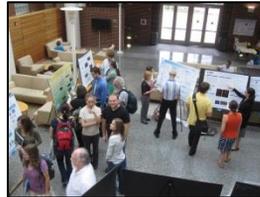
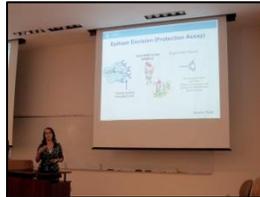
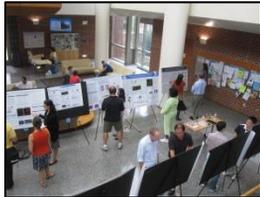
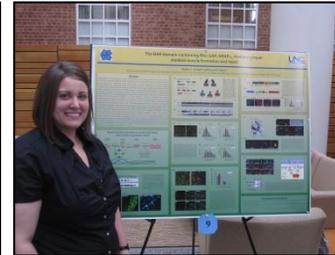
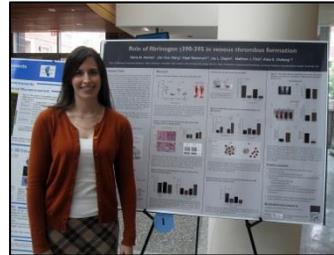
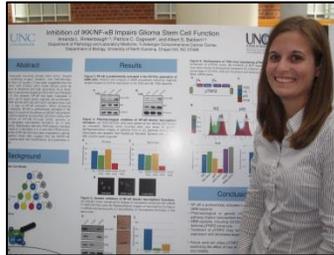
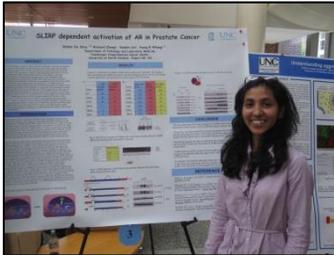
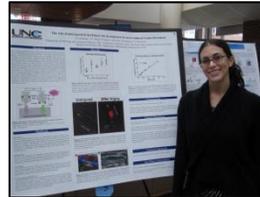
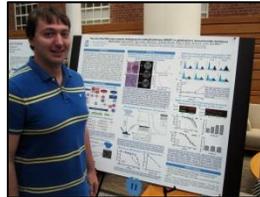
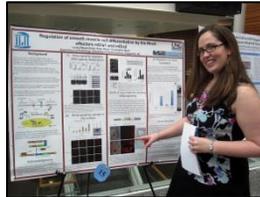
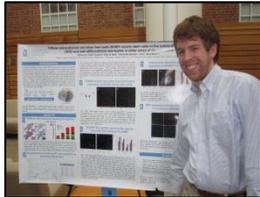
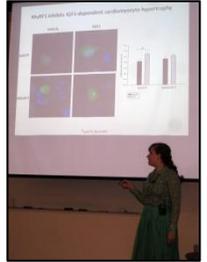
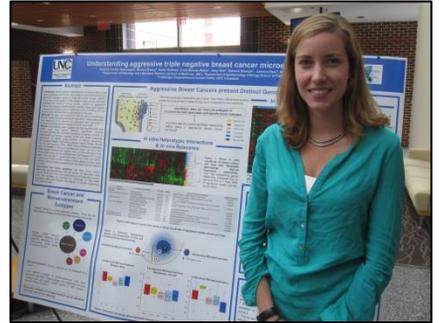
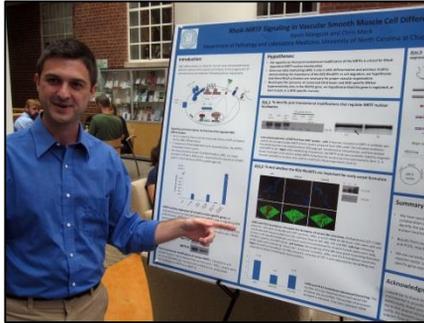
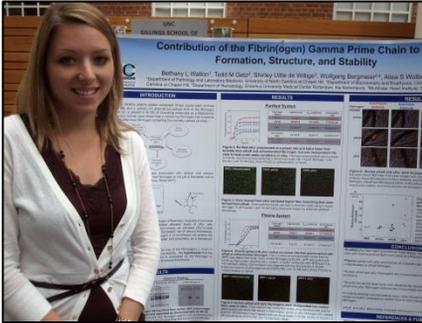


University of North Carolina School of Medicine
Department of Pathology and Laboratory Medicine
Annual Research Symposium

September 20, 2013





THE UNIVERSITY
of NORTH CAROLINA
at CHAPEL HILL

Department of Pathology and Laboratory Medicine

Annual Research Symposium

Highlighting the research conducted by our Predoctoral Students and Postdoctoral/Clinical Fellows

September 20, 2013



The Brinkhous-Bullitt Building

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In Honor of
Nancy Hamilton Nye
Associate Chair for Administration
Department of Pathology and Laboratory Medicine



Ms. Nancy H. Nye will retire in Fall 2013 after a 46 year career at the University of North Carolina School of Medicine that includes service to both the Department of Pathology and Laboratory Medicine and the Department of Biochemistry. Ms. Nye began her service to UNC in 1967 when she became a part-time typist/bookkeeper for Dr. J. Logan Irvin, the long-time Chair of the Department of Biochemistry. Dr. Irvin eventually promoted Ms. Nye to Administrative Manager of the Department. She served in that capacity for many years and throughout the tenure of Dr. Mary Ellen Jones who was the Chair of the Department of Biochemistry from 1978-1989. In 1990, Ms. Nye was recruited to the Department of Pathology by Dr. Joe W. Grisham. Upon her arrival, she worked closely with Drs. Joe Grisham and Bill McLendon to integrate the operations of the Department of Pathology and of the Department of Hospital Laboratories to form the Department of Pathology and Laboratory Medicine. Since 1999, Ms. Nye has served alongside the current Chair of the Department of Pathology and Laboratory Medicine, Dr. J. Charles Jennette. At her recent retirement reception, Dr. Grisham commented on the years he worked with Ms. Nye – *“Nancy has worked diligently to enable the administrative staff members to develop their careers independently by acquiring new skills, and she has endeavored to provide the equipment and facilities which they need to apply the new skills to cope with the expanding scope and complexity of their jobs. She has expected high performance, but she asked no more from anyone than she gave of herself. She has been a model leader. Nancy has been a dedicated advocate of the academic goals of the university and the clinical responsibilities of the hospital; she has had a major role in counseling and supporting faculty, students, postdoctoral fellows, and resident scientists/physicians to cope with the pressures of maintaining a high level of performance in their responsibilities of teaching, clinical diagnosis and interpretation, and research. She has been a fierce and effective defender of gender and racial equality among faculty, students, and staff.”* Ms. Nye is a former recipient of the **Governor’s Award for Excellence** and the **C. Knox Massey Distinguished Service Award**, and recently received the **Order of the Long Leaf Pine**. In continuing recognition and honor of Ms. Nye’s contributions to the Department of Pathology and Laboratory Medicine, the **Nancy Hamilton Nye Award** has been established to recognize and reward Departmental administrative staff excellence. Today’s Annual Research Symposium honors Ms. Nye’s many years of dedication and service to the UNC School of Medicine and the Department of Pathology and Laboratory Medicine.



Friday, September 20, 2013 - G100 Bondurant Hall

Schedule of Events

- 8:55 Introduction and Welcoming Remarks**
Dr. J. Charles Jennette, Brinkhous Distinguished Professor of Pathology and Chair
- 9:00-11:00 Oral Presentations by Graduate Students and Postdoctoral Fellows**
Moderator: Maria Aleman
- Docking of Tumor-derived Microparticles to Activated Endothelium in Cancer-induced Venous Thrombosis*
Julia E. Geddings, Todd M. Getz, Mauricio Rojas, Lorenz Wacht, Maria M. Aleman, Alisa S. Wolberg, Wolfgang Bergmeier, and Nigel Mackman
- Regulation of Smooth Muscle Cell Differentiation by the RhoA Effectors mDia1 and mDia2*
Laura Weise Cross and Christopher P. Mack
- Malignant Progression in a Genetically Engineered Mouse Model of Astrocytomas Occurs Through Stochastic Acquisition of Secondary Mutations*
David Irvin, Ralf S. Schmid, Mark Vitucci, Andrea M. Werneke, Ryan E. Bash, and C. Ryan Miller
- Elevated Gamma Prime Fibrinogen Does Not Cause Arterial Thrombosis*
Bethany L. Walton, Todd M. Getz, Wolfgang Bergmeier, Shirley Uitte de Willige, and Alisa S. Wolberg
- Solution Structure and Dynamics of Methyl-cytosine Binding Protein 4 Suggests a Mechanism to Scan for Mismatched DNA*
Ninad M. Walavalkar, J. Neel Scarsdale, and David C. Williams Jr.
- Contribution of Red Cells to Thrombin Generation in Sickle Cell Disease (SCD)*
Matthew F. Whelihan, Micah J. Mooberry, Robert Bradford, Kenneth I. Ataga, Kenneth G. Mann, and Nigel S. Key
- 11:00-11:15 Break and Refreshments**
- 11:15-12:30 Keynote Presentation**
Moderator: Britta Jones
- Autoimmunity as Viewed from the Clinic*
Ronald J. Falk, M.D., Professor, Departments of Medicine and Pathology and Laboratory Medicine, UNC Kidney Center, UNC School of Medicine
- 12:30-2:00 Poster Session, Lunch and Refreshments**
12:30-1:15 Presentation of odd-numbered posters
1:15-2:00 Presentation of even-numbered posters
- 2:10 Awards Presentation**

Graduate Student and Postdoctoral Fellow Speaker Abstracts

Docking of Tumor-derived Microparticles to Activated Endothelium in Cancer-induced Venous Thrombosis

Julia E. Geddings¹, Todd M. Getz⁴, Mauricio Rojas², Lorenz Wacht³, Maria M. Aleman¹,
Alisa S. Wolberg^{1,2}, Wolfgang Bergmeier^{2,3}, Nigel Mackman^{1,2}

¹Department of Pathology and Laboratory Medicine, ²Division of Hematology/Oncology,
Thrombosis and Hemostasis Program, UNC McAllister Heart Institute, ³Department of Medicine,
⁴Department of Biochemistry and Biophysics, University of North Carolina School of Medicine,
Chapel Hill, NC

Venous thromboembolism (VTE) is a clinical term which refers to the development of deep vein thrombosis and pulmonary embolism. Cancer patients have a high rate of VTE but the mechanism of VTE development has not been fully elucidated. Microparticles (MPs) are small membrane vesicles that are released by many cell types, including cancer cells, and those that carry tissue factor (TF) are highly procoagulant. We hypothesize that tumor-derived MPs (TMPs) trigger VTE in cancer patients. We evaluated the role of different ligand-receptor interactions in the docking of TMPs to P-selectin, E-selectin and activated endothelial cells (ECs) *in vitro* and *in vivo*. We demonstrate that TMPs isolated from the human pancreatic cell line BxPc-3 bind to immobilized P-selectin and E-selectin. In addition, TMPs bind to human umbilical endothelial cells (HUVECs) in culture in an E-selectin and P-selectin dependent manner. BxPc3 TMPs also bound to ECs in mouse cremaster venules treated with TNF α . These TMPs enhanced thrombosis in an inferior vena cava stenosis model of venous thrombosis in a MP TF-dependent manner. Currently, we are evaluating if inhibition of P-selectin or E-selectin reduces TMP-enhanced thrombosis in mice. We propose that TMP-vascular cell docking interactions represent potential new therapeutic targets for the prevention cancer-induced thrombosis.

Regulation of Smooth Muscle Cell Differentiation by the RhoA Effectors mDia1 and mDia2

Laura Weise Cross and Christopher Mack

Department of Pathology and Laboratory Medicine, University of North Carolina School of
Medicine, Chapel Hill, NC

We have previously shown that the RhoA effectors mDia1 and mDia2 promote smooth muscle cell (SMC) differentiation by regulating nuclear localization of myocardin-related transcription factors. However, their role in determining SMC phenotype in animal models is unknown, and virtually nothing is known about the mechanisms that regulate their expression in SMCs. To assess the role of mDia signaling *in vivo*, we generated an SM22 Cre-based model to express DNmDia, which inhibits both mDia1 and mDia2. Using paraffin-embedded sections and western blotting, we assessed transgene expression, SM marker expression, and general morphology of SMC-rich tissues. To determine the role of mDia signaling in phenotypic switching, we subjected mice to vascular injury and used paraffin-embedded sections to analyze SM marker expression and neointima formation. A minority of transgenic mice die perinatally and/or exhibit a runted, hairless phenotype, which we are still characterizing. Transgenic mice that survived to adulthood were protected *against* neointima formation following carotid artery ligation. mDia2 is strongly, selectively expressed in SMC and is upregulated by S1P, suggesting that regulation of mDia2 expression may partially control SMC phenotype. Using DNase I hypersensitivity (DHS) methods, we identified an SMC-

specific open chromatin region in the mDia2 promoter with high, SMC-specific transcriptional activity, dependent upon a CArG-like element within this DHS. Gel-shift experiments showed that this CArG binds SRF and myocardin. We generated a LacZ transgenic mouse, driven by the mDia2 DHS. Although it alone did not display SMC-specific expression, we are trying to identify a larger region, including the DHS, which promotes SMC-expression *in vivo*.

Malignant Progression in a Genetically Engineered Mouse Model of Astrocytomas Occurs Through Stochastic Acquisition of Secondary Mutations.

David M. Irvin¹, Ralf S. Schmid², Mark Vitucci¹, Andrea M. Werneke³,
Ryan E. Bash³, and C. Ryan Miller¹⁻⁵

¹Curriculum in Genetics and Molecular Biology, ²Lineberger Comprehensive Cancer Center,
³Department of Pathology and Laboratory Medicine, ⁴Neurology, ⁵Neurosciences Center,
University of North Carolina School of Medicine, Chapel Hill, NC

Low grade astrocytomas (LGA) inevitably progress to higher grade astrocytomas (HGA), including the most malignant variant glioblastoma (GBM). The genetic alterations present in human LGA and HGA have been established; however, the timing and sequence of mutations acquired during malignant progression have yet to be accurately defined. Using conditional genetically engineered mouse (GEM) models with GFAP driven inactivation of *Rb* (I), constitutively activated *Kras* (*KrasG12D*, R), and/or *Pten* deletion (P), we have shown that LGA→GBM progression occurs in areas of high LGA burden in 81, 91, and 75% of TR, TRP^{+/-}, and TRP^{-/-} GEM and proves increasingly fatal (median 4.9, 4.3, and 2.5 months). Array comparative genomic hybridization (aCGH) shows recurrent gains of chromosome 6, which contains *Met*, *Kras*, and *Braf* oncogenes, in 100%, 64-74%, and 25% of HGA, but only 14% of LGA from these from these genotypes. Infrequent (<10%) copy number abnormalities (CNA) on other chromosomes are evident. In TRP^{+/-} mice, a time dependent increase in tumor burden is evident. Focal areas of hypercellularity with increased proliferative index as determined by Ki-67 staining and EdU pulse labeling, as well as longitudinal magnetic resonance imaging, show that HGA masses appear stochastically. Moreover, some mice develop with multiple genetically distinct HGA masses. This model accurately recapitulates the histology and genetic features of human astrocytomas and will be useful in further dissecting the genetics of malignant progression.

Elevated Gamma Prime Fibrinogen Does Not Cause Arterial Thrombosis

Bethany L. Walton¹, Todd M. Getz², Wolfgang Bergmeier^{2,3},
Shirley Uitte de Willige⁴, and Alisa S. Wolberg^{1,3}

¹Department of Pathology and Laboratory Medicine, ²Department of Biochemistry and Biophysics,
³McAllister Heart Institute, University of North Carolina school of Medicine, Chapel Hill, NC,
⁴Erasmus University Medical Center, Rotterdam, The Netherlands

Elevated levels of the acute phase plasma protein fibrinogen are associated with increased risk of thrombosis. Using mouse models of thrombosis, we have previously shown that elevated total fibrinogen levels increase fibrin formation rate, increase thrombus fibrin content, and promote arterial thrombosis. Fibrinogen is composed of two copies of three polypeptide chains: A α , B β , and γ . The fibrinogen γ chain undergoes alternative splicing, resulting in a dominant form (γ A/ γ A) that comprises ~90% of circulating fibrinogen and a minor species (γ A/ γ ') that comprises 8-15% of

circulating fibrinogen. Epidemiologic studies have detected elevated levels of $\gamma A/\gamma'$ in patients with a history of arterial thrombosis. However, *in vitro* data show $\gamma A/\gamma'$ has anticoagulant properties due to its ability to sequester thrombin. To determine whether $\gamma A/\gamma'$ is pro- or antithrombotic *in vivo*, we purified $\gamma A/\gamma A$ and $\gamma A/\gamma'$ from human plasma, raised circulating levels in mice, and determined the effects of increased levels of these proteins on arterial thrombosis *in vivo*. We also determined the effects of $\gamma A/\gamma A$ and $\gamma A/\gamma'$ on plasma clot formation, platelet aggregation, and endogenous procoagulant activity. Compared to controls, $\gamma A/\gamma A$ shortened the time to carotid artery occlusion, whereas $\gamma A/\gamma'$ did not. Both $\gamma A/\gamma A$ and $\gamma A/\gamma'$ could be cleaved by murine and human thrombin, were incorporated into murine and human clots, and supported murine and human platelet aggregation. When $\gamma A/\gamma A$ or $\gamma A/\gamma'$ was spiked into human or murine plasma, $\gamma A/\gamma A$ increased the fibrin formation rate to a greater extent than $\gamma A/\gamma'$. Interestingly, compared to controls, mice infused with $\gamma A/\gamma'$ had lower levels of plasma thrombin-antithrombin complexes following arterial injury, whereas mice infused with $\gamma A/\gamma A$ did not. These data suggest $\gamma A/\gamma'$ sequesters thrombin *in vivo*, and decreases circulating prothrombotic activity. Together, these findings indicate that elevated levels of $\gamma A/\gamma A$ promote arterial thrombosis *in vivo*, whereas $\gamma A/\gamma'$ does not.

Solution Structure and Dynamics of Methyl-cytosine Binding Protein 4 Suggests a Mechanism to Scan for Mismatched DNA

Ninad M. Walavalkar¹, J. Neel Scarsdale², and David C. Williams Jr.¹

¹Department of Pathology and Laboratory Medicine, University of North Carolina School of Medicine, Chapel Hill, NC, ²Institute of Structural Biology and Drug Design, Center for the Study of Biological Complexity and Massey Cancer Center, Richmond, VA

Having a C-terminal glycosylase domain in addition to N-terminal methyl-cytosine binding domain (MBD) makes methyl-cytosine binding protein 4 (MBD4) a unique member of the family of MBD proteins that recognize methylated cytosine-guanine (mCpG) dinucleotide. MBD4 serves as a potent DNA glycosylase in DNA mismatch repair specifically targeting TpG•CpG mismatches arising from spontaneous deamination of mCpG. The MBD4 MBD binds TpG and mCpG dinucleotide and thus could drive specificity for the glycosylase domain. Here, we present a solution structure of the MBD4 MBD bound to dsDNA. Based on chemical shift and binding analyses, we showed that the MBD4 MBD can bind methylated as well as unmethylated, hydroxymethylated, and mismatched (TpG) DNA with preference for mCpG. Further, we probed the dynamics of methylated DNA recognition by MBD4 and found that while MBD4 exchanges slowly between separate molecules of dsDNA (inter-molecular exchange), the domain exhibits fast exchange between two sites in the same molecule of dsDNA (intra-molecular exchange). This suggested that MBD4 prefers to move along the DNA using either sliding or hopping until it finds its target rather than searching through random three-dimensional diffusion. Introducing more bases or a single-stranded defect between two sites on the same DNA molecule did not affect the fast exchange rate, indicating that MBD4 likely uses a hopping mechanism for moving along the DNA. Furthermore, we demonstrated the effect of NaCl concentration on inter- and intra-molecular exchange of MBD4. Our findings suggest that the MBD4 MBD both targets the protein to CpG islands and assists in scanning for mismatched DNA.

Contribution of Red Cells to Thrombin Generation In Sickle Cell Disease

Matthew F. Whelihan¹, Micah Mooberry², Robert Bradford¹, Kenneth I. Ataga²,
Kenneth G. Mann³ and Nigel S. Key^{1,2}

¹Department of Pathology and Laboratory Medicine, ²Department of Medicine, and, University of North Carolina at Chapel Hill, Chapel Hill, NC, ³Department of Biochemistry, University of Vermont, Burlington, VT

1 in 625 African Americans are born with homozygous sickle-cell disease (HbSS) that is associated with vaso-occlusive manifestations (pain crises) of varying severity. Crises are generally ascribed to obstruction of the microvasculature secondary to decreased deformability of hypoxia-induced sickling of red blood cells (RBCs) and ensuing activation of coagulation and inflammatory pathways. We recently demonstrated that RBCs contribute a significant fraction (40%) of the thrombin generating potential of blood and that thrombin generation on RBCs proceeds through the meizothrombin (mIIa) intermediate. Due to the markedly enhanced phosphatidylserine (PS) expression by sickled RBCs, we hypothesized that mIIa production in HbSS patients would be significantly increased compared to a healthy control group. To test this hypothesis, we recruited 7 outpatients with HbSS, in their non-crisis, “steady states,” and 6 healthy African-American controls. TF-initiated thrombin generation was assessed over time using thrombin-antithrombin complex ELISAs. The rate of α TAT generation in the HbSS cohort was slightly faster (15%) than the control group, however both groups reached a similar maximum level. The rate of mTAT generation in the HbSS cohort was significantly faster (50%) than that observed in the control group and displayed a significantly higher (36%) maximum mTAT level. When the rates of mTAT formation were plotted against the relative PS concentration, correlation coefficients of 0.51 and 0.72 were observed for the HbSS and control groups, respectively. These data support the hypothesis that the increase in PS expression observed in HbSS red cells leads to increased thrombin generation through the mIIa intermediate. Although there was no statistical difference in the absolute α TAT levels between the two groups, the higher rate of mIIa generation and prothrombin consumption observed in the HbSS patients, together with their much lower RBC numbers, suggests that a much higher proportion of thrombin is generated on RBC surfaces.

Keynote Lecturer



Ronald J. Falk, M.D.

Department of Medicine, Division of Nephrology and Hypertension, UNC Kidney Center,
Department of Pathology and Laboratory Medicine,
University of North Carolina School of Medicine, Chapel Hill, NC

Dr. Ronald J. Falk is the Allan Brewster Distinguished Professor of Medicine, Chief of the Division of Nephrology and Hypertension in the Department of Medicine, Director of the UNC Kidney Center, and Director of the Center for Transplant Care at the University of North Carolina School of Medicine. Dr. Falk graduated from Dartmouth College, attended medical school at the University of North Carolina, was trained in internal medicine and nephrology at UNC, and completed a research fellowship at the University of Minnesota. Dr. Falk has been a member of the faculty at the UNC School of Medicine since 1983 and has been closely aligned with the Department of Pathology and Laboratory Medicine for many years (serving as research preceptor for six Molecular and Cellular Pathology graduate students since 1992). Dr. Falk is an exceptionally accomplished researcher with 200 published papers, 130 published reviews and editorials, and 285 published abstracts. Dr. Falk has served as Editor for 7 books, including five editions of *Primer on Kidney Diseases* and most recently for *Diseases of the Kidney and Urinary Tract, Ninth Edition* (2012). Dr. Falk has received numerous honors over the course of his career, including being selected to deliver the *Norma Berryhill Distinguished Lectureship* at UNC in 2011.

Dr. Falk's research probes questions focused on immune mediated kidney diseases, especially glomerulonephritis. His clinical and basic science interests include both ANCA glomerulonephritis and small vessel vasculitis (SVV). A central objective of his research is elucidating the causes of ANCA necrotizing and crescentic glomerulonephritis. Unraveling the cause of this disease requires considering a number of factors involved in the development of ANCA glomerulonephritis. Dr. Falk conceptualizes this process as opening the vasculitis lock with a key that has a number of "ridges and valleys" analogous to those factors that contribute to the development of this autoimmune disease. He participates in a research group that, in a large study over the last four years has revealed a number of avenues of investigation and new approaches to ongoing questions that pertain not only to ANCA glomerulonephritis, but to the general fields of autoimmunity, inflammation, and basic neutrophil and monocyte biology.

Keynote Presentation

Autoimmunity as Viewed from the Clinic

Ronald J. Falk, M.D.

Three common questions asked by patients with any autoimmune disease are: (1) How did I get it?; (2) What will make it go away?; and (3) What will prevent it from coming back? Stated differently, (1) What is the immunopathogenesis?; (2) What are the factors that are involved in disease pathogenesis?; and (3) What causes a relapse and what sustains a remission? We have tried to explore these questions.

For decades, we have sought to understand what are now called antineutrophil cytoplasmic autoantibody (ANCA)-associated small vessel vasculitides. Once described with a wide variety of pathologic and clinical descriptors, these diseases are now proving to be caused by autoantibodies that target antigens in both neutrophils and monocytes and the granule proteins myeloperoxidase or proteinase 3. There is now tremendous *in vitro* and *in vivo* evidence suggesting that MPO-ANCA or PR3-ANCA have pathogenetic potential, especially MPO ANCA. Mounting evidence suggests that these autoantibodies have different natural history, genetics, and disease pathogenesis, with beautiful animal models of MPO-ANCA disease, but of variable quality for PR3-ANCA disease.

What induces the autoimmune response in the first place? Much discussion in our laboratories has hypothesized various theories pertaining to the antigens themselves including antigen presentation, the possibility that there are complimentary antigens that spur the immune response system, and dysregulation of the autoantigen message with respect to gene silencing and gene activation.

We have learned that there is incredible specificity with respect to epitopes, at least on myeloperoxidase. There are epitopes that are associated with active disease that appear to be different than those found in remission, or even in those who have naturally occurring autoantibodies. Even in cases of so-called ANCA-negative disease, the responsible antigen(s) are being exposed. We have studied the T cell responses in this group of diseases and have learned that there are T effectors that proliferate and do not respond as well to T regulatory cells. In fact, these T regulatory cells appear ineffective. From a clinical perspective, we have learned that the drugs aimed at removing autoantibodies are as useful as more broad-based immunosuppressive drugs. We have learned a great deal about the pathogenesis of disease from both humans and in animal studies. Finally, we have been trying to determine what factors may affect disease relapse and remission. Where is this field headed, and what will propel this field forward? The answer is simple – we aim for a far better understanding about the basic immunopathogenesis in any given person, and the interaction between genes and their environment.

The ANCA experience asks another different basic question. Are there two general types of human autoimmune disease in which there is direct attack of antibody or cell on target tissue or, as in ANCA disease, a model in which there is an autoantibody or a cell that interacts with the circulating cell that attacks target tissue when activated? We have recently begun exploring this question in another autoimmune disease, minimal change disease. On light microscopic examination, there is no evidence for immune deposition and only podocyte disruption. Again, we are learning that there is an autoantibody that is directed against very immature circulating cells and that when these autoantibodies interact with this cell, the cell becomes activated. We still have much to learn about this disease process, but it raises the real possibility that an indirect autoimmune response occurs in humans where an attack on a circulating cell damages a distant target cell.

List of Poster Presentations by Predoctoral Students

1. *Fibrin Crosslinking is Required for Retention of Red Blood Cells in Venous Thrombi*
Maria M. Aleman, James R. Byrnes, Jian-Guo Wang, Nigel Mackman, Jay L. Degen, Matthew J. Flick, and Alisa S. Wolberg
2. *Optimizing Efficiency in Direct Cardiac Reprogramming*
Kim Bird and Li Qian
3. *Binding of Coagulation Factor XIII to Fibrinogen is Disrupted by Mutations Within γ -chain Residues 390-396*
James R. Byrnes, Maria M. Aleman, Daniel G. Isom, Jay L. Degen, Matthew J. Flick, and Alisa S. Wolberg
4. *Probing the Dynamic Distribution of Bound States for Methyl-cytosine Binding Domains on DNA*
Jason Cramer, J. Neel Scarsdale, Ninad M. Walavalkar, William A. Buchwald, Gordon D. Ginder, and David C. Williams Jr.
5. *Characterizing the Role of SLIRP in AR-Ack1 Signaling in Prostate Cancer*
Dinuka De Silva, Zhentao Zhang, Yuanbo Liu, H. Shelton Earp, and Young E. Whang
6. *Elucidating the Molecular Mechanisms of Direct Cardiac Reprogramming*
Ashley M. Fuller and Li Qian
7. *Pol η -dependent Translesion Synthesis: A Novel Target for Cancer Therapy*
Alicia Greenwalt, Victoria Korboukh, Emily Hull-Ryde, William Janzen, and Cyrus Vaziri
8. *Non-viral and Integration-free Delivery of Cardiac Reprogramming Factors GMT Using oriP/EBNA-1 Episomal Vectors*
Chuner Guo, Raluca Dumitru, and Li Qian
9. *DNA Methylation in Patients with ANCA Disease: Amplicon Design and Optimization*
Britta E. Jones, Akhil Muthigi, Theresa Swift-Scanlan, Ronald J. Falk, and Dominic J. Ciavatta
10. *The BAR Domain-containing Rho-GAP, GRAF1, Associates with the Endocytic Recycling Machinery During Skeletal Myoblast Fusion and is Necessary for proper Muscle Formation*
Kaitlin C. Lenhart, Christopher P. Mack, and Joan M. Taylor
11. *Regulation of NFAT by BMP Signaling*
Pamela Lockyer, Xinchun Pi, and Cam Patterson
12. *Tissue Resident Macrophages Regulate IL-23-dependent Granulopoiesis*
Lantz C. Mackey, Eliezer Perez, Jason Rose, and Jonathon W. Homeister
13. *RhoA GTPase Signaling in Vascular Smooth Muscle Cell Differentiation and Vessel Development*
Kevin D. Mangum and Christopher P. Mack
14. *MAPK and PI3K Signaling in Gliomagenesis and Response to Clinically Relevant Targeted Inhibitors*
Robert S. McNeill, Ralf S. Schmid, Ryan E. Bash, Mark Vitucci, Andrea M. Werneke, Kristen K. White, and C. Ryan Miller

15. *Transcriptomic Classification of Genetically Engineered Mouse Models of Breast Cancer Identifies Human Subtype Counterparts*
Adam D. Pfefferle, Jason I. Herschkowitz, Jerry Usary, J. Chuck Harrell, Benjamin T. Spike, Geoff M. Wahl, Jeffrey M. Rosen, and Charles M. Perou
16. *GSK3a and TBK1 Cooperate to Promote Akt Activation Downstream of Growth Factors*
Amanda L. Rinkenbaugh and Albert S. Baldwin
17. *An Intrinsically Unstructured Domain in MBD2 Recruits the Histone Deacetylase Core Complex of NuRD and Modifies Kinetics of DNA Binding*
Leander Sinanan, Megha Desai, Ninad M. Walavalkar, Gordon D. Ginder, and David C. Williams Jr.
18. *Muscle RING Finger-1 Promotes Nuclear Membrane Accumulation of TRa and Inhibits TRa Transcriptional Activity*
Kristine M. Wadosky, Makhosazane Zungu, and Monte S. Willis

Fibrin Crosslinking is Required for Retention of Red Blood Cells in Venous Thrombi

Maria M. Aleman¹, James R. Byrnes¹, Jian-Guo Wang¹, Nigel Mackman²,

Jay L. Degen³, Matthew J. Flick³, Alisa S. Wolberg¹

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Venous thrombi contain high levels of red blood cells (RBC) and fibrin, but little is known about the mechanisms regulating venous thrombus formation and composition. Fibrin acts as a scaffold for leukocytes and platelets that mediate thrombus formation, and cross-linked fibrin networks promote clot stability. Mice expressing a mutant form of fibrinogen with residues 390-396 mutated to a series of alanines (termed Fibr^{390-396A}) exhibit normal fibrin polymerization and normal hemostasis. In this study, we analyzed the role of this region of fibrinogen in a stasis-induced model of venous thrombosis. Following inferior vena cava ligation, Fibr^{390-396A} mice had 50% smaller thrombi than wild type (WT) mice. Reduced thrombus weight was not due to reduced thrombin generation and total neutrophil, platelet, and fibrin content within thrombi were similar between groups. Strikingly, Fibr^{390-396A} thrombi had significantly fewer RBCs than WT thrombi. To determine the mechanism, we developed an *ex vivo* whole blood clot retraction assay. Interestingly, although retraction of platelet-rich plasma clots was indistinguishable for Fibr^{390-396A} and WT mice, retraction of whole blood clots resulted in dramatically reduced RBC retention and smaller clots for Fibr^{390-396A} mice compared to WT. Control experiments determined that neither abnormal RBC function nor reduced RBC binding to Fibr^{390-396A} fibrinogen was involved. To test the hypothesis that the Fibr^{390-396A} mutation disrupts a specific interaction with the fibrin-stabilizing transglutaminase, factor XIII (FXIII), we analyzed levels of FXIII that co-precipitated with WT and Fibr^{390-396A} fibrinogen. Despite normal circulating levels of FXIII in Fibr^{390-396A} mice, FXIII co-precipitated with WT, but not Fibr^{390-396A}, fibrinogen. Compared to WT, plasma clots from Fibr^{390-396A} mice exhibited slower FXIII activation and consequently, slower fibrin crosslinking. Provocatively, whole blood from FXIII-deficient mice and humans phenocopied Fibr^{390-396A} clots, with reduced RBC retention following clot retraction. Taken together, these data identify critical residues in fibrinogen that mediate FXIII activation and fibrin crosslinking. Further, these studies suggest a critical, yet previously un-described, role for FXIII in mediating RBC retention within venous thrombi.

Optimizing Efficiency in Direct Cardiac Reprogramming

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Heart failure is the leading cause of death in the United States and results in nearly 60,000 deaths and \$34 billion in healthcare costs each year. Mammalian hearts have little ability to regenerate, so cell-based therapies are an attractive approach to replace lost cells due to injury. Cellular reprogramming holds promise to be an exciting alternative to traditional treatments for heart disease. Recent advances in the field of direct cardiac reprogramming utilize endogenous cardiac fibroblasts (CF) to generate functional cardiomyocyte-like cells *in vitro* and *in vivo* with introduction of three transcription factors: Gata4, Mef2c, and Tbx5 (GMT). CFs comprise more than half of the cells in the heart and therefore provide an abundant source for cardiac regeneration. However,

reprogramming efficiency with current methods is low, with only ~6% of GMT-transduced CFs becoming fully functional cardiomyocyte-like cells *in vivo* and less than 1% *in vitro*. It was previously shown that the proliferative status of cells is important in the generation of induced pluripotent stem cells (iPSC). Based on our preliminary data and literature on iPSC research, we hypothesize that proliferation also plays a critical role in the transdifferentiation of CFs into induced cardiomyocytes (iCMs). Using both genetic and pharmacological approaches, we are investigating the effects of proliferation on direct cardiac reprogramming efficiency and the underlying mechanisms.

Binding of Coagulation Factor XIII to Fibrinogen is Disrupted by Mutations Within γ -chain Residues 390-396

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During coagulation, fibrinogen is proteolytically cleaved to form fibrin monomers, which aggregate into a clot. Once activated, coagulation factor XIII (FXIII), a circulating protransglutaminase, cross-links and stabilizes the fibrin network. Previous studies have demonstrated that FXIII binds to fibrinogen with high affinity (Kd ~10 nM), but the residues on fibrinogen that mediate this interaction are uncharacterized. Using mice bearing a mutation in fibrinogen in which residues 390-396 of the γ -chain are converted to alanines (Fib $\gamma^{390-396A}$), we have identified proteins that coprecipitate with wild type (WT), but not Fib $\gamma^{390-396A}$, fibrinogen. Using mass spectrometry-based protein identification, the coprecipitates were identified as the A- and B-subunits of FXIII. This observation led us to hypothesize these residues mediate FXIII-fibrinogen interactions and this mutation disrupts the FXIII binding site on the γ chain. To elucidate the nature of this binding site, we developed an ELISA to measure binding of FXIII-A₂B₂ to adherent WT and Fib $\gamma^{390-396A}$ fibrinogen. Preliminary experiments suggest FXIII binds WT fibrinogen with ~10-fold higher affinity compared to Fib $\gamma^{390-396A}$ fibrinogen (Kd 0.011±0.001 μ M versus 0.10±0.03 μ M, respectively). We are also utilizing quantitative cysteine reactivity (QCR) to define the FXIII-fibrinogen interaction in solution. Pilot QCR experiments reveal that isotherms for fibrinogen and FXIII can be measured using this technique. Finally, we are creating a recombinant human form of Fib $\gamma^{390-396A}$ fibrinogen. Once generated, we will assess binding of FXIII to this fibrinogen by ELISA and QCR. We will also define the importance of FXIII-fibrinogen interactions on the viscoelastic and fibrinolytic properties of clots using thromboelastography and D-dimer release studies, respectively. We anticipate these studies will show how FXIII binding to fibrinogen promotes clot stability *in vitro* and *in vivo*. We propose that disrupting the FXIII-fibrinogen interaction may be a viable therapeutic approach for the prevention of thrombosis.

Probing the Dynamic Distribution of Bound States for Methyl-cytosine Binding Domains on DNA

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Although highly homologous to other methylcytosine binding domain (MBD) proteins, MBD3 does not selectively bind methylated DNA and thus the functional role of MBD3 remains in question. To explore the structural basis of its binding properties and potential function, we characterized the solution structure and binding distribution of the MBD3 MBD on hydroxymethylated, methylated and unmethylated DNA. The overall fold of this domain is very similar to other MBDs, yet a key loop involved in DNA binding is more disordered than previously observed. Specific recognition of methylated DNA constrains the structure of this loop and results in large chemical shift changes in NMR spectra. Based on these spectral changes, we show that MBD3 preferentially localizes to methylated and, to a lesser degree, unmethylated cytosine-guanosine dinucleotides (CpGs), yet does not distinguish between hydroxymethylated and unmethylated sites. Measuring residual dipolar couplings (RDCs) for the different bound states clearly shows that the MBD3 structure does not change between methylation specific and non-specific binding modes. Furthermore, RDCs measured for MBD3 bound to methylated DNA can be described by a linear combination of those for the methylation and non-specific binding modes, confirming the preferential localization to methylated sites. The highly homologous MBD2 protein shows similar but much stronger localization to methylated as well as unmethylated CpGs. Together, these data establish the structural basis for the relative distribution of MBD2 and MBD3 on genomic DNA and their observed occupancy at active and inactive CpG rich promoters.

Characterizing the Role of SLIRP in AR-Ack1 Signaling in Prostate Cancer

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Difficulty in treating prostate cancer arises when the tumor progresses to a castration resistant stage deemed CRPC. A hallmark of CRPC is the reemergence of Androgen receptor (AR) signaling due to sensitivity to low androgens, kinase activation, and a myriad of other factors. We focus on the reactivation of AR by tyrosine kinase Ack1 via phosphorylation of Y267. Examining the complexes that are bound to AR in the presence and absence of Ack1 revealed SLIRP as a potential interactor. We explored the role of SLIRP in AR signaling and its' connection to Ack1. SLIRP over-expression reduced AR transactivation while SLIRP knockdown increased AR transactivation in reporter assays. SLIRP knockdown resulted in an increase of endogenous levels of AR dependent genes PSA and hK2. SLIRP co-immunoprecipitates with AR but its' binding to AR is lost with AR activation by Ack1 or DHT. Our model illustrates inhibition of AR signaling by a SLIRP co-repressor complex that dissociates with Ack1 phosphorylation resulting in activation of transcription. Microarray studies with SLIRP knockdown shows a broad global change in gene expression. Additional analysis

of the microarray data steered us to look at cell cycle regulation. Using siRNA studies, we discovered that SLIRP knockdown increases phospho-Rb and cell cycle cyclins and cdks in LNCaP cells but not in AR negative PC3 and DU145 cells. Flow cytometry experiments with SLIRP knockdown increased the number of cells that entered S-phase in LNCaP cells. Data obtained demonstrates that SLIRP could function as a possible tumor suppressor that is lost in CRPC.

Elucidating the Molecular Mechanisms of Direct Cardiac Reprogramming

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Heart failure currently affects approximately 5 million people in the United States, and nearly 550,000 new cases are reported annually. The development of therapies to treat heart failure is challenging due to the limited intrinsic regenerative capacity of post-natal cardiac tissue. However, it has been recently reported that cardiac fibroblasts, which comprise approximately 50% of the mammalian heart, can be directly reprogrammed into functional cardiomyocyte-like cells by the introduction of the transcription factors *Gata4*, *Mef2C*, and *Tbx5* (*GMT*). Although this technique holds significant therapeutic potential, little is known about the molecular mechanisms that underlie the cardiac reprogramming process. As a step toward understanding these mechanisms, we seek to characterize the “molecular cornerstones” that occur during the conversion of murine cardiac fibroblasts to induced cardiomyocytes. To this end, we are generating a doxycycline-inducible system that will enable us to identify the temporal requirement for *GMT* expression during direct reprogramming. We will then examine the molecular and epigenetic features of the cells during that time period, such as sarcomere assembly, chromatin remodeling, and gene methylation status, in order to identify the changes associated with the temporal progression of reprogramming. Ultimately, we anticipate that the mechanistic knowledge gained from this study will be invaluable for improving the efficiency and clinical feasibility of cell reprogramming for cardiac regeneration.

Pol η -dependent Translesion Synthesis: A Novel Target for Cancer Therapy

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While DNA repair is beneficial in order for cells to survive DNA damage, inappropriate activation can cause detrimental effects. This concern is all too apparent in the field of chemotherapy, which utilizes DNA damaging agents such as cisplatin as a means to trigger cancer cell death. Platinating chemotherapeutics are widely used to treat a multitude of cancers including testicular, ovarian and bladder cancer. A significant problem facing the clinic today is the high dose of drugs, like cisplatin, required to effectively kill cancer cells. This resistance is, in part, due to decreased drug uptake, increased drug efflux and also the DNA damage tolerance pathway, Trans-Lesion Synthesis (TLS). Through the accurate recruitment of TLS polymerase eta (Pol η) to cisplatin-induced guanine dimer lesions (Pt-GG), cells are capable of surviving moderate levels of chemotherapy. It has been demonstrated the cells that inherently lack functional Pol η (Xeroderma Pigmentosum Variant-XPV) demonstrate significant sensitivity to genotoxins such as UV and cisplatin and that this phenotype is

restored by complementing with functional wild type Pol η . Due to the key role of TLS in tolerance of cisplatin-induced DNA damage, we propose to investigate Pol η as a novel drug target for combination therapy with platinating agents. Cisplatin targets rapidly dividing cells largely through DNA damage, resulting in intra-strand bulky adducts. In non-dividing cells, however, cisplatin accumulates and can result in replication-independent cell damage and injury. Off-target side effects, such as kidney failure and peripheral nerve damage, are significant limitations to treatment in the clinic. By targeting TLS regulation in cancer cells it is possible to lower the effective dose of cisplatin required to induce replication-dependent cell death, thereby lowering the risk of toxic side effects. Our working hypothesis is that inhibition of TLS through small molecule inhibitors will confer killing of rapidly dividing cancer cells by platinating drug. We have developed and completed a 100,000 compound small molecule drug screen aimed at inhibiting TLS in cancer cells. Our long-term goal is to validate the putative TLS inhibitors identified in the screen *in vitro* and determine their mechanism of action. We anticipate that TLS inhibitors will provide us with the tools necessary to overcome current limitations of platinating chemotherapeutics, as well as improve our knowledge of an important pathway in mutagenesis and chemoresistance.

Non-viral and Integration-free Delivery of Cardiac Reprogramming Factors GMT Using oriP/EBNA-1 Episomal Vectors

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The field of cardiac regeneration has witnessed rapid advances in the past few years. We and others reported the generation of murine induced cardiomyocytes (iCMs) from cardiac fibroblasts (CFs) using cardiac reprogramming factors Gata4, Mef2c, and Tbx5 (GMT), which marked an exciting path to regenerating damaged hearts. However, current reprogramming approaches involve the introduction of GMT via retroviral transduction, which delivers the factors by genomic integration. Because integration occurs randomly, adverse effects such as disruption of biologically important endogenous genes cannot be ruled out. Our study aims to develop an integration-free delivery method for iCM generation. We intend to take advantage of episomal oriP/EBNA-1 vectors, which are large plasmids that reside and replicate in the host nuclei extrachromosomally, thus resolving the issue of integration. To this end, we generated episomal vector constructs containing the reprogramming factors Gata4, Mef2c, and Tbx5. Using a dsRed control vector, we optimized the delivery conditions for primary neonatal mouse CFs using the Neon Transfection System. Our preliminary results using fluorescence microscopy and flow cytometry suggest that the episomal system is able to confer robust expression in murine cells. In addition, the expression was found to persist for at least one week. Coupled with appropriate antibiotic selection, the episomal system would potentially confer a high reprogramming efficiency. Further characterization of iCMs using this integration-free method is underway. Taken together, our data suggest that the episomal system can be used as an alternative approach for safer generation of iCMs, making iCM technology closer to future clinical application.

DNA Methylation in Patients with ANCA Disease: Amplicon Design and Optimization

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In patients with ANCA disease the autoantigen-encoding genes, *PRTN3* and *MPO*, are inappropriately expressed. DNA methylation is a critical epigenetic mark associated with gene silencing. We propose to test whether changes in DNA methylation occur in ANCA disease patients and if this explains aberrant *PRTN3* and *MPO* expression. We will analyze DNA methylation changes at specific loci of interest using the Sequenom[®] EpiTYPER platform. Bisulphite-treated genomic DNA is PCR amplified to interrogate regions of interest. The PCR product is transcribed into RNA, cleaved in a base-specific manner, and cleavage products are analyzed by MALDI-TOF MS, which detects quantitative mass differences between methylated and unmethylated DNA. We tested multiple protocols using the Zymo EZ DNA Methylation[™] kit to obtain the highest concentration and quality of bisulfite-treated DNA as measured by NanoDrop spectrophotometer and Qubit[®] fluorometer. We designed seven primer pairs to amplicons at four different genes of interest: *MPO*, *PRTN3*, *RUNX3*, and *FOXP3*. PCR conditions for each primer pair were optimized on bisulfite-treated DNA to determine the ideal annealing temperature for amplification. A 57°C annealing PCR protocol was optimal for six of the seven pairs while our second *MPO* primer pair amplified best with a 60°C annealing PCR protocol. These seven pairs were used to generate amplicons from three ANCA disease patients and three healthy controls, which will be analyzed with Sequenom[®] EpiTYPER platform. Moving forward, we will analyze a unique cohort of ANCA disease patients in order to compare DNA methylation between disease classes and disease activity states as well as drug treatments.

The BAR Domain-containing Rho-GAP, GRAF1, Associates with the Endocytic Recycling Machinery During Skeletal Myoblast Fusion and is Necessary for Proper Muscle Formation

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Recent studies indicate that RhoA plays a significant role in skeletal muscle maturation as its activity must be tightly regulated to allow for proper differentiation and fusion of skeletal myoblasts. However, how RhoA controls skeletal muscle maturation and what factors influence RhoA activity during this process are still largely unknown. We show that the BAR domain-containing Rho-GAP, GRAF1, is particularly abundant in perinatal mammalian skeletal muscle undergoing fusion to form multinucleated muscle fibers. In support of a critical role for GRAF1 in muscle formation, depletion of GRAF1 in a novel mouse model resulted in a decrease in myofiber size. As well, our studies in C2C12 skeletal myoblasts indicated that GRAF1 induced cell membrane branching and elongation of myoblasts, and subsequently promoted fusion in both a GAP- and BAR-dependent manner. This dual role suggests that GRAF1 facilitates both actin- and membrane-based dynamics to regulate skeletal muscle cell fusion. Therefore, we hypothesize that GRAF1 mediates the intracellular trafficking of so-called “fusogenic” proteins to pre-fusion complexes to promote membrane

coalescence. It has been previously shown that pre-fusing skeletal myoblasts express a family of endocytic recycling proteins, termed ferlins, which regulate myoblast fusion and have been implicated in various skeletal and cardiac myopathies. We have shown by immunoprecipitation and immunohistochemical techniques that GRAF1 associated with these ferlin proteins in pre-fused myoblasts. Additionally, our preliminary data indicated that GRAF1 is necessary for the proper localization of fusogenic proteins at pre-fusion complexes, implicating the role for GRAF1 in skeletal muscle cell fusion. Currently, we aim to explore the mechanism by which GRAF1 regulates ferlin trafficking and the consequence this has on the fusogenic potential of muscle.

Regulation of NFAT by BMP Signaling

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Dysfunction of the vascular endothelium results in various cardiovascular, circulatory, and blood diseases, and exemplifies the importance of endothelial integrity. Bone morphogenetic protein (BMP) signaling has been identified as a vital component in the vascular response to stress. In keeping with this, BMP signaling is regulated at many different levels, and our lab has previously characterized mechanisms of BMP regulation by BMP-binding endothelial regulator (BMPER) protein. Interestingly, recent data has shown BMPER can elicit cellular effects that are independent of BMP signaling. Microarray analysis revealed 80 upregulated genes and 108 downregulated genes that are not associated with BMP. Nuclear factor of activated T cells (NFAT) is one of the genes found to be most highly upregulated by BMPER. Therefore we believe BMPER is an important regulator of NFAT and want to determine the effect of BMPER on calcium-dependent NFAT activation. Our data shows the translocation of NFATc1 from the cytoplasm to the nucleus following treatment of mouse endothelial cells (MEC) with BMPER. Additionally, when human umbilical vein endothelial cells (HUVECs) are treated with BMPER, an increase in total NFATc1 protein occurs approximately 1 hour post treatment. This data supports the idea that BMPER can regulate NFAT, but requires more investigation to determine if BMPERs regulation is through dependent or independent BMP signaling pathways.

Tissue Resident Macrophages Regulate IL-23-dependent Granulopoiesis

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Mice deficient in $\alpha(1,3)$ -fucosyltransferases 4 and 7 ($FUT^{-/-}$) lack selectin ligand activity and selectin-dependent leukocyte trafficking, resulting in altered granulopoiesis. It is not clear if the altered granulopoiesis is dependent on bone marrow-derived macrophages (BM-MCs) or a distinct population of tissue resident macrophages. We sought to determine the role of BM-MCs and tissue resident macrophages in regulating granulopoiesis. We hypothesized that the loss of selectin dependent trafficking in $FUT^{-/-}$ mice, reduces the number of neutrophils able to infiltrate into the tissues, undergo apoptosis, be taken up by tissue resident macrophages, and suppress their IL-23 production. Primary tissue resident Kupffer cells (pKC) and BM-MC were isolated from $FUT^{-/-}$ and wild type (WT) mice, stimulated *in vitro* with LPS, and assessed for IL-23 production by qPCR. Additionally, WT mice were irradiated and their hematopoietic system was reconstituted with 1×10^6 bone marrow cells isolated from WT or $FUT^{-/-}$ mice. Six weeks after reconstitution circulating WBC

counts were assessed via flow cytometry. There was no difference in gene expression levels of IL-23 between WT and FUT^{-/-} pKCs or BM-MCs, indicating that IL-23 production from both populations of FUT^{-/-} macrophages are intact. WT mice reconstituted with FUT^{-/-} bone marrow show a pronounced neutrophilia similar to that seen in FUT^{-/-} mice. These results confirm that FUT-dependent neutrophil trafficking is required for the regulation of granulopoiesis. Additionally, our results suggest that the tissue resident macrophage population may be the major phagocytic cell population responsible for clearing apoptotic neutrophils and regulating IL-23 production in granulopoiesis.

RhoA GTPase Signaling in Vascular Smooth Muscle Cell Differentiation and Vessel Development

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Proper vascular smooth muscle cell (SMC) function, which is governed by the expression of critical SMC-specific genes, underlies normal blood vessel physiology and development. Interestingly, by modulating expression of SMC-specific genes, SMCs are able to maintain and/or revert to a more synthetic phenotype, away from the more mature, contractile state. Such phenotypic plasticity underlies several significant vascular diseases, including hypertension and atherosclerosis. One of the central pathways regulating SMC function and differentiation is the RhoA/ROCK signaling axis, perturbations in which are linked to vascular disease. RhoA itself is regulated by GEFs, which promote GTP-RhoA, and GAPs, which enhance RhoA's intrinsic GTPase activity, leading to inactive GDP-RhoA. Using mutant G17A RhoA-GST, we identified the active GEF, Trio, in 10T1/2 precursor SMCs. Trio signals through the G α q family of GPCRs that stimulate RhoA in response to specific agonists (e.g., angiotensin II). Here we show that Trio positively regulates SMC differentiation and migration during early vessel development. Secondly, based on preliminary data showing that the RhoA-GAP, GRAF3, exhibits selective SMC expression, in combination with DNase I hypersensitive site (DHS), SRF ChIP-seq, ENCODE, and GWAS data sets, we have identified SNPs associated with hypertension within likely enhancer regions in the GRAF3 gene that selectively affect DHS promoter activity. Lastly, we demonstrated that a DHS in the ROCK2 gene displays SMC-specific promoter activity. Taken together, we hypothesize that integrated regulation of these specific RhoA/ROCK components are critical for SMC differentiation and function, and that aberration in any one of these modulators underlies common vascular diseases.

MAPK and PI3K Signaling in Gliomagenesis and Response to Clinically Relevant Targeted Inhibitors

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Glioblastoma (GBM) is a high-grade astrocytoma with a median survival of 12-15 months. The vast majority (92%) of GBM have receptor tyrosine kinase (RTK) mutations and/or mutations in the RAS/MAPK and PI3K effector arms of RTK signaling. Based on this mutational landscape, targeted inhibitors, including the MEK inhibitor AZD6244 and the PI3K inhibitor BKM120, are

currently being investigated as monotherapies in clinical trials for GBM. To investigate the role of RAS and PI3K signaling in gliomagenesis and response to targeted inhibitors, we developed an orthotopic allograft model of GBM using AdCre-infected primary astrocytes from a conditional genetically engineered mouse model (TRP) with inactivated Rb- (T) and Pten- (P), and constitutively activated Kras- (R). We have shown that R and P mutations activate MAPK and PI3K signaling and cooperate during GBM pathogenesis by inducing proliferation, migration, and invasion *in vitro*. Inhibition of both MAPK and PI3K signaling is required for maximal reduction of migration and invasion. Moreover, these mutations cooperate *in vivo* to enhance malignant progression to lethal GBM. TRP astrocytes are sensitive to AZD6244 or BKM120 *in vitro*. AZD6244 reduces phosphorylation of Erk, but induces a compensatory increase in PI3K signaling (Akt phosphorylation). BKM120 reduces AKT phosphorylation, but induces a compensatory increase in MAPK signaling (Erk phosphorylation). In contrast, dual inhibition of both MAPK and PI3K signaling in TRP astrocytes with AZD6244 and BKM120 is synergistic at clinically achievable low micromolar concentrations. We conclude that dual inhibition of both MAPK and PI3K signaling may be required for maximal efficacy in GBM clinical trials.

Transcriptomic Classification of Genetically Engineered Mouse Models of Breast Cancer Identifies Human Subtype Counterparts

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Human breast cancer is a heterogeneous disease consisting of multiple molecular subtypes. Genetically engineered mouse models (GEMMs) are a useful resource for studying mammary cancers *in vivo* under genetically controlled and immune competent conditions. Identifying murine models with conserved human tumor features will facilitate etiology determinations, highlight the effects of mutations on pathway activation, and should improve preclinical drug validation. Transcriptomic profiles of 27 murine models of mammary carcinoma and normal mammary tissue were determined using gene expression microarrays. Hierarchical clustering analysis identified 17 distinct murine subtypes (classes). Across species analyses using three independent human breast cancer datasets identified eight murine classes that resemble specific human breast cancer subtypes. Multiple models were associated with human basal-like tumors including TgC3(1)-*Tag*, TgWap-*Myc*, and *Trp53*^{-/-}. Interestingly, the TgWAPCre-*Etv6* model mimicked the HER2-enriched subtype, a group of human tumors without a murine counterpart in previous comparative studies. Gene signature analysis identified hundreds of commonly expressed pathway signatures between linked mouse and human subtypes, highlighting potentially common genetic drivers of tumorigenesis. This study of murine models of breast carcinoma encompasses the largest comprehensive transcriptomic dataset to date to identify human-to-mouse disease subtype counterparts. This approach illustrates the value of comparisons between species to identify murine models that faithfully mimic the human condition and indicates that multiple GEMMs are needed to represent the diversity of human breast cancers. These trans-species associations should guide model selection during preclinical study design to ensure appropriate representatives of the human disease subtypes are used.

GSK3 α and TBK1 Cooperate to Promote Akt Activation Downstream of Growth Factors

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Growth factor signaling is an essential component of normal cell biology and its dysregulation can contribute to several diseases, including cancer and diabetes. The PI3K pathway, especially Akt, propagate many of these signals, through both activation and inhibition of many downstream effectors. GSK3, consisting of α and β isoforms, is a well-established Akt target and is phosphorylated at Ser21/9 to inhibit its activity. While the two isoforms are redundant in some contexts, there is evidence for specific roles for GSK3 α or β in other situations. TBK1, typically associated with innate immunity, has been tied to oncogenic signaling, especially in combination with mutant *Ras*. Recent reports have independently shown roles for GSK3 α and TBK1 in the activation of Akt. We hypothesize that GSK3 α and TBK1 cooperate in this process, accounting for the similar Akt phenotypes. Using *GSK3 α* knockout MEFs, we have shown diminished Akt activation downstream of IGF-1, EGF, insulin, and glucose. *In vitro* kinase assays suggest TBK1 phosphorylates GSK3 α at Ser21, potentially inhibiting GSK3 α to relieve inhibition of Akt. Through endogenous co-immunoprecipitation we have shown that Akt and TBK1 interact. Treatment with IGF-1 increases the interaction, while *GSK3 α* deletion decreases it. We propose a model in which GSK3 α serves as a scaffold to bring TBK1 into a complex with Akt, leading to its full activation downstream of a number of stimuli. These results provide a novel mechanism for regulation of Akt activation. Intriguingly, this places GSK3 α upstream of Akt, though it has traditionally been thought to be a downstream effector.

An Intrinsically Unstructured Domain in MBD2 Recruits the Histone Deacetylase Core Complex of NuRD and Modifies Kinetics of DNA Binding

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MBD2 specifically recognizes methylated DNA and recruits the NuRD chromatin remodeling complex to silence expression of the associated gene. We recently solved the structure of a coiled-coil interaction between MBD2 and p66 α that recruits the Mi2 nucleosome remodeling protein to the complex (Gnanapragasam *et al.*, *Proc Natl Acad Sci.* 2011, 108:7487). In subsequent work, we have shown that a region between the methyl-cytosine binding (MBD) and the coiled-coil domains of MBD2 stably binds the histone deacetylase core complex of NuRD comprised by HDAC1/2, MTA1/2, and RbAp46/48. NMR and CD studies demonstrate that this region is largely unstructured even in the context of full-length protein and while bound to methylated DNA. Immunoprecipitation studies show that the critical determinant of protein binding resides within two small molecular recognition fragments (MoRFs) that together are sufficient to recruit the core deacetylase complex. Previous analyses showed very rapid association and dissociation rates for the isolated MBD. In contrast, incorporation of the intrinsically unstructured domain reduces the association rate and markedly prolongs dissociation from methylated DNA. Therefore, this

intrinsically unstructured domain functions both to recruit a large portion of the NuRD complex and stabilize interaction with methylated DNA.

Muscle RING Finger-1 Promotes Nuclear Membrane Accumulation of TR α and Inhibits TR α Transcriptional Activity

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Nuclear receptor transcription factors regulate critical metabolic processes in the myocardium; such that perturbation of this regulation is common in cardiac disease states. We previously identified that the muscle-specific protein Muscle RING Finger-1 (MuRF1) mono-ubiquitinates PPAR α to inhibit its transcriptional activity in cardiomyocytes. To determine if MuRF1 regulates other nuclear receptors, we tested the hypothesis that MuRF1 inhibits thyroid receptor- α (TR α), the predominant TR subtype in the heart. We used thyroid receptor response element (TRE)-driven luciferase assays to measure TR α transcriptional activity. In the presence of increased MuRF1 expression, T3-dependent TR α activity was significantly inhibited, suggesting that MuRF1 inhibits the T3-dependent TR α activity. We hypothesized that MuRF1-dependent inhibition of TR α relies on promotion of TR α nuclear export. Surprisingly, immunoblot of nuclear lysates showed that MuRF1 induced TR α nuclear accumulation (instead of export) and that MuRF1's ubiquitin ligase activity was required for this accumulation. Immunofluorescence experiments showed that MuRF1 promotes TR α localization specifically to the nuclear membrane in cardiomyocytes and that MuRF1 and TR α co-localize in that region; suggesting that MuRF1 sequesters TR α to the nuclear membrane. Co-immunoprecipitation and GST pull-down experiments confirmed that MuRF1 and TR α physically interact and showed that MuRF1 specifically binds to the DNA binding domain of TR α . These data suggest that MuRF1 may interfere with TR α 's ability to bind DNA. To our knowledge, this is the first report of TR α 's localization to the nuclear membrane interfering with its ability to bind DNA. Together, this study shows that MuRF1 regulates TR α in cardiomyocytes via novel mechanism.

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Focal Adhesion Kinase Antagonizes Doxorubicin Cardiotoxicity Via p21^{Cip1}

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Clinical application of potent anthracycline anticancer drugs, especially doxorubicin (DOX), is limited by a toxic cardiac side effect that is not fully understood and preventive strategies are yet to be established. Studies in genetically modified mice have demonstrated that focal adhesion kinase (FAK) plays a key role in regulating adaptive responses of the adult myocardium to pathological stimuli through activation of intracellular signaling cascades that facilitate cardiomyocyte growth and survival. The objective of this study was to determine if targeted elevation of myocardial FAK activity could protect the heart from DOX-induced de-compensation and to characterize the underlying mechanisms. Mice with myocyte-restricted FAK knock-out (MFKO) or myocyte-specific expression of an active FAK variant (termed SuperFAK) were subjected to DOX treatment. FAK depletion enhanced susceptibility to DOX-induced myocyte apoptosis and cardiac dysfunction, while elevated FAK activity provided remarkable cardioprotection. Our mechanistic studies reveal a heretofore unappreciated role for p21 in the repression of Bim and the maintenance of mitochondrial integrity and myocyte survival. DOX treatment induced proteasomal degradation of p21, which exacerbated mitochondrial dysfunction and cardiomyocyte apoptosis. FAK was both necessary and sufficient for maintaining p21 levels following DOX treatment and depletion of p21 compromised FAK-dependent protection from DOX. These findings identify p21 as a key determinant of DOX resistance downstream of FAK in cardiomyocytes and indicate that cardiac-restricted enhancement of the FAK/p21 signaling axis might be an effective strategy to preserve myocardial function in patients receiving anthracycline chemotherapy.

Cardiac Peroxisome Proliferator-activated Receptor - γ Activity is Upregulated in the Absence of Muscle RING Finger-2 and is Protective Against High-fat Diet-induced Cardiomyopathy

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Diabetic cardiomyopathy is characterized by structural and functional alterations leading to cardiac hypertrophy and heart failure. These alterations include metabolic disturbances in energy metabolism, increased lipid oxidation, intramyocardial triglyceride accumulation, and reduced glucose oxidation. These underlying changes are linked to peroxisome proliferator activating receptor (PPAR) signaling. We recently identified that mice lacking the ubiquitin ligase Muscle RING Finger 2 (*MuRF2*^{-/-}) have increased cardiac PPAR- γ activity *in vivo*. Therefore, we hypothesized that challenging *MuRF2*^{-/-} mice with a high fat diet, where fat (lipids) act as PPAR ligands, would enhance the severity of the diabetic cardiomyopathy due to their increased cardiac PPAR- γ activity. *MuRF2*^{-/-} mice and strain-matched controls were fed a high-fat diet (HFD) comprised of 60% fat, 20% protein, and 20% carbohydrates. Body weight, serum insulin, and serum glucose levels were measured every two weeks and conscious echocardiography was performed

every three weeks. Surprisingly, *MuRF2*^{-/-} body weights were significantly decreased at 6 weeks of HFD compared to strain-matched controls before the onset of insulin resistance. By echocardiography, left ventricular mass (LV Mass) increased by 6 weeks in in both groups, but no significant changes in cardiac function were identified ($54.3 \pm 0.8\%$ versus $50.7 \pm 3.9\%$ in *MuRF2*^{-/-} versus wild-type, respectively). Since MuRF2 is found in skeletal muscle as well as the heart, metabolic or protein synthesis alterations in the *MuRF2*^{-/-} skeletal muscle are present which are independent of MuRF2's potential role in diabetic cardiomyopathy, which begins at 13-15 weeks in this model. These findings may suggest a novel role of MuRF2 in regulating HFD-induced skeletal muscle mass pre-diabetes, where its role in cardiomyopathy is still being followed.

Multi-center Comparison of Testosterone Measurements Using Immunoassay and Mass Spectrometry

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Measurement of circulating testosterone is important in the evaluation of endocrine function as well as in the investigation of androgen disorders. These investigations encompass all ages and include females as well as males. Thus analytical methods must be able to span a broad analytical measuring range (AMR), typically 1-1000 ng/dL. The objective of this study was to compare the analytical performance of a micro-well competitive testosterone immunoassay with a testosterone measurement using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to determine the suitability of this immunoassay for all populations. Serum samples (n=45) submitted for routine testosterone evaluation were used for this comparative study as part of ongoing quality initiatives. Testosterone measurements were performed by competitive immunoassay and LC-MS/MS. Comparative analysis of testosterone VITROS automated immunoassay with testosterone LC-MS/MS yielded the regression equation: VITROS = 0.67x LC-MS/MS + 5.9, ($R^2 = 0.976$). These data demonstrated the VITROS immunoassay has a proportional bias that included a positive bias at reportable testosterone levels of ≤ 35 ng/dL and a negative bias at the higher end of the AMR as compared to the LC-MS/MS method. While the VITROS automated immunoassay is a sufficiently sensitive and specific assay for the measurement of testosterone in the adult healthy male, its use in the pediatric and female populations may be problematic in the ranges necessary for these populations. This study further supports the need for the development of communicable standards as well as harmonization of testosterone measurement procedures.

Ellagic Acid Induces Apoptosis and Cell Cycle Arrest in HeLa Cells and Inhibits HPV Oncogene Expression

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Ellagic Acid is a naturally occurring compound that demonstrates anti-cancer activity *in vitro* and *in vivo*. Ellagic acid inhibits protein kinase CK2, which has been identified as a promoter of tumorigenesis. Blocking CK2 activity can reactivate dormant cellular defensive mechanism and further block signaling pathways that induce cancer. In this study, we are testing HPV 18 positive cervical cancer cells (HeLa), to investigate the effect of ellagic acid on CK2 kinase activity, HPV oncogenes E6 and E7 expression and downstream targets leading to cell death *in vitro* and *in vivo*.

Treating HeLa cells with ellagic acid induced *in vitro* cell cycle arrest and caspase mediated apoptosis in both time and dose dependent manner. Ellagic acid inhibited CK2 phosphorylation activity, as well as the expression of viral oncogenes E6 and E7. Inhibition of HPV oncogene expression was accompanied by an increase in P53 and a decrease in cyclin A expression. Ellagic acid treatment induced cytochrome c release in the cytosol and subsequent activation of caspase 3, which led to the fragmentation of Poly (ADP) Ribose Polymerase (PARP) at the end of the caspase cascade. Finally, mice treated with ellagic acid, demonstrated inhibition of cervical cancer xenograft growth, and longer survival compared to vehicle-treated mice, confirming the potent anti-tumor activity of ellagic acid. Our results suggest that ellagic acid has anti-tumor and anti-viral properties which could make it suitable for the prevention of HPV induced cervical carcinoma.

Characterization of Microparticle Numbers and Cellular Origin in Human Endotoxemia Using High-sensitivity Flow Cytometry

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Endotoxemia causes acute systemic inflammation and activation of coagulation. Circulating microparticles (MPs), particularly those bearing tissue factor (TF⁺-MPs), have been studied in a human endotoxemia model and increase acutely after endotoxin administration. We hypothesized that MPs detected by flow cytometry would increase in parallel with MP-TF activity in this model, and that the magnitude of increase would vary by cellular origin. Sixteen subjects were infused with LPS (4 ng/kg), with blood collected at baseline, 3-, 6- and 24-hours post infusion. MP-TF activity was determined using a chromogenic assay detecting TF-dependent Xa generation on the MP fraction of plasma. MP enumeration and characterization were performed with high-sensitivity flow cytometry using a size gate of 0.2-0.9 μm . Total MPs, platelet MPs (PMPs), monocyte MPs (MMPs), RBC MPs (RMPs), and endothelial MPs (EMPs) were measured. Circulating nucleosomes (DNA-histone complexes) and thrombin anti-thrombin (TAT) complexes were measured by ELISA. MP-TF activity and TATs increased with a peak at 3 hours ($p < 0.001$), with a similar pattern seen with circulating nucleosomes ($p < 0.01$). MPs detected by flow cytometry peaked later (at 6 hours) and was significant for total MPs ($p < 0.05$) and PMPs ($p < 0.01$). Similar trends were noted for RMPs ($p = 0.090$) and MMPs ($p = 0.225$) but did not reach significance. These data further characterize the pathophysiologic processes seen in human endotoxemia. Acute increases in MPs and nucleosomes following endotoxin exposure may contribute to the observed activation of coagulation, and thus the thrombotic risk associated with certain inflammatory disorders such as sepsis.

Predicting Respiratory Distress Syndrome Using Gestational Age and Lamellar Body Counts

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We aimed to develop a predictive model for assessing the risk of developing respiratory distress syndrome (RDS) using gestational age (GA) and lamellar body counts (LBC) performed using the Advia 120 hematology system (Siemens Healthcare Diagnostics, Inc.). We conducted a

retrospective cohort study of patients who received a trans-abdominal amniocentesis with LBC analysis between January 2003 and May 2012. Based upon institutional studies, LBCs ≥ 35000 counts/ μL are considered indicative of probable maturity. Maternal and neonatal medical records were reviewed for obstetrical and neonatal outcomes. A standardized definition was used to define RDS, using clinical, laboratory and radiographic findings. Using these data, logistic regression (Stata Corp, College Station, TX) was used to predict the risk of RDS at each week of gestation based upon the LBC. 357 patients were included in the analysis. The mean GA at time of sample was 36 6/7 weeks gestation (SD 2.0). The median time between sample collection and delivery was 1 day (IQR 1-6). 31.4% of patients had preexisting or gestational diabetes, 10.6% had polyhydramnios and 62.2% were born via cesarean section. There were 18 cases (5%) of RDS. The risk of RDS was found to increase with decreasing GA and with decreasing LBC. Gestational age-specific predicted risk of RDS using LBC provides a statistical model which can aid clinicians in individually counseling patients regarding the absolute risk of their newborn developing RDS. This information will be especially useful for those whose laboratories utilize the Advia 120 for the measurement of LBC.

Spontaneous Colitis in a Recombinant Inbred Line of the Murine Collaborative Cross

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A newly established recombinant inbred line of mice was identified as having an increased incidence of rectal prolapses and reduced reproductive performance. Thirty-two retired breeders, over the age of 20 weeks and exhibiting these clinical signs, were selected for diagnostic necropsy. Marked splenomegaly, enlarged, cystic mesenteric lymph nodes, and thickened, opaque colonic walls were found in thirty-one mice (96.7%). The colonic thickening was most significant in the proximal and transverse colon but extended to the rectum on a subset of animals. Histologic findings included severe hyperplasia of the colonic epithelium with loss of mucous cells, mucosal ulcers, and occasional mucinous lakes at the bases of crypts. The lamina propria was distended with edema and a moderate to severe lymphoplasmacytic infiltrate. Adjacent mesenteric vessels exhibited varying degrees of multifocal arteritis. The spleens displayed marked lymphoid hyperplasia. Other tissues were grossly and histologically unremarkable. As the majority of existing murine models of inflammatory bowel disease (IBD) are associated with known genetic mutations altering mucosal immunity, epithelial barrier function, or an aberrant host response to the intestinal microflora, animals underwent extensive testing for microbial pathogens and parasites often associated with murine colitis. Mice were housed in sterile individually-ventilated cages and their health status monitored quarterly with dirty bedding sentinel mice which were continuously negative for commonly excluded murine pathogens and parasites. Additionally, feces from individual affected mice were negative for *Helicobacter* species by PCR and *Citrobacter rodentium* by repeated fecal culture. Given the propensity of this line to develop severe, proliferative colitis in the absence of known murine pathogens, this newly developed recombinant inbred line is a potentially valuable new model of IBD. Efforts are currently under way to determine the genetic loci associated with this line's susceptibility to colitis using the unique resources of the Collaborative Cross.

Rad18 Functions in Hematopoietic Genome Maintenance

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In cultured mammalian cells, the E3 ubiquitin Ligase Rad18 contributes to activation of several genome maintenance processes including Trans-Lesion Synthesis (TLS), Homologous Recombination (HR), and the Fanconi Anemia (FA) pathway. However, physiological roles of Rad18 and its effector pathways in DNA damage tolerance, genome maintenance and tumor suppression *in vivo* have not been studied. Because the FA pathway promotes DNA damage tolerance in Hematopoietic Stem Cells (HSC) we investigated potential roles of Rad18 in hematopoietic genome maintenance. Isolated CD34⁺ human HSC expressed high levels of Rad18 and FANCD2 compared with CD34⁻ cells, potentially consistent with a role for Rad18 in stem cell function. However, in contrast with reported phenotypes for FA patients and fanc-deficient mice, *Rad18*^{-/-} animals did not have significantly reduced percentage of Lin⁻Sca⁺Kit⁺ (LSK) hematopoietic stem cells in Lin⁻ population when compared with *Rad18*^{+/+} mice. We performed competitive transplantation assays to determine whether *Rad18*^{-/-} HSC efficiently reconstitute the immune system of lethally-irradiated mice. Our results show that *Rad18*^{-/-} HSC had no deficit in T-cells, monocytes or granulocytes reconstitution, while had 20% reduce in the ability to reconstitute B-cell (P<0.05) 8 weeks after transplantation, but no significant deficit after 16 weeks. We conclude that Rad18 plays redundant role in FA pathway function and tolerance of basal stresses experienced by HSC in the bone marrow, but plays specific role in B-cell development. Because Rad18 has multiple effector pathways in genome maintenance we have also generated new 'knock-in' mice expressing mutant *Rad18* alleles. These *Rad18* mutant mice will be used to determine the contribution of different Rad18 effector pathways to hematopoietic genome maintenance and tumor suppression *in vivo*.

Acknowledgements

The organizers of the *2013 Annual Research Symposium* would like to thank everyone who contributed to the success of this event. We appreciate the enthusiastic participation from the graduate students, postdoctoral fellows, and clinical fellows who shared their research with the Department through oral presentations and posters. Thank you to Dr. Ronald J. Falk for his willingness to deliver the keynote lecture for this symposium. We also thank Maria Aleman and Britta Jones for their willingness to serve as moderators for the oral presentations by the graduate students, postdoctoral fellows, and keynote speaker. We thank the individual faculty members who judged the presentations of the trainees. We give special thanks to Dr. J. Charles Jennette for his continued strong support of this important event and to Ms. Nancy Nye and Ms. Brenda Brock for their assistance with planning this event, preparation of the program booklet for the symposium, and for logistical support during the event itself. We also thank Mr. Paul Cox for his assistance with the poster boards and setting up for the poster session. Finally, thank you to everyone who is pictured on the program cover, and to everyone that attended this event to learn about the excellent research being conducted by our graduate students and postdoctoral fellows.

Jiandong Liu, Bill Coleman, and Jon Homeister

