

Pennsylvania State University

Annual Progress Report: 2009 Formula Grant

Reporting Period

July 1, 2013 – December 31, 2013

Formula Grant Overview

The Pennsylvania State University received \$8,412,824 in formula funds for the grant award period January 1, 2010 through December 31, 2013. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Modifications of Histone H3 in Diabetic Retinopathy - The overall purposes of this project are twofold. First we plan to define some of the changes in chromatin structure that occur in the retinas of an animal model of Type I diabetes. This will give us fundamental information about the range of retinal changes seen in this disease and will offer new insights into the types of change that need to be treated. The second objective is to monitor how treatment with insulin can reverse these changes and restore the retina to its normal state. This set of experiments will test the hypothesis that some of the retinal changes in diabetes become irreversible with time.

Duration of Project

1/1/2010 – 6/30/2011

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement Annual C.U.R.E. Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 2: Project Title and Purpose

Brain and Behavior in Early Iron Deficiency - Aggressive iron repletion following iron deficiency in infancy will result in excessive accumulation of this potential neurotoxin in the brain and is associated with alteration in brain genomic level and myelin composition. The purpose of this project is to determine if aggressive dietary iron intervention to replenish brain iron concentrations in formerly iron deficient animals is neurotoxic.

Duration of Project

1/1/2010 – 6/30/2011

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement Annual C.U.R.E. Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 3: Project Title and Purpose

Cell Signaling Mechanisms Necessary for High-Fidelity Replication of Repetitive Sequences - The integrity of an organism depends on faithful replication of the genome with every cell division. The human genome contains six billion bases, many of which can be present in structures that make it difficult for replicative enzymes to work efficiently and accurately. Human cells have 15 different DNA polymerases that function to copy the genome. Mutations caused by polymerase errors within repetitive microsatellite sequences pose a significant threat to genome integrity, as is evident in colorectal cancers. The goal of this project is to elucidate the polymerases and cell signaling mechanisms that are necessary for high-fidelity replication of microsatellites. Understanding the dynamics of microsatellite replication will lead to new approaches for detecting and treating colorectal cancer, one of the most common cancers in the United States and Pennsylvania.

Duration of Project

1/1/2010 - 12/31/2010

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement Annual C.U.R.E. Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 4: Project Title and Purpose

Impact of the Penn State Diabetes Patient Registry - Disease registries (or electronic patient databases) are being implemented extensively by health care systems with the goals of improved records management, individualized patient care, and population-based disease management. They are a central feature of improving quality care for chronic illnesses and feature prominently in the national effort towards redesigning primary care as the Patient Centered Medical Home (PCMH). Although used extensively, little data are available on the overall impact of registries on care. The Penn State Diabetes Registry (PSDR) was first implemented in the Penn State Hershey system in 2002 to achieve the aforementioned goals and to simultaneously provide a research network for patient recruitment for diabetes research. The purpose of this project is to identify the value of this chronic illness registry for quality improvement of clinical outcomes, to examine its effectiveness in promoting high quality clinical research and to identify opportunities for PSDR refinements through assessments of the impact of the PSDR.

Duration of Project

5/1/2010 - 12/31/2011

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 5: Project Title and Purpose

Epigenetic Changes in Myeloid Differentiation and Acute Myeloid Leukemia - The purpose of this project is to identify changes in epigenetic mechanisms controlling myeloid differentiation and transcription in order to predict and prevent the progression of acute myeloid leukemia (AML), a highly malignant blood cancer. Recent development of high-throughput “next-generation” genomic sequencing technologies such as ChIP-sequencing provided a dramatic breakthrough in whole-genomic mapping of epigenetic markers such as histone methylation and acetylation and their abnormalities. Here we propose to conduct a detailed whole-genomic characterization of the localization and expression of one such marker, histone H3 methylation, which is altered in AML cells, and to identify clusters of genes involved in epigenetic changes marked by this histone modification and transcriptionally deregulated in leukemia.

Duration of Project

1/1/2010 – 6/30/2013

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 6: Project Title and Purpose

Bridging the Gap between Label-Retention and Mammary Stem Cell Properties - Breast cancer patients frequently suffer relapse after prolonged periods of clinically undetectable disease. Whether dormant breast cancer is comprised of quiescent cells versus cycling cells, whose proliferation is offset by cell death, remains unknown. When modeling dormant breast cancer by reversing Wnt1-initiated mammary tumorigenesis in transgenic mice, we found that minimal residual disease (MRD) lesions harbor both latent malignant potential and mammary gland reconstitution capacity. Moreover, our preliminary data demonstrate that MRD lesions harbor a slow-cycling mammary epithelial cell (MEC) population. We propose to demonstrate that these quiescent cells are enriched for expression of validated mammary stem cell markers.

Duration of Project

7/1/2010 - 6/30/2011

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement Annual C.U.R.E. Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 7: Project Title and Purpose

Neural Control of Breathing by a Signal Encoding Venous Pressure: Implication in Heart Failure - Respiratory symptoms such as chronic hyperventilation are dominant expressions of cardiac insufficiency. Hyperventilation induced hypocapnia also has the potential to disrupt sleep architecture and to provoke central sleep apnea, further limiting quality of life in these patients. Distension of the venous system, a common feature of cardiac failure, leads to the activation of neural afferent fibers located in the adventitia of the post capillary bed, which in turn increases breathing. The proposed studies are intended to establish, in an animal model, the involvement of such a mechanism at rest and during muscle activity.

Duration of Project

7/1/2010 – 6/30/2012

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 8: Project Title and Purpose

Influence of Brain Astrocyte Glutamate Concentrations on Neurotransmission - The most abundant type of cell in the brain is the astrocyte. Astrocytes may control neurotransmitter traffic by lowering the concentration of neurotransmitter glutamate in synaptic spaces. Vesicular glutamate released from presynaptic neurons into the synaptic space stimulates depolarization and electrical signal transduction by post-synaptic neurons. The synaptic space barrier is composed of membranes from both presynaptic and post-synaptic neurons and astrocytes. Astrocytes rapidly remove glutamate from the synaptic space which would otherwise prolong signal transmission and cause excitotoxicity. The study examines the properties of the glutamate transporter in the astrocyte membrane to evaluate the influence of the intracellular astrocyte glutamate on inward transport and examines how cytosolic concentrations of glutamate are regulated by astrocytic enzymes.

Duration of Project

1/1/2010 – 6/30/2011

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement Annual C.U.R.E. Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 9: Project Title and Purpose

Identification and Function Study of Inflammatory Bowel Disease-associated DNA Methylation - Inflammatory bowel disease (IBD), with Crohn's disease (CD) and ulcerative colitis (UC) as its two major forms, is a complex human disease. In the US, about 1.4 million people are affected with IBD. Currently, there is no known specific cause and no medical cure for the disease. Although more than 32 genes have been identified as IBD-associated genes, the total number of genetic factors discovered so far can only account for less than 20% of the overall genetic risk. It is clear that other factors make a significant contribution to the disease process. In this project we will examine the role of DNA methylation in IBD. DNA methylation is a modification of DNA molecules and has emerged as an important player in human development and diseases.

Duration of Project

7/1/2010 – 6/30/2011

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement Annual C.U.R.E. Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 10: Project Title and Purpose

Allogeneic CMV CTL for Refractory Glioblastoma Multiforme - The purpose of this project is to determine the safety as well of the clinical and immunological effects of infusing allogeneic, cytomegalovirus (CMV) specific cytotoxic lymphocytes (CTL) in patients with refractory, CMV positive glioblastoma multiforme (GBM). Patient tumors will be tested for the presence of CMV pp65 and IE-1, and if the tumors are positive and the patients meet other eligibility criteria they will receive CMV CTL from an HLA partially matched donor. We will determine the duration and magnitude of CMV specific T cell function and donor cell microchimerism, the latter using PCR assays. We will also determine the incidence of clinical responses to this intervention, and correlate these clinical responses with donor cell chimerism and CMV specific immunity post-infusion.

Duration of Project

1/1/2010 - 1/17/2011

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement Annual C.U.R.E. Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 11: Project Title and Purpose

Molecular Mechanisms of Olanzapine's Metabolic Side Effects in an Animal Model - Understanding the mechanism of the metabolic side effects of atypical antipsychotics is very important to the patients taking these drugs and their families. This research will focus on how atypical antipsychotics lower free fatty acids and will provide data supporting our hypothesis that it both impairs lipolysis leading to increased adiposity and simultaneously increases lipid oxidation leading through substrate competition to hyperglycemia and apparent insulin resistance.

Duration of Project

1/1/2010 - 6/30/2011

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement Annual C.U.R.E. Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 12: Project Title and Purpose

Genetic Analysis of Papillomavirus Virion Morphogenesis - The central hypothesis of this project is that the capsid proteins of human papillomavirus (HPV) have specific functional domains for morphogenesis of infectious viral particles in a differentiation-dependent manner. When completed, this project will provide new insight into the molecular mechanisms of virion morphogenesis in a system capable of reproducing the natural complex process and differentiating environment of native HPV virion (NV) morphogenesis and infection.

Duration of Project

1/1/2010 – 11/30/2010

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the

Commonwealth Universal Research Enhancement Annual C.U.R.E. Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 13: Project Title and Purpose

Epidermal Stem Cell Properties in Mice with Altered Polyamines - Our research studies the role of the polyamine pathway in early skin cancer development. Since skin cancers are the most common form of malignancies world-wide, identification of possible targets for prevention is highly relevant to public health. Understanding the role of the polyamine pathway is also of great importance because the polyamine biosynthesis inhibitor difluoromethylornithine (DFMO) is a promising chemopreventive agent of human skin cancer.

Duration of Project

1/1/2010 - 6/30/2011

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement Annual C.U.R.E. Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 14: Project Title and Purpose

Topical Application of Isoselenocyanates for the Prevention of Melanoma – Malignant melanoma is the most fatal and invasive form of skin cancer. The cause of 60% of melanomas is yet to be determined, making development of effective targeted chemopreventive agents especially important. In spite of the widely appreciated magnitude of the problem, there is still a critical gap in knowledge regarding key deregulated signaling pathways causing melanoma and chemopreventive agents targeting these gene defects to prevent this disease. Therefore, the purpose of this project is to develop *a rationally designed chemopreventive agent targeting the Akt3 signaling pathway*. As a direct outcome of the investigation, we expect to validate the effectiveness of novel synthetic agents derived from naturally occurring chemopreventive agents as a topical agent that prevents early melanocytic lesion development.

Duration of Project

7/1/2010 – 6/30/2011

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement Annual C.U.R.E. Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 15: Project Title and Purpose

Activation of STAT3 Protects Liver from Ischemia Reperfusion Injury - Liver resection is the only curative option for liver tumors and liver transplantation is the only viable treatment for end stage liver disease. Warm ischemia/reperfusion (I/R) is a common clinical problem during both types of surgeries. Warm ischemia also occurs in trauma and shock. The liver is the most commonly injured abdominal organ in blunt and penetrating abdominal trauma. The management of trauma and shock frequently involves exposing the liver to varying periods of warm ischemia followed by reperfusion. I/R injury is significantly associated with morbidity and mortality in such conditions. Prevention or minimization of liver I/R injury is the clinical priority for our study, because there is still no safe and promising strategy to protect liver from I/R injury.

Duration of Project

1/1/2010 – 6/30/2011

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement Annual C.U.R.E. Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 16: Project Title and Purpose

Multifunctional Nanoparticles for Melanoma and Brain - Malignant melanoma and glioblastoma multiformi (GBM) are invasive and deadly forms of skin and brain cancer respectively with no effective therapy to treat advanced disease, leading to poor survival rates. A P01 Program Project Grant is proposed consisting of 5 projects using “Multifunctional Nanoparticles” to develop a new category of agents to treat these cancers. This project will focus on developing preliminary data to be used in supporting the Program Project application and to illustrate advanced integration and improved synergy between the 5 projects incorporated in the Program Project Grant (PPG).

Duration of Project

7/1/2010 – 6/30/2012

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement Annual C.U.R.E. Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 17: Project Title and Purpose

Parathyroid Hormone and Prostate Cancer Colonization of Bone - There is a fundamental gap in understanding how osteosclerotic prostate cancer (PCa) metastases colonize bone, and the contribution of bone formation and parathyroid hormone (PTH) to this process. Our long term goal is to understand what regulates PCa colonization and establishment in bone, and to aid development of treatments that prevent bone metastases. The objective of this project is to identify the requirement(s) for PTH-enhanced bone formation in osteosclerotic PCa colonization and establishment in murine bone. Our central hypothesis is that osteosclerotic PCa colonization of bone, from the vasculature, requires enhanced bone formation. The rationale for this project is that once we know how enhanced bone formation facilitates the favorable interaction(s) between osteosclerotic PCa cells and bone, novel treatments can be developed to prevent such interactions.

Duration of Project

4/21/2010 - 6/30/2011

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement Annual C.U.R.E. Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 18: Project Title and Purpose

Perlecan Regulation of Synovial Hyperplasia - In patients with arthritis, synovial hyperplasia (SH) gives rise to pannus formation, invasion and erosion of articular cartilage, leading to joint destruction and chronic pain. There is a fundamental gap in understanding what regulates SH. The objective of this proposal is to identify a role for the HSPG, perlecan, in modulating SH.

Duration of Project

4/21/2010 – 2/28/2011

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement Annual C.U.R.E. Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 19: Project Title and Purpose

A Multi-Center Randomized Single Blind Trial of Intravenous Fluids during Labor - To determine if intrapartum use of a glucose-containing solution will improve the outcome of labor and reduce operative deliveries.

Duration of Project

4/21/2010 - 6/30/2013

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 20: Project Title and Purpose

Identification and Characterization of Regulatory Factors of the Human Telomerase Gene - Normal human somatic cells age due to the fact that telomere is limiting, and telomerase is stringently repressed in these cells. The human telomerase reverse transcriptase (*hTERT*) gene, which encodes the limiting catalytic subunit of telomerase, is primarily regulated at the level of transcription. It is highly expressed in pluripotent stem cells but silenced in most somatic cells. Hypomorphic alleles of the telomerase gene, found in patients of dyskeratosis congenita, cause severe abnormalities in multiple organs and tissues. On the other hand, telomerase activation in tumor cells leads to limitless proliferation, a hallmark of all cancers. Our research is to reveal how telomerase is repressed during development and activated in cancer cells. Specifically, we have identified several candidate genes involved in telomerase regulation and plan to understand how telomerase is regulated by these genes. This study will lead to a better understanding of cancer and age-related diseases, and ultimately better therapeutic strategies for these diseases.

Duration of Project

4/21/2010 - 9/30/2011

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 21: Project Title and Purpose

Biomechanical Failure and Loosening Characteristics of a Novel Posteriorly Augmented Glenoid Component – The objective of this study is to characterize the mechanics of novel and standard cemented Total Shoulder Arthroplasty glenoid implants subject to cyclic mechanical loading. We have developed a novel glenoid implant that includes a posterior augmentation to compensate for posterior bone loss often found in shoulder osteoarthritis. We seek to understand the loosening resistance and micro-mechanics of the bone-cement-implant interface in both this implant and standard glenoid implants. Micro-computed tomography will be used to image the implant fixation after various stages of controlled cyclic loading applied to simulate physiologic shoulder activity.

Duration of Project

9/1/2010 - 9/30/2011

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 22: Project Title and Purpose

Delivery and Mechanism of Action of Novel Therapeutic Agents to Combat ALS – Amyotrophic Lateral Sclerosis (ALS) is a progressive degenerative disease causing loss of spinal cord motor neurons and is almost always fatal within 3 -5 years of diagnosis. In this project we will use mutant SOD1 transgenic mice to test the actions of a well-established neuroprotective factor Pigment Epithelium Derived Factor (PEDF) that has a number of unique features about its structure and mode of action that strongly suggest it will succeed in treating ALS where studies of different growth factors or neurotrophic factors have failed. The outcome of this project will be new knowledge on ways of delivering drugs to combat ALS as well as an understanding of the molecular mechanisms by which PEDF can exert its protective action on spinal motor neurons.

Duration of Project

9/1/2010 - 6/30/2012

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 23: Project Title and Purpose

Developing an Animal Model of Cognitive Bias to Study the Impact of Emotion on Health and Behavior – Emotional states play a fundamental role in influencing health outcomes; individuals with a bias toward negative emotions, such as anger and depression, are more prone to health related problems. Furthermore, it has recently been shown that positive emotions help to protect against certain kinds of disease. Animal models provide a powerful tool to experimentally determine the processes involved in disease pathways. Such models are used to investigate the health consequences of states such as depression or anxiety, but current models of animal emotion are poor and incomplete because they take no account of the cognitive aspect of emotion - appraisal processes that bias an individual toward a more negative or positive outlook. This project aims to develop an improved animal model of emotion.

Duration of Project

9/1/2010 - 12/31/2013

Project Overview

Using novel testing paradigms that directly measure rodent cognitive appraisal strategies, we will create a new animal model of emotional states. Our approach will quantify emotional state by simultaneously evaluating behavioral, physiological and cognitive biases. Specifically, this refined model will enhance our ability to determine the consequences of positive and negative affective states (analogous to optimism and pessimism in humans), and how these influence different health issues. There are two main objectives.

Objective (i) Demonstrate that cognitive biases in rats are associated with neuroendocrine and behavioral biases that accompany positive and negative affective states in humans. By doing so, we will develop a more comprehensive rodent model of emotion that includes assessment of cognitive as well as physiological and behavioral biases.

Objective (ii) Develop a rearing paradigm to create different populations of rats with contrasting long-term cognitive biases (i.e., groups of animals with either more positive or more negative tendencies). These rats can then be used to determine health consequences of cognitive bias. In this initial project we will investigate choice behaviors related to drugs of abuse, but future work will address how emotional state affects resilience and plasticity in response to different kinds of challenge such as compromised health, stress and pain.

In the current project, we will train rats to associate different stimuli with specific outcomes (i.e., one stimulus leads to a reward, a different stimulus leads to a punishment). We will then test rat response to ambiguous ‘in between’ stimuli. Previous work has shown that rats that appraise ambiguous events to be more like the rewarded stimulus generally have a positive affective state, whereas animals that assess ambiguous stimuli as more like the punishment stimulus have a negative affective state.

We will focus on determining how positive or negative emotional bias affects addiction to drugs of abuse. By developing a more comprehensive method to quantify animal emotional state we will devise a more sophisticated and accurate model that can be manipulated to determine how emotional states influence health trajectories over an individual’s life span.

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Expected Research Outcomes and Benefits

The novel approach described in this project will allow us to create a superior model of animal emotion that encompasses the key component of cognitive bias – a part that is so far lacking in current models. By quantifying cognitive bias, we will characterize emotion more accurately and critically. Such animal models will allow us to address the effects that cognitive bias has on health including aspects relating to addiction. Much of the current focus in human research has been on the detrimental effects of negative cognitive bias, but recent research has shown that positive cognitive bias is likely to play an equally important role. Although optimism and pessimism are usually considered opposites, it now appears that these two states, while related, are distinct constructs. Our animal models will allow us to more systematically investigate these differences to provide us with a better understanding of the role played by cognitive bias and emotion on human health and behavior. In the future, we envisage using this model to investigate the consequences of positive and negative affective states and their effects on other health issues such as depression, anxiety and thresholds of pain.

Summary of Research Completed

All experiments were approved by the Pennsylvania State University IACUC committee, protocol #35761.

To further characterize the long-term effects of experiencing chronic, unpredictable stress during adolescence, we ran trials with rats to investigate their decision-making behavior when the animals were in a negative affective state (generated by simulating an attack by a bird of prey – a bird silhouette would swoop over the test arena in a standard way). There have been suggestions that the experience of stressors during sensitive phases of development could help to prime the animal for making more appropriate decisions in adulthood under stressful conditions (such as the threat of predation). To test this we designed an open field foraging task. Foraging trials were conducted in a 122cm x 122cm x 46cm white opaque Plexiglas arena; rats began each test in a 7.6cm diameter PVC tube shelter identical to PVC tubes that the animals had access to in their home-cages.

Six cages of pair housed rats (n=12) were randomly assigned to the control condition and six cages (n=12) to the stress treatment. For the latter group, stressors were presented daily from 30 to 70 days of age, with 8 days of rest occurring intermittently. Prior studies of adolescent-stress have varied in the duration of stress exposure, due in part to the large window of time during which adolescent ontogenetic changes occur. These changes are thought to conclude at approximately 55 days of age in male rodents. To cover the entirety of the ontogenetic window of adolescence, studies have included a postpubertal “sub-adult” period. As the current study evaluated behaviors mediated by the prefrontal cortex (i.e. decision making, coping), and this region is still developing in early adulthood, the duration of stress exposure (30-70 days of age)

included a postpubertal period in early adulthood.

For the chronic unpredictable stress procedure both physical and social stressors were presented randomly across the light/dark cycle to maximize unpredictability. An average of three physical and three social stressors were presented between each rest day. Stressors noted to induce short-term changes in cognitive bias were used (e.g. cage tilt, damp bedding; crowding, confinement). An additional stressor, isolation, was chosen because it has been associated with long-term changes in behavior following exposure during adolescence.

To control for the influence of circulating corticosterone on experimental measures mediated by glucocorticoid levels, we controlled for daily rhythms in glucocorticoid production by avoiding testing during peak corticosterone production; all tests started a minimum of 2 hours after the beginning of the dark cycle and were completed within 6 hours of the start of the test. Weight and physical appearance were monitored; no changes in aggression or health related to either the unpredictable stress regimen or any of the behavioral tests were observed.

Food patches were positioned in the same position and orientation for all tests. The foraging patches consisted of discrete patches of cheerio breakfast cereal pieces covered by novel objects. The novel objects varied in texture, color, shape, and size; a silver metal bowl; a translucent red plastic triangle; a shiny yellow cylinder with a rippled surface and a translucent igloo shelter were used.

To compare the activity levels movement in the arena was evaluated over a 5-minute period using a video-recorded trial where an individual animal was allowed to freely move around the arena. To quantify activity, the number of squares crossed on an 8 x 8 grid placed on the video screen were counted.

The time to approach a novel object in the 122cm x 122cm x 46cm Plexiglas arena was quantified. More rapid approaches of novelty are indicative of increased boldness. Approaching a novel object was defined as the initial latency to physically contact one of the two novel objects in the arena with either a paw or nose.

Motivation to consume a reward

To test for differences in motivation to feed, an initial foraging trial was conducted to quantify how quickly the animals approached and fed on a food item in a familiar environment (their home cage). Both rats that had received adolescent-stress exposure and control animals (housed without exposure to stressors) did not differ in their reward consumption ($F_{1,21} = 1.59, P = 0.22$).

Foraging behavior

In a familiar environment and while foraging in a novel environment adolescent-stressed and control animals did not differ in their reward consumption ($F_{1,21} = 1.59, P = 0.22$; $F_{1,21} = 1.30, P = 0.27$). Under threat, however, adolescent-stress animals consumed more cheerios than animals naïve to threat ($F_{1,21} = 4.24, P = 0.05$). While foraging in a novel environment adolescent-stressed and control animals did not differ in their reward consumption ($F_{1,21} = 1.30, P = 0.27$). However, in the novel environment animals that experienced adolescent chronic unpredictable stress exhibit a longer latency to come into contact with a food patch than control animals ($F_{1,22}$

= 4.44, $P = 0.05$). Under control conditions animals reared in standard housing visited more food patches than animal previously exposed to threat. However, the trend reversed once the predation threat was introduced into the testing environment ($F_{1,22} = 12.53$, $P < 0.01$).

When foraging under control conditions, adolescent-stressed animals took longer to access the first patch and they visited fewer patches in total, but they consumed similar amounts of food to the control animals. However, when foraging under stressful conditions (simulated predator threat), adolescent-stressed animals visited more patches and consumed more of the reward than animals naïve to threat. This suggests that experience of threat in adolescence promotes better adaptive responses to threat in adulthood, further highlighting the impact that stressors can have on adult affective state and behavior.

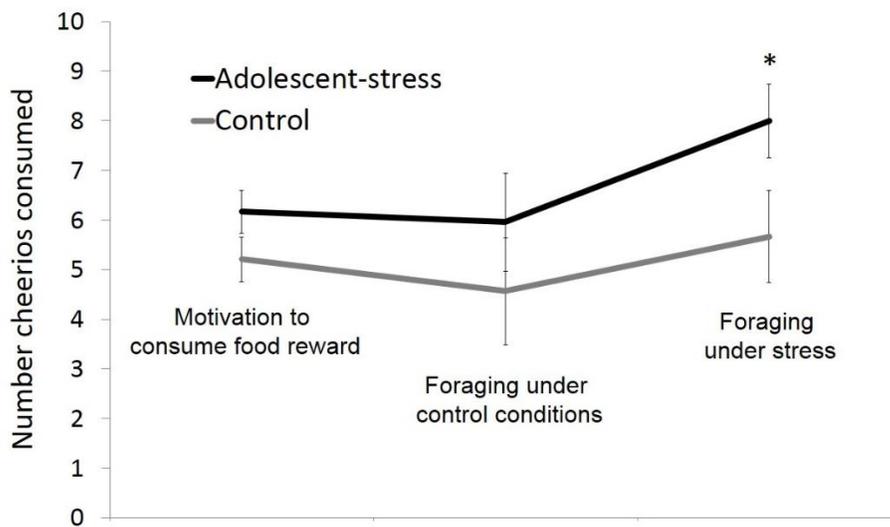


Figure 1: Comparison of amount of food consumed at three time points, (i) general motivation to consume food in a familiar environment, (ii) amount of food consumed in a novel, non-threatening environment, (iii) amount of food consumed in a threatening environment (simulated predator attack).

Research Project 24: Project Title and Purpose

Structural Studies of Tight Junction Structure Function and Regulation – Tight junctions (TJ) are supramolecular structures that form the cell-cell barriers required for tissue formation and in regulating the flow of solutes through paracellular spaces. Occludin (Occ) is a tetraspanning integral membrane protein in epithelial and endothelial tight junctions. This protein and its interacting extra- and intracellular partners are believed to be critical for regulating TJ properties. Specifically, Occ is proposed to act in signal transduction to adapt TJ properties to cellular and tissue need. Understanding the structure and function of Occ is critical for understanding its role in TJ biology. The results obtained from this project will lay the foundation for pursuing external grants, including one from the NIH membrane protein structure initiatives, where

proposals that show purified, well behaved membrane proteins are generally well received.

Duration of Project

9/1/2010 - 12/31/2012

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 25: Project Title and Purpose

Epigenetic Basis of Metabolic Memory in Diabetic Retinopathy – Recent longitudinal studies demonstrate that despite achieving good control of blood glucose for ten years patients formerly under non-intensive insulin therapy continue to have a higher rate of developing diabetic retinopathy and other complications. With this background, we hypothesize that poor glucose control causes epigenomic changes that are not fully reversed with intensive insulin therapy. Demonstration of epigenetic changes in the retina, in response to diabetes, not reversible by insulin, would be an example of an innovative, high pay-off, paradigm-shifting outcome. Such information would open new avenues for understanding diabetic complications and developing treatments that, used in conjunction with homeostatic insulin replacement, would be capable of restoring the pre-diabetes epigenetic state and reducing the risk of complication development.

Duration of Project

9/1/2010 - 6/30/2012

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 26: Project Title and Purpose

Transplantation of Human Retinal Pigment Epithelial Cells (RPEs) in the Nucleus Accumbens of Rats – The purpose of this research project is to determine, preclinically, whether rats with a history of cocaine self-administration can be rescued from the grips of this disease with focal bilateral transplant of 20 or 30 K/side of L-dopa producing fetal human (fh) Retinal Pigment Epithelial Cells (RPEs) into the nucleus accumbens. The potential effectiveness of the transplant in preventing relapse will be examined following 14, 30, and 60 days of abstinence. Given that transplant of these cells already is in Phase II Clinical Trials as a potential treatment for Parkinson's Disease, the long-term objective is to determine whether such a transplant into the reward, rather than the motor, pathway is a viable option for the treatment of addiction in

humans.

Duration of Project

9/1/2010 - 6/30/2012

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 27: Project Title and Purpose

Interrogating the Role of Myoepithelial Cells in Mammary Carcinogenesis – Breast cancers arise from mammary epithelial cells (MECs). Two distinct MEC compartments have been defined which occupy concentric layers in normal mammary ducts. An inner luminal MEC layer harbors milk-producing cells whereas an outer myoepithelial cell layer forms a contractile sheath that aids milk expulsion. The role of myoepithelial cells in breast carcinogenesis remains poorly defined. Some findings suggest that myoepithelial cells can themselves be transformed into cancer cells. Other findings suggest that normal myoepithelial cells protect against cancer by providing a barrier against luminal MEC invasion. By using novel transgenic mouse models, we will test whether myoepithelial cells promote or inhibit carcinogenesis. These studies will define whether prevention strategies ought to maximize or minimize myoepithelial cell number.

Duration of Project

7/1/2011 - 6/30/2013

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 28: Project Title and Purpose

Outcomes for Recurrent Thyroid Cancer in the Elderly – The purpose of this project is to identify factors associated with variation in treatment for thyroid cancers in the Medicare population, to estimate the impact of different treatments on cost of medical care for patients with recurrent thyroid carcinomas, controlling for tumor features, demographic characteristics, and co-morbid conditions, and to assess the impact of different treatments on overall and disease-free survival for patients with recurrent carcinomas. Results will inform and guide physicians as they consider treatment recommendations for individual patients and policy makers as they develop and refine treatment guidelines for Medicare patients. In addition, while these aims deal with recurrent thyroid cancer, the broader issues of treatment specific costs and

effectiveness are also relevant for other cancers.

Duration of Project

9/1/2010 - 6/30/2012

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 29: Project Title and Purpose

Body Weight- and Nutrition-Sensitive Regulation of Skeletal Muscle Composition in Health and Obesity – The purpose of this project is to extend preliminary findings showing that experimental manipulation of body weight results in precise adjustments to skeletal muscle *Troponin T (Tnt)* expression, and that this response is impaired in a genetic model of obesity. This response is mediated by alternative splicing and is sufficiently accurate that a unit of externally attached load has the same effect on the *Tnt* mRNA splice form profile as does a unit of actual body weight. The project will more firmly establish body weight- and nutrition-dependent causes of variation in mammalian skeletal muscle *Tnt* expression, and the generality of a mismatch between body weight, *Tnt* splicing, and skeletal muscle performance and energy expenditure during obesity.

Duration of Project

9/1/2010 – 8/31/2011

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 30: Project Title and Purpose

Role of Olfactory Cues in Addictive Behavior – In this pilot study we will be studying altered brain activity related to the reward value of eating/smoking in smokers, binge-eaters, smokers who binge-eat, and healthy controls. This project will test the following hypotheses concerning the human brain's response to food/smoking cues. We predict that responses will be 1) lowest for pictures, larger for odorants, and maximal for combined congruent pictures with odorants; 2) larger for smokers for smoking cues and larger for binge-eaters for food cues. These responses may have implications for treatments designed to change smoking or eating patterns.

Duration of Project

9/1/2010 - 6/30/2012

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 31: Project Title and Purpose

MRI Marker(s) for Neuropsychological Outcomes in Adolescents with Mild Concussion – The purpose of this study is to identify MRI markers that correlate with neuropsychological outcomes in adolescents who have suffered a mild concussion. Recent imaging results in adults with concussions have demonstrated alterations in imaging parameters that are associated with outcome measures. The current project will extend these results to adolescents with mild concussions to test the central hypothesis that axonal injuries, microbleeding, and/or functional interruption occurring during concussion may be responsible for the observed neuropsychological dysfunction and predictive of recovery patterns in adolescents.

Duration of Project

9/1/2010 - 6/30/2013

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 32: Project Title and Purpose

Epigenetic Changes in Gene Expression Associated with an Anxious Depressive like Phenotype – The purpose of this study to discover gene expression changes that could serve as diagnostic biomarkers of anxious depressive disorders. Such biomarkers hold the promise of 1) improving the speed and accuracy of diagnosis, thereby reducing the time consuming and often futile guesswork involved in finding an efficacious therapy for anxious depressive disorders, and 2) finding novel therapies for currently drug resistant forms of the syndrome.

Duration of Project

9/1/2010 - 6/30/2012

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 33: Project Title and Purpose

Tumor Associated Receptor Targeted Neuroimaging for Effective Diagnosis of HGA – Our major intention in this project is to develop tumor targeted gadolinium loaded nanovesicles which will allow us to visualize tumor initiation and growth non-invasively in a much more efficient manner compared to free gadolinium. We will also study the ability of the gadolinium loaded IL-13 receptor targeted nanoliposomes to differentiate the radiation induced necrosis and tumor recurrence which is one of the common phenomenon observed among patients undergoing radiation therapy for brain tumors. Diagnosis of radionecrosis is a challenging issue because of the resemblance of radiological pattern with tumor recurrence. In our second aim we will focus more on differentiating the normal and tumor tissues using an IL-13 conjugated nanoconjugate known as quantum dots.

Duration of Project

9/1/2010 - 6/30/2012

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 34: Project Title and Purpose

Viral RNA Trafficking in Living Cells – Mouse mammary tumor virus (MMTV) is a retrovirus that causes adenocarcinoma in mice, and MMTV-like elements have been detected in human breast cancers. This project focuses on how MMTV commandeers cellular machinery to facilitate capsid assembly with the ultimate goal of identifying targets for the treatment of retrovirus-induced cancers.

Duration of Project

9/1/2010 - 12/31/2013

Project Overview

This project focuses on the mechanism used by the oncogenic retrovirus MMTV to produce new virus particles in infected mammary cells. We have compelling new evidence that MMTV capsids associate with RNA processing machinery, which is the foundation for our hypothesis:

MMTV genomic RNA (gRNA) is targeted to PB/SG/RISCs where it binds to the structural protein Gag to encapsidate the viral genome into assembling capsids. The movement of MMTV Gag and gRNA are dynamic, transient events that can only be revealed using realtime imaging of individual living cells. Therefore, to test our hypothesis, we will develop an innovative method to track MMTV gRNA in live cells using florescence microscopy.

Specific Aim 1: Identify RNA processing factors that mediate MMTV gRNA trafficking in living cells. Following integration of proviral DNA into the host genome, retroviruses produce unspliced viral RNAs that are used for encapsidation into virions (gRNA) or as a template for protein synthesis (mRNA). To initiate capsid assembly, Gag binds the gRNA, then multimerizes to form an immature capsid. Efficient assembly requires that gRNA be separated from viral mRNA and transported to intracellular sites of Gag accumulation. However, the mechanism used to sort gRNA from viral mRNA is unknown. Our preliminary studies suggest that MMTV hijacks the mRNA-silencing pathway to sequester its gRNA into a protected environment, where Gag proteins are localized. Because micro-RNAs (mi-RNAs) bind complementary sequences in mRNAs to suppress their translation, it is feasible that viral or host encoded mi-RNA could serve as a tag to “mark” MMTV gRNA, directing it to PB/RISCs. To test the idea that PBs/SGs/RISCs serve as the staging site for MMTV capsid assembly, we propose this set of experiments:

- 1a. Track MMTV RNA in living cells to visualize sites of gRNA localization.
- 1b. Determine the role of PB/SG/RISCs in MMTV gRNA trafficking.
- 1c. Determine whether the mi-RNA pathway influences MMTV gRNA localization.

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Expected Research Outcomes and Benefits

Approximately 15–20% of malignancies worldwide are linked to infection. HTLV-I (human T-cell leukemia virus type-I), the cause of T-cell leukemia, was the first human retrovirus discovered. More recent work implicates a murine-derived retrovirus in prostate cancer, and MMTV-related sequences and antibodies were detected in women with breast cancer. The possibility that an MMTV-like agent may contribute to human breast cancer highlights the need for a more thorough understanding of how MMTV replicates to identify novel therapeutic targets. Through this work, we will identify cellular pathways used by MMTV for virus replication; these pathways may serve as novel targets for the development of new antiviral

drugs to treat virus-induced cancers.

An ongoing mystery in retrovirology is how viral mRNA is distinguished from genomic RNA. Because MMTV Gag proteins localize to RNA-induced silencing complexes, as do micro-RNAs (mi-RNA), we propose the innovative hypothesis that MMTV genomic RNA might be sequestered in cytoplasmic SGs/PBs/RISCs so that the genomic RNA is available for packaging into virus particles. Although targeting foreign RNA (e.g., MMTV viral genomic RNA) to SGs/PBs could be a defensive strategy to promote degradation of MMTV RNA, we propose that MMTV has evolved a counter-offensive measure by protecting genomic RNA prior to its destruction by binding to MMTV Gag in PB/SG/RISCs. This project will test these intriguing models of MMTV gRNA encapsidation, with a focus for this reporting period on host factors associated with SGs.

Summary of Research Completed

Aim 1a. Track MMTV RNA in living cells to visualize sites of genomic RNA (gRNA) localization. This aim was described in prior annual reports. The results are described in our publications (Bann and Parent, *Viruses* and Bann et al., *Journal of Virology*) and will not be repeated here.

Aim 1b. Determine the role of PB/SG/RISCs in MMTV gRNA trafficking. This aim was described in prior annual reports. The results are described in our publications (Bann and Parent, *Viruses* and Bann et al., *Journal of Virology*) and will not be repeated here.

Aim 1c. Determine whether the mi-RNA pathway influences MMTV gRNA localization. To address this question, we first characterized the subcellular localization of MMTV RNA and its association with host proteins. We used the lambdaN-eGFP reporter to label the MMTV-derived RNA construct described in Aims 1a and 1b to visualize the localization of the subviral RNA (svRNA) as a surrogate for full-length MMTV RNA. See Figures 1 and 2 for the data we obtained for this Aim.

We have also received plasmids expressing transdominant mutants of the Microprocessor, which carries out miRNA-mediated RNA silencing. The Microprocessor mutants were successfully expressed in MMTV-infected cells and experiments are still in progress. We also found that Mov10, a component of P bodies and RNA-induced silencing complexes (RISC) did colocalize with MMTV Gag and MMTV svRNA (Figure 3). Further analysis of PB and RISC proteins involved in MMTV Gag and svRNA localization are in progress.

Aim 2a. Develop methods for detecting endogenous LINE-1 activity

We obtained a plasmid expressing the murine LINE-1 element and a LINE-1 mutant that is defective in retrotransposition. We have been testing conditions to detect retrotransposon activity in a number of different cell types, including HeLa, U2OS, and NMuMG cells. We have encountered difficulties with the detection of LINE-1 activity, as the cells have not tolerated expression of LINE-1. We are continuing to troubleshoot the assay by titrating the concentration of the plasmid, trying different transfection reagents, and using various drug concentrations for

selection of LINE-1 expressing cells. In addition, we will continue to develop a PCR-based assay to detect endogenous LINE-1 RNA levels.

We have also cloned LINE-1 Orf1 protein (Orf1p) fused to mCherry to study the localization of Orf1p in MMTV-infected cells. Our preliminary data indicate that MMTVsvRNA colocalizes with LINE-1 Orf1p. Further experiments are underway to look for colocalization of MMTV Gag, svRNA, and LINE-1 Orf1p.

Aim 2b. Develop a packaging assay to quantitate MMTV genomic RNA in virions.

To detect MMTV RNA in virus particles, we have developed a reverse-transcriptase (RT) PCR-based assay. Virus particles are collected in the supernatant of infected cells, pelleted through a sucrose cushion, and viral RNA is extracted. The RNA from virions is subjected to RT-PCR and run on an agarose gel. Using this method, we can detect MMTV viral RNA. We have decided not to pursue a ribonuclease-protection assay because we have had initial success with the RT-PCR packaging assay. For most purposes, detecting viral RNA using a semi-quantitative approach will be adequate, in which we will perform dilutions of the viral RNA template prior to RT-PCR. If needed, we will perform quantitative RT-PCR using an instrument available in our core facility.

Aim 2c. Determine whether components of RNA silencing complexes (RISCs) inhibit LINE-1 retrotransposition and/or MMTV replication. We performed siRNA mediated knockdown of expression of Mov10, finding that reducing the intracellular levels of Mov10 significantly impaired MMTV virus particle assembly (Figure 4). We will use this approach with siRNAs directed against Ago1 and Ago2 to determine whether depleting these RISC proteins have a similar effect on MMTV particle release.

Publications submitted or published during this period:

1. Stake MS, Bann DV, Kaddis RJ, Parent LJ. "Nuclear trafficking of retroviral RNAs and Gag proteins during late steps of replication." *Viruses*. 2013 Nov 18;5(11):2767-95. doi: 10.3390/v5112767. PMID: 24253283.
2. Bann DV, Beyer AR, Parent LJ. "A Murine Retrovirus Co-opts YB-1, a Translational Regulator and Stress Granule-associated Protein, to Facilitate Virus Assembly." *J Virol*. 2014 Feb 5. [Epub ahead of print] PMID: 24501406.

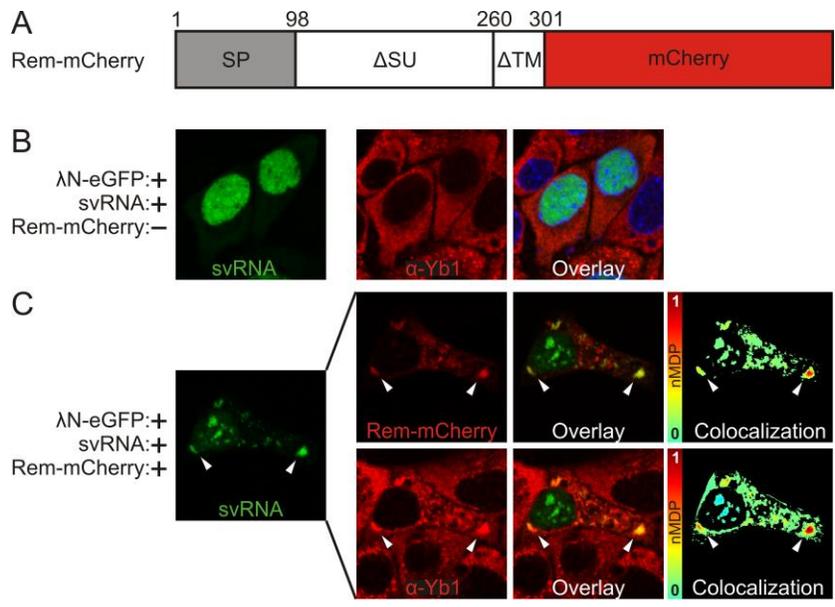


Figure 1. MMTV subviral RNA colocalizes with the MMTV Rem protein and the cellular factor YB-1 in the cytoplasm. MMTV Rem is required for the export of unspliced viral RNA from the nucleus and Rem remains colocalized with viral RNA in the cytoplasm. (A) Schematic diagram showing the domains of MMTV Rem protein with SP (signal peptide), partial deletion of surface (SU) and TM (transmembrane) domains, and C-terminal fusion of the mCherry protein. (B) Expression of λ N-eGFP reporter construct is confined to the nucleus due to the insertion of a nuclear localization signal (left panel) and the svRNA remains in the nucleus. Cells were immunostained for endogenous YB-1 (CY3, red; middle panel). Overlay of GFP, Cy3, and DAPI channels are shown in the right image. (C) Single left panel: Expression of MMTV Rem-mCherry with svRNA induced the export of svRNA from the nucleus. Note the presence of svRNA in the cytoplasm, indicated by white arrowheads. Top set of panels: Rem-mCherry (red) remains associated with svRNA (green) as demonstrated by yellow color in the Overlay. Colocalization colormapping (far right image) shows colocalization as a color gradient, with highest levels of colocalization as red and lower levels as green. The svRNA-Rem foci are red, indicating a high intensity of colocalization. Lower set of images: The same cells as shown in the upper panel were immunostained with anti-YB1 antibody to detect endogenous YB-1 (red). The overlay and colocalization colormapping indicate that YB-1 accumulates with Rem and svRNA in discrete cytoplasmic foci.

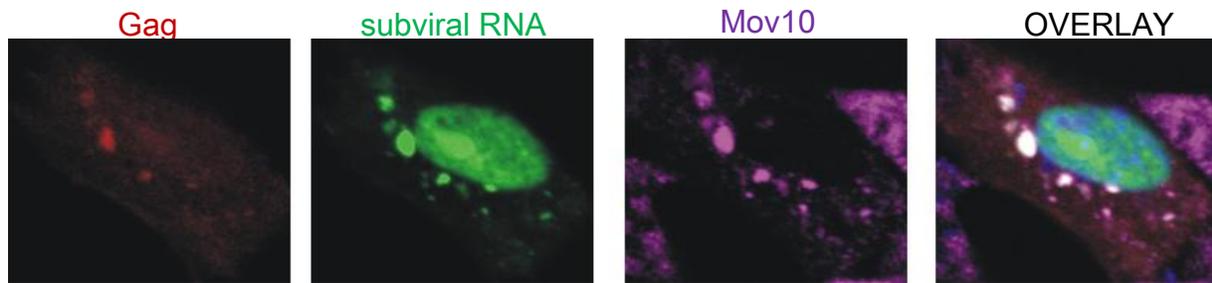


Figure 2. The P body and RISC (RNA-induced silencing complex) protein Mov10 colocalizes with MMTV Gag and svRNA in cytoplasmic complexes. Confocal images were obtained through the center of the cell demonstrating that Gag, svRNA, and Mov10 colocalize in the cytoplasm. Mov10 plays an important role in the replication of several retroviruses (e.g., HIV-1 and murine leukemia virus) and endogenous retroelements (LINE-1).

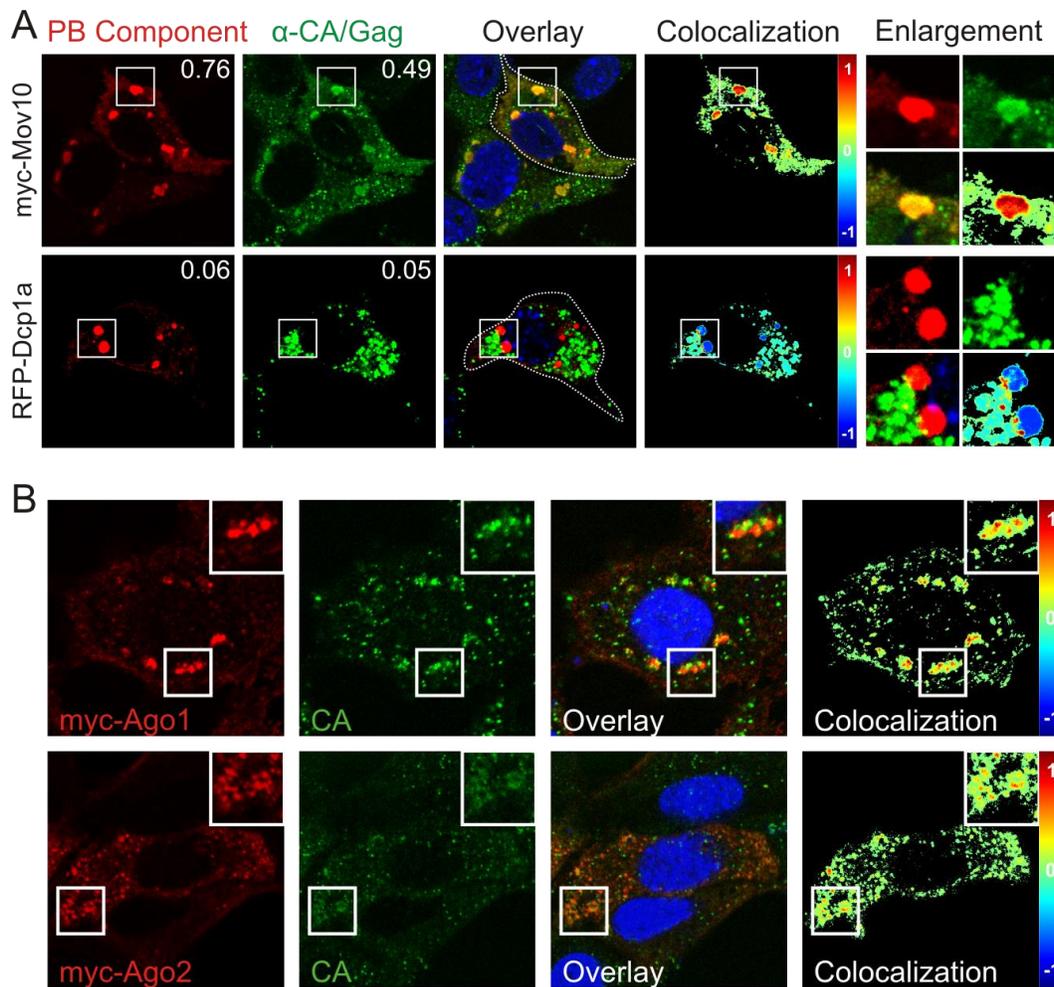


Figure 3. The MMTV Gag protein colocalizes with components of cellular RISC machinery. (A) MMTV-infected cells were transfected with myc-tagged Mov10 or RFP-tagged DCP1a (decapping enzyme 1a) and immunostained for Gag using an anti-CA antibody/ The overlay images and colocalization colormapping show that myc-Mov10 colocalizes strongly with Gag whereas RFP-Dcp1a appear to be adjacent to gag cytoplasmic foci. (B) RISC components Ago1 and Ago2 (Argonaute proteins) were expressed in MMTV-infected cells and detected using anti-myc antibodies. The images demonstrate that both Ago1 and Ago2 colocalize with MMTV Gag in cytoplasmic aggregates. The functional relevance of these interactions is being tested.

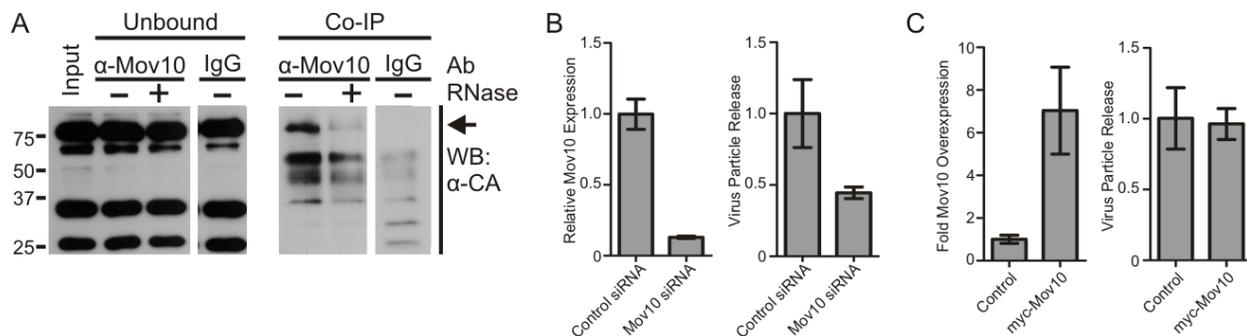


Figure 4. MMTV Gag interacts with RISC protein Mov10, which is required for efficient virus particle production. (A) Co-immunoprecipitation was performed in MMTV-infected cells using anti-Mov10 antibody to pull down endogenous Mov10. Proteins were transferred to a membrane and Western blotting was performed using anti-CA antibody to detect Gag. Gag was co-immunoprecipitated with Mov10 in the absence of RNase, but the interaction was disrupted when the cell lysates were pre-treated with RNase. This result indicates that Gag-Mov10 interaction is RNA dependent. (B) MMTV-infected cells were transfected with a si-RNA specific for Mov10 or with a nonspecific control siRNA. Mov10 expression was significantly reduced by the anti-Mov10 siRNA (~15% of baseline levels). In the same cells, cell culture supernatants were collected and virus particles were isolated. Detection of viral proteins was performed using Western blotting with anti-CA antibody. The ratio of viral proteins present in the supernatant was compared to the amount of viral gag protein expressed in the cells to yield the virus particle release. In the Mov10 knockdown cells, MMTV particle release was reduced by approximately 50%, indicating that Mov10 plays a role in MMTV particle assembly. (C) The effect of Mov10 overexpression on MMTV particle production was tested. In the left panel, the Mov10 protein was expressed ~7-fold higher in the myc-Mov10 transfected cells. In the right panel, the data show that there was no change in the efficiency of virus particle production with or without myc-Mov10 overexpression. We plan to test the effects of Mov10 knockdown on murine LINE-1 expression once the retrotransposition assay has been optimized. We will also examine whether the localization of MMTV Gag and LINE-1 Orf1p is altered in the Mov10 knockdown cells.

Research Project 35: Project Title and Purpose

Social Networking for Activity Promotion with Cell Phones (SNAP-C) – Programs designed to help sedentary adults increase their physical activity are often effective in the short-term, but these effects are rarely maintained. Technology-based physical activity programs, such as those using cell phones, may help expand social networks that could support maintenance of physical activity. This project aims to explore the efficacy of using cell phones to engineer social networks that could provide long-term support for active lifestyles. We plan to conduct a randomized controlled trial that compares an intervention group (Social Networking for Activity Promotion with Cell Phones: SNAP-C) to a minimal treatment control group (Usual Care).

Duration of Project

9/1/2010 - 6/30/2012

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 36: Project Title and Purpose

Cerebral Malaria as a Cause of Epilepsy – Malaria is a widespread problem in much of the world. In malaria endemic regions, there is a high rate of childhood epilepsy, approaching a 2-10 fold higher incidence (5-10% prevalence) over developed countries with temperate climates. Clinical studies have clearly linked malarial infection with future chronic seizure disorders in children. There is no animal model for post-malarial epilepsy. The purpose of this project is to develop a mouse model of post-malarial epilepsy that will permit us to explore the feasibility of using modulation of the immune response, in addition to antimalarial medication, to reduce post-malarial epilepsy.

Duration of Project

9/1/2010 - 6/30/2012

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 37: Project Title and Purpose

Mechanisms of Excess Dietary Salt on Sympathetic-Cardiovascular Function – Both preliminary and recently published data in our laboratory demonstrate the ingestion of excess dietary salt enhances sympathetic-cardiovascular reflexes and increases blood pressure variability in laboratory animals – blood pressure variability is a major risk factor for end-organ damage and cardiovascular disease including hypertension. The purpose of this project is to determine whether excess dietary salt intake alters sympathetic-cardiovascular regulation in humans.

Duration of Project

9/1/2010 - 12/31/2011

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 38: Project Title and Purpose

Chemokines and Immune Cells in Diabetic Nephropathy – Inflammation and endothelial cell dysfunction are central pathophysiologic mechanisms that contribute to diabetes and diabetic nephropathy (DN). CCR2 chemokine receptors are required for monocyte emigration from the bone-marrow and play pivotal roles in the pathogenesis of inflammatory disorders, endothelial dysfunction and fibrosis. We hypothesize that inhibition of CCR2 receptors expressed on kidney derived cells, such as endothelial cells or podocytes will ameliorate renal injury associated with DN. An understanding of the interaction between diabetes, CCR2 signaling, inflammation, and specific cell types in the kidney may help to better elucidate the pathologic mechanisms involved in diabetic renal disease and lead to the development of new therapeutic strategies to manage the disease process.

Duration of Project

4/10/2012 – 12/31/2013

Project Overview

Diabetes mellitus (DM) is a global health problem. The prevalence of diagnosed and undiagnosed diabetes in the US is progressively increasing, from 7.8% in 2007 to 14.5% in 2010, and is expected to rise to between 24.7% and 32.8% by 2050. Diabetes is the most common cause of end stage renal disease, responsible for more than 40% of all cases in the US. Diabetic Nephropathy (DN) demonstrates infiltration of inflammatory cells such as monocytes/macrophages, and the number of kidney macrophages correlates directly with the duration and severity of renal injury in diabetes. CCR2 regulates monocyte/macrophage migration into injured tissues. However, the direct role of CCR2 in the pathogenesis of DN is not known. Our recent publication clearly shows that genetic deficiency or pharmacological blockade of CCR2 confers kidney protection in diabetic mouse models. Furthermore, our preliminary data suggest that activation of CCR2 may alter podocyte and/or glomerular endothelial cell integrity and function that may contribute to the glomerular permeability defects associated with DN. Therefore, we hypothesize that inhibition of CCR2 receptors expressed on kidney derived cells, such as endothelial cells or podocytes will ameliorate renal injury associated with DN.

Our experimental approach will use genetically altered mice (mice with complete absence of CCR2 expression and their wild type littermates, and transgenic mice that overexpress CCR2 specifically in endothelial cells (Tie2-CCR2) and podocytes (pNephrin-CCR2) to fully define the role of CCR2 in DN.

Aim 1: Test the hypothesis that CCR2 expressed on endothelial cells and podocytes directly contributes to diabetic renal injury. Our preliminary data suggest that activation of CCR2 may alter podocyte and/or glomerular endothelial cell integrity and function that may contribute to the glomerular permeability defects associated with DN. Thus we will use mice transgenic for CCR2 driven by the Tie2 promoter (endothelial specific; Tie2-CCR2) and by the pNephrin promoter

(podocyte specific; pNephrin-CCR2) to test whether this overexpression of CCR2 in either of these cell types is sufficient to induce renal damage *in vivo*.

Outcome: Results of these studies will provide a basis for the development of new therapeutic modalities in the development and progression of DN.

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Expected Research Outcomes and Benefits

This project is innovative because it capitalizes on new concepts in the pathogenesis and therapeutic modalities of DN. The PI and Co-investigators provide a unique and powerful combination of expertise in renal pathophysiology, in genetic approaches and in renal inflammation to elucidate mechanisms involved in diabetic renal complications. This combination of expertise will ultimately contribute to the overall success of the proposal. Our initial results indicating that renal CCR2 expression contributes to the renal pathophysiology in diabetes and that a CCR2 antagonist can largely prevent development of diabetic renal complications are exciting and innovative. The use of transgenic mice to elucidate relative contributions of CCR2 expression levels on endothelial cells and podocytes in DN *in vivo* represents the development of new research tools to explore mechanisms involved in diabetic renal complications.

Therefore, this project will investigate, for the first time, the direct role of CCR2 on podocytes and endothelial cells. These data will reveal important signaling mechanisms for CCR2 on these cells. We anticipate that elevated CCR2 expression *per se* in control Tie2-CCR2 and pNephrin-CCR2 mice will be sufficient to induce renal damage compared to wild type mice. Therefore, we anticipate more aggressive kidney damage in streptozotocin (STZ)-induced diabetic Tie2-CCR2 and pNephrin-CCR2 mice compared to STZ-diabetic WT mice. These data could reveal a new model for DN that mimics human nephropathy. We also expect to find that endpoints of diabetic renal injury, including renal hypertrophy, glomerular filtration rate, albuminuria and glomerular matrix deposition, will be reduced by treatment with the CCR2 antagonist. Positive results would strengthen the resubmission of the NIH application and form a basis to develop clinical trials in humans for treatment of DN.

Summary of Research Completed

Role of MCP-1/MCP-5 in DN

Experiments were conducted in male $Ins2^{Akita}$ and their wild type littermate mice (DBA background) starting at 8 weeks of age until 17 weeks of age. At 8 weeks of age, animals began treatment with vehicle, MCP-1/MCP-5 (0.5/0.3 mg) inhibitors biweekly or captopril (24 mg/L in drinking water) for 9 weeks (n=4-8 each group). Renal function, histology, and kidney macrophage recruitment were monitored throughout the study. Selected data are shown:

MCP-1/MCP-5 inhibition ameliorates renal dysfunction in $Ins2^{Akita}$ mice. Vehicle-treated $Ins2^{Akita}$ mice had a significant increase in albuminuria compared to controls at 17 weeks of age. Albuminuria was significantly reduced in $Ins2^{Akita}$ mice treated with MCP-1/MCP-5 inhibitors or captopril at 17 weeks of age (Figure 1). Furthermore, $Ins2^{Akita}$ vehicle-treated mice show increased plasma creatinine (0.48 ± 0.07 ; $p < 0.0001$) compared to control (0.21 ± 0.03) mice; effects significantly reduced using MCP-1/MCP-5 inhibitors (0.25 ± 0.03 ; $p < 0.01$) or captopril (0.24 ± 0.05 ; $p < 0.001$) treatment despite comparable blood glucose levels, hemoglobin A1C and blood pressure levels.

MCP-1/MCP-5 inhibition decreases renal histological changes in $Ins2^{Akita}$ mice. PAS staining of kidney sections (Figure 2) showed increased glomerular cellularity and mesangial expansion (score: 0.8 ± 0.2 vs. 0.4 ± 0.2 , $p < 0.01$) at 17 weeks of age in vehicle-treated $Ins2^{Akita}$ (Panel B) vs. control (Panel A), respectively. MCP-1/MCP-5 inhibitors (Panel C); but not Captopril (Panel D) treatment in $Ins2^{Akita}$ mice significantly reduced glomerular changes (scores: 0.4 ± 0.1 , $p < 0.05$ and 0.5 ± 0.1); respectively compared to vehicle-treated $Ins2^{Akita}$.

MCP-1/MCP-5 inhibition decreases macrophage recruitment in $Ins2^{Akita}$ mice. To determine whether MCP-1/MCP-5 is critical for kidney macrophage infiltration in DN, we show the distribution and quantitation of macrophages in kidneys by immunohistochemistry (Mac-2 positive macrophages) (Figure 3). The number of glomerular macrophages in control mice was low and increased significantly in vehicle-treated $Ins2^{Akita}$ mice (0.17 ± 0.01 and 2.4 ± 0.25 macrophages/glomerulus; $p < 0.0001$; respectively). Both MCP-1/MCP-5 inhibitors and captopril treatment in $Ins2^{Akita}$ mice displayed significantly reduced glomerular macrophage recruitment (0.99 ± 0.19 and 0.53 ± 0.03 macrophages/glomerulus; $p < 0.001$; respectively) compared to vehicle-treated $Ins2^{Akita}$ mice; at 17 weeks of age.

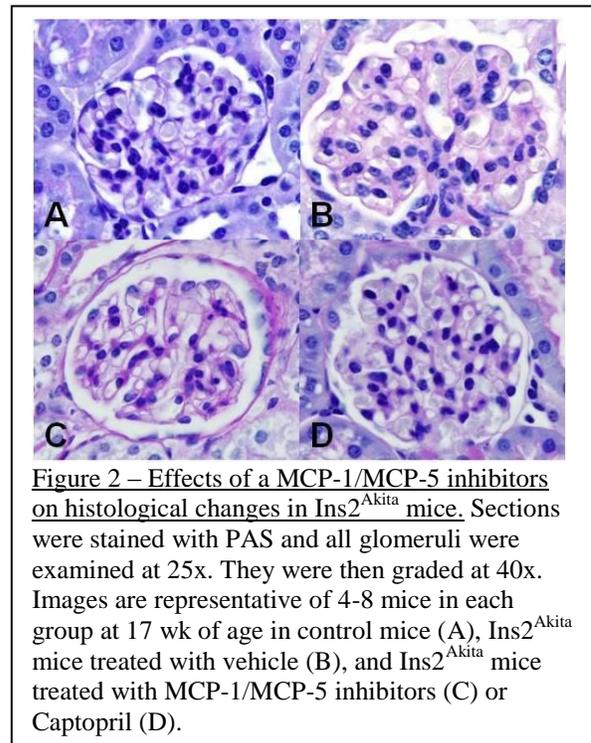
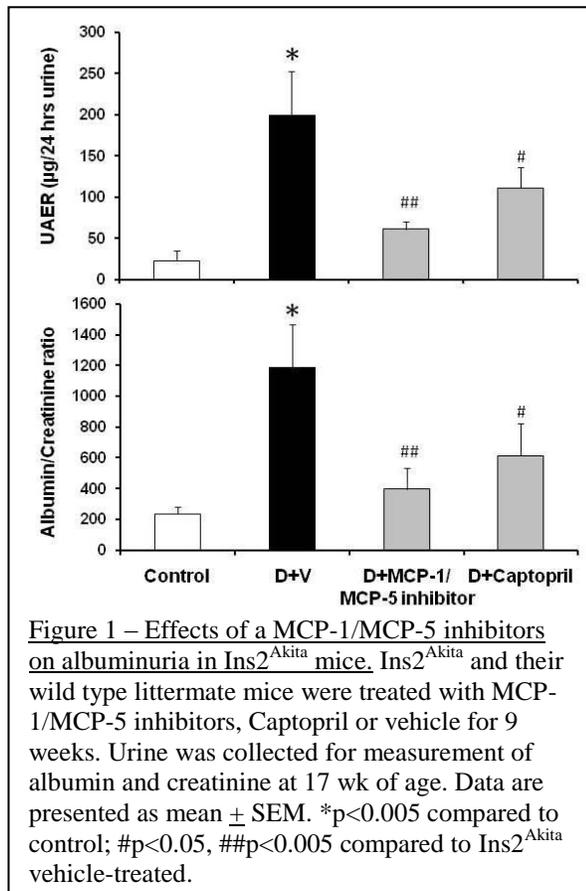
What is the direct contribution of CCR2 expressed on podocytes to the progression of DN?

During this 6 months period, we focused our effort to successfully generate Podocin-CCR2 mice (mice that overexpress CCR2 in podocytes). Schematic diagram for generating this mouse is shown in Figure 4.

- During the end of the first annual reporting period, we performed our first micro-injection. DNA was prepared for micro-injection by precipitation in 100% Ethanol. Quality control of DNA samples were assessed using a 2100 bioanalyzer in the Penn State core facility. DNA was diluted to 4ng/ul, and micro-injection was performed in Penn State transgenic mouse core facility. Genomic DNA was isolated from mouse tails using a Qiagen DNeasy Kit. PCR was performed to detect transgene in mouse genomic DNA. While initial genotyping PCR

results indicate that 3 pups might carry podocin-CCR2 transgene, our further characterization was negative.

- We next performed a second micro-injection at the Penn State core facility. However, this micro-injection was unsuccessful.
- We next sent our DNA to Cyagen Company which specializes in micro-injection and generating transgenic mice. After 2 micro-injections experiments, we finally were able to successfully generate a total of 5 (4 females and 1 male) podocin-CCR2 transgenic mice *Figure 5*.



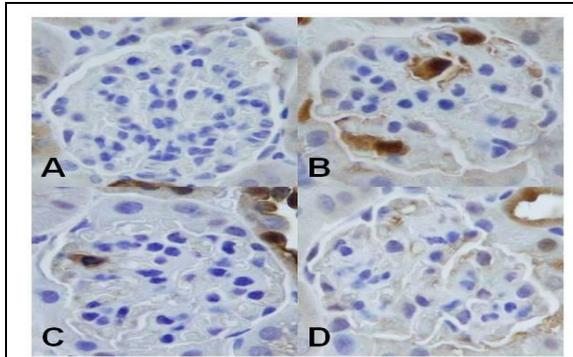


Figure 3 – Effects of a MCP-1/MCP-5 inhibitors on macrophage recruitment in *Ins2^{Akita}* mice. Immunohistochemical staining for Mac-2 positive macrophages in glomeruli at 17 wk of age. Images are representative of 4-8 mice in each group in control mice (A), *Ins2^{Akita}* mice treated with vehicle (B), and *Ins2^{Akita}* mice treated with MCP-1/MCP-5 inhibitors (C) or Captopril (D).

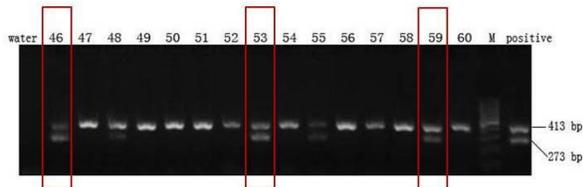
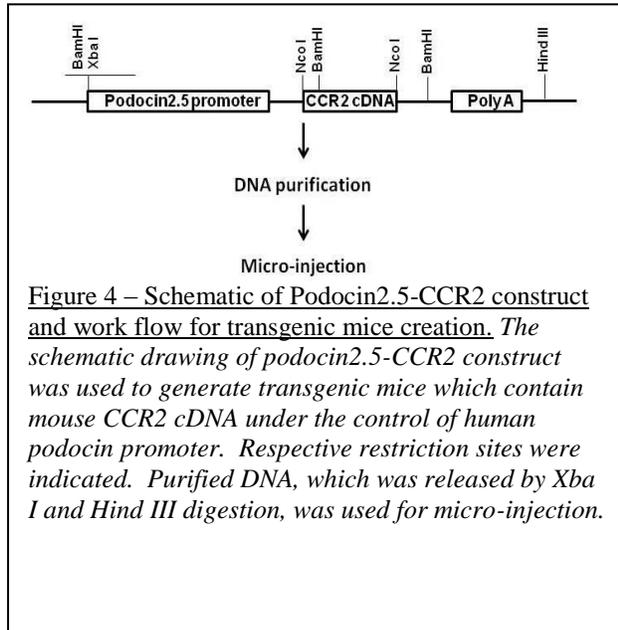


Figure 5 – Genotyping PCR. Pups genomic DNA was isolated and subjected to genotyping PCR. The 413 bp PCR product represents internal control β -actin. The 273 bp PCR product represents podocin2.5-CCR2 transgene. The podocin2.5-CCR2 construct was used as a positive control; and water was used as a negative control. Figure shows representative 3 podocin-CCR2 transgenic mice out of total 5 generated.

Research Project 39: Project Title and Purpose

Spectroscopic Studies of Cardiac Myosin Structure Function – The purpose of these studies is to investigate how mutations in the cardiac myosin heavy chain associated with inherited cardiomyopathies alter cardiomyocyte contraction and the development of specific disease phenotypes. Our approach will be to express and purify a motor fragment of human beta cardiac myosin in the muscle derived C₂C₁₂ cells that can be fluorescently labeled at strategic sites. We will examine key structural changes in the enzymatic cycle of cardiac myosin using fluorescence spectroscopy, kinetic analysis, and fluorescence resonance energy transfer. Our analysis of the impact of mutations on structural transitions and contractile function will provide insight into disease pathogenesis and potential therapies.

Duration of Project

4/10/2012 – 12/31/2013

Project Overview

Inherited cardiomyopathies are the leading cause of sudden death in young people and affect one in five hundred individuals worldwide. A large fraction of the mutations are associated with human beta-cardiac myosin, the molecular motor that drives contraction in ventricular cardiomyocytes. The mutations are located throughout the motor domain and can lead to a variety of different phenotypes including hypertrophic, dilated, and restricted. It is critical to determine how the different cardiomyopathy mutations alter key structural changes, and how this results in specific defects in motor function and disease pathogenesis. The atomic level structures of myosin in various stages of its enzymatic cycle have provided a framework of the molecular mechanism of force generation utilized by myosin. These structures as well as other biochemical and structural data suggest that myosin generates force by coupling small conformational changes in the nucleotide-binding region to a large swing of the light-chain binding region while myosin is strongly bound to actin. However, there is a lack of information about the subdomain coordination that is necessary for actin to activate the release of the products of ATP hydrolysis (phosphate and ADP) and trigger the force generating swing of the lever arm. Myosin V, a non-muscle myosin that has unique structural and biochemical properties, is used by our group as a model to examine specific conformational changes in the actin-binding, nucleotide-binding, and lever arm regions. We have developed a system in myosin V in which fluorescence probes are strategically placed to measure conformational changes in these three critical regions using fluorescence resonance energy transfer (FRET). We will develop a similar system of examining key conformational changes in cardiac myosin by engineering the fluorescence labeling sites at the corresponding locations in cardiac myosin. The engineered cardiac myosin will be used to examine the impact of cardiomyopathy mutations on key structural changes to determine how these changes alter motor function. Overall, our studies will be instrumental in developing therapeutic drugs that target myosin motor activity in heart failure and establishing the structural defects associated with cardiomyopathy mutations in myosin.

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Other Participating Researchers

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Expected Research Outcomes and Benefits

The research aims of the project will lead to a better understanding of how cardiomyopathy mutants in cardiac myosin lead to specific defects in heart function. The contractile activity of the heart relies on the finely tuned motor activity of cardiac myosin that drives contraction in the heart. Our studies will define how cardiomyopathy mutations impact important structural changes in cardiac myosin that alter the force generating properties of the motor. One of the mysteries of inherited cardiomyopathies is how the many different mutations can lead to different disease phenotypes. Our studies will provide insights into how certain structural defects can lead to disease phenotypes and provide opportunities for the development of treatments for the various forms of cardiomyopathies. The methods established for examining structural changes in cardiac myosin will be extremely valuable for designing high throughput screening studies designed to develop drugs capable of specifically modulating cardiac myosin motor activity. These drugs would also be helpful in treating contractile dysfunction associated with various forms of heart disease.

Summary of Research Completed

Progress on AIM #1 *To establish a system of examining key structural changes in beta-cardiac myosin utilizing site specific fluorescence labeling and spectroscopy.*

We have successfully established as spectroscopic system to examine key structural changes in human beta cardiac myosin. We describe the details of the development of the project below.

Recombinant Adenovirus and Expression System: The only system to express recombinant beta cardiac myosin is the adenovirus based C₂C₁₂ expression system. The method requires culturing embryonic derived muscle cells and differentiating them into myotubes. The myotubes are infected with a high titer adenovirus that drives the expression of cardiac myosin. We first generated the cardiac myosin cDNA containing sites for fluorescent labeling, and a FLAG tag for purification. The recombinant adenovirus was produced with the AdEasy system which utilized the 293 cell line. High titer adenovirus was produced with a large scale virus amplification and CsCl preparation. We determine the titer of the virus using viral titer plaque assays. The protein purification of cardiac myosin from the C₂C₁₂ cells required using immunoaffinity chromatography. In Figure 1A we demonstrate successful purification of human beta-cardiac myosin subfragment 1 (M2β-S1) resolved by coomassie stained SDS-PAGE gels. We also introduced a tetracysteine site in the upper 50 kDa domain (M2β-S1.U50) for labeling with the bis-arsenical probe FAsH. Figure 1A also demonstrates that we can label cardiac myosin at this site, and the fluorescence is observed following SDS-PAGE. We demonstrate successful expression and purification of a beta cardiac myosin construct that contains the motor domain and a C-terminal GFP as well as an N-terminal tetracysteine site (M2β-GFP.NT). Figure 1B demonstrates a coomassie stained and fluorescent gel showing purification and ReAsH labeling of this construct.

Biochemical Results: We performed actin-activated ATPase assays to demonstrate the activity of purified M2β-S1.U50 both unlabeled and labeled with FAsH (Figure 2). The ATPase values were found to be minimally affected by FAsH labeling and similar to published values for tissue

purified cardiac myosin. We also performed in vitro motility assays to examine the motile properties and found that the velocity of motion generated by both labeled and unlabeled M2 β -S1.U50 was similar. Thus, we successfully demonstrated purification of cardiac myosin that can be site-specifically labeled without altering the ATPase and motor properties.

Spectroscopic Results: We have begun to examine the structural changes in FIAsh labeled M2 β -S1.U50 using fluorescence spectroscopy. We propose to examine the opening and closing of the nucleotide binding pocket with the U50 FIAsh and Cy3ATP, donor-acceptor pairs. We demonstrate preliminary data that highlights the decrease in FIAsh fluorescence in the presence of Cy3ATP (Figure 3). We also monitored the rate constant of Cy3ATP binding to M2 β -S1.U50.FIAsh in the stopped flow and find it is linearly dependent on ATP concentration (Figure 3, inset). We examined the FRET between IAEDANs labeled actin and M2 β -S1.U50.FIAsh, which is proposed to measure the conformation of the actin binding cleft. Figure 4 demonstrates the quenching of IAEDANs-actin fluorescence when M2 β -S1.U50.FIAsh is bound in the absence of nucleotide. To measure the conformation of the lever arm we proposed to examine the FRET between ReAsH labeled at the N-terminus and GFP at the C-terminus. We observed strong quenching of GFP fluorescence when the N-terminus was labeled with ReAsH and an increase in GFP fluorescence upon ATP binding. Thus, our strategy for measuring three important conformational changes in human cardiac myosin is successful and will allow us to examine the impact of cardiomyopathy mutations.

Progress on AIM #2. To examine the structural and functional impact of cardiomyopathy mutations in beta-cardiac myosin with fluorescence spectroscopic analysis.

We have developed a strategy to examine the impact of cardiomyopathy mutations on cardiac myosin structure function. We inserted the disease associated mutations at appropriate locations in the cDNA. We also generated high titer recombinant adenovirus of the mutant constructs. We are currently in the process of expressing and purifying the mutant constructs. Soon after that we will begin characterizing their impact on motor activity and key structural changes.

Impact of point mutations. In order to further develop our FRET based technology for examining key conformational changes in myosin we performed experiment with myosin V, which has served as a model for developing our approach. We also examined the impact of point mutations in myosin V which has enhanced our overall spectroscopic approach as well as understanding of the actomyosin-based force generation mechanism. We examined two point mutations in the conserved switch II region of myosin by measuring FRET changes associated with the opening and closing of the nucleotide binding pocket and actin binding cleft. We found that introduction of the G440A mutation results in an uncoupling between these two domains during the ATP-binding step. We also found that disruption of the salt bridge that stabilizes the interaction between the switch I and switch II regions is important for mediating the opening of the nucleotide binding pocket during the ADP release step. We published this work in *Biophysical Journal* (Trivedi et al., 2012). Our results have uncovered a coupling pathway between the actin and nucleotide binding regions, which will be important for assessing the impact of cardiomyopathy mutations associated with this pathway.

Impact of magnesium. A long standing question in the actomyosin field is understanding how

coordination of the magnesium ion (Mg) in the active site alters the structural and functional properties of myosin. We found that Mg is critical for forming the closed nucleotide binding pocket conformation in the presence of ADP and is the main step in ATPase cycle that is altered by physiological Mg concentrations is the ADP release steps. Thus, myosin V is highly Mg dependent and its ATPase activity and motor properties are altered by Mg in the physiological range. Our results were published this year in the journal *Biochemistry* (Trivedi et al., 2013).

We examined the Mg dependence of several muscle (skeletal, cardiac, smooth) and non-muscle myosins and these results have been submitted to the *Journal of Biological Chemistry*. We find that all of these myosins have a similar mechanism of Mg-dependences (Figure 5). Overall, our results have led to novel insights into the regulation of myosin by physiological Mg concentrations. In addition, cardiomyopathy mutations that impact Mg coordination may alter the motor properties of cardiac myosin.

Publication: Trivedi, D.T., Muretta, J.M., Swenson, A.M., Thomas, D.D, and Yengo, C.M. Magnesium impacts myosin V motor activity by altering key conformational changes in the mechanochemical cycle. *Biochemistry* 52, 4710-4722.

Presentations - Posters presented at:

Regional Biophysical Society Meeting in University Park, PA , October 4, 2013.

1. Trivedi, D., Swenson, A., and Yengo C.M. Dynamics of lever arm swing in myosin V.
2. Swenson, A.S., Winkelmann, D., and Yengo, C.M. Spectroscopic systems for examining structural changes in beta cardiac myosin.

Pennsylvania Muscle Institute Annual Retreat, Philadelphia, PA , October 28, 2013.

1. Trivedi, D., Swenson, A., and Yengo C.M. Dynamics of lever arm swing in myosin V.
2. Swenson, A.S., Winkelmann, D., and Yengo, C.M. Spectroscopic systems for examining structural changes in beta cardiac myosin.

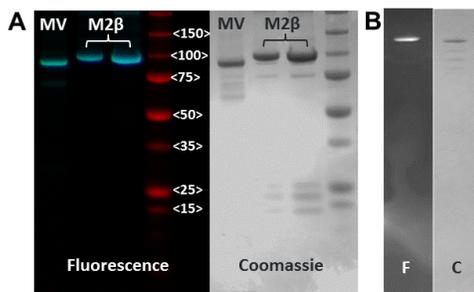


Fig. 1. A) Purified M2β-S1.U50 labeled with FIAsh compared to MV FIAsh. B) Purified M2β-GFP. NT labeled with ReAsH. Both coomassie (C) and fluorescence (F) gels are shown.

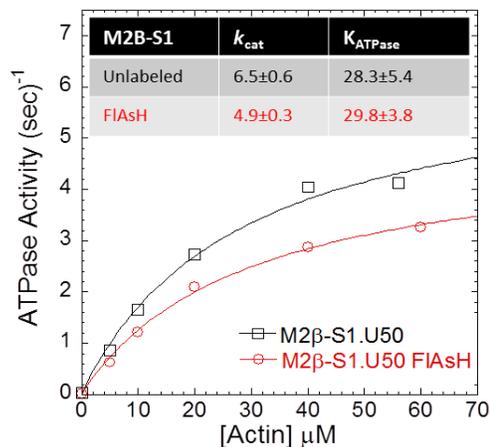


Fig. 2. Actin-activated ATPase activity of purified M2β-S1.U50. (FIAsH labeled and unlabeled)

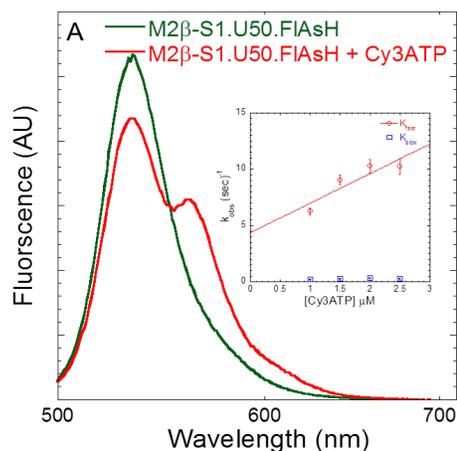


Fig. 3. FRET observed upon Cy3ATP binding to M2β-S1.U50 FIAsH. Inset, stopped-flow rate constants observed upon mixing M2β-S1.U50 FIAsH with Cy3TP.

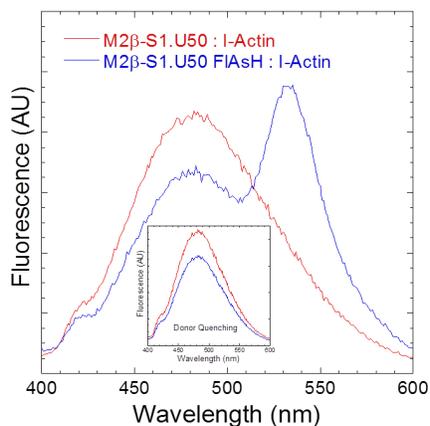


Fig. 4. FRET observed upon M2β-S1.U50 FIAsH binding to IAEDANS-Actin. Inset is donor quenching used to quantify FRET efficiency.

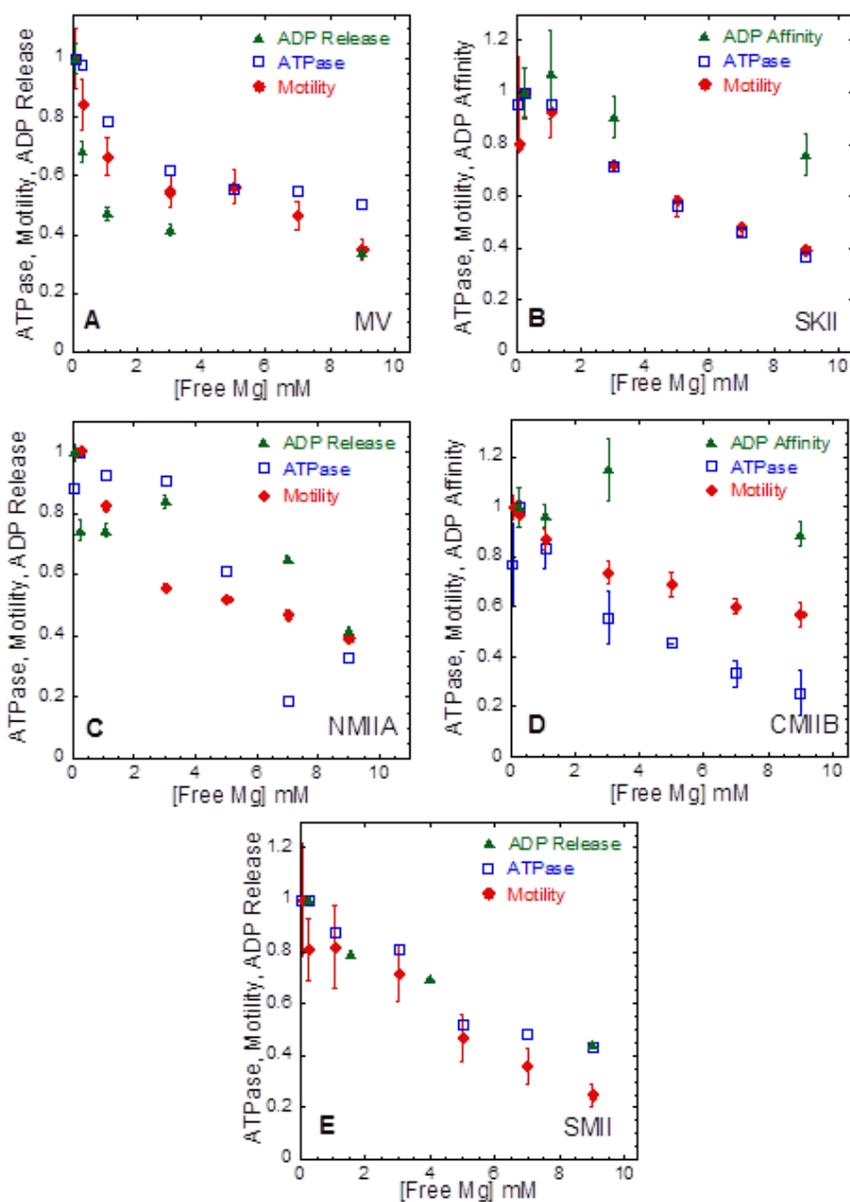


FIGURE 5: Influence of Mg on actomyosin ATPase, motility, and ADP release/affinity. Steady-state ATPase (*open blue squares*), motility (*filled red circles*), and ADP release/affinity (*filled green triangles*) were performed in ionic-strength controlled conditions with 0.5-10 mM $MgCl_2$ for (A) MV and members of the myosin II family: (B) SKII, (C) NMIIA, (D) CMIIIB, and (E) SMII. The ATPase assays were performed at 25°C with fixed actin concentrations in MV (20 μM actin) and MII (60 μM actin). Actomyosin motility was performed at 27°C. The data are plotted as relative values. The ADP release rate constant was examined with *mant*dADP and ADP affinity measured by competition with ATP-induced dissociation.

Research Project 40: Project Title and Purpose

Oncogenic Mutations/Rearrangements in Patients with Advanced Adenocarcinoma Lung - Most patients present after non-small-cell lung carcinoma (NSCLC) has spread to regional or distant sites. Patients presenting with advanced, unresectable disease (stage IIIB or IV) and patients who develop recurrent or metastatic disease following surgical resection are candidates for systemic therapy. There is an urgent need to develop novel and effective regimens as the current therapies do not offer a curative potential. Testing for mutations/gene rearrangements as predictive biomarkers of efficacy to both chemotherapy and targeted approaches with integration of molecular diagnostics in tumor samples and circulating tumor cells will be key to personalized treatments. The current project is designed to pave the way for individualizing therapy for patients with lung cancer.

Duration of Project

7/20/2012 – 12/31/2013

Project Overview

We hypothesize that approximately 50% of the patients with advanced adenocarcinoma of the lung will have the presence of one of the driver mutations/gene rearrangements that is related to clinical outcome (clinical features and overall survival). In addition, the study will characterize prognostic markers in adenocarcinoma lung and identify new drugable targets. This will subsequently lead to individualized approaches to treatment and overall increased efficacy.

Specific Aim 1: To determine the frequency of oncogenic mutation/gene arrangements in tumors from patients with advanced adenocarcinoma of the lung. The mutations of interest include those seen in EGFR, HER2, BRAF, MET, MEK1, EGFR, Ross1 and rearrangement of the EML4-ALK fusion gene.

Specific Aim 2: To correlate the presence of the oncogenic mutation/gene arrangements with clinical outcome. The clinical features associated with these mutations are not well characterized as the testing for these abnormalities has not been done in the current clinical setting. As some of these mutations/rearrangements are drugable, the ultimate goal will be to develop effective therapies with better understanding of the biology of lung cancer.

Specific Aim 3: To enumerate circulating tumor cells (EpCAM+CD45) from the same patients with adenocarcinoma of the lung and correlate with clinical outcome and detect the presence of the oncogenic mutations/gene rearrangements (e.g. ALK fusions) as outlined above. The tumor cell heterogeneity in the CTC population will be examined and compared with that seen in primary tumor samples.

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Expected Research Outcomes and Benefits

The overall research outcomes and benefits of this project are to develop effective therapeutic strategies which may result in elimination of adenocarcinoma lung with the understanding of the biology of the cancer. We are expecting to precisely outline the key driver mutations/gene rearrangements and define drugable targets that will improve the overall efficacy and outcome. In addition, we want to see whether these mutations/gene rearrangements can be evaluated in enumerated circulating tumor cells and compare them to those seen in the tumor tissue from these patients.

Molecular markers for treatment selection of patients with NSCLC will be necessary to personalize therapy. The presence of an activating mutation in the EGFR is associated with high response rates and improved PFS with EGFR TKI's. This has already led to the use of an EGFR TKI as first line therapy in patients with a sensitive EGFR mutation as opposed to standard combination therapy. On the other hand, mutations in the k-ras oncogene lead to decreased effectiveness of EGFR TKIs. EGFR mutations are more common in females, non-smokers and with adenocarcinoma histology while mutations in k-ras are more common in smokers. For patients with wild-type EGFR (or if the EGFR status is unknown) chemotherapy remains as the 'standard of care' for first-line therapy of advanced NSCLC. The accelerated approval of crizotinib targeting anaplastic lymphoma kinase (ALK) in NSCLC, based on objective response rates in parallel with a diagnostic test is a true example of a personalized approach. Other markers of interest in NSCLC include: ERCC1, RRM1, TS, Ross1, k-ras mutation, Her-2, BRAF, EGFR, MEK1 c-met expression/mutation, etc. Thus the major focus of current research is to identify various driver mutation/gene rearrangements of interest in adenocarcinoma of the lung and correlate with clinical features and outcome. Identification of these correlative markers (mutations/gene rearrangements) will prove to be significant in developing personalized therapy for lung cancer.

Summary of Research Completed

We opened a clinical trial entitled "Oncogenic Mutations/Gene Rearrangements in Patients with Advanced Adenocarcinoma Lung" (PSHCI-12-064, IRB #40267EP) for this project. Target

accrual is 40 patients and we have successfully enrolled 33 patients to the trial. Among them, 29 subjects were recruited from Penn State Hershey Cancer Institute. Research blood, FFPE-derived tumor tissues as well as clinical data were collected from these patients. In addition, four eligible patients' samples along with clinical data were requested from Lung Cancer Biospecimen Resource Network (LCBRN). Next generation sequencing technology has been used to detect oncogenic mutations in FFPE samples of tumor DNA from 18 patients with lung adenocarcinoma (*Specific Aim 1*). The association of common mutation statuses with clinical data was analyzed in these 18 patients (*Specific Aim 2*). Finally, circulating tumor cell (CTCs) counts were determined in ten patient bloods over their courses of therapy (*Specific Aim 3*).

Specific Aim 1: To determine the frequency of oncogenic mutation/gene arrangements in tumors from patients with advanced adenocarcinoma of the lung. The mutations of interest include those seen in EGFR, HER2, BRAF, MET, MEK1, EGFR, Ross1 and rearrangement of the EML4-ALK fusion gene.

Material and Methods: The purpose of this study was to identify and quantify somatic mutations in archived FFPE tumor tissues from patients with advanced-staged lung adenocarcinoma, utilizing Ion AmpliSeq™ Cancer panel in Ion Torrent platform. The Ion AmpliSeq™ Cancer Panel represents a fast and easy approach to generating target sequence for next-generation sequencing. It requires very little input DNA, and high read depths can be generated to support good sensitivity. In addition, Ion AmpliSeq™ technology is a new tool for enriching tens to thousands of genomic targets in a single tube from 10ng of input DNA. The Ion AmpliSeq™ Cancer Panel consists of a pool of 190 primer pairs that are designed to amplify mutation hotspot regions (including 739 known somatic mutations) across 46 genes. 14 FFPE-derived (from PSHCI) and 4 frozen resected (from LCBRN) lung adenocarcinoma samples of tumor DNA were amplified using this panel, and the products were then ligated with barcodes, subjected to emulsion PCR and sequenced on an Ion Torrent PGM using 316 chips (6 samples per chip).

Results: Among 18 lung adenocarcinoma patient specimens, we identified 37 genetic alterations in 19 genes by Ion Torrent variant caller software (Table 1). The sequencing analysis revealed frequent mutations in *TP53* (61%), *KDR* (44%), *KRAS* (28%), and *KIT* (17%) genes in these lung cancer patient samples. Moreover, distinctive patterns and the combination of mutations in various sets of genes, including *TP53*, *KDR*, *KRAS*, *KIT*, *SMO*, *MET*, *FGFR3*, *PTEN*, *ATM*, *PTPN11*, *APC*, *ABL1*, *HNF1A*, *SMARCB1*, *CDKN2A*, *STK11*, *ERBB4*, *ERBB2* and *IDH1* were also identified. The range of somatic mutation frequencies was 5.76% to 98.99% and variant coverage ranges from 100× to 3249×. The average amplicon read length was 76 bp and the average read depth was 669; more than 87.5% of the amplicons had a read depth >200. In addition to the known sequence variants, a new frameshift mutation for gene *GNAI1* in patient 01-018 and a new nonsense mutation for gene *STK11* in patient LCBRN_V0120 were detected. Interestingly, 72% of specimens harbored at least one “druggable” alteration that has been linked to a target treatment or is currently being investigated in clinical trials. Those pairs of drug and targeted genes include AEE 788 to *KDR*; selumetinib to *KRAS*; sunitinib to *KIT*; vismodegib to *SMO*; Tivantinib to *MET*. These preliminary results indicate the necessity of targeted sequencing of key cancer related genes enabling identification of “druggable” mutations for individual lung adenocarcinoma patients.

Specific Aim 2: To correlate the presence of the oncogenic mutation/gene arrangements with clinical outcome. The clinical features associated with these mutations are not well characterized as the testing for these abnormalities has not been done in the current clinical setting. As some of these mutations/rearrangements are drugable, the ultimate goal will be to develop effective therapies with better understanding of the biology of lung cancer.

Material and Methods: Two sample T test was used to analyze the potential association between the most common *TP53*, *KRAS*, *KDR* mutations and clinical factors including gender, age, race, disease stage, body surface area (BSA), ECOG performance status, tumor burden, systolic BP, diastolic BP, height, weight, pulse, respiration rate, temperature, alcohol assumption, smoking history, family lung cancer history, levels of LDH, albumin, alkaline phosphatase, bicarbonate, bilirubin, glucose, BUN, calcium, chloride, creatinine, RBC, WBC, ANC, platelets, and percentage of basophils (%), eosinophils (%), hematocrit (%), hemoglobin, neutrophils (%), WBC - Lymphocytes (%), WBC - Monocytes (%). In addition, the effect of *TP53*, *KRAS*, *KDR* mutations on overall survival in lung adenocarcinoma patients were determined by Kaplan–Meier survival curve.

Results: Overall survival of patients with stage IIIB or IV lung adenocarcinoma was statistically significantly higher in those with *TP53* mutant tumors (medium survival 1075 days) than in those with *TP53* wild-type tumors (medium survival 366 days) ($P= 0.023$) (Figure 1). In addition, the tumor burden evaluated by RECIST is larger in those with *TP53* mutant tumors (11.31 ± 1.76 cm) than in those with *TP53* wild-type tumors (7.1 ± 1.02 cm) ($P= 0.045$) (Figure 2). No clinical factor was found to be significantly associated with *TP53*, *KRAS*, *KDR* and *KIT* mutations (data not shown), due to relatively small sample size ($n=18$).

Specific Aim 3: To enumerate circulating tumor cells (EpCAM+CD45) from the same patients with adenocarcinoma of the lung and correlate with clinical outcome and detect the presence of the oncogenic mutations/gene rearrangements (e.g. ALK fusions) as outlined above. The tumor cell heterogeneity in the CTC population will be examined and compared with that seen in primary tumor samples.

Material and Methods: The dissemination of circulating tumor cells (CTCs) that cause metastases in distant organs accounts for the majority of cancer related deaths. CTCs have been established as a cancer biomarker of known prognostic value. The enrichment of viable CTCs for *ex vivo* analysis could further improve cancer diagnosis and guide treatment selection. We collaborated with Dr. Siyang Zheng, whose Micro & Nano Integrated Biosystem Laboratory at Penn State University Park designed a new flexible micro spring array (FMSA) device for the enrichment of viable CTCs independent of antigen expression (Harouaka RA, et al. *Clinical Chemistry*, 60:2, 2014). CTCs were enriched and counted in blood samples obtained from ten lung adenocarcinoma patients throughout their chemotherapies.

Results: FMSA device can enrich tumor cells with 90% capture efficiency, higher than 10^4 enrichment, and better than 80% viability from 7.5 mL whole blood samples in less than 10 minutes on a 0.5 cm^2 device. Varying trends were observed in CTC counts from these lung cancer patients over the course of therapy (Harouaka RA, et al. *Clinical Chemistry*, 60:2, 2014). Baseline CTC counts were obtained prior to the initiation of therapy. Overall Survival was calculated

based on measured baseline values for 10 patients undergoing therapy and followed up for 300 days. The patients were split into two groups: one with ≤ 5 CTCs detected at the baselines measurement (n=5), and another with ≥ 22 CTCs at baseline (n=5). Patients with ≤ 5 CTCs were observed to have a greater probability of survival beyond 200 days, though a greater sample size is required to establish statistical significance (Figure 3). Interestingly, patient 01-005 initially showed a sharp decline in FMSA detected CTCs after the first cycle of treatment, but then CTC counts increased sharply and the patient died within two months of the final blood draw. Patients 01-001 and 01-004 with declining or comparatively low CTC counts over the course of therapy are alive, and have survived at least 19 and 32 months respectively since diagnosis (Harouaka RA, et al. Clinical Chemistry, 60:2, 2014). Additional blood samples obtained during follow up visits were analyzed to monitor CTC counts during the course of therapy. Most patients with declining or comparatively low CTC counts over the course of therapy survived for 300 days after the baseline measurement, as indicated by the green lines in Figure 4. All patients except one with an initial CTC count greater than 5 died within 300 days.

Table 1. Detection of somatic mutation in lung adenocarcinoma patient tumor samples.

patient #	GENE_NAME	Chro	Zygoty	POS	Residue_Ref	Residue_Alt	TYPE
01-001	KRAS	12	Het	13	G	R	Single AA Change
	TP53	17	Het	274	V	D	Single AA Change
01-003	KIT	4	Hom	852	V	I	Single AA Change
01-004	TP53	17	Het	173	V	L	Single AA Change
01-005	KDR	5	Het	472	Q	H	Single AA Change
	CDKN2A	12	Het	124	G	V	Single AA Change
	KRAS	14	Het	12	G	A	Single AA Change
01-006	KDR	4	Het	472	Q	H	Single AA Change
	TP53	17	Het	215	S	G	Single AA Change
01-007	N/A						
01-008	APC	5	Het	1105	R	W	Single AA Change
	KRAS	12	Het	12	G	C	Single AA Change
	TP53	17	Het	277	C	W	Single AA Change
	STK11	19	Het	163	G	V	Single AA Change
01-009	KDR	4	Het	472	Q	H	Single AA Change
	TP53	17	Het	215	S	G	Single AA Change
01-010	N/A						
01-011	KDR	4	Het	472	Q	H	Single AA Change
	ABL1	9	Het	266	K	R	Single AA Change

	KRAS	12	Het	61	Q	L	Single AA Change
01-013	KDR	4	Het	472	Q	H	Single AA Change
	MET	7	Het	359	R	Q	Single AA Change
	KRAS	12	Het	12	G	C	Single AA Change
01-016	TP53	17	Het	158	R	L	Single AA Change
01-017	ERBB4	2	Het	362	I	M	Single AA Change
	TP53	17	Het	245	G	V	Single AA Change
01-018	GNA11	19	Het	27	TG	T	frameshift
LCBRN_V0007	KIT	8	Het	677	D	E	Single AA Change
	KDR	10	Het	472	Q	H	Single AA Change
	MET	18	Het	162	F	L	Single AA Change
	SMO	19	Het	404	V	M	Single AA Change
	HNF1A	30	Het	202	N	D	Single AA Change
	TP53	34	Het	242	C	W	Single AA Change
	TP53	37	Het	105	G	C	Single AA Change
	SMARCB1	40	Het	155	F	L	Single AA Change
LCBRN_V0120	KDR	4	Het	472	Q	H	Single AA Change
	SMO	7	Het	532	M	T	Single AA Change
	TP53	17	Het	248	R	Q	Single AA Change
	ERBB2	17	Het	862	T	A	Single AA Change
	STK11	19	Het	48	K	*	Nonsense
LCBRN_W0024	IDH1	2	Het	115	K	E	Single AA Change
LCBRN_W0076	ERBB4	2	Het	945	C	Y	Single AA Change
	FGFR3	4	Het	699	G	S	Single AA Change
	KIT	4	Het	566	N	S	Single AA Change
	KDR	4	Het	1196	T	I	Single AA Change
	PTEN	10	Het	173	R	H	Single AA Change
	ATM	11	Het	3045	L	P	Single AA Change
	PTPN11	12	Het	61	D	A	Single AA Change
	TP53	17	Het	286	E	Q	Single AA Change

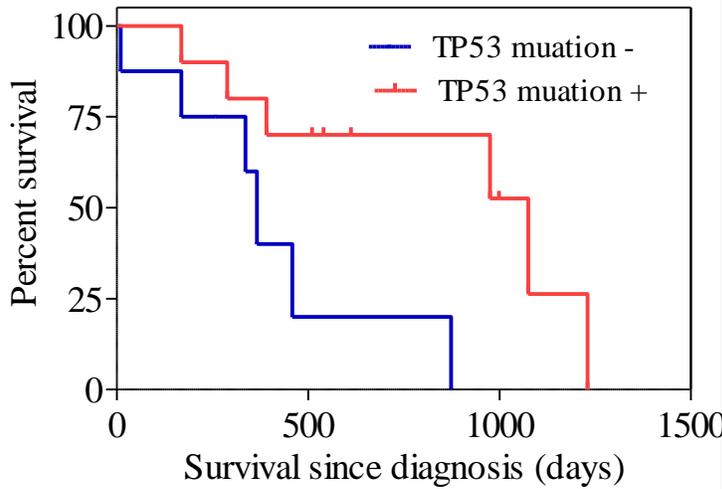


Figure 1. Effect of *TP53* mutations on overall survival in lung adenocarcinoma. Kaplan–Meier survival curve for each patient group was compared using the log-rank test. Overall survival of patients with stage IIIB or IV lung adenocarcinoma was statistically significantly higher in those with *TP53* mutant tumors than in those with *TP53* wild-type tumors ($P= 0.023$).

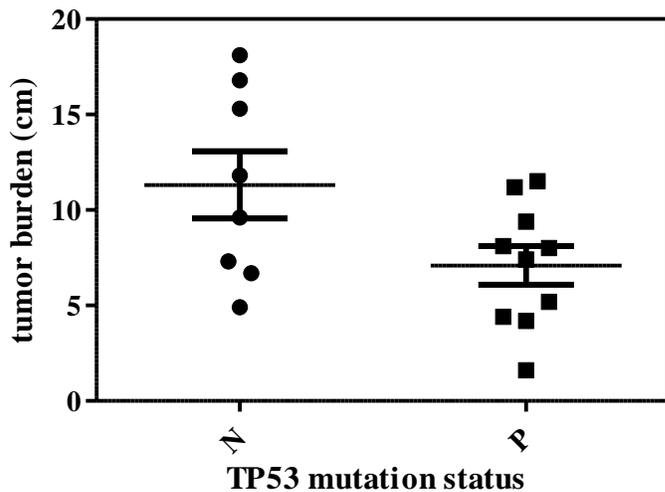


Figure 2. Effect of *TP53* mutations on tumor burden in lung adenocarcinoma. Tumor burden of patients with stage IIIB or IV lung adenocarcinoma was statistically significantly larger in those with *TP53* mutant tumors than in those with *TP53* wild-type tumors by unpaired T-test ($P= 0.045$). N=TP53 mutation negative, P=TP53 mutation positive.

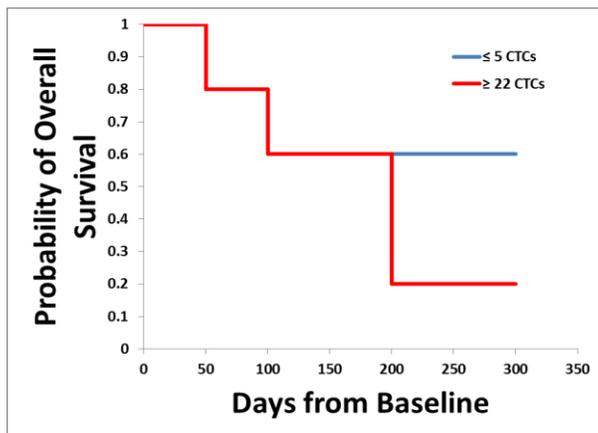


Figure 3. Kaplan-Meier estimates of Overall Survival probabilities for patients determined to have ≤ 5 CTCs at the baseline measurement ($n=5$) and for patients with ≥ 22 CTCs at the baseline measurement ($n=5$).

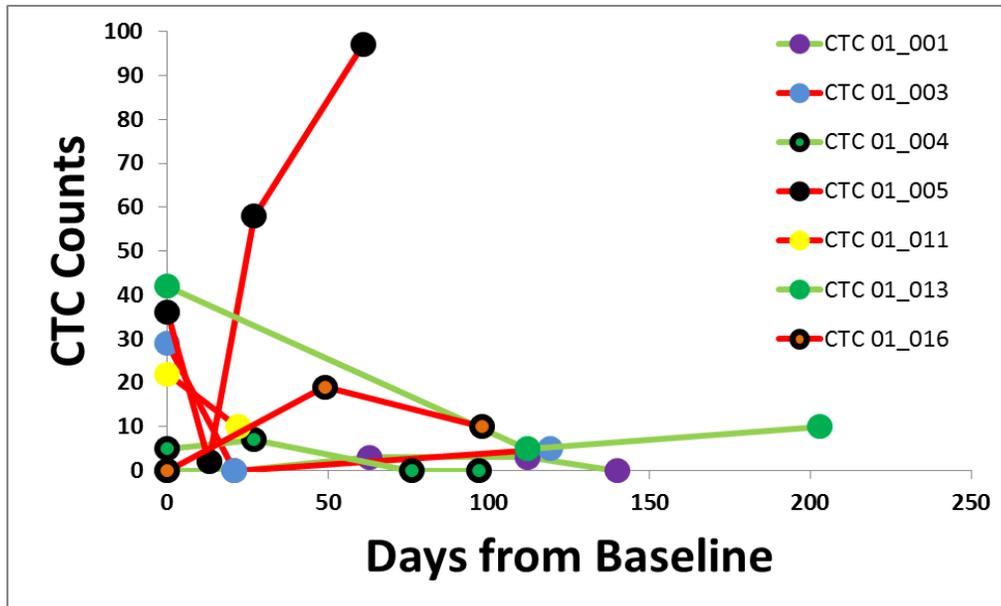


Figure 4. CTC counts are plotted for 7 patients through the course of therapy. Baseline counts obtained prior to initiation of therapy occurred at Day 0. Green lines represent patients that have survived 300 days from baseline, while red lines represent patients that have deceased.

Research Project 41: Project Title and Purpose

Development of a Nanotechnology Platform for Delivery of siRNA to Cancer Cells - The purpose of this project is to develop a nanotechnology platform that will deliver an effective dose of siRNA to a cancer cell thereby increasing the vulnerability of the cancer cells to therapeutic intervention. We have selected glioblastoma multiforme (GBM) as our initial target for this research. We will build on our established and successful liposomal formulation that selectively targets GBMs and delivers effective doses of chemotherapeutic agents to the cancer cells. Specifically, we have developed an innovative approach taking advantage of the selective expression of interleukin 13 receptors on glioma cells to selectively target drug and contrast enhancing agents in liposomes to cancer cells. We will modify these liposomes to carry siRNA for ferritin, an intracellular iron storage and cytoprotective protein to the cancer cells. We have shown that decreasing expression of ferritin through siRNA therapy will increase the vulnerability of the cancer cells to chemotoxins and radiation thus allowing more efficient killing of cancer cells while limiting side effects to normal tissue. We have developed liposomes to carry the siRNA for ferritin and the efficacy data have been published. This project will focus on optimizing the technology for targeted delivery of the siRNA carrying liposomes to tumors and showing efficacy in an orthotopic tumor model.

Duration of Project

7/20/2012 - 6/30/2013

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at <http://www.health.state.pa.us/cure>.

Research Project 42: Project Title and Purpose

Molecular Analysis of Breast Cancer CTC Heterogeneity and Drug Sensitivity in Preclinical Models and Patients - Circulating tumor cells (CTCs) in patients with metastatic breast cancer are shed into the blood circulation and provide valuable information about a patient's prognosis as well as their response to therapy. We believe that a molecular analysis (gene mutations and proteins) of CTCs and a study of our ability to kill living recovered tumor cells from a patient's blood sample (drug sensitivity) will provide important information to the treating physician to guide therapy decisions. Because cancer cells even within a given patient are heterogeneous and may require combination therapy to target all the different kinds of malignant cells, we believe that a 'liquid biopsy' of the circulating cells in patients with advanced aggressive breast cancer will allow us to personalize therapy by testing treatments on the patient's own tumor cells before treatments are given to patients.

Duration of Project

7/20/2012 - 12/31/2013

Project Overview

The objective of the project is to isolate circulating tumor cells (CTCs) from a mouse model of breast cancer as well as from patients with metastatic breast cancer and perform two types of analysis on the recovered cells including (1) single cell analysis of gene mutations, gene expression, protein marker expression, and (2) analysis of drug sensitivity on the isolated growing CTCs. The heterogeneity of the molecular analysis will be unraveled among the initially isolated CTCs and this will be compared with the growing cells as well as the primary tumor from either the mouse model or the human patients. We have developed a mouse model of breast cancer using H-Ras transformed human mammary epithelial cells and BRCA1 knockdown. The tumors that result are rapidly growing, show features of the epithelial-to-mesenchymal transition, invasiveness and angiogenesis. For analysis of drug sensitivity we will evaluate effect of dose of all FDA approved drugs in a high-throughput 384 well plate format. Our preliminary data suggests that as few as 20 recovered tumor cells can grow to over a million cells in 2-3 weeks and that as few as 25 cells in a well can reliably provide information regarding tumor cell drug sensitivity. Our preliminary data shows clearly that many patients with advanced colorectal or breast cancer can have dozens and sometimes hundreds or thousands of CTCs in a single tube of blood. The objectives will be accomplished through:

Specific Aim #1: Molecular analysis of heterogeneity of isolated CTCs from a mouse model or from patients with advanced breast cancer, and

Specific Aim #2: Analysis of drug sensitivity (IC50) against hundreds of FDA-approved drugs and drug combinations on recovered CTCs from a mouse model or from patients with advanced breast cancer.

Principal Investigator

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Expected Research Outcomes and Benefits

The expected outcomes are two-fold:

1. Knowledge of the molecular heterogeneity of circulating tumor cells from patients with breast cancer, and the relationship of that heterogeneity to the original tumor from which these cells emerged will provide important basic information regarding the nature of breast cancer. Such information will become available for the first time as a result of these studies, and may help us as well as the scientific community to develop better strategies to treat breast cancer.
2. Evaluation of the sensitivity of isolated circulating tumor cells from patients with advanced breast cancer to all the FDA-approved drugs used to treat cancer, as well as combinations of such agents, is feasible and will help provide potentially critically useful information to the treating physician to personalize the treatment options and plan for patients.

Modeling these studies in mice and doing experiments on patient samples will greatly facilitate progress in the effort to understand and treat breast cancer. The proposed studies are intended to pilot these efforts and positive results will be used to expand the scope of the work in order to develop a clinical trial for patients with breast cancer.

Summary of Research Completed

Specific Aim 2: To investigate the changes in breast cancer specific-markers expression pattern on CTCs and in primary tumors.

Result: Breast cancer arises mainly due to mutations in different tumor suppressor genes such as BRCA1, BRCA2, p53, PTEN, etc. or due to alterations in receptor signaling such as ER, Her-2, etc. It will be interesting to find out the changes, if any, associated with breast cancer specific-

markers on both primary tumor and on CTCs. During cancer progression and metastasis, one may speculate that biomarker expression pattern may be altered in CTCs, contributing to more stemness and resistance against chemotherapeutics. To better understand the breast cancer specific-biomarkers expression, we are applying Qdot conjugated antibodies for immunostaining and for multiplexing breast cancer markers as emission from Qdot nanocrystals is narrow and symmetric, therefore, overlap with other colors is minimal.

At present, as shown in Figure 1, different breast cancer cells, cultured on slides are stained successfully with Qdot conjugated antibodies specific for breast cancer biomarkers. Efforts are underway to stain these markers on primary tumor tissue specimens and on isolated CTCs from clinical samples.

Specific Aim 3: To investigate the sensitivity of breast cancer cells in primary tumors and in CTCs against chemotherapeutic drugs.

Result: Breast cancer chemotherapy drugs may be used individually or in combination to increase the effectiveness of the treatment. Lapatinib is an orally active drug used in treating breast cancer. It is a dual tyrosine kinase inhibitor which blocks HER2/neu and epidermal growth factor receptor (EGFR) pathways and used in triple positive (ER+/EGFR+/HER2+) breast cancer. We have tested the sensitivity of different breast cancer cells against Lapatinib in sub G1 FACS analysis. After 24 hrs of drug treatment with different doses of Lapatinib in vitro, cells are harvested and subjected for sub G1 FACS analysis (Figure 2).

During this reporting period, we have continued to make progress in isolating circulating tumor cells (CTCs) from breast cancer patients and multiplexing with multiple markers. In a metastatic breast cancer (estrogen receptor negative) patient with metastasis to the bone we have identified breast cancer cells both by size-based enrichment followed by multiple marker analysis as well as by Veridex Cell Search.

Consistent with breast cancer, this patient had at least two Cytokeratin 7 (CK7) positive (green), Cytokeratin 20 (CK20) negative and Thyroid transcription factor 1 (TTF1) negative cancer cells in the blood (Figure 3 & 5).

Patient blood (roughly 7.5mL) was enriched for CTCs using a flexible microspring array (FMSA) device. After fixation of the cells on the FMSA device, permeabilization and blocking, the FMSA was incubated with TTF-1 antibody overnight (4 Degrees Celsius) followed by washing and exposure to anti-rabbit Alexa Fluor 680 secondary antibody. After washing, the slide was further exposed to CK7 antibody conjugated to DyLight 488 and CK20 antibody conjugated to DyLight 594. The nucleus was stained for identification. Using this approach (Figure 4A) we identified at least 2 CK7-positive CTCs in a tube of blood. Figure 5A appears to show a clump of 3 cells. The CTC in Figure 1C was flanked on each side by 2 CK7-negative cells, which are likely white blood cells. Furthermore, this patient had 2 CTCs from a 7.5mL tube of blood analyzed by Veridex Cell Search (Figure 4C).

Discussion: We have established successfully, pre-clinical mouse animal models bearing xenograft human breast tumors which are facilitating our understanding of CTC detection as well

as analysis methods and also in understanding of human breast cancer metastasis. Though our experimental system with mammosphere cultures clearly enhances the stem-like MDA-MB-231 cells in vitro, it does not fully reflect on enhancing the CTCs in vivo in mouse model. As human breast cancer CTCs are very much short-lived in different host system, timing of sampling mouse blood is critical and different sampling periods are being tested to enhance the CTC detection. Also, blood clotting arising due to mixing of mouse blood with human blood needs to be sorted out before subjecting those blood samples for CTC detection in Veridex. Generation of ZSGreen and CellVue tagged breast cancer cells help in designing much simpler microscopic ways to detect CTCs in pre-clinical mouse models though it may not be practical with clinical blood samples.

We have successfully developed different Q-dot antibody and DyLight antibody conjugates in laboratory to test in breast cancer specific immunostaining assays. Multiplexing of different breast cancer specific-biomarkers is being conducted using Qdot and DyLight conjugated antibodies for cells grown in vitro and for CTCs from clinical samples, recovered through Veridex CellSearch System. Comparing the biomarkers expression pattern on primary tumors and on CTCs may shed more light on to our understanding of the mechanism of higher stemness and chemo-resistance commonly associated with metastatic CTCs.

Efforts are also in progress to test the sensitivity of different chemotherapeutic drugs against breast cancer cells in vitro and also in xenograft animal models in vivo. Further efforts will be directed to analyze the chemo-sensitivity of CTCs isolated on different timings from patients subjected to chemotherapy.

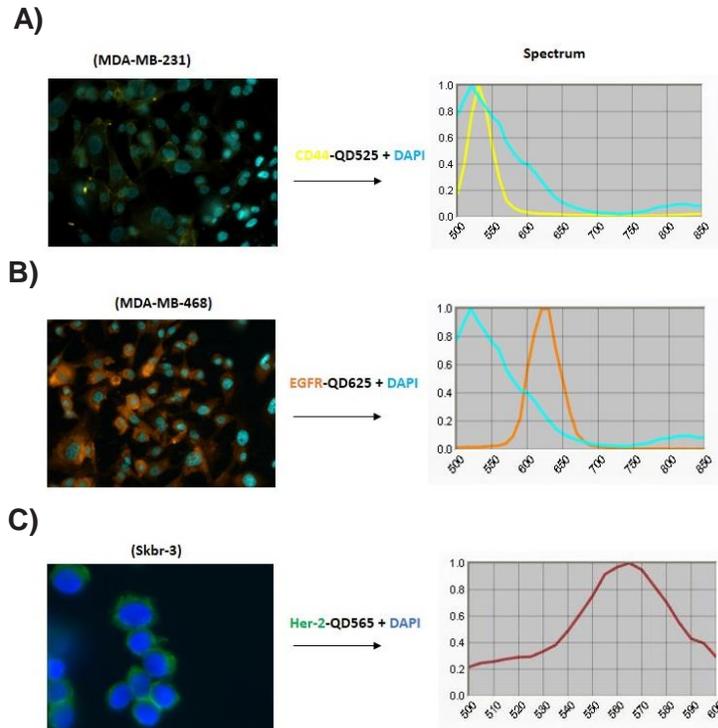


Figure 1. Q-Dot immunostaining of different breast cancer specific-markers. **A)** MDA-MB-231 cells are grown on chambered slides and stained with CD44-Qdot525nm antibody. **B)** MDA-MB-468 cells are similarly stained with EGFR-Qdot625nm antibody. **C)** Skbr-3 cells grown on chambered slides are stained with Her2-Qdot565nm antibody.

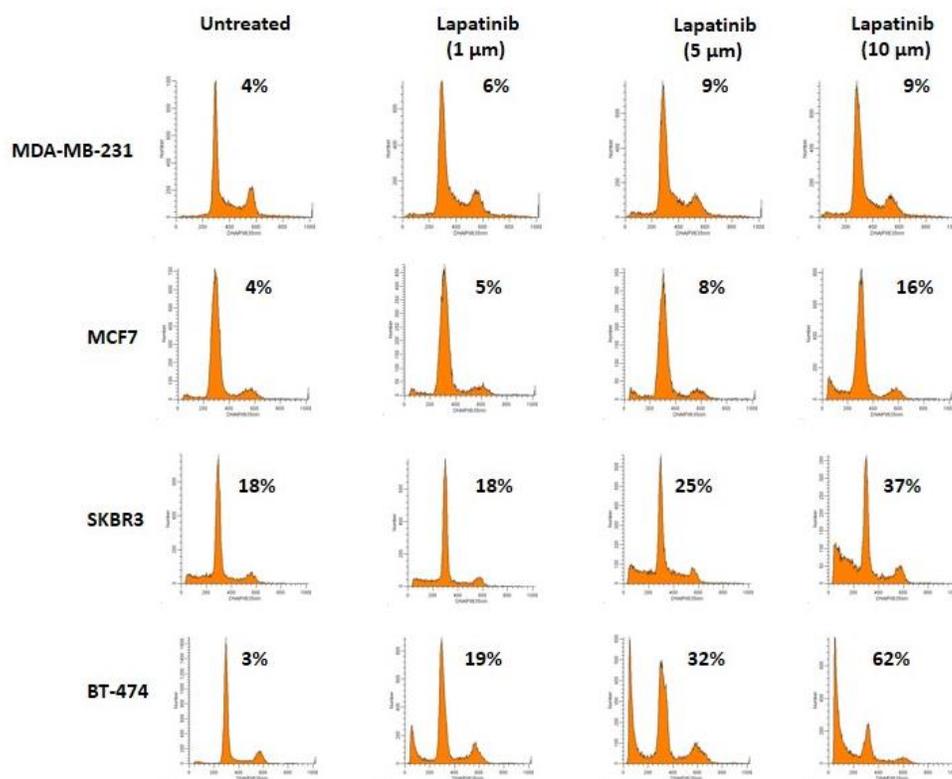


Figure 2. Varying sensitivity of breast cancer cells in vitro against Lapatinib drug, in sub G1 FACS analysis.

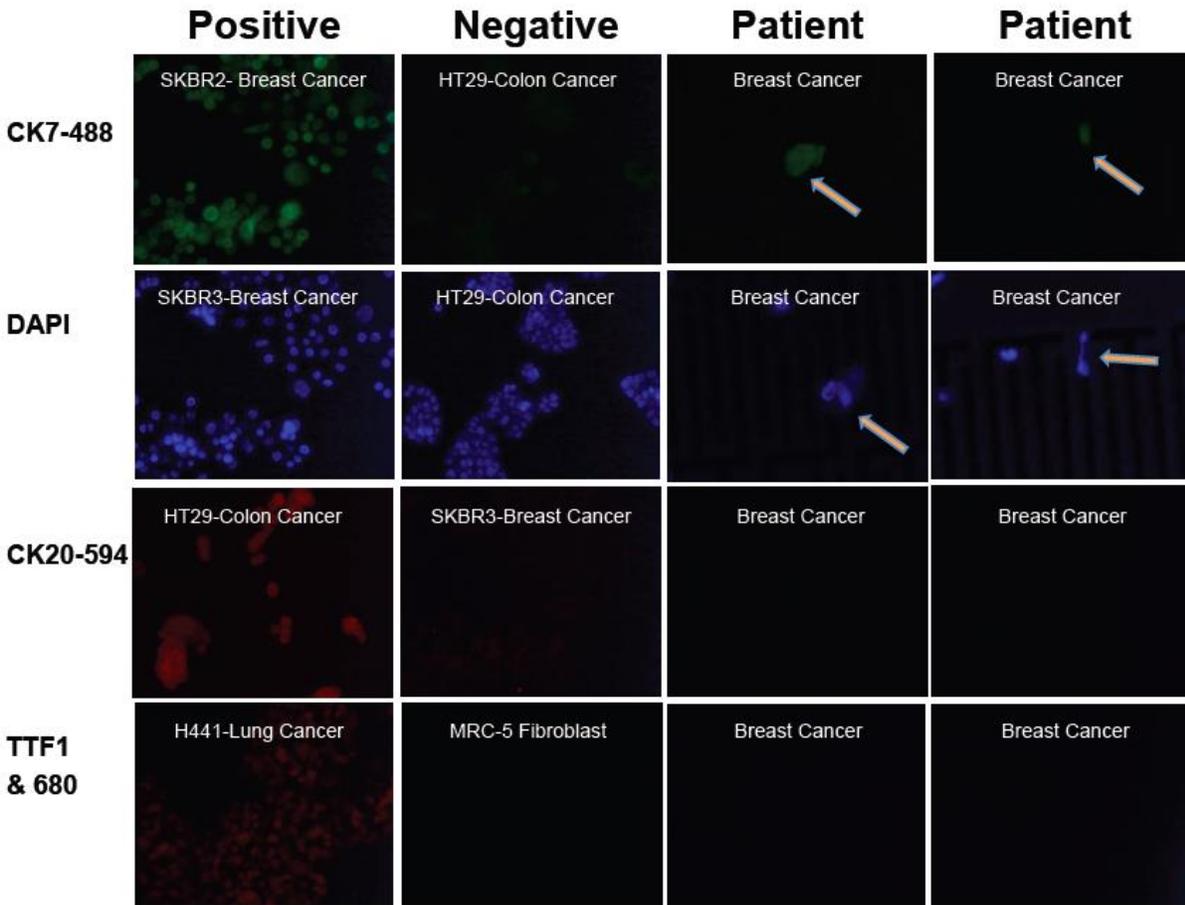


Figure 3. CTCs from metastatic ER-negative breast cancer patient. A patient demonstrated Cytokeratin 7 (CK7) positive (green), Cytokeratin 20 (CK20) negative and Thyroid transcription factor 1 (TTF1) negative cancer cells in the blood. Positive and negative control cancer cells are shown for each of the markers.

Breast Cancer Patient CTC Immunophenotyping

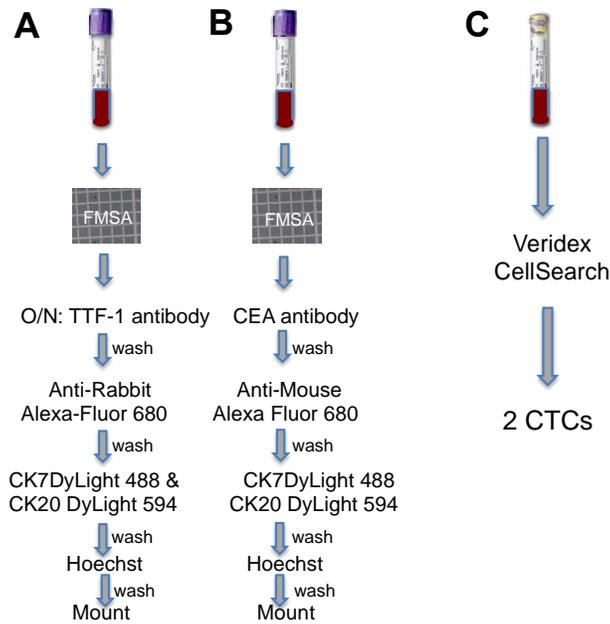


Figure 4. Breast Cancer Patient CTC Immunophenotyping. Three tubes of blood from a metastatic breast cancer patient were subject to size-based enrichment and multiple marker analysis (A-B) and Veridex CellSearch Analysis (C). CTCs from tube A are shown in Figure 1.

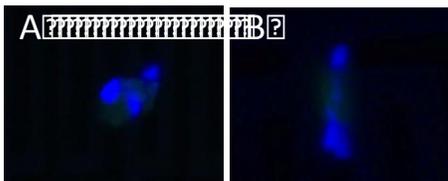


Figure 5. CTCs from Metastatic Breast Cancer Patient. Zoomed imerged (blue nuclear stain & green CK7 expression) images of CTCs from breast patient cancer patient.

Research Project 43: Project Title and Purpose

UGT Genetic Variants and Colorectal Cancer Risk - We aim to identify genetic variants in the UDP-glucuronosyltransferase (UGT) genes associated with carcinogen metabolism and risk. The findings will result in a significant advance in our understanding of colorectal cancer (CRC) and help to identify high-risk individuals for the development of colorectal cancer screening and prevention strategies.

Duration of Project

7/20/2012 - 12/31/2013

Project Overview

We *hypothesize* that the risk of colorectal cancer (CRC) is associated with carcinogen levels, and that the risk is modified by individual differences in detoxification of carcinogens due to genetic variants in the carcinogen-metabolizing UDP-glucuronosyltransferase (UGT) genes. We aim to identify genetic variants in the UGT genes associated with carcinogen metabolism and CRC risk. The specific aims are to:

1. *Perform genetic association studies of UGT polymorphisms with colorectal cancer risk and perform functional assays of associated polymorphisms (i.e. gene expression or enzyme activity).* We will obtain preliminary results by testing some of the polymorphisms most likely to be functional such as the whole gene deletion polymorphisms (copy number variants) in UGT2B17 and UGT2B28. We will use real-time PCR assays for genotyping of 2400 CRC cases and controls, and we will use logistic regression analysis for genetic association studies adjusting for known risk factors (i.e. age, gender, diet).
2. *Recruit additional colorectal cancer cases and controls.* Our current population of ~2400 individuals provides excellent power for examining the association of common (>5%) polymorphisms with CRC risk. However, our research would be strengthened by being able to detect associations of rare variants or increasing power for testing associations in stratified analyses (i.e. colon vs. rectum, males vs. females). We expect approximately 300 additional subjects will be recruited with this project funding.

The findings will result in a significant advance in our understanding of colorectal cancer and help to identify high-risk individuals for the development of colorectal cancer screening and prevention strategies. For example individuals with these variants may benefit from more frequent screening or personalized chemoprevention or intervention programs on diet or smoking.

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Terry Hartman, PhD – employed by Penn State University

Expected Research Outcomes and Benefits

We expect to identify genetic variants in the UGT genes that are associated with carcinogen metabolism and colorectal cancer (CRC) risk. UDP-glucuronosyltransferase (UGT) enzymes are involved in the detoxification of the carcinogens 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and benzo(a)pyrene (BaP). Individuals that are lacking a particular UGT (for example individuals homozygous for the UGT2B17 gene deletion polymorphism) may have a lower capacity to detoxify these carcinogens and therefore have an increased risk for cancer. We expect to determine that individuals with these polymorphisms have reduced carcinogen detoxification and increased CRC risk. We also expect that the preliminary results for this project will lead to sufficient evidence supporting involvement of UGTs in colorectal cancer, which will strengthen our DOD and NIH applications upon re-submission.

Summary of Research Completed

In July 2013 we published a manuscript in *CANCER* including most of the data described in our previous annual report:

Angstadt AY, Berg A, Zhu J, Hartman TJ, Lesko SM, Muscat JE, Lazarus P, Gallagher CJ. The effect of copy number variation (CNV) in the phase II detoxification genes, UGT2B17 and UGT2B28, on colorectal cancer risk. *CANCER* 2013; Jul 1;119(13):2477-85

In addition we have submitted a second manuscript to *Genes, Chromosomes, and Cancer* (this is new data and will be covered in this report):

Angstadt AY, Hartman T, Lekso SM, Muscat JE, Zhu J, Lazarus P, Gallagher CJ. The effect of *UGT1A* and *UGT2B* polymorphisms on colorectal cancer risk: haplotype associations and gene-environment interactions.

Genetic polymorphisms in combination with environmental exposures have been hypothesized to affect colorectal cancer (CRC) risk. Metabolizers of environmental carcinogenic and endogenous compounds affecting CRC risk include the UGTs which are phase II detoxification enzymes. UGT2B17 and UGT2B28 are two of the most commonly deleted genes in the genome, so we examined their CRC associations first and concluded that the UGT2B17 gene deletion

polymorphism is associated with decreased CRC risk (please refer to last year's annual report for details of that study). We next wanted to perform a more comprehensive study of polymorphisms in all of the UGT genes. To study the effect of UGTs on CRC risk we genotyped polymorphisms in 14 additional UGT1A and UGT2B genes on 1800 Caucasian subjects (CRC cases and controls) that had completed extensive demographics and lifestyle questionnaires, and then performed genetic association studies of SNPs and haplotypes.

SNP selection and genotyping

Genotypes for SNPs in the *UGT1A* and *UGT2B* genes representing people with European Ancestry (CEPH) were downloaded from the International HapMap Project. Linkage disequilibrium (LD) in the *UGT1A* and *UGT2B* genes was determined using Haploview Software. LD was estimated between all pairs of SNPs using the D' statistic and haplotype block structure was determined using the Solid Spine of LD option, with the block extended if pairwise D' between SNPs was greater than 0.80. From this analysis, 96 tag, coding, and additional SNPs within and surrounding the nine *UGT1A* genes on chromosome 2q37 to fill in gaps greater than 10kb, were used to design an Illumina GoldenGate genotyping assay (Illumina San Diego, CA). A separate GoldenGate genotyping assay was designed containing 16 tagging and coding SNPs and one deletion/insertion polymorphism within five *UGT2B* genes on chromosome 4q13; two *UGT2B* genes, *UGTs 2B17* and *2B28*, were not profiled by the SNPs in the present study as these genes had been previously examined for associations with CRC risk using the same sample set (Angstadt, et al. 2013) (described in last year's annual report). DNA isolation and quantification was described in last year's annual report.

Statistical Analysis

Dietary characteristics between cases and controls were compared using the χ^2 -test for categorical variables and non-parametric Wilcoxon Rank-Sum test for continuous variables. If continuous dietary variables appeared non-normally distributed then the appropriate transformation was performed (for example, log-transformation) to normalize their distributions. T-tests were used on transformed data and then confirmed by the Wilcoxon Rank-Sum test. Likelihood ratio tests were used to evaluate the fit of each model. A total of 854 Caucasian cases and 969 Caucasian controls were genotyped on the *UGT1A* assay, and 897 Caucasian cases and 955 Caucasian controls were genotyped on the *UGT2B* assay. Although the final number of genotyped samples is similar between datasets, 470 of the samples in the *UGT2B* dataset do not overlap with the *UGT1A* dataset due to limited quantities of DNA. In order to control for implausible dietary data, individuals who reported < 500 or >5000 kcal/day ($n = 53$ for *UGT1A*, 50 for *UGT2B*) were excluded from the analysis along with individuals ≤ 35 years of age ($n = 13$). After this exclusion, a total of 816 cases and 941 controls were analyzed from the *UGT1A* assay and 857 cases and 932 controls were analyzed from the *UGT2B* assay.

Hardy-Weinberg Equilibrium, allele frequencies, and identification of haplotype blocks in the study dataset were conducted using the control sample set in the Haploview software, defining blocks by the solid spine of LD. SNPs were excluded if the call rate was <90% and/or a Hardy-Weinberg Equilibrium (HWE) $p < 1 \times 10^{-3}$. This study is powered to detect associations with SNPs as low as 5% frequency for an OR >2.0 (>95% power) and can additionally detect lower effect sizes (OR>1.5) for common SNPs (>30%) with >80% power. The SAS PROC HAPLOTYPE procedure (Czika and X 2004) was used to conduct the haplotype analysis using

the haplotype block definitions from the Haploview software. The procedure utilizes the Expectation Maximization (EM) algorithm to generate maximum likelihood estimates of haplotype frequencies given a multilocus sample of genetic marker genotypes under the assumption of HWE. The initializing method was INIT=RANDOM, which initializes haplotype frequencies with random values from a Uniform (0,1) distribution. The haplotype frequency threshold was set to 5%, and haplotypes with a lower frequency were excluded from subsequent logistic regression analysis. The standard errors and the confidence intervals for each haplotype were estimated under binomial assumption, by default. The total probability of an individual having a particular haplotype compared to all other haplotype possibilities was determined. These values were used in the following statistical analysis assuming an additive statistical model (comparing the probability of one haplotype to all other haplotypes combined).

Unconditional logistic regression models were used to calculate odds ratios (ORs) and 95% confidence intervals (CIs) for associations between individual SNPs and haplotypes and CRC risk. Three statistical models were tested for individual SNP logistic regression analysis: additive: (BB) > (BA) > (AA), dominant: [(BB + (BA))] vs (AA), and recessive: (BB) vs [(BA) + (AA)], with B being the minor allele. Multivariate models were used adjusting for potential confounding variables that were selected *a priori*: age (continuous), sex (male, female), total energy intake (kcal/d, continuous), body mass index (BMI; kg/m², continuous), smoking status (never, current, or former), family history of CRC (yes, no; first degree relative), alcohol (g/d, continuous), physical activity (yes, no; ≥ 1 hr/week of vigorous activity), education (no college degree, college degree or above), and regular non-steroidal anti-inflammatory drug (NSAID) use (yes, no; regular use defined as at least 3 times a week for 1 year prior to diagnosis for cases and 1 year prior to interview for controls). According to a 10% change-in-estimate criterion, age, education, sex, BMI, family history, NSAID use, and physical activity were included in the final multivariate model. Dietary carcinogen levels (PhIP, MeIQx, DiMeIQx, and BaP) were adjusted for total energy intake (kcal/day) by the nutrient density method (grams per 1000 kcal) (Willett, et al. 1997) and separated into quintiles of intake based on the distribution among controls. The lowest level (quintile 1) for each respective dietary carcinogen served as the referent quintile. Since the UGT gene family is known to metabolize environmental carcinogens, high intake of each carcinogen was individually tested as a possible confounding covariate and added into the model if necessary according to a 10% change-in-estimate criterion (Greenland and Rothman 2008). Associations stratified by sex, anatomical sub-site (colon and rectum), and high carcinogen intake (quintile 5 of each dietary carcinogen) were also investigated. Reported p-values are 2-sided after correcting for the effects of multiple testing within each genomic region using SAS PROC MULTITEST. The False Discovery Rate (FDR) method described by Benjamini and Hochberg (Benjamini and Hochberg 1995) was used and controls the rate at level $\leq \frac{m_0}{m} \alpha \leq \alpha$ when you have independent *p*-values that are uniformly distributed under their respective null hypotheses. An adjusted *p*-value < 0.05 was considered significant for all tests.

The PheWAS-View software was used to visually integrate study results, to discover novel relationships between SNPs and phenotypes, and to produce forest plots (Pendergrass, et al. 2012). All statistical analyses were performed with SAS version 9.2/9.3 (SAS Institute, Inc., Cary, NC) and JMP Pro 10 (SAS Institute, Inc., Cary, NC).

UGT1A SNP associations with CRC risk

Unconditional logistic regression analysis using an additive, dominant, and recessive statistical model was conducted for individual SNPs and haplotypes within the *UGT1A* gene family for the effect on CRC risk controlling for age, education, sex, BMI, family history, NSAID use, and physical activity. Three of the 96 SNPs did not amplify and 8 of the SNPs (rs2741028, rs11893247, rs6706988, rs17863773, rs10176426, rs12474980, rs12463641, rs17862878) failed HWE in the controls, leaving a total of 85 SNPs for analysis. No significant associations were found between individual *UGT1A* SNPs and CRC risk overall or after stratification analysis by sex, cancer sub-site (colon versus rectum), or levels of carcinogen intake when applying the FDR multiple testing correction for all SNPs genotyped.

UGT1A Haplotype associations with CRC risk

Haploview software was used on the controls to divide the *UGT1A* gene region into eleven haplotype blocks, and each haplotype was then analyzed for impact on CRC risk. Haplotype block 7 was divided into four blocks (7.1, 7.2, 7.3, and 7.4) due to computational power for the analysis. SAS PROC HAPLOTYPE was then used to assign the probability that each individual possesses a particular haplotype compared to all other haplotype possibilities, which was then analyzed in unconditional logistic regression analysis controlling for the same covariates in the individual SNP analysis. Therefore, the analysis, assuming an additive statistical model, reported the risk associated with a specific haplotype when compared to all other haplotypes in the population at a frequency greater than 5%. Several *UGT1A* haplotype blocks were associated with cancer risk in this analysis. In the overall analysis, the T-T-A-G-A haplotype in block 4 was found to significantly increase CRC risk (OR= 2.44, 95% CI=1.29-4.6). In addition, stratifications by sex and cancer sub-site also yielded FDR-adjusted significance for haplotypes in blocks 2, 5, 6, 7.1, 7.4, 9 and 11. The significant decrease in cancer risk for the T-G haplotype (rs17864678, rs10929251) in block 2 was found in both proximal (OR = 0.29, 95% CI = 0.11-0.69) and distal (OR = 0.32, 95% CI = 0.12-0.95) colon cancer patients making it associated with colon cancer risk and not rectal cancer risk. The significance found between decreased proximal colon cancer risk and haplotypes in blocks 7.1 (OR = 0.24, 95% CI = 0.085-0.69) and 7.4 (OR=0.26, 95% CI= 0.091-0.71) is similar, as the two blocks were divided within one block created by Haploview and they contain three overlapping SNPs (rs1604144, rs12988520, and rs7240193). The C-T-G haplotype in block 11 (rs7578153, rs10203853, rs6728940) was found in males to increase CRC risk overall (OR = 2.56, 95% CI = 1.10-5.95) and the risk of proximal colon cancer (OR = 4.06, 95% CI = 1.30-12.6). No association with cancer risk was observed for any *UGT1A* blocks specifically in females even after stratification by sub-site.

UGT2B SNP associations with CRC risk

As conducted for SNPs in the *UGT1A* gene family, unconditional logistic regression analysis using an additive, dominant, and recessive statistical model was analyzed on individual SNPs and haplotypes within the *UGT2B* gene loci for the effect on CRC risk controlling for age, education, sex, BMI, family history, NSAID use, and physical activity. Two of the 16 SNPs did not amplify, 1 SNP was not a polymorphism in our population (rs7439366), and 1 SNP was genotyped in only 84% of our patients (rs7668258), leaving a total of 12 SNPs and one deletion/insertion polymorphism (rs35922514) for analysis. All SNPs were consistent with HWE. Overall, no significant associations were found between individual *UGT2B* SNPs and CRC risk even after stratification analysis by sex and high carcinogen intake, when applying the

FDR multiple testing correction for all SNPs genotyped. In rectal cancer patients, a few SNPs yielded borderline associations (rs4148269, rs61750900, rs835317, rs11737566) but only one SNP in *UGT2B15*, rs6837575 (minor allele frequency of 0.386 in controls) was found to significantly decrease risk after multiple testing correction using a dominant statistical model (OR= 0.47, 95% CI = 0.29-0.74, FDR p = 0.020; Figure 1, panel A).

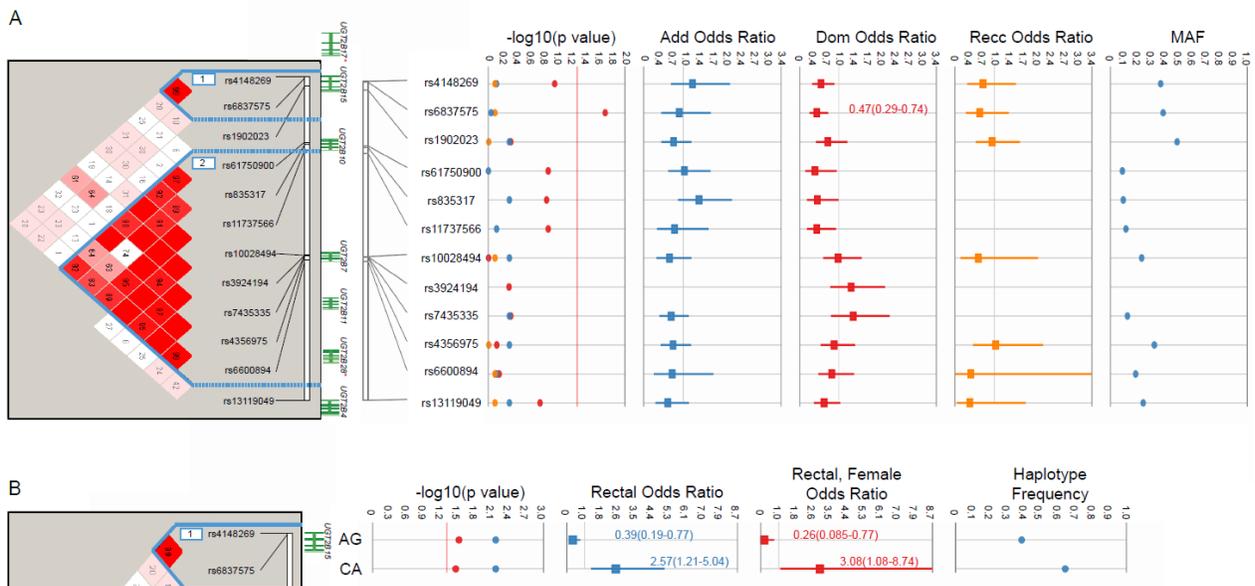
UGT2B haplotype associations with CRC risk

SNPs within the *UGT2B* region were divided into two haplotype blocks using Haploview and SAS PROC Haplotype to calculate the probability that a particular individual possesses a certain haplotype compared to all other haplotype possibilities (Figure 1). The rs35922514 polymorphism was excluded from the haplotype analysis because it was a deletion/insertion polymorphism and the insertion was only present in 0.6% (11/1761) of the population with no individuals exhibiting the homozygous rare genotype. While no *UGT2B* haplotypes were significantly associated with overall CRC risk, a significant decreased risk was found for the A-G haplotype (rs4148269; minor allele (MA) = A; rs6837575, MA = A) in block 1 (OR=0.39, 95% CI=0.19-0.77, FDR p = 0.01) in patients with rectal cancer in an additive statistical model (Figure 1, panel B). An increased risk for rectal cancer was found for the same haplotype block with a C-A haplotype (OR=2.57, 95% CI=1.21-5.04, FDR p = 0.01). After further stratification by gender, the A-G (OR=0.26, 95% CI=0.085-0.77, FDR p = 0.03) and C-A (OR=3.08, 95% CI=1.08-8.74, FDR p = 0.035) haplotypes in block 1 were found to significantly alter rectal cancer risk in females in the same directions; no significant associations were observed specifically in males. The two SNPs that make up block 1 are located in *UGT2B15*; rs4148269 is a missense polymorphism (c.C1568A, K523T) in exon 1 and rs6837575 is in intron 1.

NSAID use and *UGT1A* polymorphisms

Gene x environment (GxE) interactions were tested for all SNPs and haplotypes in both the *UGT1A* and *UGT2B* locus with high carcinogen intake (PhIP, MeIQ_x, DiMeIQ_x, and BaP) as well as high NSAID use. No significant GxE interactions were found with carcinogen intake after conducting a multiple testing correction. In the *UGT1A* gene cluster, the interaction between high NSAID use and the A-G-T haplotype (rs6717546, rs1500482, rs7586006) combined had a significant (p = 0.027) interaction after multiple testing correction, leading to decreased CRC risk. The homozygous recessive allele of rs1500482 was significant prior to multiple testing correction (p = 0.0007) but did not remain significant after the FDR correction (p = 0.051) for the interaction between high NSAID use and decreased CRC risk. No other gene x NSAID interaction was found between individual SNPs and haplotypes.

Figure 1: Schematic representation of the effect the UGT2B gene family on rectal cancer risk produced by the PheWAS software (Pendergrass, et al. 2012). Forest plot showing the odds ratios and 95% confidence intervals of the effect of individual SNPs (A) and haplotypes in block 1 (B) in the UGT2B genomic region on rectal cancer risk. LD plot demonstrates the haplotype blocks within the region and the position of each UGT2B gene (* on genes indicates that this gene has been previously studied for CRC risk associations using the same sample set (Angstadt, et al. 2013)). The p -value graphed as the $-\log_{10}$ (p -value) was adjusted for multiple testing by the FDR method and the red line denotes the $p < 0.05$ cutoff. Abbreviations are as follows: B = minor allele; Add [Additive Statistical Model, (BB) > (BA) > (AA)]; Dom [(Dominant Statistical Model, (BB + (BA) vs (AA))]; Recc (Recessive Statistical Model, (BB) vs (BA) + (BB)]; MAF (minor allele frequency in population controls). Blank plots in the Additive and Recessive Statistical Model are provided to focus the graphical scale because these SNPs were insignificant and contained a large confidence interval. In addition, rs3924194 and rs7435335 only had two alleles and therefore could not be analyzed in a recessive model.



Research Project 44: Project Title and Purpose

Determining the Neurochemical Profile of Addiction in Near Real Time - As we see it, drug addiction is a two-part problem involving drug-induced devaluation of essential natural rewards and cue-induced craving and relapse. Our paradigm models both of these key features, allowing for us to begin to discern the underlying neurocircuitry. *Specific Aim 1* of the project will test whether a non-gustatory cue can elicit the onset of the conditioned aversive state known to contribute to drug-seeking and ‘relapse’. *Specific Aim 2* seeks to block the onset of this conditioned aversive state with the infusion of a known anxiolytic 5-HT1A agonist, 8-OH-DPAT, into the pariacqueductal gray. Finally, *Specific Aim 3* will adopt a new technique to allow for a minute by minute simultaneous assessment of all major neurotransmitters in the nucleus accumbens during the onset of the conditioned aversive state and subsequent drug-seeking and taking. This new methodology offers a very powerful, cutting edge technique that will revolutionize our understanding of the neurochemical basis of cue-induced craving, withdrawal,

and relapse.

Duration of Project

7/20/2012 - 12/31/2013

Project Overview

Currently, we view addiction as a two-part problem. First, while natural rewards can protect against drug addiction under some circumstances, a prominent symptom of the disease is the devaluation of very important natural rewards. Second, addiction is a disease of chronic relapse where the drug, stress, or drug-associated cues can repeatedly initiate drug-seeking and drug-taking, even following very prolonged periods of abstinence. Here, we describe a paradigm that models both aspects of the disease and, as such, can be used to begin to identify the associated underlying neural substrates. Specifically, in our paradigm, rats avoid intake of a taste cue that predicts the availability of a drug such as morphine or cocaine. We have found that avoidance of the taste cue is associated with an elevation in levels of the stress hormone, corticosterone, a blunting of the reward transmitter, dopamine, in the nucleus accumbens, and the onset of frank aversive taste reactivity behaviors (i.e., gapes) when the taste cue is infused directly into the oral cavity. Cue-induced anticipation of drug availability (i.e., waiting for drug), then, is highly aversive. Importantly, greater aversiveness on these measures is associated with a shorter latency to take drug, greater load up on drug at the start of the session, and faster acquisition of the drug self-administration behavior.

Specific Aim 1 of this project will test whether a non-gustatory cue (i.e., an audiovisual cue) also can elicit the onset of the conditioned aversive state and whether the magnitude of this effect will predict drug-seeking and drug-taking. Until now, this effect has only been demonstrated with a gustatory cue and (with the exception of alcohol) audiovisual cues play a greater role than gustatory cues in relapse to drug-seeking in addicted humans. Therefore, it is important to demonstrate that the conditioned aversive state occurs in anticipation of drug delivery, regardless of the nature of the drug-paired cue. *Specific Aim 2* will test whether onset of the conditioned aversive state, and subsequent cocaine-seeking behavior, can be blocked by the infusion of a 5-HT1A agonist, 8-OH-DPAT, into the parvocellular paraventricular nucleus (PVN). Finally, *Specific Aim 3* will adopt a new technique that will allow for minute by minute assessment of all major neurotransmitters (e.g., dopamine, norepinephrine, serotonin, GABA, glutamate, acetylcholine) in the nucleus accumbens while the subject avoids the taste cue and then seeks and takes drug. This is a very powerful, cutting edge technique that will revolutionize our understanding of the neurochemical basis of cue-induced craving, withdrawal, and relapse.

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Expected Research Outcomes and Benefits

According to DSM-IV, substance abuse and dependence involve a failure to fulfill major obligations at work, school, or home, the giving up of important social, occupational, or recreational activities, and continued drug use in spite of recurrent physical, legal, social, or psychological problems. Addiction, then, has devastating consequences on the addict, his or her family, and on society as a whole. Indeed, the impact of this disorder on the individual and on society is compounded by the fact that addiction is now recognized as a chronic relapsing disease of the brain. Indeed, 50 – 90% of those addicted to drugs and alcohol will relapse at least once and the total financial burden to the US is an astounding estimated \$484 billion dollars per year. It is, then, imperative that we better understand how an individual transitions from use to abuse and addiction. Can we pinpoint this transition? What is the underlying neurochemistry? Can we intervene? We are using our rodent model to address just these questions in a way that cannot be addressed in humans. Our published data suggest that the onset and development of the conditioned aversive state indicates who will take drug, when, and how much. The present project is designed to ensure that this process is not specific to the use of a gustatory cue (i.e., that it will be evident even when using audiovisual cues known to contribute to relapse in humans). The project will explore for the first time the involvement of the pariacqueductal gray, a region known to be involved in anxiety, fear, and dread in humans. Finally, we will be able to link the transition from use to abuse and addiction with a comprehensive, near real-time, neurochemical profile in brain. Such an understanding is essential for the development of reasonable and effective treatment strategies.

Summary of Research Completed

Specific Aim 1 tests whether a non-gustatory cue (i.e., an audiovisual cue) also can elicit the onset of the conditioned aversive state and whether the magnitude of this effect will predict drug-seeking and drug-taking. *Hypothesis.* We predict that the audiovisual cue will elicit the onset of the learned aversive state and greater aversiveness will predict greater drug-seeking and taking.

As described previously, given the use of a non-gustatory cue, we proposed to use the measurement of ultrasonic vocalizations (USVs) to test whether the non-gustatory cue, like the gustatory cue, will elicit the onset of an aversive state when presented 30 min, rather than immediately, prior to drug access. To this end, we first needed to set up the system to measure USVs and then to verify that we could, in fact, measure both negative (i.e., 20kHz) and positive (i.e., 50kHz) calls. Having reported on this new capability in our last progress report, here we replicated our first experiment designed to examine how USVs change with consumption of a palatable saccharin cue that predicts experimenter delivered morphine, cocaine, or LiCl. In this study, 80 (n = 32 for Replication 1 and n = 48 for Replication 2) naive water restricted (5 min a.m., 20 mls overnight) rats were placed in the operant chambers and given 5 min to lick a palatable 0.15% saccharin solution. After a 5 min interstimulus interval, the rats were injected intraperitoneally (IP) or subcutaneously (SC) with saline or drug (see below). The rats were then

placed back into the operant chambers for an additional 5 minutes before returning to their home cage. They received 20 ml overnight access to water. There was one such taste-drug pairing/trial for a total of 6 daily trials, and each occurred at 48 h intervals. On trial 1 and 2 rats were injected IP or SC with saline (n=16), SC with 10 mg/kg cocaine (n=24), IP with 15 mg/kg morphine (n=24), or IP with .009M LiCl (n=16). On trial 3 and 4 the LiCl dose was increased to 0.075 M LiCl. On trial 5 and 6, drug doses were increased to 20 mg/kg cocaine, 30 mg/kg morphine, or 0.15 M LiCl. USVs were summed across the 5 min saccharin access period, the 5 min wait before injection, and the 5 min after injection. Here we show the behavioral data from both Replication 1 and 2, but the USV data from only Replication 1 (The data from Replication 2 are still being scored). *Hypothesis.* Because rats emit aversive orofacial responses (i.e., gapes) to a gustatory cue when paired with a drug of abuse, we hypothesized that aversive USVs also would be emitted following presentation of the cocaine- and morphine-paired saccharin cue.

Results and Discussion. The results shown in Figure 1 indicate that our hypothesis was not correct. Aversive USVs were emitted in response to the LiCl-paired saccharin cue, but not to the morphine- or cocaine-paired saccharin cue. Figure 1 shows data for rats in the saccharin-saline, saccharin-morphine, saccharin-cocaine, and saccharin-LiCl conditions on the first and last trial: Trial 1 and Trial 6. *Top panel.* The results show that while rats in the saccharin-saline condition increased intake of the saccharin cue from Trial 1 to Trial 6, other rats suppressed intake of the drug-paired saccharin cue when paired with morphine, cocaine, or LiCl, $ps < .05$. *Second panel.* In addition to drinking less of the saccharin cue, rats in the cocaine and LiCl conditions also exhibited a longer latency to lick the drug-paired saccharin cue on Trial 6, $ps < .05$. As shown in *Panel 3*, ingestion of the saccharin cue was associated with emission of positive 50 kHz vocalizations and the number of appetitive calls increased from Trial 1 – Trial 6 for rats in the saccharin-morphine condition, $p < .05$. There was a tendency for an increase in aversive calls when saccharin was paired with the aversive illness-inducing agent, LiCl, but this trend did not attain statistical significance, $p > .05$ (see *Bottom Panel*). Interestingly, there were few if any aversive calls emitted to the saccharin cue when paired with morphine or cocaine. In summary, we can now detect both appetitive and aversive calls. Rats appear to make aversive calls to a gustatory cue that has been paired with the illness-inducing agent, LiCl, but not when paired with a drug of abuse such as morphine or cocaine. If this pattern persists when combined with the USV data from the second replication, we will conclude that, under these test conditions, avoidance of the drug-paired cue is mediated more by a drug-induced devaluation of the otherwise palatable saccharin cue, rather than the onset of a conditioned taste aversion.

Specific Aim 3 adopts a new technique that will allow for a minute by minute simultaneous assessment of all major neurotransmitters (e.g., dopamine, norepinephrine, serotonin, GABA, glutamate, acetylcholine) in the nucleus accumbens while the subject avoids the taste cue and then seeks and takes drug. *Hypothesis.* We predict that a key role for dopamine, GABA, glutamate, and Ach will be revealed.

Although this technique can be used to assess changes in brain chemicals from minute to minute, we chose to use a more standard 15 min time period to increase our initial chances for success. Of course, while a 15 min sample period is common for microdialysis assessments, as mentioned, standard procedures allow for the measurement of only one neurochemical at a time. Here we successfully implemented the innovative technique described by Bob Kennedy at the

University of Michigan and we were able to measure 15 neurochemicals simultaneously from the same perfusate. For the first time, this allowed us to begin to examine the complexity with which these many neurochemicals change, in concert, during drug-induced devaluation of an otherwise palatable saccharin cue and during cue-induced craving and/or anticipation of drug availability.

Subjects. The subjects were 8 male Sprague-Dawley rats obtained from Charles River. Rats weighed between 293-339 g on the day of surgery. They were housed individually in standard, metal cages in a temperature-controlled (21 °C) animal care facility with a 12:12 hour light:dark cycle (lights on at 7:00 am). All experimental manipulations were conducted during the light phase of the cycle. The rats were maintained with free access to dry rodent chow (Harlan 2018) at the beginning of the experiment.

Surgery. Microdialysis Guide Cannulae. Implants were performed under ketamine/xylazine anesthesia/analgesia. Each rat was implanted with a guide cannula (CMA/Microdialysis AB, Stockholm, REF 8309025) aimed at the right nucleus accumbens. Craniotomies were made at the following coordinates (relative to bregma) in a level-skull preparation: 1.2 mm lateral, 1.2 mm rostral. The guide cannula was inserted to a depth of 5 mm ventral to the skull surface and secured in a dental acrylic headcap for the duration of the experiment. *Self-administration catheter.* The self-administration catheter was prepared and implanted as described previously by Twining et al. (2009), as were the intraoral cannulae (see Colechio, Imperio, and Grigson, 2014). Each rat received 300000 units of GPenicillin (sq) at the beginning of the procedure, and 10 mL saline (sq) when it was complete. Rats recovered for 13-14 days before habituation began.

Apparatus. Each rat was trained in one of 4 identical operant chambers (MED Associates, St. Albans, VT) described previously (see Colechio et al., 2014). Gustatory stimuli were delivered into the oral cavity using a computer controlled syringe pump system and orofacial responses were recorded using video monitoring (these data have not yet been scored). Operant responses for the IV infusion of saline or drug were made by contacting, via licks or nose pokes, an empty spout operant and completing a fixed ratio requirement. Thereafter, a computer controlled syringe pump delivered IV drug or saline. Events in the chamber and collection of data were controlled on-line with a Pentium computer that used programs written in the Medstate notation language (MED Associates).

Procedure. Once having recovered from surgery, rats were handled, fluid deprived (5 min a.m./20 ml access to fluid p.m.), and then habituated to the operant chambers for 5 min/day for 9 days. Water was available during this time. Using a within subjects design, the fluid deprived rats then received one intraoral (IO) infusion per minute for 30 min of one of two Kool-Aid flavored saccharin solutions. On day 1, the IO infusion of the CS+ flavor (e.g., orange) was followed by 2 h to self-administer cocaine (0.33 mg/infusion). On day 2, the IO infusion of the CS- flavor (e.g., grape) was followed by 2 h to self-administer saline. There were 5 such CS+ and 4 such CS- trials, occurring on alternating days, followed by one microdialysis test day.

Microdialysis Test Day. On the day of testing, each rat was briefly anesthetized with .1 mL ketamine (i/m). The guide cannula was removed and replaced with a probe (CMA/Microdialysis AB, Stockholm, PAES membrane, 2 mm membrane length, .5 mm membrane OD, 20 kDa cut-

off, 14 mm shaft length, REF 8010432). Each rat equilibrated for 3h at 1 μ l/min. Three 15 min baseline samples (samples 1-3) were then collected. One sample each was collected during the CS- (sample 4), ISI (sample 5, “inter”), and CS+ (sample 6) periods, followed by 5 post-injection samples (7-11). Ten μ l from each sample were immediately pipetted from the collection tube into a vial and mixed with 5 μ l each borate buffer and derivitizing agent before being placed onto dry ice. Samples were stored at -80 C and then analyzed using HPLC and Mass Spec. Rats were later sacrificed and the location of the guide cannulae in the nucleus accumbens verified.

Results and Discussion. Figure 2 shows data comparing neurochemical levels in the nucleus accumbens of one small drug-taker and one large drug-taker during the 15 min CS- period and averaged across the five 15 min post periods (referred to as Post) following exposure to the CS+. The Post period was used as the best indication of the impact of CS+ delivery because the CS+ sample was accidentally lost for the rat with the history of the most drug-taking (and the highest gaping behavior to the CS+). We believe that the Post period is a reasonable indicator of the response to the IO delivery of the CS+. First and foremost, the data shown In Figure 2 reveal that while Dopamine levels are low for both the low and the high drug-taker following the IO infusion of the CS-, infusion of the CS+ leads to a marked increase in dopamine, but only in the rat with a history of high drug-taking. This elevation in dopamine was surprising and may relate to the use of the within subjects design. Later in testing, using very similar methodology, we know that the direction of this response is reversed, at least when assessed using voltammetry (i.e., dopamine levels are low in the highest of drug-takers following the IO infusion of the CS+). It will, then, be critical to determine how and when the transition from reward (high dopamine) to aversion (low dopamine) occurs – as least as indicated by brain neurochemistry. Also noteworthy is the finding that GABA levels are increased in the high drug-taking rat, while acetylcholine levels and aspartate levels are reduced. Not shown are histamine, serine, taurine, glycine, the 5-HT precursor, 5-HIAA, and the dopamine metabolites, DOPAC, HVA, and 3-MT. Taken together, these are the first data of their kind. They will help us to compete in the current climate at the NIH and, importantly, they promise to reveal highly novel information about the brain and drug seeking and taking behavior.

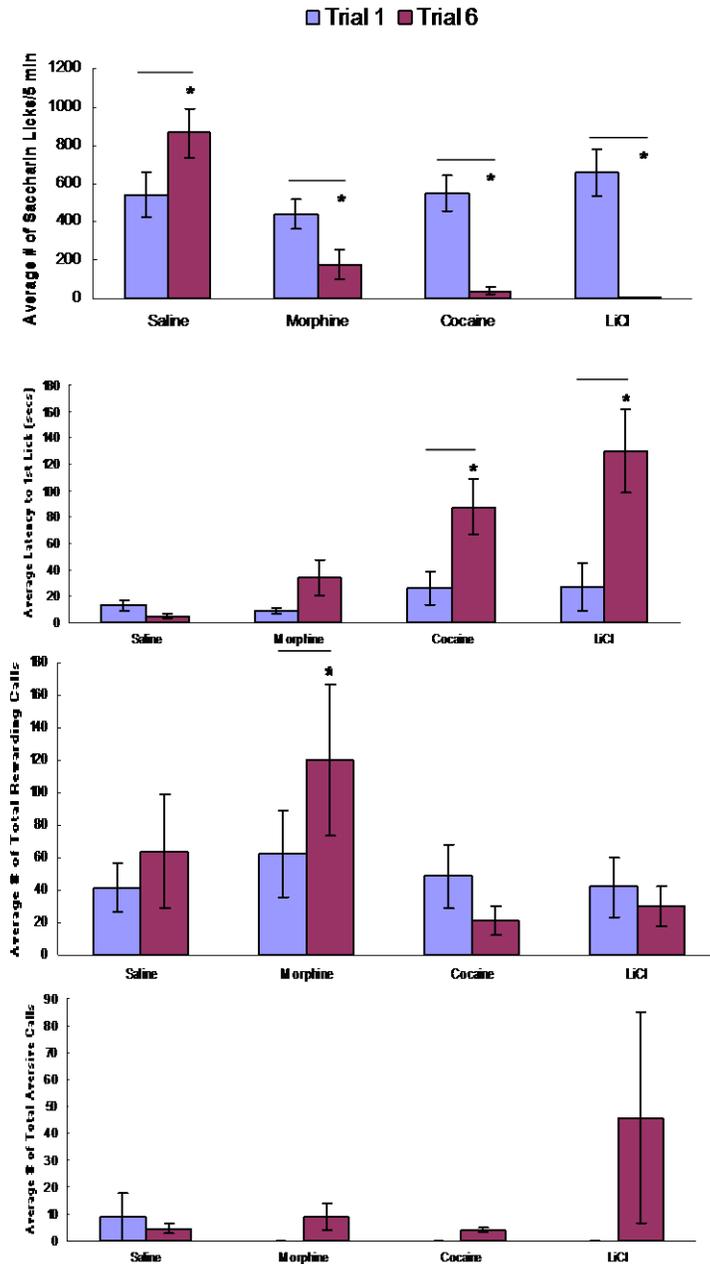


Figure 1. Top panel. Mean number of licks of 0.15% saccharin/5 min on Trial 1 and Trial 6 for rats in the saccharin-saline, saccharin-morphine, saccharin-cocaine, and saccharin-LiCl condition. Panel 2. Mean latency (s) to lick the saccharin cue on Trial 1 and Trial 6 for rats in the saccharin-saline, saccharin-morphine, saccharin-cocaine, and saccharin-LiCl condition. Panel 3. Total number of appetitive calls (50kHz) on Trial 1 and Trial 6 for rats in the saccharin-saline, saccharin-morphine, saccharin-cocaine, and saccharin-LiCl condition. Bottom panel. Total number of aversive calls (20kHz) on Trial 1 and Trial 6 for rats in the saccharin-saline, saccharin-morphine, saccharin-cocaine, and saccharin-LiCl condition. Appetitive and aversive calls were summed across the 5 min access period, the 5 min interval, and the 5 min period following injection of saline or drug.

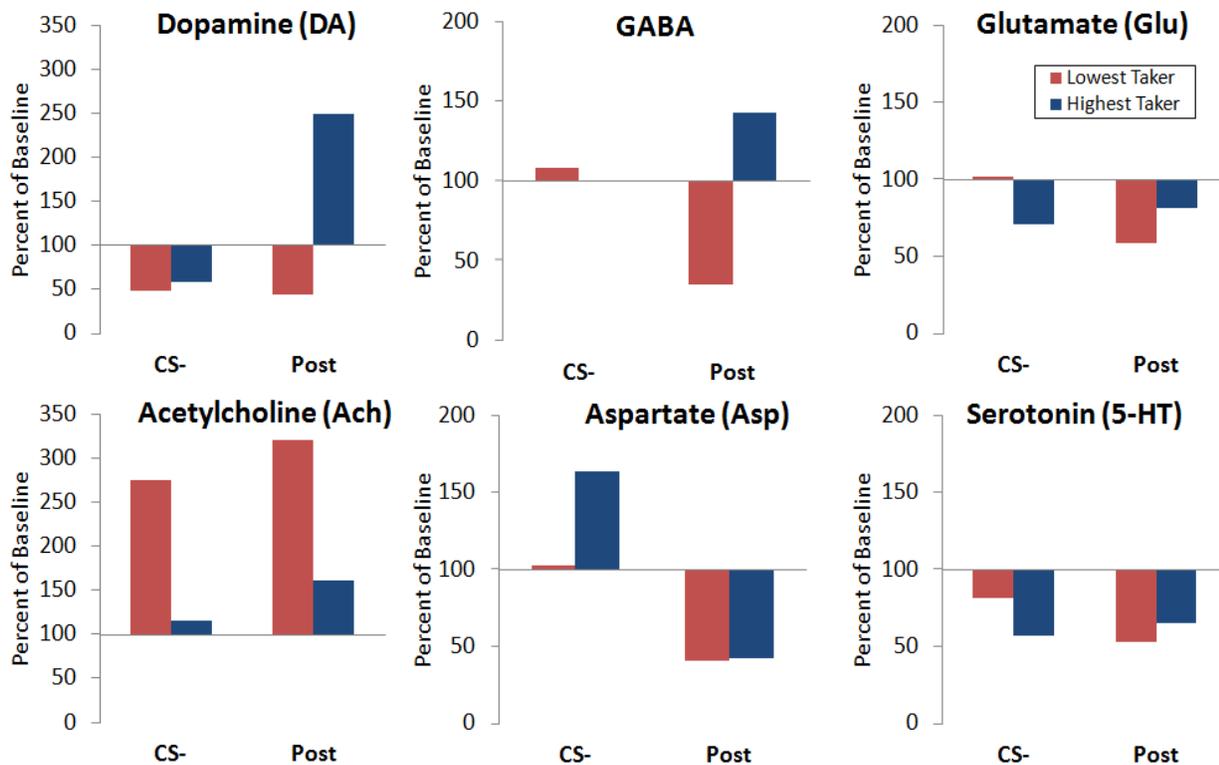


Figure 2. Percent change from Baseline for DA, GABA, Glutamate, Aspartate, Acetylcholine, and Serotonin for the lowest (red) and the highest drug-taker during infusion of the saline-paired CS- and during the period following the IO infusion of the cocaine-paired CS+ (referred to as Post).

Research Project 45: Project Title and Purpose

Mechanisms for km23-1 Control of Ras/TGF β Signaling in Human Colon Cancer and Noncancer Cells - km23-1 was only recently identified as a novel Ras adaptor and critical target for regulating Ras and transforming growth factor-beta (TGF β) signaling. Further, km23-1 knock-down (KD) can reduce TGF β -mediated mitogen-activated protein kinase (MAPK) signaling events and TGF β production in noncancer/untransformed epithelial cells (UECs), as well as in human colon carcinoma cells (HCCCs). This project will address the precise nature of the intracellular (IC) regulation of TGF β receptor (T β R)/km23-1/Ras signaling complexes, as well as how these pathways (PWs) are altered in human colon carcinoma cells (HCCCs). This research will also provide a better understanding of how inhibition of km23-1 can diminish major Ras/Raf-regulated events, particularly in HCCCs harboring mutations in KRas or BRAf.

Duration of Project

7/20/2012 - 12/31/2013

Project Overview

Colorectal cancer is the second leading cause of cancer-related deaths in the U.S., accounting for approximately 20% of all cancer deaths. Nearly 50% of colon cancers harbor activating mutations in K-Ras. In addition, somatic point mutations in the Ras effector B-Raf have been reported in 5-12% of colorectal cancers. As alterations in the Ras and mitogen-activated protein kinase (MAPK) pathway (PW) components occur frequently in human cancers, these targets have attracted attention for anticancer therapy. Unfortunately, efforts to develop direct inhibitors of either Ras or Raf have fallen short of expectations, with no direct inhibitor showing meaningful clinical activity to date. Thus, more efficacious alternatives are needed.

Tumor cells that are resistant to the growth inhibitory effects of TGF β can still secrete TGF β , which enhances tumorigenesis via the paracrine effects of TGF β in the tumor microenvironment (TME). Thus, it is advantageous to block this constitutive, secreted TGF β in late-stage tumors that have lost the TGF β growth inhibitory signals. We have previously shown that the signaling PWs mediating production of TGF β involve Ras and MAPKs. Further, these signaling PWs are distinct in noncancer epithelial cells (UECs) compared to human colon carcinoma cells (HCCCs), permitting selectivity in targeting such events. For example, components may be mutated in the HCCCs (i.e., Ras, B-Raf), or selectively utilized in the HCCCs vs. the UECs (i.e., c-Fos). Additionally, we have shown that the T β R-interacting protein km23-1 affects the TGF β production PW upstream at the level of Ras and Raf. Further, blocking km23-1 can decrease the constitutive TGF β 1 production in HCCCs harboring Ras and Raf mutations. This project will address the mechanisms underlying this unique ability of km23-1 to reduce TGF β production and its paracrine tumor-enhancing effects in both K-Ras and B-Raf mutant HCCCs. The future goal of the project is to advance new TGF β /Ras PW-based drug combinations towards clinical trials. We anticipate that these novel inhibitors will provide much more efficacious alternatives for colon cancer treatment than those currently in use.

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Expected Research Outcomes and Benefits

There is considerable evidence implicating Ras signaling in the conversion from the anti- to pro-oncogenic actions of TGF β . For example, Ras is known to interfere with TGF β /Smad signaling at several levels. However, the majority of the reports do not take into account that TGF β can activate and signal through Ras in normal cells as part of normal physiological programs. For

example, one physiological response to TGF β that requires normal Ras signaling is TGF β autoinduction. It is critical to understand how cells alter this normal TGF β /Ras signaling to achieve the unbalanced activation of components of the Ras/MAPK PWs, constitutive TGF β production, and aberrant regulation of the normal physiological processes that utilize these signaling events [i.e., cell motility, metastasis, angiogenesis, and epithelial-mesenchymal transition (EMT)]. Accordingly, it is necessary to identify the changes that have occurred in HCCCs relative to non-cancer cells, as will be done in this project, specifically with respect to the altered scaffolding and trafficking of Ras/MAPK complexes. Further, since altered compartmentalization is a key determinant of aberrant effector outcomes, especially for Ras signaling, studies related to this aspect are required for the success of therapeutic strategies. The results of the project will provide proof-of-principle evidence for the utility of km23-1 inhibitors in the treatment of colon cancer. Thus, the overall goal of this project is to reduce the production of a factor that enhances the growth and malignant properties of human colon cancer cells. The results will lead to the development of novel approaches to treat colon cancer.

Summary of Research Completed

Specific Aim 1. Specific Aim 1 was completed in the previous reporting period.

Specific Aim 2. We have shown that km23-1 knock-down (KD) diminished the malignant potential of KRAS- and BRAF-mutant human CRC cells in vitro and in vivo via several mechanisms (Jin et al, 2013c: *Plos One* 8(6): e66439). These include a reduction in constitutive extracellular signal-regulated kinase (ERK) and Elk-1 activity, diminished production of the pro-invasive cytokine TGF β 1, decreased paracrine effects of CRC cell-secreted factors on fibroblast mitogenesis and migration, and attenuated CRC motility and invasion (Jin et al, 2013c). We had previously shown that an ERK/Elk-1/c-Fos pathway mediates TGF β 1 production in human CRC cells, which can be attenuated by c-Fos KD, thereby reducing the in vivo growth of the human tumor cells (Liu et al, 2006, *Molec Carcinogen* 45(8): 582; Pandey et al, 2012, *Int J Cancer* 130: 213). Our data from the current project, therefore, support and extend this previous work. In addition, with regard to the scaffold Ezrin, we have shown that km23-1 depletion reduced Ezrin expression in invading human CRC cells. Additional data to address the mechanisms by which km23-1 depletion reduces the malignant potential of KRas and BRaf-mutant CRC cells demonstrated that km23-1's role in CRC invasion and tumorigenicity is not directly linked to effects on cell growth (see abstract below).

Fig. 1

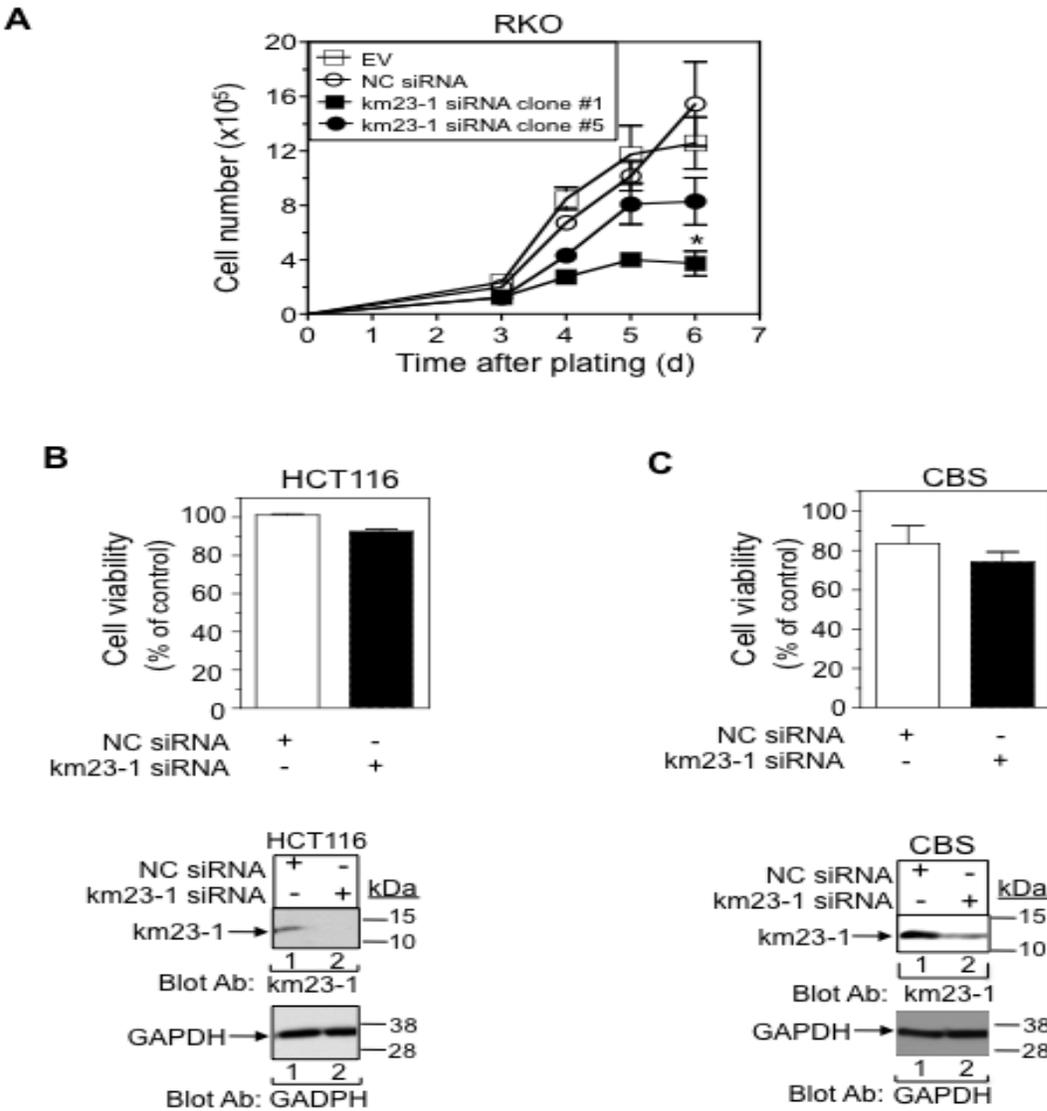


Fig. 1. km23-1 silencing inhibits the growth of RKO cells, but not of HCT116 and CBS cells, suggesting that km23-1's role in CRC cell motility and invasion are not directly associated with changes in cell growth. **A:** RKO cell clones stably expressing EV, NC siRNA, or km23-1 siRNA were plated and analyzed for cell number by trypan blue exclusion staining over the indicated days after plating. Mean \pm SE (n = 3), *p<0.05 compared to the NC siRNA on day 6. **B: Top,** HCT116 human CRC cells stably transduced with either pilitenti NC siRNA-GFP or pilitenti km23-1 siRNA-GFP pools were subjected to XTT assays. Mean \pm SE (n = 3). **Bottom,** HCT116 stable pools were grown and harvested for Western blotting to detect endogenous km23-1. GAPDH expression was used as a loading control. **C: Top,** similar XTT assays were performed in CBS cells stably transduced with either pilitenti NC siRNA-GFP or pilitenti km23-1 siRNA-GFP pools. **Bottom,** CBS stable pools were analyzed by Western blotting as for the bottom panel of **B**.

Research Project 46: Project Title and Purpose

Risk Profiling in Lupus - These studies will develop an approach to profiling risk for development of systemic lupus erythematosus (SLE) and evaluate whether hydroxychloroquine (HCQ) might have properties that could decrease this risk. Our preliminary data indicate that measures of autoantibodies and genes that are expressed in peripheral blood cells of patients with incomplete lupus (ILE) syndromes can detect changes that look like those seen in SLE. We hypothesize that when these markers are expressed in ILE, risk for disease progression is increased. The markers that are identified will be used to develop a risk profiler for prediction of lupus onset and to assess the impact of treatment with HCQ on the risk profile components. The long term goal is to develop strategies that can be used to identify patients in early preclinical stages and then to design appropriate preventive treatments.

Duration of Project

7/20/2012 - 12/31/2013

Project Overview

The objectives of this research are to develop insights into profiling immune features in individuals who are at high risk for development of systemic lupus erythematosus (SLE). The long term goals are to develop a Lupus Risk Calculator and then to design an early intervention trial to ameliorate or abort this disease. The risk calculator will be modeled on similar quantitative scales that are useful for personalizing risks of cardiovascular events and that are being currently applied to the early identification and preclinical treatment of patients with Type I diabetes mellitus. Elements of the calculator tool will include demographic features and immunologic measures including autoantibodies, cytokines, lymphocyte subsets and levels of genes expressed in peripheral blood cells. The high risk population we propose to study is composed of individuals who have sought medical care for evaluation of antinuclear antibody (ANA) positivity and have some additional elements suggestive of lupus. These individuals are classified as having incomplete lupus (ILE). Since ANA is generally considered a requirement to establish a diagnosis of SLE, this population is already enriched for individuals who are at risk for developing this disease. The problem of ANA positivity is relatively common, and 12-15% of our requests for new patient consultations in rheumatology are for evaluation of the significance of ANA positivity, which assures sufficient subjects for study. Developing tools to better assess the significance of a positive ANA in terms of disease risk will permit early disease detection and effective triage of patients from primary care to specialty clinics. An advantage of identifying high risk patients in the early stages of lupus is that interventions to ameliorate, abort or prevent disease could be tested. A significant body of available data suggests that hydroxychloroquine is an excellent candidate therapeutic for an early intervention trial. The one year period of support requested will enable collection of sufficient data to establish feasibility of this approach and support an application for extramural funding to proceed with the long term objectives.

For this project, we propose the following two specific research aims:

1. To determine the prevalence of high risk markers for SLE in 50 subjects with ILE who will be studied at two different time points, separated by at least 4 months.
2. To determine the immunologic and gene expression correlates of hydroxychloroquine treatment in ILE patients.

The results of these studies will provide insights into the variables that will be useful to develop as biomarkers of lupus risk in early patients and will define a suitable population and approach for a subsequent intervention trial.

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Expected Research Outcomes and Benefits

The expected outcome is the development of insights into which patients who come to rheumatology clinics with ANA positivity but who do not satisfy diagnostic criteria for SLE are at high risk for disease progression and are in need further evaluation and treatment. The blood markers of autoantibodies and gene expression that will be measured and that are observed to model those seen in patients with SLE are likely to indicate individuals with a high risk of progression. The prevalence of ANA positivity is high, with low titers observed in up to 25% of the population. So even though SLE is a relatively rare disorder, affecting about 1/2000 individuals, the issue of appropriate triage for specialty care is a significant public health issue, especially since there are only 5000 rheumatology specialists in the country to evaluate potential lupus patients. Furthermore, organ damage from SLE can be sudden and have devastating consequences like renal failure. SLE is in fact an important cause of end stage renal disease at younger ages. Therefore, early identification and treatment is a major target goal for improving outcomes and decreasing medical costs. Safe and effective treatments that can modulate early immune responses, such as hydroxychloroquine, would be of potential use in high risk individuals even prior to an SLE diagnosis. The project will permit development of paradigms for risk stratification and approaches to designing early therapeutic interventions.

Summary of Research Completed

Ten additional patients have been enrolled during this reporting period. This is added to the 40 who were reported previously for a total of 50. When added to patients who were enrolled prior to the beginning of the grant period, the total number of subjects is now 110. These include

individuals with incomplete lupus (ILE), defined as having less than four of the classification criteria defined in 1982 and modified in 1997, and the remaining patients have SLE, with at least 4 of these criteria. All of these patients were enrolled from the Rheumatology clinics at Hershey Medical Center. The ILE patients have an average of 2 criteria, compared to an average of 5.4 in the SLE patients, a highly significant difference ($P=3 \times 10^{-26}$). Each patient has had assessment of disease activity using the SLEDAI, which is a validated tool. The average SLEDAI score is significantly lower in ILE (1.29) than in SLE (3.81; $P=1 \times 10^{-5}$).

Previously we reported that antibodies to C1q might be of interest in detecting risk of lupus progression in ILE patients. This is based on a significant body of published literature indicating that anti-C1q autoantibodies may be a useful biomarker for risk of renal flares in lupus patients. We have now carried out some additional analyses on the clinical associations with C1q antibodies that were measured in ILE and SLE patients using a commercially-available ELISA kit (Abcam). The ILE patients had significantly lower mean values for anti-C1q (2.44 ± 0.61 U/ml) than the SLE patients (13.24 ± 6.26 U/ml; $P=0.044$; Figure 1).

Consistent with previous reports, the highest levels of anti-C1q in our sample were seen in SLE patients who had a history of renal involvement related, but not all of these patients were active with renal disease at the time of sampling. The criteria association shown suggests that anti-C1q positivity would not be expected in an ILE patient; there is a clear uptick of values in those patients with 7 or more criteria. Of interest, the highest anti-C1q value in the ILE group (8 U/ml) was in a patient who at the baseline visit satisfied only 1 lupus criterion, had a SLEDAI score of 2 (alopecia) and a physician global assessment of disease activity of 0.5. At follow-up one year later, she had accumulated one additional criterion (total of 2), had a SLEDAI of 4 and a PGA of 1.5. Subsequently she was evaluated by two other rheumatologists for central nervous system abnormalities and was started on treatment for presumed CNS lupus. This small sample result suggests that antibodies to C1q may be an important biomarker for disease progression in ILE patients; observing a high level would possibly suggest risk for additional illness.

Another potential source of biomarkers is levels of gene expression, which can be readily measured in peripheral blood using the PAX™ tube system. Initial focus has been on those genes that are regulated by Type I interferon (IFN), which is associated with viral illness but which has also been associated in many studies with SLE. In previous studies we also have shown that some patients with ILE and some first-degree relatives of SLE patients show elevations of at least some components of this signature. For the present study, we chose to measure three genes in this signature: MX1, OAS1 and IFI27. Previous studies had suggested that MX1 and OAS1 are rather broadly elevated in lupus-like syndromes but that IFI27 was more closely associated with SLE itself. This was further investigated in our collected samples including healthy controls (HC; N=10) and patients with ILE (N=64) or SLE (N=27).

Starting material was peripheral blood (2.5 ml) drawn into PAXgene™ tubes at the time of the visit and then stored frozen until RNA was prepared using the manufacturer's protocol. Two such samples are routinely drawn and stored for replication or future tests. Preparation of cDNA was done using the High Capacity RNA-to-cDNA Kit (Applied Biosystems/ Life Technologies, Carlsbad CA USA) with 100-200 ng RNA per synthesis reaction. RT-PCR analysis was

performed for selected genes using TaqMan Gene Expression Assays (Life Technologies) with GAPDH as the housekeeping control gene with an ABI-7300 Real Time PCR instrument. Expression values are normalized to GAPDH levels using the following formula: $2^{-(\text{GAPDH Ct} - \text{Test gene Ct})}$.

As expected, the IFN signature genes were generally correlated with each other when all three subject groups (HC, ILE, SLE) were analyzed together (Figure 2). However the correlation between MX1 and OAS 1 ($R^2=0.6$) is higher than for the other two pairs $R^2=0.16$ and 0.19 . This may become an important consideration when limiting the number of tests in the final risk profile for feasibility and cost reasons. These findings suggest that including either OAS1 or MX1 with IFI27 might provide broader coverage of the signature without unnecessary duplications.

The three measured genes were then compared for the three subgroups: HC, ILE and SLE (Figure 3). Data were analyzed by first comparing all three groups using the Kruskal-Wallis test which yielded statistically significant differences for MX1 and IFI27, but not for OAS1. Dunn's multiple comparisons test was then used in the post hoc analysis to compare each of the patient groups (ILE, SLE) to the HC group. For both MX1 and IFI27, the SLE group but not the ILE group was significantly different from HC ($P<0.05$).

To further explore differences in the patient datasets, the ILE and SLE groups were compared using a Mann-Whitney test, as a probe to see how different these two groups were in overall expression levels (Table 1). Each of the three specificities was significantly higher in SLE than ILE, but the level of significance was less for OAS1 than for the other two. Inspection of the scatterplot supports this, showing in fact that highest levels of OAS1 were seen in some of the ILE patients. By contrast, the highest IFI27 values were in SLE patients, as seen previously. One implication of these findings is that if an ILE patient expresses high levels of IFI27, the prediction would be that this carries a higher risk of progression to SLE.

An additional analysis was carried out to determine the prevalence of significant elevation of all 3 IFN genes in the ILE and SLE groups. The upper limit of normal expression for each gene was defined as greater than 2 standard deviations above the HC mean value. Using this definition, 12 of 64 ILE patients (19%) had uniform IFN elevations compared to 19/28 (70%) of the SLE patients ($P<0.0001$, Fisher's exact test). This result suggests that elevation of all 3 genes is more characteristic of SLE and that the ILE patients who show this pattern might be at elevated risk of disease progression. Evaluation of the clinical features of ILE patients who showed elevation of all 3 gene markers in this signature is in progress.

One of the major interests in this project is the potential for treatment of ILE patients with hydroxychloroquine (HCQ) to delay or abort development of SLE and associated immune abnormalities. In our recent review of the effects of HCQ in disease, it was noted that HCQ has many effects on intracellular processes. We would like to know which such changes might be tracked in a patient with ILE as a measure of how HCQ affects the immune system. To get some idea of what pathways might be a focus of such in vivo measurements, we carried out some exploratory in vitro analyses using HCQ with human cell lines. These studies show that HCQ does not increase apoptosis of immune cells, and differs in this regard from effects of another

immunotherapeutic agent, methotrexate (MTX; Figure 4). Furthermore, in terms of alterations in gene pathways that might be markers of HCQ actions, the in vitro results suggest that the oncogenes JUN and FOS are not altered by HCQ, but the related pathway components JNK1 and JNK2 might be increased by this drug (Figure 5). As a follow-up to this observation, the residual PAX tube material available on our patients will be used to assess the utility of these genes as components of a drug response signature.

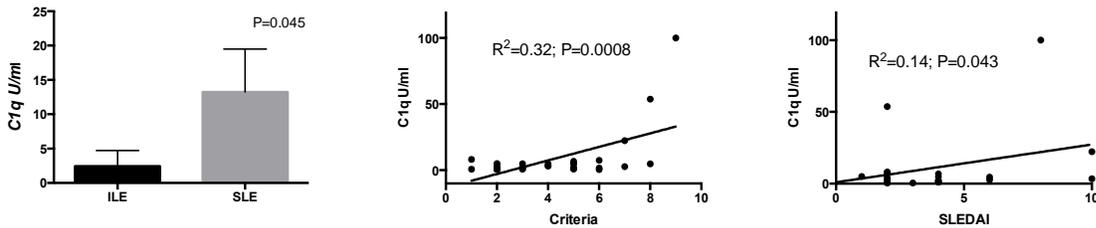


Figure 1 Correlations with antibodies to C1q in ILE and SLE patients. Levels of anti-C1q are on average significantly lower in ILE than SLE (left panel). Correlations of this autoantibody with disease features show a stronger association with numbers of lupus criteria (middle panel) than with the SLEDAI measure of disease activity (right panel).

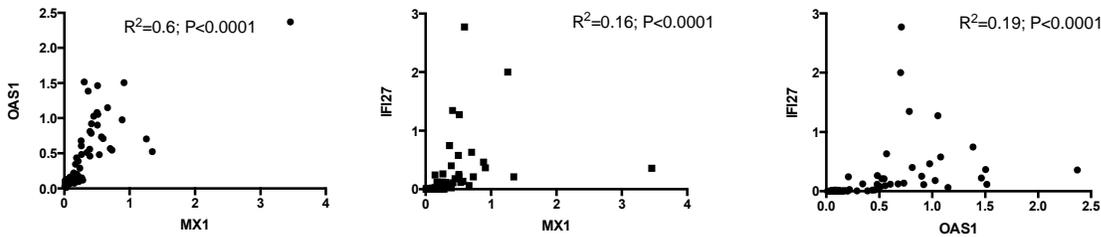


Figure 2 Gene expression values for OAS1, MX1 and IFI27 were compared to each other, pairwise. HC, ILE and SLE samples were included. Statistical analysis utilized Pearson's R.

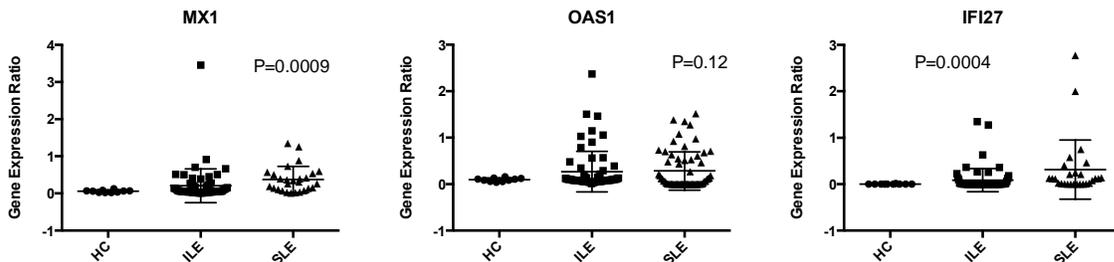


Figure 3 Expression of MX1, OAS1 and IFI 27 in three study groups, healthy controls (HC) and patients with ILE or SLE. Values for gene expression were calculated as described in the text. P values were determined by the Kruskal Wallis test.

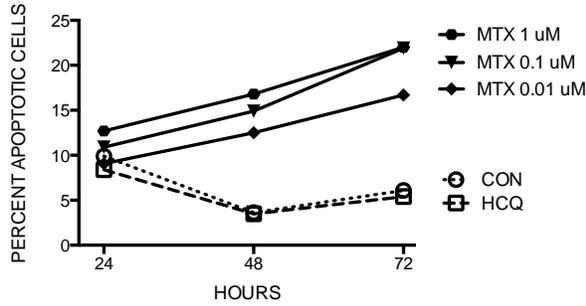


Figure 4: Measurement of apoptosis in the monocytoic human cell line U937. Cells were cultured with methotrexate (MTX) at indicated concentrations or with HCQ at 1 micromolar. Apoptotic cells were detected by flow cytometry using the PE Annexin V Apoptosis Detection Kit (BD Biosciences).

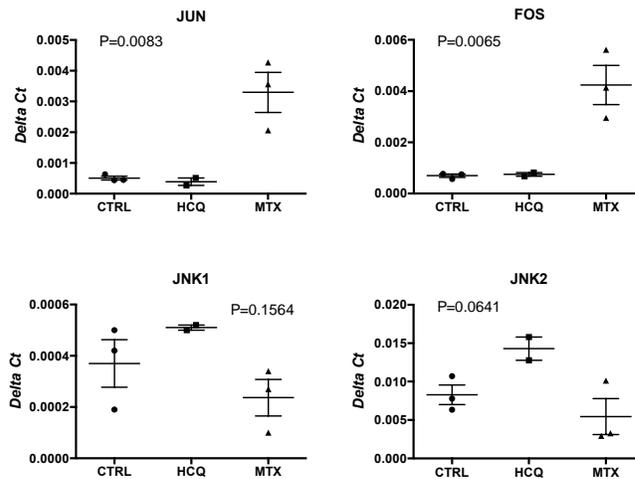


Figure 5 Measurement of gene expression levels in the human U937 cell line cultured for 72 hours with either HCQ or MTX. Levels are expressed using the delta Ct formula described in the text. Mean and SEM values are shown and P values were calculated using the Kruskal-Wallis test.

Table 1: Gene expression values in three study groups

	HC	ILE	SLE	P **: ILE vs SLE
MX1	0.059 (0.010)*	0.207 (0.057)	0.375 (0.068)	0.0012
OAS1	0.094 (0.012)	0.287 (0.055)	0.283 (0.051)	0.0364
IFI27	0.003 (0.001)	0.086 (0.031)	0.314 (0.122)	0.0012

* Values represent mean (SEM) expressed as delta Ct values, as described in the text.

** P values compare the ILE and SLE groups using a Mann-Whitney test.

Research Project 47: Project Title and Purpose

Basal Ganglia Neurophysiology During Drug Induced Dyskinesia - Parkinson’s disease (PD) is characterized by a disabling complication called drug induced dyskinesia (DID). Our previous studies suggest that dopaminergic grafts into the brain can possibly normalize the abnormal electrophysiology and thus prevent DID. This project will examine the feasibility of using a

rodent model of PD to understand the pathophysiology and electrophysiology of DID using variety of techniques including tetrode recordings that have never been attempted previously. These studies, to be performed in collaboration with tetrode recording experts, will potentially open new avenues of research in PD.

Duration of Project

7/20/2012 - 12/31/2013

Project Overview

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the loss of dopamine-producing cells in the substantia nigra (SN) and its connections. PD manifests itself with tremor, slowness of movement (bradykinesia) and stiffness (rigidity). Most PD patients develop a complication of therapy called drug induced dyskinesias (DID), 2-5 years after initiation of treatment. DID are difficult to treat and finding the pathophysiological basis of DID is cardinal to finding new treatments. The pathophysiological basis of current treatments for DID are not well understood and in most cases these treatments are short lived. Although loss of continuous dopaminergic stimulation has been implicated as one potential cause of DID, many recent studies indicate that the pathophysiology is inherently related to problems with neuronal circuits in the basal ganglia and surgical stimulation or lesions of specific areas in the basal ganglia can actually mitigate DID. These findings suggest that understanding abnormalities in the electrical properties of the basal ganglia circuits during and around DID will lead to a better understanding of DID pathophysiology and treatments.

The specific aim of this project will be to test the hypothesis that the anti-PD and DID effects of LD/DDCI are mediated by altering the electrophysiology of the basal ganglia in a unique and different manner and that DID has a electrophysiological signature in the basal ganglia. Hemiparkinsonian (HP) rats will be used for these recordings from the subthalamic nucleus (STN), Substantia nigra reticulata (SNr) and the entopeduncular (EP) nucleus. Single cell recordings, local field potentials (LFP) and electroencephalogram (EEG) recordings will be obtained with and without various treatments. Experiments will be performed in awake behaving rats while exhibiting DID and while not exhibiting DID and compared to identical recordings from normal animals.

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Other Participating Researchers

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Expected Research Outcomes and Benefits

We expect a clear electrophysiological hallmark for DID from the basal ganglia structures. Our data to date from other studies indicate that the electrophysiological changes due to PD in the basal ganglia remain unmitigated with oral levodopa therapy that is intermittent. This data was obtained in awake behaving parkinsonian primates and in anesthetized parkinsonian rats. To our knowledge the proposed experiment on rats exhibiting DID has never been attempted. We will be collaborating with an experienced team from the University of Michigan to make tetrode recordings from the STN, SNr and EP. The expected results are that electrophysiological patterns are abnormal in the parkinsonian state and that they remain unmitigated with levodopa intermittent therapy. However, we expect these recordings to be different when the animal exhibits DID versus when not exhibiting DID. In addition, our recent data suggest that information transfer from the cortex to the basal ganglia is uniquely effected during parkinsonism and that transfer entropy a non-linear measure of informational transfer is the most sensitive measure in such studies. Therefore, we expect that information transfer from the cortex to the basal ganglia as detected by transfer entropy will show that synchronous activity predominates. In contrast, in normal animals such stereotypic informational transfer does not exist. In animals with DID recordings are likely to show periods of complete silence or mitigated neuronal activity. We predict that these changes are chronic and require neuronal plasticity. We predict that continuous dopaminergic stimulation will alter this neuronal plasticity. The benefits of this line of research are that it will improve preclinical drug research for PD and DID. We expect our research to lead the way to the inclusion of electrophysiological outcomes to all preclinical drug and therapy studies for PD and DID. Upon completion of these studies we expect to publish high-impact journal articles that are likely to influence all future DID pathophysiology studies and drug discovery or treatment discovery in PD.

Summary of Research Completed

During this annual report period we had the opportunity to investigate the usefulness of optogenetic techniques in the context of understanding its influence on drug induced dyskinesias (DID). Although this was not originally proposed and we had planned only on using tetrode recordings in the context of the 6-OHDA lesioned hemiparkinsonian rats, it became obvious to us that neuronal plasticity in the brain which is a crucial component of the changes seen in PD and DID requires additional techniques. This became more apparent after our publication (Gilmour, TP., Piallat, B., Lieu, CA., Venkiteswaran, K., Ramachandra, R., Rao, AN., Petticoffer, AC., Berk, M., Subramanian, T., The effect of striatal dopaminergic grafts on the neuronal activity in the substantia nigra reticulata and subthalamic nucleus in hemiparkinsonian rats, *Brain*, 134(Pt 11):3276-89, 2011. PMID: 21911417 and Gilmour, T, Lieu, CA, Nolt, MJ, Piallat, B, Deogaonkar, M, Subramanian, T, The effects of chronic levodopa treatments on the neuronal firing properties of the subthalamic nucleus and substantia nigra reticulata in hemiparkinsonian rhesus monkeys, *Exp Neurol.*, 228(1):53-8, 2011. PMID 21146527) were replicated for other investigators. This necessitated us to explore what other mechanisms besides the well-known CDS hypothesis may play a role in the genesis of DID in PD. Specifically, the idea is that if, indeed, levodopa treatments cause DID in PD and that intermittent levodopa treatments have a unique electrophysiological signature that is distinct from what happens when constant infusion of dopamine (e.g., a dopaminergic graft) in terms of electrophysiology, this

would provide a new pathophysiological basis for DID. To test this scenario, a paradigm that allows intermittent dopamine depletion and repletion without the use of synthetic levodopa is essential. We were somewhat lucky that Gradinaru, et. al., described recently eNpHR 3.0, a modified halorhodopsin that can inhibit selectively pathways in the brain in a reversible manner when combined with the “cre-lox” system and wheat germ agglutinin (WGA) that allows trans synaptic protein trafficking. Taking advantage of this system and the availability of optogenetic tools, we adapted this to the current project and combined it with the specific aims of the current project. To clarify, the funds from this project and the research remained focused on what was proposed originally. We obtained additional separate funding for acquiring the tools and techniques for optogenetics from the Grace Woodward Foundation.

We used a dual viral vector injection system into normal rats to label interhemispheric nigrostriatal neurons and the nigrostriatal pathway completely on one side. Rats received 3 injections of AAV recombinant gene vector that expressed the transsynaptic WGA-Cre (“switch gene”) into the left striatum and one injection of AAV recombinant gene vector that expressed eNpHR 3.0 (opsin driven chloride channel, halorhodopsin) and eYFP (enhanced yellow fluorescent protein) into the left nigra and 1 injection into the right nigra. After 3 weeks, we implanted fiberoptic implants targeted to the left SNpc and right SNpc. After surgical recovery, animals were habituated to the tethering systems for several days. Laser activation of eNpHR was performed 1 month after the implantation of the laser fiberoptic into the brain. Following a mean of 4.5 hours of laser activation, these animals developed right hemiparkinsonism that persisted despite turning off the laser light source. This right HP state remained in place for a mean duration of 14 days after laser activation was stopped and then showed complete recovery to the normal baseline state without any residual parkinsonism. This laser activation experiment was repeated in the same set of animals (N=5) with the same results and without any permanent residual effects. The opposite SN (that received eNpHR 3.0 to label the interhemispheric SN neurons was separately inactivated using laser light application at the same lengths of time). As expected, the solo activation of the interhemispheric right to left nigrostriatal interhemispheric fibers that represents a very small population of cells did not elicit any behavioral effects or significant parkinsonism. This finding is exactly as we anticipated from the inhibition of the interhemispheric fibers. At the end of the experiment, we histologically examined the entire brain including the nigral brain sections. We found a large number of eNpHR+ cells (yellow) within the left SNC and a small number of interhemispheric nigral neurons on the right SNC, much as expected. No evidence of phototoxicity was seen. Stereological estimate of eNpHR+ cells in the nigra indicated that virtually 100% of the SNpc TH +ve dopaminergic neurons were successfully expressing eNpHR. No evidence of tyrosine hydroxylase (TH) neuronal loss or local evidence of phototoxicity was noted in the SNpc, its vicinity or along the tract of the optic fiber. These preliminary results demonstrate the capability to accurately label the nigrostriatal pathways using optogenetics labeling techniques, and the ability to develop a tethering system for laser fiberoptic experiments to cause reversible hemiparkinsonism. Thus far this animal model has not exhibited DID despite fluctuating between hemiparkinsonism and normal state via optogenetic inhibition. Additional tests are ongoing to evaluate DID in this system and to perform electrophysiology and microdialysis in this new animal model system that has the ability to completely reverse to the normal behavioral state. This is an important new discovery in the field of PD experimental therapeutics. Ongoing experiments will evaluate further the mechanisms involved in onset of hemiparkinsonism and its slow recovery. We anticipate the

discovery of a completely reversible model of PD to be a major scientific resource to the field of experimental therapeutics in PD.

Research Project 48: Project Title and Purpose

Targeting Survival Pathways in Treatment of Large Granular Lymphocyte (LGL) Leukemia –

The purpose of this project is to assess the potential therapeutic efficacy and the mechanisms of targeted drug therapy with HuMax-IL15, a human IgG1 anti-IL-15 monoclonal antibody, PDGF-RTK inhibitor (Nilotinib/Imatinib), NF-κB inhibitor (MLN9708/Bortezomib) and ERK/Survivin pathway inhibitors (LCL161 and YM155) in treatment of LGL leukemia of both T and NK subtypes.

Duration of Project

11/26/2012 – 12/31/2013

Project Overview

Large granular lymphocyte (LGL) leukemia is a disorder of clonal expansion of cytotoxic lymphocytes resulting from dysregulation of cell death, clinically characterized by severe/symptomatic neutropenia or anemia. No standard treatment for patients with LGL leukemia is available because of the absence of prospective clinical trials. We discovered that overexpressed PDGF-BB and IL-15 are the key molecules that trigger multiple downstream survival pathway activations in leukemic LGL. We also found that the TRAIL/DcR2 pathway plays a decisive role in mediating NF-κB pathway constitutive activation. The activated NF-κB pathway is an upstream regulator, and regulates multiple downstream transcript expressions such as Mcl-1, c-FLIP and survivin and multiple cytokines/lymphokines such as IL-8, IL-18, INF-γ, Fas/FasL and TRAIL, which are all implicated in the pathogenesis of LGL leukemia. We observed that inhibition of IL-15, PDGF-RTK and downstream NF-κB and ERK/survivin pathways resulted in an elevated level of apoptosis in LGL leukemia cells but not in normal counterpart cells.

Hypothesis: We hypothesize that the targeted therapy with multiple drugs listed above will eliminate the pathologic LGL clones via inhibition of NF-κB activation, resulting in downregulation of overexpressed anti-apoptotic proteins and the dysregulated cytokines/lymphokines production that are known to be responsible for initiating/sustaining leukemia cell survival.

Specific Aim 1: To determine clearance of LGL pathologic clones using targeted drugs listed above in PBMCs from patients with LGL leukemia and LGL leukemia cell lines. We will evaluate the tumor selective cytotoxicity of these targeted drugs in LGL leukemia cells versus primary CD3⁺/CD8⁺CD57⁺ T cells or CD3⁻/CD56⁺CD16⁺ NK cells from normal donors.

Specific Aim 2: To determine the mechanisms of LGL leukemic clonal elimination in response to the drugs as it correlates with NF-κB activity in leukemic cells. Correlative studies proposed here are designed to determine the regulatory mechanisms of NF-κB activity by inhibition of

upstream IL-15 pathway, PDGF-RTK phosphorylation or direct targeted inhibition of NF- κ B. We also propose to evaluate the activities of other transcription factors, including Stat-3 and T-bet which are constitutively activated in leukemic LGL as alternatives.

Specific Aim 3: To determine the contributions of NF- κ B pathway in regulating cytokine/lymphokine production in LGL leukemic cells. Correlative studies proposed here are designed to determine the transcriptional and translational regulatory mechanisms of key cytokine/lymphokine production by leukemic cells.

Principal Investigator

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

The overall research outcomes and benefits of this project stem from the proposed work to discover effective therapeutic drugs for LGL leukemia. There is no standard therapy for this disease and no curative treatment. By defining how these medicines work in killing the leukemia cells in the test tube, we hope to develop better treatment for this leukemia.

Summary of Research Completed

The following specific aim was accomplished within this period:

Research goal 1: We performed experiments for the determination of NF- κ B pathway derived cytokine/lymphokine production using PBMC from patients with LGL leukemia of T and NK subtypes.

Work Performed:

PBMC Isolation:
Multiplex Cytokine/Chemokine Bio-Plex (Luminex) Assay:

Results and Conclusions:

One milestone was in progress for this period:

Milestone 1: Partial completion of the experiments in determination of NF- κ B-derived cytokine/Chemokine production in PBMC from patients with T and NK -LGL leukemia patients.

PBMC Isolation- PBMC from four LGL leukemia patients (T-LGL n=3, NK-LGL n=1) were isolated by Ficoll-Hypaque gradient separation. PBMC from LGL leukemia patients were seeded in 24-well plates at 20×10^6 cells per well in 200ul of RPMI 1640 with 10% of FBS. Cells were kept in a tissue culture incubator at 37° C with 5% CO₂. PBMC were treated with recombinant human TRAIL ligand (rh-TRAIL) at 10ng/ml concentration in the presence or absence of Bortezomib or Ixazomib (MLN9708). Recombinant human TNF-alpha (rh-TNF- α , 10ng/ml) and DMSO were used as positive control and vehicle control, respectively. All treatments were administered in triplicate. Samples from each treatment were pelleted at 1,200 RPM for 5 minutes; and the resultant tissue culture supernatants were harvested for experiments. Cell pellets were stored at -80° C for later use.

Bio-Plex Assay- Two customized Bio-Plex Assay kits were designed using Bio-Plex Assay Builder software (Bio-Rad Life Science Research) to query the target genes of interest. The following NF- κ B downstream transcripts were included in the company defined Group I or Group II human cytokine/chemokine assay kits. Group I: IL-1 β , IL-2, IL-4, IL-6, IFN- γ , MIP-1 α , MIP-1 β , PDGF-BB, RANTES and TNF- α ; Group II: INF- α , IL-1 α , IL-2R α , IL-18, and TNF- β . Cytokine/chemokine concentrations in tissue culture supernatants were determined using Bio-Plex 200. Data analysis was performed using the Bio-Plex system in combination with the Bio-Plex Manager software (BIO-RAD Life Science Research).

Results and Conclusions

In specific aim 3 we proposed to determine the contributions of the NF- κ B pathway in regulating cytokine/chemokine production in LGL leukemia cells. Correlative studies were designed to determine the transcriptional and translational regulatory mechanisms of key cytokine/chemokine production by leukemic cells. The data generated from this study led to the following results and conclusions:

1] High Expression Levels of Multiple Cytokines/Chemokines in T and NK -LGL Leukemia –

Data from this study shows that T and NK -LGL leukemia patient cells share similar cytokine/chemokine expression profiles. The results of this experiment are outlined below. High protein concentrations of PDGF-BB, IL-8, MIP-1 β and RANTES were observed in the tissue culture medium. Concentrations of other targets were either relatively low or undetectable in relation to the standard curves generated for each target. Additionally, data from this current study closely correlates to our previous observations concerning LGL leukemia cells, sera and plasma samples from patients with T and NK -LGL leukemia subtypes (Yang, et al; Blood, 2010, Vol 115, pp51-60, Kothapalli/Nyland, et al; Int J. Oncology, 2005, Vol 26 pp529-535). Table 1 details how these proteins are expressed in LGL leukemia.

Data showed that the NF- κ B transcripts (Boston University Biology, www.bu.edu/nf-kb/gene-resources/target-genes/) IL-8, MIP-1 β , RANTES and PDGF-BB are highly expressed in leukemic LGLs. Data from the current study suggest a rational connection between NF- κ B activity and the elevated transcript expression in LGL leukemia cells.

2] rh-TRAIL Induces Elevated Multi-Cytokine/Chemokine Production- Transcription factor NF- κ B includes a group of key molecules, and together with other members of the NF- κ B family

forms homo- or heterodimers in the process of activation. The dimers play important roles in regulating expression of multiple downstream targets. In turn, downstream molecules play crucial roles in regulating immune functions, tumor genesis, cell proliferation and survival. Data generated from this *in vitro* study strongly support our experimental model of NF- κ B activation via TRAIL/TRAIL-DcR2 interactions. Upon measuring the protein concentrations for the downstream targets of interest in the supernatants, we observed that IL-6, IFN- γ , soluble IL-2 receptor alpha (sIL-2R α), IL-2, and IL-18 were significantly upregulated in the rh-TRAIL - treated T-LGL leukemia PBMC. The expression levels of these proteins were either relatively low or undetectable in samples with no treatment and in vehicle control samples. Similar results were observed in tissue culture supernatants of PBMC treated with rh-TNF- α at 10ng/ml concentration. Rh-TNF- α was widely used as a positive control for the canonical NF- κ B pathway activator in different cell types. Importantly, the proteasome inhibitors Bortezomib and Ixazomib effectively downregulated cytokine/chemokine and growth factor production as measured in tissue culture supernatants. In contrast to the results observed in T-LGL leukemia cells, sIL-R α remained undetectable in NK-LGL leukemia samples regardless of whether they were treated with rh-TRAIL, with rh-TNF- α , or with vehicle. Furthermore, rh-TRAIL and TNF- α mediated significant increases of IL-1 β , IL-18, IL-2, INF- γ , RANTES, IL-6, IL-8 and PDGF-BB production by PBMC into the tissue culture medium. Treatment with the second generation proteasome inhibitor Ixazomib (MLN9708) dramatically eliminated the TRAIL-mediated increases of IL-1 β and RANTES, and downregulated IL-18 and IL-6. Ixazomib showed no efficacy in downregulating the expression of IL-2, INF- γ , IL-8 and PDGF-BB. Collectively, data from this study strongly supports our hypothesis that TRAIL/TRAIL-DcR2 interaction plays a decisive role in triggering and sustaining NF- κ B pathway activation in T-LGL leukemia cells. Importantly, our work with NK-LGL leukemia patient specimens also provided preliminary data which lays a valuable foundation for directing our future studies.

Table 1.

Concentration (pg/ml)	IL-8	MIP-1 β	PDGF-BB	RANTES
T-LGL Pt. # 1	124.20	230.30	269.62	2109.51
T-LGL Pt. # 2	745.95	560.34	46.33	2384.96
T-LGL Pt. # 3	595.96	92.71	75.40	1260.97
NK-LGL Pt. # 1	96.90	152.26	567.26	701.20

Research Project 49: Project Title and Purpose

Research Infrastructure: Penn State Center for Computational and Integrative Biomedical Research – We propose to renovate facilities to house the Center for Excellence in Computational and Integrative Biomedical Research at Penn State. The renovated facility will create critical infrastructure and the support of key shared resources that are needed to catalyze the discovery of new knowledge and the development of novel methods to detect, prevent and treat disease. This will be accomplished by the development of current open and unfinished space on the 3rd floor of MSC into high quality collaborative workspaces that will house

computational scientists and scholars engaged in interdisciplinary research in biomedical, life, materials, bioengineering and information sciences. The newly developed space will be able to house more than 20 research scientists, including bioinformaticians, cyberscientists, postdoctoral fellows and graduate students. In addition, we propose to enhance infrastructure in the West wing of MSC to enable the safe and secure conduct of biomedically relevant research, especially in the areas of infectious diseases, genomics and bioinformatics, neurosciences, imaging, and biomedical (neural) engineering through the installation of keycard entries and upgrading of critical research infrastructure that will support the activities of an additional 20 faculty led laboratories involving over 100 research scientists within the MSC.

Duration of Project

12/12/2012– 12/31/2013

Project Overview

The role of interdisciplinary research in enabling basic and translational health research is well established. The Millennium Sciences Complex on the University Park campus of Penn State is a newly constructed facility (\$297 million, 295,000 sq. ft.) that promotes interdisciplinary research with broad impact at the interface of biomedical (life) and materials science.

The broad objective and overall goal of this project is to develop a Center for Excellence in Computational and Integrative Biomedical Research at Penn State. To accomplish this goal, an unfinished portion of the 3rd floor of the Millennium Sciences Complex (MSC) at the University Park campus of Penn State will be developed to support interdisciplinary computational modeling and bioinformatics research. Activities in this center will be integrated with key biomedical research facilities at the Huck Institutes of the Life Sciences so as to enable the discovery of new knowledge and approaches to the diagnosis, prevention and treatment of diseases. Together, we believe that our activities will not only positively impact health related research through biomedical resource and capacity building, but also anticipate that this program will embrace new approaches in social sciences and lead to the translation of basic scientific research to directly improve patient health.

Specific Aim 1. To develop current, open and unfinished space on the 3rd floor of MSC into high quality collaborative workspace that will house computational scientists and scholars engaged in interdisciplinary research that applies information sciences, material & bioengineering research to address biomedical issues.

Principal Investigator

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Expected Research Outcomes and Benefits

The development of collaborative, computational workspaces on the 3rd floor of MSC will enable interdisciplinary computational modeling and bioinformatics research and support activities of the Huck Institutes of the Life Sciences. In addition, the enhancement of infrastructure within the Life Sciences (West) wing of MSC will enable the safe and secure conduct of biomedically relevant research, particularly in the areas of infectious diseases, genomics and bioinformatics, neurosciences, imaging, and biomedical (neural) engineering in MSC and at Penn State.

Summary of Research Completed

The Center for Excellence in Computational and Integrative Biomedical Research at Penn State provides a facility with state of the art technology to support the research needs of combining biological research with computational science. The newly developed space includes 3 additional conference rooms, one permanent office, nine graduate and post graduate workstations along with a central visualization area to support large collaborative sessions between researchers and computational scientists. The new space includes; under-floor power and data-distribution (for easy servicing), workstations, energy-efficient air handling and lighting systems, a video conferencing area, an integrated 16-tile touch screen visualization wall, along with a 72 inch computational modelling display table. The facility will be able to house more than 20 research scientists, including bio-informaticians, cyber-scientists, postdoctoral fellows and graduate students. In addition, the area located between the North and West wing of MSC offers a secure location to support biomedical relevant research, especially in the areas of infectious diseases, genomics and bioinformatics, neurosciences, imaging, and biomedical (neural) engineering.

This space is designed to create an environment that fosters collaboration in themed areas of research. By including other Penn State Institutes, research can be expanded to include “big-data” and information from bio-medical studies to the impact on social, environmental and material research. Multiple data sets can now be shared simultaneously allowing for translational studies, embracing the individual variations, interactions and identifying personalized solutions.

The additional 2,800 square feet of work related space delivers state of the art technology to support large scale visualization of computational “big-data” and imaging. The space allows multiple users to connect simultaneously through a single system to display data. The added technological feature gives researchers the opportunity to share massive amounts of data, overlapping results through one centralized mechanism. With the support of touch screen technology, users are able to easily move data around the screens and overlay new screens by simply touching the wall. This advancement in technology allows large quantities of data to be compared and analyzed by multiple Principal Investigators in one central location. The space

also serves as a training location for investigators to explain large data set variants to students and visitors, by closely interacting with the visualization wall.

In addition to the visualization wall, the Center houses an Ideum Pano touch table. Ideum touch table is an integrated 100” multi-touch table with dual 55” 4k Ultra HD displays and a continuous touch surface that supports over 40 touch points. The table display and applications are supported by a high-performance graphics workstation. The paired 55” ultra HD displays offer display resolution of 7680 pixels wide by 2160 pixels tall (8.3 megapixels), allowing display of finely detailed images that often are required for dense data visualization applications. The table allows touch-enabled interactive control of the desktop, supported applications, and data displays so that users can directly, intuitively and simultaneously work with multi-model application workspaces and data representations of various types. The collective feature of the Pano table provides a rich venue for natural display management and collaborative data exploration by researchers, with the goal of facilitating more immediate and intuitive understanding of data being studied.

With the addition of the Tele-presence room adjacent to the visualization area, researchers in Hershey and other academic institutions can communicate through real-time interactions allowing for world-wide collaboration between Penn State scientists and investigators located throughout the globe. The goal is to bring research scientists closer together without the barriers of distance.

The newly created space offers critical infrastructure and the support of key shared resources that are needed to catalyze the discovery of new knowledge and the development of novel methods to detect, prevent and treat disease. The previous unfinished space on the 3rd floor of MSC has now been transformed into high quality collaborative workspaces that will house computational scientists and scholars engaged in interdisciplinary research in biomedical, life, materials, bioengineering and information sciences.

The new Center for Excellence has been put in place to perform “big-data” analytics at its finest. In the interdisciplinary setting of MSC, the aim is to facilitate sifting through information from various projects and layering data sets to expand the scope of current research. The strategy, however, would be incomplete without vigorous wet-bench research to both generate raw data as well as validate hypothesis generated in-silico. Additional research instruments purchased and included in this project to assist researchers in creating analytical data include the Spectrophotometer and Biorad Chemidoc XRS system.

The Spectrophotometer and Biorad Chemidoc XRS system will be shared by faculty belonging to the Center for Infectious Disease Dynamics (CIDDD), Center for Molecular Immunology of Infectious Diseases (CMIID) and Center for Neuro Engineering (CNE) for research and analysis. These groups will use both instruments for generating data specific to diseases their research is focused on, such as herpes, malaria, and whooping cough. The Chemidoc XRS system will be used in the process of generating genomic data such as SNPs between different sets of pathogenic strains and in preparative aspects of genome sequencing. The Spectrophotometer will be used to generate additional “omics” data via high-throughput enzymatic/ELISA type

analysis. Further, these instruments will be used for testing the hypothesis generated by in-silico analysis of the big-data.

MSC facilities include both purpose-built (specialized) and general laboratory space as well as common areas that are designed to encourage discussion and the sharing of physical and intellectual resources in the life and materials sciences. We now seek to use this unique opportunity to help realize the full potential of this interdisciplinary approach to advancing biomedical and health research by utilizing the Center for Excellence in computational and integrative biomedical research. The Center provides workspace for computational scientists and students that work at the disciplinary boundaries of health, life, and materials sciences research, to conduct biomedical relevant research, particularly in the areas of infectious diseases, genomics and bioinformatics, neurosciences, metabolomics, imaging, and biomedical (neural) engineering in MSC and at Penn State.

Currently, there are several researchers that are using the Center for Excellence along with the new instrumentation to facilitate research and education. Dr. Moriah Szpara, who studies virus DNA and investigates the staggering complexity of the herpes simplex genome, is using the large-scale representations as an important step in training students to notice the comparisons, variations and gaps in genetic sequencing through the variations and subtleties in enormous number sets.

Dr. Matt Ferrari, an assistant professor of biology, who is working to prevent the spread of measles in children around the globe, will begin using the visualization wall this month to eliminate the back and forth process of sharing data among researchers. Instead, Dr. Ferrari and a team of researchers will utilize the new Center as a location to meet and share data in real time, allowing them to look at different variables simultaneously in order to predict and prevent outbreaks.

Dr. Marylyn Ritchie, the Director of the Center for System Genomics, has begun to use the Center of Excellence to enhance the progress of the ATHENA project (Analysis Tool for Heritable and Environmental Network Associations). ATHENA is a solution for the dissection of genetic architecture in common, complex disease. ATHENA will provide a mechanism to 1) perform variable selection from categorical and continuous independent variables, 2) model single factor and/or interaction effects to predict continuous or categorical outcomes, and 3) interpret or annotate the significant statistical models for use in biomedical research.

The promotion of the Center's technology and capabilities to key researchers is integral to the success of the Center. With the Center having been completed at the end of December, faculties are now beginning to take advantage of the technological capabilities. Workshops and seminars are being planned for this summer and fall semester as a key location for researchers to showcase their work and findings. The space will also serve as a training ground for students and post-doctoral fellows to hone skills and techniques beyond previous capabilities. The addition of the Center for Excellence meets the specific aim of developing an interactive space within the confines of MSC that offers advanced visual technology that can promote and engage interdisciplinary research among researchers, computational scientists, and students.

Research Project 50: Project Title and Purpose

Characterizing a New Mouse Model to Study Diabetes and Stroke – The immediate objective of this project is to obtain sufficient preliminary data on our new RCS10 mouse model of type II diabetes to justify our proposed R01 study into the role anti-diabetic agents have on stroke outcome. Diabetics are at a 3-fold higher risk of experiencing a stroke and have a greater incidence of mortality and morbidity following an ischemic stroke and currently there are no known therapies that are specifically targeted at diabetics to improve stroke recovery. This project will investigate: 1) the role a compromised inflammatory response plays in the stroke recovery in the diabetic RCS10 and how this might be modulated by anti-diabetic agents and 2) the behavioral consequences associated with stroke recovery in the diabetic RCS10 mice and the efficacy of the anti-diabetic agents in ameliorating behavioral outcomes.

Duration of Project

12/12/2012 – 12/31/2013

Project Overview

The overall objective of this project is to address specific deficiencies identified in our R01 renewal submission, in which we proposed to determine the role of anti-diabetic agents on stroke outcome in a new mouse model of type II diabetes. The reviewers of the renewal application raised three significant reservations that will be addressed in this project: 1) the viability of the RCS10 mice to serve as a better model to investigate stroke outcomes than the other rodent models of Type II diabetes, 2) the failure to provide any mechanistic approaches to investigate what underlies the impaired recovery in the diabetic mice, and 3) the absence of behavioral studies to assess the distinctions in stroke recovery in the diabetic and non-diabetic mice and the ability to determine the efficacy of the proposed therapeutic interventions. Reservations 1 & 2 will be addressed in Specific Aim 1 in which we propose to obtain more preliminary data characterizing the RCS10 mouse response to stroke with a specific focus on 1) the extent to which the inflammatory response is compromised in the diabetic mice and how this leads to a breakdown in the blood-brain barrier (BBB) and 2) investigate what underlies these changes and how they are modulated by the various anti-diabetic agents. In Specific Aim 2 we propose to develop a battery of behavioral tests that will enable a non-invasive assessment of stroke damage and subsequent recovery and the efficacy of therapeutic interventions.

Specific Aim 1. To investigate the inflammatory responses initiated in male RCS10 mice following a hypoxic/ ischemic (H/I) insult. The hypothesis to be tested is that the increased cell loss observed in the diabetic RCS10 mice compared to their non-diabetic littermates and closely related NON mice controls following an H/I insult will result from an impairment in inflammatory responses that mediate recovery, resulting in a breakdown in the BBB. We propose to monitor the temporal activation of astrocytes and microglia and the recruitment of neutrophils, macrophages and mast cells and determine what role these cells might play in mediating endothelial cell proteolysis and the permeation of the BBB. Specific Aim 2. To develop a battery of behavioral tests to assess the extent of neurological impairment in diabetic and non-diabetic RCS10 mice following a hypoxic/ischemic insult. The hypothesis to be tested is that behavioral

changes will accurately reflect differences in ischemic damage between diabetic and non-diabetic RCS10 mice following a stroke and will serve to assess the efficacy of therapeutic intervention with anti-diabetic agents.

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Expected Research Outcomes and Benefits

There are an estimated 24 million Americans living with Type II diabetes. This diabetic population has a 2-4 times higher risk of experiencing an ischemic stroke and a 2.8 times greater risk of mortality. Stroke is the third leading cause of death and the major cause of long-term disability in the USA. The confluence of the dramatic increases in the number of diabetic patients coupled with aging baby boomers predicts a dramatic increase in the occurrence of strokes. The objective of this project is to characterize the responses and subsequent recovery following stroke of a newly created NONcNZO10/LtJ (RCS10) mouse model of Type II diabetes. These mice represent a potentially superior model to study stroke recovery as their diabetes more closely parallels the human situation. For example, their diabetes is polygenic and not monogenic and the onset of diabetes is induced by feeding male mice a moderate 10-11% fat diet that becomes fully apparent by 12-14 weeks of age. Moreover, unlike other mouse models of Type II diabetes, these mice are responsive to anti-diabetic agents. Thus, it is now possible to investigate which class of anti-diabetic drugs that are commonly prescribed (metformin, sulphonylureas or thiazolidinediones (TZDs)) is most efficacious in reducing damage from a subsequent stroke. The current studies are specifically investigating why the stroke outcome is worse in the diabetic mice and are focusing on how the immune system is compromised in the diabetic mice with the objective of identifying specific therapeutic targets. The other objective of this project is to identify a battery of behavioral tests that will non-invasively characterize the extent of stroke damage in the control and diabetic mice and allow the investigators to assess the efficacy of any therapeutic interventions. This represents an entirely novel approach to studying the effects of diabetes on stroke outcomes, which could have important consequences when attempting to translate therapeutic interventions to the human situation.

Summary of Research Completed

Figure 1 represents a summation of the behavioral experiments in which we investigated the effects of inducing a stroke on the right hemisphere of C57Bl6 mice on the ability of the mice to perform the Corner, Cylinder, Foot Fault and SNAP behavioral tests described in the earlier

report. The completion of these initial behavioral studies has not only enabled us to comply with a very specific request from the reviewers of our RO1 grant that we include a behavioral component in our resubmission but has enabled us to use the methodology and data to apply for additional grants from both the NIH and AHA.

As indicated in the previous Annual Report, we were somewhat confounded by the requirements outlined by the American Heart Association and reviewers of our most recent RO1 proposal that provided the basis for the current proposal. The AHA have strongly advocated that stroke research be conducted on older animals to more closely conform with human population and our reviewers indicated that if we are to study meaningful interventions then the duration of the diabetes be extended to at least 1 month. To comply with these prerequisites required the NONcNZO10/LtJ (RCS10) be at least 20 weeks old and as we demonstrated in the previous report at this age the numbers of non-diabetic mice that were to serve as controls dwindled to almost zero. Thus we proposed to use NONShiLtJ (NON) that has an 88% genomic identity to the NONcNSO10/LtJ (RCS10) mice. These mice become equally obese as the RCS10 mice, but do not develop diabetes. In collaboration with Dr. Sarah Bronson, we were able to establish a breeding colony for these mice and have now begun to complete the necessary comparative experiments with the RCS10 mice to establish the significance of the overt hyperglycemia on the stroke outcome. These experiments are ongoing, however it should be noted that it takes 6-7 months to generate each set of experimental animals and the number of successful matings, pups /litter and the ratio of males to females are variables that are far from predictable. We currently anticipate that sufficient experimental animals will be generated within the next 4-6 months to complete our characterization experiments.

We were able to initiate an alternative approach to investigate the role of hyperglycemia on stroke outcome in which euglycemia could be restored in the RCS10 mice with Metformin. Metformin is a widely prescribed agent to treat Type II diabetes and as illustrated in Figure 2 when included in the water is able to establish and maintain euglycemia in the RCS10 mice over a 4-week period, which addressed a concern raised by the reviewers regarding the ability to sustain therapy. The Metformin was administered to the mice in their water to which 0.15% saccharin was added to ensure that mice consumed sufficient metformin/water to normalize their blood sugar (0.8-1.3 g/kg/D). The saccharin levels in the water of control mice were diluted to normalize saccharin consumption. Figure 3 describes the effects of the Metformin on stroke outcome, which was determined at 48 h after the infarct by H/E staining. These results in some respects are disappointing as our expectations were that the metformin would elicit a comparable effect to that we had obtained when euglycemia was induced in the *ob/ob* mouse with darglitazone, a *ppar* γ agonist. In that study, darglitazone not only normalized the blood glucose levels, it dramatically reduced the infarct volume in both diabetic and non-diabetic animals. The data described in Figure 3 is comparable to that obtained by (Tureyen et al., 2007) who found that Metformin treatment in the *db/db* mouse had no effect on stroke outcome. However, in those studies the *db/db* mice were too insulin resistant to fully restore euglycemia and thus the observations were always considered equivocal. It should also be noted that the levels of Metformin used in this study and that of Tureyen et al. is significantly higher on a mg/kg basis than used in patients, suggesting marked difference in sensitivity between rodents and humans which was not the case with the *ppar* γ agonists. This represents a very important observation that will alter the scope of our RO1 resubmission, as we will now not propose to investigate the

mechanistic aspects of metformin but we have clearly demonstrated unlike the *db/db* mice the glycemic state in RCS10 can be modulated.

Figure 1 Behavioral Deficits in C57 Bl6 Mice at Day 7 Post Stroke

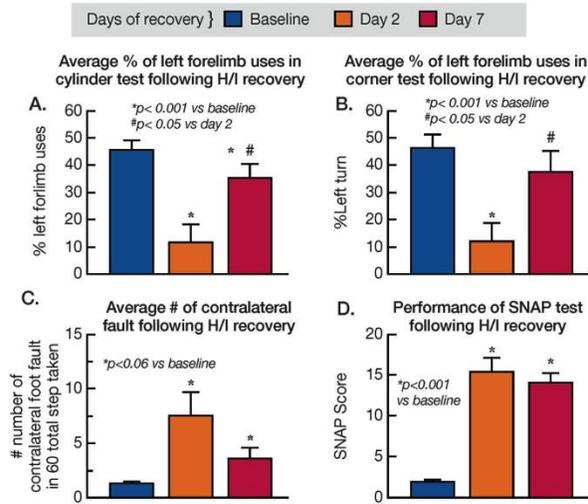


Figure 1. Studies were performed on 16 week-old C57 Bl6 mice (n=20). For the cylinder test a 5 min. trial period was used to observe each mouse at each time point. 5 trials at each time point were observed for the foot fault test and the corner test. The SNAP tests were conducted at each time point.

Figure 2 Effects of Metformin on Blood Glucose in RCS10 Mice

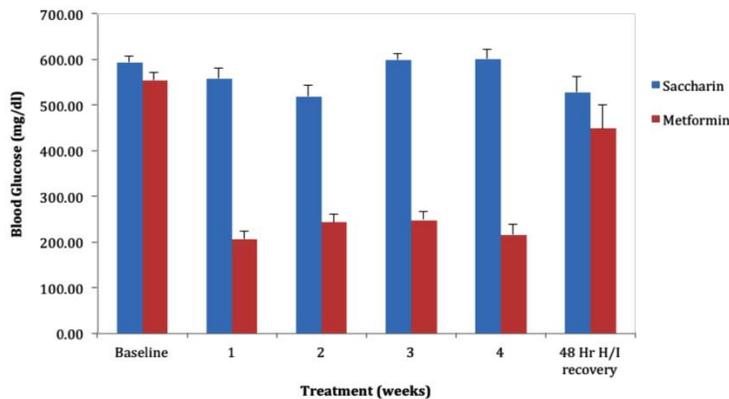


Figure 2 describes the time course for the normalization of blood glucose in the RCS10 mice. The RCS10 mice were 20 weeks old when Metformin treatment was initiated and had been diabetic for at least 4 weeks. To maintain euglycemia the Metformin concentration in the drinking water was increased from 100 -120 mg/ml over the course of the 4 weeks. 0.15% saccharin was added to mask Metformin and the concentrations were adjusted to ensure equal saccharin intake in the control RCS10 mice. The Metformin was unable to maintain euglycemia following the stroke presumably due to the increased insulin resistance.

Figure 3

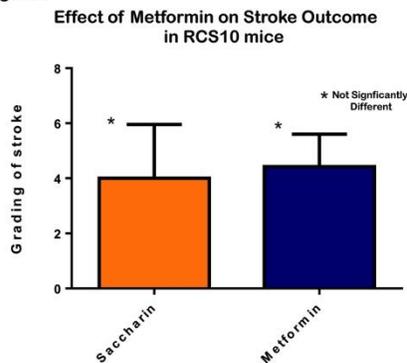


Figure 3 illustrates the effects of maintaining RCS10 mice euglycemic for four weeks on the subsequent outcome following stroke in the mice maintained as described in Figure 2. Both sets of animals were exposed to 22 min of 9% oxygen. The extent of the stroke damage was determined at 48 h post stroke infarct from H&E sections (n=8 Saccharin control mice and 15 Metformin mice). There was no significant difference in the extent of the insult between the diabetic and euglycemic mice.

Research Project 51: Project Title and Purpose

Defining the Neonatal Septisome and Postinfectious Hydrocephalus – Neonatal sepsis kills over 1 million newborn infants each year, with cases highly concentrated between sub-Saharan Africa and southern Asia, and in survivors accounts for most of the world’s infant hydrocephalus. We have assembled a unique collection of samples for microbial genomic analysis from 80 mother-infant pairs with neonatal sepsis from Uganda, and a set of samples from matched cohorts of 25 patients with postinfectious and 25 with non-postinfectious hydrocephalus. This project will complete the metagenomic analysis on these infant samples in order to further delineate the microorganisms responsible for neonatal sepsis and its sequelae in this setting.

Duration of Project

12/12/2012 – 12/31/2013

Project Overview

This project seeks to test the hypothesis that: The pathogenic bacteria and viruses responsible for neonatal sepsis in sub-Saharan Africa have not been well characterized. A comprehensive evaluation of the responsible bacteria and viruses is the starting point to develop more rational methods of improving treatment and prevention to meaningfully reduce the morbidity and mortality from this syndrome.

Over 1 million neonates die each year from preventable and treatable infections in the neonatal period, and many of those that survive give rise to a cohort with substantial sequelae. The vast majority of these neonatal infections are in the developing world, about half in sub-Saharan Africa. Survivors of such infections also appear to constitute a substantial fraction of infant

hydrocephalus. Nevertheless, we know little about the underlying bacterial and viral agents involved in neonatal sepsis in such settings, and have no rational basis to optimize treatment or prevention.

This project seeks to begin to characterize the neonatal sepsisome – the collection of invasive microorganisms that underlie neonatal sepsis. We further seek to characterize the particular agents associated with postinfectious hydrocephalus in survivors of neonatal sepsis. We will do this by a bacteriological and metagenomic evaluation of mother-infant pairs with neonatal sepsis, and a comprehensive metagenomic evaluation of postinfectious hydrocephalus.

The specific aims are:

1. Characterize the Neonatal Sepsisome from 80 mother-infant pairs with neonatal sepsis from the Mbarara Regional Hospital in Uganda using a metagenomic analysis of Bacterial 16S rRNA gene
2. Characterize an age matched cohort of postinfectious hydrocephalus infants (25) and non-postinfectious hydrocephalus patients (of known congenital cause and without a history of neonatal sepsis) using metagenomic analysis of Bacterial 16S rRNA gene from infant blood and cerebrospinal fluid (CSF).

This will be the first such characterization of the neonatal sepsisome, and the first in a developing country. Our findings will lay the groundwork for future optimization of treatment of neonatal sepsis in such settings, which will reduce both the deaths and sequelae such as hydrocephalus in the survivors. Long-term, identifying the routes of infection of the agents will enable the construction of rational public health policy to better prevent these cases. The potential impact on global infant health is substantial.

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Expected Research Outcomes and Benefits

- 1) Develop more optimal DNA and RNA extraction protocols for our existing samples.

Research Outcome: The variability and artifacts of metagenomic sample sequencing from such samples can be high and the diagnostic yields low. We will optimize our sequencing protocols against our known bacteriological culture results from the same samples, in order to validate our sequencing results.

2) Complete the sequencing analysis on our specimens already in hand. We will complete the processing of our mother-infant paired specimens with neonatal sepsis (80 total), and complete analysis of our matched cohorts of postinfectious and non-postinfectious hydrocephalus patients (50 total).

Research Outcome: This unique dataset comprises the world's most complete set of samples from mother-infant pairs with neonatal sepsis in the developing world, and the only existing matched control set of samples from post-infectious hydrocephalus. Our outcomes will be the first comprehensive characterization of the spectrum of microorganisms that are the candidate pathogens from this syndrome – the Neonatal Septisome – and the most serious sequelae in the survivors, postinfectious hydrocephalus. Once such organism characterization is complete, we can proceed towards more effective treatment and prevention strategies aimed at reducing the morbidity and mortality in these infants.

This project scored very well as a finalist for an NIH Director's Pioneer Award in 2012. This present project seeks to gather additional preliminary data and to keep the specimen collection team going until substantial NIH funding is secured to comprehensively complete the full scope of the work required. We expect to secure substantial and large scale NIH funding within PA, and PA physicians and scientists will be responsible for saving the lives of so many around the world. Our findings will lay the groundwork for future optimization of treatment of neonatal sepsis, which will reduce both the deaths and sequelae such as hydrocephalus in the survivors. Long-term, identifying the routes of infection of the agents will enable the construction of rational public health policy to better prevent these cases. The potential impact on global infant health is substantial.

Summary of Research Completed

We have successfully extracted bacterial 16S DNA from filter papers spotted with 100 ul of blood and cerebrospinal fluid (CSF) from all of the patients planned in this project.

Analysis of the data from 80 patients with neonatal sepsis is shown in Figure 1. Multiple negative control samples were included for the extraction, amplification, and libraries preparation steps; any that yielded 16S DNA were also sequenced. The most common classifiable contaminant is the genus *Ralstonia*.

Leptospira species (*Broomii* and *Indai*) were the dominant organisms identified to the genus level in these patients, in 31 of 80 patients. We have further confirmed *Leptospira* with separate gene analysis to sequencing non-16S genes in a subset of these specimens.

These data are important at several levels. First, we have spent over a year now optimizing and controlling our pipeline for extracting DNA from these filter card samples. Our prior analysis, done in collaboration with a well-known genomics institute, had revealed a broad range of apparent organisms in these samples, which we have now eliminated as contaminants. Second, we have now been able to validate single species molecular identifications against bacterial culture and chemical analysis results from the Microbiology laboratory at Mbarara. Third, using

patients as internal controls, we are identifying the same organism from molecular techniques in blood and spinal fluid, when our bacteriological culture results (published in 2013 in PLoS ONE) the same organism cultured from both blood and CSF. Fourth, we are failing to confirm Staphylococcal species from samples that the limited microbiological laboratory chemical and microscopic testing had classified as Staphylococcus aureus. This is both an important source of clinical microbiology quality control, as well as a potentially important way to improve patient outcomes. Fifth, the finding of Leptospirosis in these infants appears to be a major finding demonstrating the value of molecular techniques. *Leptospirosis is likely the most common zoonosis in the world, and is vastly underreported because it cannot be recovered through routine bacterial culture in most laboratories* (WHO report, 2011). Our preliminary findings further suggest that we are specifically seeing this as a co-infection in patients with evidence of both early and late neonatal sepsis, suggesting maternal vertical transmission in some, and de novo environmental acquisition, in other infants. Leptospirosis fits with our previous fieldwork regarding the close proximity of these infants to domestic animals in rural Uganda. Furthermore, our findings of a clear association of cases of postinfectious hydrocephalus (PIH) with levels of rainfall would fit with what we know about the rainfall association with infectivity of environmental Leptospirosis. Lastly, to our knowledge, Leptospirosis has never been documented in neonatal sepsis (NS) before. Although one would typically wish to institute screening with antibody tests to confirm Leptospirosis in adult patients, the documented low sensitivity of these tests in clinical practice with Leptospirosis, suggests that molecular techniques may be particularly valuable in these diagnostics with neonates.

Several important additional questions arise in light of these new preliminary data. First, the role of polymicrobial infections in humans is gaining increasing attention, and our molecular data supports that possibility here. Second, are the organisms responsible for PIH a select subset of the NS cases, with neurotropic predilection? Third, what is the potential role of viruses in both NS and PIH in this setting?

The typical intraventricular findings from African PIH infants are the presence of considerable purulent debris within the loculated ventricular system, almost always coating the choroid plexus, as well as diffuse hemosiderin staining. Such findings may not be new – data from South Africa in the 1970s demonstrated loculated debris within the ventricles of such infants, accompanied by recovery of Gram-negative coliforms (including Acinetobacter). Nevertheless, the appearance of the CSF cistern beneath the brain in such patients is typically pristine without evidence of purulent material or even scarring in the fluid spaces around the basilar artery. Following bacterial meningitis, this fluid space would be typically extensively scarred and obstructed. What we are therefore finding is that these infants appear in large numbers with evidence of a purulent intraventricular infection without evidence of meningitis. We make this statement with great care, because our clinical findings are stereotyped and unusual. There are several hypotheses we offer: 1) there are bacteria that are capable of primary ventricular infection through choroid plexus seeding. We note that primary ventriculitis from group B Streptococcus in humans has been documented, and choroid plexitis with *H. influenzae* has been shown in primates; or 2) there are neurotropic viruses that play an important role in NS in these cases, but fit no previously known human viral infection pattern (demonstrating the purulence typically associated with bacterial ventriculitis); or 3) an inflammatory response to a prior NS

infection alters the BBB allowing subsequent entry of bacteria; or 4) there are true polymicrobial infections creating the PIH syndrome.

The molecular tools to identify bacteria are relatively straightforward. All bacteria have a set of ribosomal genes that are partially conserved, such as the 16S rRNA gene, yielding mutation variability rates that permit speciation. We are now using select species specific protein-coding genes to confirm our 16S findings. But viral discovery using molecular tools requires deep sequencing approaches when the viral species are not known ahead of time. A very relevant recent study of the human febrile virome has demonstrated that such metagenomic approaches to viral discovery can be productive. Instructive in this viral metagenomics work was the availability of a control group of children undergoing elective surgery. In our case, we have defined a control cohort of PIH children with congenital causes of infantile hydrocephalus and no evidence of previous NS – a non-postinfectious hydrocephalus group that have nevertheless shared the same neonatal environments and are age and environmentally matched to the PIH infants. Although we do not have an explicit control group for NS at Mbarara (there is little neonatal surgery in Uganda at present), we are using the comparison of blood and CSF fluids as internal consistency checks when the same pathological organism is expected to be present in both fluids in cases of meningitis and many cases of blood sepsis. We are presently developing a workflow to develop extraction of RNA from these filter paper cards in order to proceed with deep sequencing for viral pathogen identification.

Pending NIH Grant Applications:

NIH Director's Pioneer Award, Schiff(PI) \$2,500,000 (pending as finalist)

“Control of the Neonatal Septisome and Hydrocephalus in sub-Saharan Africa”

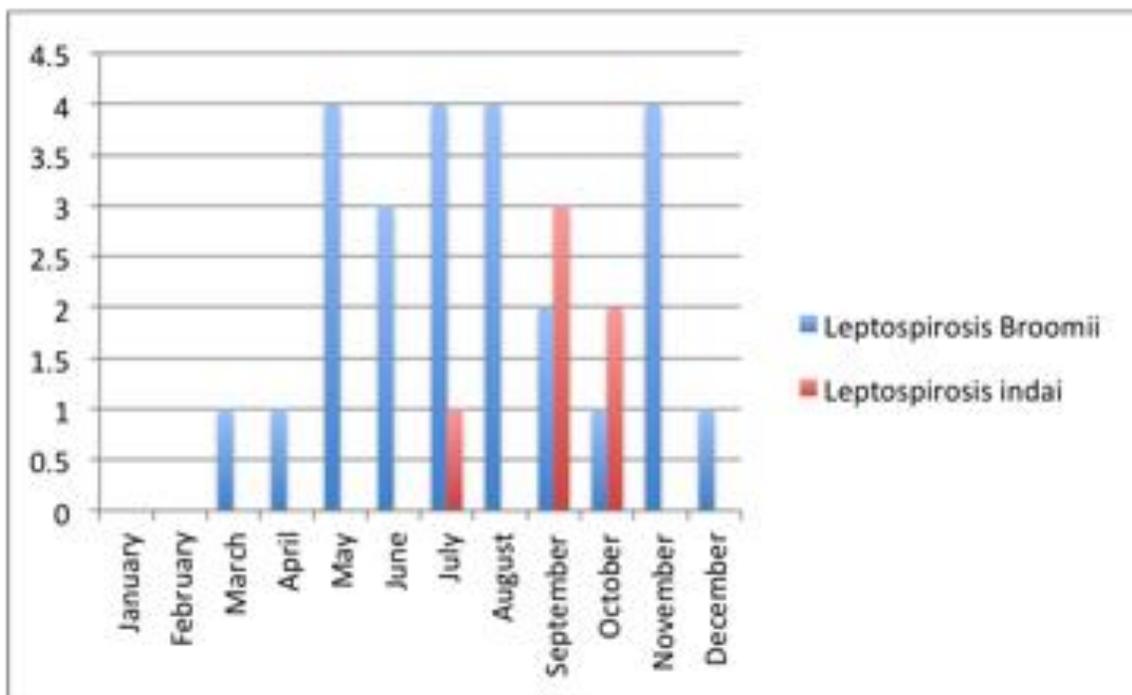


Figure 1. Cases of Leptospirosis in first 80 neonatal sepsis cases.

Research Project 52: Project Title and Purpose

The Gastrointestinal Microbiome in Parasitic Infections - Towards Testing the “Hygiene Hypothesis” - Diseases have been proposed to disrupt the composition and function of the commensal microbiota and to alter the homeostatic interactions between the microbial community and the host. How this happens and how the microbiota influences disease dynamics and severity is still unclear and largely based on correlation studies. This project seeks to identify how changes in the intensity of parasitic infections impact microbiome assembly and composition in the gastrointestinal tract. This study aims to provide fundamental knowledge for understanding disease development and pathogenicity and for developing targeted approaches to the prevention and treatment of the adverse health consequences of these common parasitic infections.

Duration of Project

3/12/2013 – 12/31/2013

Project Overview

The broad objective of this study is to investigate the impact of parasitic infections on the dynamics and composition of the commensal microbiome of the gastrointestinal (GI) tract. We will leverage ongoing studies using the well-established model of helminth infections (*Trichostrongylus retortaeformis*) of the rabbit (*Oryctolagus cuniculus*) by performing experimental animal manipulation and high-throughput DNA sequencing of the host GI bacterial community. Bioinformatic analysis will be applied to the genomic data to characterize the microbiome assembly and statistical analysis will be used to examine the relationship between microflora community and helminth dynamics.

This host-parasite system has many similarities with the helminth infections of humans, and findings can be used to identify general rules driving parasitic-commensal interactions and to seek alternative methods for controlling infections. Importantly, this system is unique in that host's coprophagy allows individuals to naturally boost themselves with their own microbiome and potentially modulate the severity of GI helminth infections. The project has two specific goals.

Goal 1. To determine the dynamical association between intensity of infection and changes in commensal microbiome in the GI tract. By sampling rabbits over the course of the infection we anticipate to clarify how changes in helminth load influence microbiome assemblage and composition.

Goal 2. To prevent host's coprophagy and examine the consequences on microbiome assemblage and infection severity. We propose that coprophagy is a form of 'self-medication' from gastrointestinal infections and expect to identify differences in the microbiota by manipulating this rabbit behavior.

By comparing temporal changes in the helminth-microbiome association during normal and restrained settings and over the course of the infection this study will strengthening fundamental

theory on host-parasite-microbiome interaction and provide a fertile ground for developing new hypotheses on the role of the gastrointestinal microbiome in infectious diseases.

Principal Investigator

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Expected Research Outcomes and Benefits

Approximately a quarter to a third of the world's population is infected by gastrointestinal helminths. Parasitic helminths remain amongst the most common and prevalent infections on the planet. These infections are surprisingly common in the US, with more than 4 million individuals affected annually. A better understanding of how gastrointestinal parasitic infections interact with the host microbiome and how this affects disease pathogenesis is essential for developing new approaches to treat debilitating and persistent infections that can have a profound impact on gastrointestinal health and overall nutrition. The microbiome is complex and shaped by a wide range of factors.

The application of genomics and bioinformatic methods along with parasitology and epidemiology will provide a robust tool for understanding the contribution of host microbiome in health and disease. More broadly, this study will allow us to provide a more mechanistic understanding of the helminth-microbiome interactions and advance the groundwork for assessment of infection risk based on host properties.

Summary of Research Completed

The current report summarizes our progress in the last 6 months.

We have completed the animal trial using New Zealand White rabbits and a factorial design where groups of 16 rabbits were subjected to one of the following treatments: i- infected with the gastrointestinal worm *Trichostrongylus retortaeformis*, ii- infected with the helminth and wearing an Elizabethan collar to prevent coprophagy, iii- only wearing the collar, and iv- not manipulated and used as control individuals. A group of 4 rabbits from each treatment were sacrificed at 15, 30 and 60 days. 4 rabbits were also sampled at day 0. Host tissues and worm specimens were collected at each sampling point. Tissue samples from the small intestine have been processed as follows: a. to extract DNA for high-throughput DNA sequencing to identify the microbiota community in the duodenum; b. to extract RNA for cytokine gene expression

involved in the host immune response to the parasitic infection; c. to collect mucus for host antibody detection against the helminth; d. to quantify the host lipid profile using a metabolomic approach; and e. to collect helminths for parasitological work (parasite abundance, development and fecundity). Additionally, we also quantified the bacterial microbiota and the lipid profile in the caecum as well as the concentration of systemic antibodies in the blood and the amount of parasite eggs shed in the environment. The next paragraphs, briefly report the results/current progress of these activities.

a. Microbiome in the duodenum and caecum

We had a few initial technical delays in the high-throughput DNA sequencing. All the issues have now been resolved, the protocol has been optimized and all the small intestine samples have been processed. Briefly, a 5 cm piece of small intestine-duodenum was cut, the ingesta gently washed with PBS and the piece was cut open longitudinally. The mucosa, lining the inside of the small intestine, was then gently collected using a sterile swab applicator. The DNA extraction was performed using the Powersoil isolation kit (Mo Bio). The concentration of the extracted DNA was evaluated using a NanoDrop Spectrophotometer and a *Qubit 2.0* Fluorometer (Life Technologies). The procedure was performed in duplicate for each animal. As reported in other microbiome studies, individual variation can be very large and obscure the ‘treatment effect’. To reduce the between-individual variation in microbiome composition within treatments, bacterial genome was run using these two replicates for every rabbit.

Genomic data have been processed using the "mothur" software package by Schloss P.D. and colleagues (2009), an open-source, platform-independent software for describing and comparing microbial communities. The methods implemented in this software follow widely accepted data analysis protocols. Specifically for our dataset we have: split reads into samples, filtered and trimmed data to retain high quality measurements, realigned the sequences to 16S regions of all known bacteria, filtered for sequences that align in the expected 16S regions and classified the sequences via the RDP Multiclassifier. Following this quality control and data classification, we need to repeat a small fraction of samples to provide better genome coverage and robust taxonomy. Analyses are still in progress but a preliminary investigation using a subset of data already shows a number of patterns. At Phylum level, we found that some bacteria taxa are more common in some treatments than others (Fig. 1). Similarly, some taxa are well represented while others are rare or completely missing across all the treatments. There is a temporal variation in the occurrence and percentage of some of these taxa -from 15 to 60 days into the trial- although this trend is not particularly strong. These general patterns are also detected at the taxonomic level of Order and Class (figures not presented). The caecum samples are in the pipeline and will be processed using the same approach.

b. Cytokine gene expression in the duodenum

In addition to the cytokines we previously found important for the rabbit immune response to helminth infections, we have also checked for a number of additional cytokines (IL2, TGF β), identified as good standard markers for immune mediated tolerance in general and to parasites in particular, and cytokines involved in host resistance to helminth infections (e.g. IL5, IL13). Cytokines from the small intestine duodenum have been quantified using Quantitative Real Time PCR and commercially available or custom made primers and probes (Applied Biosystems). RNAs have been isolated and samples are almost ready to be PCR processed.

c. Mucus antibody detection in the duodenum

Mucus and serum antibodies (IgA and IgG) against helminth adults and total Ig concentration have been processed using an ELISA approach. Based on our previous experience with this system, these antibodies have been selected because they contribute to helminth clearance and affect parasite development and fecundity. We have not tested IgE, commonly produced against parasites in other system, as we found that the concentration of this antibody is hardly detectable to this worm species in the rabbit. Preliminary findings indicated a somatic IgA and IgG response to the worm infection, both in the mucus (Fig. 2) and the serum. In the mucus both IgG and IgA increased with time and values were significantly higher in infected than control individuals. Results are currently under additional scrutiny for outliers and possible technical artifacts.

d. Host lipid profile in the duodenum and caecum

Samples have been processed at the Metabolomics Core Facility at Penn State. We concentrated on the detection of the short chain fatty acid profile, using the GC-MS method, and the total lipid profile using the LC-MS/MS method. The short chain fatty acid results were not particularly useful and we now plan to change the protocol for the sample preparation in order to obtain meaningful results. The total lipid profile in both positive and negative ion modes is now available and we are currently running the statistical analysis. Preliminary results show clear clustering of small intestine and caecum samples, as expected, and for the small intestine there is a difference between infected and control treatments, irrespective of whether rabbits wear collars, although sample variability tends to be large.

e. Parasitology and host body mass

Sections of the duodenum were collected, helminth extracted and counted, and a subsample, 40 females and 40 males, randomly collected. These specimens were further analyzed using standardized procedures we have applied before, specifically, a digitalized system of a camera connected to a microscope. We measured worm length and width and counted the number of eggs *in utero* directly under the microscope. Twice a week, fresh feces have been collected from each rabbit, parasite eggs extracted and counted. In summary, for every infected rabbit we have collected: worm abundance at each stage to estimate establishment and survival; body length and width at different stages for worm development; eggs *in utero* for fecundity and eggs in the feces for parasite shedding. Worm measurements are close to completion, below we report results on abundance and eggs shed in feces (epg).

Helminth abundance was comparable between infected and infected+collar individuals (mean±s.e.: 283.89±64.69 vs 249.22±67.14) (Fig. 3 left side), abundance was consistently higher in the first segment of the small intestine and lower in the third segment, in agreement with our previous findings using this system. Similarly, the amount of parasite eggs shed in rabbit feces was comparable between infected and infected+collar individuals (Fig. 3 right side). For both treatments, shedding consistently dropped at around 40 days post infection but increased again by 60 days post infection, with infected rabbits showing a tendency to shed more than infected+collar hosts. This result, and the lack of clear temporal trend in parasite abundance in the small intestine segments, was unexpected and worth more careful analyses, which are currently in progress.

We also collected rabbit body mass and values increased with the course of the experiment for

all the 4 treatments, based on a fixed amount of 125g of pellet per day (Fig.4). In general, infected rabbits tend to weigh more than control individuals and collared cases were slightly lighter than non-collared ones especially towards the end of the experiment. The drop in body weight around 20 days was caused by a dysfunctional balance and should be not be biologically relevant; we believe that a similar issue also occurred around 40 days.

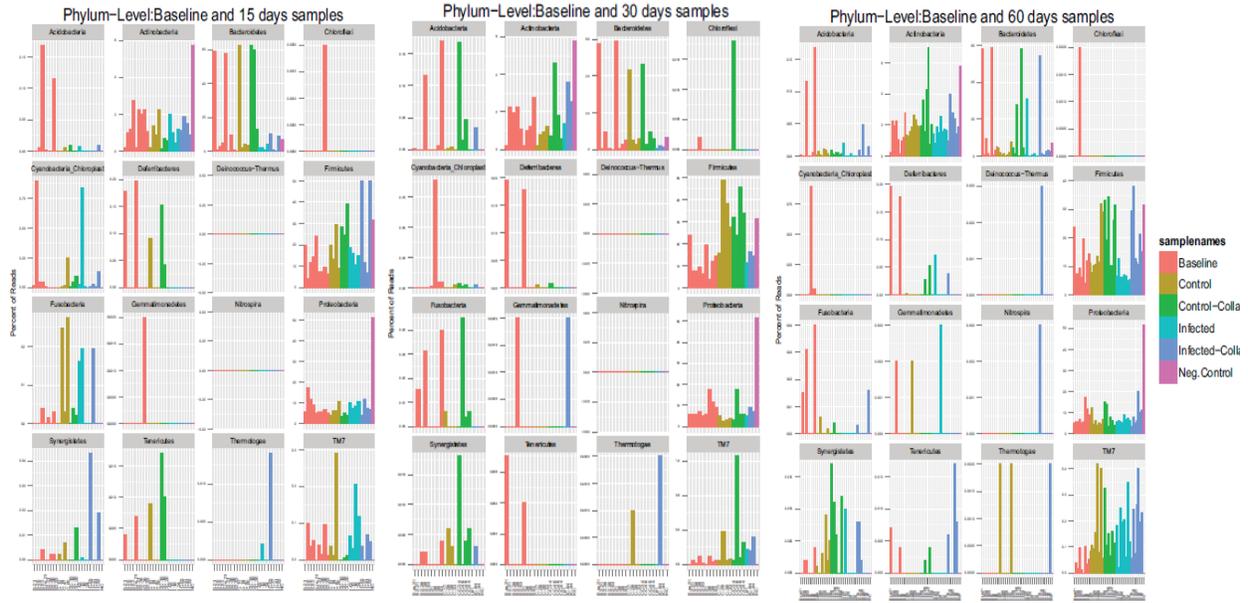


Fig. 1 Percentage of reads (y-axis) by bacteria taxa -different plots- at phylum level in every rabbit (x-axis) sampled at day 15, 30 and 60 (from the left to the right side of the figure). Treatments are highlighted in colors (negative controls and baseline cases at time point 0 are also included).

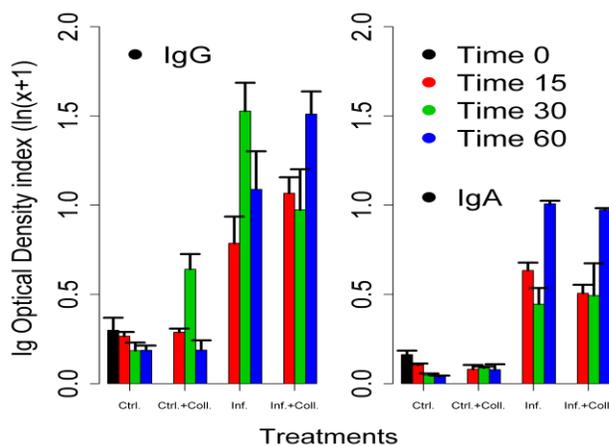


Fig. 2 Mean optical density index for IgG and IgA in the small intestine mucus of rabbits by experimental treatment and experimental time.

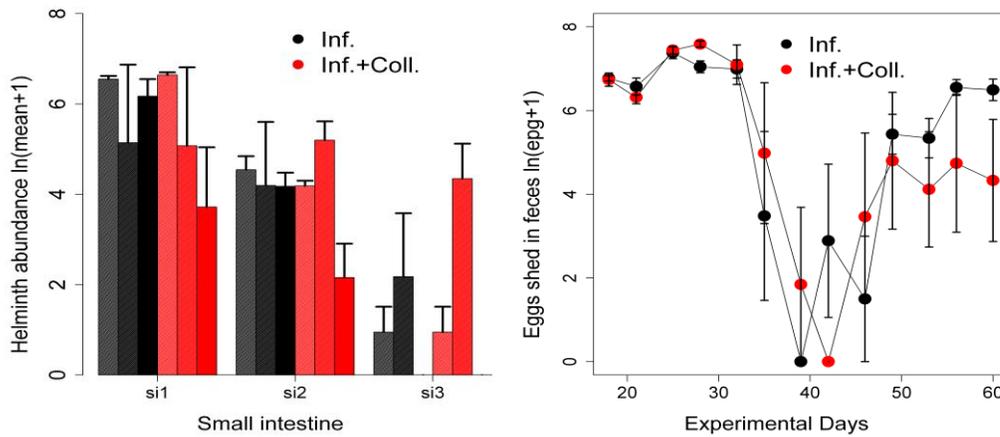


Fig. 3 Left: Mean helminth abundance by small intestine segment (SI1 to SI3) in infected and infected+collared individuals and by sampling time (color gradient from high to low 15, 30, 60 day of sampling, respectively). Right: Mean eggs shed per gram of host feces over the course of the infection by infected and infected+collared individuals.

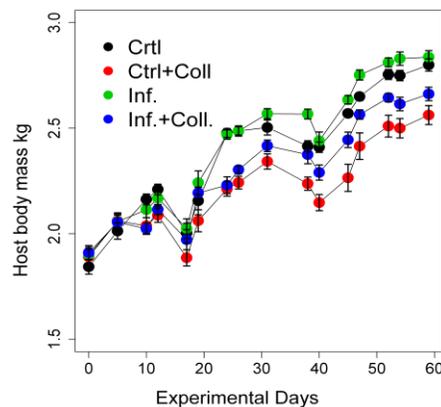


Fig. 4 Mean rabbit body mass by sampling time and experimental treatment.

Research Project 53: Project Title and Purpose

Targeting the Oncogenic Mutant p53 Signaling in Colorectal Cancer Therapy - Tumor suppressor p53 is a transcription factor that is frequently mutated or inactivated in colorectal cancer (CRC). Small molecules that can activate the p53 family member p73 in mutant (mt) p53 expressing tumors can elicit a p53-like tumor suppressive function. Utilizing a luciferase reporter based functional screen we have identified Prodigiosin and CB-7587351 (Chembridge library # 7587351) as potent p53-family transcriptional activators. Our goal is to evaluate these compounds as lead candidates to target mt p53 in therapy of CRC. We will test the efficacy and safety of p53 restoration in preclinical models of colorectal cancer harboring mt p53. Currently there are no FDA-approved drugs targeting activation of p73 for CRC therapy. Our project addresses this unmet need for safe and effective small molecules in this class.

Duration of Project

3/12/2013 – 12/31/2013

Project Overview

Our goal is to specifically target mutant p53 in colorectal cancer by an indirect mechanism involving the p53 family member p73. We aim to evaluate the safety and efficacy of the highly potent small molecules Prodigiosin, Prodigiosin-related compound and CB-7587351 with *in vitro* and *in vivo* studies. We will optimize and improve the *in vivo* experiments by using various mutant p53 expressing cell lines to demonstrate robust anti-tumor activity with Prodigiosin, its related compound and CB-7587351 in xenograft tumor models. HPLC/ Mass spectrometry will be used to develop and optimize a method for detection of Prodigiosin, its related compound and CB-7587351 that will be utilized for pharmacokinetic analysis of these small molecules in *in vivo* studies. The p53 pathway restoration and apoptotic effects of the compounds will be demonstrated in a dose dependent manner.

Specific aims:

Aim 1: Demonstrate effects in p53 null cells and evaluate the effect of varying doses of compounds on p53 reporter activation and apoptosis

Aim 2: Perform *in vivo* pharmacokinetic analysis of p53 restoring compounds using HPLC detection of small molecules.

Aim 3: Optimize *in vivo* studies to demonstrate robust anti-tumor efficacy with p53-pathway restoring compounds in various xenograft tumor models.

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Expected Research Outcomes and Benefits

According to the American Cancer Society, nearly 150,000 new cases of CRC and over 50,000 deaths due to CRC are expected to occur in 2012 in the United States. Despite new treatment regimens, the 5-year survival remains very poor for patients with CRC that are not diagnosed at an early stage. Mutant p53 is a major cancer-specific target for therapy development. p53 is

mutated in more than 50% of all human cancers. The impact of this work is the potential to provide novel alternatives for cancer therapy by specifically targeting colon cancer cells with minimal toxicity through restoration of wild-type p53 function with small molecules. Currently, there are no FDA-approved drugs in the clinic targeting specific activation of p73 for CRC therapy. Our project aims to validate CB-758731, Prodigiosin and its related compound as safe and effective small molecules in this class for CRC therapy. The molecular mechanism of p73 activation is unclear for currently available p73 activators such as RETRA. Our project will validate p73-dependent effects of CB-7587351, Prodigiosin and its related compound and identify potential mediators of p73 activation for these compounds including blockade of interaction between mutant p53 and p73. Also, the results of this project will provide preliminary data for re-submission of an NIH grant application.

Summary of Research Completed

Specific Aim 1: Work on Aim 1 was completed and described in a previous report.

Aim 2: Perform *in vivo* pharmacokinetic analysis of p53 restoring compounds using HPLC detection of small molecules.

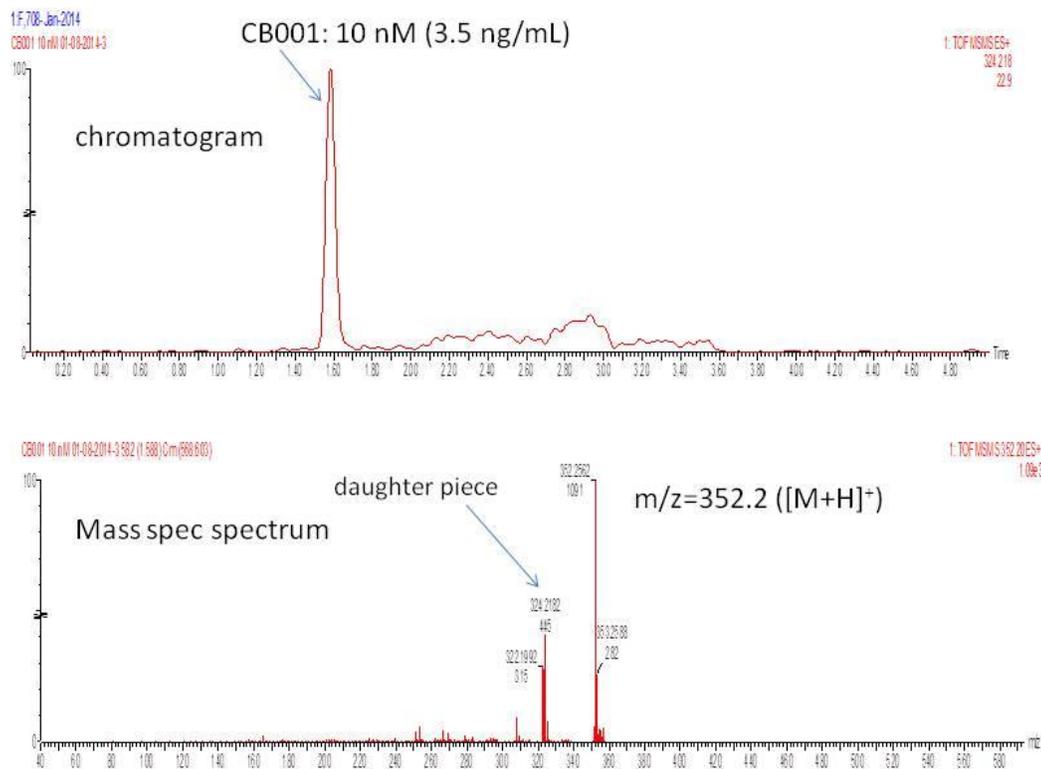
Method: p53 pathway restoring compound CB-7587351 was provided to the Penn State Hershey Mass Spectrometry core facility for chromatography and mass spectrometry detection.

Results: The compound was detected by HPLC and its identity was confirmed by mass spectrometry (Figure 1). We have established a robust HPLC/Mass spectrometry detection method to obtain the pharmacokinetic profile for the p53 pathway restoring compounds to be tested. The identity of the compounds was confirmed by mass spectrometric detection.

Aim 3: Work on Aim 1 was completed and described in a previous report.

The RO1 grant related to this work was resubmitted to the NIH in June 2013 and scored in the top 2 percentile in December 2013. Work related to this grant was published in the journal *Cancer Research*: Hong B, Prabhu VV, Zhang S, van den Heuvel AP, Dicker DT, Kopelovich L, El-Deiry WS. Prodigiosin Rescues Deficient p53 Signaling and Antitumor Effects via Upregulating p73 and Disrupting Its Interaction with Mutant p53. *Cancer Res.* 2014 Feb 15;74(4):1153-65.

Fig 1: Determination of CB001 by UPLC/MS/MS



Research Project 54: Project Title and Purpose

Therapeutic Targeting of Malarial Placental Cytoadherence - Infection by malaria parasites, particularly *Plasmodium falciparum* infection, during pregnancy leads to several pathological conditions, including low birth weight, spontaneous abortion, stillbirth, and a significant number of infant deaths and maternal morbidity and mortality. These conditions are collectively called placental malaria (PM) or pregnancy-associated malaria (PAM) and are caused by the sequestration of *P. falciparum* in the placenta via binding of parasite-infected red blood cells (IRBCs) to the placental chondroitin sulfate proteoglycan (CSPG) receptor. This results in the massive accumulation of IRBCs in the placenta, leading to infiltration of mononuclear cells, inflammation and placental dysfunction. Understanding the molecular details of parasite placental sequestration is expected to provide strategies for the development of a vaccine and/or small molecule inhibitor-based therapeutics for PM.

Duration of Project

3/12/2013 - 12/31/2013

Project Overview

The malaria parasite protein, VAR2CSA, is a member of the *Plasmodium falciparum* erythrocyte binding protein-1 family that is expressed on the surface of parasite-infected red blood cells (IRBCs). It mediates the placental sequestration through binding to placenta-specific chondroitin 4-sulfate (C4S) chains of low sulfated chondroitin sulfate proteoglycan (CSPG) receptor. Thus, the key host-IRBC interaction in PM is between the parasite protein VAR2CSA and the host receptor C4S. The high affinity binding of VAR2CSA to C4S involves a dodecasaccharide (12-mer) motif containing 2 sulfate groups. VAR2CSA is a single span transmembrane protein with a large functional ectodomain, consisting of six adhesive domains called Duffy-binding like (DBL) domains. How VAR2CSA interacts with placental C4S is not known.

Specific Aims: (1) To express the full-length ectodomain and the N-terminal half of VAR2CSA in a mammalian expression system. (2) To localize the C4S-binding site in VAR2CSA and identify the VAR2CSA peptide motifs that interact with C4S. Our overall approach here is to obtain codon-optimized parasite *var2csa* DNA, clone and transiently express the entire ectodomain and the N-terminal portion encompassing DBL1x-DBL-3x into HEK293 cells as secretory molecules, and purify and characterize the recombinant proteins. Using the purified recombinant VAR2CSA proteins, we will localize the region in VAR2CSA involved in specific binding of C4S by electron cryomicroscopy using N-terminal and C-terminal tag-specific antibodies and gold particles conjugated C4S probes. We will also identify the VAR2CSA peptide motifs that interact with C4S using photoreactive C4S probes and mass spectrometry. These studies will demonstrate the feasibility of obtaining meaningful structures of the large VAR2CSA-C4S complex that can be used to guide inhibitor-optimization.

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Expected Research Outcomes and Benefits

According to the 2010 World Health Organization report, 50-60 million pregnant women yearly are at risk of contracting placental malaria (PM), resulting in ~200,000 infants and many maternal deaths. Regardless of gender, people in the endemic areas develop protective immunity to malaria during childhood years and thus are resistant to developing severe malaria. Despite this, during pregnancy, women are susceptible to malaria pathogenesis, resulting from sequestration of a variant *P. falciparum* strain that specifically expresses a protein called

VAR2CSA, in the placenta. During the first or second pregnancies, women are either immunogenically naive or have only a low level of immunity to this pathogen and thus, they and their fetus are a high risk for PM. Multigravid women, who were infected during their previous pregnancies and have acquired placental sequestration blocking antibody responses, are resistant to developing PM. Since placental sequestration of parasites involves VAR2CSA-C4S interaction, VAR2CSA based vaccines or therapeutics that inhibit VAR2CSA-C4S interactions are useful for preventing/treating PM. To this end, understanding the structure of VAR2CSA and how it interacts with C4S is important. The results of this project will provide preliminary data for re-submission of an NIH grant application. The anticipated outcomes of this research project also will provide insight into the C4S-binding site of C4S. In the long term, this knowledge will help in developing novel vaccine and/or therapeutics for PM. Malaria parasite also sequesters into other organs by expressing different *Plasmodium falciparum* erythrocyte membrane protein-1 family of variant adhesive proteins, contributing to multi-organ pathologies often seen in falciparum severe malaria. Thus, the knowledge gained from this project will also be useful in designing vaccine and/or therapeutics for other organ-related severe and fatal malaria.

Summary of Research Completed

In our previous annual report, we presented results showing the expression of the N-terminal half (DBL1x-3x; ~178kDa) of the extracellular portion of VAR2CSA as a secreted protein by HEK293F cells. During the current period, we have expressed the C-terminal half (DBL4ε-6ε; ~138 kDa) of VAR2CSA (Figure 1). Further, we scaled up the expression of both the N-terminal (DBL1x-3x) and C-terminal (DBL4ε-6ε) portions of VAR2CSA in HEK293F cells, and standardized purification procedures, which involve an initial Ni-affinity chromatography followed by cation-exchange chromatography and size-exclusion chromatography. We found that DBL1x-3x protein expressed at moderate levels (1-2 mg/L), whereas DBL4ε-6ε protein expressed 7-8 mg/L. Using these procedures, we have been able to purify (see Figure 2 below) ~16 mg of DBL4ε-6ε. In our planned efforts to determine the crystal structure of this protein, we performed crystallization trials at various conditions using 6 mg protein/ml solution. We found that the protein crystallizes in 100 mM TAPS buffer, pH 9.0, containing 100 mM potassium thiocyanate and PEG 400 (40% v/v) (Figure 3). Based on these results, we are planning to optimize crystallization conditions and scale up the screen to obtain crystals suitable for structural studies. Additionally, we are planning to assess the C4S-binding ability of DBL4ε-DBL6ε. We have also purified DBL1x-3x (Figure 4) expressed in HEK293F, but noticed that this protein has a tendency to aggregate. About half of the expressed protein appears to be improperly folded. We are currently trying to resolve this problem and trying to analyze its C4S-binding property using the protein that is purified.

The cloning of full length VAR2CSA (DBL1x-6ε; ~300 kDa) and the N-terminal portion comprising DBL1x-4ε (~215 kDa) to an expression plasmid for protein expression in HEK293F cells was found to be more challenging than we anticipated. GenScript, the company that we originally employed for this work, found that the cloning of full length VAR2CSA gene into the expression vector in a single step is not possible because of complications due to DNA recombination. However, currently another company, NorClone, is cloning both DBL1x-6ε and DBL4ε-6ε genes into the expression plasmid using a two stage cloning strategy. We are

anticipating that this strategy will work and we will be able to obtain plasmids in a month's time.

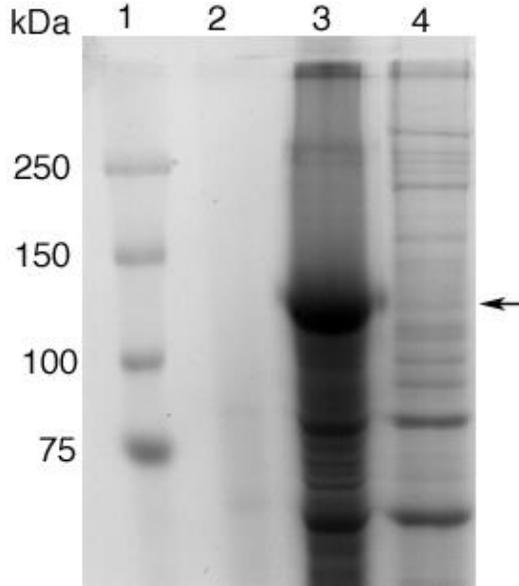


Figure 1. SDS-PAGE analysis of HEK293F cells expressing DBL4 ϵ -6 ϵ . Lane 1, Molecular mass (kDa) marker proteins; lane 2, Culture supernatant of untransfected cells; lane 3, Culture supernatant of cells transfected with DBL4 ϵ -6 ϵ expression plasmids; lane 4, lysate of cells transfected with DBL4 ϵ -6 ϵ expression plasmids. Arrow shows the DBL4 ϵ -6 ϵ protein band.

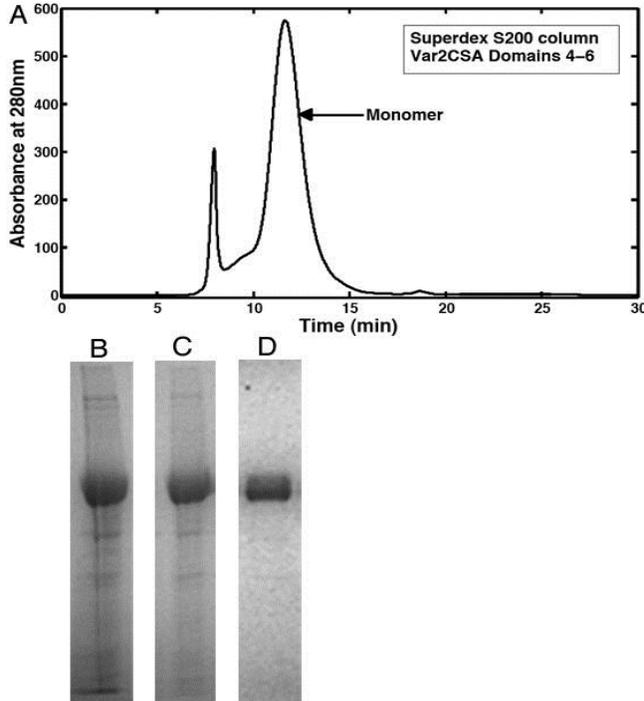


Figure 2. Purification of VAR2CSA DBL4 ϵ -6 ϵ protein. (A), Elution pattern of DBL4 ϵ -6 ϵ protein on size-exclusion column; (B), DBL4 ϵ -6 ϵ protein bound to Ni-affinity column; (C), DBL4 ϵ -6 ϵ protein bound to cation-exchange column; (D), DBL4 ϵ -6 ϵ protein purified by size-exclusion chromatography (panel A).



Figure 3. DBL4 ϵ -6 ϵ crystallization screen. Image shown is 24 h after initial set up. Needle-shaped crystals are observable, growing from a nucleation center in the bottom middle of the figure and extending diagonally. Fifteen hundred and thirty six individual conditions were screened, encompassing pH 2-11 units and low and high ionic strengths. Crystals were clearly observable in 100 mM potassium thiocyanate, 40 % PEG 400 and 100 mM TAPS at pH 9.0 after the first 24 h and remained at the fourth time point of 2 weeks. These crystallization trials were performed under oil using very small sample sizes. We are beginning experiments to screen around these conditions and scale up the screen to volumes where we can extract the crystals and collect data on them to assess their diffraction limit.

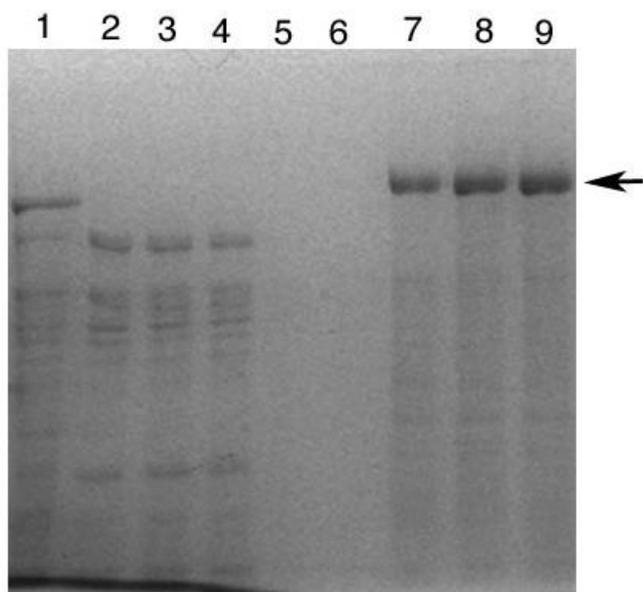


Figure 4. Purification of VAR2CSA DBL1x-3x protein. The DBL1x-3x protein bound to Ni-affinity column was chromatographed on cation-exchange column and the bound protein was eluted with 100-1.0 M NaCl gradient. The Fractions were analyzed by SDS-PAGE. Lane 1, Culture supernatant of HEK293F cells expressing DBL1x-3x; lane 3-4, column flow through fractions; lanes 5 and 6, column wash; lanes 7-9, fractions containing bound DBL1x-3x eluted with NaCl gradient. Arrow shows the DBL1x-3x protein band.

Research Project 55: Project Title and Purpose

Genetic Determinants in Familial Diverticulitis - The purpose of this project is to identify specific, measurable genetic determinants that can: 1) eventually be used as a clinical decision making tool for the identification of diverticulitis patients who will require surgery for their disease; and 2) be used as a basis for studying the pathophysiology of diverticulitis.

Duration of Project

6/1/2013 - 12/31/2013

Project Overview

Diverticulitis is a common inflammatory condition of the colon usually affecting older individuals with about 1 - 2% of the American population requiring surgery that can be life-threatening and disabling. However, it is also seen in youthful individuals with affected family members suggesting a genetic basis for the disease. Our hypothesis is that there are *host genetic factors that predispose the individual to the development of diverticulitis, the identification of which can help in disease management and decision making as well as directing future research into the pathophysiology of the ailment.*

The research aim of this project is to identify single nucleotide polymorphisms (SNP's) associated with diverticulitis requiring surgery. We plan to do this by studying a group of individuals very likely to have such genetic SNP's, specifically youthful individuals with severe disease (requiring surgery) and their family members some of whom are also affected with diverticulitis. From our well established, IRB approved biobank, we presently have approximately 20 such individuals from 4 different families.

Our specific aims for this project are:

- A) To perform total exome sequencing of approximately 22 individuals from 4 family cohorts containing at least one individual below the age of 50 with diverticulitis and at least one other first degree relative with diverticulitis.
- B) To perform statistical analysis on the genomic data so developed from (A) to identify single nucleotide polymorphisms (SNP's) or insertion/deletions that are associated with diverticulitis.

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Expected Research Outcomes and Benefits

This project expects to identify genetic determinants that are associated with a severe form of diverticulitis, namely diverticulitis requiring surgery. Although approximately 50% of Americans have diverticulosis, or outpouchings of the colon that form the anatomic basis for the disease, only about 20% of diverticulosis patients will suffer from infection, or diverticulitis. Most will be treated with antibiotics, but 15% will require surgery which can be highly morbid and will often need a colostomy bag especially when done under emergent circumstances. Using government data, in 2007 there were approximately 276,000 admissions for diverticulitis reflecting its significant impact on the healthcare system. Presently, there is no method to prospectively identify high risk patients who should have elective colon surgery that would minimize mortality, complications and the creation of a colostomy. Thus, this project seeks to take the first step towards identifying a patient specific genetic determinant that may be associated with the need for surgery in diverticulitis. Such a determinant may then be used as an aid in surgical and medical decision making, identifying patients who should have surgery under elective, safer circumstances so minimizing the complications associated with urgent surgery.

Summary of Research Completed

Specific Aim A:

We completed whole exome sequencing and genetic analysis looking for SNP's and genes of interest on 3 of the 4 families (190, 231, 242) from the original proposal. Family 159 was not investigated because there was no recruited member that did NOT have diverticular disease and thus represented a much less informative study group. We have pursued further recruitment of individuals from this family 159 to make the family more informative from a genetic standpoint, but divorce, death, distance and poor relations between family members have prohibited further recruitment during the period of this grant. However we continue to pursue recruitment, hoping to contact a family member (divorced spouse, living at a distance) that would render this family more informative. This family however may still be utilized in our subsequent studies to possibly confirm or refute SNP's/genes found in other investigations.

The process/methodology of whole exome sequencing of study patients used the Hi-Seq sequencer present in the Institute of Personalized Medicine. Steps of specimen preparation and assay included:

- a) **Sample DNA Fragmentation:** Genomic DNA was prepared/isolated from whole blood using a QiaSymphony DNA robot and quality/concentration assessed using pico green quantification. Genomic DNA was fragmented using a Covaris E220 focused-ultrasonicator.
- b) **Illumina Library Preparation:** The Apollo 324 Library Prep System was used to prepare libraries for sequencing.

- c) Exome Enrichment Cluster Generation: SeqCap EZ Human Exome Library v3.0 enrichment was used.
- d) Sequencing and Basecalling: Sequencing was performed on an Illumina HiSeq 2500 machine.

Specific Aim B:

Raw data generated from HiSeq analysis was analyzed as follows. We first performed quality control on the FASTQ files (raw reads) by using FastQC software to make sure the sequencing data was of high quality and there was no systematic bias (such as GC content). Next we aligned the FASTQ files to human genome (hg19) with BWA. The alignment results were then converted to SAM format (Sequence Alignment/Map), which contains many features that are critical for downstream analysis. The SAM files were then converted to BAM (binary Alignment/Map) format, which is essentially a compressed file and indexed for faster data access. We then used the Picard tool to remove the PCR duplicates due to amplification biases in PCR. Small Indels at the end of sequence reads are known to lead to false positive SNPs and therefore need to be identified. To solve this problem, we performed local realignment around indels using local realignment tools from the Genome Analysis Tool Kit (GATK). The base quality score for sequencing reads could contain bias and thus lead to false positive SNP calls. We recalibrated the base quality score with the following functions (CountCovariates and TableRecalibration) from GATK. Finally, we made the SNP calling with GATK UnifiedGenotyper program and saved the output in a VCF file.

The single nucleotide variants (SNVs) obtained from the above described ‘pipeline’ consisted of the SNVs that matched the pattern of inheritance for each family. The data was further truncated by removing all SNVs that were present in the population with a frequency greater than 1/1000 for all families. For families 190 and 231, the remaining SNVs were then condensed down to a list of genes that the variants were present in. Family 242, due to its multiple diseased and non-diseased members across multiple generations was particularly informative. It was subjected to two methods of analysis. The first was performed using SnpEff, a software which categorizes the variants and predicts the effect of those variants on genes (<http://snpeff.sourceforge.net/SnpEff.html>). Using this technique, a list of highly damaging variations matching the inheritance pattern of diverticulitis within the family was produced (Table 1 below). Following this, a second analysis was performed in a similar fashion for families 190 and 231, yielding the tables displayed below for the three families.

Further Studies:

Using the above approach, LamB4 stood out as a likely candidate gene as the causal variation for the inherited diverticulitis observed in family 242. In order to verify the validity of the SNV observed in LamB4 in family 242, the exome sequencing results from the Illumina HiSeq were confirmed via manually inspection using the Borad Institute’s Integrated Genome Viewer. Following this *In silico* verification, Sanger sequencing was performed on family 242 to confirm the same SNV in LamB4 (Figures below). The results affirmed that the inheritance pattern matched that of an autosomal dominant pedigree, with each affected family member being heterozygous for the variant.

We then attempted to identify variations in the tissue expression of LamB4 by performing

immunohistochemistry (IHC). Unfortunately we had tissue only from the index affected patient from family 242 and no related unaffected individuals. Thus, tissues evaluated for LamB4 expression were from 7 unrelated control patients, 7 unrelated patients with sporadic diverticulitis (both taken from the Colorectal Biobank) and the one sample from the index affected member of family 242.

The control patients showed heavy staining of LamB4 antibody in the epithelial cells of the colon and regions of the myenteric plexus. The sporadic diverticulitis samples showed similar staining as the controls in the regions of the myenteric plexus. However, the epithelial cells showed decreased staining relative to the controls. The patient from family 242 showed decreased staining in epithelial cells and near absent staining in the myenteric plexus (see figures below). To verify the location of staining within the myenteric plexus, S100 neuronal staining was performed, confirming the LamininB4 co-localization in the myenteric plexus.

Table 1.

Family 190

Chromosome	Location	Gene	Gene Name	Gene Description	Possible Relation to Diverticulitis
1	1	12175729	TNFRSF8	Tumor Necrosis Factor Receptor Superfamily, Member 8	Regulates immune response
2	5	112439941	MCC	Mutated In Colorectal Cancers	Regulates cell growth
3	6	30993377	PBMUCL1	Mucin 22	Epithelial cytoprotection
4	8	110376861	PKHDLL1	Polycystic Kidney And Hepatic Disease 1 (Autosomal Recessive)-Like 1	Polycystic Kidney disease is a known risk factor for Diverticulosis/It is
5	11	125659371	PATE3	Prostate And Testis Expressed 3	Causes neurontis
6	11	1857173	SYT8	Synaptotagmin VIII	Causes neurontis
7	12	10149851	CLEC1B	C-Type Lectin Domain Family 1, Member B	Regulates immune response
8	18	21511089	LAMA3	Laminin, Alpha 3	Cytoskeletal rearrangement
9	18	47563299	MYO5B	Myosin VB	Cytoskeletal rearrangement
10	21	46314907	ITGB2	Integrin, Beta 2 (Complement Component 3 Receptor 3 And 4	Cytoskeletal rearrangement

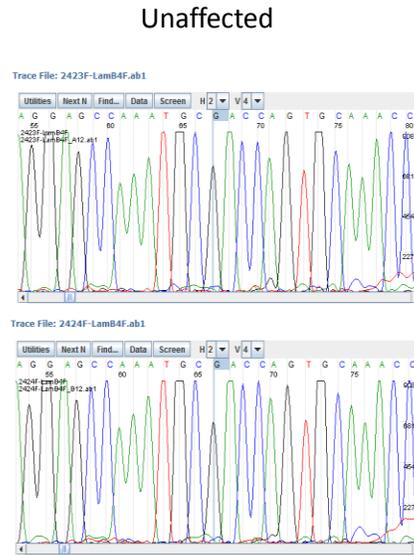
Family 231

Chromosome	Location	Gene	Gene Name	Gene Description	Possible Relation to Diverticulitis
1	1	240370985	FMN2	Formin 2	Cytoskeletal rearrangement
2	5	150121691	DCTN4	Dynactin 4 (P62)	Associated with muscular dystrophy
3	6	136582417	BCLAF1	BCL2-Associated Transcription Factor 1	Associated with muscular dystrophy
4	8	144940230	EPPK1	Epiplakin 1	Cytoskeletal rearrangement
5	8	23056956	TNFRSF10A	Tumor Necrosis Factor Receptor Superfamily, Member 10a	Regulates immune response
6	10	135440216	FRG2B	FSHD Region Gene 2 Family, Member B	Associated with muscular dystrophy
7	11	77937657	GAB2	Growth Factor Receptor Bound Protein 2-Associated Protein 2	Regulates immune response
8	11	1017783	MUC6	Mucin 6, Oligomeric Mucins/Gel-Forming	Epithelial cytoprotection
9	12	123200334	HCAR3	Hydroxycarboxylic Acid Receptor 3	Heredity motor neuropathy
10	17	48276811	COL1A1	Collagen, Type I, Alpha 1	Associated with Ehlers-Danlos syndrome

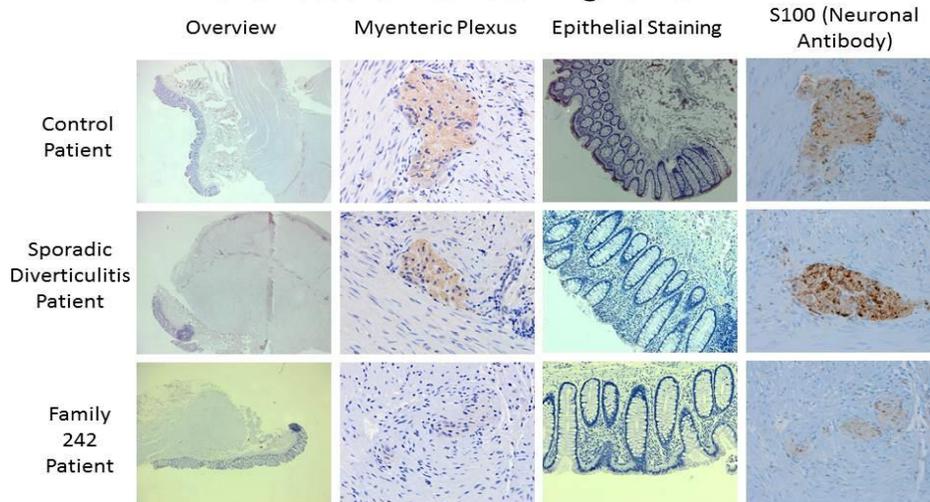
Family 242

Chromosome	Location	Gene	Gene Name	Gene Description	Possible Relation to Diverticulitis
1	5	115394456	ARL14EPL	ADP-Ribosylation Factor-Like 14 Effector Protein-Like	Cytoskeletal rearrangement
2	7	100028159	PPP1R35	Protein Phosphatase 1, Regulatory Subunit 35	Cytoskeletal rearrangement
3	7	107738905	LAMB4	Laminin Beta 4	Associated with myopathies
4	12	54686662	NFE2	Nuclear Factor (erythroid-derived)	Regulated erythroid maturation
5	14	23240713	OXA1L	Oxidase (cytochrome C) Assembly 1-like	Regulates expression of complex 1 in mitochondria
6	14	55169050	SAMD4A	Sterile Alpha Motif Domain Containing 4A	Posttranscriptional regulator
7	17	17250157	NTSM	5',3'-Nucleotidase, Mitochondrial	Regulates pool of mitochondrial dTTP
8	17	76694990	CYTH1	Cytohesin 1	adhesiveness of integrins in lymphocytes
9	19	55263886	KIR2DL3	Killer Cell Immunoglobulin like Receptor, 2 Domains, Cytoplasmic Tail 3	Transmembrane glycoproteins
10					Associated with neurogastrointestinal encephalopathy

Verification of G1303A variation observed in family 242 by Sanger sequencing of affected(left) and unaffected(right) patients



Immunohistochemical Staining for Lamb4



Research Project 56: Project Title and Purpose

Posttranscriptional Mechanisms Regulating Epstein-Barr Virus Latent Infection - The purpose of this project is to provide insight into the basic mechanisms of action of two families of Epstein-Barr virus (EBV) long non-coding RNAs (lncRNAs) that are expressed during EBV infections associated with several human cancers. Cellular lncRNAs are known to function in many ways and likely affect virtually all cell processes, e.g., differentiation and development, gene expression and immune responses. Viruses have no doubt conscripted lncRNAs for their own purposes, however very few viral lncRNAs have thus far been identified, and their functions in virus infections are unclear. Elucidation of the mechanisms of action of the two EBV lncRNAs we have identified should provide greater insight into the contribution of EBV to several human cancers, information that could ultimately be harnessed to inhibit EBV-dependent tumor development and growth.

Duration of Project

5/1/2013 – 12/31/2013

Project Overview

Epstein-Barr virus (EBV) is an extremely successful pathogen, being able to establish a lifelong latent infection within B lymphocytes of its human host, with overt disease in healthy individuals restricted to a self-limiting mononucleosis in ~40% of individuals when infection is delayed until the second decade of life. However, a breakdown in cellular immunity, particularly as a consequence of immunosuppressive therapy or AIDS, remains a significant risk factor for development of EBV-associated lymphoma and lymphoproliferative disease, underscoring the highly evolved equilibrium that exists between EBV and its host. Establishment of this equilibrium is dependent upon the concerted actions of nine EBV proteins and a subset of the EBV microRNAs (miRNAs) expressed during latency. In addition to EBV proteins and miRNAs, our findings have revealed that during latency EBV also expresses transcripts that likely function as long non-coding RNAs (lncRNAs): the abundant polyadenylated RNA encoded by the viral *BHLF1* gene, and a family of novel RNAs that overlap the transcriptional promoter Cp that is responsible for driving expression of the six latency-associated EBV EBNA proteins, a family of transcriptional regulators and a virus-genome-maintenance protein critical for EBV persistence and pathogenic potential. Our long-term objective is to elucidate the contributions of these lncRNAs to EBV latency, and thus the oncogenic potential of the virus. We hypothesize that the Cp-associated transcripts (CpATs) contribute to latency through regulation of EBNA-gene transcription. Our preliminary results suggest that at least one function of the *BHLF1* lncRNAs is to maintain proper splicing of the EBNA mRNAs that originate from Cp, a function we hypothesize occurs as a consequence of these lncRNAs being bound by splicing regulatory proteins that would otherwise suppress appropriate splice-site usage within the EBNA primary transcripts.

Specific aims:

Specific Aim 1: Identify the means for *BHLF1* mechanism of action.

Under Aim 1, we propose to identify the cellular proteins that bind the *BHLF1* RNA so that the

functional consequence of these RNA:protein interactions can be elucidated.

Specific Aim 2: Define the structure of the CpATs.

Under Aim 2, we propose to complete our structural analysis of the CpATs, so that we will then be able to design experimental approaches to interrogate the specific contribution(s) of these RNAs to EBV latency.

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Expected Research Outcomes and Benefits

Expected Outcomes

This is a completely new area of research in the EBV field, and overall from this research project we expect to begin gaining insight into the roles that EBV lncRNAs contribute to the biology and pathogenic potential of the virus. Specifically, we expect to learn: 1) the identity of the proteins that are bound to the EBV BHLF1 transcripts (Aim 1); and 2) the complete structures of the apparent ncRNAs associated with the EBV EBNA promoter Cp, whose function is critical to successful colonization of EBV's human host.

Expected Benefits

Based on what we now know about the mechanisms of action of cellular lncRNAs similar in structure to BHLF1 transcripts and which act largely through RNA:protein interactions, we believe the benefit of identifying the proteins that bind to BHLF1 RNA is that this information will provide important clues to the role these viral lncRNAs play in EBV infection. Specifically, it will provide a “jumping off point” for subsequent research to determine the functional consequence of these RNA:protein interactions. With respect to the Cp-associated transcripts (CpATs) to be addressed in Aim 2, our findings thus far strongly suggest that these fall into the class of lncRNAs known as promoter-associated transcripts, and are thus likely to play a transcriptional regulatory role. While the mechanisms of action of this newly described class of ncRNA are unclear, the benefit of knowing the structures of the CpATs is that it will enable us to begin probing their function in the context of EBV latent infection, namely by introduction of mutations/short deletions that inactivate their expression from the viral genome. The overall benefit of this work to improvement of health status is that it may reveal new biochemical pathways that the virus is dependent upon for persistent infection and its oncogenic potential,

and therefore knowledge necessary for development of new anti-viral/tumor therapies.

Summary of Research Completed

Specific Aim 1. Our hypothesis is that during *latent* EBV infection, *BHLF1* functions exclusively as a lncRNA, possibly restricted to the nucleus, and that expression of any polypeptide encoded by a translational open reading frame (ORF) within its mRNA (not all EBV isolates have conserved the *BHLF1* ORF) is limited to the virus replication/lytic cycle due to dependence on the EBV SM protein for expression of the *BHLF1* protein (our unpublished observation). Further, our preliminary data indicated that the *BHLF1* gene locus is required to sustain the Latency III pattern of EBV gene expression, possibly through (at least in part) regulation of splice-site selection within the common 5' termini of the mRNAs encoding the EBV EBNA proteins (deletion of the *BHLF1* gene resulted in a specific intron retention event that correlated with a loss of EBNA protein expression, but not of the mRNAs themselves).

While lncRNAs function in many cellular processes via a growing list of mechanisms, most act in part through an interaction with protein(s). Thus, our objective in Aim 1 was to begin to elucidate which cellular and possibly viral proteins bind to *BHLF1* RNA. Based on our preliminary observations, one class of RNA-binding proteins targeted by a *BHLF1* lncRNA may be the splice regulatory proteins, particularly the hnRNPs that negatively regulate splice-site selection. For example, sequestration of these by the *BHLF1* RNA might prevent intron retention within the 5' termini of the EBNA mRNAs (the alternative splicing that does occur here under normal conditions with wild-type (wt) virus suggests that splicing is highly regulated at this locus).

Our preferred approach was to introduce a structured RNA element, the so-called S1 aptamer, within the *BHLF1* RNA that could be bound with high specificity by streptavidin. Once expressed in cells, the RNA and its associated proteins could be isolated (with streptavidin-coated beads), and the proteins identified by mass spectrometry. We planned to introduce the aptamer within a recombinant EBV (rEBV) genome cloned in a bacterial artificial chromosome (BAC) by recombineering in *E. coli*. Upon infection of an appropriate EBV-negative B-cell line or primary B cells with this rEBV (produced in HEK293 cells), this would allow us to isolate RNA:protein complexes that form *in vivo* in the context of an actual EBV infection. Prior to this reporting period we identified, by prediction of RNA secondary structure *in silico*, a relatively non-structured domain of the *BHLF1* RNA for insertion of the aptamer element. Our second objective - to introduce the aptamer element into the EBV genome by recombineering; during this reporting period - was unfortunately met with technical difficulties, namely that our targeting construct was inserting instead into the highly homologous gene *LF3* (an apparent paralog of *BHLF1*) in another region of the EBV genome. (*LF3* also encodes a lncRNA and, along with *BHLF1* is the focus of research supported by a new NIH R01 grant.) We are currently taking a more laborious approach to build a longer targeting construct that will incorporate, on one end of the construct, a unique sequence domain not present in *LF3*.

Finally, while addressing the technical issues described above, we elected to further probe the potential significance of *BHLF1*'s contribution to EBV latency and ability to establish a persistent infection within B cells. This was prompted by our findings obtained upon infection of

BL2 cells with our *BHLF1*-negative mutant rEBVs. First, BL2 is a long-term BL-tumor-cell line that is naturally EBV-negative, and thus does not require EBV for growth. Further, while we were excited by our finding that *BHLF1* was required to sustain the Latency III program of EBV gene expression in BL2 cells, in the absence of *BHLF1* the conversion to Latency I required nearly 2 months post infection (this seemed a bit long, though if the mechanism were known it may not be unexpected). We had repeated this experiment with the same result nearly a half-dozen times with two independently derived and distinct *BHLF1*-mutant rEBVs. Nonetheless, we felt that if this was indicative of a biologically relevant function of *BHLF1*, then we must demonstrate it in the context of an EBV infection of primary B cells.

To do this, we isolated human primary B cells (by CD19 selection) from whole blood obtained from the Penn State Hershey Medical Center Blood Bank (donors anonymous to us), and performed *in vitro* immortalization assays with wt and two different *BHLF1*-mutant rEBVs (Δ B-S & Δ BHLF1), all generated from their respective BAC clones in HEK293 cells. Note that Δ B-S rEBV lacks the entire *BHLF1* RNA-coding region and ~1 kbp of upstream (promoter) DNA, whereas Δ BHLF1 lacks only the “ORF” (within the EBV genome that we work with [Akata isolate], the *BHLF1* ORF has been disrupted by a single-nucleotide deletion that results in a premature stop codon). Briefly, 5×10^4 B cells in each well of a 96-well plate were infected with 2-fold serially diluted virus (8 replicates/dilution/plate x 2 or 3 plates); both wt and mutant rEBVs were carefully titered beforehand to ensure equivalent multiplicity of infection (MOI) per respective virus inoculum. Cells were monitored for several days and then at least weekly for expression of green fluorescent protein (GFP; encoded by the rEBV genomes and an indication of infection) and characteristics indicative of immortalization, i.e., utilization of media and clumping (due to EBV-induced expression of cellular adhesion molecules). At 6 weeks post-infection immortalization was scored, and cells from up to 12 positive wells were expanded and assessed for EBV latency-gene expression by immunoblotting.

Our first experiments compared both Δ B-S and Δ BHLF1 to wt rEBV using B cells from the same donor. A subsequent experiment compared Δ BHLF1 to wt rEBV, but used B cells from a second donor. We were very excited to find that we observed no immortalization by the Δ B-S rEBV. By contrast, the same cells infected with wt rEBV were efficiently transformed, with 50% immortalization (TD_{50}) occurring at a MOI of $\sim 2.55 \times 10^{-2}$ (i.e., $\sim 1,275$ infectious virus per well). At the highest MOI possible with our preparations of Δ BHLF1 ($6.75 \times 10^{-2}/3375$ infectious virus per well), we did observe immortalization in 3/16 wells (18.75%) with this mutant virus that lacks just the “ORF” segment of the gene, but this was too low to calculate an accurate TD_{50} . Although we were able to eventually expand these into continuously growing LCLs, this was not as efficient as for the wt rEBV-infected B cells. Note that Δ B-S and Δ BHLF1 rEBVs were able to efficiently infect the primary B cells, as indicated by GFP expression shortly after infection.

Using B cells obtained from a second donor, we also observed immortalization with Δ BHLF1 rEBV (the Δ B-S mutant was not tested), but the efficiency was ~ 64 -fold higher than that which was observed with the first donor's B cells (same virus preparation). However, it is important to note that immortalization with wt rEBV (same virus preparation) was similarly more efficient with B cells from the second donor than those of the first donor. We have also been able to obtain LCLs from these Δ BHLF1 rEBV infections (and positive for Latency III protein

expression). Note that this is not an uncommon finding - that B cells from some individuals are more easily immortalized - and is why it is important to assess immortalization potential using B cells from at least two independent donors. Presumably this is due to genetic factors and/or stochastic or individual-specific differences in the expression of cellular genes that positively or negatively influence immortalization efficiency. Importantly, as we would expect from our observations with BL2 cells, *BHLF1* was required for efficient B-cell immortalization by EBV. The question of why the Δ B-S mutant had no measurable immortalization potential (at least in the context of the first donor's B cells), may be due to the fact that the deletion in this virus removes one of the BHRF1 miRNAs (miR-BHRF1-1), and/or because all of the coding region/promoters for the *BHLF1* RNAs that are expressed during latency have been deleted. Presently we are attempting to rescue the defects associated with *BHLF1* loss, by expression of its lncRNA following infection with *BHLF1*-mutant virus.

Specific Aim 2. During our studies to define the contribution that the chromatin boundary protein CTCF plays in directing epigenetic silencing of the common EBNA promoter Cp, we discovered (by RT-PCR) that transcripts we believed to originate from the Cp transcription start site (TSS), which had originally been mapped by S1 nuclease-protection assay, actually initiated upstream. Further analysis of these Cp-associated transcripts (CpATs) is described in a previous reporting period.

Research Project 57: Project Title and Purpose

Role of mTOR in Repair of UVB-Induced DNA Damage - The goal of this project is to investigate the role of the two mTOR complexes, mTORC1 and mTORC2, in the repair of DNA damage, specifically global-genome nucleotide excision repair (GG-NER), in keratinocytes exposed to UVB. UVB wavelengths account for the majority of the biologically damaging effects from sun exposure. UVB is a complete carcinogen, acting as a tumor initiator by inducing mutations in critical target genes, and a tumor promoter by inducing cell cycle progression and proliferation. Better understanding of the pathways activated by UVB in keratinocytes is essential for the effective prevention and treatment of skin cancer. If keratinocyte GG-NER is enhanced when mTOR activity is limited, this would provide a strong rationale for using mTOR inhibitors in skin cancer prevention.

Duration of Project

5/1/2013 – 12/31/2013

Project Overview

Ultraviolet (UV) radiation is the major risk factor for developing skin cancer, the most prevalent cancer worldwide. Several recent studies from our lab and others indicate that mammalian target of rapamycin (mTOR) signaling is activated by UVB and may play an important role in skin tumorigenesis. mTOR exists in two functionally and compositionally distinct protein complexes: the rapamycin-sensitive mTOR complex 1 (mTORC1) and the rapamycin-resistant mTOR complex 2 (mTORC2). The goal of the project is to investigate the role of the two mTOR complexes in the repair of DNA damage, specifically global-genome nucleotide excision repair

(GG-NER), in keratinocytes exposed to UVB. Direct absorption of UVB irradiation by DNA within the epidermis causes damage through the formation of covalent linkages that result in products such as cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts (6-4PPs), which are repaired by GG-NER. Several reports in the recent literature have suggested that the tumor suppressor PTEN and putative tumor suppressor AMPK promote GG-NER in the skin by regulating expression of the xeroderma pigmentosum group C (XPC) protein. XPC is essential for GG-NER, and the XPC gene is lost or inactivated in a large percentage of sporadic SCC cases, perhaps providing a selective advantage for initiation of UVB-induced SCCs. Both PTEN and AMPK are key negative regulators of mTOR signaling, with loss of either PTEN or AMPK leading to mTOR activation.

Specific Aim 1: To establish the contribution of mTOR to global genome nucleotide excision repair (GG-NER) capacity in human keratinocytes exposed to UVB.

Using HaCaT cells, an established human keratinocyte cell line, as a model, Aim 1 will address the hypothesis that activation of mTOR impairs efficient GG-NER of UVB-induced DNA damage. We will use the specific mTORC1 inhibitor rapamycin and the mTOR kinase inhibitor Torin-2 to determine whether mTORC1 or mTORC2 inhibition protects UVB-treated cells from accumulating mutations by maintaining efficient GG-NER. The rate of repair will be evaluated by measuring removal of CPD and 6-4PP lesions.

Specific Aim 2: To determine whether mTOR activation leads to downregulation of xeroderma pigmentosum group C protein (XPC).

A possible mechanism by which uncontrolled stimulation of mTOR signaling in the skin might impact the survival of UVB-treated cells will be addressed in Aim 2, which will use HaCaT cells with activation or disruption of mTOR signaling to determine whether expression of XPC is regulated by mTOR-dependent pathways.

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None

Expected Research Outcomes and Benefits

Our research studies the role of mTOR-dependent pathways in development of non-melanoma skin cancer. Since skin cancers are the most common form of malignancies world-wide,

identification of possible targets for prevention and therapy is highly relevant to public health. Investigating the potential benefit of inhibiting both mTOR protein complexes, mTORC1 and mTORC2, is also of great importance because specific mTORC1 inhibitors, while showing great promise in early studies, have had limited success as chemotherapy in a variety of human cancers. The work described in this project tests the hypothesis that mTOR-dependent pathways influence the survival of DNA-damaged keratinocytes after exposure to UVB by controlling the process of global-genome nucleotide excision repair (GG-NER). If we find that keratinocyte GG-NER is enhanced upon inhibition of mTORC1 and/or mTORC2 activity, this would not only present a strong rationale for using such inhibitors in skin cancer prevention, but also provide evidence of a new pathway contributing to regulation of GG-NER in the skin that has not been previously proposed.

Summary of Research Completed

Using treatment with the mTOR complex 1 (mTORC1) inhibitor rapamycin, and mice with conditional deletion of mTOR in the skin, we showed previously that UVB activates signaling downstream of both mTOR complexes, mTORC1 and mTORC2. Inhibition of mTORC1 with rapamycin effectively blocked the hyperproliferation response that occurs with UVB exposure, while keratinocytes were sensitized to UVB-induced apoptosis when both mTORC1 and mTORC2 activities were deleted, suggesting separate but complementary roles for the two mTOR complexes in prevention of photocarcinogenesis. The overall goal of the CURE studies was to determine whether mTOR inhibition also protects surviving UVB-treated cells from accumulating DNA damage.

Figures 1, 2, and 3 describe experiments designed to address Aim 1. Repair of DNA damage was assessed by measuring removal of CPD lesions after UVB exposure in HaCaT cells treated with either rapamycin to inhibit mTORC1 or Torin-2 to inhibit both mTOR complexes. This was followed by performing the same experiment in Rictor^{-/-} mouse embryo fibroblasts (MEFs), to directly assess the effect of blocking mTORC2 activity. Surprisingly, rather than suggesting increased repair of surviving cells after UVB, the results showed that DNA damage increased over time when mTORC2 was inhibited. Western blot analysis of checkpoint proteins showed that treatment of cells with Torin-2 delayed the phosphorylation of Chk-1, Chk-2 and p53 in response to UVB, while rapamycin had no effect. Phosphorylation of histone H2.AX, which localizes to sites of DNA damage, was also delayed in Torin 2-treated cells. Aim 2 experiments, which are shown in Figures 4, 5 and 6, showed that in the presence of PTEN knockdown, XPC levels were decreased. Treatment with rapamycin, Torin-2 or the PI 3-kinase inhibitor LY294002 returned XPC to levels seen in control cells, suggesting the change in XPC protein levels observed with PTEN knockdown may be mediated by mTORC1.

Methods:

Cell culture and drug treatment: HaCaT keratinocytes (obtained from The German Cancer Research Center) or Rictor^{+/+} and Rictor^{-/-} mouse embryo fibroblasts (a generous gift of Dr. Mark Magnuson, Vanderbilt University) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals), and penicillin/streptomycin (100µg/ml, Invitrogen), and cultured for less than 20 passages at 37°C with 5% CO₂. Drug treatments were added 1 hour before UVB exposure at a

concentration of 50 nM for Rapamycin (Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD) and Torin 2 (Tocris Bioscience, Bristol, UK) and 1 μ M for LY 294002 hydrochloride (LY; Tocris Bioscience, Bristol, UK). Vehicle dimethyl sulfoxide (DMSO) was added as a control.

UVB Treatment: Cells were cultured until 70% confluence on 12 well cell culture plates (Greiner Bio-One) under normal culture conditions. Before UVB treatment, cells were washed twice with PBS, and then exposed to UVB (FS20 UVB bulbs, National Biological) emitting light between 290nm and 320nm in minimal PBS at dose described in the figure legends. A UVB 500C meter (National Biological) was used to monitor irradiation intensity. Following UVB irradiation, PBS was removed and replaced with the saved media containing the drugs treatments. For DNA damage assays, cells were incubated overnight in low serum (2%) prior to UVB to minimize cell cycle progression. When inhibitors were used, cells were pre-incubated for 2 h before UVB at concentrations described in the figures.

DNA Damage Repair Assay: Cells were collected at different time points post-UVB and DNA was isolated using a QIAamp DNA Mini Kit (Qiagen). DNA concentration was calculated from the absorbance at 260 nm. The presence of CPDs in DNA was quantified by ELISA using a monoclonal antibody (TDM-2, COSMO BIO) according to the manufacturer's instructions. The absorbance of colored products derived from o-phenylene diamine (Sigma) was measured at 492 nm. For examining repair kinetics, the percentage (%) of repair was calculated by comparing the absorbance at the indicated time to that of the corresponding absorbance at time=0 when there was no opportunity of repair and 100% of the CPDs were present post-UVB.

siRNA Transfection: HaCaT cells were transfected with siRNA (On-TARGETplus SMARTpool, Dharmacon) targeting PTEN or with 1x siRNA Buffer as a negative control. The procedure was carried out according to the 12 well plate format in the Thermo Scientific DharmaFECT 1 siRNA transfection protocol for HEK293 cells.

Western blot: Cells were subjected to Western blot analysis as described previously. Briefly, cells were harvested and lysed using 1x SDS sample buffer. Samples were boiled and run on either a 7.5% or 10% polyacrylamide gel and transferred to a PVDF membrane. Imaging of proteins was performed by customary western blot procedure. Antibodies utilized in the assay comprised of anti-XPC (H-300) (Santa Cruz), anti-GAPDH (New England Biolabs), anti-alpha/beta-tubulin, anti-Phospho-Akt (Ser473) and total Akt, anti-Phospho-p70 S6 Kinase (Thr389) and total p70 S6 kinase, anti-Phospho-Chk1 (Ser345), anti-Phospho-Chk2 (Thr68), anti-Phospho-p53 (Ser15), anti-Phospho-Histone H2A.X (Ser139), and anti-rabbit IgG, HRP-linked Antibody (all from Cell Signaling).

Results:

Aim 1

Measurement of DNA damage in HaCaT cells exposed to UVB

Much of the mutagenic and carcinogenic effects of UV irradiation are a consequence of DNA damage in the form of cyclobutane pyrimidine dimers (CPDs). To assess the effect of mTOR inhibition on repair of CPDs after exposure to UVB, HaCaT cells (human keratinocytes) were

pre-incubated in vehicle or 50 nM Torin-2, a TOR kinase inhibitor that blocks pathways downstream of both mTORC1 and mTORC2, then exposed to either a low, non-apoptotic (5 mJ/cm²) or higher, apoptotic (25 mJ/cm²) dose of UVB (Figure 1). As expected, HaCaT cells exposed to low dose UVB completely repaired UVB-induced DNA damage within 24 h, as measured by decreased CPDs remaining (Figure 1), indicating efficient GG-NER. Significant repair was also seen at higher dose UVB (Figure 1). Contrary to our original prediction that mTOR inhibition would protect surviving cells from DNA damage, inhibition of mTOR with Torin-2 significantly reduced the repair of CPDs at 6h post-UVB in cells exposed to either a sub-apoptotic or apoptotic UVB dose. Interestingly, in cells exposed to the higher, apoptotic dose of UVB, treatment with Torin-2 actually accentuated DNA damage by almost 40% at 6 h post-UVB compared to cells at zero time (Figure 1).

Assessment of DNA damage signaling checkpoints in HaCaT cells treated with Torin-2

A very recent study has shown that, in addition to its inhibition of mTOR, Torin-2 exhibits inhibitory activity against the mTOR-related PIKK family kinases ATM and ATR, major regulators of the DNA damage response in cells exposed to UVB.

We therefore analyzed the effect of Torin-2 treatment on UVB-induced checkpoint function. DNA damage-mediated growth arrest acts through phosphorylation and activation of the checkpoint kinases Chk-1 and Chk-2, followed by p53 accumulation and activation. Exposure of cells to 20 mJ/cm² UVB induces phosphorylation of Chk-1 by ATR and Chk-2 by ATM, as well as p53 phosphorylation, in a time-dependent manner (Figure 2). Phosphorylation of all three proteins was markedly inhibited in cells exposed to Torin-2. In contrast, exposure to rapamycin had no effect (Figure 2). Similarly, phosphorylation of histone H2A.X, which upon phosphorylation localizes to the site of DNA damage, was also delayed in Torin-2 treated cells but not in cells treated with rapamycin. These data imply that the increased DNA damage response seen with Torin-2 treatment may be the result of ATM/ATR inhibition rather than changes in mTOR activity. However, they do not rule out a role for mTORC2 in the DNA damage response.

Measurement of DNA damage repair in mouse embryo fibroblasts with deletion of Rictor

To directly address the question of whether inhibition of mTORC2 affects the DNA damage response, we assayed CPD repair in MEFs with deletion of Rictor expression, and wild-type controls (Figure 3). The results show that absence of Rictor augments DNA damage in the form of CPDs after UVB exposure, and this effect occurs at both low and high dose UVB. Thus, inhibition of mTORC2 causes increased sensitivity to UVB-induced DNA damage, providing a mechanism for the increased apoptosis seen in our earlier studies. These results suggest that mTORC2 (or Rictor) plays an important role in regulating cell survival under DNA damaging conditions.

Aim 2

Knockdown of PTEN expression in HaCaT cells by siRNA.

To establish the necessary dosage of siPTEN needed for the successful knockdown of PTEN in HaCaT cells, a dose response was performed using 3 concentrations of siPTEN (25µM, 50µM, & 100µM) transfected into HaCaT cells. The cells were incubated with the siRNA for 72 h and levels of PTEN expression were measured using western blot analysis. It was determined that

100nM of siPTEN was sufficient to successfully knockdown PTEN expression in HaCaT cells (Figure 4), and this concentration was used for subsequent experiments.

Verification of the inhibitory effects Rapamycin, Torin 2, & LY on control cells and cells with PTEN knockdown.

HaCaT cells were treated with each inhibitor as described in the Methods and Western blot analysis was performed on each group to evaluate the levels of phosphorylated AKT-Ser473 (p-AKT (473)) and phosphorylated p70 S6 Kinase (p-S6K) as a measure of mTORC1 and mTORC2 activity, respectively, in the presence or absence of PTEN knockdown. Figure 5 shows that, without inhibitor treatment, siPTEN resulted in increased phosphorylation of both AKT and S6K. Torin 2 and LY both lowered P-AKT (S473) levels while rapamycin, Torin 2, and LY all blocked phosphorylation of S6K expression (Figure 5). Inhibition occurred in both the presence and absence of PTEN knockdown.

Inhibitor treatment restores baseline XPC expression in siPTEN HaCaT cells following UVB exposure.

To ascertain how the manipulation of the PTEN/AKT pathway affects levels of XPC following UVB exposure, the siPTEN HaCaT cells were subjected to 50 mJ/cm² UVB exposure after either DMSO, rapamycin, Torin 2, or LY were applied to the cells. Cells were harvested and Western blot analysis was performed to determine XPC protein levels (Figure 6). Levels of XPC in the siPTEN-DMSO group decreased at 2 h post UVB irradiation. The addition of rapamycin, Torin 2 or LY restored XPC expression to the levels seen in HaCaT cells without PTEN knockdown (Figure 6), suggesting that XPC expression in cells exposed to UVB may be under control of mTORC1 signaling pathways. These data extend previous findings characterizing regulation of DNA damage repair by PTEN.

Summary and conclusions.

Our data indicate that inhibition of mTORC2-dependent pathways accelerates accumulation of UVB-induced DNA damage, which ultimately may promote elimination of UV-damaged cells through apoptosis and subsequent inhibition of skin cancer development. Further, our PTEN knockdown studies suggest that the synthesis of DNA repair proteins may be under control of mTORC1 in UVB-treated cells. Future studies will use these preliminary results to further define the roles of both TOR complexes in the DNA damage response to UVB.

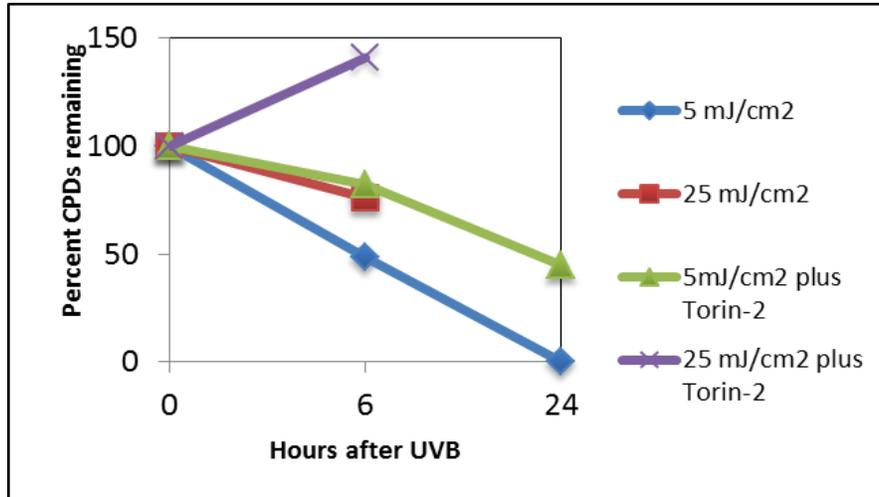


Figure 1. Treatment of HaCaT cells with Torin-2 reduces repair of DNA damage by GG-NER in response to UVB. Cells were exposed to either sub-apoptotic or apoptotic doses of UVB with or without preincubation with Torin-2 and repair of CPDs was measured over 24 h.

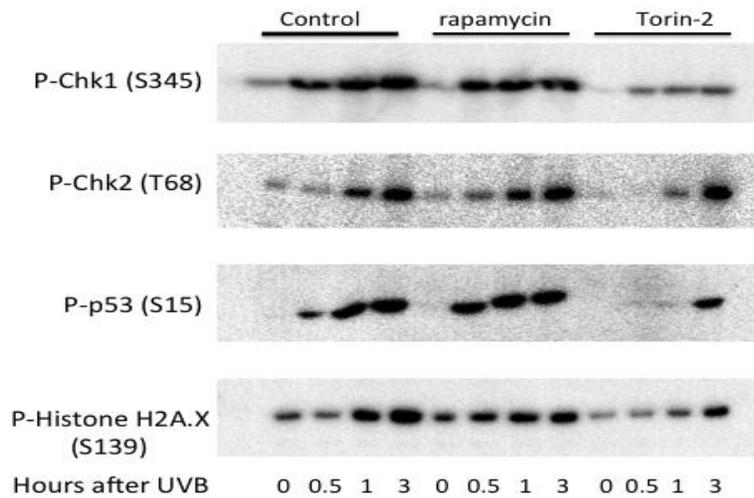


Figure 2. Western blot analysis of DNA damage response checkpoint proteins in UVB-treated cells with and without inhibitors of mTOR. Cells were exposed to 20 mJ/cm² UVB and harvested at the indicated time points. For inhibitor treatments, rapamycin (50 nM) and Torin-2 (50 nM) were pre-incubated for 2h before UVB.

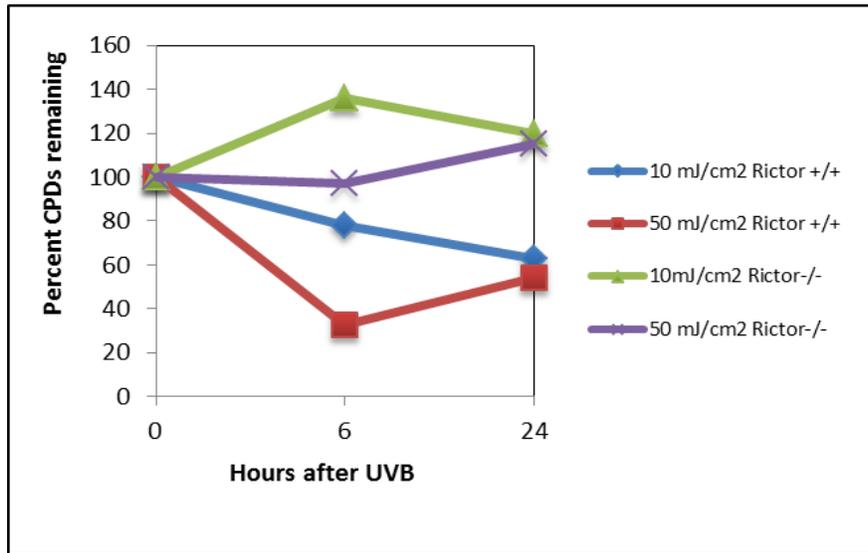


Figure 3. Deletion of Rictor reduces repair of DNA damage by GG-NER in response to UVB. Cells were exposed to either sub-apoptotic or apoptotic doses of UVB and repair of CPDs was measured over 24 h.

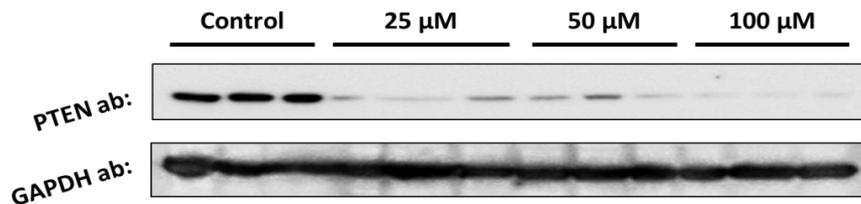


Figure 4. siRNA PTEN dose response in HaCaT cells. PTEN expression in HaCaT cells transfected with 25μM, 50μM, or 100μM siRNA targeting PTEN following 72 h of incubation. GAPDH used as a loading control.

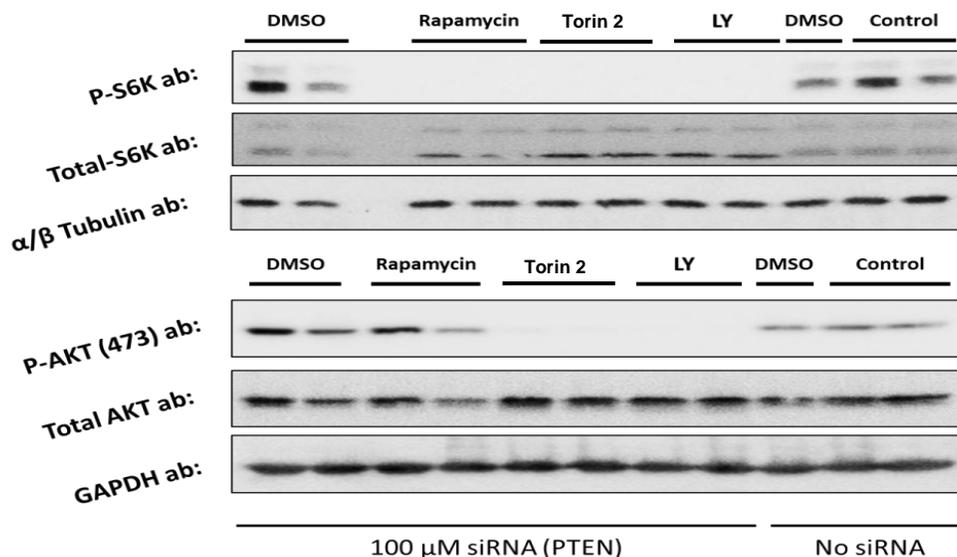


Figure 5. PTEN siRNA activates mTORC1 and mTORC2 signaling, which is repressed by inhibitor treatment. (Top) p-S6K/Total S6K expression and (Bottom) p-AKT (S473)/total AKT expression in vehicle- or drug-treated siPTEN HaCaT cells at 2 h post UVB irradiation (50 mJ/cm²). α/β Tubulin (Top) and GAPDH (Bottom) were used as controls.

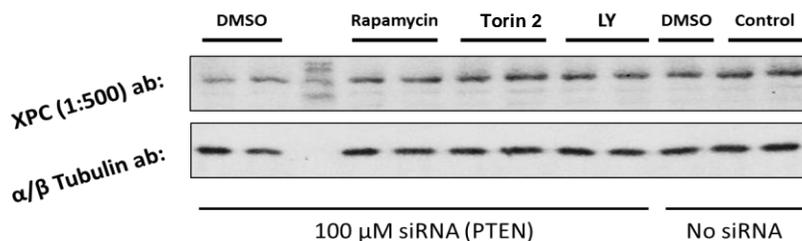


Figure 6. Rapamycin, Torin 2 and LY restore XPC expression in siPTEN HaCaT cells exposed to UVB. XPC expression in drug-treated siPTEN HaCaT cells 2 h post-UVB irradiation (50 mJ/cm²). α/β Tubulin used as a control.

Research Project 58: Project Title and Purpose

Research Infrastructure - Central Research Sample Storage Facility - The purpose is to create a centralized facility to store biological specimens from both the new Institute for Personalized Medicine as well as the larger institutional research community. Biological specimens are important in clinical research because they make it possible to correlate laboratory science with clinical response. While researchers may store aliquots of specimens in their own labs, storing aliquots of non-commercial and unique cell-lines in these freezers will allow for samples to be protected by the safeguards built-in to protect them from possible power-outages, leaks, etc. This is also true for tissue samples that the labs would store in this facility. The cell-lines and tissue samples can be used for comprehensive projects ranging from genetic therapies to drug affinity protocols.

Duration of Project

6/1/2013 – 12/31/2013

Project Overview

The scope of the project is to design and build an essential research sample storage facility that will allow researchers to store their non-commercially available and other unique biological specimens in freezers for long-term storage. Collection of biological specimens is time-consuming and expensive, but critical to effectively test the efficacy of compounds, map biological processes and pathways, and obtain genetic information. One of the most important and vulnerable aspects of this research is storage of the specimens, which may be irreplaceable. Failure to store the specimens at strictly regulated temperatures will result in their rapid degradation, sometimes within hours. The aims of this project are to design and renovate a research sample storage facility that will house multiple freezers and will allow researchers to store duplicate aliquots of their samples in a secondary location for long-term storage, and to create a core facility. Creation of a central freezer facility in a location with safeguards will allow the samples to be protected against threats like power outages, flooding, excessive heating and/or humidity, amongst others. Based on an engineering study, a location on the ground floor of the Penn State Hershey Cancer Institute will be renovated to meet these standards. The facility will be composed of multiple freezers for storage of biological samples from various institutional labs as well as the biorepository of the Institute for Personalized Medicine. The types of freezers will range from -80°C freezers to liquid nitrogen cryovial containers for long-term deep-freezing of samples. This facility will be built to meet the unique needs of the institution and is a much needed resource for the research community.

Principal Investigator

Bruce Stanley, PhD
Director, Section of Research Resources
Penn State University College of Medicine
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Hershey PA 17033

Other Participating Researchers

None

Expected Research Outcomes and Benefits

The Research Sample Storage Facility will provide space for multiple freezers and provides optimal storage conditions for biological samples, allow tracking and easy access of specimens for multiple investigators, and act as a backup for biological specimens stored in individual laboratories. Because this facility will be monitored and alarmed, research projects may continue even if an unforeseen event destroys samples stored at the laboratories themselves. The division and distribution of samples into two distinct locations reduces the probability that an

event would damage and destroy all of the samples. The benefits include reduced power interruptions through the integration of an auxiliary power source, reduced possibility of water damage through the use of water detection systems, and controlled HVAC to prevent freezer malfunction, among others. By concentrating duplicate aliquots of biological specimens in one area and creating an oversight structure compatible with a core facility for distribution, central management of samples will lead to improved scientific outputs and better safeguards for these valuable resources.

Summary of Research Completed

The scope of the project was to design and build an essential research sample storage facility that allows researchers to store their non-commercially available and other unique biological specimens in freezers for long-term storage. Collection of biological specimens is time-consuming and expensive, but critical to effectively test the efficacy of compounds, map biological processes and pathways, and obtain genetic information. One of the most important and vulnerable aspects of this research is storage of the specimens, which may be irreplaceable. Failure to store the specimens at strictly regulated temperatures will result in their rapid degradation, sometimes within hours. The aims of this project are to design and renovate a research sample storage facility that will house multiple freezers and will allow researchers to store duplicate aliquots of their samples in a secondary location for long-term storage, and to create a core facility. Creation of a central freezer facility in a location with safeguards will allow the samples to be protected against threats like power outages, flooding, excessive heating and/or humidity, amongst others. Based on an engineering study, a location on the ground floor of the Penn State Hershey Cancer Institute was renovated to meet these standards.

Specific aim 1: To design and renovate a research samples storage facility that will house multiple freezers and will allow researchers to store duplicate aliquots of their samples in a secondary location for long-term storage.

This aim has been achieved. The facility has been fully designed and built. A centrally located room of approximately 1216 square feet was identified (Figure 1). The location was first surveyed to determine the necessary design steps that would be needed to ensure that the specific safety and security measures as detailed in the strategic plan are implemented. These include back-up CO₂ access, Wi-Fi Alarm system, temperature monitoring system, water detection system, and card readers for access control. The freezers are also connected to the back-up power grid for the Cancer Institute building. This will ensure that the facility will function even in the event of a power outage.

One of the primary reasons that the location was chosen was based on ease of access for retrieval of the samples during an emergency should the need arise. The location is also within a few feet of a freight elevator and a wide access staircase that will allow movement of the samples up to the main entrance where freezer trucks could be waiting should evacuation of the building be necessary. The area is not near an inpatient ward and hence the sample retrieval efforts will not be interfered by significant patient traffic during an evacuation. The facility will also use a sample logging system that will allow the samples to be retrieved successfully during day to day operations and in the event of a disastrous incident. The water detection system that

has been installed will alert the respective individuals immediately so that the appropriate steps can be taken.

Six freezers have been purchased and installed to meet the initial demand for freezer space. There are two freezers that operate at -80°C , three that operate at -140°C and one that operates at -20°C . The -140°C cryostorage units are fed with LN_2 from the LN_2 generator (Figure 2) that was purchased with project funds. This allows for deep, long-term storage of samples which are not required for current research but allows for archiving of samples from recently completed research projects. The -20°C freezer allows for back-up storage if any of the other freezers fail. Wi-Fi monitors and alarms are also connected to each freezer that allows for remote monitoring of each freezer's temperature and also allows for the notification of the appropriate parties in the event of a failure. The water detection system that has been installed will trigger an alarm at the central facilities department if any trace of water is detected in the premises. An institutional -80°C , 25cu. ft. emergency freezer has also been incorporated into the facility for labs to use should any of the freezers fail. This will allow for the appropriate corrective action to be taken without any damage to the samples.

Measures have been incorporated into the facility design to ensure the security of the samples as well. There will be samples from various departments and labs housed at this location, some relating to unpublished data. A badge reader has been placed at the entrance that will only grant access to pre-approved individuals from the labs that have opted to place their samples in the freezers. The individual freezers also have locks on them that will prevent unauthorized individuals from gaining access to freezers that are holding samples from other labs and departments.

Policies have been developed for the operations of the facility. Principal Investigators and Departments who wish to place samples into the facility will have to submit applications stating the type of samples and the justification for placing it in the facility. The Research Quality Assurance Office will be reviewing these applications and also managing the day to day operations of the facility like monitoring access, temperatures, alarm status, maintaining the chemical fire suppressant system, etc.

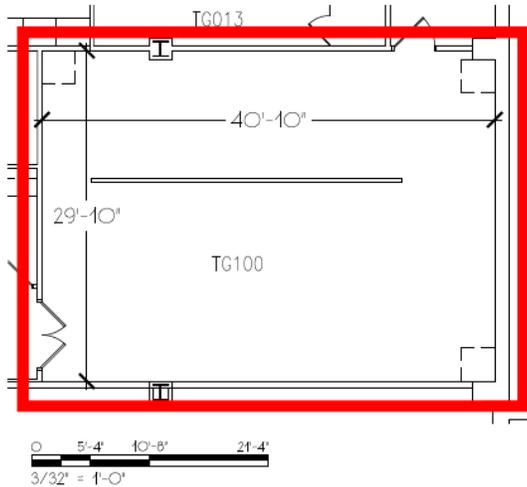
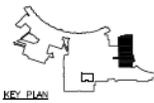
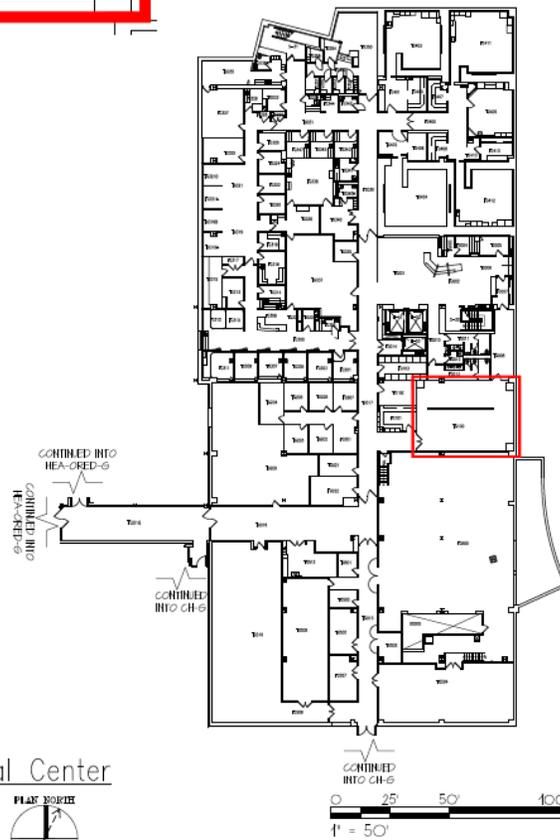


Figure 1. Map of the Facility



Penn State Hershey Medical Center
 College of Medicine
 PSHCI GROUND FLOOR- TG100
 1,216 Sq. Ft.





Figure 2. Liquid Nitrogen Generator

Research Project 59: Project Title and Purpose

Small Peptide Eye Drops for Diabetic Retinopathy - Although diabetic retinopathy (DR) is a leading cause of blindness with a projected incidence of ~350 million worldwide by 2020, there are no effective treatments for this condition. DR includes a spectrum of pathologies including cell death, inflammation, and vascular leakage. We have developed a 29-mer bioactive peptide (serpxA1), the first targeted DR drug that reduced both early and advanced DR pathologies in rodents when administered as an eye drop. Our goal in this proof-of-concept study is to improve this technology by designing a series of structurally novel and potentially patentable analogs that show equal or better activity as well as improved bioavailability, and metabolic stability in rodents. We will then confirm bioavailability of lead compounds in primates.

Duration of Project

3/12/2013 – 12/31/2013

Project Overview

Specific Aim 1. *We will generate 10-15 structurally different analogs of serpxA1 and –*
(a) Identify metabolic sites within the 29-residue peptide
(b) Determine whether shorter analogs have better bioavailability and maintain the same bioactivity

(c) Assess the effect of site-specific mutations on peptide metabolism, bioavailability, and bioactivity

Specific Aim 2. All synthesized compounds will be tested in a series of rigorous quantitative in vitro screens to select up to the 5 most effective and structurally diverse analogs of serpxA1.

(a) To test efficacy of the compounds in preventing cell death, we will screen for their ability to

(i) Increase cellular bioenergetic levels in an ATP assay; (ii) Reduce cell death in an LDH assay

(b) To test their action on inflammatory processes, we will examine their ability to decrease production of inflammatory cytokines using Luminex bead arrays

(c) To test their potential to reduce vascular leakage, analogs will be screened by qPCR for their ability to increase mRNA levels of ZO1 and occludin, two junction proteins essential to vascular integrity.

(d) To test peptide stability in the vitreous, compounds will be incubated with dissected vitreous humor and samples analyzed at various time points using Maldi TOF.

Specific Aim 3. We will test efficacy of the 5 lead compounds from SA2 in longitudinal studies in vivo through the period when vascular leakage is ophthalmoscopically evident in rodent DR.

Vascular leakage is first noticed in diabetic mouse retinas ~13 wks after onset of hyperglycemia (HG). Lead peptides will be tested at the effective dose of P78 for 15 wks at the onset of HG and the following measured -

(a) Reduction in vascular leakage will be assessed by fluorescein angiography using the Micron III retinal imaging real time acquisition system equipped with StreamPix 5.8.1.4. Fluorescein extravasation from vessels will be quantitated immediately after IP injections of AK-FLUOR (n=5)

(b) RGC death in the retina will be calculated by morphometric analysis and levels of inflammatory markers

Specific Aim 4. Because rodent and primate eye are quite different in size, the bioavailability of 2-3 of the most effective analogs from SA3 will be determined at two time points in African Green monkeys.

Principal Investigator

Joyce Tombran-Tink, PhD

Professor

Penn State University College of Medicine

Department of Neural and Behavioral Sciences

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Hershey, PA 17033

Other Participating Researchers

Colin J Barnstable, DPhil – employed by Penn State University College of Medicine

Expected Research Outcomes and Benefits

We anticipate that the lead application for the compound(s) will be DR, a disease that affects millions worldwide. Other possible applications include age-related macular degeneration (AMD) and retinitis pigmentosa (RP). The target market will include both non-proliferative and proliferative diabetic retinopathy patient populations. During the non-proliferative stage, treatment may be viewed as preventative to development of the proliferative stage of the disease. Treatment during the proliferative stage of the disease will be viewed as restorative therapy. The most likely profile will be a diabetic person > 40 year old whose visual function has been tracked by an ophthalmologist over the course of the disease. We envision treatments as a benefit to both patient and physician. Patients will benefit by receiving a treatment option with a higher degree of delivery ease in prescription eye drops instead of monthly ocular injections. They may be able to decrease visits to the ophthalmologists as well. Physician will see value in cost-savings of the drug(s) compared to Lucentis through the reimbursement model. We see the ophthalmologist as a customer and prescriber of the drug with health insurance reimbursement options.

The value of the product is based on four key factors: 1). delivery as an eye drop rather than injection; 2.) it is a multi-hit drug that is effective in reducing vascular leakage, inflammation, and cell death in DR; 3) it has the potential to be used as both add on and stand alone treatment for DR; 4) it has both preventive and restorative potential in non-proliferative and the proliferative stages of the disease.

Summary of Research Completed

From in vitro peptide screenings of Aims 1 & 2 (Jan 1-June 31, 2013), we selected five analogs with best biological activity to test efficacy in vivo in the Ins2Akita mouse model of DR.

SA3. Male hyperglycemic mice with blood glucose levels >300 mg/dL were used and treatments carried out essentially as described ((Liu Y et al., Mol Med 2012). Diabetic mice were treated 2x/wk for 15wks at the onset of HG using a single dose of 5µg/5µl artificial tears for each drug. Both eyes received the same treatment. At the termination of the experiment, vitreous and retina samples were collected for analysis. Bioavailability of the analogs was also tested.

Bioavailability: Bioavailability of peptides was determined by MALDI TOF in vitreous samples after 1-6 hr (n=8 eyes) topical application and spectral intensities compared to spectra of known concentrations of each analog to calculate vitreous concentrations. All samples showed peak levels at ~1 hr (Fig 1). Confocal microscopy of retinas immunolabeled with a PEDF peptide antibody (Fig 2) show intense labeling in the retina after peptide eye drops were given and largely represents peptides delivered to the retina by topical routes. Labeling of the choroidal vasculature suggests they may be delivery route of the peptides (Fig 2). This data provide evidence that the peptides are delivered to the retina when administered topically.

Reduction of inflammation, VEGF levels and vascular leakage: Vitreous samples harvested at 15 wks of treatment were analyzed to detect levels of proinflammatory cytokines using Luminex

beads. We show a marked reduction in VEGF, TNF α , IFN γ , and IL-6 by the P78 peptide analogs compared to controls (Fig 3). 81-5 and 81-3 showed slightly better effects in reducing IL-6. All peptide reduced levels of VEGF relative to diabetic controls (Fig 4). Vascular hemorrhaging, measured by albumin extravasation in the retina by leaky retinal vessels using an ELISA (Fig 5), indicated an increase in diabetic retinas and a significant reduction by the peptide and confirmed by immunolabeled for albumin (Fig 6; arrows, 20x; 40x). In Fig 7, the effects of the peptides on albumin extravasation into the retina are shown.

RGC survival: RGC loss occurs early in diabetic retinopathy. Stained nuclei in the RGC layer of non-treated and treated retinas were counted from 6x250 mm zones along the length of the retina from centrally located fields adjacent to the optic nerve to the periphery. 6 fields/retina were analyzed and data presented as the avg # cells/400 mM (n=6). The results (Fig 8,9) show a decrease in number of surviving RGCs in diabetic controls (DC) and neuroprotective actions of the peptide on these cells. From these studies we selected 2 active, structurally diverse peptides 81-5 and 81-13 to test bioavailability in the primate eye.

SA4. There are substantial differences between the eyes of rodents and primates. These include size of the aqueous and vitreous fluid compartments and fluid flow forward which may influence drug concentrations in the vitreous. We tested access of two our lead compound(s) to the retina using the RxGen facility, St Kitts Biomedical Research Foundation. We scaled up doses by 8-20 fold to account for differences in eye volume (Table 1). Animals were fasted overnight then sedated with ketamine (8mg/kg, I.M) and xylazine (1.6 mg/kg, I.M) prior to all procedures. Eyes were manually blinked after dosing and prior to vitreous humor collection, topical local anesthesia was administered and eyes disinfected with 5% Betadine. Vitreous samples were collected and immediately analyzed by mass spectrometry. The results indicated that peptides in eye drops were delivered to the posterior eye in primates as well. This study is encouraging and holds promise for delivery of therapeutic peptides to the human eye to treat ocular diseases.

Hurdles and Alternate approaches: One of the hurdles encountered in the study was that we did not predict animal loss during live retinal imaging we planned to detect vascular leakage during the late stage pathology. This method worked safely during early stage diabetes but the animals were unable to withstand the anesthesia required for us to obtain the fluorescein angiograms and died before or during the retinal imaging. Because of this, we deviated from the original study and instead assessed vascular leakage quantitatively using an ELISA measurement of albumin content in the retina and confirmed leakage by confocal microscopy.

Commercial Potential

A patent application for the peptide technology and its uses for retinal diseases and diabetic complications was filed in September 2013. We are currently in negotiations with several interested parties to license the technology.

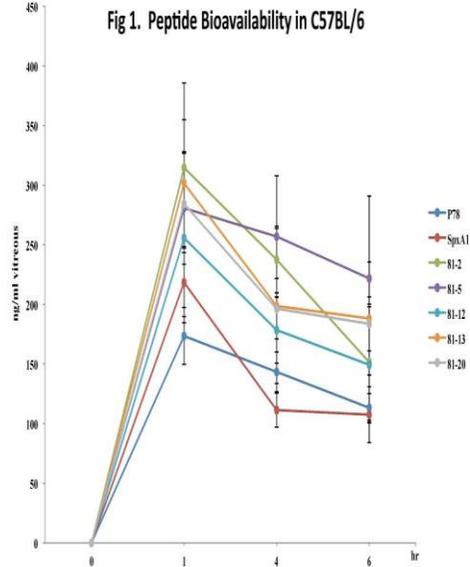


Fig 2 Peptide Delivery to the retina – 1 hr eye drop treatment

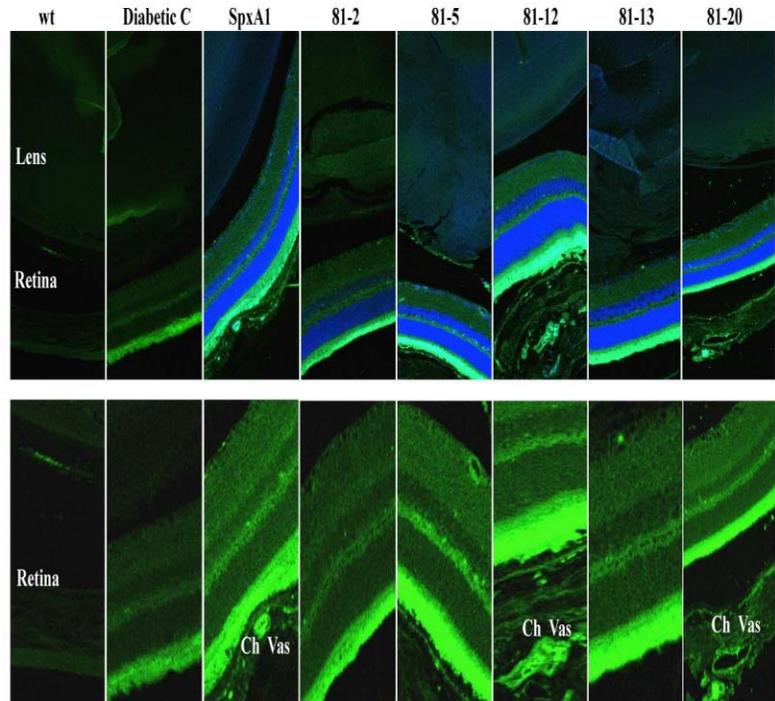


Fig 3. Inflammatory cytokines

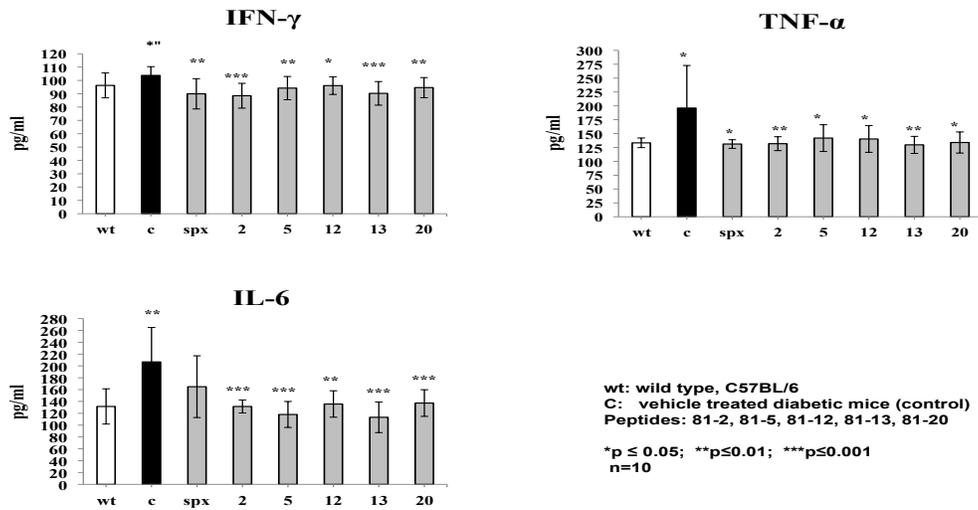
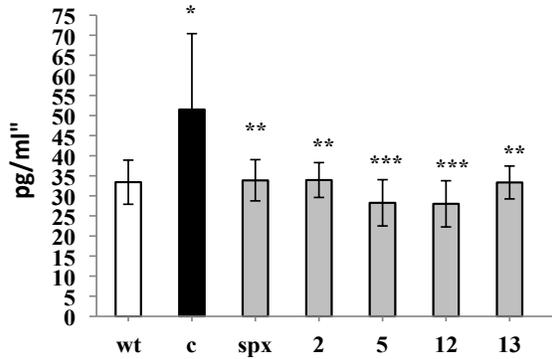


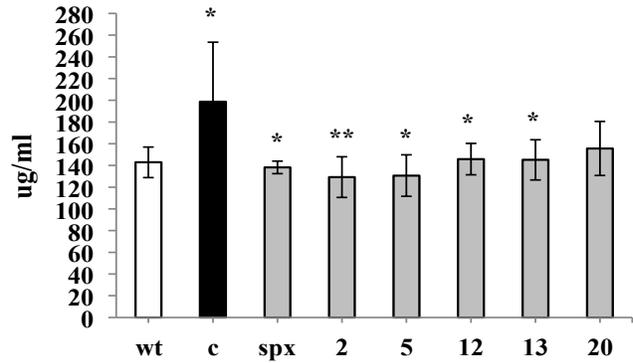
Fig 4. VEGF Levels



wt: wild type, C57BL/6
 C: vehicle treated diabetic mice (control)
 Peptides: 81-2, 81-5, 81-12, 81-13, 81-20

*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001
 n=10

Fig 5. Albumin Leakage in the retina



wt: wild type, C57BL/6
 C: vehicle treated diabetic mice (control)
 Peptides: 81-2, 81-5, 81-12, 81-13, 81-20

*p ≤ 0.05; **p ≤ 0.01
 n = 10

Fig 6. Vascular leakage: Albumin content

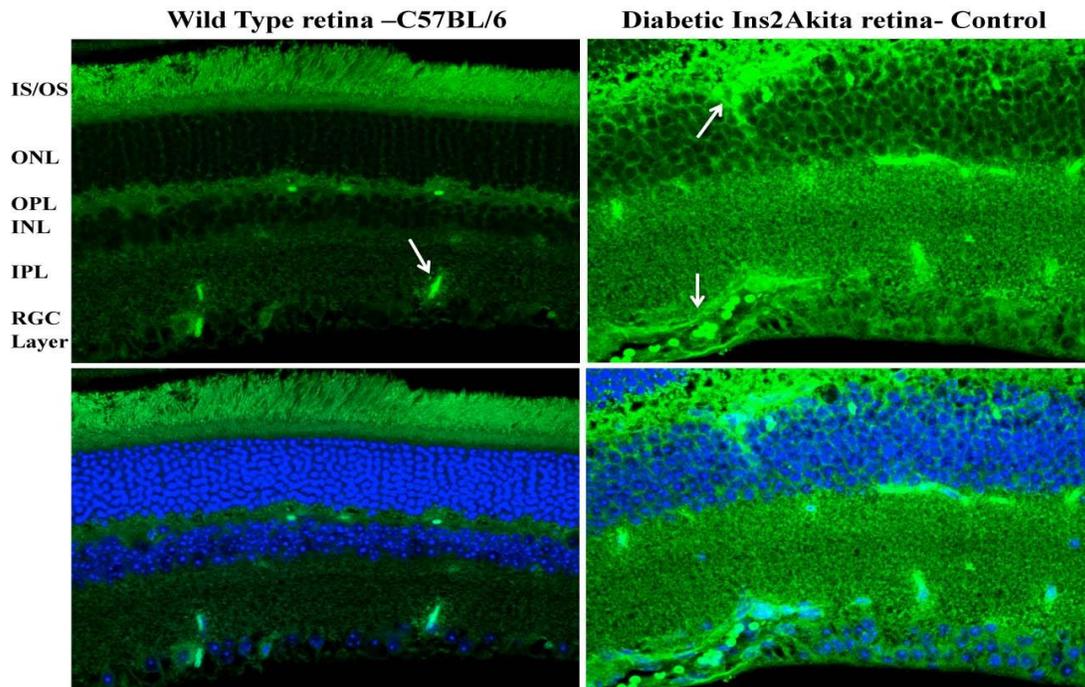


Fig 7. Vascular Leakage after peptide treatment – 15 weeks : Albumin immunolabeling

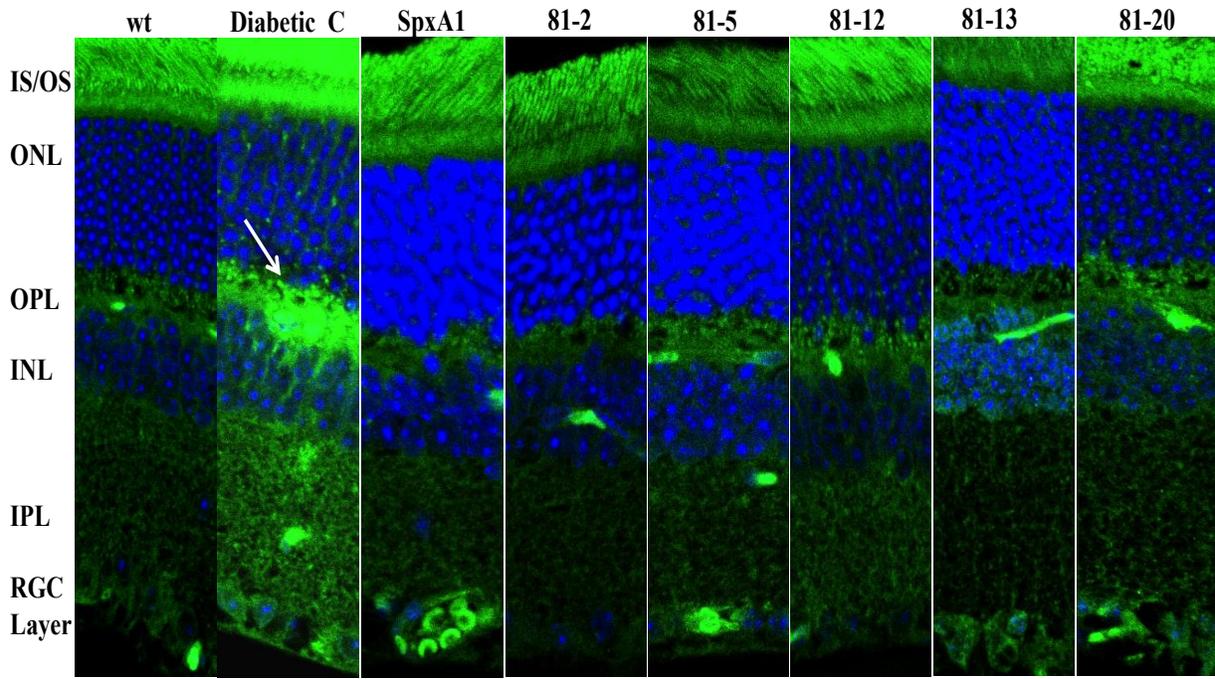


Fig 8. RGC survival

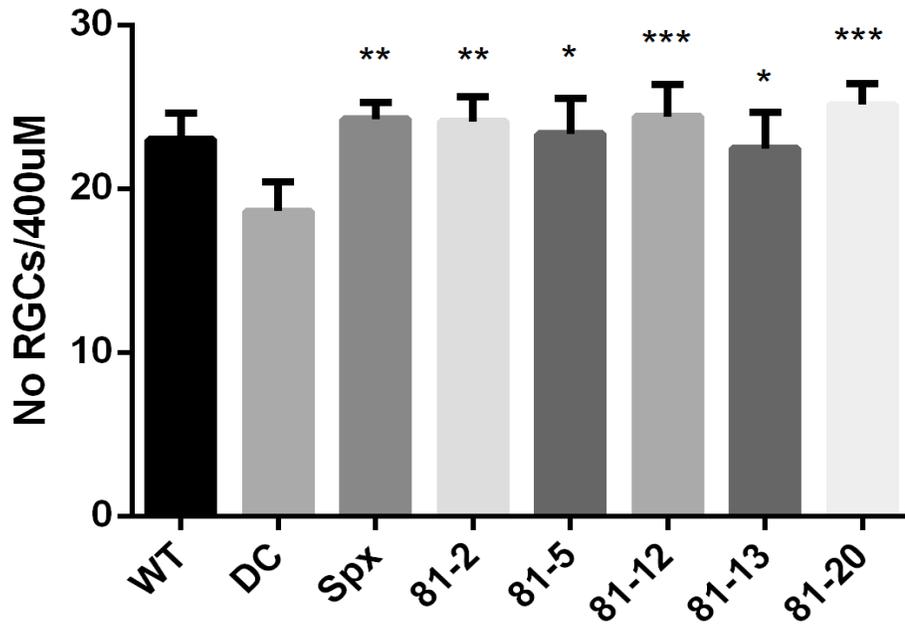


Fig 9. Retinal Ganglion Cell Survival

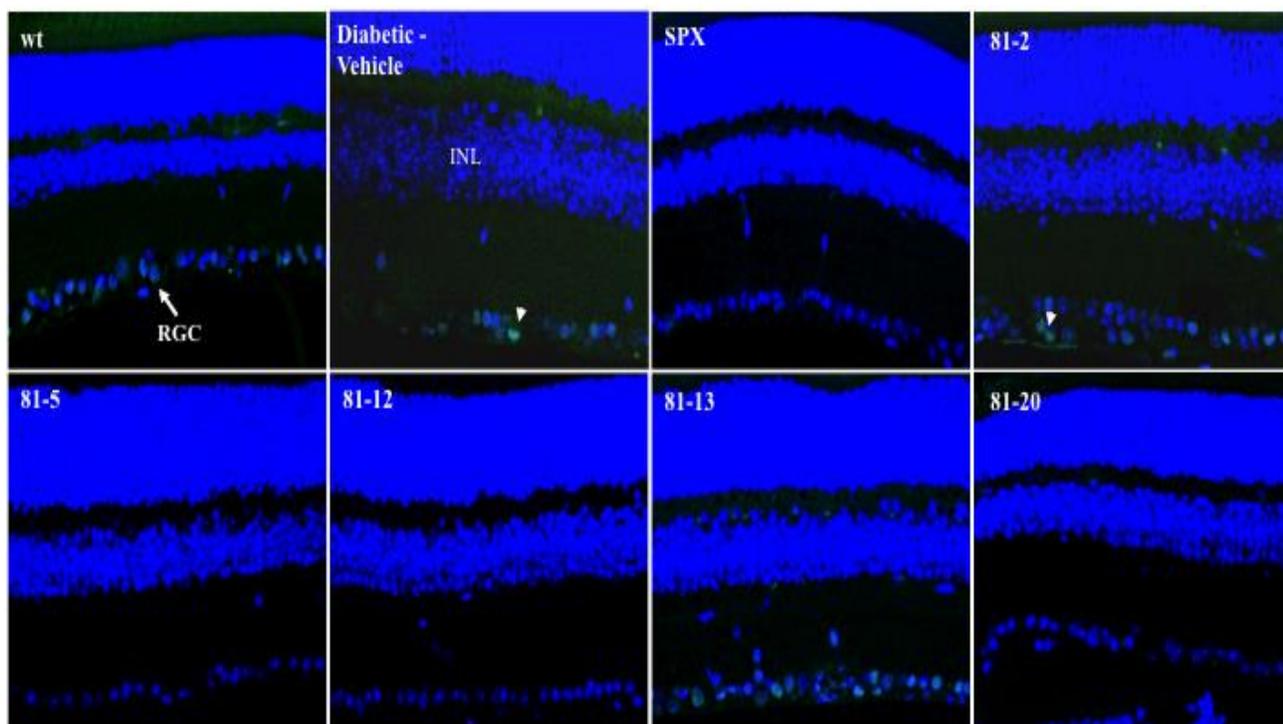


Table 1. Peptide bioavailability in primate (Vervets) eyes

Group	Animal ID	Sex	Body weight (kg)	Eye	Test article	Dose/ Topical	Vitreous humor collected after dosing	Volume collected	Mass Spec Avg Conc ng/ml
1	Z998	Male	6.54	OU	81-5	40µg/40µL/eye	OD:67 min OS:69 min	OD:100 µL OS:100 µL	
1	K099	Female	3.78	OU	81-5	40µg/40µL/eye	OD:65 min OS:66 min	OD:100 µL OS:100 µL	Group 1 (n=4) 158.5±15.5
2	V715	Female	4.68	OU	81-13	100µg/50µL/eye	OD:59 min OS:60 min	OD:100 µL OS:100 µL	
2	K146	Male	5.92	OU	81-13	100µg/50µL/eye	OD:58 min OS:64 min	OD:100 µL OS:100 µL	Group 2 (n=4) 237.5±32.5
3	X429	Female	4.98	OU	81-13	100µg/50µL/eye	OD:120 min OS:118 min	OD:100 µL OS:200 µL	
3	K169	Male	8.28	OU	81-13	100µg/50µL/eye	OD:125 min OS:128 mi	OD:100 µL OS:100 µL	Group 3 (n=4) 212.5±15.0

Research Project 60: Project Title and Purpose

Research Infrastructure: Animal Biosafety Level-2 Facility - This project will renovate existing space to provide additional animal biosafety level-2 (ABSL-2) housing in the Central Animal Quarters (CAQ) of the Penn State College of Medicine. Modifications of the exit and entrance of the facility will also be part of this project and will greatly enhance the security and biocontainment of the facility. The overall goal of the renovations and equipment purchases is to provide housing for “humanized” mouse models, i.e., immunodeficient mice ingrafted with various human cells. These immunocompromised animals require special housing condition, including autoclaved cage setups and sterilized, autoclaved drinking water to minimize infection with adventitious pathogens.

Duration of Project

6/1/2013 – 12/31/2013

Project Overview

The specific aim of this infrastructure project is to renovate and expand additional housing space for ABSL-2 work, and enhance security and biocontainment. The objective of the project is to meet the increasing demand for work with animal models involving BSL-2 agents, specifically severely immunocompromised mice engrafted with human cells. Some of the renovations include installing wall-mounted ventilated caging systems and biosafety cabinets in addition to relocating and adding security systems to the access doors. The proposed renovations provide an excellent opportunity to expand research requiring ABSL-2 housing conditions as well as to enhance the safety and security of the animal facility. Specifically, this project will address housing and husbandry needs for a newly emerging animal model, humanized mice.

The renovations and purchase of equipment proposed in this application are part of an ongoing strategy by the Director, Penn State Hershey Animal Resource Program, supported by leadership, to upgrade the entire animal care program of the institution to meet immediate and future needs of the organization’s research program. With the modifications requested, this objective can be met and help to support quality biomedical research conducted under the highest standards of animal care while providing operational efficiency.

Principal Investigator

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Professor and Chair, Department of Comparative Medicine
Director, Animal Resource Program
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Hershey PA, 17033

Other Participating Researchers

Penny L. Devlin, MLAS – employed by Penn State University College of Medicine, M.S.
Hershey Medical Center

Expected Research Outcomes and Benefits

The ability to engraft severely immunocompromised mice with cells from other mice and other species including human immune cells and stem cells is advancing research in immunology, cancer biology and therapy, and drug discovery. These animal models are currently used by approximately 10 research groups at Penn State Hershey with anticipation that a growing number of researchers will use this animal model in the future. Furthermore, the security and operational improvements to the CAQ facility will benefit all researchers who house animals in this facility, currently numbering approximately 30 research groups.

This project will provide the necessary upgrades to work safely with BSL2 agents in animals. This includes specialized individual ventilated caging (IVC), class II bio-safety cabinets, special air flow at the room level and standard operating procedures to ensure proper handling of the agents, animals, caging, and waste products, facility flow patterns and personal protective equipment. New entrance and exit doors give us the opportunity to restrict personnel, who are not essential to the functioning of the facility, from entering.

Summary of Research Completed

A room, CG766 in the Central Animal Quarters (CAQ) facility which previously housed -70°C ultra-low freezers was vacated and renovated to provide ABSL-2 housing for mice. NIH and CDC (*Biosafety in Microbiological and Biomedical Laboratories*, 5th edition) guidelines were followed in design and renovation of the space. The resulting animal room is 492 net square feet. Individually-ventilated caging systems were purchased and installed in the space. Two biosafety cabinets (BSC), one Class II type A2 and one Class II type B2 (hard-ducted exhaust) were also purchased and installed in the space. The biosafety cabinets provide protection for personnel working with the animals and biohazardous agents and also protect the health status of the animals during manipulations. The Class II type A2 BSC is used primarily for cage changing and handling of the mice, while the Class II type B2 BSC is used by researchers for surgical and other experimental procedures on the mice. Total housing capacity of the renovated space is 720 cages/3,600 mice. Occupancy of the renovated space occurred in early January, 2014. The space primarily houses animals of researchers in the Penn State Hershey Cancer Institute. Current research projects focus primarily on brain cancer, melanoma, lymphoma and leukemias.

A bottle filling station with acid proportioner was purchased and installed in the CAQ clean cagewash facility. The station provides for time and labor efficient filling of water bottles with acidified (pH 2.7 – 3.4) drinking water. Autoclaved acidified drinking water is the industry standard for husbandry of severely immunocompromised mice. It inhibits the colonization and infection of immunodeficient mice with opportunistic bacteria, especially *Pseudomonas*, *Streptococcus* and *Staphylococcus* species. With this equipment, the pH can be accurately

adjusted and eliminated the direct handling of hydrochloric acid thus providing safety for personnel.

The entrance and exit to the CAQ animal facility are currently open to the adjoining space. Adding entrance and exit doors with proximity card readers and cameras will greatly enhance the security of the animal facility as access can be limited to personnel who have a need to utilize the facility. Furthermore, solid doors will provide a barrier to airflow into the facility from adjacent areas thus improving biosecurity for the animals housed in the facility. All doors, hardware, badge readers and cameras have been purchased. Final completion of this phase of the project is anticipated in April 2014. Occupancy of the renovated space occurred in early January, 2014. The objective of the project is to meet the increasing demand for work with animal models involving BSL-2 agents, specifically severely immunocompromised mice engrafted with human cells.