14 and 21 days post-treatment. However, results of the 3
independent trials have not shown significant differences in the tumor growth kinetics between the three groups. Additionally, there was no difference in survival between the groups. To evaluate if HERV-E T-cells survive, circulate and home to the tumor site, mice were re-treated and subsequently bled and their organs (lungs, liver, spleen) and tumors harvested and analyzed by flow cytometry. No HERV-E T-cells were found, which is the suggested reason of the tumor treatment failure. Future directions with this animal model include conducting studies aimed at monitoring the in vivo survival of TCR-transduced T-cells, with the goal of decreasing the tumor burden in animals following HERV-E T-cells adoptive transfer.

Daniel Beury  
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Gene Therapy

**Genetically-engineered myeloid cells alter the metastatic microenvironment, enhance anti-tumor immunity, and limit metastasis.**

Primary tumor triggers microenvironmental changes at future metastatic sites. These changes are generated by the secretion of hematopoietic and inflammatory mediators from the primary tumor and occur before the arrival of disseminated tumor cells and facilitate the development of a metastasis-promoting microenvironment. This metastatic niche is characterized by an influx of myeloid cells that enhance metastasis by inhibiting tumor-specific T cells and enhancing tumor cell proliferation, survival, and invasiveness. Therefore, we hypothesized that altering the immune milieu at future metastatic sites such as the lung could limit metastasis. We propose that genetically-engineering myeloid cells (GEMys) to constitutively express IL-12, an established anti-tumor cytokine, and adoptively transferring GEMys into tumor-bearing mice can reprogram the metastatic niche. We synthesized GEMys by culturing bone marrow-derived stem cells in Stemspan media supplemented with IL-6, SCF, FLT3-L, and an IL-12-encoding lentivirus, yielding primarily monocytes and macrophages with a 10%-35% transduction efficiency. To characterize immune changes in the metastatic niche, we injected mice orthotopically in the gastrocnemius muscle with M3-9-M, a syngeneic rhabdomyosarcoma model which spontaneously metastasizes to the lungs, and harvested the lungs of tumor-bearing and tumor-free mice at various time points for analysis by flow cytometry. We observed that the lungs of tumor-bearing mice had more myeloid cells and less T and natural killer cells, and these changes were correlated with increasing tumor burden. When we gave GEMys or non-transduced myeloid cells to M3-9-M-bearing mice, only GEMys increased the number of activated T cells in the lungs. GEMy-treated mice had less lung metastasis and primary tumor growth, and increased survival time. GEMys also treat established metastasis as mice receiving M3-9-M i.v. or surgical-resection of primary tumor had increased survival time and decreased metastasis with GEMy transfer compared to untreated mice. Since publications have shown anti-tumor effects of IL-12 are enhanced when combined with chemotherapy, we combined GEMys with cyclophosphamide and observed that cyclophosphamide + GEMy treated mice lived significantly longer than cyclophosphamide or GEMy monotherapy mice and that >50% of mice were cured of disease. Together, GEMys offer a novel immunotherapy for targeting metastasis, and have the potential to be an adjunct therapy.
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Genetics

Mechanisms of Tandem Repeat Formation at HPV Integration Sites in Cervical Carcinoma Cells

Integration of oncogenic human papillomavirus (HPV) genomes into host chromatin is common in HPV-associated cancers. Integration is not part of the normal HPV life cycle and often results in dysregulation of the viral E6/E7 oncogenes, which promotes oncogenesis. HPV integration is classified as Type I, a single integrated viral genome; Type II, tandem head-to-tail copies of HPV integrated at a single genomic locus; or Type III, tandem copies of HPV integrated with intervening host DNA. We have characterized a Type III HPV16 integration site in CIN612 cervical cells that, in synergy with the viral promoter, generated a viral-cellular super-enhancer-like element that drives high viral oncogene expression from the integrated locus. This â€œenhancer hijackingâ€ is a novel mechanism by which HPV integration can promote oncogenesis. Treatment of CIN612 cells with BET-inhibitors, which disrupt binding of the super-enhancer factor Brd4 to the integration site, results in E6/E7 oncogene downregulation and induction of cellular senescence. This has great therapeutic potential against HPV-positive tumors. To address whether enhancer hijacking and amplification are common at HPV integration sites in cervical carcinomas, we compiled cervical cancer genomic datasets and assigned each integration as Type I, II or III based on HPV copy number and amplification status at insertion breakpoints. We found that approximately 40% HPV-positive cervical tumors displayed host genome amplifications at HPV integration sites. HPV Integration is known to occur frequently in common fragile sites (CFS) and/or transcriptionally active regions of the host genome. CFS are susceptible to copy number variations that could increase the frequency of tandem repeat formation at associated HPV integration sites. We performed correlation analysis to address overlap between integration type, CFS and active cellular enhancers defined in the FANTOM5 (Functional Annotation of the Mammalian Genome) dataset. We show that cellular enhancers are significantly over-represented in host sequences co-amplified with HPV, relative to size-matched random control regions. Furthermore, 30% HPV integration sites are associated with CFS, and >70% of Type III integrations also capture cellular enhancer elements. We propose that integration adjacent to active cellular enhancers can drive high levels of viral oncogene expression through an â€œenhancer-hijackingâ€ mechanism.

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Genetics

DNA variation in SNCA and TMEM175 loci as modifiers of Parkinsonâ€™s disease age of onset

Increasing evidence supports an extensive and complex genetic contribution to Parkinson's disease (PD). PD is pathologically characterized by the loss of dopaminergic neurons in the substantia nigra and a-synuclein (encoded by SNCA) protein aggregates. Previous genome-wide association studies (GWAS)
have shed light on the genetic basis of risk for this disease. The genetic determinants of PD age of onset are largely unknown, despite a clear age-dependent prevalence. Here we performed the largest PD age of onset GWAS to date including 28,568 PD cases. We estimated that the heritability of PD age of onset due to common genetic variation was ~11%, lower than the overall heritability of risk for PD (~27%) likely in part because of the subjective nature of this measure. We found two genome-wide significant association signals, one at SNCA and the other a protein-coding variant in TMEM175, both of which are known PD risk loci. Both variants are resulting in an earlier age of onset of ~0.6 years. Additionally, Bonferroni corrected significant effects were identified at other known PD risk loci, INPP5F/BAG3, FAM47E/SCARB2, and MCCC1. In addition, we identified that GBA coding variant carriers had an earlier age of onset compared to non-carriers. Notably, SNCA, TMEM175, SCARB2, BAG3 and GBA have all been shown in vitro to either influence alpha-synuclein aggregation or are implicated in alpha-synuclein clearance via lysosomal degradation. Remarkably, other well-established PD risk loci such as GCH1, MAPT and RAB7L1/NUCKS1 (PARK16) did not show a significant effect on age of onset of PD. While for some loci, this may be a measure of power, this is clearly not the case for the MAPT locus; thus genetic variability at this locus influences whether but not when an individual develops disease. This provides a compelling picture, both within the context of functional characterization of disease linked genetic variability and in defining differences between risk alleles for age of onset, or frank risk for disease. We believe this is an important mechanistic and therapeutic distinction. Furthermore, these data support a model in which alpha-synuclein and lysosomal mechanisms impact not only PD risk but also age of disease onset and highlights that therapies that target alpha-synuclein aggregation are more likely to be disease-modifying than therapies targeting other pathways.

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Genetics

Genome-wide association study in patients with pulmonary Mycobacterium avium complex disease
Rationale: Pulmonary nontuberculous mycobacteria (NTM) disease is a chronic progressive lung disease caused by environmental mycobacteria. Although epidemiological data indicate a potential genetic predisposition to NTM disease, the nature of this remains unclear. Objectives: The present study aimed to identify host genes associated with susceptibility to Mycobacterium avium complex (MAC), the most common NTM pathogen. No genome-wide association study (GWAS) has been published for pulmonary NTM or MAC) diseases. Methods: We conducted a genome-wide association study in Japanese patients with pulmonary MAC and healthy controls, followed by genotyping of candidate SNPs in another group of patients and controls. For verification in European populations, we performed imputation to estimate genotypes of a candidate SNP from exome sequencing data. Results: The GWAS discovery set included 475 pulmonary MAC patients and 417 healthy subjects. After quality control filtering of genotyped variants, 622,723 autosomal variants were analyzed. Both GWAS and replication analysis of 591 pulmonary MAC patients and 718 controls identified the strongest association with chromosome 16p21, and particularly with rs109592 (p = 1.60E-13, odds ratio = 0.54). This SNP is located in an intronic region of the calcineurin like EF-hand protein 2 (CHP2) gene, and expression quantitative trait locus analysis showed its association with lung CHP2 expression. Further, radiological findings showed that this SNP
was associated with the nodular bronchiectasis disease subtype. This SNP was also significantly associated with the development of the disease in European populations (p = 5.08E-06, odds ratio = 0.23). Discussion and Conclusions: This is the first pulmonary MAC GWAS, performed on 1066 patients and 1135 controls. Furthermore, we discovered that the disease risk of this SNP was shared between Japanese and European populations. The results of our study suggest that CHP2, which encodes a protein involved in cellular pH and ion homeostasis, may play an important role in host susceptibility to pulmonary MAC disease. This study provides the basis for conducting further research with a larger sample size and for functional validation of the identified regions to better understand the pathogenesis of pulmonary MAC disease.

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Genetics

A Transcriptome-Wide Association Study (TWAS) Identifies Novel Candidate Susceptibility Genes for Pancreatic Cancer

Background: Pancreatic cancer is currently the third leading cause of cancer deaths in United States, and seventh worldwide. Established risk factors include tobacco smoking, long-standing diabetes, obesity, chronic pancreatitis, and family history of pancreatic cancer. Over the past decade, 20 pancreatic cancer association signals at 17 risk loci have been identified in populations of European ancestry using genome-wide association studies (GWAS). However, these loci explain a small fraction of genetic heritability for pancreatic cancer, and the genes underlying the associations of most of these loci are unknown. Most susceptibility alleles discovered through GWAS reside in noncoding regions of the genome and likely function through allele specific regulation of gene expression. Transcriptome-wide association studies (TWAS) build on this premise by imputing genetically predicted gene expression levels into GWAS datasets to discover genes whose cis-regulated expression is associated with complex traits. Methods: We performed a transcriptome-wide association study (TWAS) by integrating pancreatic cancer GWAS summary statistics from 9,040 pancreatic cancer cases and 12,496 controls of European ancestry, and gene expression prediction models that were built using transcriptome data from histologically normal pancreatic tissue samples (the NCI Laboratory of Translational Genomics, LTG (n=95) and Genotype-Tissue Expression, GTEx v7 (n=174) datasets), and 48 different tissues (GTEx v7, n=74-421 samples). Results: We discovered 25 genes whose genetically predicted expression was significantly associated with pancreatic cancer risk (FDR < 0.05), including 14 candidate genes at 11 novel loci (1p36.12: CELA3B; 9q31.1: SMC2, SMC2-AS1; 10q23.31: RP11-80H5.9; 12q13.13: SMUG1; 14q32.33: BTBD6; 15q23: HEXA; 15q26.1: RCCD1; 17q12: PNMT, CDK12, PGAP3; 17q22: SUPT4H1; 18q11.22: RP11-888D10.3; and 19p13.11: PGPEP1) and 11 at 6 known risk loci (5p15.33: TERT, CLPTM1L, ZDHHCl1B; 7p14.1: INHBA; 9q34.2: ABO; 13q12.2: PDX1; 13q22.1: KLF5; and 16q23.1: WDR59, CDFP1, BCAR1, TMEM170A). Among these genes, the association for 12 genes (CELA3B, SMC2, PNMT, TERT, CLPTM1L, INHBA, ABO, PDX1, KLF5, WDR59, CDFP1 and BCAR1) remained statistically significant after Bonferroni correction. Conclusions: We performed the first large TWAS for pancreatic cancer risk and identified novel risk loci and candidate functional genes for pancreatic cancer that warrant further investigation.
New mouse model with IFITM5 S42L connects types V and VI osteogenesis imperfecta

Osteogenesis Imperfecta is a rare bone disorder that occurs in 1:20,000 births. About 85% of cases are the result of dominant mutations in COL1A1 or COL1A2. Type V OI, caused by a recurrent dominant mutation in the plasma membrane protein IFITM5/BRIL, and type VI OI, caused by recessive null mutations in the anti-angiogenic factor PEDF, have distinct features. IFITM5 S40L, a substitution in the coding region of IFITM5 reported in 6 patients, causes severe dominant OI with phenotype, bone histology and decreased cellular secretion of PEDF similar to type VI OI, rather than Type V OI. Our objective is to understand the role in bone development of the pathway connecting IFITM5 and PEDF. We generated a conditional knock-in mouse model to investigate atypical type VI OI. The mutation, located at murine BRIL S42L, was activated using E2A-CRE mating. Heterozygous and homozygous mutant mice were analyzed at 1 and 2 months of age. BRIL S42L is non-lethal in both heterozygous and homozygous mice. Newborn heterozygous and homozygous S42L pups have flared rib cage, shoulder and knee dislocations, and homozygotes have rib fractures and unmineralized calvaria. In radiographs, S42L heterozygous mice exhibit 50% humeral fractures in 1- and 2-month-old mice, while homozygotes incur fractures in 96% of humerii, as well as femora and pelvis. Serum alkaline phosphatase was increased in 1 and 2 month-old heterozygous males (p<0.01) wrt WT, as occurs in typical and atypical OI type VI. On mechanical testing, femora of 2-month old heterozygous males showed reduced stiffness, yield and ultimate load, with marked increase in brittleness. Biomechanics are not explained by change in bone size, suggesting material differences. Pore volume/BV was increased on uCT, consistent with increased vascularity. Whole body DXA aBMD was significantly decreased in 1 and 2 month-old heterozygotes (p<0.01) and 1 month-old S42L homozygotes. qBEI revealed increased mineralization in cancellous and cortical bone of 2 month-old heterozygous males. This is consistent with in vitro osteoblast studies from heterozygous mice yielded increased mineralization by alizarin red staining (p<0.05) and increased expression levels of osteoblast genes throughout differentiation. Taken together, the altered bone fragility, mineralization, vascularity and serum ALP indicate that the IFITM5 S42L mouse is a valid model to investigate the bone cellular and tissue mechanisms of atypical type VI OI.
functions as a chaperone protein during 26S proteasomal assembly. Mutations in genes encoding the non-ATPase subunits of the 19S regulator base of 26S proteasome complex have been associated with ophthalmological and vertebral defects in humans. The purpose of this study was to evaluate the role of psmd5 during optic fissure closure and vertebral development in zebrafish. Zebrafish psmd5 CRISPR mutants were created by co-injection of Cas9 protein with sgRNAs targeting specific regions of psmd5 into 1-2 cell stage ABTL zebrafish embryos. Sanger sequencing was used to confirm the mutations in the psmd5 gene. qRT-PCR, in situ hybridization (ISH) and H&E staining were used for morphological and expression analysis. Cartilage and bone defects were determined by Alcian blue and Alizarin red staining, respectively. CRISPR induced phenotypes were verified by injecting morpholinos targeting psmd5. ISH revealed maternal expression of psmd5 with continued expression during the early stages of the developing zebrafish embryo. At 26 hours post fertilization, expression was observed in the entire eye and brain with less intense expression in other parts of the body. CRISPR targeted disruption of psmd5 gene resulted in different mutations including, single/few base pair (bp) changes to indels carrying 23 or 25 bp deletions. A significant reduction up to 50 - 60 % of psmd5 expression was detected by qRT-PCR in the mutant embryos. F1 embryos obtained by intercrossing F0 mutant fish showed coloboma and other vertebral defects. Coloboma was observed in the ventral retina/retinal pigment epithelium (RPE) and in the optic stalk. Vertebral defects including bone fusions, multiple transverse processes on vertebral bodies, and missing vertebrae were detected in psmd5 mutants. No coloboma or vertebral defects were observed in control ABTL zebrafish adults. F3 homozygous mutant embryos carrying 23 bp deletions showed coloboma and other ocular defects. Injection of wild type zebrafish embryos with two different splice-blocking morpholinos targeting the 2nd and 3rd exons of the psmd5 gene showed dose-dependent severity of the coloboma phenotype along with other body defects in zebrafish embryos. We conclude that mutations in psmd5 gene result in ocular coloboma and vertebral defects in zebrafish, suggesting an important role for psmd5 during optic fissure closure and vertebral development.

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Genetics
The role of chromosome axis protein enrichment in the initiation of meiotic recombination
Homologous recombination is important for the proper segregation of homologous chromosomes during meiosis. Errors in this process can lead to miscarriages, genetic disorders and in some cases cancer. Sites of meiotic recombination initiation are non-random and have a high correlation with sites of enrichment for meiosis-specific chromosome axis proteins, but the role of axis proteins in recombination remains poorly understood. We developed a method to change axis protein levels to study whether or not meiotic recombination is directly influenced by local axis protein levels. The two axis proteins in Saccharomyces cerevisiae, Red1 and Hop1, are essential for meiosis and spore viability. To understand the relationship between these proteins and recombination, we used the ParB-parS system to increase the levels of either Red1 or Hop1 in a region that is usually depleted for these proteins (like URA3). Similar to the lac-operon system, ParB protein binds to a ~20bp parS sequence. The advantage of this system is that ParB self-oligomerizes, so it can cover a region of several kilobases
around a parS insert site with minimal genome disruption. We used the ParB-parS system with Red1-ParB and Hop1-ParB fusions to increase axis protein levels at URA3, an axis protein poor region with low recombination. Genetic analysis showed that increasing Hop1-ParB fusions, but not Red1-ParB fusions, increased the rate of meiotic recombination almost 5-fold at URA3 making it similar to the rate of recombination observed at the hottest meiotic recombination site yet identified in yeast. This suggests that Hop1 levels directly influence rates of recombination. We plan to use this system to identify domains of Hop1 that are important for recombination, and to determine the mechanism by which Hop1 plays its role in meiotic recombination. Moreover, since Red1 is thought to be important for Hop1 loading, it would be interesting to see if the requirement for Red1 in meiotic recombination is bypassed at the parS site in the presence of Hop1-ParB. In conclusion, our results not only show that elevated Hop1 levels are crucial for a higher rate of recombination but also gives us a great system to study the rate of recombination at a specific-genetic-loci without interfering with global meiotic recombination.

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Genomics

Systematic Prediction of Novel Anti-CRISPR Proteins

There is an everlasting battle between prokaryotes and viruses that infect prokaryotes, often described as an arms-race. Prokaryotes continually evolve defenses against viruses while viruses continually evolve weapons to thwart those defenses. A key prokaryotic defense system is CRISPR-Cas, which explicitly targets viral nucleic acids. In turn, viruses have evolved counter-defenses to CRISPR-Cas. One such counter-defense system is a set of recently discovered proteins that interact with CRISPR-Cas and render it ineffectual. These proteins are termed anti-CRISPR (Acr). Acrs have a clear biotechnological use: as CRISPR-Cas has been appropriated by researchers to edit DNA, Acrs allow a higher degree of control on the DNA editing process, by providing the ability to stop it at will. However, Acrs have very little sequence similarity to each other. Thus, detecting novel Acrs is a daunting challenge, and to date only a handful have been discovered. We have therefore developed an approach to detect novel Acrs. Specifically, we have created a set of quantifiable protein features and assessed those features in known Acrs (n=3,666). We then trained a random forest model on this data set, resulting in a trained model that can predict whether or not a candidate protein is an Acr. To assess our model, we applied it to an unseen test set and found it to be significantly predictive of novel Acrs (ROC AUC: 0.81; p-value: 0.001), successfully predicting Acrs from non-Acrs. As a secondary screen, we implemented an iterative search that consists of searches for homologs of proteins that are adjacent to previously discovered Acrs, in an effort to detect novel Acr genomic neighborhoods. We ran this iterative search exhaustively, combined with our model, across our dataset of prokaryote and phages genomes. In total, we found 9,823 novel Acr candidate proteins that cluster into 2,770 novel Acr candidate families. Thus, we developed an approach to predict novel Acrs, an elusive class of proteins that are key components of the weaponry deployed by viruses of prokaryotes against their hosts and have potential in genome engineering.
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Genomics  

*Using Deep Learning to understand the grammar of enhancers.*  
Enhancers are one of the key non-coding functional elements in the human genome. Recent studies have reported that Deep Learning can predict numerous epigenetic and open chromatin regions with unprecedented accuracy. In this work, we employed Deep Learning to use 2000 different signals (DNasel hypersensitivity sites (DHS), transcription factor binding sites (TFBS) and histone marks (HM)) from 127 different cell lines and tissues to define and predict enhancer regions. We built a two-step Convolutional Neural Network, where the first step is trained to recognize the DHS, TFBS and histone marks, and the second step fine-tunes the outcome of the first layer to formulate enhancer regions defined by H3K27ac and H3K4me1 marks. The average AUC for the predictors of DHS, TFBS, HM, and enhancers were 0.93, 0.91, 0.88 and 0.87 respectively, whereas the cutting-edge SVM-based method predicted with AUC of 0.71. We then used our method to profile the entire mutation space within the enhancer regions. Analysis of predicted scores generated through in-silico mutagenesis approach revealed that the positions which correspond to the binding sites of transcription factors show high correlation with the binding site motifs of the given transcription factor. The scores generated with our method fit binding site profiles significantly better than the corresponding average conservation scores (phyloP/phastCons). As the benefit of using the two-step approach, we can detect which input signals (DHS, TFBS, HM) contributed to the outcome of the mutagenesis. For instance, we observed that %65 of the enhancer regions in the HepG2 cell line can be explained by the TFBS or the combinations among them. Furthermore, we validate the accuracy of our method using the publicly available datasets for MPRA, GWAS, and eQTLs.

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*Elucidating Functional Enhancers Governing Pancreas Cell Identity*  
The genetic and epigenetic regulatory mechanisms underlying human disorders have recently been a focus for etiological models of human diseases, including diabetes mellitus. All forms of diabetes result
from loss or impairment of beta-cells, the sole source of the principle glucose homeostasis hormone, insulin. Delineating the molecular pathways underlying the acquisition or maintenance of pancreatic cell fates is instrumental for developing targeted regenerative therapies. Enhancers are principle regulators of cell identity; however, the number of predicted enhancers greatly surpasses the number of coding genes, and a single enhancer can regulate multiple gene targets. Thus, studying enhancer function in human tissues has remained a challenge. To begin addressing this issue, in our prior work we combined cell sorting with ATACseq, ChIPseq and RNAseq to identify cell-type specific genomic regions in normal human pancreas cells. We discovered putative regulatory regions corresponding to specific pancreas lineages. More than 50% of these cell type-specific regions are linked to lineage-specific gene expression and coincide with active enhancer chromatin state. However, mechanisms linking these regions to lineage-specific gene regulation, particularly in endocrine cells, remain unclear. In this study, we performed Hi-C linked with chromatin immunoprecipitation (HiChIP) in EndoC-bH1, an insulin-producing human beta cell line, to elucidate the enhancer-interacting genomic regions. We also established CRISPR-interference systems in EndoC-bH1 cells to determine the functionality of putative enhancers to be identified by HiChIP. By integrating the HiChIPseq and ATACseq data, we identified unique enhancer-target interaction loops in EndoC-bH1 cells, whose anchors correspond to beta-cell specific accessible chromatin regions. To test whether these looping and accessible chromatin regions control transcription, we systematically targeted several regions around the PCSK1 gene, which encodes a rate-limiting enzyme essential for insulin biosynthesis, using the CRISPR-i (KRAB) and CRISPR-a(VP64) systems. We found that perturbation of enhancers that loop to the PCSK1 TSS (transcription start site) significantly decreased (KRAB) or increased (VP64) its transcription, while those with no loops had no effect. Our data will provide critical knowledge on gene regulatory mechanisms governing beta cell identity and function to advance cell replacement therapies for diabetes.

Alexandre Girard
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HIV and AIDS Research
Dynamic Interactions Between CD4 and Integrin a4ß7 on Primary CD4+ T Cells

Human gut associated lymphoid tissue (GALT) is a major site of HIV replication. During the acute phase of HIV infection, the virus preferentially infects a4ß7high CD4+ T cells. HIV gp120 interacts with both the CD4 receptor and a4ß7, the gut homing receptors on CD4+ T cells in gut tissues. By confocal microscopy, we demonstrated that CD4 and a4ß7 colocalize on the surface of a peripheral and gut CD4+ T cells. Additionally, a complex of a4ß7 and CD4 can be immunoprecipitated from CD4+ T cells with an anti-CD4 mAb. Although a4ß7 and CD4 are not known to function in a coordinated way, we find that HIV gp120 binds to and signals through both. In this study we employed high-resolution confocal microscopy, Förster resonance energy transfer (FRET), fluorescence lifetime imaging microscopy (FLIM) and a proximity ligation assay (PLA), to evaluate the relationship between CD4 and a4ß7 on the surface of primary CD4+ lymphocytes. We find that a4ß7 and CD4 interact in a dynamic fashion. Following stimulation by either MAdCAM (the natural ligand of a4ß7) or HIV gp120, these to receptors rapidly form a complex on the surface of primary CD4+ T cells. Memory a4ß7+ CD4+ T cells show higher CD4-a4ß7 complex formation than naive CD4+ T cells. Surface plasmon resonance analysis show a high
affinity (nanomolar) between soluble CD4 and soluble α4β7. The specific high-affinity interaction between these two cell surface receptors, both of which bind to HIV gp120, suggests a novel functional coupling that is likely unique to gut associated lymphoid tissues, that HIV has evolved to exploit.

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Alice Duchon  
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*Exploring the requirements for RNA packaging through a non-viral RNA binding protein*

To generate infectious virus, two copies of the RNA genome must be packaged into new particles. HIV-1 Gag polyprotein selects and incorporates the RNA genome into assembling virions at the plasma membrane. The nucleocapsid domain (NC) of Gag is integral to the RNA selection process; however, little is known about potential contributions of other domains of Gag during packaging. To define the HIV-1 RNA packaging mechanism, we designed an experimental system to examine the influence of individual Gag domains on RNA packaging in the absence of NC. Replacement of NC with a leucine zipper, GagLZ, forms morphologically normal virus-like-particles that lack viral RNA genomes. Thus, to package RNA in this context, we fused a bacterial RNA-binding protein, BglG, to an internally mCherry-labeled GagLZ to generate GagLZ-C-Bgl. BglG specifically binds to a stem-loop RNA structure (BSL). To generate virus particles, we used three constructs expressing unlabeled GagLZ, GagLZ-CFP, and GagLZiC-Bgl. Additionally, these Gag-expressing constructs contain two sets of stems-loops: BSL, and sequences recognized by bacterial phage PP7 coat protein. To detect viral RNA, we coexpressed a yellow fluorescent protein fused to PP7 coat protein (PP7-YFP). Viral particles were harvested and imaged using fluorescent microscopy. We observed that most particles were CFP+ and mCherry+, indicating efficient incorporation of GagLZC-Bgl. Additionally, ~40% of the particles were also YFP+, indicative of PP7-YFP labeled RNA brought into the particles by the GagLZ-C-Bgl protein. To determine trans-acting requirements for RNA packaging, we next created eight GagLZiC-Bgl truncation mutants. All mutant Gag proteins were able to co-assemble with GagLZ-CFP; however, RNA packaging was significantly reduced when regions of Gag critical for oligomerization were deleted from GagLZ-C-Bgl. Inhibition of GagLZ-C-Bgl myristylation also conferred a packaging defect, underscoring the importance of RNA bound Gag to traffic and anchor to the plasma membrane during assembly. Together these results reveal that trans-acting elements that facilitate Gag-bound RNA
multimerization at the membrane are needed for efficient genome packaging. Currently, there are no therapies that target the assembly stage of HIV-1 infection and understanding basic mechanisms behind selection and packaging of the RNA genome are essential for the development of future treatments.

Feng Li
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Identification of an integrase-independent pathway of retrotransposition

Retroelements, such as long terminal repeat (LTR)-retrotransposons and retroviruses, insert their reverse transcribed cDNA into the host genome through the activity of integrase (IN). However, human immunodeficiency virus type 1 (HIV-1) is able to insert its cDNA into chromosomal sites independent of IN, albeit at lower frequencies. The IN-independent insertion may act as a virus reservoir that compromises the antiretroviral therapy which currently relies on IN inhibitors. To determine how cDNA is inserted into the genome in the absence of integrase we studied retrotransposition in the model organism fission yeast. Retrotransposon Tf1 is dependent on a life cycle that parallels that of retroviruses, including particle formation, reverse transcription, and integration. We found that Tf1 mutants lacking IN are capable of inserting into the chromosomes, albeit with 20 to 30-fold lower frequencies than wild type Tf1. To determine the mechanism of integrase independent events, we used a deep sequencing method capable of mapping 10,000 Tf1 insertions produced by Tf1 with an IN frameshift (INfs). DNA logo analysis, which is aimed at identifying sequence bias, showed that the sequences just after the insertion sites had a prominent representation of ATAACTGA. Surprisingly, this unique sequence matches that of the primer binding site (PBS), an 11 bp sequence that is retained at the 3′ end of the cDNA after reverse transcription but is removed by IN. The presence of the PBS sequence downstream of the insertions strongly suggested that single stranded PBS (ssPBS) sequence mediates IN-independent insertion, probably by homologous recombination (HR). To examine the HR function in the IN-independent events, we conducted a genetic assay to test Tf1 insertion in HR mutants. Interestingly, the deletion of single strand recombination protein Rad52, and not the double strand factor Rad50, caused a dramatic reduction of insertions produced by Tf1 INfs. This indicates that Rad52 plays a critical role in IN-independent Tf1 insertions by binding to the ssPBS at the end of the cDNA causing invasion at genomic sites with similar sequence. Importantly, I found that IN-independent insertion sites of HIV-1 previously published show PBS sequence. In summary, our findings demonstrate that IN-independent insertions are mediated by homologous recombination via the ssPBS. More importantly, the PBS mode of cDNA insertion occurring with HIV-1 may be a novel target for anti-HIV therapy.

Ruth Hunegnaw
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Alveolar Macrophage Dysfunction and Increased PD-1 Expression During Chronic SIV Infection of Rhesus Macaques

HIV infected individuals have been shown to be predisposed to pulmonary infections even while receiving anti-retroviral therapy. Alveolar macrophages (AMs) play a critical role in lung innate immunity, but contradictory results have been reported regarding their functionality following HIV infection. Here, using the SIV rhesus macaque model, we document the effect of SIV infection on the phenotypic and functional properties of AMs. Following infection with SIVmac251, AMs in bronchoalveolar lavage (BAL) sampled over 2- to 20-weeks post-infection (wpi) were compared to those in BAL samples from naïve macaques. AM expression of proinflammatory cytokines TNF-α, IL-6, IL-1β, and chemokine RANTES drastically increased 2-wpi compared to AMs of naïve macaques (p<0.0001 for all) but dropped significantly with progression to chronic infection. Phagocytic activity of AMs 2-and 4-wpi was elevated compared to AMs of naive animals (p=0.0005, p=0.0004, respectively) but significantly decreased by 12-wpi (p=0.0022, p=0.0019, respectively). By 20-wpi the ability of AMs from chronically infected animals to perform SIV-specific antibody-dependent phagocytosis (ADP) was also diminished (p=0.028). Acute SIV infection was associated with increased FcgammaRIII expression which subsequently declined with disease progression. Frequency of FcgammaRIII+ AMs showed a strong trend toward correlation with SIV-specific ADP, and at 2-wpi FcgammaRIII expression negatively correlated with viral load (r=-0.6819; p=0.0013), suggesting a contribution to viremia control. Importantly, PD-1 was found to be expressed on AMs and showed a strong trend toward correlation with plasma viral load (r=-0.8266; p=0.058), indicating that similar to over-expression on T-cells, PD-1 expression on AMs may also be associated with disease progression. These findings provide new insight into the dynamics of SIV infection leading to AM dysfunction and alteration of pulmonary innate immunity. Our results suggest new pathways to exploit in developing therapies targeting pulmonary disease susceptibility in HIV-infected individuals.

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Immunology - Autoimmune

Tcell-exosome-derived miR-143-3p impairs glandular cell function in Sjögren’s syndrome

Sjögren’s syndrome (SS) is a systemic autoimmune disease that mainly affects salivary and lacrimal glands. Mechanisms of SS pathogenesis are poorly understood. It is thought that inflammation leads to destruction of exocrine glands, however the triggers of autoimmunity and the mechanisms by which inflammation drives immunopathology are not characterized. Our work identifies T cell-exosome-derived miR-143-3p as a pathogenic driver of immunopathology in SS. MicroRNAs (miRNAs) are endogenous small noncoding RNA molecules that regulate the expression of target genes through translational repression of mRNAs. Through transcriptomic profiling studies our group had previously documented a significant upregulation of miR-142-3p in patient SS tissues and in serum exosomes. In our current work we document miR-142-3p in the salivary glands of SS patients but not healthy volunteers, both in the epithelial gland cells and within T cells of the inflammatory infiltrate. We go on to show that activated T cells secrete exosomes containing miR-143-3p which can impair the function of
salivary gland epithelial cells (both protein production and secretion). We further identify and validate target genes downregulated by miR-142-3p in epithelial cells. We demonstrate that miR-142-3p binds to the 3' untranslated region of SERCA2b and RyR2. Indeed, transfection of miR-142-3p in epithelial cells, as well as treatment with exosomes from activated T cell supernatants led to significant decrease in the mRNA and protein levels of SERCA2b, RyR2 and AC9. Importantly, overexpression of miR-142-3p impaired glandular cell function as indicated by restricted cAMP production and altered calcium signaling upon isoproterenol and carbachol stimulation, respectively. This study provides evidence for a functional role of the miR-142-3p in SS pathogenesis and promotes the concept that T cell-activation directly may impair epithelial cell function through secretion of mi-RNA containing exosomes.

Na Rai
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Immunology - Autoimmune

Chronic type I interferon excess disrupts tissue macrophage homeostasis in vivo

Overproduction of type I interferon is characteristic of autoimmune diseases but its impact on tissue phagocyte function is poorly understood. Tissue macrophages, in particular in the peritoneum and lung, play a key role in preventing autoimmunity through tonic clearance of apoptotic cells ("efferocytosis"). We hypothesized that type I interferon might be responsible for the deficits in macrophage efferocytosis commonly observed in autoimmune disease. To address this, we used mice deficient in immunity-related GTPase M protein 1 (Irgm1), a model that we recently reported has hallmarks of autoimmunity including lymphocytic tissue lesions, autoantibodies, and type I interferon overproduction. Here, we report that macrophages in the naïve Irgm1-/- peritoneum are reduced in number and exhibit higher retention of the circulating monocyte marker Ly6C and reduced expression of the maturation markers MHCII, F4/80, and CD11c, collectively suggesting defective monocyte-to-macrophage differentiation. Increased Ly6C and decreased MHCII, CD88, and CD11b was also observed in interstitial macrophages of Irgm1-/- lungs, suggesting a systemic deficit in tissue phagocyte differentiation. Single cell RNA-seq revealed that a genomically distinct population of macrophages had replaced the conventional yolk sac-derived F4/80hi resident macrophages of the naïve Irgm1-/- peritoneum. Gene set analysis indicated widespread induction of interferon-stimulated genes in the new macrophage cluster, indicating a robust type I interferon signature. Irgm1-/- peritoneal macrophages expressed reduced levels of the efferocytosis receptor Tim4 and showed decreased ability to uptake apoptotic cells, as well as decreased delivery of internalized cell corpses to the acidified lysosome. Deletion of the type I interferon receptor (Ifnar) in Irgm1-/- animals normalized the maturation phenotype of Irgm1-/- peritoneal macrophages and restored their efferocytic ability. Moreover, Ifnar deletion abolished autoimmune tissue pathology and elevated autoantibodies in vivo in Irgm1-/- mice. Taken together, our studies suggest that type I interferon dysregulates the ontogeny and differentiation of tissue macrophages and may thereby promote autoimmunity by obstructing the developmental seeding of efferocytosis-competent tissue phagocytes.
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NIDCR  
Immunology - Autoimmune  

*High-glucose intake exacerbates autoimmune through ROS-mediated TGF-β activation*

Autoimmune diseases arise from abnormal immune responses attacking normal tissues of the body, which results in chronic destruction of these tissues and a serious diminishment in the quality of patients’ life. The incidence of autoimmune diseases, including Crohn’s disease and multiple sclerosis (MS), has increased tremendously over the past few decades, especially in western countries. Diet has been suggested as a potential environmental risk factor for the increasing incidence of autoimmune diseases, but the underlying mechanisms remain largely unknown. The consumption of high salt, fat, and sugar has already been suggested and associated with increasing prevalence in various diseases. However, whether high-sugar intake affects the development of autoimmunity remains unclear. Here we show that high-glucose intake exacerbated autoimmunity in mouse models of T cell transfer colitis (a model of Crohn’s disease) and experimental autoimmune encephalomyelitis (EAE, a model of MS). We demonstrated that high-glucose intake exacerbated autoimmunity by induction of Th17 cells, as high-glucose intake specifically increased Th17 cells in the mouse models, and autoimmunity exacerbated by high-glucose intake was completely reversed when Th17 cells were abolished. Mechanistically, we elucidated that high levels of glucose specifically promote Th17 cell differentiation by activating TGF-β from its latent form in vitro. We further determined that the high glucose-induced TGF-β activation was mediated by increased reactive oxygen species (ROS) in T cells, as elimination of ROS completely reversed Th17 cell differentiation. We have thus revealed a previously unrecognized mechanism underlying the adverse effect of high-glucose intake in the pathogenesis of autoimmunity and inflammation. Our findings warrant further exploration of the immunological mechanisms of high-sugar intake and should have potential clinical significance.

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Immunology - Autoimmune  

*Prolonged antibiotic treatment reverses the protective effect of gut microbiota depletion on autoimmune uveitis*

Autoimmune uveitis, thought to be driven by retina-specific T cells, is a major cause of blindness. We and others have shown that gut microbiota act as a trigger and/or modulator of spontaneous uveitis, at least in part by antigenic mimicry. In a model of experimental autoimmune uveitis (EAU), where an antigenic trigger is provided by immunization with the retinal protein IRBP, long-term elimination of commensals by oral antibiotic treatment (ABX) did not affect susceptibility to EAU. In apparent contradiction, others reported that short-term ABX in the immunization-induced EAU model was protective. To reconcile these findings, we compared how long vs. short ABX affected the composition of gut microbiota, systemic host-immune responses, and susceptibility to EAU. EAU-susceptible B10.RIII mice were continuously treated with oral broad-spectrum antibiotics starting 12-weeks (long ABX) or 1-week (short ABX) before induction of EAU. Monitoring disease development by fundus examination, we
confirmed that short ABX delayed the onset of EAU, whereas long ABX displayed disease kinetics and severity similar to untreated controls. Metagenomic 16S rRNA analyses of fecal pellets of ABX mice revealed time-dependent depletion of gut microbial communities (dysbiosis), including effects on Lactobacillus and other taxa. Interestingly, we observed a progressive disappearance of CD4+ and CD4+CD8+ intraepithelial lymphocytes (IELs) from the gut epithelium, such that long ABX mice had markedly reduced IELs compared to short ABX mice. Notably, these IELs, which are induced by Lactobacillus reuteri and thought to regulate gut barrier integrity, exhibited cytotoxic activity in vitro towards activated IRBP-specific T cells and B cells. We propose that gut microbiota play dual roles in uveitis development: they provide stimuli for uveitogenic effector cells, but also maintain a "regulatory" IEL population, whose progressive loss reverses the protective effect of short ABX. A greater loss of "protective" IELs during long ABX may counterbalance the benefit of the reduced bacterial mass, resulting in net zero effect on disease. Ongoing work is focused on linking aspects of gut mucosal integrity and IEL function to EAU susceptibility. Our findings may have implications for extended use of antibiotics in clinical settings and may lead to a better understanding of the role of IELs in the microbiota-gut-eye axis.

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Immunology - General

Regulation of pyruvate kinase activity is required for normal IL-2/12-induced Natural Killer cell metabolism and function

Natural Killer (NK) cells are cytotoxic lymphocytes that are crucial for defense against viruses and cancer. In cytokine activated NK cells, elevated rates of glycolysis are important for anabolic processes, as glycolytic intermediates contribute to the synthesis of nucleotides, lipids and amino acids. Pyruvate Kinase Muscle (PKM) is the last enzyme of glycolysis and converts phosphoenolpyruvate to pyruvate. Two isoforms exist, PKM1 and PKM2. Data suggests that PKM1 exists as a tetramer that is fully active. In contrast, PKM2 can adopt two configurations, active tetramers or inactive mono/dimers. Thus, PKM2 has the ability to adopt different activity levels whereas PKM1 does not. In cancers, inactive monomers limit the rate of pyruvate production resulting in accumulation of upstream glycolytic intermediates to support biosynthesis and cell growth. Our data show that in vitro cytokine stimulated murine NK cells and in vivo Poly(I:C) activated NK cells up-regulate the expression of PKM2. In order to dissect the role of PKM2 in NK cell physiology, we generated an NK cell specific PKM2 knock out mouse line (Ncr1Cre x Pkm2fl/fl). NK cells lacking PKM2 are present in normal frequencies and respond normally to stimulation with IL-2/12 or Poly(I:C). Interestingly, baseline levels of glycolysis and OXPHOS are similar in WT and Ncr1CrePkm2fl/fl NK cells. Biochemical analysis shows elevated levels of PKM1 in Ncr1CrePkm2fl/fl NK cells but unexpectedly these cells possess overall PK activity comparable to that of WT cells. This observation indicates that regulation of pyruvate kinase activity is likely important for NK cell metabolism. To dissect this hypothesis we acutely dysregulated PK activity using a specific pharmacological activator of PKM2, TEPP-46. We find that TEPP-46 promotes PKM2 tetramerization and increases glycolytic flux indicating that NK cells do not use their full pyruvate kinase potential during IL-2/12 stimulation and that PKM2 is a key regulator of pyruvate production. Interestingly, TEPP-46
treatment of NK cells also reduces cytokine-induced activation of metabolic signaling pathways and leads to considerable defects in OXPHOS. Moreover, elevated PK activity leads to functional defects in the form of reduced blastogenesis and proinflammatory cytokine production. Overall, our data indicate that NK cells can utilize a pool of inactive PKM2 to maintain metabolic plasticity and that aberrant engagement of this pool is detrimental.

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Immunology - General  
The bone marrow protects and optimizes immunological memory during dietary restriction  
Mammals evolved in the context of fluctuating food availability. However, how fundamental aspects of the immune system are preserved when calories and nutrients are reduced remains unclear. We have previously shown that white adipose tissue (WAT) is a preferred site of lodgement for memory T cells. During dietary restriction (DR), WAT is used as an energy source and so is reduced throughout the body, whereas adipocytes in bone marrow (BM) are paradoxically increased in number. As such, we postulated that the BM compartment may play an important role in protecting memory T cells in the context of nutritional stress. We found that 50% DR resulted in a sharp reduction in number of memory T cells in secondary lymphoid organs (SLO) and adipose tissue. In contrast, memory T cells rapidly increased in number in BM throughout the skeleton, which included an increase in both central and effector memory subsets. This cellular redistribution was coordinated by the steroid hormones glucocorticoids (GC), factors that play a critical role in regulating energy balance during times of stress, but that can cause T cell death at high concentrations. BM was found to have low levels of GC at baseline and during DR and as such provided a niche that promoted cell survival. Furthermore, DR was associated with a profound remodeling of the BM compartment, which included an increase in T cell homing factors and adipogenesis. Adipocytes, as well as CXCR4-CXCL12 and S1P-S1P1R interactions contributed to enhanced T cell accumulation and survival in BM. During DR, memory T cells exhibited a reduced metabolic state associated with a decrease in homeostatic proliferation and a reduction in signaling via the nutrient sensor mammalian target of rapamycin (mTOR). As such, memory T cells appeared to have entered into a state resembling quiescence or energy conservation, with this phenotype more pronounced in BM compared to SLO. Surprisingly, DR promoted memory T cell function and resulted in an enhanced ability of the host to control secondary infections. This enhanced functional response during DR was extended beyond infectious disease, as shown by the ability of these cells to restrict the growth of melanoma during DR and promote host survival. Together, this work uncovers a fundamental host strategy to sustain and optimize immunological memory during nutritional challenges that involved a temporal and spatial reorganization of the memory pool within a safe haven compartments.
Djalma Lima
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Immunology - General

Retroviral control of skin inflammation

Obesity is a growing and significant healthcare problem worldwide. Clinical research have shown that obesity is associated with an increased severity of inflammatory skin disorders. However, the mechanisms underlying these negative outcomes remain unclear. Here, we investigated the impact of a diet enriched in fat on skin physiology and how such responses may alter host-microbiota interactions.

We fed mice with a control (CTRL) or high fat diet (HFD/ containing 60% fat) for 6 weeks then analyzed the skin inflammatory profile. At steady state, HFD induced the accumulation of lymphocytes, including CD4+, CD8+, gama delta T cells and innate lymphoid cells, in skin. This occurred prior to the development of metabolic syndrome and was not seen in other organs. We also found an increase in keratinocyte proliferation and higher frequencies of lymphocytes producing the inflammatory cytokines TNF-a, IFN-g and IL-17. In contrast, HFD did not affect the frequency or function of macrophages, neutrophils or dendritic cells in the skin. Such an effect could be partially recapitulated with oral supplementation of the free fatty acid palmitic acid. Interestingly, high lipid content in the diet was associated with a significant increase in the expression of several endogenous retrovirus (ERV) families in the skin. Looking for the mechanism involved in this process, the cellular infiltrate following feeding of a HFD was abolished in the absence of DNA sensor stimulator of interferon genes (STING), the Nlrp3 inflammasome and following treatment with antiretroviral drugs. Given that skin commensals can lead to the induction of T cell infiltration in the absence of inflammation, we assessed whether HFD could disrupt the skin immune response to commensal microbes. We found that HFD induced aberrant inflammatory responses following exposure to S. epidermidis, a response that could be prevented by antiretroviral treatment. Taken together, our data propose a link between the diet and ERV in controlling the threshold of immune cell activation in skin, a phenomenon that has consequences on the ability of the host to develop beneficial responses to the microbiota. Also, these studies provide a new understanding of the etiology of skin inflammatory disorders during obesity. This could allow the development of novel therapeutic strategies with tailored clinical interventions that reshape our microbial communities in order to limit tissue damage and/or promote skin disease control.

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Mining immune memory to identify causative gut microbiota members in inflammatory disease in mice and humans

Numerous studies have identified shifts in the gut microbiota associated with human inflammatory states including diseases such as arthritis, inflammatory bowel disease, asthma, and multiple sclerosis and with beneficial inflammatory states such as in the response to cancer immunotherapy. However, such association studies are limited in their capacity to discern causality in the host-microbe relationship, and tools to probe such relationships in humans and mice are limited. We have developed
a novel technique to identify gut microbes that have specifically elicited potent systemic immune activation via the B cell arm of the peripheral adaptive immune system (IgG-seq). We have utilized this technique to identify pro-inflammatory gut bacteria in murine models of gut barrier disruption including infection with Toxoplasma gondii and Yersinia pseudotuberculosis, as well as the non-infectious colitis model of dextran sodium sulfate (DSS). In these contexts, IgG-seq identified previously uncultured gut bacteria (including Erysipelotrichaceae gen. sp. nov. and Porphyromonadaceae gen. sp. nov.). Using high-throughput culture techniques, these bacteria were isolated and found to exacerbate T cell and innate immune activation when supplemented into mice subjected to the respective models of gut barrier injury. To test applicability in humans, we applied IgG-seq to identify pro-inflammatory gut bacteria in subjects infected with HIV as a prototypical inflammatory disease, wherein chronic immune activation is associated with early death and shifts in the gut microbiota are linked to this persistent inflammation. Utilizing primary human gut microbiota samples and the endogenous, systemic immunoglobulin G repertoire via IgG-seq, we have found that a conserved signature of robust systemic immune memory responses against members of the Erisypelotrichaceae family is unique to progressive HIV disease. Supplementation of mice with these previously uncultured novel taxa and treatment with DSS was found to elicit systemic innate immune and T cell activation, supporting a possible role for this gut microbe in HIV-associated chronic inflammation and HIV disease progression. Identification of human inflammation-promoting gut microbiota members in HIV thus provides a framework from which to explore the host-microbiota relationship in a variety of chronic inflammatory diseases, and opens doors to precision microbiome editing strategies for alleviating pathologic inflammation.

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Immunology - General
Single-Cell Transcriptomics Identifies TOX as a Key Transcriptional Regulator of Progenitor-Like CD8 T Cells in Chronic Infection
During chronic viral infection and cancer T cells become exhausted, which is characterized by progressive loss of T-cell function and memory potential, upregulation of inhibitor receptors, and reduced proliferation. Our group and others recently identified a TCF1+ CD8 T cell subset during chronic infection, the progenitor-like CD8 T cells, that is crucial for long-term antiviral immunity by self-renew and repopulating the terminally differentiated CD8 T cells. The progenitor-like CD8 T cells share some common transcriptional regulatory pathways with memory precursor cells generated after acute viral infection. However, it is not clear whether the progenitor-like CD8 T cells are transcriptionally and epigenetically distinctly regulated for adaption of immune suppressive environment during chronic infection. We first compared single cell transcriptome of virus-specific CD8 T cells isolated from mice 4.5 days or 7 days after infection with lymphocytic choriomeningitis virus (LCMV) Armstrong, which causes acute infection, or LCMV clone 13, which causes chronic viremia in mice. Single-cell RNA sequencing (scRNA-seq) is a powerful tool to subset cells independent of previous knowledge of known markers, and to uncover transcriptional differences that might be masked by averaging gene expression of pooled cells. Our scRNA-seq data showed that the transcriptomes of virus-specific CD8 T cells generated after acute and chronic infections diverged prior to the termination of clonal expansion. We also found that
progenitor-like CD8 T cells distinguished themselves from memory precursor effector cells by higher expression of a gene module containing Tox, which encodes TOX, belonging to a family of high mobility group (HMG) transcription factors. Using chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq), we found that progenitor-like CD8 T cells exhibited more active histone markers at genes co-expressed with Tox. To determine the function of TOX, we adoptively transferred TOX-overexpressing viral-specific T cells into mice followed by chronic viral infection, and found that TOX promoted the long-term persistence of virus-specific CD8 T cells. Conversely, using TOX knock out (KO) mice, we found that TOX was required for the progenitor-like CD8 population, and maintenance of antiviral T cell response. Thus, TOX is critical for the longevity of progenitor-like CD8 T cells and long-term antiviral CD8 response during chronic viral infection.

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Immunology - General

Identification of CD28 homolog as a strong activating receptor of NK cells that promotes anti-tumor responses

Background: Natural killer (NK) cells are regulated by activating and inhibitory innate receptors. Cancer and infected cells upregulate ligands for NK activating receptors, and trigger NK cell cytotoxicity. NK cells are promising candidates for cancer immunotherapy due to their intrinsic antitumor activity without causing graft-versus-host disease (GVHD). However, a major constraint of NK cells is the suppression of their activity by inhibitory receptors for MHC class I. My goal was to study novel NK cell activating receptors, and modulate them to override MHC class I inhibition. The B7 protein family includes important positive (e.g. NKp30 ligand B7H6) and negative (e.g. PD-1 ligand B7H1) regulators of immune responses. Recently, B7H7 (HHLA2), which is highly expressed on tumors, was identified as a novel member of the B7 family, and binds receptor CD28 homolog (CD28H). I studied whether CD28H could activate NK cells to recognize B7H7+ tumor cells. Methods: Using freshly isolated human NK cells, CD28H was crosslinked along with other NK activating receptors and synergetic NK cell activation was measured by degranulation, cytokine secretion and killing of tumor cells. Several CD28H chimeric antigen receptors (CD28H-CARs) were engineered to test the cytotoxic activity of NK cells toward B7H7+ cells, and whether it could override MHC class I inhibition. Results and conclusions: I found that CD28H is expressed on freshly isolated NK cells. CD28H selectively synergized with receptors 2B4 and NKp46 to induce robust NK cell degranulation and cytokine production. Using NK cells transfected with CD28H mutants, I identified tyrosine 192 in the cytoplasmic tail as being essential for CD28H-mediated activation. NK cells killed B7H7+ non-Hodgkinâ€™s lymphoma cells through CD28H-dependent activation. I found that expression of CD28H-CAR1 (full-length CD28H fused to CD3 zeta chain) in NK cells triggered efficient lysis of B7H7+HLA-E+ tumor cells by overriding inhibition by NKG2A, a receptor for HLA-E. CD28H-CAR2, which incorporated the signaling domain of 2B4 between CD28H and CD3 zeta, led to even greater resistance to inhibition. When Compared to a CAR signaling domain designed for T cells (CD28-41BB-CD3zeta), CD28H-CAR2 induced stronger NK cell activation. In conclusion, I have shown that CD28H is a novel and powerful activator of NK cytotoxicity, which can be used to construct NK-optimized CARs that overcome MHC class I inhibition.
Rajan Guha
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Immunology - Infectious Disease

Repeated malaria exposures skew monocytes/macrophages toward a regulatory phenotype

In malaria-naïve individuals, P. falciparum (Pf) infection results in high levels of Pf-infected red blood cells (iRBCs) that trigger systemic inflammation and fever. Conversely, individuals in endemic areas who are repeatedly infected are often asymptomatic and have low levels of iRBCs, even children who have yet to acquire fully protective antibodies. The molecular mechanisms underlying these clinical observations remain unclear. We previously showed that PBMCs collected from healthy Malian children before the malaria season responded to iRBCs by producing pyrogenic, pro-inflammatory mediators such as IL-1ß, IL-6 and IL-8. However, following febrile malaria there was a marked shift in the response to iRBCs with the same children's PBMCs producing lower levels of pro-inflammatory cytokines and higher levels of anti-inflammatory cytokines (IL-10, TGF-ß). Moreover, genome-wide expression analysis showed that molecules involved in phagocytosis and intracellular killing were upregulated in PBMCs after malaria as compared to before. Together, these data suggested that malaria-induced epigenetic reprogramming of innate immune cells might play a role in immunity to malaria. In follow-up studies, age-stratified analysis of monocytes collected before the malaria season showed an inverse relationship between age and pro-inflammatory cytokine production capacity. Accordingly, monocytes of Malian adults expressed higher levels of CD163, CD206 and arginase 1 ; molecules associated with a regulatory phenotype. These observations were recapitulated with an in vitro system of monocyte-macrophage differentiation whereby re-exposure to iRBCs was associated with diminished expression of pro-inflammatory mediators and a corresponding decrease in epigenetic markers of active gene transcription (i.e. H3K4me3) at the TSS and promoter regions of the same pro-inflammatory mediators. Together these data support the hypothesis that epigenetic reprogramming of monocytes/macrophages toward a regulatory phenotype contributes to the acquisition of clinical immunity to malaria.

Jigar Desai
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Immunology - Infectious Disease

C5ar1 regulates metabolism in myeloid phagocytes and promotes their antifungal effector functions

Candida albicans is responsible for >400,000 invasive-infections per year globally, leading to renal failure and mortality of >40% despite antifungal therapy. A detailed understanding of anti-Candida immune responses is thus necessary in order to identify high-risk individuals for early prophylactic interventions and uncover novel molecules for adjunctive immunotherapy. The anaphylatoxin C5a is the penultimate product of the activated complement system, which regulates chemotaxis, cell-survival and antimicrobial effector-functions in diverse hematopoietic and stromal cell-types, via its receptors-C5ar1/C5ar2. We found that C5a was induced in sera of candidemic patients and of mice post-infection.
C5a signaling through C5ar1, was found to be critical for protection during invasive candidiasis as C5ar1 knockout (KO), not C5ar2 KO, mice displayed dramatically increased mortality and tissue fungal-burden post-infection. Among the different cell-types that express C5ar1, we found that neutrophils and macrophages are essential for protection, as determined using bone-marrow chimera, C5ar1-reporter and cell-type specific conditional C5ar1 KO mice such as Lyz2-Cre, S100a8-Cre and Cx3cr1-Cre/C5ar1[flox/flox] mice. Interestingly, C5ar1-dependent protection is independent of its roles in cell-recruitment, as infected C5ar1KO kidneys showed no defect in leukocyte accumulation, which was confirmed using mixed bone marrow-chimeras. Instead, C5ar1 promotes phagocyte-specific, cell-intrinsic effector functions, being critical for neutrophil-mediated Candida-uptake and macrophage-mediated Candida-killing, as assessed using (1) a novel Candida reporter-strain that allows simultaneous evaluation of phagocytic-uptake/fungal-viability in vivo, (2) real-time intravital kidney imaging in vivo and (3) primary phagocytes ex vivo. Moreover, we observed that C5ar1 regulates glucose utilization via glycolysis in neutrophils and macrophages which we observed to be essential for C5ar1-dependent Candida uptake and killing, as pharmacological inhibition of glycolysis leads to impaired uptake and killing by neutrophils and macrophages, respectively. Thus, we uncovered that activated C5a signaling regulates cell-type-specific anti-Candida effector functions of myeloid phagocytes via regulation of glucose utilization. Ongoing experiments will comprehensively evaluate the broader impacts of C5ar1-dependent metabolic-reprogramming on myeloid phagocyte biology.

Jaekeun Park
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Immunology - Infectious Disease

Pre-existing immunity to the conserved hemagglutinin stalk of influenza virus drives selection for an escape mutant virus in humans.

Influenza viruses are a major public health threat due to their unpredictable and frequent escape mutations and pandemic potential of novel animal viruses. Recently, the conserved influenza hemagglutinin (HA) stalk has gained much attention as a potent target for universal influenza vaccines. While this strategy has shown potential in different experimental settings, it remains unclear if immune pressure applied to the conserved HA stalk would result in the emergence of escape mutants in humans. This is particularly important because influenza viruses have been successful at escaping pre-existing immunity, and answering this question would help advance universal influenza vaccine development. We performed a series of experiments using an influenza human challenge study where study participants were challenged with an influenza virus stock containing a polymorphism in the HA stalk. The mutant virus with the stalk mutation did not show any evidence of decreased viral fitness as measured in different cell lines and in mice and ferrets as compared to the wild type virus, showing that the HA stalk mutant virus is as infectious and pathogenic as wild type. The mutation resulted in a significant conformational change, measured by ELISA and flow cytometry, and the mutant virus showed a significantly increased resistance to a potent broadly neutralizing monoclonal antibody. Immune pressure applied to the HA stalk robustly selected for the mutant virus over the wild type virus in a co-culture setting, suggesting that pre-existing immunity to the HA stalk might effectively support the emergence of escape mutants in humans. Importantly, we demonstrated that pre-existing immune
pressure selects for the mutant virus during replication in humans by 1) showing a statistically significant association between a higher level of pre-existing anti-HA stalk antibodies and the selection for the mutant virus in human influenza challenge participants; 2) showing that human serum with higher anti-HA stalk antibodies selects for the mutant virus in a co-culture setting while serum with lower anti-HA stalk antibodies did not show any evidence of immune selection pressure. This study shed light on a potentially serious hurdle for success of stalk-targeting universal influenza vaccines and calls for intense investigations to overcome this hurdle for the success of truly universal influenza vaccines.

Emily Speranza
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Immunology - Infectious Disease

Defining the role of RLR-MAVS signaling in orchestrating protective immune responses to RNA viruses at a single cell resolution.

One of the earliest responses to infection with RNA viruses is the expression of type I interferon (IFN). This response is vital to host survival by limiting virus replication and regulating innate and adaptive cellular immunity. Replication of RNA viruses is detected by host cells through the RIG-I-like receptor (RLR) pathway that signals through a critical adaptor protein called mitochondrial antiviral signaling protein (MAVS). A thorough understanding of the early IFN response after virus infection requires determining how individual cell types within complex tissues respond at a transcriptional level. Significant advances in single cell technologies are facilitating this type of detailed investigation, but no study to date has used single cell sequencing (scRNA-Seq) in the context of virus infection in vivo. We are coupling scRNA-Seq with high content imaging studies in mouse lymph nodes (LNs) to understand how cell-specific transcriptional responses are organized by cell-to-cell interactions following infection with vesicular stomatitis virus (VSV) and Ebola virus (EBOV). VSV induces a strong IFN response and is also highly sensitive to the antiviral effects of IFN, thus serving as a model for induction of a protective antiviral response. In contrast, EBOV encodes antagonists of the RLR pathway, linked to virus virulence through dysregulation of IFN transcription and signaling. We first infected wildtype (WT) and MAVS-knockout (KO) mice with VSV via the ear pinna and isolated the draining LNs at 8 and 24 hours post infection (hpi). Using scRNA-Seq, we observed that RLR-MAVS signaling is required to initiate responses to IFN specifically in macrophages and plasmacytoid dendritic cells (pDCs) by 8 hpi as determined using a novel algorithm to identify cell types in an unbiased manner. pDC function is thought to be highly dependent on toll-like receptor signaling, and is rarely connected to RLRs suggesting new conduits of pDC communication. The defect in pDC activation observed in MAVS KO mice was likely associated with a failure of NK cells to upregulate genes involved in T cell and neutrophil recruitment. Thus, our work is providing unprecedented resolution of the biological consequences of RLR-MAVS-IFN signaling to antiviral immunity. We are currently conducting these studies with EBOV to determine how dysregulation of RLR-MAVS signaling by highly pathogenic RNA viruses disrupts these earliest of immune responses.
Ai Ing Lim
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Immunology - Infectious Disease

Transient Maternal Infection Prenatally Imprints Long-Term 'Immunological Scar' on Offspring

Mammalian pregnancy is a unique physiological context in which the mother undergoes profound hormonal, metabolic, microbiota and immunological changes. Clinical evidence has associated defined maternal infections during pregnancy with long-term adverse effects on offspring health, including increased risk of inflammatory diseases. With the exception of pathogens that invade the placenta, a mechanistic explanation for these adverse effects remains elusive. Our present work explores how maternal exposure to a transient infection during pregnancy affects the offspring. We found that infection of pregnant mice with the attenuated gastrointestinal pathogen YopM mutant Yersinia Pseudotuberculosis (YptbYopM) produces an infection restricted to maternal tissues, without dissemination across the placenta, or vertical transmission to the offspring. Strikingly, offspring delivered by YptbYopM-infected dams displayed alterations in gut immunity, with a significant increase of pro-inflammatory T helper 17 cells (Th17) in both the large and small intestine. Altered gut immunity was observed after weaning and remained long term into adulthood. Consequently, in line with elevated accumulation of intestinal Th17 cells, offspring delivered by YptbYopM-infected dams displayed higher susceptibility to experimentally-induced intestinal inflammation. Through microbiome profiling, fecal transplant and co-housing experiments, we demonstrated that induction of intestinal Th17 in maternally-infected offspring was microbiota-independent. Strikingly, multiplex quantitative imaging of the gastrointestinal tract demonstrated an extensive and persistent activation of intestinal epithelial cells in offspring delivered by YptbYopM-infected dams. Furthermore, cross-fostering and serum transfer experiments revealed that soluble factors from the circulation of infected pregnant mothers was sufficient to prenatally imprint this proinflammatory intestinal microenvironment. On-going work suggest maternal IL-6 is a potential upstream of this long-term inflammatory imprinting. Together, our data revealed that a transient maternally restricted infection during pregnancy can prenatally condition intestinal barrier function, perturb gut immunity, and lead to increased susceptibility to intestinal inflammation in offspring. Our work provides insight into the factors predisposing tissue specific inflammation and how prenatal events could impose a long term "immunological scar" on the offspring.

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Immunology - Infectious Disease

Structurally Conserved Bat IgG Antibodies Show a High Abundance of Sialylated Glycans: Potential Implications for Disease Resistance in Bats

The prototypic henipaviruses, Nipah and Hendra, are emergent paramyxoviruses capable of causing highly fatal outbreaks associated with extreme neurological and respiratory disease. Despite the severe disease caused in spillover hosts, reservoir host bats do not show negative clinical symptoms in response to natural or experimental infection. This striking difference in disease pathology raises questions about
how features of the bat immune system differ from those of humans in the context of control of viral replication and clinical outcome, and presents the need to further classify the immune components of the host species in detail. This project aims to provide a structural basis for the host response to henipavirus infection by characterizing the antibodies of reservoir bats, particularly the Fc region, using a crystallography approach. Although bats are the primary reservoir for several viral diseases, this is the first in-depth analysis of bat Fc proteins. The Fc region of IgG is involved in antiviral immunity via binding to Fc receptors, which either activates or inhibits immune effector cells, depending on the receptor subtype and the presence and composition of N-linked glycans on the Fc molecule. The IgG Fc region from Pteropus alecto, a confirmed host for Hendra virus, was expressed and crystallized individually as well as bound to the bat high affinity Fc receptor, FcyRI. The P. alecto Fc showed an overall conserved structure and mechanism for receptor binding to human Fc. A conserved feature of the binding site is a hydrophobic pocket on FcyRI into which a specific leucine residue on the Fc molecule is inserted. This contact is critical for the high affinity of the FcyRI â€“ IgG interaction in the human complex, and it appears that this mechanism for high affinity binding is conserved in P. alecto. In addition, as the Fc glycans are crucial to Fc effector function, we sought to analyze the native Fc glycan composition of IgG purified from bat serum using Ultra Performance Liquid Chromatography (UPLC). Unexpectedly, the bat serum IgG displayed an abundance of sialylated glycans on both the Fc and the Fab regions, which is not typically seen on human antibodies (the level of sialylated IgG is ~10% in human serum). As sialylated antibodies are associated with an anti-inflammatory immune profile, their apparent preponderance on bat IgG may have implications for how the bat immune system responds to and tolerates viral infection.

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Immunology - Innate and Cell-mediated Host Defenses

Immunity and Memory Against Malaria: An Atlas of the Mosquito Immune System at Single-Cell Resolution
Malaria is yearly responsible for 219 million cases and over 400,000 deaths. Mosquitoes of the Anopheles gambiae species complex are the main African vectors for the most virulent malaria parasite: Plasmodium falciparum. Mosquitos are not mere bystanders however, and rely on both humoral and cellular innate immune responses to defeat invading pathogens, including malaria. These efforts are coordinated by hemocytes, the insect equivalent to vertebrate’s white blood cells. Yet, hemocyte biology is largely unknown, mainly due to the low number and fragility of mosquito immune cells. In order to identify previously unknown cell types, their gene signatures, and their spatial-temporal localization in the mosquito we isolated Anopheles hemocytes and characterized them by single-cell RNA sequencing. A total of 5,218 individual Anopheles hemocytes were profiled 1,3 and 7 days after sugar-feeding, blood-feeding, or infection with Plasmodium berghei. At least 10 cell sub-types were identified, including novel effector, inhibitory, phagocytic, and anti-microbial peptides’ secreting cell subtypes. Bulk RNAseq of Anopheles hemocytes, guts, and carcasses in the same conditions was also performed. Genes that were both specific to each cell type in single cell RNA-seq data, and exclusively expressed in hemocytes in bulk RNAseq data were selected as cell markers. The putative cell types were validated with fluorescence in situ hybridization (RNA-FISH) in mosquito sections, whole guts and
carcasses, and isolated hemocytes, showing an increase in active granulocytes and novel effector cells with malaria infection. After validation, challenged hemocytes’ transcriptomic changes with time were investigated to understand hemocyte lineage and development. Both a rapidly dividing hemocyte progenitor pool and a more general trajectory of cell activation were identified, showing a progressive increase in immunity, signal transduction, spliceosome, and cell cycle genes from day 1 to 2 and 3, before returning to baseline at day 7. Our results are the first comprehensive transcriptomic study of a whole invertebrate organism’s immune system, demonstrating hemocytes’ complexity far exceeds what is currently described in the literature. The new understanding could prove useful in developing vector control strategies for malaria, Zika, and Dengue. In addition, our methods and results will serve as a resource for many entomologists, from Anopheles and Aedes, to Culex and Drosophila.

Forrest Jessop
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Immunology - Innate and Cell-mediated Host Defenses

Reprogramming Host Cell Metabolism Using Mitochondrial Inhibitors Controls Francisella tularensis Infection

In the age of antibiotic resistance, control of bacterial infections requires development of new drugs and therapeutic strategies. Traditional approaches for drug development have relied on direct targeting of microbial components. However, these targets may mutate rendering the drug ineffective. There is a growing appreciation that pathogens modulate host metabolism to successfully cause disease. Thus, an alternative antimicrobial strategy could include repurposing currently available drugs that modulate host metabolism, e.g. mitochondrial function or glycolysis. Francisella tularensis subsp. tularensis (Ftt) is a highly virulent intracellular bacterium that maintains mitochondrial function and impairs glycolysis in macrophages as a central virulence mechanism. We hypothesized inhibition of specific mitochondrial components would control Ftt infection. Using extracellular flux analysis, we assessed the ability of a range of compounds to modulate host metabolism in primary macrophages. Rotenone and metformin (CI inhibitors) and dimethyl malonate (DMM) and meclizine (MCL) (CII inhibitors) promoted a specific metabolic state characterized by reduced CI and/or CII activity and increased glycolysis. In contrast, TLR agonists promoted glycolysis but caused minimal CI/II impairment. Treatment of macrophages with complex I or II inhibitors controlled intracellular replication of Ftt. However, treatment with TLR agonists not only failed to control Ftt infection but transiently improved bacterial growth. Metformin, MCL, and DMM did not impair Ftt replication in broth, supporting the notion that antimicrobial activity was host driven. We utilized MCL as a proof of principle agent to test control of Ftt infection in vivo. C57Bl/6 mice were intranasally infected with Ftt followed by daily intraperitoneal injections of MCL or vehicle control. Mice treated with MCL had significantly increased mean time to death compared to controls, corresponding with delayed dissemination of Ftt from the lung to the spleen. Furthermore, unrestricted cytokine production associated with fatal pathology at the end stages of Ftt infection was significantly reduced in MCL-treated mice, consistent with moderate control of disease progression. Together, our data suggest targeting host cell metabolism is a promising approach for control of virulent bacteria, and drugs which appropriately manipulate host metabolic response may be repurposed to serve as effective anti-microbials.
Allergen pre-sensitization drives an eosinophil-dependent lung-specific arrest of helminth development and dictates the outcome of helminth infection

Allergic diseases and helminth infections are two distinct health concerns, which together affect more than 3 billion people worldwide. This study takes a novel look at the relationship between helminth infection and allergic sensitization by assessing the influence of pre-existing allergic sensitization on the outcome of helminth infections rather than the more traditional approach whereby the helminth infection precedes the onset of allergy (hygiene hypothesis). Here we used a murine model of house dust mite (HDM)-induced allergic asthma inflammation followed by Ascaris (helminth nematodes) infection to demonstrate that allergic sensitization to environmental aeroallergens drives an eosinophil-rich pulmonary type-2-immune response (Th2 cells, M2 macrophages, ILC2s, IL-33 and mucus). When the Ascaris larvae migrate from the liver circulation to the lung tissue in their quest to reach the airways in these HDM-sensitized mice, the type-2-dominated response leads to a 70% reduction in the number lung-stage Ascaris larvae and a profound larval developmental delay. This effect is dependent on the presence of eosinophils, as eosinophil-deficient mice were unable to limit parasite development or numbers. RNA-seq analyses of Ascaris larvae from allergen pre-sensitizes mice compared to larvae from non-allergic mice demonstrated marked alterations between the 2 sets of Ascaris larvae with 1330 genes being significantly differentially expressed. Using L3 and L4 larval stage-specific signature mRNA profiles, it could be shown that the Ascaris larvae recovered in the airways of non-allergic mice showed a transcriptomic-signature of a larvae transitioning between the L3-lung stage and that felt to represent the L4 intestinal stage; in marked contrast however, the larval stages recovered in the airways of HDM-pre-sensitized mice had a molecular signature of L3-liver stage. Our data suggest that HDM-induced allergic sensitization drives a response that mimics a primary Ascaris infection, such that lung-specific type-2 mediated eosinophil-dependent helminth larval killing in the tissue occurs. This study provides a window through which the mechanisms underlying tissue-specific responses that can drive a protective response against the early stages of helminth parasites. It also helps identify at a molecular level potential genes product crucial for larval development that could be used as potential targets for the prevention of chronic infections in the host.

Patrolling ILCs Restrain Mucosal Viral Replication Through Tissue-Wide Delivery of IFN-gamma

Smallpox, caused by infection with variola virus (VARV), led to devastating human pandemics with high mortality until eradication through a global vaccination campaign. Despite eradication, the precise
immune mechanisms underlying recovery from infection are incompletely understood. The most common model employed to study smallpox pathogenesis is murine infection with vaccinia virus (VACV), the virus used for human smallpox vaccination. VACV models of smallpox infection commonly employ intranasal routes of infection, resulting in widespread viral dissemination. However, VARV infected humans through the oropharyngeal mucosa, after which oral lesions developed and ruptured, spilling copious infectious virus into the saliva (the route of human-to-human spread). Currently, there are no animal models of poxvirus infection of the oral mucosa, and only a handful of reports of infection of the oral cavity with any virus. To understand protection in this critical barrier site, we developed a mouse model of lip (labial mucosal) infection. After labial administration, VACV replicates to high titers and two waves of innate lymphoid cells (ILCs) are recruited in response to infection. Intravital microscopy reveals highly motile ILCs patrol the infected tissue, however, they do not form contacts with virus-infected cells. Although, we did not directly visualize ILC-mediated killing of virus-infected cells in the tissue, depletion of NK1.1+ cells enhance viral replication and spread. In addition to direct lysis of target cells, ILCs can secrete potent antiviral cytokines including IFN-gamma. Using immunohistochemistry, we show that ILCs produce IFN-gamma throughout the infected tissue. This widespread disruption of IFN-gamma producing ILCs led us to question whether ILCs could endow the epithelium with infection resistance. Antiviral signaling pathway qPCR arrays using isolated RNA from infected tissues reveal a number of upregulated IFN-regulated genes. Furthermore, IFN-gamma neutralization greatly enhances viral infection, suggesting that ILCs deliver IFN-gamma to the mucosal tissue to restrain viral replication. Together, these data illustrate the complex nature of immune protection in a critical yet overlooked tissue during viral pathogenesis. Further, with the recent understanding that many viruses including HSV, Ebola, and Zika can be shed for prolonged periods in the saliva, we believe our data provides a new system for the evaluation of these viruses.

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Immunology - Innate and Cell-mediated Host Defenses

*NETosis proceeds via a defined series of cytoskeleton and endomembrane disassembly events including PAD4-mediated chromatin decondensation and nuclear envelope rupture*

Neutrophil extracellular traps (NETs) are web-like DNA structures decorated with histones and cytotoxic proteins released by activated neutrophils to trap and neutralize pathogens. NETs also form in sterile inflammation. Peptidylarginine deiminase 4 (PAD4) citrullinates histones and is required for NETosis in mouse neutrophils. While the in vivo impact of NETs is accumulating, the cellular events and mechanisms driving NETosis are unclear. Here, we determined the sequence of cellular events in NETosis, and examined the role of PAD4 in these events. Using high resolution time-lapse spinning disc confocal microscopy we examined the morphodynamics of Hoechst-stained HL60-derived neutrophils (HdN), mouse and human neutrophils (PMN) stimulated with ionomycin or LPS to induce NETosis. Actin, microtubule and endoplasmic reticulum (ER) networks were visualized with vital dyes in PMN and cDNA-encoding fluorescent proteins in HdN. Vimentin, Lamin A/C, B and Lap2B dynamics were imaged in HdN. Plasma membrane (PM) integrity was probed by calcein and dextran cellular influx. We found that minutes after stimulation, the actin cytoskeleton disassembles, followed by PM vesiculation and vesicle
shedding, coincident with the disassembly of vimentin, microtubule and ER vesiculation, but leaving the nuclear envelope (NE) intact. Subsequently, chromatin decondenses, the lobulated nucleus rounds up and PM permeability increases. The lamina network and NE then rupture, leading to rapid expulsion of decondensed chromatin into the cytosol. Finally, most cells rupture their PM resulting in NET release. To examine the role of PAD4 in NETosis we isolated neutrophils from PAD4-deficient mice and generated a PAD4-knock down (KD) HL60 CRISPR line. We found that while PM vesiculation, cytoskeletal disassembly and ER vesiculation occur independently of PAD4, efficient chromatin decondensation, NE, lamina rupture and extracellular DNA release require PAD4. Defects in chromatin decondensation, NE rupture and NETosis were rescued in PAD4-KD HdN cells to the WT level by re-expression of WT-PAD4 but not with enzymatically dead or nuclear localization signal-deficient PAD4 mutants. Our findings reveal that NETosis proceeds by a striking and stereotypical step-wise sequence of cellular events culminating in the PAD4 enzymatic activity and nuclear localization-mediated chromatin decondensation, nuclear envelope rupture and extracellular DNA expulsion.

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Immunology - Lymphocyte Development and Activation

An unbiased transcriptomic analysis identifies Zfp281 as a novel controller of CD4 T cell development

CD4 helper T lymphocytes are a critical component of the adaptive immune response, demonstrated by the fact that patients with a deficiency in CD4 T cells cannot fight infections typically controlled by a replete immune system. CD4 T cells develop in the thymus from cells that can become either helper CD4 T cells, or cytotoxic CD8 T cells. The â€œdecisionâ€ is tied to whether the cell is signaled by MHC class I or class II molecules, both of which engage the T cell receptor. Class II-signaled cells become CD4 T cells, and class I-signaled cells become CD8 T cells. The transcription factor Thpok is required to generate CD4 T cells, in that disruption of Thpok abrogates CD4 T cell development. However, simply over-expressing Thpok does not generate bona fide CD4 T cells, suggesting that Thpok is not sufficient to generate CD4 T cells. We wondered whether additional factors could promote CD4 T cell development in the thymus, and to address this question, we took an unbiased approach to define the gene expression program of developing T cells by RNA-seq. We compared cells signaled by either class I or class II MHC molecules during various stages of their development. We identified a set of genes that are preferentially upregulated in CD4 T cells over CD8 T cells during development. We coupled the expression data with genome accessibility analyses to find genes that gain expression and locus accessibility as measured by ATAC-seq. Amongst these genes, we found that the transcription factor Zfp281 is highly expressed early during CD4 T cell development. We questioned whether Zfp281 was involved in CD4 T cell development. We found that deletion of Zfp281 in T cells had a mild effect on CD4 T cell development and Thpok expression. However, disruption of both Zfp281 and the highly homologous protein Zfp148 resulted in a 50% reduction in the number of CD4 T cells, and a 45% reduction in the expression of Thpok. Preliminary experiments suggest that Zfp281- and Zfp148- double-deficient MHC II-signaled T cells redirect into the CD8 lineage. Additionally, we found that both Zfp281 and Zfp148 bind to two distinct regions of the Thpok locus. Ongoing experiments are investigating whether Zfp281 and Zfp148 function to control CD4 T cell development solely through their effect on Thpok, or through additional mechanisms. Thus, by
mapping the gene expression changes of developing CD4 T cells, we have identified Zfp281 and Zfp148 as novel controllers of CD4 T cell development.

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Immunology - Lymphocyte Development and Activation  
title/abstract not published at request of author  
title/abstract not published at request of author

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Natalia Kunz  
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Immunology - Lymphocyte Development and Activation  
A "sweet spot" for complement receptor CD46 in T cell immunity via direct regulation of GAPDH moonlighting functions  

Th1 cells are critical for the host defense against intracellular pathogens and viruses. A crucial step for successful Th1 induction in humans is T cell receptor (TCR)-driven autocrine engagement of the cell-surface complement receptor CD46 on CD4+ T cells. CD46 is present in different isoforms on human T cells which are characterized by the expression of two distinct cytoplasmic domains, CYT-1 or CYT-2. Upon TCR-driven autocrine CD46 stimulation, the intracellular domains of CD46 are cleaved by gamma-secretase and translocate to the nucleus where particularly CYT-1 drives the expression and surface shuttling of the glucose transporter GLUT1 and the amino acid transporter LAT1. These events induce increased glycolysis and oxidative phosphorylation - a metabolic reprogramming fundamentally required for cell growth, expansion and effector function. Yet, the molecular pathways underlying specific CYT-1 activities remain largely unexplored. To probe for the exact CYT-1 functions, we generated a novel antibody recognizing cleaved CYT-1 and performed co-immunoprecipitation experiments using resting and activated T cells. Unexpectedly, Mass Spec analysis of CYT-1-bound proteins revealed direct association with several key metabolic enzymes, including glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Subsequent ELISA and Microscale Thermophoresis experiments with recombinantly-produced purified enzyme and synthetic CYT-1 or scrambled peptides confirmed dose-dependent, direct and specific interactions between CYT-1 and GAPDH. Functional studies revealed that CYT-1 modulates the activity of a key "moonlighting function" of GAPDH, namely the posttranscriptional regulation of mRNA stability/translation: CYT-1 binding fostered des-oligomerization of glycolytically active GAPDH tetramers to monomers with altered mRNA binding abilities particularly for mRNAs encoding metabolic enzymes. These data identify CD46 as a 'shape-shifter' of GAPDH activity, critically balancing its canonical (glycolysis) versus non-canonical (posttranscriptional regulation) activity further substantiating the notion that autocrine complement plays an integral role in immunity.
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Informatics/Computational Biology
*Long Intergenic Non-coding RNA Profiles of Pheochromocytoma and Paraganglioma: A Novel Prognostic Biomarker*

Abstract: Background: Pheochromocytoma and paraganglioma (PCPG) are rare neuro-endocrine cancers. Malignant cases of PCPG show poor prognosis. Long intergenic noncoding RNAs (lincRNA) are an abundant class of RNA which are implicated in multiple cancer types. To get an understanding of the role of this major class of non-coding RNAs in PCPG, we performed extensive characterization of lincRNA expression profiles from The Cancer Genome Atlas (TCGA) data. Methods: We compared the RNA-SEQ expression profiles of the lincRNAs annotated in GENCODE (v22) in PCPG with 17 other tumor types from TCGA and other published sources. Machine-learning was used to identify a candidate set of lincRNAs associated with established PCPG molecular subtypes as well as aggressive tumors. The role and clinical relevance of lincRNAs in the presence of select driver mutations in PCPG were evaluated using uni-variate and multi-variate survival analysis. Potential targets of the identified lincRNAs were calculated from lincRNA-mRNA expression correlation analysis. Results were validated in independent samples using RT-PCR. Results: Unsupervised clustering of lincRNA expression profiles across eighteen different cancer types identified PCPGs to be clustered with gastro-entero-pancreatic neuroendocrine tumors, adrenocortical and thyroid cancers. Within PCPGs, five different molecular subtypes were identified corresponding to the established molecular classification. Up-regulation of 13 lincRNAs was found to be associated with aggressive/metastatic PCPG. RT-PCR validation in independent set of PCPGs confirmed the overexpression of 4 lincRNA in metastatic compared to non-metastatic PCPGs. Kaplan-Meier analysis identified expression of 5 lincRNAs to be prognostic markers for metastasis-free survival of patients in SDHx-mutated, non SDHx-mutated, and kinase-signaling PCPGs. A prognostic index combining the expression profiles of 18 lincRNAs, two driver mutation status, tumor location and hormone profiles was formulated that can predict the metastasis-free survival in PCPG patients. Conclusions: PCPG have different lincRNAs expression profiles that showed potential to identify aggressive/metastatic PCPGs.

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Informatics/Computational Biology
*Deep Learning from Incomplete Data: Detecting Imminent Risk of Hospital-acquired Pneumonia for ICU Patients*

Hospital acquired pneumonia (HAP) is the second most common nosocomial infection in the ICU and costs an estimated $3.1 billion annually. The ability to predict HAP could improve patient outcomes and reduce costs. Traditional pneumonia risk prediction models rely on a small number of hand-chosen signs
and symptoms and have been shown to poorly discriminate between low and high risk individuals. Consequently, we wanted to investigate whether modern data-driven techniques applied to respective pneumonia cohorts could provide more robust and discriminative prognostication of pneumonia risk. Specifically, we were interested in harnessing the clinical narratives documented in ICU notes. Clinical narratives provide a rich but underutilized source of information for clinical decision support potentially documenting and/or summarizing the main observations about the patient, relevant procedures and important positive and/or negative laboratory results. Unfortunately, processing clinical narratives requires overcoming several barriers, including the prevalence of missing, inconsistent, or underspecified information. Moreover, while typical risk predictors such as vital signs are continually recorded through a patient’s stay, clinical notes are produced at irregular intervals and often in bursts (that is, there are often multiple days during a patients ICU stay in which no clinical notes are produced). Consequently, inferring pneumonia-risk from clinical notes requires accounting for (1) incomplete or sparse information, (2) gaps in the patients time line in which no notes were generated, (3) limited availability of data, and (4) lack of direct (ground-truth) pneumonia risk labels. We developed the Pneumonia Risk predictiOn NeTwOrk (PRONTO) which harnesses modern deep learning techniques to infer and predict when and if an at-risk ICU patient will progress to imminent pneumonia risk within a given time window based on the content of his or her longitudinal ICU notes. We show how PRONTO can be trained from a retrospective cohort (n=1137) without direct supervision to predict future pneumonia risk in a held-out cohort (n=147) with 96% Sensitivity, 72% AUC, and 80% F1-measure, outperforming previous models using the same features by 22% (relative; 15% absolute).

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Crossing fitness valleys via double substitutions within codons

Single nucleotide substitutions in protein-coding genes can be divided into synonymous (S), with little fitness effect, and non-synonymous (N) ones that alter amino acids and thus generally have a greater effect. Most of the N substitutions are affected by purifying selection that eliminates them from evolving populations. However, additional mutations of nearby bases can modulate the deleterious effect of single substitutions and thus might be subject to positive selection. To reconstruct the history of nucleotide substitutions in protein-coding DNA we used 37 triplets of closely related prokaryotic species. Each triplet consists of two closely related species and an outgroup allowing to calculate the frequency of each codon change. To elucidate the effects of selection on double substitutions in all codons, it is critical to differentiate selection from mutational biases. We approached this problem by comparing the fractions of double substitutions within codons (the double substitution frequency divided single and double frequencies) to those of the equivalent double synonymous substitutions in adjacent codons, used as controls. Under the assumption that substitutions occur one at a time, all within-codon double substitutions can be represented as ?ancestral-intermediate-final? sequences and can be partitioned into 4 classes: 1) SS: S intermediate - S final, 2) SN: S intermediate - N final, 3) NS: N intermediate - S final, 4) NN: N intermediate - N final. We found that the selective pressure on the second substitution markedly differs among these classes of double substitutions. Analogous to single S
substitutions, SS evolve neutrally whereas, analogous to single N substitutions, SN are subject to purifying selection. In contrast, NS show positive selection on the second step because the original amino acid is recovered. The NN double substitutions are heterogeneous and can be subject to either purifying or positive selection, or evolve neutrally, depending on the amino acid similarity between the final or intermediate and the ancestral states. The results of the present, comprehensive analysis of the evolutionary landscape of within-codon double substitutions reaffirm the largely conservative regime of protein evolution. However, the second step of a double substitution can be subject to strong positive selection when the first step is deleterious. Such positive selection can result in frequent crossing of valleys on the fitness landscape.

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Making sense of biomedical literature at sentence level

Literature search is a routine in scientific work as new discoveries build on knowledge from the past. Most current tools dedicated to this task such as PubMed or PubMed Central are however publication-centric and do not allow direct retrieval of specific statements, which is key for biomedical researchers, healthcare professionals, biocurators and others to quickly compare/validate new findings with previous knowledge or perform evidence attribution from the literature. In such cases, a sentence-level search is preferable to scanning entire documents returned by those tools. In response, we introduce the first web-based search engine that specializes in sentence retrieval for both abstracts and full-texts in biomedical literature. By integrating the entire 29 million abstracts in PubMed and nearly 3 million full-text articles in PMC Open Access Subset, our system indexes over half a billion sentences. To provide the best search results, we combine the traditional Inverse Document Frequency (IDF) approach that up-weights sentences containing more of the rare (i.e. more specific) terms in the user query, with cutting-edge and challenge-winning neural sentence embedding techniques, which allows us to retrieve semantically relevant results that may not explicitly mention query keywords. In addition to optimizing for search quality, our system provides a user-friendly interface allowing users to (1) filter returned sentences by normalized article sections (e.g. Show only sentences from â€œConclusionâ€) and/or publication date, (2) highlight biomedical entities (e.g. gene/proteins) identified via state-of-the-art text mining tools, and (3) examine each returned sentence in the context of its original publication. To evaluate our search algorithm, we created a gold-standard dataset containing 100 queries and ~10 retrieved sentences for each query. Our tests showed that the proposed algorithm achieved ~10% improvement over traditional term-weighting approach. Our system is updated regularly and has been tested by users for different use cases. For example, when a database curator is searching the scientific evidence for describing a protein superfamily: â€œBasic leucine zipper (bZIP) factors comprise one of the most important classes of enhancer-type transcription factorsâ€, our system not only shows already curated publications (PMID16731568), but also suggests other publications that may be of interest for manual review and further curation (e.g., PMID28955639).
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ProteoGE: A proteogenomic tool for integrating proteomic, genomic and epigenomic data to discover novel protein-coding genes

Accurate gene annotations are critical to infer proteins they encode and the functional roles they play in human health and diseases. The widely used sources of reference gene annotations (NCBI, UCSC, and Ensembl) for a variety of organisms utilize automated prediction pipelines to identify the locations of genes and coding regions in a genome. However, recent studies report widespread inaccuracies with reference gene annotations, e.g., incorrect gene/exon/CDS boundaries and incomplete catalogue of tissue-specific splicing events and genes. Although technological advances in mass spectrometry (MS)-based proteomics and next-generation sequencing now allow for near-comprehensive characterization of proteomes and transcriptomes respectively, development of algorithms to integrate multi-omic data to annotate/re-annotate genes is still in its infancy. Here we present ProteoGE, a proteogenomic tool to refine eukaryotic gene annotations through integration of data from MS-based proteomics, transcriptomics (RNA-Seq) and epigenomics (ChIP-Seq) experiments. ProteoGE ensures high sensitivity and specificity while refining genome-wide annotations by utilizing input DNA (ChIP-seq) and decoy database (proteomics) as negative controls. To demonstrate its utility, we applied ProteoGE on in-house generated high-resolution data from mouse embryonic stem (ES) cell differentiation representing early embryo development. Through integration of ~1B RNA-Seq reads, ~500M ChIP-Seq reads, and ~10M MS/MS spectra, ProteoGE reports comprehensive annotations and protein products for 9,358 annotated genes. More importantly, ProteoGE uncovered 27 novel protein-coding genes and 183 novel splice variants with high-confidence unique peptide identifications. Additionally, our effort also revealed ~400 putative long non-coding RNAs (>200 bp) originating from un-annotated regions of the mouse genome. Through comparative genomics, we show that the novel protein-coding genes we discovered are highly conserved in mammals, contain functional protein domains, show tissue specific expression, and carry mutations in various cancers highlighting their functional significance and potential role in development and/or pathogenesis. In summary, ProteoGE enables discovery of novel protein-coding genes and should serve as an excellent resource to refine gene annotations in other biological settings (cell-types/tissues and organisms).

Kuo-Hui Su
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Metabolomics/Proteomics

HSF1 is a direct AMPK antagonist to promote protein cholesteroylation and tumor growth

Rationale: AMP-activated protein kinase (AMPK), a key cellular metabolic sensor and downstream effector of the tumor suppressor LKB1, activates catabolic pathways but suppresses anabolic pathways, including fatty acid and cholesterol biosynthesis, thereby maintaining energy homeostasis. Heat shock
factor 1 (HSF1) is the master transcriptional regulator of the evolutionarily conserved proteotoxic stress response, thereby preserving proteomic stability upon environmental insults. HSF1 is also a potent pro-oncogenic factor, suggesting that proteomic stability enables oncogenesis. We previously showed that upon activation by metabolic stressors, AMPK interacts with and phosphorylates HSF1 to inactivate it and cause proteomic instability, leading to tumor suppression. Intriguingly, we also discovered that HSF1 suppresses AMPK and controls body fat mass. Aims: To investigate the mechanisms by which HSF1 suppresses AMPK and the roles of this suppression in lipid metabolism and tumorigenesis. M&R: By utilizing recombinant proteins, HSF1 peptide libraries and transcription-deficient HSF1 mutants, our in vitro studies reveal that through physical interactions HSF1 imposes multilayer regulations on AMPK, including blocking AMP binding to gamma subunits, impairing LKB1-mediated Thr172 phosphorylation, promoting Thr172 de-phosphorylation by PP2A, and impeding ATP binding to catalytic alpha subunits. Circular dichroism spectroscopy reveals that HSF1 induces global AMPK conformational changes. Biologically, Hsf1 deficiency suppresses lipogenesis and decreases lipid content via AMPK activation. Moreover, Hsf1-deficient cells and mice display reduced cholesterol levels. Interestingly, this defect leads to impaired cholesterolylation of cellular proteins, including sonic hedgehog (SHH). Consequently, the oncogenic SHH signaling is impaired in HSF1-deficient cells. In vivo, Hsf1 deficiency reduces body fat mass in mice, which can be markedly rescued by either pharmacological or genetic inhibition of AMPK. Importantly, the transcription-deficient HSF1 mutant, through AMPK suppression, enhances the lipid content and protein cholesterolylation, and promotes the in vivo growth of xenografted human melanomas. Conclusions: HSF1 is a direct AMPK antagonist. This transcription-independent interaction of HSF1 with AMPK epitomizes a reciprocal kinase-substrate regulation whereby lipid metabolism and proteomic stability intertwine and promotes cancer anabolism and oncogenesis.

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Metabolomics/Proteomics

Metabolic rewiring mediated epigenetic reprogramming as a mode of resistance to EGFR-tyrosine kinase resistance in lung cancer

Despite the clinical use of tyrosine kinase inhibitors (TKIs) such as osimertinib for treatment of epidermal growth factor receptor (EGFR) mutant lung cancer, resistance is common. Resistance results from genomic alterations or even without a genetic cause, raising the possibility of a non-genetic mode of resistance, such as alterations in enzyme activities or by epigenetics. The study aimed to identify pathways that mediate drug resistance and target them using drugs. We generated osimertinib resistant cell lines from parental sensitive cells, both in vitro and in vivo as xenografts. Using these models, we demonstrated that sensitive cells were glucose dependant; however, resistant were glutamine dependant and overexpressed glutaminase (GLS), that converts glutamine to glutamate. 13C tracing using glucose and glutamine showed an increased rate of alpha-ketoglutarate (?KG) accumulation and reductive carboxylation in resistant cells. Inhibition of GLS by CB839, a GLS inhibitor, led to selective death of resistant cells, which rescued by ?KG but not by citrate, succinate or fumarate. Moreover, fumarate, a competitive inhibitor of histone demethylases (HDMs) which use ?KG as a cofactor, inhibited the viability of resistant cells. RNAseq analysis showed an active transcription program in
glutamine dependant resistant cells compared to glutamine independent resistant cells. Given $\gamma$KG is essential for HDMs, we investigated the activity of HDMs. Resistant cells exhibited decreased histone methylation, which upon treatment with CB839 showed increased methylation, suggesting reduced intracellular $\gamma$KG due to the inhibition of GLS could have reduced the activity of HDMs. Furthermore, resistant cells were sensitive to GSK J4, an HDM inhibitor, which also increased methylation in resistant cells. These findings were validated in our clinical study of EGFR mutant lung adenocarcinoma patients treated with osimertinib (NCT02759835). We discovered GLS overexpression upon osimertinib resistance in 50% (n=10; pre-, post- RNA sequencing) of patients. Further, we confirmed that combination therapy of CB839 with osimertinib significantly reduced resistant xenograft tumors over 50days. Together, these results suggest that increased glutaminase expression and activity increases $\gamma$KG, which activates demethylases resulting in the transcriptional reprogramming that drive the osimertinib resistance. Inhibition of this mechanism by CB839 and GSKJ4 overcomes the resistance to therapy.

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Regulation of the Escherichia coli multidrug efflux pump AcrB by the small protein AcrZ

Multidrug resistant bacteria are a global health problem. Resistance can be caused by increased expression and/or activity of multidrug efflux pumps. These transporters include RND-family efflux pumps, which are overexpressed in some multidrug resistant pathogens such as Escherichia coli and Pseudomonas aeruginosa. The E. coli RND-family pump AcrB was known to bind the 49-amino acid a-helical small transmembrane protein AcrZ, which is required for resistance to some AcrB substrates including chloramphenicol and tetracycline. However, the mechanism by which AcrZ binding affects AcrB activity and the determinants for the AcrZ-AcrB interaction were unknown. To address these questions, AcrB alone and the AcrBZ complex were reconstituted in native E. coli nanodiscs and the structures were solved by cryo-EM. These structures revealed that AcrZ causes conformational changes in AcrB in not only the transmembrane binding site, but also in the distally located drug binding pocket and access channels. The changes were only observed in the structures derived from cryo-EM in lipid nanodiscs and not in previous crystal structures, suggesting that the local lipid environment is important for AcrB function. Cardiolipin is a modulator of other membrane proteins, and molecular dynamics
simulations predicted cardiolipin enrichment around the AcrBZ complex. In cryo-EM structures, the most pronounced changes in AcrB were seen in the presence of both AcrZ and cardiolipin. To examine the effects of cardiolipin and AcrZ on AcrB, the corresponding genes were deleted, resulting in an additive effect on chloramphenicol sensitivity. Together, these results suggest that AcrZ and cardiolipin cause allosteric changes in AcrB to affect drug transport. Mutational analyses to identify the AcrZ determinants that affect AcrB interaction and activity showed that proline 16 in the transmembrane domain of AcrZ is required. Proline is a helix breaker that confers a kink to α-helices. Interestingly, replacing the proline further down the helix and increasing flexibility with glycine substitutions rescued both binding and resistance. Together, these results suggest that the overall hydrophobic character and bent shape of AcrZ rather than specific AcrB-AcrZ contacts are important for regulation. These studies help explain the mechanism by which AcrZ regulates AcrB. Further elucidation of this mechanism will be useful in informing drug design experiments for inhibitors of multidrug efflux pumps.

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Microbiology and Antimicrobials
A pathogenic bacterium targets the mammalian protooncogene NRas during infection
Intracellular bacterial pathogens have evolved to efficiently manipulate vital functions of their eukaryotic host cells to evade host immune responses and control host cell biology. The gram-negative human pathogen, Legionella pneumophila (Lp), secretes over 300 effector proteins into host cells via its type IV secretion system to alter host signaling and trafficking pathways to promote infection. Targeting small guanosine triphosphatases (GTPases) is a common virulence strategy of Lp and other pathogens, as these proteins are central platforms for an array of cellular signaling pathways. While studying intracellular trafficking of Lp, I discovered that the small GTPase NRas, but not its homologs KRas or HRas, accumulates on the Legionella-containing vacuole (LCV). Ras GTPases are GDP/GTP molecular switches that control cell survival and differentiation in eukaryotes, with activating mutations found in 30% of all human cancers. Because the localization of Ras dictates its signaling capacity, I investigated the mechanism of NRas recruitment to the LCV. The NRas C-terminus contains sites for the post-translational modifications (PTMs) farnesylation and palmitoylation, which anchor it at the plasma membrane and Golgi. Site-directed mutagenesis of these sites indicates that both the farnesylation and palmitoylation sites are required for NRas recruitment to the LCV. Interestingly, while HRas is not recruited to the LCV, it differs from NRas in having a second palmitoylation site. By mutating this site, HRas is recruited to the LCV, indicating that the selectivity for NRas recruitment is due to the differential localization caused by NRas-specific PTMs. Like all GTPases, NRas actively signals when bound to GTP. The constitutively active NRas-G13V accumulates on the LCV, indicating that the LCV is a unique NRas signaling compartment during infection. To identify the bacterial effectors involved, a novel CRISPR interference approach was used to knock-down expression of Lp effectors and screen for failure to recruit NRas to the LCV. This approach identified three effectors with Ras-guanine nucleotide exchange factor domains, which stimulate GTP-binding to activate Ras signaling. Further work will characterize how exactly these effectors influence NRas signaling from the LCV. Together, these results provide the
first evidence for an intracellular pathogen exploiting Ras GTPase function and reveal an unexpected link between NRas signaling and microbial infection.

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Microbiology and Antimicrobials

**CD153 signaling into T cells is required for pulmonary control of Mycobacterium tuberculosis infection**

*Mycobacterium tuberculosis* infection (Mtb) remains a leading cause of death worldwide, accounting for nearly 1.3 million patients succumbing to disease in 2018. The CD4 T cell response is essential for resistance to Mtb infection, and current dogma holds that IFNg production is the primary mechanism of CD4 T cell-mediated protection. However, our group has previously shown that IFNg responses do not correlate with bacterial burdens, and several other reports demonstrate additional anti-mycobacterial CD4 T cell effector functions remain unaccounted for. Recently we demonstrated a key role for TNF superfamily ligand, CD153 (TNFSF8), expression on CD4 T cells in host resistance to Mtb infection as validated in mice, non-human primates, and in human patients. To understand the mechanism behind CD153 mediated protection, here we compare mice deficient in both CD153 and its receptor CD30 (TNFRSF8). We show that CD30 expression by CD4 T cells is also required for control of pulmonary Mtb. Mice deficient in either CD30 or CD153 develop high pulmonary bacterial loads and synchronously succumb to infection early. Deficient mice have no alterations in myeloid cell frequencies but do show decreased terminal differentiation of activated CD4 T cells. Additionally, deficient T cells are able to produce similar, if not more, IFNg compared to wild-type (WT) T cells suggesting that this defect in protection is likely independent of IFNg. To compare deficient T cells in a normalized environment, we irradiated and reconstituted mice with an equal mixture of WT and CD30-/- or WT and CD153-/- bone marrow. Following infection with Mtb, T cells deficient in CD30, but not in CD153, fail to accumulate in the lungs after priming and clonal burst. While naive cells originate at a 1:1 ratio, only ~10% of activated CD4 T cells in the lungs are CD30-/- . Comparatively, CD153-/- and WT T cells in the lung retain a 1:1 ratio. Moreover, CD30 deficient T cells have a drastic reduction in KLRG-1 expression. These results indicate that CD30 signaling may modulate differentiation during activation of CD4 T cells. Finally, reconstitution of T cell-deficient hosts with either CD30-/- or CD153-/- CD4 T cells cannot protect from early death after Mtb infection demonstrating that T cells may require CD30/CD153 signaling from other T cells in order to mediate protection. Thus, we have identified a major role for the CD30/CD153 axis in CD4 T cell mediated control of Mtb infection.

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Microbiology and Antimicrobials
A genome-scale reverse genetic screen to identify functional determinants controlling Toxoplasma gondii sexual cycle

Toxoplasma gondii transmission in nature is highly dependent on the parasite’s sexual cycle that occurs exclusively in felids. Infected felids shed high numbers of infectious oocysts, which are known to be the main source of toxoplasmosis outbreaks in the human population. The molecular pathways promoting sexual stage development are unknown, thus, identifying essential molecules for oocyst formation is essential to block T. gondii transmission to humans. Our previous work using RNA-Seq to identify merozoite transcripts critical for sexual development has identified a series of gene families that are stage specific, conserved across the Apicomplexa, and thought to facilitate sexual competency. These include the SRS/6-Cys proteins and AP2 transcriptional factors. T. gondii encodes well over 180 SRS proteins orthologous to the 6-Cys proteins that affect male-female gamete recognition in Plasmodium, and 32 AP2 factors thought to regulate T. gondii gene families that are sexual stage-specific, but their individual role in contributing to oocyst formation remains unknown. We have undertaken a genome-scale reverse genetic signature-tag mutagenesis screen, using CRISPR/Cas9, to generate a library of 200 Toxoplasma strains that each possess a unique barcode and are each deficient in a single SRS or AP2 gene. Our aim is to perform an input/output screen to identify relevant sexual stage-specific proteins that are critical for the oocyst formation. In preliminary experiments to test the sufficiency of our screen, we pooled together 14 knockout strains deficient in SRS and AP2 factor genes expressed in asexually replicating parasites and infected either human foreskin fibroblasts (HFF) or mice. Mi-Seq sequencing was used to identify KO strains that failed to expand. Three genes, SRS26J, AP2IX-1 and AP2IX-6 were shown to competitively facilitate tachyzoite growth in vitro in infected HFF cells, as these bar-codes were identified 2 to 3.6 times over the WT control, whereas three genes, PLP1-, SRS22G- and AP2IV-3-KO showed impaired tachyzoite growth. In mice, parasites deficient in SRS15A, SRS26B and AP2IV-3 failed to migrate to the spleen, whereas they had no defect in expansion in vitro. With the methodology in place, mice are currently infected with a pool of 25 knockouts for a proof-of-
principle cat challenge experiment. We will present parasite genes identified as critical for Toxoplasma sexual development in order to block parasite transmission by cats.

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Molecular Biology - Prokaryotic and Eukaryotic

A cytoplasmic role for the nuclear transcription factor TAF7

In eukaryotes, transcription of most protein-encoding genes is initiated by recognition of the promoter by the general transcription factor TFIID. TFIID is a multi-subunit complex consisting of the TATA-binding protein (TBP) and a set of TBP-associated factors (TAFs), including TAF7. Although TAF7 was originally considered to be solely a structural component of TFIID, we have provided evidence that it actively contributes to transcription regulation. Previously we found that TAF7 controls the orderly progression of events during transcription by interacting with and regulating the activities of the various enzymes required for transcription initiation, early elongation and productive elongation. We have now identified two novel TAF7 characteristics, a nucleocytoplasmic shuttling ability and an RNA-binding ability. Using immunofluorescence and cell fractionation, we found that TAF7 exists at significant levels in the cytoplasm of multiple cell lines in addition to its nuclear localization. Analysis of TAF7 mutants identified a nuclear localization signal (NLS) in the middle region and a nuclear export signal at the C-terminus of TAF7. Moreover, we showed that the transport receptor CRM1 interacts with TAF7 and mediates its nuclear export. To investigate whether TAF7 shuttles RNA transcripts from the nucleus into the cytoplasm, we examined TAF7-RNA interaction. Both in vivo RNA immunoprecipitation experiments and in vitro RNA-binding assays indicate that TAF7 binds a variety of RNA species through an RNA-binding domain that overlaps the NLS. Importantly, cytoplasmic TAF7 associates with polysomes through the RNA. Furthermore, using a combination of proximity ligation assay, co-immunoprecipitation and pull-down assays, we demonstrated RNA-dependent interactions between TAF7 and the ribosomal proteins RPL5 and RPL8. Deletion of cytoplasmic TAF7 results in the reduction of global protein synthesis without affecting global transcription, indicating that TAF7 functions in promoting protein translation. Together, these results characterize TAF7 as a new RNA-binding protein that shuttles between the nucleus and the cytoplasm, uncovering a novel role for TAF7 in linking transcription and translation.

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Molecular Biology - Prokaryotic and Eukaryotic

Homeostatic Control of Cellular Polyamine Levels in S. cerevisiae through Translational Regulation of Hol1, the First High-affinity Polyamine Transporter

Cellular polyamines (PAs) putrescine, spermidine, and spermine play critical roles in various processes including cell proliferation, development, transcription and translation. In eukaryotes, intracellular PA
pools are homeostatically regulated by PA control of the synthesis of key biosynthetic and regulatory proteins. For example, PA-induced ribosomal frameshifting governs the synthesis of antizyme (OAZ1), an inhibitor of ornithine decarboxylase (ODC), which catalyzes the first step in polyamine synthesis. Moreover, PAs act via regulatory upstream open reading frames (uORFs) to control the synthesis of both S-adenosylmethionine decarboxylase (AdoMetDC), an enzyme required for PA synthesis, and antizyme inhibitor (AZIN1). The AZIN1 uORF, referred to as a uCC (for upstream conserved coding region), encodes a conserved peptide terminating with the sequence motif EPPWxPS* (* indicates a stop codon). We have shown that translation elongation and termination on this sequence motif are controlled by PAs via altered function of the eukaryotic translation elongation factor eIF5A. In addition to de novo synthesis of PAs, cells acquire PAs from extracellular sources via membrane transporters. However, as of yet, no high-affinity polyamine transporters have been identified in eukaryotes. In this study, we have identified Hol1 as the major high-affinity PA transporter in S. cerevisiae. Our Studies revealed that GFP-tagged Hol1 localizes to the plasma membrane in S. cerevisiae, and (14C)spermidine uptake assays demonstrated that Hol1 is required for PA transport. Ribosome profiling studies in low and high PA conditions revealed translational repression of the HOL1 mRNA in high PA conditions. Moreover, similar to uORF-mediated regulation of AZIN1, we identified a conserved uORF encoding the peptide MLLLPS* in the 5′ leader of HOL1 mRNA. Using a HOL1-FLuc reporter, we found that the PS* motif in the uORF is critical to mediate PA control of HOL1 expression via altered function of eIF5A. Thus, similar to the feedback mechanisms regulating the expression of PA metabolizing enzymes and regulators, we have identified Hol1 as the first high-affinity PA transporter and shown that its expression in S. cerevisiae is autoregulated by PAs.

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DISOME PROFILING REVEALS GENOME-WIDE TARGETS OF RIBOSOME QUALITY CONTROL
To prevent formation of aberrant proteins, ribosomes must tightly control the speed and accuracy of translation. Ribosomes can encounter obstacles during protein synthesis, such as mRNA structures, inhibitory codon pairs and premature poly-A tracts, which slow the ribosome or cause it to stall, blocking further translation of the message. Failures in resolving unwanted ribosome slowdowns result in mitochondrial dysfunction, aggregation of abnormal proteins and neurodegeneration. To address this
problem, cells have evolved a ribosome-associated quality control (RQC) system that can recognize stalled ribosomes and degrade aberrant proteins and mRNAs. The conundrum for the RQC system is to discriminate between transient or programmed ribosome pauses, such as those required for protein localization or protein folding, from detrimental ribosome stalling cases. Recent structural and biochemical data proposed that the complex formed by two collided ribosomes (disome) is the substrate detected by the RQC pathway and the RQC protein Hel2/ZNF598 plays a major role in recognizing disomes. However, the extent of disome formation in the cell and the repertoire of endogenous mRNAs where the disomes are formed have not been characterized. To study this question in yeast, we used ribosome profiling, a method for deep sequencing of ribosome-protected footprints and revealing a global translational snapshot of the cell. By specifically isolating the mRNA footprints protected by disomes rather than single ribosomes, we were able to obtain genome-wide positioning of disomes in the cell. Our results showed that disomes are formed on many endogenous mRNAs at specific sites encoding poly-Arg and poly-Lys peptide sequences and inhibitory codon pairs. These sites are known targets of RQC and may be important for regulating the mRNA stability. In other cases, we found disomes enriched on sequences, such as poly-Pro, that are known to slow the ribosome but not thought to be triggers for RQC. These findings hint that recognition of disomes by RQC machinery depends on sequence context; some disomes may evade RQC and have alternative roles in translation regulation. By modulating the level of Hel2 in yeast, we are currently investigating which disomes are specifically targeted by RQC. Our studies are important for understanding the molecular basis for discrimination between problematic vs programmed ribosome slowdown and to reveal how dysregulation of RQC triggers disease phenotypes.

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Astrocyte-derived extracellular vesicles as carriers of complement-mediated neurotoxicity in Alzheimer’s disease

In Alzheimer’s disease (AD), neuroprotective astrocytes are transformed into reactive astrocytes that mediate neuronal death through mechanisms that are yet not fully understood. Findings in postmortem AD brains show that 60% of reactive astrocytes overexpress complement proteins suggesting deregulation of innate neuroinflammatory mechanisms. More recently, our group has shown that extracellular vesicles of astrocytic origin (AEVs) isolated from the plasma of AD patients carry abnormally high levels of potentially neurotoxic complement proteins, including the terminal membrane attack complex (MAC) known to cause plasma membrane disruption. In this study, we sought to elucidate the mechanisms by which complement-laden AEVs are involved in AD pathogenesis. To this end, we isolated AEVs circulating in the plasma of AD patients and age-matched controls using an immunocapture technique pioneered by our laboratory that targets the glial glutamate transporter, an astrocyte-specific transmembrane protein. The potential neurotoxicity of immunoprecipitated AD AEVs was assessed in vitro using cultures of rat cortical neurons and human iPSC-derived neurons. Neurons incubated with AD AEVs exhibited significantly decreased neurite density by beta-tubulin immunoreactivity, decreased cell viability by the MTT assay, and disruption of the plasma membrane as indicated by fluorescent nuclear
staining using the membrane impermeable nucleic acid probe EthD-1 compared to neurons treated with AEVs from control subjects or with EVs from AD and control subjects immunocaptured by the non-specific EV marker CD81. The AD AEV-mediated neuronal membrane disruption and decreased viability were rescued by co-incubation with the endogenous MAC inhibitor CD59, firmly implicating the complement system and MAC formation in the neurotoxicity of AD AEVs. These observations provide the first demonstration that AEVs cause neurodegeneration in AD, reveal it to be complement-mediated, and stimulate the search for novel therapeutics targeting complement in AD.

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*Electrophysiological and neurotransmitter release properties of VTA combinatorial glutamate-GABA neurons*

The ventral tegmental area (VTA) is a midbrain structure that contains dopamine neurons involved in diverse motivated behaviors. In addition to dopamine neurons, VTA contains glutamate neurons expressing the vesicular glutamate transporter 2 (VGLuT2) and GABA neurons co-expressing glutamate decarboxylase (GAD), and vesicular GABA transporter (VGAT). Both VGLuT2 and VGAT neurons mediate motivated behaviors by establishing synapses on neighboring dopamine neurons or on neurons within brain structures implicated in reward or aversion. Additionally, VTA contains combinatorial neurons co-expressing VGLuT2 and VGAT and shown to co-release glutamate and GABA. Due to the lack of tools for the specific targeting of combinatorial VGLuT2-VGAT neurons, their electrophysiological and functional properties are unknown. In this study, we combined a new double transgenic mouse with a novel intersectional viral vector to selectively tag combinatorial VTA VGLuT2-VGAT neurons (combinatorial glutamate-GABA), VTA-VGLuT2 neurons (glutamate-only) and VTA-VGAT neurons (GABA-only); and determined their electrophysiological properties by patch clamp recordings. We found that combinatorial glutamate-GABA neurons had hyperpolarized resting potential, lower firing frequency and lower excitability than GABA-only neurons. Moreover, combinatorial glutamate-GABA neurons expressed lower Ih current amplitude than either glutamate-only or GABA-only neurons. Based on their firing properties, we determined that the combinatorial glutamate-GABA neurons and the glutamate-only neurons fall into two categories: a) rapid onset firing, and b) delayed onset firing (firing property conferred by a potassium type A current). In contrast, we only identified the category of rapid onset firing within the population of GABA-only neurons. In addition to the electrophysiological properties of combinatorial glutamate-GABA neurons, we determined their neurotransmitter release properties, and found that they released more GABA than glutamate. In summary, we found that (1) combinatorial VTA glutamate-GABA neurons are heterogeneous and share electrophysiological properties with both VTA glutamate-only and VTA GABA-only neurons, and (2) while combinatorial VTA glutamate-GABA neurons co-release glutamate and GABA, they release more GABA than glutamate, conferring them an inhibitory phenotype. These findings dispute the idea that VTA neurons can be differentiated by their electrophysiological properties.
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Neuroscience - Cellular, Molecular, and Glia  
*Gene network of dopamine receptor type 2 (Drd2) in addiction*

The dopamine (DA) reward system is central to decision-making, as it modulates neural plasticity to mark important life events and shapes future behavior. Low availability of type 2 DA receptors D2Rs in the striatum has been observed in addiction, obesity and ADHD. We hypothesize that low expression of D2R gene (Drd2) in indirect Medium Spiny Neurons (iMSNs) affects the expression of other genes, and therefore the functionality of iMSNs and the decision-making process. We performed whole transcriptome RNAseq analysis, from mice with wt or heterozygous expression of Drd2 (iMSN-Drd2-WT and HET). RNA from iMSNs was isolated by Translating Ribosome Affinity Purification (TRAP). Differential gene expression analysis resulted in 31 genes enriched in features such as signal, potassium transport and calcium. Most genes were validated by qPCR on independent samples, and are currently being quantified in subregions of the striatum by RNAscope, as well as in iMSN-Drd2-KO mice. Among the top findings, Fxyd2, a modulatory subunit of the Na/K pump, showed a negative correlation to the expression of Drd2 in our mouse model. To translate our results to human health, gene expression correlations between FXYD2 and DRD2 were performed on human postmortem brain tissue from individuals with a verified cocaine abuse history, and on publicly available (GTEx and BrainSpam) data. In all datasets, a significant negative correlation was found. We are currently running Gene Association Studies on FXYD2 on over 1300 patients with alcohol use disorder (AUD) from the NIAAA Clinical Center. Preliminary results suggest an association (p<0.04) between an intronic (and regulatory region) genetic variant (rs17121264) of FXYD2 and an impulsivity phenotype. Electrophysiological studies will be performed on brain slices from iMSN-Drd2-HET and WT mice with an Fxyd2 inhibitor (cyclothiazide). Further in vivo studies are required to understand the role of Fxyd2 in behavior. Ongoing follow-up studies include RNAseq of TRAP-purified Drd2 and Drd1 neurons, in mice subjected to a 5-week chronic cocaine exposure paradigm. All together this work allowed us to identify a gene network that is affected by low expression of Drd2 in a mouse model. We further studied Fxyd2 in several sets of human data, which support a strong association with Drd2. This gene network points to possible drivers of addictive associated behaviors, and potentially new targets for treatment.
**Dorien Roosen**  
Doctoral Candidate  
NIA  
Neuroscience - Cellular, Molecular, and Glia  

*Parkinson's disease-associated Auxilin mutations impair clathrin trafficking at synapses and the Golgi and underlie neurological phenotypes in mice*

Clathrin-coated vesicles (CCVs) selectively transport cargo from the plasma membrane and the trans-Golgi network (TGN) to intracellular destinations in all eukaryotic cells. In neurons, Auxilin is required for the uncoating of CCVs and therefore for the successful delivery of cargo to its destination compartments. Whereas its role in the uncoating of CCVs at the synapse has been well-documented, the role of Auxilin in the uncoating of TGN-derived CCVs is less established. Recently, homozygous recessive mutations in Auxilin have been found to cause an aggressive form of young onset Parkinson’s disease (PD), a neurodegenerative motor disorder characterized by lesions in dopaminergic neurons in the nigrostriatal pathway. How pathogenic Auxilin induces PD development remains unclear and we have attempted to dissect this mechanism by generating a CRISPR-based mouse model with an endogenous pathogenic Auxilin mutation. We observed neurological phenotypes that phenocopy clinical features seen in patients, including seizures and motor impairment. Additionally, dopaminergic neurotransmission in the nigrostriatal pathway was drastically impaired due to impaired clathrin trafficking. Super-resolution microscopy analysis of mutant mouse brain showed dystrophic ultrastructural alterations at both the synapse and the Golgi-apparatus. Using unbiased proteomics, we identified novel bona fide Golgi-resident interactors of Auxilin and RNAseq-based transcriptome analysis revealed the activation of the Golgi stress response in mutant Auxilin primary neurons. Taken together, our findings provide a near-complete model of PD pathogenesis in Auxilin mutation carriers. We find that impaired clathrin trafficking at the synapse and Golgi apparatus results in dystrophic neuronal defects underlying PD-relevant neurological phenotypes in a novel mouse model. These results underscore an important role for clathrin trafficking in PD. Moreover, we have gained further insight in the physiological role of Auxilin at the Golgi apparatus.

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**Saumitra Choudhury**  
Visiting Fellow  
NICHD  
Neuroscience - Cellular, Molecular, and Glia  

*High resolution in vivo detection of synaptic proteins using a novel nanotag-nanobody system*

The chemical synapse is the fundamental communication unit connecting neurons to one another and designed to mediate rapid transmission of signals across the synaptic cleft. Crucial to this function is the organization and fine-tuning of the presynaptic and postsynaptic structures, both composed of densely packed multi-protein assemblies. Our present understanding of synaptic architecture comes mostly from elegant electron microscopy studies. However, the subsynaptic distribution of many synaptic components and their dynamics remain obscure, partly due to unavailability of suitable antibodies and the low level of synaptic proteins. Adding fluorescent tags may interfere with protein function and conventional immunohistochemistry could add about 30 nm between the epitope of interest and detectable chromophore. Super-resolution microscopy cannot unequivocally differentiate between pre-
and postsynaptic locations across a synaptic cleft of 20 nm. With a length of 2-4 nm, nanobodies may offer a solution to this problem. We have used a novel nanotag-nanobody system, called ALFA-tag and NbALFA, to examine the distribution of synaptic proteins at the Drosophila larval neuromuscular junction (NMJ). As proof of concept, we chose to tag Neurexin 1 (Nrx1), a highly conserved cell-adhesion molecule with multiple extracellular modules, and a short intracellular domain bearing a C-terminal PDZ-binding motif. We used phylogenetic analysis and secondary structure prediction to guide the insertion of the ALFA-tag (AT) in between functional motifs. We generated tagged (Nrx1-AT, Nrx1-GFP) and non-tagged Nrx1 transgenes, then used a comprehensive set of genetics, histology and electrophysiology approaches to compare their distribution and function at the larval NMJ. Neuronal expressed Nrx1-AT co-localizes with endogenous Nrx1 at the active zone. More importantly, when provided in motor neurons, Nrx1-AT rescues the lethality and the physiological defects of nrx1 mutants, demonstrating that the Nrx1-AT is fully functional. NbALFA binds the ALFA-tag with high affinity both inside and outside the cells. The ability of NbALFA to function as an intrabody allowed us to generate a genetically encoded transgene that could be differentially expressed and used for detecting ALFA tagged proteins in vivo in distinct cellular compartments. In conclusion, this unique system offers a versatile cell biology tool towards studying protein distribution within a tight and crowded cellular milieu.

Irina Evsyukova
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Neuroscience - Developmental

Developmental Disruption of Locus Coeruleus-Norepinephrine Signaling Results in Male-Specific Behavioral Phenotypes Relevant to Neurodevelopmental Disorders

Autism spectrum disorder (ASD) is a heterogeneous group of neurodevelopmental disorders that affects boys four times more frequently than girls. In addition to the clinically characterized deficits in social interaction and communication, a number of other conditions – including attention deficit/hyperactivity disorder, learning disorders, and seizures – frequently co-occur with ASD. While it has been proposed that developmental dysregulation of the locus coeruleus-norepinephrine (LC-NE) system underlies behavioral deficits associated with these conditions, supporting evidence is lacking. Unfortunately, current strategies aimed at testing this developmental hypothesis are restricted to manipulation of the adult LC-NE system, due to the difficulty of targeting the developing LC without affecting other central and peripheral NE neurons. To circumvent this problem, we exploited our finding that LC-NE neurons are uniquely defined by embryonic expression of the transcription factor Engrailed 1 (En1) and later expression of dopamine beta-hydroxylase (Dbh), the enzyme required to convert dopamine to NE. We generated a conditional knockout allele of Dbh and crossed it with En1cre to selectively eliminate NE synthesis in LC neurons during embryonic development (LC-NE mutants). Unlike the full Dbh knockout, which is embryonic lethal, LC-NE mutants survive to adulthood, allowing us to evaluate the consequences of embryonic disruption of LC-NE on adult behavior. We subjected LC-NE mutants and littermate controls to a battery of behavioral tests to assess sociability, general activity, and learning. We found that male, but not female, LC-NE mutants exhibit reduced sociability, as well as hyperactivity, impaired contextual learning and increased incidence of seizures. Surprisingly, results from mass spectrometry revealed that only female LC-NE mutants have elevated dopamine levels in the
cortex consequent to Dbh loss. These data suggest that the behavioral phenotypes observed in male mutants are likely due to loss of LC-NE, and that elevated dopamine in females may compensate for NE loss. Taken together, our data demonstrate that LC-NE mutants exhibit several behavioral deficits observed in ASD and associated disorders, providing a new experimental system to investigate how developmental dysregulation of LC-NE drives these sex-specific phenotypes.

Mingan Sun  
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NICHD  
Neuroscience - Developmental

**Determination of spinal motor neuron specificity by Hb9-mediated repression of genes for alternative neuronal types**

Motor neuron (MN), together with interneuron, are the two major neuronal types in spinal cord. MNs control muscle movement, and are essential for breathing, walking and fine motor skills. Further, malfunction of MNs is frequently associated with neuronal diseases such as spinal muscular atrophy and amyotrophic lateral sclerosis. To establish normal function, MNs need to be differentiated properly from neural progenitor cells. Thus, it is of great importance to study the mechanism for MN specification. This study focuses on Hb9 - a conserved homeobox transcription factor gets expressed during MN specification. Hb9 has MN-specific expression and it is also the most widely used MN marker. Previous studies reported that Hb9 mutation in mouse causes early lethality - probably by affecting the controlling over respiratory system. However, the exact function of Hb9 for MN specification remains poorly understood. In this study, taking advantage of a recently developed model for efficient MN induction from mouse ES cells, we combined experimental and OMICs techniques to systematically investigate the function of Hb9 during MN specification. We identified more than ten thousand of Hb9 binding sites at genome-wide - most lacking active histone modifications (e.g. H3K4me3 and H3K27ac), indicating that Hb9 may repress instead of activate transcription. To validate if it really behaves as transcription repressor, we applied CRISPR to knockout Hb9 and then generated transcriptome to examine its effect on gene expression. As expected, the disruption of Hb9 causes derepression of almost one thousand genes - in contrast, only three hundred genes get down-regulated. Gene ontology analysis showed that derepressed genes are significantly associated with neuronal functions such as neuron migration and brain development. Interestingly, many of these derepressed genes normally should be expressed in spinal interneuron (Chx10, Shox2), brain neurons (Chrna3-5, Sorcs3) or neural progenitor cells (Nkx1, Olig2, Sox1). In summary, this study represents the first systematic investigation about the function of the core MN transcription factor Hb9 during MN specification. Our results indicated that Hb9 determines MN specificity by repressing genes for alternative neuronal types, and highlight the importance of transcription repression mechanisms during neuron specification.

Dahong Chen  
Visiting Fellow
Excitatory regulation from the parabrachial nucleus to the ventral tegmental area mediates unanticipated long-term memory of aversion

The ventral tegmental area (VTA) is a brain structure well known for containing dopamine neurons that have been implicated in motivation, addiction, decision making, aversion and pain. While it has been suggested that different types of VTA dopamine neurons participate in these different behaviors, emerging findings suggest that some of these behaviors may also be mediated by VTA non-dopamine neurons. In this regard, we have recently demonstrated that the VTA has a subset of glutamatergic neurons (expressing vesicular glutamate transporter 2, VGlut2 mRNA) that are involved in aversion. Here, we conducted monosynaptic rabies tracing studies to determine the upstream neurons that may regulate the activity of VTA glutamatergic neurons. From these tracing studies, we found that the VTA glutamatergic neurons received inputs from the parabrachial nucleus (PBN), a brain area implicated in aversive behavior. Next, we determined the phenotype of PBN neurons innervating the VTA by using a combination of chemical neuronal tracing and in situ hybridization and found that the vast majority of PBN neurons that innervate the VTA expressed VGlut2 mRNA. Given that these findings suggest that PBN-glutamatergic neurons excite VTA-glutamatergic neurons, we combined viral tracing techniques with immuno ultrastructural analysis, and found that axon terminals from PBN neurons (containing VGlut2-protein) established asymmetric (excitatory type) synapses on VTA VGlut2-positive neurons. To confirm the excitatory nature of the PBN synapses on VTA-glutamatergic neurons, we used a combination of viral tract tracing, optogenetics and slice electrophysiology and found that glutamate released from PBN-glutamatergic innervations evoked the firing of VTA-glutamatergic neurons. Next, by combination of optogenetics and behavior analysis, we found that VTA laser-induced release of glutamate from PBN inputs resulted in place aversion. Moreover, in the absence of laser-stimulation, this aversion was maintained for up to 60 days. In summary, we provide compelling evidence indicating that glutamatergic inputs from the PBN to the VTA are part of a neuronal network that mediates long-lasting memory of aversive signaling.
Dennis Burke  
Doctoral Candidate  
NIAAA  
Neuroscience - General  

*Dopamine release in the nucleus accumbens shapes lateral inhibition*

The nucleus accumbens (NAc) is a brain region that subserves healthy motivated behaviors and is an important site of dysfunction in addiction and depression. Acting as a limbic-motor interface, projection neurons in the NAc, medium spiny neurons (MSNs), integrate excitatory glutamate signals from cortex, amygdala, and hippocampus along with dopamine (DA) signals from midbrain to facilitate motivation and reward learning. However, NAc also contains an extensive network of inhibitory GABA synapses between MSNs, which remains understudied. It is currently unknown how DA interacts with this network of MSN GABA synapses. We hypothesize that by modulating synaptic strength within this network of lateral inhibition, DA influences NAc information processing by shifting the balance of excitation/inhibition at the micro circuitry level. MSNs express either Gs coupled D1 receptors or Gi coupled D2 receptors (D2MSNs). Due to the link between D2 receptor (D2R) signaling and multiple psychiatric disorders, we begin by focusing on D2MSNs. Here we ask: how does DA acutely affect GABA transmission from D2MSNs onto neighboring MSNs? We hypothesize that endogenously released DA from the midbrain can inhibit local GABA transmission from D2MSNs to MSNs. Using a dual optogenetic approach to test this hypothesis in brain slices, I show that DA neuron stimulation that precedes D2MSN GABA release by 500-1000ms can acutely depress GABA transmission from D2MSN synapses. To further probe this DA inhibition of D2MSN synaptic transmission, I applied exogenous DA at different concentrations and found the IC50 to be in the micromolar range, an order of magnitude higher than previous studies using heterologous systems. We next hypothesized that this DA depression of D2MSN GABA transmission is mediated presynaptically by D2Rs expressed on D2MSNs. I found that indeed this depression is driven by a presynaptic mechanism, but surprisingly it is only partially explained by D2R activation. Genetically deleting or pharmacologically blocking D2Rs only attenuates DA mediated depression by ~50%. Moreover, D1 receptor antagonists did not block the depression, suggesting a novel target of DA. Taken together, these results shed light on the actions and mechanisms by which DA acutely modulates the network of lateral inhibition in the NAc. This insight is necessary for understanding how DA shapes NAc circuitry and information processing to promote motivated behaviors, both in health and disease.

Shana Silverstein  
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Neuroscience - General
Observational fear learning: a behaviorally translational approach from mouse to man

Learning from others’ experiences is a highly evolutionarily conserved mechanism providing necessary information about threat and safety. This behavior is observed across species, including humans. Various behavioral tasks have been established to assay socially acquired fear, but the literature on observational fear learning (OFL) remains scarce. First, we established a paradigm in mice using a modified version of standard cued-Pavlovian fear conditioning, but with two mice—one is exposed to a footshock-tone pairing (demonstrator), while the other watches (observer). We then assessed the associative memory of the observer. Employing complementary behavioral, anatomical, and in vivo optogenetics, we defined a novel mechanism subserving observational fear that has not previously been identified. We demonstrated a critical contribution of the dorsomedial prefrontal cortex (dmPFC) to OFL and showed that ventral hippocampal (vHPC) inputs to the dmPFC negatively gate OFL. This suggests that the inhibitory vHPC-dmPFC pathway is critical for OFL. Second, we developed a human version of the OFL paradigm and applied detailed computational modelling of trial-by-trial variation of behavior, supported by Bayesian model comparison, to examine learning rates and variability during OFL. In this paradigm a video of a man in a similar setup to participants was presented with two conditioned stimuli—one was associated with a high probability of shock, and another with a low probability association. Participants were asked to predict whether each stimuli would result in a shock. The Bayesian model comparison revealed participants’ choices were best characterized combining learning rates and variability parameters as opposed to a more simplistic or separate model. We also observed a negative correlation between learning rate and trait anxiety level indicating individuals with higher levels of anxiety were slower to update their beliefs as to the likelihood a shock would be delivered. We are currently running a fMRI study to assess the brain regions engaged during OFL compared to the circuit identified in mice. We are coupling this information with a computational model to see if learning rate is modulating vHPC or dmPFC activity. Together, our findings reveal a novel translational model of OFL in both mice and humans with therapeutic implications for conditions associated with atypical fear learning, including generalized anxiety and post-traumatic stress disorders.

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Ji Chen
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CC Neuroscience - General

Toward a Wearable Pediatric Robotic Knee Exoskeleton for Real World Overground Gait Rehabilitation in Ambulatory Individuals

Crouch gait, or excessive knee flexion, is a debilitating gait pathology in children with cerebral palsy (CP). Surgery, bracing and therapy provide only short term correction of crouch and more sustainable solutions remain a significant challenge in children with CP. One major hurdle is achieving the required dosage and intensity of gait training necessary to produce meaningful long term improvements in walking ability. Rather than replace lost or absent function, gait training in CP population aims to improve the participant’s baseline walking pattern by encouraging longer bouts of training and exercise, which is different than in those with paralysis. Wearable robotic exoskeletons, as a potential strategy, can assist individuals with CP to gradually regain knee extension over time and help maintain it
for longer periods through intense task-specific gait training. We previously tested our initial prototype which produced significant improvement in knee extension comparable in magnitude to reported results from orthopedic surgery. Children continued to exert voluntary knee extensor muscle when walking with the exoskeleton which indicated the device was assisting but not controlling their gait. These positive initial results motivated us to design second prototype to expand the user population, and to enable its effective use outside of the laboratory environment. The current version has individualized control capability and device portability for home use as it implemented a multi-layered closed loop control system and a microcontroller based data acquisition system. The benchtop evaluation shows that the exoskeleton has very small torque assistance activation latency (<33ms). The torque bandwidth was 12 Hz with a 10 Nm amplitude chirp in knee flexion and extension. The actuator had low output impedance (<1.5 Nm) at low frequencies expected during use. Three different control strategies were developed for use with the exoskeleton: stated-based assistance, impedance based assist-as-needed control, and a real-time adaptive control. Exoskeleton performance was validated in a healthy subject. These three control strategies had different effects on knee angle, and spatiotemporal parameters (cadence, step length and step width). Future work will investigate the effects of providing children with CP assistance during walking on gait biomechanics and identify the optimal individualized control strategies for exoskeleton prescription in this population.

Zheng Nordman
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Neuroscience - Integrative, Cognitive, Sensory and Behavioral Neuroscience

Traumatic stress induces long lasting violent aggression through synaptic potentiation of two medial amygdala circuits

Traumatic stress has long been shown in humans and rodents to promote excessive and recurring violent aggression, co-morbid with psychiatric diseases such as PTSD, intermittent explosive disorder, and schizophrenia. In individuals suffering from PTSD, hyperactivity of the amygdala is strongly correlated with stress responses that trigger anger and violence. The medial amygdala (MeA) is an evolutionarily conserved subnucleus of the amygdala that regulates attack behavior and has been implicated in stress induced aggression. The precise contribution of the MeA in traumatic stress induced aggression, however, requires further elucidation. In this study we used a modified foot shock protocol, a common method for inducing aggression in rodents, to evaluate the circuit level mechanisms of the MeA in driving long lasting attack behavior associated with traumatic stress. We observed that foot shock increased aggression for weeks after training. In a previous study we showed that the MeA produced a phenomenon known as aggression priming by potentiating two canonical members of the aggression circuit, the ventromedial hypothalamus (VmH) and bed nucleus of the stria terminalis (BNST). We hypothesized that a similar mechanism might be operating in foot shock induced aggression. In vivo electrophysiological recordings, a method for detecting neural activity in an awake and freely moving animal, revealed that foot shock potentiates these same circuits, but for far longer periods of time than aggression priming, consistent with our behavioral data. Intriguingly, low frequency photostimulation (LFPS), an optogenetic strategy for inducing synaptic depression in the brain, suppressed long-lasting aggression induced by our foot shock protocol when applied directly to the MeA, confirming that
potentiation of these circuits is integral for the observed attack behavior. Notably, these effects were irrespective of non-violent social behavior or fear memory, suggesting that these MeA circuits are dedicated aggression pathways. These results reveal an important function for the MeA in traumatic stress induced aggression and may be useful in developing therapeutic strategies to treat excessive aggression associated with traumatic stress and psychiatric disease.

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**Chloe Jordan**  
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*Dopamine D3 Receptor-Based Medication Strategies to Combat Opioid Addiction*

Opioid addiction is a mounting public health crisis with massive personal and economic costs. Prescriptions opioids, including oxycodone, are critical in pain management but are often misused, diverted, and abused. Identifying non-opioid-based pharmacotherapies for the treatment of opioid abuse that do not interfere with analgesia is vital. The addictive liability of opioids is due in part to their action in the brain, where they increase dopamine (DA) activity in key reward-related regions, such as the ventral tegmental area (VTA). Of the five DA receptor subtypes, D3 receptors (D3Rs) exhibit the highest binding affinity for DA and have restricted distribution in the brain reward system, making these receptors attractive therapeutic targets for the treatment of opioid addiction. Previous work by our group and others has shown that D3R antagonists, which block DA from binding D3R, can reduce the motivation to self-administer cocaine and relapse to cocaine-seeking behaviors. However, despite the ongoing opioid crisis, little research to date has examined whether D3R antagonists are effective in reducing opioid abuse. Here, we determined whether a new, highly selective and metabolically stable D3R antagonist, R-VK4-40, alters gold-standard preclinical models of opioid addiction, including intravenous oxycodone self-administration, DA-driven brain stimulation reward, and oxycodone-induced analgesia (reduction of pain sensation). We found that R-VK4-40 dose-dependently reduced intravenous oxycodone self-administration and blocked oxycodone-induced increases in brain reward, as measured by responding for optogenetic intracranial self-stimulation of VTA DA neurons. Furthermore, R-VK4-40 enhanced oxycodone-induced analgesia and exerted analgesic effects when administered alone, without altering locomotor activity or cardiovascular function (e.g., heart rate, blood pressure). These results indicate that R-VK4-40 attenuates opioid reward through a DA-dependent mechanism, while simultaneously augmenting opioid-induced analgesia. Taken together, these findings indicate R-VK4-40 has translational therapeutic potential for the treatment of prescription opioid use disorders, and support the utility of D3R-based medication strategies in combatting the ongoing opioid crisis.

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**Ann Ida Fredriksson**  
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Incubation of oxycodone craving after conflict-induced voluntary abstinence

High relapse rates perpetuate opioid addiction and are a major obstacle in addressing the current U.S. opioid epidemic. The main limitation of most current animal models of drug relapse is that prior to relapse testing, abstinence is forced (i.e., experimenter-imposed). This contrasts with the human condition in which abstinence is often self-imposed. Furthermore, relapse episodes often involve conflict situations where the addict chooses between experiencing the drug’s rewarding effect and the potential adverse consequences of pursuing the drug. To mimic this human condition, we recently developed a rat model of oxycodone relapse and craving after conflict-induced voluntary abstinence. In this model, we induce abstinence by introducing an electric barrier near the drug-paired lever that the rats must cross to gain access to oxycodone. As shock intensity is increased over days, the rats decrease their oxycodone intake and eventually completely stop taking oxycodone. We then assess relapse to drug seeking during early and late withdrawal in the absence of oxycodone or shock. We found time-dependent increases in drug seeking (incubation of drug craving), an effect that was potentiated by conflict-induced abstinence compared to homecage forced abstinence. We then examined the role of ventral subiculum (vSub) in incubation of oxycodone seeking after this form of abstinence because vSub is critical for context-induced reinstatement of heroin seeking and relapse to alcohol seeking after punishment-imposed abstinence. We trained male and female rats to self-administer oxycodone (0.1 mg/kg/infusion, 6-h/d) for 14 days. We then introduced an electric barrier near the drug-paired lever of increasing intensity over several days (0.1 to 0.4mA) to produce cessation of oxycodone self-administration (conflict phase). Next, we tested the rats (n=6-7/group) for relapse to oxycodone seeking during late withdrawal on abstinence day 15 and extracted the brains for Fos (neural activity marker)-immunohistochemistry or tested the rats (n=11/group) after inactivation of vSub using GABA A+B receptor agonists. We found that relapse after conflict-induced abstinence was associated with increased neural activity in vSub, and local inactivation of vSub decreased oxycodone seeking. Together, these data demonstrate a role of vSub in incubation of oxycodone craving after cessation of drug taking due to adverse consequences of drug seeking involving a conflict situation.

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Neuroscience - Integrative, Cognitive, Sensory and Behavioral Neuroscience

Reading the thinking brain: verbal and visual thinking decoded from magnetoencephalographic signals

Recent advances in computational neuroscience allow us to decode from neuroimaging signals what information content a participant is seeing, imagining, or even dreaming about. On the other hand, similar methods have been used to distinguish different brain states, such as quietly resting, remembering, doing calculations. Nevertheless, within a single brain state, such as quietly resting, one can be engaged in various forms of spontaneous thinking, such as internal dialogue or visual imagery. In this study we aimed to read the modality of spontaneous thinking. We recorded magnetoencephalography (MEG) signals from participants while they were cued to think about either a cow or a bicycle (Exp. 1) or about anything they wanted (Exp. 2). They were free to choose between two modalities of thought: the verbal modality, which involves thinking in words, and the visual modality,
which involves picturing a scene. After developing their thought during 3.5 s, they pressed a button to report the modality they chose. The button-response association was only given at the end of the trial so that motor response was orthogonal to the modality of thinking. Furthermore, eye fixation was controlled during the thinking period to minimize interference from eye movements or blinks. In Exp. 1, the association between the visual cue and the object of thought was varied across two sessions to make these two variables independent. Data were analyzed with multivariate linear modeling to compute the time-course of decoding performance for the visual cue, the object of thought and the modality of thinking. Our results revealed that the visual cue can be significantly decoded from MEG signals shortly after its presentation, whereas the object of thought in Exp. 1 (cow vs. bicycle) can be significantly decoded for a longer period of time during the thinking period. Most interestingly, the decoding performance of the modality of thought (visual vs. verbal) significantly increased up to ~60% during the thinking period for both Exp. 1 and 2, while chance decoding performance was at 50%. This study revealed our ability to infer from MEG signals the object and modality of thought of participants, independent from the visual input and overt motor response. These results will contribute to a better understanding of mental states and might be particularly useful for non-communicative patients, including those with locked-in syndrome.

Justin Siemian
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Neuroscience - Integrative, Cognitive, Sensory and Behavioral Neuroscience
Lateral hypothalamic fast-spiking parvalbumin neurons modulate nociception through connections in the periaqueductal gray area

The lateral hypothalamus (LH) contains a diverse collection of cell types crucial for orchestrating behaviors that facilitate survival. Over the past decade, tremendous progress has been made on new methods that allow systematic characterization of the function and connectivity of these heterogeneous neuronal subtypes. While studies have begun to identify LH circuits that regulate food intake and reward-related behaviors, less attention has been given to the contributions of genetically-identified LH circuits that modulate nociceptive behaviors. Here we examined how lateral hypothalamic neurons that express the calcium-binding protein parvalbumin (PVALB; LH-PV neurons), a small cluster of neurons within the LH glutamatergic circuitry, modulate nociception in mice. Using optogenetics to modulate neuronal activity, we found that photostimulation of LH-PV neurons suppressed nociception to an acute, noxious thermal stimulus ($p = 0.007$), whereas photoinhibition potentiated thermal nociception ($p = 0.0145$). Next, brain slice electrophysiology recordings using optogenetics revealed that LH-PV axons form functional excitatory synapses on neurons in the ventrolateral periaqueductal gray (vIPAG), a critical brain region for pain modulation. Moreover, photostimulation of LH-PV axons in the vIPAG suppressed nociception to both thermal ($p = 0.0007$) and chemical stimuli ($p = 0.0212$). Interestingly, this antinociceptive effect appears to occur independently of opioidergic mechanisms, as antagonism of mu-opioid receptors with systemically-administered naltrexone did not abolish the antinociception evoked by activation of this LH-PV to vIPAG pathway. Importantly, none of the optogenetic manipulations significantly affected generalized locomotor activity or anxiety-like behavior as measured by the open-field and elevated plus maze tests, suggesting the role of LH-PV neurons may be specific to
nociception. Together, these results directly implicate LH-PV neurons in modulating nociception, thus identifying a potential novel target for pain therapies and expanding the repertoire of survival behaviors regulated by LH circuits.

**Michael Authement**  
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Neuroscience - Neural Circuits  
*Cocaine actions on cortico-striatal circuitry: a focus on cholinergic interneurons*  
Cholinergic interneurons (CINs) of the striatum are thought to play a critical role in behavioral flexibility and dysfunction of CINs may underlie the pathology of compulsive behaviors that are expressed in drug abuse. Although CINs account for only 1% of striatal neurons, they are the major source of acetylcholine in the striatum. Furthermore, recent studies have identified another critical function of CINs: triggering dopamine release. Direct and indirect activation of CINs through cortical and thalamic inputs can evoke dopamine release independent of midbrain dopaminergic neuron firing. Therefore, we hypothesize that if drugs of abuse have effects on CIN physiology, they would not only affect the levels of acetylcholine in the striatum, but also affect this novel form of striatal dopamine signaling. The central goal of this study is to identify the mechanisms underlying the acute and chronic effects of cocaine, a stimulant drug of abuse, on CIN activity and on synaptic inputs to CINs. Specifically, we are focusing on glutamatergic inputs from the prefrontal cortex (PFC) onto CINs based on the well-known role of the PFC in behavioral flexibility and inhibitory control. Recordings from CINs in ex vivo brain slices showed that cocaine potently and dose-dependently depressed excitatory transmission from PFC inputs onto CINs. The mechanism underlying this acute depression appeared to be presynaptic and was not blocked with the D2 dopamine receptor antagonist sulpiride. Cocaine did increase the spontaneous action potential firing in CINs by two-fold, and this effect was blocked with a serotonin receptor antagonist, ketanserin, or D1/D5 dopamine receptor antagonist, SCH-23390. After repeated administration of cocaine over a 5-day period, the excitability of CINs was decreased and remained lower for up to 21 days after the last cocaine administration. Thus, while cocaine acutely increases CIN firing, chronic exposure to cocaine produces a long-lasting depression of firing, a plasticity in the opposite direction. We are now investigating the mechanism underlying this long-lasting, cocaine-induced decrease of CIN excitability and whether synaptic transmission from the PFC inputs is similarly affected by chronic cocaine exposure. These studies are revealing novel actions of cocaine on cortico-striatal circuitry that we speculate may contribute to the loss of inhibitory control and the behavioral inflexibility that characterizes compulsive cocaine use.

**Patrick Piantadosi**  
Postdoctoral Fellow  
NIAAA  
Neuroscience - Neural Circuits
Cortico-limbic-striatal regulation of risky decision-making

In healthy individuals, the risk of an aversive outcome occurring following a given action biases subsequent behavior towards other, safer actions. Such adaptive cost/benefit analyses are disturbed in substance-abuse and anxiety disorders, and have been suggested to be mediated by interactions between cortico-limbic-striatal circuits. Yet, how these circuits signal during discrete phases of the decision-making process remains unclear. Here, we utilized projection-specific fiber photometry and optogenetics to examine the relevance of basolateral amygdala (BLA) neurons projecting to the nucleus accumbens shell (NAcShell) during decision-making in safe and risky contexts. We designed a touchscreen-based risky decision-making task (RDT) where mice could choose between two screens that differed in the volume of milkshake reinforcement they produced. Mice initially chose between these options in the absence of risk, during which calcium-dependent GCaMP6m-based fluorescence was monitored. BLA-NAcShell activity encoded task events including touchscreen selection, reward delivery, and reward consumption. Optogenetic silencing in mice expressing the inhibitory opsin eArchT3.0 in the BLA-NAcShell projection had no impact on reward-seeking in the absence of risk (as compared to eYFP-expressing controls), suggesting that, although this pathway encodes relevant behaviors, it is not necessary for their execution. Mice were then exposed to the RDT, whereby a minor footshock (0.1 mA) was paired with the large reward option across 3 discrete trial blocks of ascending shock probability (0, 50, 75% probability of shock on the large/risky option). BLA-NAcShell activity encoded the aversive footshock, consistent with this pathway providing a “loss”-like signal during risky decision-making. Support for this contention was provided by our finding that silencing this projection markedly attenuated the shock-mediated shift towards the small/safe option observed in eYFP control mice, an effect that resulted from an insensitivity to negative-feedback in the eArchT animals. Control experiments suggested that the loss of risk-sensitivity observed in eArchT mice was not due to changes in motivation or pain sensitivity. Future experiments will determine whether BLA provides negative-feedback information to the NAcShell via projections to specific neuronal subtypes, including dopamine D2-receptor expressing neurons that have been shown to signal losses during risky decision-making.

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Hindbrain catecholaminergic neurons control glucoprivation-induced feeding via projections to the paraventricular nucleus of the thalamus

Glucose metabolism is crucial for proper brain function. Indeed, inadequate brain glucose can result in cognitive impairments, unconsciousness, and even death. Given the importance of maintaining optimal glucose levels, glucoregulatory responses are in place to support glucose homeostasis. These include behavioral (e.g. increased food seeking/feeding) and physiological responses (e.g. decreased insulin and increased corticosterone secretion). Past research showed that A1/C1 catecholamine neurons within the ventrolateral medulla are potently activated by glucoprivation and support glucoprivation-induced food seeking/intake. However, how this neuronal subpopulation orchestrates feeding behavior is unclear. Here, we investigate the circuit mechanism for glucoprivation-induced food seeking/intake in mice. Previous anatomical studies show that A1/C1 neurons project to the paraventricular nucleus of the
thalamus (PVT), a brain region known to participate in adaptive behavioral responses to stress. Thus, we hypothesized that PVT is a critical node linking the glucoprivation-induced activation of A1/C1 neurons with food seeking. First, using optogenetics we found that activation of A1/C1 projections to PVT was sufficient to induce feeding in sated mice. Because previous research has linked neuronal inhibition in PVT with feeding, we next combined in-vivo Ca2+ imaging with optogenetics to test the impact of optogenetically activating A1/C1 terminals on PVT neuronal activity. Surprisingly, we found that optogenetic activation of A1/C1->PVT projections leads to a robust increase in the activity of PVT projection neurons. Notably, the concomitant feeding behavior triggered by light stimulation attenuated the activity of PVT neurons suggesting that activation of PVT neurons may drive feeding through negative reinforcement. This A1/C1 projection-driven excitation of PVT neurons was further confirmed in vitro using a slice preparation and patch clamp electrophysiology. In addition, these results revealed that A1/C1-evoked effects in the PVT are mediated by the beta-adrenergic receptor, consistent with the catecholaminergic nature of this cell group. Lastly, optogenetic inactivation of A1/C1 projections to PVT attenuated pharmacological-induced glucoprivation feeding. Together, these results demonstrate that the A1/C1->PVT pathway is critical for glucoprivation-induced food seeking and that activation of A1/C1->PVT projections mimics this process.

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*In vivo labeling of active neurons in the prelimbic cortex during a cocaine discrimination task using a photo-activatable genetically encoded calcium integrator*

Environmental stimuli paired with cocaine experience can trigger relapse long after a person’s last drug exposure. We developed a trial-based discrimination task and found that a rat’s response to a discriminative stimulus (predicting cocaine availability) progressively increases over 60 days of abstinence and persists up to 300 days (almost half the rats’ lifespan). The identification of in vivo neural activity and circuits underlying the formation, maintenance and expression of these persistent maladaptive learned associations are essential to addiction research. The expression of immediate early genes such as Fos is typically used as a proxy for identifying active neurons following behavior. Fos gene induction is thought to be due to strong persistent neural activity-induced calcium influx integrated over several minutes. Our lab and others have shown that specific patterns of neurons, called Fos-expressing ensembles, mediate distinct long-term memories and learned behaviors involving complex sets of cues and rewards. Unfortunately, Fos protein regulation is slow: levels increase over 2-3 hours and return slowly to baseline. This makes it difficult to identify neurons underlying individual stimulus-drug associations with high temporal precision or track these neurons across time. To address this problem, we developed a technique to permanently label activated neurons in vivo with high temporal specificity (<1 minute) in rats during behavior. We use a calcium integrator protein named CaMPARI (calcium modulated photo-activatable ratiometric integrator) that can be rapidly and permanently converted using UV light from its native green state to an “active” red state only in strongly activated cells (high calcium influx). We first inject an AAV packaged construct to express the protein in the prelimbic cortex of rats and implant an optical fiber to deliver the UV excitation light into the region. Following
discrimination training for cocaine reward, we label either neurons activated by cues predicting cocaine availability or lack of availability during drug-taking. We are currently using fluorescence activated cell sorting (FACS) to compare molecular alterations in these two active neuron populations during drug-taking. The permanent nature of the label allows us to identify molecular alterations that are specific to the drug-cue neurons during abstinence for the ultimate purpose of identifying the engram underlying maladaptive memories in addiction.

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A potential role of iron in the repair of experimental inflammatory demyelinating lesions
Inflammatory destruction of iron-rich myelin is characteristic of multiple sclerosis (MS). Although iron is needed for oligodendrocytes to produce myelin, its deposition has also been linked to neurodegeneration and inflammation, including in MS. Indeed, some MS lesions harbor iron, which is detectable with magnetic resonance imaging (MRI) in vivo, as well as by histopathology. Despite these observations, the spatiotemporal dynamics of iron deposition and the cellular pathways involved in intrallesional iron accumulation in MS have not been elucidated. Fundamentally limiting our understanding of the role of iron in MS is that most knowledge derives from histopathology-based studies of patients with long disease duration, potentially many years after iron first appears. Characterizing the spatiotemporal patterns of iron deposition using a relevant animal model therefore may help identify the timing, origin, and pathophysiological significance of intrallesional iron. Experimental autoimmune encephalomyelitis (EAE) in the common marmoset recapitulates pathobiological and radiological features of focal MS lesions in the cerebral white matter. This provides a window of opportunity to investigate how lesions form, as well as the relative timing of factors, including iron, involved in lesion pathogenesis. To study the role of iron in marmoset EAE, we used proton-density-weighted MRI for lesion detection and T2*-weighted MRI for iron detection. We tracked EAE lesions in vivo to determine their age and location, then performed histopathology for characterization of iron, iron-regulating proteins including transferrin receptor (TfR), and cells including microglia and astrocytes. We report perivascular iron deposition in multiple sclerosis lesions that was mirrored in 72 lesions from 13 marmosets with experimental autoimmune encephalomyelitis. Iron accumulated mainly inside microglia/macrophages from 6 weeks after demyelination. Consistently,
expression of TfR, the brain’s main iron-influx protein, increased as lesions aged. Iron was uncorrelated with inflammation and oxidative stress, and post-dated initial demyelination, suggesting that iron is not directly pathogenic. Iron homeostasis was at least partially restored in remyelinated, but not persistently demyelinated, lesions. Taken together, our results suggest that iron accumulation in the weeks after inflammatory demyelination may contribute to lesion repair rather than inflammatory demyelination per se.

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Background: The dominant LIM Domain Binding protein 3 (LDB3) mutation p.A165V is the most common cause of myofibrillar myopathy type 4 (MFM4) which is also known as Markesbery-Griggs distal myopathy. Disease mechanisms underlying MFM4 are not yet defined, partly due to lack of mouse models. Here, we aimed to generate a p.A165V mouse model and characterize disease mechanisms underlying skeletal muscle degeneration in MFM4. Materials and methods: LDB3-A165V mutant knock-in mice were generated by homologous recombination. We examined histological, biochemical and physiological characteristics of LDB3 knock-in mice to determine the morphological, functional and molecular consequences of the p.A165V mutation. Mutant mice were compared to wildtype littermates in a longitudinal follow up over 10 months. Histological and biochemical and protein expression changes were examined using Gomori trichrome (GT), mitochondrial enzyme-histochemistry, Immunofluorescence and Reverse Phase Protein Array (RPPA). Autophagy flux was measured in mice following colchicine administration. Results: Beginning at 3 months of age, four paw grip strength was significantly reduced in mutant mice (p < 0.01, n = 35). Maximal isometric contractile force was 147.8+/-5.2 mN/mm2 in mutant and 187.4+/-3.2 mN/mm2 in wildtype mice (p < 0.01, n = 3-4) in EDL ex-vivo without evidence of fatigability. Muscle mass was reduced in mutant mice (p < 0.05, n = 5). By 6 months, hallmark pathology changes of MFM were observed by immunostaining and electron microscopy, followed at 8 months by vacuolar degenerative myopathy on histological staining. Mainly, aggregation of sarcoplasmic protein including MFM proteins and CASA components were observed. RPPA identified mTOR dysregulation at 4 months in mutant mice. Colchicine treatment showed an
impaired autophagy in mutant mice as compared to wildtype. Conclusions: LDB3-A165V mice develop progressive myofibrillar myopathy reminiscent of human patients. Our studies in heterozygous and homozygous mice indicate a dominant gain of function by mutant LDB3. Impaired autophagy and CASA pathway lead to the filamentous aggregates and myofibrillar disintegration associated with disease. Changes in signaling pathways precede MFM pathology in LDB3-A165V mutant mice and suggest a role in pathogenesis and, potentially, will lead to identification of therapeutic targets in MFM.

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Silencing Dopamine Neurons During Sleep as a Potential Therapeutic Strategy for Parkinson’s Disease
Parkinson’s Disease (PD) is the second most prevalent neurodegenerative disorder, and the most prevalent movement disorder in the US. The loss of dopamine neurons in the substantia nigra pars compacta (SNc) and subsequent reduction of dopamine levels in the striatum underlie the cardinal motor symptoms. Thus, the dopamine precursor, L-DOPA, is the primary treatment for PD. However, L-DOPA is a symptomatic treatment and there are currently no treatments that modify PD progression. Dopamine neurons have many structural and functional characteristics that require high energy expenditure, which can result in mitochondrial dysfunction, proteostatic stress, high calcium load, and excitotoxicity. Therefore, we hypothesized that silencing the activity of dopamine neurons during sleep will conserve energy and reduce dopamine cell death in PD models. To silence dopamine neuron activity, we used a combined drug approach of the D2/D3R agonist pramipexole with the GABAB receptor agonist baclofen. Brain slice electrophysiology and spectrally resolved fiber photometry were used to evaluate pramipexole + baclofen reduction of dopamine neuron firing in vitro and in vivo, respectively. In the acute 6-OHDA PD model, we unilaterally lesioned the dopaminergic terminals in the striatum of C57BL/6J mice and administered pramipexole + baclofen daily by i.p. injections for 4 weeks at the start of the sleep cycle. Pramipexole + baclofen administration significantly reduced the loss of dopamine neurons in the SNc compared to saline controls. These results suggest that silencing dopamine neuron activity during sleep may be a promising novel therapeutic approach, which could be tested in humans for stopping the progression of PD.

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Neuron-derived extracellular vesicles predict cognitive decline in cognitively intact people at risk for Alzheimer’s disease  
The amyloid hypothesis for Alzheimer’s disease (AD) posits that beta-amyloid (Aβ) aggregation precedes hyperphosphorylated Tau pathology and neurodegeneration, but recent Tau PET studies have called this sequence of pathogenic events into question. Established AD biomarkers have so far failed to predict cognitive decline at the preclinical phase of AD. Extracellular vesicles (EVs) are membranous particles 50 - 300 nm in size that are continuously secreted by all cells including brain neurons and astrocytes. Their cargos reflect the physiological or pathological states of the cells of origin. Our Lab has pioneered a technique for isolating enriched populations of neuronal derived (ND) EVs from peripheral blood and have used them as a source of biomarkers for AD. In this study, which leverages a large set of Wisconsin Registry for Alzheimer’s Prevention (WRAP) samples, we hypothesized that Tau proteins in NDEVs can predict cognitive decline in middle aged cognitively intact individuals at higher risk for AD due to family history. We blindly analyzed 431 repeated serum samples from 297 participants (mean age 63.4±6.4 years old; 69% females; 45% ApoE4 carriers), classified as cognitive decliners vs. cognitively stable based on their longitudinal cognitive performance. We isolated total EVs followed by enrichment for NDEVs and measured pTau(T181), pTau(S231) and total tau using electrochemiluminescence assays. We found significantly higher levels of pTau(T181) and pTau(S231), but comparable total tau, in cognitive decliners compared to cognitively stable participants. We also examined longitudinal changes in the two groups and found that pTau(T181) and pTau(S231) levels did not change significantly over time in either group; however, total tau levels increased significantly over time in decliners compared to stable participants. These results provide tantalizing evidence that NDEV Tau biomarkers may identify future Alzheimer’s patients at the preclinical stage. In ongoing experiments seeking to explain the basis for these findings, we are measuring NDEV biomarkers reflecting insulin resistance, kinases involved in Tau phosphorylation and synaptic degeneration.

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*Developing novel antibodies against the conformationally-exposed extracellular loop of oncogenic EGFR*

The epidermal growth factor receptor (EGFR) is a transmembrane receptor kinase that is central to the growth and development of epithelial tissues. Dysregulation of EGFR has been implicated in various cancer malignancies such as glioblastoma, breast carcinoma, lung cancer and colorectal cancer. EGFR related oncogenesis can occur by the overexpression of EGFR, which is often linked with gene amplification. Amplification is often associated with the expression of a mutant form of EGFR known as EGFRvIII. EGFRvIII is a tumor-specific mutant form of EGFR that results from the in-frame deletion of 267 amino acids from the extracellular domain. Although this truncated mutant is unable to bind ligands, its presence appears to maintain a low-level constitutive kinase activity resulting in aggressive tumor behavior. EGFRvIII-expressing cancer cells are often both radiotherapy and chemotherapy resistant. Given its prominent role in malignancy, EGFRvIII is an important molecular target for therapy. However, cells expressing EGFRvIII appear to be relatively resistant to current conventional EGFR-targeted therapies including tyrosine kinase inhibitors. And given the side effects of treatments that target wildtype EGFR, the identification of novel antibodies that specifically target EGFRvIII will be very valuable in treating a wide array of EGFR-associated cancers. We approached the discovery of novel anti-EGFR antibodies by targeting a conformational loop in the extracellular domain that is exposed in EGFRvIII, as well as misfolded oncogenic EGFR, but is normally masked in wtEGFR. Using mouse hybridoma techniques, we produced seven new monoclonal antibodies to EGFRvIII which do not bind wtEGFR. These antibodies bind to various malignant cell lines that are associated with EGFR over expression. Sequence analysis indicates that all seven of the antibodies differ from previously identified EGFRvIII antibodies. Binding analysis using amino acid substitutions within the conformational loop indicates that our newly developed antibodies bind to different regions of the exposed loop. This will provide us with various therapeutic strategies based on the antibody binding characteristics. Development of these antibodies as therapeutics may allow tumor efficacy without damaging side effects to normal epithelial tissue.

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*Arf1 Ablation Kills Cancer Stem Cells and Induces DAMP-mediated Anti-tumor Immune Responses in Mice*

Immunotherapies, particularly those with checkpoint blockage, are beginning to change the paradigm for treating cancer patients. However, most patients receive little or no benefit, because their tumors have already developed immune evasion mechanisms in the tumor microenvironment, including dysfunctional T cells and a lack of T cell infiltration. Cancer stem cells (CSC) as a subpopulation of cells in a tumor that are in a stem cell state and have stem cell characteristics. CSCs may be responsible for treatment resistance, tumor metastasis, disease recurrence, and eventually patient death. Here, we generate the Arf1 conditional knockout mice to study the Arf1 function in the cancer stem cells. The
intestine stem cells and liver stem cells specific CreER mice, Lgr5-CreER and Axin2-CreER mice, Apc floxP
tumors and Cebpb-tTA/TetO-Myc liver tumor mice were selected to cross with Arf1 floxp
mice to generate the Lgr5/Arf1f/f/Apcf/f intestine cancer mouse model and Cebpb-tTA/TetO-Myc/Axin2-
CreER/Arf1f/f liver cancer mouse model. Using the biochemistry, immunohistochemistry, flow cytometry,
electron microscopy etc. methods, we demonstrated that ablating Arf1 in mice disrupts lipolysis and
causes the necrosis of cancer stem cells (CSCs) and induces anti-tumor immune responses. Notably, Arf1
ablation in cancer cells induced mitochondrial defects, endoplasmic-reticulum stress, and the release of
damage-associated molecular patterns (DAMPs) that recruited and activated dendritic cells (DCs) at
tumor sites, which further activate IFN-gamma-secreting by cytotoxic T lymphocytes (CTLs) to kill CSCs.
The activated immune system finally elicited antitumor immune surveillance by stimulating T-cell
infiltration and activation. TCGA data analysis showed an inverse correlation between Arf1 expression
level with patient survival time in human cancers. Thus, Arf1-pathway knockdown not only kills CSCs but
also elicits a tumor-specific immune response. In summary, we identified a novel connection that links
lipid metabolism, CSC death, and anti-tumor immunity. CSCs may be responsible for the treatment
resistance and immune evasion of tumors. CSC signals can modulate lymphocyte infiltration into a
tumor and alter the tumor microenvironment.

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*Improving drug delivery to central nervous system tumors by targeting canonical WNT/β-catenin
signaling in the blood-brain barrier*

The blood-brain barrier (BBB) is a dynamic capillary bed that strictly regulates exchange of materials
between the blood and brain. High expression of tight and adherens junctions and a lack of
fenestrations in the endothelium result in a poorly permeable barrier, limiting drug delivery to the
central nervous system (CNS) and posing a major obstacle for chemotherapy. The BBB is partially
maintained by WNT/beta-catenin-mediated transcription of growth factors, multidrug resistance
proteins, and junctional proteins. In the WNT-subtype of medulloblastoma, a pediatric brain tumor,
activating mutations of beta-catenin lead to reciprocal secretion of WNT antagonists such as WIF1 and
DKK1 into the tumor microenvironment. These WNT antagonists act upon the surrounding endothelium
and induce a leaky BBB. Interestingly, patients with WNT-medulloblastoma respond exceptionally well
to chemotherapy compared to patients with other medulloblastoma subtypes. Therefore, we
hypothesize that pharmacological inhibition of WNT/beta-catenin signaling in brain endothelial cells will
decrease BBB integrity, enabling enhanced paracellular drug delivery to CNS tumors. We first
recapitulated the WNT-medulloblastoma phenotype in glioblastoma (GBM), the most lethal adult brain
tumor, by activating WNT/beta-catenin signaling in primary human glioma stem cells (GSC). By
immunoblotting, we detected induced downstream WNT antagonists in the conditioned-medium (CM)
of WNT-activated GSCs. Human brain microvascular endothelial cells (HBMEC) exposed to WNT-GSC-
CM, but not control GSC-CM, upregulated fenestration related protein, PLVAP, and downregulated
junctional proteins claudin-5, ZO-1, and VE-Cadherin. Next, we directly inhibited WNT/beta-catenin
signaling in HBMECs with the small molecule inhibitor ICG-001. As previously seen with WNT-GSC-CM,
expression of junctional proteins was decreased and PLVAP was increased. Lastly, using electrical cell impedance monitoring, we determined that endothelial barrier function was transiently disrupted by ICG-001. Pre-clinical studies are underway to evaluate the functional impact of WNT/beta-catenin inhibition on BBB integrity and permeability in animal models of CNS tumors. Through this, we will identify reasonable therapeutic dose and time-window to promote effective drug delivery while limiting toxicity. Altogether, these results support targeting WNT/beta-catenin signaling in brain endothelial cells to enhance drug delivery to CNS tumors.

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Identifying modulators of synthetic lethal interactions from CRISPR Screening

Synthetic lethality (SL) refers to a type of genetic interaction, where the inactivation of either gene alone is viable but the perturbation of both genes results in the loss of viability. This is highly clinically relevant because SL provides a natural framework to develop selective treatment that kills only tumor cells by inhibiting the synthetic lethal partners of the genes that are inactivated specifically in tumor (e.g. tumor suppressor). Due to the increasing volume of high throughput functional screens and molecular profiles of cancer cells and tumors, it recently became possible to infer SL interactions at a large scale via experimental and computational screens. However, most SL interactions are likely to be effective in a specific background, as manifested in the clinic by the 20-30% response rate of BRCA-deficient patients to treatment via a PARP inhibitor. Thus, one of the most important emerging challenges now for effectively advancing SL based treatments is to identify the context in which a given SL interactions are functional, a challenge proposed in multiple works, and is in dire need to be addressed. This will enable better patient stratification for SL-based targeted therapies, advancing both patients treatment and drug development. Here, we develop and apply a computational approach for analyzing a genome-wide CRISPR-Cas9 knockout viability screening profile of 558 cancer cell lines from 28 tissue types, to identify the genes (Modulators) whose activity determines the strength of a given SL interaction. Demonstrating the proof of concept, we used these modulators to predict cell line-specific double-knockout effect on viability, for 128 SL gene-pairs performed in two cell lines originating from different tissue types. We further showed support for these identifications at the clinical level in patients’ tumor from TCGA by showing that, for a modulator-SL pair interaction, modulators activity can be a strong determinant of the negative selection of a SL pair in patient’s tumors, a hallmark of SL-interaction strength defined by their extent of co-inactivation. Finally, we successfully predicted drug synergy for 21 drug pairs via these modulators in a novel drug combination screenings in 80 lung cancer cell lines. Further, to accelerate the reach to patients in clinics, we provided modulators for 11 currently clinically available SL-based targeted therapies to potentially identify responders.
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*Pancreatic Cancer Immunotherapy through beta-1,3-glucan AuNP Conjugates*

Pancreatic cancer (PC) is a deadly disease with an extremely low 5-year survival rate. The two main factors that contribute to such a poor prognosis are 1) the often late-stage diagnosis of advanced disease and 2) the lack of optimum treatment regimens for PC. A promising biomarker that has emerged is Mucin 4 (MUC4), which is overexpressed in PC but absent in normal pancreatic tissue. Our lab has been interested in immunological targeting of MUC4 by developing novel vaccine constructs and antibodies directed to the aberrantly glycosylated tandem repeat (TR) sequence of MUC4 that is displayed in multiple copies at the PC cell surface. These TRs contain several residues that are O-glycosylated with Tumor-Associated Carbohydrate Antigens (TACAs); abnormal glycans that are targets of the immune system during PC. We have prepared vaccines and antibodies to a 16-residue TR glycopeptide that are potential therapeutics for PC. We are currently investigating a third means of advancing immunotherapies for PC by developing a delivery system for our glycopeptide immunogens via targeting a specific C-type lectin expressed on antigen presenting cells (APC). We have prepared a nanoparticle construct comprised of a polysaccharide (beta-1,3-Glucan, B13GAuNP) that binds to Dectin-1, a C-type lectin expressed on APCs via a single step synthesis. Combined with conjugation of our glycopeptides, these B13GAuNP will engage Dectin-1 resulting in uptake and stimulation of APCs for MHCIImediated presentation to T-cells. As a proof of principle, we opted to screen preliminary immune activation through a model system using Ovalbumin peptide (OVA323-339) and its available cognate T cell hybridoma with APCs. A key component involved in the B13GAuNP processing is a lysosomal cathepsin B protease cleavage sequence (GLFG) that is stable in serum but is cleaved during immune processing yielding linker free peptide. Our studies have demonstrated that our engineered B13GAuNPs enhanced binding to Dectin-1 and that treatment of Dectin-1-expressing macrophages with an OVA323-339/GLFG peptide conjugate stimulates IL-2 production, suggesting both uptake and presentation of the OVA peptide in our system. The significance of this project is the induction of enhanced antigen uptake and immunity towards novel glycopeptides such as our TACA-MUC4 antigens through the increased adjuvating capabilities of our B13GAuNPs.

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*Targeting delta133p53 isoform with small molecule compounds to modulate cellular senescence*

Cellular senescence (CS) and senescence-associated secretory phenotype (SASP) contribute to aging and age-related diseases. The stress sensor p53 plays a pivotal role in the initiation and maintenance of CS. In humans, TP53 is expressed as 12 isoforms that contribute to the fine-tuning of p53 activity. Delta133p53 is generated from an alternative promoter located in intron 4. It is predominantly located in the nucleus, and is largely regulated at the protein level through chaperone-assisted selective autophagic degradation. Delta133p53 counteracts p53-mediated replicative CS and reduces the
secretion of SASP cytokines. Delta133p53 is expressed in most normal tissues, but its expression is deregulated in age-associated diseases such as cancer, neurodegenerative diseases and premature aging disorders. Delta133p53 is downregulated in exhausted CD8+ T-cells, nearly senescent fibroblasts from Hutchinson-Gilford progeria syndrome patients, and astrocytes from irradiated brains, Alzheimer’s disease and amyotrophic lateral sclerosis patients. Delta133p53 overexpression extends the replicative lifespan in normal cells, but does not cause immortalization or malignant transformation. Hence, delta133p53 appears to be a safe novel therapeutic target to regulate CS. The present study aims at identifying small molecule compounds that induce delta133p53 protein expression and inhibit CS. To identify activators of delta133p53, we developed a cell-based assay using an inducible translational reporter system containing the delta133p53 cDNA sequence cloned in frame with the GFP. The biological activity of the fusion protein was confirmed by evaluating its i) nuclear localization, ii) autophagic degradation, and iii) ability to delay replicative CS. In collaboration with NCATS, we recently screened a collection of ~8,350 compounds that includes all drugs that have been approved for use by the US FDA. Based on fluorescence analysis, we identified 95 compounds able to increase delta133p53 protein level without cytotoxicity. The selected compounds were then screened in a secondary assay to evaluate their efficacy at reducing SASP cytokines secretion. Thirteen compounds are currently being tested to confirm their effect on endogenous delta133p53 expression and CS. We have established a robust high throughput screening assay and identified candidate compounds that could potentially develop into novel therapeutic leads to treat major life-threatening diseases.

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*Understanding the health benefits of quercetin and other dietary flavonoids: selective inhibition of an inositol polyphosphate kinase.*

Quercetin and related flavonoids are bioactive, polyphenolic compounds often found in fruits and vegetables. Many studies have concluded that flavonoids reduce risks of cardiovascular disease, type 2 diabetes, and cancer. Flavonoids block ATP-binding by some protein kinases, but physiological relevance is debated, in part due to frequent experimentation with supraphysiological flavonoid concentrations. The novelty of our study is our identification of a new, biologically-relevant flavonoid target, which can be exploited for future drug development. Our work arose from recent studies describing that the ATP-binding sites of protein kinases are unexpectedly well-conserved in a member of the inositol phosphate (IP) kinase family: inositol hexakisphosphate kinase (IP6K). The latter generates an intracellular signal “IP7” that is involved in the etiology of cancer metastasis, as well as the metabolic imbalance that plagues obesity and diabetes. We performed IP6K activity assays with recombinant enzyme; our assays included 0.01% Brij to prevent the artifact of colloidal flavonoid aggregation. We discovered that quercetin and related flavonoids potently inhibit IP6K activity, by competing for ATP-binding. We next modeled cell exposure to dietary flavonoids in a physiological context: we incubated human colonic epithelial (HCT116) cells with each flavonoid at a concentration (2.5 µM) that is known to occur in the gastrointestinal tract, even without dietary supplementation. We used HPLC to assay cellular IP levels, which are reporters for individual IP kinase activities. We found inhibition of IP7 synthesis by IP6K
(>70%) occurred within 20 min of flavonoid addition to HCT116 cells; inhibition was sustained for up to 3 hours. No other cellular IP kinase was affected. Identification of IP6K as a flavonoid target may help explain the well-reported protective effects of flavonoids upon colorectal cancer. We also performed X-ray analysis of multiple kinase/flavonoid crystal structures, which provided detailed structure—activity data, including the identification of individual hydrophobic and polar ligand/protein interactions, the flexibility of key amino acid side chains, and the importance of active-site water molecules. Such highly-detailed information on the pharmacophore properties of the inhibitor-binding site is key to future rational design of IP6K inhibitors with improved potency and target selectivity, for use as research tools, and to assist future drug development.

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Pharmacology and Toxicology/Environmental Health

A nongenomic mechanism for â€œmetalloestrogenicâ€ effects of cadmium in human uterine leiomyoma cells through G protein-coupled estrogen receptor

Uterine leiomyomas (fibroids) are highly prevalent gynecologic neoplasms that are estrogen-responsive, which can make them the target of environmental xenoestrogens. Cadmium (Cd) is a toxic heavy metal that is ubiquitous in the environment. Major sources of exposure to Cd result from occupational inhalation, seafood and rice, drinking water, as well as cigarette smoking. Cd is reported to be a â€œmetalloestrogenâ€ that possesses estrogenic properties and can activate estrogen receptors. Epidemiologic studies have shown a positive correlation between blood Cd concentrations and fibroids in women. Our previous study showed that Cd can stimulate proliferation of human uterine leiomyoma (ht-UtLM) cells, not by classical estrogen receptor (ER) binding, but through EGFR and MAPK. Whether nongenomic ER pathways are involved in Cd-induced proliferation is unknown. G protein-coupled estrogen receptor (GPER) and ERalpha36 have been identified as novel membrane-associated ERs mediating rapid nongenomic pathways. The present study aimed to investigate the effects of Cd on ht-UtLM cells and whether nongenomic ERs (GPER or ERalpha36) signaling pathways are involved in Cdâ€™s effects. We evaluated both human tissues and cells in vitro. In human tissues, we found that GPER, ERalpha36, and phospho-EGFR, were highly expressed in fibroids compared to patient-matched myometrial tissues. In ht-UtLM cells, low doses of Cd (0.1 µM and 10 µM) increased cell proliferation, and this effect could be inhibited by GPER-specific antagonist (G15) pretreatment, or silencing (si) GPER, but not by siERalpha36. Cd-induced cell proliferation was through an activated MAPK pathway, which was dependent on GPER/EGFR transactivation. Cd increased phospho-Src through GPER and then transactivated EGFR by both indirect and direct ways. We found that phospho-Src transactivated EGFR indirectly by enhancing the activity of matrix metalloproteinase-2 (MMP2) and MMP9, which resulted in elevated expression of HB-EGF available to bind and activate EGFR. Also, we confirmed by coimmunoprecipitation that phospho-Src could interact directly to phosphorylate EGFR initiating downstream MAPK signaling. Overall, Cd-induced proliferation of human fibroid cells was through a nongenomic GPER/p-src/EGFR/MAPK signaling pathway that did not directly involve ERalpha36. This suggests that Cd may be a risk factor for uterine fibroids and its effects are mediated through crosstalk between hormone and growth factor pathways.
Bevin Blake  
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Pharmacology and Toxicology/Environmental Health

*Gestational exposure to the perfluorooctanoic acid replacement, GenX, induces adverse responses in maternal weight gain and placental health in CD-1 mice*

Perfluoroalkyl substances (PFAS) comprise a diverse class of industrially useful chemicals that are environmentally ubiquitous and detectable in the blood of nearly all US citizens. PFAS chemicals make products resistant to stains, grease, oil, and water and have become persistent drinking water contaminants across the globe. The PFAS compound perfluorooctanoic acid (PFOA) is associated with adverse maternal-fetal health outcomes in humans including increased gestational weight gain (GWG), preeclampsia, and fetal growth restriction (FGR), but the mechanism through which it induces these adverse pregnancy outcomes is not known. PFOA has been phased out of production in the US due to its adverse effects on human health and replaced with new compounds, including GenX. GenX was designed as a safer alternative to PFOA but the animal toxicity data are limited and community-based concerns have surfaced in response to widespread GenX contamination of major drinking water sources such as the Cape Fear River in North Carolina. To determine a potential mechanism of action of FGR for PFOA and evaluate whether its replacement, GenX, has similar effects on FGR and GWG, pregnant CD-1 mice were exposed daily via oral gavage to PFOA (1 or 5 mg/kg) or GenX (2 or 10 mg/kg) from embryonic day (E) 1.5 to E17.5. In analyses adjusting for litter size and gestational day, GWG was significantly increased in exposed mice relative to controls, with effects most prominent in mice exposed to 10 mg/kg GenX (10.7% increase), followed by 5 mg/kg PFOA (8.9% increase) and 2 mg/kg GenX (5.4% increase). Placentae exhibited significantly increased weight relative to controls (a 21 mg and 15.5 mg average increase in placental weight for 5 mg/kg PFOA and 10 mg/kg GenX exposed mice, respectively) as well as reduced fetal:placental weight ratios. Histopathological analyses of the placenta showed treatment-related placental lesions, including labyrinth atrophy and labyrinth congestion. Placental lesions were most prevalent in mice exposed to 10 mg/kg GenX (87% evaluated placentae affected), 5 mg/kg PFOA (60%), 2 mg/kg GenX (54%), and 1 mg/kg PFOA (6%). Preliminary placenta gene expression data suggest that GenX may induce adverse effects through a different biological pathway than PFOA. These findings suggest GenX has the potential to adversely affect maternal-fetal health in the human population, possibly via placental or gestational weight-related mechanisms.

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Physiology

*title/abstract not published at request of author*
A large conductance ion channel on the malaria parasite digestive vacuole is a new and unexploited antimalarial target

Malaria parasites are important pathogens with disease resulting from consumption of host erythrocytes during a replicative intracellular cycle. Large-scale consumption of hemoglobin and other macromolecules occurs in the parasite’s digestive vacuole (DV), a specialized organelle that resembles mammalian lysosomes. The DV is the target of highly active antimalarial drugs such as chloroquine and mefloquine in the virulent human pathogen, Plasmodium falciparum; acquired resistance to these drugs has compromised global health and is thought to result from drug extrusion at the DV membrane. Various transmembrane transporters have been proposed as either drug resistance pathways or routes for amino acids released by DV hemoglobin digestion; these transporters and their candidate genes remain poorly characterized despite decades of study because the DV’s small size (1-2 µm) and intracellular localization have prevented direct biochemical examination. We now provide the first direct study of solute transport at the DV membrane through patch-clamp of intact organelles, enriched with a rapid (10 min) protocol that preserves DV integrity and physiology. High resistance seals (> 10 Gohms) were obtained after optimizing pipette glass composition and geometry. A large-conductance channel (450 pS in 320 mosm salt solutions) was identified as the primary channel, but additional smaller channels have also been detected. The primary channel exhibited a bell-shaped voltage-dependence with near 100% open probability at the DV resting membrane potential as well as unusual gating that suggests a two-pore configuration; this atypical behavior appears to be regulated by parasite cytosolic Na+ concentration. Channel activity is not affected by chloroquine, mefloquine, or artemisinin, implicating an unexploited antimalarial drug target. Our surveys have identified weak inhibitors, providing scaffolds and a path toward development of new antimalarials at interfere with DV membrane transport. We are making progress on the longer-range goal of identifying the channel’s molecular basis with DiCre-based conditional knockout of candidate genes that encode DV integral membrane proteins. Identification and molecular characterization of transporters on the DV membrane will provide foundational insights into vacuolar biology, clarify the resistance mechanisms for several antimalarial drugs, and guide development of new therapies for malaria.

The Impact of Dietary Iron Restriction on Intracellular Hemoglobin Concentration and Sickling Kinetics

Sickle cell disease (SCD) is caused by polymerization of deoxy-hemoglobin-S (HbS) within the red blood cell (RBC). The time from deoxygenation to polymerization of HbS (i.e., sickling) is inversely related to
the intra-erythrocytic concentration of HbS. We hypothesized that iron-deficient erythropoiesis would decrease the intracellular HbS concentration in RBCs, resulting in delayed sickling and reduced disease severity. To test this hypothesis, we studied Townes transgenic mice expressing beta globin-S (SCD-mice). To induce iron deficiency, we introduced an iron-restricted (0-3 ppm iron) or control diet (48 ppm iron) at weaning. After 2 months, we measured RBC indices and ex vivo RBC sickling kinetics at 3% oxygen. SCD mice on the iron-restricted diet had lower mean cell hemoglobin (MCH 8.4 pg vs 7.5 pg, p&lt;0.001) and mean corpuscular hemoglobin concentration (MCHC 18.6 g/dL vs 17.0 g/dL, p&lt;0.01). Surprisingly, the ex vivo sickling kinetic assay showed faster time to 50% sickled in the iron restricted group (t_50 97 vs 137 min., p&lt;0.0001) - the opposite of what we hypothesized. To confirm and extend this finding, next we studied separate cohorts of 10 male and 10 female SCD mice after 2, 4, or 6 months of iron restricted or control diets. At 6 months, iron restricted male mice had lower Hb (5.5 vs 6.2 g/dL p&lt;0.05), lower MCH (8.7 vs 9.1 pg, p&lt;0.05), lower MCHC (26.6 vs 27.8 g/dL, p&lt;0.05), and lower reticulocyte hemoglobin (RET-He 13.1 vs 13.5 pg, p&lt;0.01). Iron restriction had no effect on these indices in female SCD mice. Analysis of the hemoglobin-oxygen dissociation curve after 6 months' iron restriction revealed an increase in oxygen affinity among iron-restricted male and female SCD mice (P_50: 25.8 vs 27.4 mm Hg, p&lt;0.01). Dietary iron restriction lowered the intracellular Hb concentration in male Townes-SCD mice, as evidenced by reduced MCH, MHC, and RET-He; furthermore, the Hb oxygen affinity was increased. Each of these changes is predicted to delay the polymerization of HbS and reduce sickling of RBCs. We have performed the quantitative sickling assays at 2, 4, and 6 months; image analysis is currently underway to quantify the impact of the dietary iron restriction-induced changes in Hb concentration and oxygen affinity on sickling kinetics. These results will guide and inform human clinical investigations into the optimal management of iron availability among people living with sickle cell disease.

George Amanakis
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Physiology

Cysteine 202 of Cyclophilin D is a site of multiple post-translational modifications and plays a role in cardioprotection

Cardiomyocytes die when they are deprived of blood supply (ischemia). Upon restoration of blood supply (reperfusion) cell death is paradoxically increased for the first minutes due to oxidative stress and cytoplasmic calcium overload. The overall phenomenon is known as ischemia/reperfusion (I/R) injury and its main effector is the opening of the permeability transition pore (PTP), a complex of proteins localized in the inner mitochondrial membrane. Cyclophilin-D (CypD) is a well-known regulator of PTP; it binds to it and triggers its opening which leads to ATP depletion and cell death. However, the binding of CypD to the PTP is poorly understood. We previously reported that short non-lethal cycles of I/R lead to the attachment of nitric oxide to cysteine 202 (C202) of CypD, protecting it from oxidation, and reducing cardiac injury. Thus, we now hypothesized that a post-translational modification of C202, specifically oxidation, targets CypD to the PTP. To study the importance of C202, we developed a knock-in mouse model using CRISPR where CypD-C202 is mutated to a serine (C202S) which cannot undergo any of the post-translational modifications of cysteines. In an isolated perfused heart model, infarct size is reduced
by 51% in CypD-C202S hearts compared to WT (n=4, p=0.003). Isolated cardiac mitochondria from CypD-
C202S mice exhibit delayed PTP opening under calcium overload (n=5, p=0.011) and also display less
binding of CypD to the proposed PTP component ATP-synthase by 48% compared to WT under oxidative
stress (p=0.012, n=4). Another post-translational modification cysteines can undergo is the attachment
of a fatty acid (S-acylation). To assess S-acylation of CypD-C202 we employed a resin-assisted capture.
Briefly, free cysteines are blocked, then S-acylated cysteines are freed and captured by a resin. CypD-
C202 is abundantly S-acylated, but CypD-C202S hearts exhibit less S-acylation than WT by 63% (n=6,
p<0.001). We next investigated the importance of S-acylation of CypD-C202 in cell death in a mouse
embryonic fibroblast (MEFs) model. CypD-C202S MEFs exhibit 20% less oxidative cell death than WT
(n=5, p=0.003). Inhibition of S-acylation with 2-bromopalmitate attenuates oxidative cell death in the
WT by 20% (n=5, p=0.018), while CypD-C202S MEFs are unaffected. Thus, S-acylation and oxidation
of CypD-C202 possibly target CypD to the PTP, rendering them potent regulators of cardiac I/R injury and
potential targets for cardioprotective strategies.

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Physiology

Signal transduction and nutrient balance: regulation by inositol pyrophosphates of the cellular transport
of inorganic phosphate

Inorganic phosphate (Pi) homeostasis - balancing cell uptake and efflux of this macronutrient - is
critical for life: Pi is an essential structural element of bones and teeth, and in its organic form it is a
component of genomic material, an energy currency, and fundamental to cell signaling. Yet, excess
intracellular Pi is acutely pathological, disrupting mineral metabolism and promoting calcification of
cardiovascular and soft tissues. Critical to mammalian Pi homeostasis is the atypical GPCR, XPR1, a
transmembrane carrier for cellular Pi efflux; here, we provide the first description of XPR1 regulation.
We performed [32P] Pi efflux assays from HCT116 and HEK cells, and found that CRISPR knockout of
XPR1 reduced Pi efflux by 90%, revealing its quantitative significance. The cytosolic N-terminal SPX
domain of XPR1 has electropositive zones - candidate binding-sites for electronegative cell-signals, the
most polar in Nature being an inositol pyrophosphate, â€˜IP8â€™: a six-carbon ring with four
monophosphates and two diphosphates. Emerging literature shows IP8 acts at the interface of cell-
signaling and metabolic balance. IP8 binding to recombinant SPX domain was validated by isothermal
titration calorimetry (Kd=200 nM); binding reduces retention upon gel filtration, implying IP8 alters XPR1
conformation. To study XPR1 regulation in intact HCT116 and HEK cells, we performed CRISPR-knockout
of the kinases (PPIP5Ks) that synthesize IP8; [32P] Pi efflux fell 70-90%. The potential pathological
impact is 2.5-fold higher expression of alkaline phosphatase, a calcification marker. We next stably
expressed wild-type and kinase-dead PPIP5K constructs in PPIP5K-/- cells. Pi efflux was rescued by
restoring IP8 synthesis with wild-type PPIP5K; the kinase-dead mutant has no effect. Next, we
developed a novel strategy to deliver into cells liposomes containing IP8. This IP8 delivery completely
restored Pi efflux from PPIP5K-/-cells, in a dose-dependent manner. Significantly, Pi efflux was not
restored by delivery of IP7, which has just one diphosphate less than IP8; this control experiment adds
specificity to this mode of XPR1 regulation. Our data provide the first example of a regulatory ligand for
cellular Pi homeostasis. We are now characterizing atomic level interactions of IP8 with XPR1 by X-ray crystallography, and screening dbSNP-cataloged genetic variants of human PPIP5Ks for possible impacts on IP8 synthase activity and perturbed cellular Pi homeostasis.

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Structural Basis of Potent HIV-1 Entry Inhibitors That Block Conformational Changes Required for Membrane Fusion.

The entry of HIV-1 into target cells is a critical event in the viral life cycle and an attractive target for drug development. The HIV-1 envelope protein (Env), comprised of three gp120 subunits and three gp41 subunits, binds to cell-surface receptors before triggering the fusion of viral and host cell membranes. Entry inhibitors targeting the gp41 (Enfuvirtide) or co-receptor CCR5 (Maraviroc) have been approved by FDA for HIV-1 treatment. To date, no entry inhibitors targeting the gp120 have been FDA-approved although a promising small-molecule lead, fostemsavir (the prodrug of active compound BMS-626529), is currently in phase III clinical trials. We previously reported the crystal structure of BMS-626529 in complex with HIV-1 Env trimer, revealing its molecular basis of entry inhibition. This drug binds to a conserved pocket beneath the β20-β21 hairpin between the inner and outer domains of gp120, suggesting that drug binding blocks the conformational changes required for viral fusion to occur. This hypothesis was supported by the inhibition of antibody binding to a CD4-binding induced intermediate state when BMS-626529 is present. Here, we screened a derivative library of BMS-626529 to identify
potent next-generation entry inhibitors. A new compound, BMS-818251, was shown to have >10-fold higher potency than BMS-626529 on the neutralization of a cross-clade HIV-1 panel. To provide a high-resolution structural basis of the potent inhibition, we adopted a crystal engineering approach to improve diffraction limits. Removal of flexible regions in the crystallization chaperone 35O22 scFv (single-chain Fv), along with the introduction of stabilizing mutations to 35O22 scFv and another crystallization chaperone 3H109L Fab, improved resolution from ~3.5 Å to ~2.5 Å. The structure of BMS-818251, determined in the engineered crystal, revealed a functional group extending from the binding pocket to form additional interactions with Env residues 429 and 432, enabling its high neutralization potency against the cross-clade virus panel. Our data support further development of BMS-818251, which represents a novel class of HIV-1 drugs targeting gp120, as a next-generation entry inhibitor. Ongoing cell culture passage studies will determine if the higher potency of BMS-818251 can translate into higher genetic barrier for drug resistance to emerge. Our work prompts structure-based drug design for further optimization of a promising class of entry inhibitors.

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Unraveling the mechanism of substrate processing by the AAA-ATPase Rix7

Rix7 is an essential AAA-ATPase (ATPase associated with various cellular activities) required for the formation of the large ribosomal subunit. Rix7 has been proposed to utilize the power of ATP hydrolysis to drive the removal of assembly factors from pre-60S ribosomal particles, but the mechanism of release is unknown. Rix7’s mammalian homolog, NVL2 has been linked to cancer and mental illness disorders, highlighting the need to understand the molecular mechanisms of this essential machine. Our goal is to uncover the molecular role of the AAA-ATPase Rix7 in ribosome biogenesis. Rix7 is a type-II AAA-ATPase containing a unique N-terminus followed by tandem AAA domains known as the D1 and D2 domains respectively. We first performed yeast complementation assays to distinguish the role of the D1 and D2 domains of Rix7, which revealed that, in vivo, the ATPase activity in the D2 AAA domain is strictly required for cell cycle progression. Then we further asked the question as to if ATPase activity of Rix7 in the D1 and D2 domains affects ribosome synthesis. We recorded polysomal profiles from cytosolic extracts prepared from tetO7-RIX7 yeast strains expressing Rix7 WT and variants. We observed an obvious defect in mature 60S formation upon expression of a Rix7 mutant that is deficient in ATP hydrolysis in the D2 AAA domain. Moreover, in mammalian co-IP analysis together with sucrose gradient centrifugation indicate that Nsa1, the substrate of Rix7, which revealed that, in vivo, the ATPase activity in the D2 AAA domain is strictly required for cell cycle progression. Then we further asked the question as to if ATPase activity of Rix7 in the D1 and D2 domains affects ribosome synthesis. We recorded polysomal profiles from cytosolic extracts prepared from tetO7-RIX7 yeast strains expressing Rix7 WT and variants. We observed an obvious defect in mature 60S formation upon expression of a Rix7 mutant that is deficient in ATP hydrolysis in the D2 AAA domain. Moreover, in mammalian co-IP analysis together with sucrose gradient centrifugation indicate that Nsa1, the substrate of Rix7, accumulates with translating ribosomes upon expression of an ATP-hydrolysis deficient mutant. Also, Nsa1 has a greater binding affinity to Rix7 ATP-deficient mutant. Thus these data suggests ATP hydrolysis drives release of Nsa1 from both Rix7 and pre-60S ribosomal particles. Recently, we solved the first structure of Rix7 using cryo-Electron Microscopy to 4.5 Å resolution which revealed the unexpected structure of a substrate-bound intermediate in complex with ATP. The substrate stabilizes Rix7 as an asymmetric stacked homohexameric ring. Five of the six Rix7 protomers grip the substrate through conserved pore loops that line the central channel formed by the two ATPase rings. This arrangement suggests a processive hand-over-hand mechanism of substrate unwinding. Taken together, our results suggest that Rix7
functions as a molecular unfoldase using the energy from ATP hydrolysis of D2 AAA domain to pull on substrates and drive the release of assembly factors from the immature 60S particles.

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*Neural correlates of conditioned inhibition in children with and without anxiety disorders*

Cognitive-behavioral therapy (CBT) is the first-line treatment for pediatric anxiety disorders but does not work in 40 percent of children. An important aspect of CBT is to expose children to their feared scenarios and teach them how to regulate their fear. Exposure is based on principles of fear learning and extinction, especially inhibitory learning. In fear learning studies, a threat cue is paired with an aversive stimulus (electric shock or loud noise), whereas a safety cue is never paired with the aversive stimulus. Animal studies have shown that presenting threat and safety cues simultaneously (conditioned inhibition) may be a promising approach to augment fear reduction. Moreover, conditioned inhibition could provide information about how safety cues from the environment are incorporated. There is a critical need to extend these data to youth, because most anxiety disorders develop during childhood. Studying neural correlates of conditioned inhibition might advance understanding of mechanisms underlying CBT, and eventually improve treatment. The goal of this study was to investigate neural correlates of conditioned inhibition in children with and without anxiety disorders. 16 children with anxiety disorders and 17 healthy children performed our novel conditioned inhibition task in the MRI scanner. During the fear learning phase, children were shown threat cues paired with an aversive noise and safety cues unpaired with the noise. During the testing phase, threat and safety cues were presented simultaneously (conditioned inhibition), or the threat cue was combined with a novel cue (novel compound). Children with anxiety disorders showed increased skin conductance response across all stimuli, compared to healthy children, F(1, 28)=7.47, p=0.01. This establishes the relevance of our imaging context for evoking disorder-relevant differences in physiology. fMRI analyses indicated that children with anxiety disorders show less activity in the dorsomedial prefrontal cortex than healthy children during conditioned inhibition compared to the novel compound, Z>2.3, p=0.01. This suggests a failure to effectively represent relevant inhibitory cues from the environment; healthy children showed such evidence, manifesting as greater responding to safety than novel cues. The results of the present study will provide insights into altered conditioned inhibition in pediatric anxiety disorders, which could eventually inform exposure-based treatment.

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*Glucagon-like peptide-1 system is modulated by acute and chronic exposure to alcohol: Findings from*
**human laboratory experiments and a brain postmortem study**

The gut communicates with the brain through neural, immune, and endocrine pathways, such as glucagon-like peptide-1 (GLP-1). GLP-1 is a 30-amino acid peptide primarily produced by endocrine cells in the intestines. As an incretin, GLP-1 regulates glucose homeostasis, appetite, and food intake via central (e.g., hypothalamus) and peripheral (e.g., pancreas) pathways. GLP-1 also acts as a neuropeptide, and both the peptide and its receptor (GLP-1R) are expressed in the brain. Previous data indicate that GLP-1 signaling may play a role in biobehavioral mechanisms underlying reward processing related to not only food but also alcohol and drugs of abuse. Indeed, rodent studies suggest that GLP-1R may represent a novel therapeutic target for alcohol use disorder (AUD). Accordingly, it is important to understand how excessive alcohol drinking may affect the endogenous GLP-1 system in humans. In an effort to answer this question, we first examined the effect of alcohol administration on peripheral blood GLP-1 concentrations in heavy-drinking individuals. Four separate alcohol administration sessions were conducted in a human laboratory setting: oral self-administration (variable dose), oral fixed dose, intravenous self-administration (variable dose), and intravenous fixed dose. Repeated blood samples were obtained during each session and GLP-1 concentrations were measured via a bead-based multiplex enzyme-linked immunosorbent assay (ELISA). In all four experiments, acute administration of alcohol consistently resulted in significant reduction of peripheral GLP-1 concentrations (p’s < 0.001).

Next, we looked at the GLP-1R gene expression in postmortem brain tissue from patients with AUD and healthy controls. GLP-1R mRNA was extracted from five brain regions (i.e., prefrontal cortex, ventral tegmental area, nucleus accumbens, amygdala, and hippocampus), and real-time quantitative polymerase chain reaction (PCR) with TaqMan gene expression assay was run. Results showed that fold change in GLP-1R mRNA in the hippocampus was significantly higher in patients with AUD, compared to healthy controls (p = 0.007). Collectively, these data indicate that exposure to alcohol modulates the GLP-1 system, both in the periphery and in the brain. Future studies should replicate the present findings and investigate whether targeting the GLP-1 system may represent an effective pharmacological approach to treat AUD.

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Psychiatry

*Neuron-derived extracellular vesicles reveal neuroinflammatory mechanisms of action of immunotherapy in Bipolar disorder*

Bipolar disorder (BD), a mood disorder of unknown etiology characterized by dramatic shifts in mood and activity levels, has been associated with deregulated neuronal and systemic inflammation and increased plasma levels of the pro-inflammatory cytokine tumor necrosis factor (TNF). This observation motivated a 12-week randomized double-blind, placebo-controlled, clinical trial of the human TNF-specific neutralizing antibody infliximab for BD. The study was conducted at the University of Toronto and assessed the safety, tolerability and efficacy of infliximab in individuals with bipolar I/II depression. Infliximab significantly reduced the severity of depressive symptoms, as measured by the Montgomery-Asberg Depression Rating Scale (MADRS), in a sub-group of patients with history of physical abuse, which has been associated with early onset and a more difficult course of BD. Our
laboratory has pioneered a technique for isolating enriched populations of neuronal-derived extracellular vesicles (NDEVs) from blood and have used them as a surrogate to show target engagement in neurons in response to experimental therapeutics. Leveraging samples from the clinical trial, we investigated changes in NDEV biomarkers over time to uncover the mechanism of action underlying the anti-depressive effects of infliximab in BD. We blindly analyzed 201 repeated plasma samples (baseline, 2, 6, and 12 weeks) from 55 participants (44 +/- 11 years old) randomized to placebo or infliximab. We isolated total EVs followed by enrichment for NDEVs and measured the protein levels of TNF receptors 1 (TNFR1) and 2 (TNFR2) and effectors of the nuclear factor kappa B (NFkB) pathway, which regulates inflammation under TNF stimulation. At baseline, we found significant positive associations between biomarkers and scales of physical abuse. NFkB and TNFR1 decreased over time in infliximab-treated compared to placebo-assigned participants with history of physical abuse (p = 0.018 and 0.02, respectively). Decreases in TNFR1 levels with infliximab were associated with decreases in MADRS scores (p = 0.005). In summary, infliximab ameliorated depression in BD patients with physical abuse in association with modulation of the TNFR1/NFkB signaling cascade, thus providing mechanistic proof of concept for a potential new therapy for depression in BD. This is the first study demonstrating that NDEV biomarkers may be used to assess target engagement in clinical trials in psychiatry.

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*Magnetic Resonance Spectroscopy Reveals Age-Related Elevations in Brain Glucose*

In the global context of an aging population, it is crucial to understand the implications of brain metabolism abnormalities known to occur with aging and Alzheimer’s disease (AD). Emerging evidence points towards brain glucose hypometabolism as a common pathophysiologic mechanism underlying aging, diabetes, and AD. In light of this, interventions aiming to normalize brain glucose metabolism such as intermittent fasting are being investigated as treatments. We sought to answer the question of how the concentration of glucose in the brain changes over the course of normal human aging. To provide in vivo measures directly reflecting brain glucose metabolism, we used a cutting-edge magnetic resonance spectroscopy (MRS) technique called J-Modulated Point-Resolved Spectroscopy (J-PRESS), which allows us to accurately measure metabolites elusive to earlier methods due to signal overlaps, such as glucose, glutamine, and glutamate. For our region of interest, we selected the precuneus due to its high resting metabolic activity and prior implication in aging, AD and diabetes. We then used J-PRESS MRS to measure the concentration of glucose and other salient metabolites in the precuneus of 54 cognitively normal human participants ranging from 23 to 84 years of age (mean 55 +/- 18 yrs.). We found that the precuneal glucose concentration (normalized to creatine) rises monotonically with increasing age and that these reliable stepwise glucose elevations were accompanied by similar age-related changes in other key brain metabolites. Specifically, precuneal levels of the neurotransmitter glutamate were lower in older participants while the neurotransmitter precursor glutamine was higher, a result consistent with current theories regarding age-related imbalance in the glutamine-glutamate cycle. Likewise, the membrane components myo-inositol and phosphocholine were also lower in older participants, reflecting age-related atrophy and...
neurodegeneration. This is the first in-vivo study to quantify this expanded set of brain metabolites in normal aging, and convincingly demonstrate elevations in brain glucose over the course of normal aging. In addition to enhancing our understanding of the changes that occur in brain metabolism with aging, these results lead to the possibility that non-invasive MRS-measured glucose can serve as a diagnostic biomarker for brain aging and AD, and as therapeutic response biomarkers for interventions targeting brain glucose metabolism.

Qinglei Meng  
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A Novel Transcranial Magnetic Stimulator for Focal Stimulation of Rodent Brain

Transcranial magnetic stimulation (TMS) is emerging as a therapeutic tool for treating many neuropsychiatric disorders, but its underlying mechanisms remain poorly understood. Preclinical rodent studies are critically important in this regard. TMS systems, in their most basic form, are made of a treatment coil and a driving circuit that sends high current pulses to the coil to generate a Tesla-level magnetic field. The field penetrates brain tissue and induces eddy current when the coil is located over the skull. This stimulates the neurons causing the release of neurotransmitters and clinical effects. However, commercial rodent TMS coils lack the spatial focality to mimic human TMS treatment conditions - a large volume of brain tissue is stimulated even with the best available rodent TMS coils which prevents spatially-relevant mechanistic understanding of preclinical models and its translation to human studies. Conventional TMS coil design is limited by coil-to-brain size ratio; very high electric current is needed if a human TMS coil is scaled to rodent brain size, causing excessive heating and demanding power supply requirements. We have developed an innovative concept to significantly improve the focality for rodent brain stimulation. In our coil design, a stack of long silicon steel sheets is used as a magnetic core due to high permeability and high magnetic saturation. Individual sheets are insulated to minimize eddy current generated in the core during stimulation. Litz wires are used for coil wrapping, eliminating the “skin effect” of the current in the coil. The coil is wrapped with a tilted angle and induces a tight focal spot in its asymmetric electric field distribution. The design has been tested in both computer simulations and pilot experiments. We have also developed an impedance-matched TMS circuit to drive this coil, providing high current pulses. In in vivo experiments on mice, motor-evoked potential was recorded while TMS targeted the hindlimb motor cortex. Here, TMS induced limb twitches only on the contralateral hindlimb, but not any other body part, suggesting a much better focality than commercial rodent coils. This is the first TMS system that can induce unilateral movements on rodents, providing important access to spatially-relevant mechanistic understanding of preclinical models and great possibilities for translation to human studies.

Zijian Zhou  
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Orthogonal computations on T1-T2 dual-modal MRI improve sensitivity and accuracy in cancer diagnosis

Precision medicine relies heavily on applicable diagnosis that can provide accurate pathological characteristics for individuals. Magnetic resonance imaging (MRI) is one of the most widely used clinical tools due to the non-invasive, non-ionized, and radiation-free features. MRI diagnosis is better assisted by contrast agents that can augment the signal contrast in the imaging appearance. Generally, there are two types of contrast agents, T1 (positive) or T2 (negative), which can generate bright or dark contrast for targets in MRI. However, this approach is still limited by the inherently low sensitivity on the recorded signal changes using single-modal T1 or T2 contrast agents, which is further hampered by the occurrence of false-positive signals in MRI. Therefore, a reliable method that can augment the sensitivity and accuracy in MRI diagnosis is highly desirable. Here, we explored a new paradigm of MRI diagnosis that employs T1-T2 dual-modal MRI contrast agents for post-imaging orthogonal computations on both T1 and T2 relaxation time changes. First, we studied a laboratory-made compound as a clinically translatable T1-T2 dual-modal MRI contrast agents. This compound, denoted as Mn-NEB + BSA, is made of 1,4,7-triazacyclononane-N,N',N''-triacetic acid (NOTA) conjugated truncated Evans blue (NEB) structure which chelates with manganese ions and binds with bovine serum albumin (BSA). We further explored an orthogonal computation method on T1 and T2 relaxation maps to combine the T1 and T2 relaxation changes. This method was then examined in both subcutaneous and orthotopic brain tumor mouse models, yielding greatly enhanced (up to 3-fold) signal-to-noise ratios (SNR) and contrast-to-noise ratios (CNR) compared with traditional methods based on signal-intensity and/or single-factor relaxation changes. Furthermore, we showed that our method has great potential to eliminate suspicious artifacts and false-positive signals in mouse brain imaging, demonstrating great feasibility in precision diagnosis. This study presents that orthogonal computations integrating both T1 and T2 relaxation changes by T1-T2 dual-modal MRI contrast agents can greatly improve the ability of lesion detection in MRI, which may find usefulness in clinical diagnosis especially for precision diagnosis of clinically occult diseases using MRI.

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First-In-Human Evaluation of 11C-PS13 for Imaging Cyclooxygenase-1 in Brain and Peripheral Organs

Objectives: Cyclooxygenase (COX)-1 is constitutively expressed in most organs and produces prostanoids that mediate physiological functions. However, recent studies suggest that, because of its proinflammatory properties, COX-1 may also be a potential therapeutic target in brain disorders such as Alzheimer’s disease. Our laboratory recently developed 11C-PS13 as a novel PET radioligand for COX-1. Human whole blood assays indicated that PS13 was highly potent and selective for COX-1 versus COX-2. This human study used 11C-PS13 to evaluate: 1) whole body in vivo selectivity of COX-1, and 2) test-retest reproducibility in brain. Methods: Approximately 740 MBq of 11C-PS13 was injected intravenously into 28 healthy human subjects for dynamic positron emission tomography (PET). Seventeen whole-body scans and 11 brain test-retest scans were obtained. To measure radioligand
selectivity in vivo, whole-body scans were also obtained after administration of blocking drugs that preferentially target COX-1 or COX-2. Concurrent venous samples for whole-body scans and arterial samples for brain scans were obtained to measure concentrations of parent radioligand and radiometabolites. Results: Under baseline conditions, substantial uptake of radioactivity from 11C-PS13 was observed in most major organs, including the spleen, gastrointestinal tract, kidneys, and brain. Uptake of radioactivity in these organs was blocked by preferential COX-1 inhibitors (aspirin or ketoprofen) but not by a preferential COX-2 inhibitor (celecoxib). In the brain, 11C-PS13 showed high uptake in the bilateral hippocampus and occipital cortices as well as bilateral pericentral cortices. When total distribution volume was calculated in the brain, the overall test-retest variability was within 15%, and the intraclass correlation coefficient was about 0.9. Conclusions: Our results suggest that COX-1 is constitutively expressed in major organs such as the spleen, gastrointestinal tract, kidneys, and brain; in the latter, uptake was particularly high in the hippocampus and occipital and pericentral cortices. The in vivo selectivity of 11C-PS13 was well demonstrated by pharmacological blockade in whole-body scans, and the test-retest reproducibility was also validated in brain scans. 11C-PS13 is the first radioligand for COX-1 that acts directly at this target. It is a potential probe for measuring neuroinflammation in brain disorders as well as target engagement by therapeutic drugs.

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Reducing motion sensitivity in high-resolution T2*-weighted MRI by navigator-based motion and nonlinear magnetic field correction

Magnetic resonance imaging (MRI) is the most sensitive tool in detecting lesions in the central nervous system in multiple sclerosis (MS) patients. At high field strength of 7 T, T2* relaxation time of brain tissue revealed by high-resolution (submillimeter) images is a sensitive biomarker for detecting cortical lesion in MS, which has been largely overlooked previously due to its small size and subtle contrast. However, the clinical potential of high-resolution T2*-weighted MRI is seriously impeded by inevitable patient motion during its minutes-long scan. Here, we proposed a practical solution to correct motion artifact in T2*-weighted MRI from two sources: (1) spatial misalignment directly due to motion and (2) motion-induced changes in the magnetic field (B0). The second source is a significant component at 7 T as confirmed in our previous study and is not corrected for in existing MRI motion correction methods. In our approach, we acquired additional MRI signals in parallel with the main acquisition to construct so-called navigators which were used to track head motion and measure B0 changes. A fast image reconstruction algorithm was developed to facilitate the correction utilizing the measured B0 changes. In 5 healthy subjects and over 25 different head poses, the accuracy of the navigator in estimating head motion was evaluated to be better than 0.2 degree in rotation and 0.1 mm in translation, exceeding the need of the resolution around 0.5 mm in current high-resolution MRI. It also showed an excellent accuracy in measuring B0 changes with less than 2 Hz variance throughout the brain at 7 T. The navigator could achieve a temporal resolution of 0.54 s which is sufficient to track head motion. The navigator and reconstruction algorithm were evaluated in correcting motion-corrupted high-resolution T2*-weighted MRI with voxel size of 0.5×0.5×1.5 mm3 on healthy human subjects at 7 T. Excellent image
quality was demonstrated by correcting for both motion and B0 changes using the proposed method. Acquiring the navigator signals did not impose substantial burden on the overall scan time or the time for acquiring the high-resolution MRI signals. The method is readily applicable to clinical scanners without the need of additional hardware. Therefore, upon further development, it can have a significant clinical impact on MS and other neurological disorders such as Parkinson’s and Alzheimer’s diseases which cause T2* changes.

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RNA Biology

_H/ACA snoRNAs are determinants of stem cell homeostasis_

RNA modifications, such as pseudouridine, regulate RNA stability, function, and translation. H/ACA small nucleolar RNAs (snoRNAs) are noncoding RNAs that guide RNA pseudouridylation as part of a small nucleolar ribonucleoprotein complex (snoRNP) by base-pairing with target RNAs. In mammals, >70 distinct H/ACA snoRNAs, allow for the formation of >70 H/ACA snoRNPs with unique target specificity. The H/ACA snoRNP is essential for development and health, as mutations within the core protein components or dysregulation of H/ACA snoRNAs is observed in a variety of diseases including developmental and hematological disorders and cancer. However, the means by which the H/ACA snoRNP contributes to normal development remain largely unknown. The goal of this study is to reveal critical links between H/ACA snoRNA expression, H/ACA snoRNP function, and differentiation. Using NanoString and small RNA-sequencing, we quantified H/ACA snoRNA levels before and after mESC differentiation. While most snoRNAs were unchanged, 5 snoRNAs were significantly upregulated and 7 snoRNAs were significantly downregulated upon differentiation with retinoic acid. We observed that 6 of the downregulated snoRNAs were also downregulated when mESCs were differentiated into cardiomyocytes, suggesting that they are important for maintaining stem cell homeostasis. To assess the role of the H/ACA snoRNP in snoRNA regulation and stem cell homeostasis, we depleted two protein components of the H/ACA complexes: Dkc1, the catalytic subunit, and Nop10, which anchors the snoRNA. Knockdown of either protein disrupted mESC homeostasis and promoted differentiation, but the effect was more severe upon depletion of Dkc1. Analysis of H/ACA snoRNA expression after knockdown revealed that all H/ACA snoRNAs require Dkc1 for stability, but only a few snoRNAs are sensitive to Nop10 depletion, including 4 of the snoRNAs that are downregulated upon differentiation. The 28S ribosomal RNA (rRNA) is the known RNA target of 5 of the 6 downregulated H/ACA snoRNAs. Northern blot analysis of rRNA processing revealed significant change in 28S processing upon retinoic acid differentiation. We are investigating whether downregulation of H/ACA snoRNAs leads to differences in pseudouridylation of target sites in the 28S rRNA and alterations in ribosomal function. Together these data suggest that individual H/ACA snoRNA levels and H/ACA snoRNP activity are important determinants of stem cell homeostasis or differentiation.
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*The transcription factor CEBPD regulates the global translational switch.*  
Coordinated control of transcription and translation impels cellular phenotypes. Cancer cells can usurp the regulatory machinery to adapt to stress posed by either microenvironment or therapies. Therefore, it is important to understand the regulation of these processes at all levels. The integrated stress response mitigates cellular stress and includes transient repression of global translation through phosphorylation of the eukaryotic initiation factor 2a (p-eIF2a) by either of four kinases PERK, PKR, HRI or GCN2, which in turn induces translation of specific stress-response mRNAs such as ATF4. The transcription factor CEBPD is induced by various stresses which also modulate protein translation. RNA-seq analysis of a CEBPD-silenced human melanoma cell line showed decreased expression of 12 aminoacyl-tRNA synthetase (aaRS) genes, suggesting accumulation of uncharged tRNA, which is known to activate GCN2. Therefore, we hypothesized that CEBPD is a regulator of protein translation. Indeed, deletion of CEBPD by CRISPR/Cas9 (CEBPD-KO) compromised aminoacyl tRNA synthetase (aaRS) expression and global protein synthesis, while increasing internal ribosome entry site (IRES)-mediated and ATF4-5’UTR-mediated reporter gene expression. CEBPD-KO cells also showed increased p-eIF2a and ATF4 expression corroborated with better survival in amino acid-reduced media, indicating possible GCN2 activation. Indeed, silencing of GCN2 or its coactivator GCN1 restored global protein synthesis and attenuated ATF4-reporter activity in the CEBPD-KO cells, suggesting that CEBPD inhibits GCN2 activity. Interestingly, over-expression of a DNA-binding deficient mutant of CEBPD only partially rescued KO phenotypes, suggesting additional transcription-independent functions of CEBPD. A proteomic interactome screen with CEBPD indicated that CEBPD may bind to GCN1, which was supported by co-immunoprecipitation of CEBPD and GCN1 proteins. Taken together, these data provide a model of...
attenuation of stress responsive mRNA translation by CEBPD. Thus, CEBPD may act as a brake to cancer cell growth under microenvironmental stress, and loss of CEBPD provides a survival advantage. In line with these findings, CEBPD expression correlates with longer survival of breast and liver cancer patients while ATF4 expression indicates poor outcome. Our results propel further investigation of cancer cell adaptation in vivo through translational rewiring.

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NIA  
RNA Biology  
circSamd4 promotes myogenesis by repressing PUR protein function  
Circular (circ)RNAs lack free 5' and 3' ends and are thus believed to be quite stable. By interacting with microRNAs and proteins, they have been proposed to be key players in gene regulation influencing many biological processes, but their impact on muscle cell differentiation is poorly understood. In this study, we used RNA sequencing (RNA-seq) to identify differentially expressed circRNAs during myogenesis that might be implicated in this process. Validation experiments confirmed that circSamd4 expression was robustly elevated in mouse C2C12 myoblasts differentiating into myotubes. Further analysis revealed that silencing circSamd4, which is conserved between human and mouse, delayed myogenesis and lowered expression of myogenic markers in cultured myoblasts from both species. Using antisense oligomers to perform affinity purification followed by quantitative mass spectrometry analysis, we pulled down circSamd4 and identified associated proteins in mouse and human myoblasts. Prominent among them were PUR proteins (PURA and PURB), which are negative regulators of myogenesis and impair the transcription of the myosin heavy chain (MYH) protein family. In keeping with the notion that circSamd4 might form ribonucleoprotein complexes with PUR proteins, possibly sequestering them away from DNA, silencing circSamd4 enhanced the association of PUR proteins to MYH promoter regions. Conversely, overexpression of circSamd4 interfered with the ability of PUR proteins to form complexes at the MYH promoter regions; this effect was abrogated if a mutant circSamd4 was overexpressed that lacked PUR protein binding sites. Together, our results indicate that the association of PUR proteins with circSamd4 is important for myogenic progression and uncover a new function for circRNAs as modulators of myogenic transcription factor function.

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Signal Transduction - G-protein and Ion Channels  
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Single-cell sequencing of NF1 associated Plexiform Neurofibromas identifies IL34 as a candidate driver of the tumor microenvironment

Patients with Neurofibromatosis 1 (NF1) present a number of clinical manifestations, one of which is the development of Plexiform Neurofibroma (PN). While PN are considered benign tumors, these lesions are frequently large, painful, disfiguring and can become symptomatic due to local mass effect. In addition, PN are now understood to be precursor lesions and 10-15% of PN transform to Malignant Peripheral Nerve Sheath Tumors (MPNST), which are associated with a dismal clinical outcome. It has long been appreciated that PN are composed of a complex tumor microenvironment composed of distinct cellular compartments that promote tumor expansion in a self-reinforcing feed forward loop. The tumor compartment is composed of a neoplastic Schwann cells that have damage to both alleles of the NF1 gene; the stromal compartment is composed of secretory cells that generate excessive extracellular matrix; and a complex immune compartment composed of T cells, macrophages and mast cells. While previous efforts have used a candidate gene approach to dissect this complicated cellular network, in this work, we applied single cell RNA sequencing to human primary PN to comprehensively evaluate these cellular populations and their interactions. Fresh tumors taken directly from the operation room were minced into 1 mm cubes and dissociated into single cell suspensions in the presence of enzymatic digestive medium. Data was generated using the 10X Chromium platform as a pooled analysis of 3 separate PN using Seurat. In total 103,446 cells were included in this analysis. There were 57,952 sequencing reads generated per cell for a mean of 3473 genes per cell. Graph-based clustering was utilized and 30 clusters with distinct transcription pattern were identified. Interestingly, while our in-depth analysis of PN confirmed the cellular heterogeneity including neoplastic, immune and stromal components as previously reported, the subpopulation within each component and the interactions among them are much more complicated than previously understood. We further develop a new informatic algorithm to annotate and summarize the observed ligand and receptor interactions. Finally, we observe the production of IL34 by the neoplastic Schwann cells and verify the specificity of this novel discovery using multiplex RNAish assays. We speculate that the generation of this interleukin by the Schwann cells may contribute to the recruitment and activation of the immune cells driving PN growth.

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Single Cell Sequencing

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Stem Cells - General and Cancer

*Post-transcriptional Control of mouse embryonic stem cell maintenance by Ccr4-Not complex*

The Ccr4-Not complex is the main deadenylase in eukaryotic cells, and it regulates mRNA poly(A)-tail length to influence mRNA stability and/or translation. The complex is formed by two modules: the NOT module (by Cnot1, Cnot2, Cnot3) and the deadenylase module (by Cnot6, Cnot6l, Cnot7, Cnot8). It has been postulated that the NOT module may regulate substrate binding and the activity of the complex, and the deadenylase module enzymatically deadenylates mRNA poly(A)-tails. Interestingly, different subunits of the complex have been implicated in various development phenotypes and diseases. Thus, it is important to better understand the exact function of each subunit, as well as its specific role in a given biological context. We have previously shown that Ccr4-Not is required for mouse Embryonic Stem Cell (ESC) maintenance. To systematically dissect the role the complex in ESCs, we generated conditional deletion ESC lines for subunits in the NOT and deadenylase modules (both individually and in combination) using the CRISPR/Cas9 technology. We found that individual deletion of Cnot1, Cnot2, or Cnot3 or simultaneous deletion of Cnot7 and Cnot8 led to ESC differentiation. In contrast, individual deletion of Cnot6, Cnot6l, Cnot7, Cnot8, or double deletion of Cnot6 and Cnot6l had no obvious impact. While deletion of individual subunits did not appear to interfere with the assembly of the other subunits in the complex, NOT subunits deletions often resulted in decreased stability of others and deadenylase subunits deletions increased the stability of the NOT module. We are currently examining mRNA steady-state abundance, decay rate, and polysome association to determine the impact of Ccr4-Not deletions. We are also carrying out PAR-CLIP (photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation) experiments to determine Ccr4-Not-bound mRNAs to determine its direct targets and the role of each subunit in target recognition. Finally, we are developing a novel improved technique to measure poly(A)-tail length of all detectable transcripts. We will use this technique to investigate how Ccr4-Not regulates poly(A)-tail length to control gene expression post-transcriptionally during stem cell fate specification.

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*Diurnal geckos as a model to study foveal specialization of the human retina*

The fovea is a central depression of the retinal surface containing high density cone photoreceptors that provides high visual acuity in humans and other animal species. Failure of foveal development, such as foveal hypoplasia, is associated with several heritable diseases, including aniridia and oculocutaneous albinism. Despite its critical role in central vision, the molecular and cellular mechanisms behind the development of the fovea have not been well elucidated. In part, this is due to the absence of a fovea-like structure in commonly used laboratory animals. Certain diurnal gecko species possess a fovea. These lizards have a well-defined life-cycle that allows study of different stages of embryonic development from fertilized egg to maturation. As a result, they may serve as a more accessible platform for studying foveal development than non-human primate models. By doing embryonic developmental studies of the species *Lygodactylus picturatus* we found the formation of a foveal structure. Therefore, we sought to develop an in vitro model of foveal development of this and other foveate geckos, including *Lygodactylus conraui* and *Gonatodes albogularis.* First, we established a primary fibroblast cell line from biopsy of primary or regenerating tail. Our results demonstrated that in comparison to human fibroblasts, gecko fibroblasts have a higher cytoplasmic:nuclear ratio, smaller diameter and a slower passage rate, occurring approximately every 12-14 days. Next, we attempted to generate induced pluripotent stem cells (iPSCs) by genomic reprogramming of gecko fibroblasts with human Yamanaka factors (OCT3/4, KLF4, cMYC and SOX2) using the Cytotune 2.0 Sendai virus reprogramming kit. Efficient transduction of fibroblasts with GFP-expressing Sendai virus was observed at multiple cell densities. Reprogramming attempts resulted in rounded colonies in gecko fibroblasts, similar to human. Cell morphology from suspected gecko colonies demonstrated a high nuclear:cytoplasmic ratio with decreased polarity. Efforts are underway to successfully propagate and characterize reprogrammed cells in culture. To our knowledge, this is the first attempt at establishing a stem cell line in vitro from a gecko species. This approach will allow us to potentially model disease states involved in human foveal development and study the conservation across species of genes by genomic sequencing with hopes of providing a new tool across different fields.

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*Quiescence in early embryonic development: Understanding diapause*

Embryonic stem cells (ESCs) derive from pre-implantation embryos or blastocysts and are characterized by a high proliferation rate. In fact, compared to somatic cells they have a very short G1 phase, are lacking a G1-to-S phase checkpoint and have a constitutive CDK activity throughout the entire cell cycle. Thus, it has long been postulated that rapid and continuous proliferation was intimately associated to pluripotency and self-renewal, which are intrinsic characteristics of the ESC identity. However, the process of embryonic diapause, a reversible state of quiescence observed in pre-implantation embryos,
challenges this idea. Blastocysts in diapause show growth arrest and decreased metabolism while maintaining their full developmental potential. Although the process of diapause is not well understood, it is a widespread phenomenon in mammals and it has been observed in more than 130 different species. Myc-inactivation and mTOR-inhibition have recently been identified as chemical strategies to induce reversible developmental arrest without compromising the pluripotent state in mouse blastocysts. Still, little is known about how blastocysts enter, maintain or exit the state of diapause and whether ESCs could be induced to quiescence. To better understand this fascinating process, we generated fluorescence-based reporter ESC lines to identify quiescent ESCs in vitro. This cellular tool allowed us to perform a primary screen using an FDA-drug approved library, in which we identified 9 compounds able to induce quiescence. Surprisingly, we also observed a basal, small population of quiescent, non-cycling cells within our ESC cultures. By combining the fluorescent ubiquitination-based cell cycle indicator (FUCCI) system to monitor cell cycle progression with our quiescence reporter system, we could establish the existence of a unique population of non- or slow-dividing cells in pluripotent cell cultures. We are performing now single cell RNAseq to define and identify the molecular properties of this subpopulation of ESCs and explore whether their levels can be modified by the compounds identified in our screen. Finally, we are extrapolating our observations in human ESCs. In summary, we are convinced that understanding how ESCs can enter, maintain or exit from this dormant state not only will provide exciting and novel insights into the field of stem cell biology but will also contribute to understand the nature of dormant cancer stem cells.

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Stem Cells - General and Cancer

ID2 is Required for Hematopoietic Stem Cell Maintenance, Quiescence and Self-Renewal Through Sustained Expression of HIF1alpha

Steady-state hematopoiesis is maintained by hematopoietic stem cells (HSCs), which proliferate and differentiate to give rise to all blood cells. Inhibitor of DNA binding proteins (IDs) are required for normal myeloid, erythroid and lymphoid development, and dysregulation of Id gene expression has been linked to the initiation and progression of hematopoietic malignancies and other cancers. ID proteins (ID1-4) are members of the helix-loop-helix family of transcriptional regulators that regulate proliferation and differentiation of endothelial, muscle, neural and hematopoietic cells. We found that ID2 is highly expressed in HSCs and decreases as the cells differentiate, suggesting ID2 may be required for HSC function. Using Mx1-Cre ID2Fl/Fl mice, we investigated the cell intrinsic requirement of ID2 in adult HSCs through bone marrow transplantation and genotoxic stress. Upon loss of Id2, we found a significant reduction in the number HSCs, which lead to HSC exhaustion and bone marrow failure over time in vivo. To determine the effects that loss of Id2 had on HSCs, we performed RNA-SEQ on Id2WT and Id2KO HSCs. Bioinformatic analysis of differentially expressed genes showed elevated expression of genes involved in cell proliferation and oxidative phosphorylation in Id2KO HSCs. We found that Id2KO HSCs show elevated levels of ROS, increased mitochondrial activity and increased DNA damage confirming the increased metabolic activity and proliferation of Id2KO HSCs. Single-cell HSC divisional kinetics and proliferation assays in vitro support the conclusion that that Id2KO HSCs are less quiescent.
Mechanistically, bioinformatic analysis revealed that Id2KO HSCs show a significant loss of HIF1alpha signaling, a key regulator of glycolysis and required for HSC quiescence. We found that HIF1alpha protein expression was significantly decreased in isolated Id2KO HSCs by flow cytometry and immunofluorescence assays. We demonstrate that chemical stabilization of HIF1alpha in Id2KO HSCs fully rescues HSC exhaustion in vitro. Further, we show that enforced expression of Id2 increases HIF1alpha expression in vitro. Collectively, our data reveals that ID2 is essential for maintaining HSC quiescence and self-renewal via increased HIF1alpha expression. Our findings suggest that sustained ID2 expression could improve HSC expansion and gene editing in vitro by improving HSC survival, and may be important for the survival and maintenance of leukemic stem cells.

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Stress, Aging, and Oxidative Stress/Free Radical Research
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Stress, Aging, and Oxidative Stress/Free Radical Research
Interleukin-22 ameliorates nonalcoholic steatohepatitis through induction of metallothionein and suppression of p38 mitogen-activated protein kinase in mice
BACKGROUND & AIMS: Nonalcoholic steatohepatitis (NASH) is emerging as a major cause of end-stage liver failure worldwide; however, there are no FDA-approved drugs for NASH. The goal of the current study is to determine the therapeutic potential of the approach that inhibits ROS-induced stress kinases and the efficacy of the candidate agents that work through this mechanism. METHODS: To mimic the phenotypes of NASH patients, mice fed a high-fat diet for 3 months were injected via tail vein with an adenovirus overexpressing Cxcl1, a chemokine that recruits neutrophil. For drug screening, the mice were treated with candidate agents for 2 weeks following Cxcl1 overexpression. RESULTS: Hepatic overexpression of Cxcl1 increased the neutrophil infiltration, oxidative stress, hepatocyte injury, inflammation, and fibrosis in the liver, with an elevated phosphorylation of stress kinase p38 mitogen-activated protein kinase (p38 MAPK) mediated by neutrophilic ROS production. Pharmacological inhibition of p38 MAPK by LY2228820 reduced liver injury, inflammation, and fibrosis caused by Cxcl1 overexpression. Using this model, we explored the therapeutic potential of interleukin (IL)-22, which is under clinical trials for alcoholic hepatitis. IL-22 treatment attenuated liver injury, inflammation, and fibrosis caused by Cxcl1 overexpression with a reduction of ROS levels, p38 MAPK phosphorylation, and caspase-3 cleavage. The beneficial effect of IL-22 was diminished in mice lacking metallothionein (MT), an antioxidant enzyme induced by IL-22-STAT3 pathway. This indicates that IL-22 ameliorates NASH
through MT-mediated ROS reduction and suppression of p38 MAPK. As apoptosis signal-regulating kinase 1 (ASK1) is an ROS-activated upstream regulator of p38 MAPK, we tested if pharmacological inhibition of ASK1 improves Cxcl1-induced NASH and if the beneficial effect of IL-22 is mediated through ASK1 inhibition. Alleviation of liver injury by selonsertib, an ASK1 inhibitor, was not as potent as that by LY2228820 or IL-22. Also, IL-22 effect was not diminished in ASK1-null mice. These collectively indicate that IL-22 ameliorates NASH through inhibition of p38 MAPK, independently of ASK1. This is in line with the recent failure of selonsertib in phase III clinical trial and highlights the therapeutic potential of IL-22.

CONCLUSIONS: We identified IL-22 as a potential therapeutic agent for NASH in a mouse experimental model of NASH.

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Stress, Aging, and Oxidative Stress/Free Radical Research

Transcriptome Signature of Cellular Senescence

Cellular senescence has been studied using different cell models and inducers, including telomere attrition, damage to DNA and other molecules, and stress conditions such as oncogene activation. Given the impact of senescent cells in cancer and aging, there is escalating interest in identifying senescent cells in vivo. At present, senescent cells are identified by the combined presence of multiple markers, such as proteins p16, p53, and p21, as well as the enzymatic activity of senescence-associated (SA)-beta-galactosidase; unfortunately, these markers are not exclusively nor universally present in senescent cells. To identify robust biomarkers of senescence, we have performed RNA-sequencing analysis across a range of cell models: replicative senescence (in WI-38 and IMR90 fibroblasts), ionizing radiation-induced senescence (in WI-38, IMR-90, HUVEC, and HAEC), and doxorubicin-induced and oncogene-induced senescence (in WI-38 fibroblasts). The intersection of these models revealed 73 transcripts consistently up- or downregulated among all senescence paradigms. Among the upregulated transcripts, we identified ELMOD1 mRNA (encoding engulfment and cell motility 1, ELMOD1) and PTCHD4 mRNA (encoding patched domain-containing 4, PTCHD4) and long noncoding RNA (lncRNA) PURPL (p53-upregulated regulator of p53 levels). RT-qPCR analysis confirmed these changes and IPA analysis further revealed that senescence-related interferon and IL7 signaling pathways were prominent in senescent cells. PURPL was consistently and dramatically upregulated up to hundreds-fold higher in senescent cells across the panel studied. We propose that these RNAs can be used as reliable markers to characterize cellular senescence, enabling the analysis of senescent cells in vivo, their implication in aging and malignancy, and the development of therapies aimed at targeting senescent cells.

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Tumor Biology and Metastasis
The Uncharacterized Regulatory Role of the Dopamine Signaling Network in Immune Oncology

The five-year survival rate for lung cancer is among the lowest of all cancer types. Recent advances in immunotherapy have yielded some success in extending survival and, in extraordinary cases, reducing mortality. Immune checkpoint inhibition (ICI) is a form of immunotherapy that inhibits checkpoint proteins, such as PD-L1. ICI disables mechanisms that tumor cells use to evade the patient’s immune response against cancer, and ultimately re-sensitizes them to attack by T-cells. Although ICI drugs have demonstrated efficacy in lung cancer, durable responses are observed in only a fraction of patients. Identifying costimulatory partners for these drugs could dramatically increase the effectiveness of the ICI therapies currently used to treat lung cancer. We recently demonstrated that Dopamine Receptor D1 (DRD1) can regulate Epidermal Growth Factor Receptor (EGFR) signaling. As EGFR is a known modulator of PD-L1, we hypothesized that DRD1 could also regulate PD-L1. Indeed, we found that PD-L1 protein, but not mRNA, levels are controlled by dopamine signaling in lung cancer cells. Overexpression and pharmacological activation of DRD1 both caused a significant reduction of PD-L1 on lung cancer cell membranes. We are currently investigating whether this signaling axis involves EGFR pathway effectors, AKT and YAP1. Moreover, we are conducting a series of in vitro T-cell killing assays to verify that altering PD-L1 expression, through the dopamine signaling axis, will affect interactions between tumor cells and T-cells. Interestingly, our study also shows that DRD1 decreases membrane expression of PD-L1 but did not reduce PD-L1 expression in the cells’ nuclei. The nuclear localization of PD-L1 by DRD1 was reversible when cells were treated with a DRD1 inhibitor. Lastly, we discovered that PD-L1 is involved in chromatin-binding within the nucleus and that it reduces apoptosis in lung cancer cells. This dynamic nuclear localization of PD-L1, and it’s regulation by DRD1, are both novel and are undergoing detailed follow up studies to assess clinical relevance. Collectively, our data suggest that PD-L1 expression can be modulated by the dopamine signaling axis, and that PD-L1 exerts cell-intrinsic activity beyond its interaction with T-cells. This implicates a new role for dopamine modulators (many are already FDA-approved with more favorable safety profiles than chemotherapies) as potential costimulatory partners for immune checkpoint inhibitors in lung cancer.

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SURAJIT Sinha
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Tumor Biology and Metastasis

Metabolic Reprograming by Translational Activator GCN1 promotes Metastatic Reactivation and Outgrowth of Pancreatic Cancer in the Liver

Introduction: Liver metastasis arising from disseminated tumor cells (DTCs) is a major cause of relapse and subsequent mortality for patients with pancreatic cancer. Upon extravasation into the liver, DTCs encounter a hostile nutrient microenvironment like glutamine deprivation and undergo dormancy or temporary growth arrest. The molecular drivers that enable DTCs to reprogram cellular signaling mechanisms to overcome dormancy and revert to a proliferative stage are unknown. Method: To identify the molecular drivers, a retroviral cDNA library was generated from a highly metastatic pancreatic cell line (M-4964Liv) derived from a KPC mouse which forms macroscopic liver lesions upon splenic injection in mice. The cDNA library was then transduced into a dormant pancreatic KPC cells (D-4964Liv) cells which do not give liver lesions upon splenic injection in mice. With validation, this
methodology allowed us to assign causality to the transduced cDNAs. Results: This screening strategy led to the identification of the translational activator GCN1. GCN1 has been shown to regulate the activation of mammalian amino acid sensor GCN2 under amino acid starvation in budding yeast. GCN1’s role in the regulation of reactivation and outgrowth at a metastatic site such as the liver is not known. We show that GCN1 is transcriptionally induced and activated at physiologic glutamine levels in the liver which is around 0.5 mM compared to supraphysiologic levels of 4 mM used in standard tissue culture. Mechanistic interrogation revealed that GCN1 activates ATF4, SOX2, and mTORC1. While ATF4 induces the serine biosynthetic pathway to generate precursors for macromolecular synthesis, the activation of Sox2 and mTORC1 induces self-renewal and drives tumor growth. Knockdown of GCN1 or overexpression of Impact, a negative regulator of GCN1 function, dramatically abrogates pancreatosphere formation and liver metastasis in immunocompetent mice. Conclusion: Physiologic glutamine concentrations activate GCN1 to reprogram DTCs in the liver to generate biosynthetic precursors indispensable for macromolecular synthesis and metastatic outgrowth by activating ATF4 and mTORC1. Thus, inhibitors of the serine biosynthetic pathway or mTORC1 may benefit patients with pancreatic cancer by inhibiting the progression of their metastatic disease.

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Tumor Biology and Metastasis
MSLN positively facilitates peritoneal metastases initiation in pancreatic cancer by enhancing angiogenesis
Peritoneal metastases develop in majority of patients with advanced pancreatic ductal adenocarcinoma (PDAC), and the onset of ascites, i.e. extra fluid built up due to pancreatic cancer metastases, is associated with rapid demise. Mesothelin (MSLN) is a glycoprotein expressed in >90% of PDAC. High tumor cell expression of MSLN increases aggressiveness of PDAC in laboratory models, and shortens survival in patients, but the pathobiological role of MSLN is poorly understood. This intrigued us to investigate the role of MSLN in pancreatic peritoneal metastases. Here, we have used CRISPR-Cas9 gene-editing to delete MSLN from the KLM1 PDAC cell line (KO) and examined KO cell growth in culture and in nude mice compared to mock deleted cells (Mock). Co-expression of Luciferase(Luc) allowed for early localization and tracking of tumor deposits in the intraperitoneal (IP) cavity by bioluminescence imaging (BLI). IP tumors were harvested 24h-6w post IP injection and histologic features were examined. RNA deep sequencing of MSLN KO and Mock cells was performed to identify new molecular mediators of MSLN activity. MSLN KO and Mock cells grew similarly in culture and as subcutaneous xenografts in nude mice. But when cells were inoculated IP, KO cells grew poorly compared to Mock. While Mock cells grew metastases throughout the IP compartment, MSLN KO tumors were located primarily at the inoculation site, if they grew at all. If MSLN KO tumors did take, fatal spread throughout the IP cavity was delayed from 6w to 9w. Histology showed that by 72h, MSLN KO tumors had significantly decreased microvascular density. By 1w, decreased proliferation and invasion were also seen. These differences resolved by 6w suggesting MSLN confers a growth advantage solely during metastatic initiation. The mechanism for MSLN action has been previously ascribed to signaling through MAPK or NF-kB pathways or by MSLN binding to MUC16. However, we found no changes in p38 or ERK activation in our MSLN KO
model, nor in levels of the NF-kB target OCT-2. Since overexpression of MSLN in the MUC16(-) MIA-PaCa2 PDAC cell line also enhanced tumor growth, MUC16 cannot be entirely responsible for the protumorigenic activity of MSLN. RNA deed seq revealed changed expression of PPP1R1B, a protein phosphatase inhibitor and a pro-angiogenic signaling mediator, in KO cells. In summary, MSLN enhances PDAC peritoneal carcinomatosis by positively regulating angiogenesis during tumor initiation.

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Tumor Biology and Metastasis

COX2 Inhibition Improves Efficacy of Radiation Therapy through Immuno-modulation in the Tumor Micro-Environment

Background and Hypotheses Radiation therapy (RT) is a commonly used treatment option in more than 60% of cancer patients including breast cancer, a major health hazard among women. Focal radiation limits systemic side effects commonly associated with chemotherapy. Eventually the tumor come out of the growth delay and tends to show more aggressive phenotype leading to metastasis and chemoresistance. We found that RT induced inflammation associated bio-markers nitric oxide synthase2 (NOS2) and cyclooxygenase2 (COX2) in the tumor micro-environment (TME), specifically in the tumor cells. We recently showed that co-expression of pro-inflammatory enzymes NOS2 and COX2 is a powerful prognostic marker of poor outcome among ERα− patients which in turn drive major oncogenic pathways. This commonality between RT and poor patient prognosis led us to hypothesize that modulation of inflammation associated bio-markers in the TME would increase the efficacy of RT by amplifying anti-tumor immunity. Method To assess the role of inflammation on radiation therapy and gain mechanistic insight into aggressiveness of triple negative breast cancer (TNBC), we used mouse TNBC cell line 4T1 implanted in flank of Balbc mice. The animals were divided into four groups; Control, 6Gy RT, 30mg/L indomethacin (preferentially inhibits COX2) in drinking water and RT + indomethacin. When the tumor reached 100 mm3, group 2 and 4 animals were given 6 Gy RT and then group 3 and 4 were started on 30 mg/L indomethacin containing drinking water. We used confocal microscopy, CO-Detection by indEXing (CODEX) technology and flow cytometry to investigate the role of COX2 in radiation induced tumor growth delay and metastasis. Results and Conclusions We showed that COX2 inhibition using commercially available inhibitors significantly prolonged radiation induced tumor growth delay and reduced lung metastasis in murine models of ERα− breast cancer. Different immune cell populations in the TME was also investigated. COX2 inhibition along with RT induced proliferation of cytotoxic CD8, dendritic cells and pro-inflammatory macrophages while immunosuppressive Treg activity was reduced. Furthermore, CODEX data showed correlation between decreased macrophage population and increased CD8 infiltration suggesting role of macrophages in inhibiting CD8 infiltration. Thus, we demonstrated that co-treatment of COX2 inhibitor with RT effectively activated the immune system leading to improved efficacy.
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*Integrated proteogenomics utilizing mass spectrometry to identify MHC-associated neoantigens in EGFR mutant lung adenocarcinoma tumor*

The epidermal growth factor receptor (EGFR) is one of the most frequently mutated oncogenes in human lung cancer. Although immune checkpoint inhibitors have been therapeutically effective in multiple cancers, patients harboring EGFR mutations have not responded well to this immunotherapy. Adoptive T cell therapy (ACT) has resulted in complete and durable regression of metastatic cancers. ACT requires that neoantigens are presented by major histocompatibility complex (MHC) class I molecules on tumor but not normal cells. The MHC binding algorithms, such as IEDB, most commonly used for antigen prediction on MHC molecules is far from perfect; thus, experimental proof of MHC-bound peptides is needed to provide direct evidence of antigen presentation. We developed a mass spectrometry (MS)-based proteogenomic platform integrated with a whole exome sequencing (WES)-based protein database to identify tumor-specific mutant peptides presented by MHC class I.

Experiments were conducted using EGFR mutant PC9 lung adenocarcinoma cell line that harbors the EGFRDel746-750 mutant. MHC class I proteins and their associated peptides were immunoprecipitated, and the peptides separated from MHC proteins; subsequently, Class I-associated peptides underwent C-18 separation and tandem MS analysis. A PC9-specific database was built adding all SNVs and INDELs identified by WES to the normal human database and used to search MS data. Peptides identified by MS were subjected to IEDB algorithm search using PC9 specific HLA types. Then, we further apply this platform to identify neoantigens in one human lung adenocarcinoma tumor. The MS HLA typing identified A*02, A*24, and B*39 in PC9 cells. We identified 13,765 MHC bound peptides. 10,711 peptides were 7~15 amino acid residues in length (6~15mer), which are generally considered as class I epitopes. Using the PC9-specific protein database, 11 variant peptides (neo-peptides) were identified. However, the IEDB results predicted only 3 out of 8 identified variant peptides. We identified an insertion neoantigen (GTAAAAAAAAYAAAK) from 60S ribosomal protein L14 (RPL14Insertion A150-152A). In conclusion, our MS data provided direct experimental evidence for MHC class I presentation of 11 neoantigens in PC9 cells and 1 neoantigen in human lung tumor. Neoantigens discovered by this proteogenomic platform could potentially trigger T cell response, which needs to be validated by immunological assays.
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Tumor Immunology and Immunotherapy

*Genetically-Engineered Myeloid Cells Activate T Cells In The Pre-Metastatic Niche*

Tumor metastasis is a critical step in the progression of solid tumors that is associated with patient mortality. The pre-metastatic niche is the tumor-promoting microenvironment that is established at distant sites in response to primary tumor growth. We characterized immune populations in the pre-metastatic lungs of tumor-bearing mice by flow cytometry and RNA sequencing. In addition to validating the dramatic increase of myeloid cells and immunosuppressive pathways that has been previously reported, we discovered that T cell populations and T cell function pathways are markedly reduced in the lungs of tumor-bearing mice compared to naïve mice. We hypothesized that reversing the immunosuppressive microenvironment in pre-metastatic lungs would restore T cell function and antitumor immunity. To take advantage of the infiltration of myeloid cells into pre-metastatic lungs, we designed a novel approach in which we generated Genetically-Engineered Myeloid cells (GEMys) to deliver IL-12, a potent antitumor molecule, into the pre-metastatic microenvironment. Tumor-bearing mice were treated with unmodified myeloid cells or GEMys and lungs were harvested at primary tumor endpoint. GEMy-treated mice had significantly reduced metastasis compared to untreated mice and mice treated with unmodified myeloid cells. Further, we evaluated the lungs by flow cytometry and observed that GEMy-treated mice had increased numbers of T cells and enhanced expression of activation markers compared to untreated mice and mice treated with unmodified myeloid cells. To determine which immune populations are important for GEMy function, we administered CD8 or CD4 depletion antibody with or without GEMy therapy. We determined that CD8+ cells are necessary for GEMy function, while CD4+ cells are not required but do contribute to GEMy efficacy relative to isotype-treated controls. The adoptive transfer of tumor-specific CD8+ T cells in combination with GEMys resulted in reduced tumor burden and prolonged survival of mice relative to T cell or GEMy therapy alone. When combined with chemotherapy pre-conditioning, GEMy therapy cured mice of established tumors and generated long-lived T cell memory, as these mice were immune to subsequent tumor challenge over 100 days post-treatment compared to naïve age-matched controls. These studies demonstrate that reprogramming myeloid cells is a viable strategy to activate antitumor T cell responses in immunosuppressive microenvironments.

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Tumor Immunology and Immunotherapy

*Microbiota modulate response to cancer therapy via training of neutrophils in the tumor microenvironment*

Neutrophils can be pro- or anti-tumorigenic, however, their role in response to cancer therapy and the signals that regulate their function are unclear. Here we studied the role of neutrophils in therapy efficacy and their regulation by microbiota using implanted tumors with different susceptibility to chemo and immunotherapy in animals with an intact microbiota, treated with antibiotics (Abx) or germ-
free (GF). We showed that tumors with different susceptibility to chemo differ in their neutrophil quantity and quality, and that tumor-infiltrating ROS-producing neutrophils are required for the optimal response. We confirmed this by neutrophil depletion and recruitment blockade, which resulted in impaired response. We identified a subset of neutrophils characterized by low expression of ICAM1 and high ROS present in chemo-sensitive but not resistant tumors. We showed that absence of microbiota leads to a significant reduction in neutrophil number and frequency only in the tumor microenvironment (TME), impaired ROS production and increased ICAM1 and PD-L1 expression, resembling chemo-resistant tumors. RNA sequencing of neutrophils purified from the TME of mice lacking microbiota after chemo revealed reduced expression of key genes associated with trained immunity including mTOR signaling, cholesterol biosynthesis and DNA repair pathways. Multiplex protein analysis of the same tumors showed reduced pro-inflammatory cytokines, consistent with impaired neutrophil training in absence of microbiota. Administration of MDP, a bacterial product known to induce trained immunity, before chemo restored ROS production and reduced ICAM1 expression in tumor neutrophils from Abx-treated mice. Analysis of Flow Repository CyTOF data from mouse tumors after immune checkpoint blockade (ICB) therapy showed a positive correlation between therapy efficacy and neutrophil frequency in the TME, suggesting that neutrophils also play a role in the efficacy of ICB. To further study this, we transplanted feces from melanoma patients that responded (R) or not (NR) to ICB into GF mice implanted with syngeneic melanomas. We showed increased frequency of neutrophils only in the TME of mice transplanted with R feces, which correlated with reduced tumor volume and better ICB response. Our data reveal the critical role of microbiota-trained neutrophils in response to cancer therapy and underscore the importance of dissecting their heterogeneity when designing successful therapeutic strategies.

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Tumor Immunology and Immunotherapy

Tumor-targeted gene delivery using Human Papillomavirus pseudovirions

Human Papillomavirus (HPV) L1 virus-like particle (VLP) vaccines will dramatically change the landscape of HPV-associated diseases over the next decade. Given their established safety and their recently discovered broad tumor-specific binding tropism, the VLPs are also candidates for therapeutic interventions in cancer patients, particularly as gene delivery vectors. Current protocols for intracellular production cannot be used to generate HPV vectors encoding cytotoxic agents. Therefore, we developed a novel cell-independent technique for generation of HPV pseudovirions (PsVs), in which purified L1 and L2 VLPs are disassembled and reassembled in the presence of bacterial plasmid DNA or mRNA. This method allows for the generation of high titer HPV vectors expressing bacterial toxins that maintain tumor-specific tropism. In this study, we generated PsVs expressing Pseudomonas aeruginosa exotoxin (PE). PE conjugated to an anti-CD22 antibody has recently been approved for treatment of Hairy Cell Leukemia. To potentially deliver PE to a broad range of cancer types, we encapsidated plasmid DNA or mRNA coding for PE toxin in HPV16 PsV. Transduction of either PE DNA or RNA dramatically reduced viability of multiple tumor cell lines in vitro. These findings were extended in an in vivo setting, where intratumoral (IT) injection of PE expressing PsV led to a 50% reduction in the tumor volume.
Additionally, flow cytometry data indicated a decrease in the number of regulatory T cells and granulocytic myeloid-derived suppressor cells in mice treated with PE PsV compared to those transduced with PsV encoding mutant PE. We are currently investigating whether IT injection of the PE toxin induces immunogenic cell death, and thereby generates cellular immunity against released tumor antigens. To that aim, we are using a murine colon carcinoma model, MC38, which has several well-defined neoantigens. Induction of tumor antigen spreading would be of a particular importance for the advanced disease patients with multiple tumors.
by laser speckle imaging; n=45, p=0.001). To test intrinsic vessel function, we isolated small cerebral arteries (max diameter 120µm; control n=4, ECM n=7) and quantified their ability to vasodilate using a pressure myography system. We discovered that cerebral arteries from ECM mice dilated appropriately to the vasodilator methacholine (MCh) (difference to control=0.1%, p=.99), implying no intrinsic vascular injury. Furthermore, dilation to MCh was abrogated by L-NAME (95% reduction, p=0.0001), a NO synthase inhibitor, indicating that NO synthesis was intact. We then directly measured NO release from isolated cerebral arteries using a DAF-2 fluorescence assay and confirmed that L-NAME nullifies MCh-stimulated NO production in ECM arteries (n=6, 105% reduction, p=0.008). Seeing no evidence for intrinsic vascular injury in ECM, we then tested whether circulating factors in ECM plasma could inhibit the release of NO. However, by applying ECM plasma to isolated cerebral arteries in our DAF-2 assay, we discovered that ECM plasma stimulated NO release (3X increase, n=6, p=0.003). These surprising results indicate that NO signaling in cerebral arteries remains intact during ECM, and that circulating plasma factors stimulate, rather than inhibit, vascular NO production. Cerebral perfusion impairments during CM may not stem from impaired NO bioavailability or intrinsic vascular dysfunction; instead, we propose that a NO stimulating factor is produced during CM, leading to systemic vasodilation, redistributing blood flow, and causing cerebral hypoperfusion.

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Vascular Disease and Biology

3D bio-printed Vascularized Human Skin as a Novel Drug Screening Assay Platform for Atopic Dermatitis

In vitro cell assays and animal models are extensively used in early stage drug development for efficacy, risk and toxicity tests. However, these in vitro and in vivo models lack human physiological relevance, and therefore, they have limited predictive value of drug responses in humans. Three-dimensional (3D) tissue models are currently being developed as alternative, more predictive assays for high throughput screening (HTS) for drug discovery. In this study, we have developed a novel bio-printed vascularized full-thickness skin equivalent (VFTS) and a reconstructed human epidermis (RhE) in a multi-well plate platform for the purpose of drug testing. The morphology and functionality of the skin tissue equivalents were confirmed with H&E staining, immunohistological characterization, and transepithelial electrical resistance (TEER) measurements. In the dermis of the VFTS, collagen IV and CD31 showed the formation of a 3D microvascular network and basement membrane of microvessels. Late epidermal differentiation markers illustrated that the epidermal layer was fully differentiated in both VFTS and RhE tissues. Interleukin (IL)-4 treatment was used to induce atopic dermatitis (AD)-like phenotype in the skin equivalents constructed. These AD models were able to reproduce several clinical hallmarks of AD including (i) spongiosis and hyperplasia; (ii) early and terminal expression of differentiation proteins; and (iii) increases in levels of pro-inflammatory cytokines. The assay system for the AD VFTS model includes measurements of tight junction barrier values, quantification of inflammatory cytokines, pro-angiogenic factors measurements and structural morphology changes in the microvasculature. Janus Kinase (JAK) inhibitors have been reported to be efficacious for the treatment of AD. Three JAK inhibitors were shown to correct the disease phenotypes. In our study, we first provide an innovative and reproducible use of the bio-printing technology to create a vascularized human skin equivalent. Second, this construct
combined with RhE is used in screening and developing pre-clinical drugs for skin disease. Third, endpoint readouts are quantifiable, robust, AD relevant, and can be scaled up to allow HTS for compound discovery. Thus, the skin equivalents developed in this study offers an in vitro approach for understanding pathological mechanism, efficacy of action and toxicity of dermal drugs.

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*The Role of Unpaired Guanosines in Retroviral Genome Packaging*

A key step in the retroviral lifecycle is the packaging of genomic viral RNA into nascent particles. The viral protein Gag mediates this process through interactions with cis-acting sequences in the leader region of unspliced viral RNA. The current major questions in the field are how Gag specifically recognizes viral RNA and how the Gag:RNA interaction drives genome packaging. Previous studies of murine leukemia virus and HIV-1 showed that unpaired guanosines in the viral leader RNA are important for packaging. We hypothesized that retroviral Gag interacts with unpaired guanosines in the RNA leader sequence to direct genome packaging. To test this hypothesis, we mutated predicted unpaired guanosines in the HIV-2 leader RNA and examined the effects on RNA packaging by visualizing HIV-2 genomes in viral particles. Using fluorescence microscopy, we detected viral particles with cerulean fluorescent protein fused to Gag, and viral RNA with yellow fluorescent protein fused to a bacterial protein, BglG, that specifically binds sequences engineered into the HIV-2 genome. We found that RNA packaging is not driven by the number of guanosines in the leader region, rather it is mediated by synergistic effects among specific guanosines. Based on the severity of their effects on RNA packaging, we identified a key cluster of guanosines that act as a primary site and two clusters of guanosines that act as secondary sites. Mutating individual primary or secondary sites does not affect RNA packaging. However, mutating the primary plus a secondary site causes packaging defects, and mutating all three sites caused the most severe packaging defects. Furthermore, these mutations did not alter levels of unspliced RNA or predicted RNA structure, suggesting that the packaging defects are caused by impaired Gag:RNA interactions. These data define the distinct cis-acting elements important for HIV-2 RNA packaging and support our hypothesis that retroviral RNA packaging is mediated by Gag interacting with specific guanosines in the leader sequence of the viral RNA. Approaches that disrupt Gag:RNA interactions leading to RNA packaging defects can be developed to block replication of deadly
pathogens like HIV. These results also have wider implications in the use of retroviruses as gene delivery vehicles in human gene therapy applications.

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Virology - DNA, RNA, and Retroviruses

Monocyte Chemoattractant Protein-Induced Protein 1 Degrades Specific KSHV and EBV miRNA Precursors and Inhibits Herpesvirus Infection

Kaposi’s sarcoma-associated herpesvirus (KSHV) is a member of ?-herpesvirus family and it is closely associated with Kaposi sarcoma (KS) and two rare lymphoproliferative disorders, primary effusion lymphoma (PEL) and multicentric Castleman’s disease (MCD). KSHV expresses 12 pre-miRNAs during latency that are converted into mature miRNAs. The recent discovery that monocyte chemoattractant protein-induced protein 1 (MCPIP1) is induced by multiple inflammatory cytokines and can cleave some human miRNA precursor molecules (pre-miRNAs) raised the possibility that the host could inhibit biogenesis of viral miRNAs in the context of inflammatory signals. If MCPIP1 could inhibit biogenesis of viral miRNAs, then we hypothesized that KSHV may possess mechanisms to inhibit MCPIP1-mediated host defense mechanisms. We analyzed gene expression changes after KSHV infection in primary endothelial cells and observed that upon KSHV infection the miRNA biogenesis inhibitor, MCPIP1, was repressed and pro-biogenesis factors (Dicer, TRBP) were increased in expression. MCPIP1 degraded the majority of KSHV miRNA precursors, but a specific KSHV pre-miRNA, miR-K6, which was resistant to MCPIP1 degradation, generates a mature miRNA that directly targeted the MCPIP1 3’ UTR resulting in decreasing MCPIP1 expression. This mature miRNA is abundant in patient-derived cell lines. Interestingly, we found that MCPIP1 degraded specific human, KSHV, and EBV pre-miRNAs with the different efficiencies. Our gel shift assays revealed that MCPIP1 binding strength was not the factor determining MCPIP1 cleavage specificity. To further characterize the features of pre-miRNA that determine MCPIP1 specificity, we analyzed the loop structures of pre-miRNAs and made point mutations within our predicted motif associated with MCPIP1-mediated degradation. We found that mutations in key locations inhibited MCPIP1-mediated degradation of both KSHV and human pre-miRNAs. In addition, we found that increased MCPIP1 expression caused a decrease in KSHV latent infection. Viral genome copies were reduced following MCPIP1 expression. Ongoing experiments are investigating the mechanisms of how MCPIP1 inhibits infection. Taken together, these results demonstrated that MCPIP1 inhibited KSHV infection and suppressed viral miRNA biogenesis by directly degrading KSHV pre-miRNAs. However, KSHV can circumvent the antiviral consequences of MCPIP1 functions through repression of MCPIP1 expression by the MCPIP1-resistant miRNA.

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Single-cell resolution of metabolic control over HIV-1 entry and a role for membrane lipid order and tension  

Recent studies have highlighted cellular metabolic activity as a critical factor driving HIV-1 infection in T cells. However, deciphering how the metabolic state of single cells affects virus entry remains to be fully characterised. We developed an assay utilising FRET-based biosensors of various metabolites to evaluate the influence of global metabolic processes on the success rate of virus entry in single cells. Lifetime fluorescence images of single cells were recorded immediately before and after addition of HIV-1 pseudovirions (i.e. HIV-1JR-FL) or non-enveloped HIV-1 with incorporated BLaM-Vpr. Lifetime measurements of cells expressing biosensors for ATP:ADP ratio or lactate were utilised to determine relative metabolite concentrations before and during entry. The same cells were subsequently screened for fusion and productive infection to determine whether baseline intracellular metabolite concentrations were correlated with these processes. Interestingly, cells with a lower ATP:ADP ratio prior to virus addition were less permissive to virus fusion and infection. These results indicated a relationship between host metabolic state and the likelihood for virus-cell fusion to occur. To confirm this, we show that cells treated acutely with 2-deoxy-d-glucose (2-DG), an inhibitor of glycolysis, permitted substantially fewer fusion events. Single particle tracking (SPT) revealed that virions were arrested at hemifusion in 2-DG-treated cells. Interestingly, cells treated with 2-DG also possessed less surface membrane cholesterol, while the addition of cholesterol to the plasma membrane rescued the block to fusion. Further investigation with additional reporters revealed a link between host glycolytic activity and membrane tension and order, with cells treated with 2-DG exhibiting lower plasma membrane lipid order and higher tension values. These data suggest that low glycolytic activity results in a deficiency of membrane cholesterol. Finally, SPT illustrated that virions were less likely to enter cells at areas of high membrane tension. We are currently performing similar experiments in T cells. We have identified a connection between host glycolytic activity and membrane tension which may influence HIV-1 fusion at the single-cell level. Our results indicate that HIV-1 fuses with glycolytically-active cells and that this activity is linked to cell surface membrane cholesterol and membrane tension.