

Pennsylvania State University

Annual Progress Report: 2007 Formula Grant

Reporting Period

July 1, 2010 – June 30, 2011

Formula Grant Overview

The Pennsylvania State University received \$7,538,293 in formula funds for the grant award period January 1, 2008 through December 31, 2011. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

High Field MRI - Limitations and Solutions - We are in the process of developing new techniques and technology that will make Magnetic Resonance Imaging (MRI) more accurate, more effective, more versatile, faster, and safer in the future. Because of the wide utility of MRI, this will have major benefits on many areas of medicine. We have recently ended a period of funding from the NIH regarding this work and have received very good scores with easily-addressable concerns in our first renewal application. We anticipate further NIH funding upon review of our revised renewal application. Pennsylvania Department of Health (PA DOH) funding will allow us to continue progress without major interruption during a gap in federal funding and allow for continued demonstrable progress should a second revised application for federal funds be necessary.

Duration of Project

9/1/2008 – 6/30/2010

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 2: Project Title and Purpose

Interactions of the CA Protein in the Retrovirus Core - In retroviruses such as HIV, the human T-cell lymphotropic virus and the Rous sarcoma virus, a stage of the virus life cycle known as maturation involves very dramatic structural changes in the interior of the virus particle, leading to the activation of its infectious potential. The studies conducted as part of this project will use a combination of genetic and protein structural approaches to examine the molecular mechanisms that control this process in the formation of the functional core in the interior of the

infectious particle. A detailed understanding of this essential step of virus infection will allow development of better inhibitors of capsid assembly for use as anti-retroviral drugs.

Duration of Project

1/1/2008 – 6/30/2009

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

Evaluation of Patient-Driven Playbook (A Patient Education Tool) - Diabetes has devastating consequences in terms of morbidity, mortality and health care costs. Between 2003 and 2004, two out of three Americans did not meet clearly identified evidence-based treatment goals. Yet the vast majority (84%) of patients believed that they were doing a good job managing their disease. A clear knowledge gap and educational opportunity thus emerged. By using a patient advocacy group, patient authors, and patient focus groups, a new tool, called the Penn State Diabetes Playbook, was developed to teach patients diabetes disease awareness and self-management skills. This project is designed to evaluate the Playbook alone or with nurse dialogue using motivational interviewing when it comes to educating, motivating and altering the behavior of patients with diabetes in a primary care setting.

Anticipated Duration of Project

1/1/2008 - 12/31/2011

Project Overview

The Playbook was created via a social marketing format which is a technique used to design programs that promote behavior change. Social marketing dictates that all program planning decisions must emanate from a consideration of the consumers' wants and needs which is divergent from more traditional, expert-driven approaches in which public health professionals determine what consumers need to know. This project will evaluate if providing patients with a patient-driven educational tool will decrease the emotional distress related to diabetes, increase medical knowledge of the disease, improve self-care behaviors and result in better clinical outcomes. The addition of a nurse educator using basic motivational interviewing techniques to explore use of this tool should increase success rates even further within these parameters. It is designed as a prospective, randomized-controlled trial. It has 3-arms with one group adhering to usual care, one group receiving the Playbook and one group receiving the Playbook with 15-30 minutes of nurse dialogue using motivational interviewing techniques. It will be important to determine what the incremental benefit of adding a nurse to the intervention is in order to help inform policy makers of the potential cost-benefit of such staff. Patients will be randomly

assigned to one of the three arms. One hundred patients between the ages of 18-75 with type 2 diabetes seen by a primary care clinic will be identified and followed for six months. The four specific aims are (1) Determine the efficacy in decreasing emotional distress. (2) Demonstrate the ability in increasing medical knowledge. (3) Evaluate the capability to improve self-care behavior. (4) Examine the effect on clinical outcomes. The first three aims will be evaluated by use of surveys distributed at baseline, 4-weeks, 3-months and 6-months. These surveys include: (1) Problems Areas in Diabetes (PAID scale to assess emotional distress), which has been found to be highly reliable (>.90) and responsive to changes during brief psychosocial and educational interventions, (2) the Summary of Diabetes Self Care Activities (SDSCA) survey, a reliable and valid measure of usual self-care behavior engaged in by persons with type 2 diabetes, which has been used to evaluate individuals' self-care behavior and adherence to diet, exercise, blood glucose testing, foot care, smoking, and self-care recommendations, (3) a Knowledge Questionnaire designed for this study to assess general disease knowledge, carbohydrate understanding and treatment of low blood glucoses, and (4) a Baseline demographics questionnaire. The fourth aim will be evaluated by Hemoglobin A1C checked at baseline, 3-months and 6-months.

Principal Investigator

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Expected Research Outcomes and Benefits

In the past decade studies such as the Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) showed that intensively controlling glucose significantly reduces the risks of heart, eye and kidney disease. Yet patients throughout the country fail to achieve these goals. In order to reach treatment goals, patients with diabetes need to adhere to a healthy diet, obtain regular exercise, use medications appropriately, be consistent with glucose monitoring and possess the ability to make daily management decisions. These daily self-care behaviors, along with disease awareness and self-management skills, have proven critical to good diabetes outcomes. The current medical model with limited practitioner time, inadequate and at times poorly focused patient education material and lack of self-management training, has proven poor at teaching these skills. In addition, current patient education material fails to account for health literacy levels - the ability to read, understand, and

act on health information. One out of five American adults reads at the 5th grade level or below, and the average American reads at the 8th to 9th grade level, yet most health care materials are written above the 10th grade level. Better tools are needed. Social marketing may be one avenue to create these tools. While successful in commercial aspects and many health care settings, social marketing has yet to be introduced into an approach for chronic disease care. If patient driven tools are successful at promoting behavior change and making information more accessible to those with low health literacy, it could improve the psychosocial and clinical outcomes associated with diabetes that our current model of care has failed to achieve. This approach could be globally used to redesign patient materials for other chronic diseases such as Congestive Heart Failure, Coronary Artery Disease, and asthma where patient self-management is essential. And as more pay-for-performance programs disseminate across the country, cost effective tools such as this one and the incremental benefits of adding nurse education will play a large role in clinical care.

Summary of Research Completed

Substantial progress has been made on the Interactive Web-Based Diabetes Self-Management Tool project.

We finished recruitment of 60 people that completed the consent forms, the baseline survey, the five learning modules and the follow-up survey. After the completion of this part of the study, the participants joined the social networking program in NING© for 90 days. At 90 days, the applicable participants were contacted to complete the final survey.

After recruitment, consent and access to the internet program, participants in the trial were asked to complete four surveys (PAID, SDSCA, MDRTC and Demographics) for baseline. They were led through a series of interactive, tailored modules based upon information from the Playbook. Researchers found that most participants were able to navigate through the online modules with relative ease.

As there were multiple stages to participant involvement, it sometimes became necessary to remind participants to continue to the next stage. Investigators monitored participant progress through the stages and sent email reminders. This proactive communication increased participant continuation in the online modules and the overall number of subjects who completed all stages of the research.

During the last year, researchers enriched the program adding video content and identified several technical challenges that they were able to surmount over time. The playbook portion was aimed at being a linear process where subjects had to proceed from one section to the other. Appropriate computer coding was needed to prevent individuals from skipping forward and/or skipping the surveys at each designated portion. Investigators worked with IT developers to address these issues.

Data Analysis

A transcript containing all of the NING© conversations was compiled and reviewed. The researchers used line-by-line identification of key ideas that were labeled into codes and defined.

The codes were then organized into conceptual groupings (categories). Categories were assigned based on the overall gestalt of the comment as determined by the researcher reviewing the transcript and a qualitative researcher. A single comment could be placed into more than one category. Categories were based loosely on definitions of social support as summarized. The quotes related to the categories were analyzed to determine if they were used in the appropriate context of social support. The frequency of categories appearing in the transcript was totaled.

We are currently in the process of analyzing the data from the study. In analyzing the NING© discussions line by line, four categories of social support summarized how participants interacted with each other. The four categories were: (1) affirmation of feelings about diabetes; (2) sharing of personal stories about diabetes; (3) information or knowledge sharing about diabetes; and (4) statements of encouragement.

A manuscript that represents a qualitative analysis of the content of the NING© social networking site is in preparation. Overall, patients found the site very helpful. Key topics discussed focused on meal planning and meeting the daily challenges of diabetes.

We are in the process of analyzing the survey data on patients to assess the overall impact of the educational component of the registry and have extended the project for 6 months to complete this aspect.

We also plan to investigate options for future grant opportunities to study the use of social networks further. Finally, we have now made the web-based interactive Diabetes Playbook available free to the public where it has been used by a significant number of individuals worldwide.

Research Project 4: Project Title and Purpose

DeltaFosB and Reward Comparison in Mice - Drug addiction often leads to decreased motivation for things that were once pleasurable before drug use such as friends, family, work, hobbies, and even personal hygiene. This devaluation of naturally rewarding stimuli in the environment, in favor of the drug of abuse, leads to personal and public costs as the addict is no longer able to beneficially function in society. The present project combines the only animal model of this phenomenon (i.e., drug-induced devaluation of natural rewards) with a rich mouse model to elucidate a possible molecular mediator of the neural plasticity that leads to this potentially devastating behavioral change.

Duration of Project

7/1/2009 - 6/30/2011

Project Overview

Rats and mice avoid intake of a saccharin conditioned stimulus (CS) when it is paired with a drug of abuse such as morphine or cocaine. Apparently the rats avoid intake of the natural reward cue because the value of the gustatory stimulus pales in comparison to the subsequent

reward produced by the drug. In fact, the rats that reduce their intake of the drug-associated taste cue the most; self-administer the most of a drug of abuse. Moreover, avoidance of the saccharin cue is exaggerated in Lewis rats and in Sprague-Dawley rats subjected to chronic morphine treatment, both models that are known to be drug sensitive. The augmented response to drug reward observed in these rats has been attributed to a range of neural adaptations, including elevated levels of the nuclear transcription factor Δ FosB. DeltaFosB levels increase and remain elevated after chronic drug exposure. Increases in Δ FosB sensitize a number of behavioral responses to drug rewards including conditioned place preference, acquisition and maintenance of drug self-administration, and performance for drug on a progressive ratio schedule of reinforcement. These data led us to hypothesize that transgenic mice with elevated Δ FosB in the striatum also would demonstrate exaggerated avoidance of a saccharin cue following saccharin-cocaine pairings. To our surprise, the opposite occurred. We concluded that the mice probably are more sensitive to all rewards, both drug induced and naturally occurring. The proposed studies will test this hypothesis in mice by selectively increasing Δ FosB in the nucleus accumbens using adeno-associated viral mediated gene transfer (AAV- Δ FosB mice). *Specific Aim I* tests whether elevation of Δ FosB will lead to an increase in preference for saccharin or a neutral salt stimulus, but not for an aversive (quinine) taste stimulus. *Specific Aim II* tests whether elevation of Δ FosB will augment (as initially predicted) cocaine-induced suppression of CS intake when a neutral or aversive tastant, rather than a rewarding tastant, serves as the gustatory CS. Finally, *Specific Aim III* tests whether ‘impulsivity’ is responsible for the failure to suppress intake of a natural reward cue following saccharin-cocaine pairings in mice with elevated Δ FosB.

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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, is a two-part problem involving chronic relapse and the concurrent devaluation of natural rewards. There are a number of critical animal models of

craving and relapse. There are, however, no recognized animal models for the study of drug-induced devaluation of natural rewards. The present project describes our development of just such an animal model and the investigation of the underlying neural substrates.

Summary of Research Completed

The original project, entitled “*DeltaFosB and Reward Comparison in Mice*” supported the salary of then graduate student, Christopher S. Freet, and combined the only animal model of this phenomenon (i.e., drug-induced devaluation of natural rewards) with a rich mouse model to elucidate a possible molecular mediator of the neural plasticity that leads to this potentially devastating behavioral change. At the time that Dr. Freet completed his portion of the project, a second student, Jennifer Nyland, received word that her proposal would be funded. The overarching goal of her project is the same, to use drug-induced devaluation of natural rewards to elucidate the neural underpinnings of this devastating consequence of addiction. In this case, however, Ms. Nyland was using rats rather than mice and focused on central lesions rather than over expression of brain DeltaFosB. In specific, the present project sought to test the hypothesis that an intact thalamic orosensory area is essential for drug-induced devaluation of natural rewards. The results of this study will further our understanding of addiction, its neural underpinnings, and the development of successful treatments.

Experiment 1. An intact thalamic orosensory area is required for cue induced withdrawal when a Polycose taste cue predicts morphine.

Methods. The subjects were 30 male Sprague-Dawley rats housed individually in suspended, stainless steel cages in a temperature-controlled (21°C) animal care facility under a 12:12 hour light-dark cycle. Food and water were available ad libitum, except where noted otherwise. Subjects were anesthetized and received bilateral electrophysiologically-guided ibotenic acid lesions of the thalamic trigeminal orosensory area (TOAx, n=14) or sham lesions (Sham, n=16). The subjects were assigned to a saline or a morphine treatment group. *Baseline Intake.* All subjects were placed on a water-deprivation regimen (5 min am/1 h pm) which continued for 7 days until 5 min morning intake stabilized. *Polycose-Morphine Pairings.* Subjects were given 5 min access to 0.03M Polycose (CS), followed 5 min later by an intraperitoneal injection of either 15 mg/kg morphine or an equal volume of saline. There was one such pairing a day with 48 hours in between pairings for a total of 6 pairings. On the test day following the 6th pairing, rats were allowed 5 min access to the Polycose cue, followed by injection of a subthreshold dose of Naloxone (1mg/kg), which is sufficient to cause a loss in body mass in rats that experience cue-induced withdrawal several hours after the injection. Body mass was recorded before Naloxone administration as well as 1 and 2 hr following Naloxone administration.

Results. Five min Polycose intake and change in body mass data on the test day were analyzed separately for each lesion group (Sham and TOAx) using a one way ANOVA comparing condition (saline vs. morphine). In the Sham lesioned morphine treated rats, intake of the Polycose CS was suppressed compared with the saline treated Sham controls ($F(1,14) = 8.08$, $p < 0.05$, see Figure 1(left)). The TOAx group treated with morphine, however, failed to suppress intake of the Polycose CS compared with intake by the TOAx saline treated controls, $F < 1$. Additionally, the Sham lesioned rats with a history of morphine treatment had a significant

difference in body mass change 2 hours following Naloxone administration compared with the Sham controls with a history of morphine treatment, $F(1,14) = 9.91$, $p < 0.01$, see Figure 1 (right). As with CS intake, the 2 hour change in body mass in the Naloxone injected TOAx group with a history of morphine treatment did not differ from the Naloxone injected TOAx rats with a history of Polycose-saline pairings, $F < 1$. Thus, the lesion not only prevents avoidance of the morphine-paired taste cue, but also appears to inhibit the development of cue-induced withdrawal. This is important because cue-induced withdrawal is a primary precipitating factor for relapse in drug-addicted humans.

Experiment 2. Change in body mass following Naloxone administration is not dependent on the intake volume of a taste cue that predicts morphine.

Methods. In a within-subjects study, 16 male Sprague Dawley rats were obtained and housed as described above. *Baseline Intake.* All subjects were placed on a water-deprivation regimen (5 min am/1 h pm) which continued for 9 days until 5 min morning intake stabilized. *CS+/CS- Conditioning.* Following stabilization of morning water intake, rats were divided into Grape CS+ and Orange CS+ groups (the CS+ flavor is that which is paired with morphine and the CS- flavor is that which is paired with saline). Thus, one flavor (orange or grape) was presented each day in a 0.15% saccharin solution, alternating across days. All rats were given 5 min of access to the CS, followed 5 min later by an IP injection of either 15 mg/kg morphine (if given access to the CS+ solution) or saline (if given access to the CS- solution). All subjects had one flavor paired with morphine and the other paired with saline. There were 7 Grape and 7 Orange CS days for a total of 14 conditioning days. To maintain proper hydration, dH₂O continued to be provided for 1 hour every afternoon. *Testing.* Following conditioning, there were 2 test days, one for each flavor of CS. On each day, subjects were weighed, and then given 5 min access to the CS, followed 5 min later by a SC injection of 1 mg/kg Naloxone. A second body mass was recorded 2 hr after the Naloxone injection. To control for the volume of CS intake (i.e., for the possibility that reduced body weight was simply a consequence of low CS intake), half of the rats in the CS- (saline) group were only given as much CS as was consumed by the rats in the CS+(morphine) group. This group is hereafter referred to as the Yoked group.

Results. Five min CS intake and change in body mass data were analyzed using a one way ANOVA varying condition (CS+, CS-, Yoked). As was expected, intake during the CS- condition was greater than intake during either the CS+ condition and during Yoked access ($F(2,28) = 3.64$, $p < 0.05$, see Figure 2 (left)). That said, rats injected with the opiate antagonist, Naloxone, following intake of the CS+ exhibited a significant decrease in body mass compared with those for whom Naloxone treatment followed access to the CS- or the Yoked controls, $F(2,28) = 13.53$, $p < 0.0001$, see Figure 2 (right). Therefore, we conclude that the change in body mass is not a mere consequence of low CS+ intake, but rather depends upon the valence of the taste cue. When the cue predicts the subsequent administration of morphine (i.e., the CS+), body weight is reduced following the unexpected injection of the opiate antagonist, Naloxone. The drug-paired taste cue, then, can elicit the onset of a state of conditioned withdrawal.

Experiment 3. An intact thalamic orosensory area is not required for cue induced withdrawal when a contextual cue (i.e., runway cues) superseded the saccharin taste cue in the prediction of the morphine.

Methods. The subjects were 34 male Sprague Dawley rats, obtained and housed as described above. Fifteen subjects received TOA lesions and 19 served as Sham operated controls. All experimental manipulations were conducted in a runway (7.85 x 7 x 5.5 inches) made of Plexiglas. A start box and goal box (19.5 x 9.25 x 5.5 inches) of identical dimensions are located at opposing ends of the alleyway. The start box was equipped with an automated, retractable sipper dispenser, such that a bottle could be advanced through a 1.3cm diameter hole in the end wall. A closed circuit lick-o-meter was used to monitor licking behavior. The latency to leave the start box and enter the goal box was detected by interruption of 6 pairs of infrared photocell emitters. *Procedure.* All rats underwent a water deprivation schedule as described above. After morning water intake stabilized, testing began. The rats were run in the runway one at a time. To begin each session, each rat was placed into the start box where they received 5 minute access to a 0.15% saccharin taste cue. After the 5-minute period was over the doors to the start box and goal box opened. The rats were then given a maximum of 15 minutes to break the photobeam at the far wall of the goal box. Once this photobeam was broken the door to the goal box closed and the rats immediately received an IP injection of morphine or saline. The rat was then placed back into the Goal Box for 5 minutes. Once the 5 minutes were up the rat was immediately removed from the runway and placed back in its homecage. One hour after being removed from the runway, the rat was given 1 hour access to water on its homecage. On the 7th day, rats were run the same as described above with the exception that all rats received SC injections of Naloxone rather than morphine. Two hours after the rats were injected with naloxone their weight was recorded. Thereafter, they were returned to free access to water.

Results. CS Intake. Five min Saccharin intake on the test day was analyzed using a 2 x 2 factorial ANOVA varying lesion (Sham vs. TOAx) and condition (saline vs. morphine). There was a significant main effect of condition ($F(1,30) = 46.9, p < 0.0001$), with saline treated subjects consuming more saccharin than rats in the morphine condition. Neither the main effect of lesion, $p = 0.15$, nor the Lesion x Condition interaction, $p = 0.61$, was significant. The data were also analyzed separately for each lesion condition. For the both groups there was a significant main effect of condition (Sham: $F(1,17) = 21.93, p < 0.001$; TOAx: $F(1,13) = 26.48, p < 0.001$, see Figure 3 (left)) indicating that both groups learned to suppress intake of the taste cue when taste-drug pairings occurred in the runway. *Change in Body Mass.* The change in body mass 2 hours following Naloxone administration on test day was analyzed using a 2 x 2 factorial ANOVA varying lesion (Sham vs. TOAx) and condition (saline vs. morphine). There was a significant main effect of condition ($F(1,30) = 43.87, p < 0.0001$), with saline treated subjects gaining body mass and morphine treated subjects losing body mass following Naloxone administration. This was found to be significant for each lesion group (Sham: $F(1,17) = 25.01, p < 0.001$; TOAx: $F(1,13) = 19.21, p < 0.001$, see Figure 3 (right)). The lesion, then, did not disrupt either morphine-induced suppression of CS intake or the development of cue-induced withdrawal (as indexed by a naloxone-induced loss in body weight) when the context cues of the runway superseded the gustatory stimulus as a cue for drug.

Conclusion: Bilateral ibotenic acid lesions of the TOA prevent avoidance of a natural reward cue when paired with morphine. Additionally, when the cue is presented followed by a subthreshold dose of Naloxone, an aversive withdrawal state causes a loss in body mass for several hours thereafter in the Sham (i.e., non-lesioned) rats. This drop in body mass is not seen in TOAx rats with a history of taste-morphine pairings. This change in body mass was not due to the decreased

volume of cue intake, as was shown in the second experiment. Finally, the TOAx rats were able to associate the taste cue with morphine in the presence of contextual cues, and to exhibit evidence for cue-induced withdrawal, indicating that the lesion is specifically interfering with the association of a taste cue with a drug. Other nuclei, then, must mediate the context-drug association.

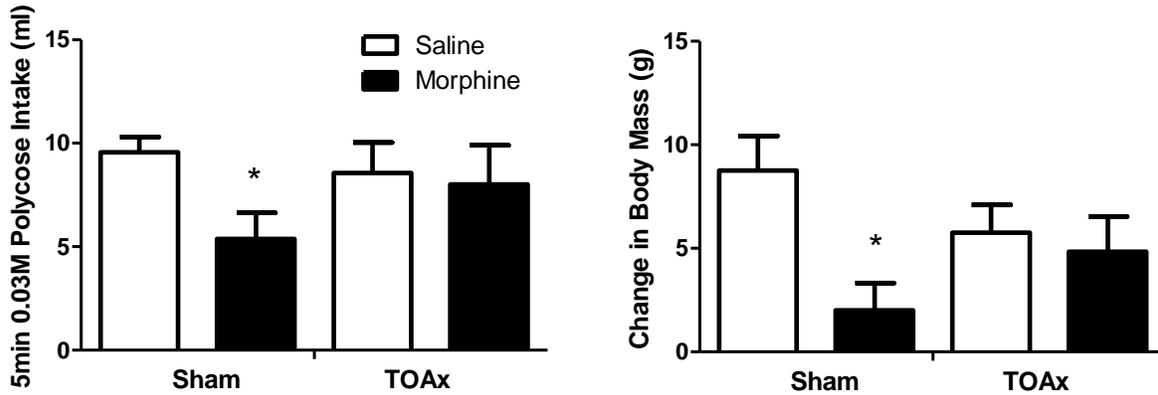


Figure 1. Taste Cue Suppression and Change in Body Mass when Polycose Predicts Morphine Mean (\pm SEM) 0.03M Polycose intake (left) and change in body mass following Naloxone administration (right) on test day. Sham rats in the morphine group had lower intake and lower body mass change compared with the Sham saline group. This difference was not seen in the TOAx group. * = $p < 0.05$

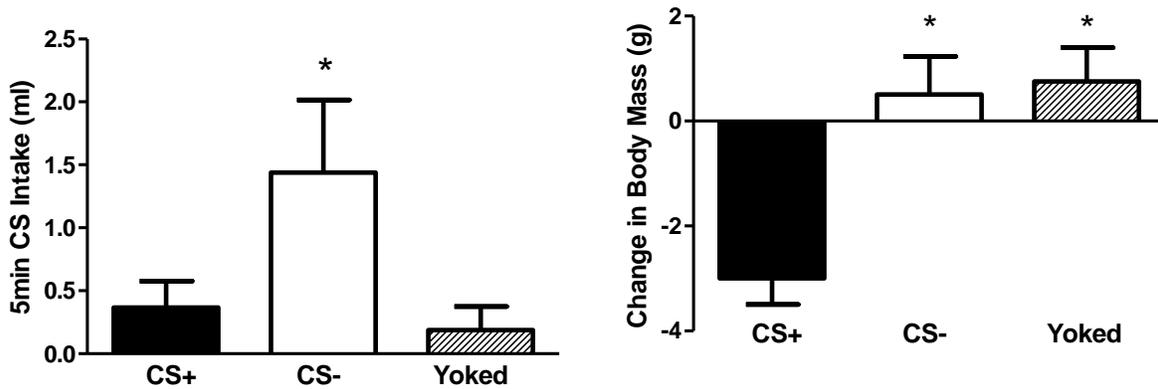


Figure 2. Within-Subjects Taste Cue Suppression and Change in Body Mass Mean (\pm SEM) CS intake (left) and change in body mass following Naloxone administration (right) on test day. When the taste cue predicted morphine (CS+ condition), subjects suppressed intake of the taste cue and lost body mass after Naloxone administration, compared to rats tested in the CS- condition (i.e., when the cue predicted saline). The Yoked group, which consumed an equal amount of the taste cue as the CS+ group, did not show a decrease in body mass following Naloxone administration. Therefore, the change in body mass following Naloxone administration was independent of the CS volume consumed. * = $p < 0.05$

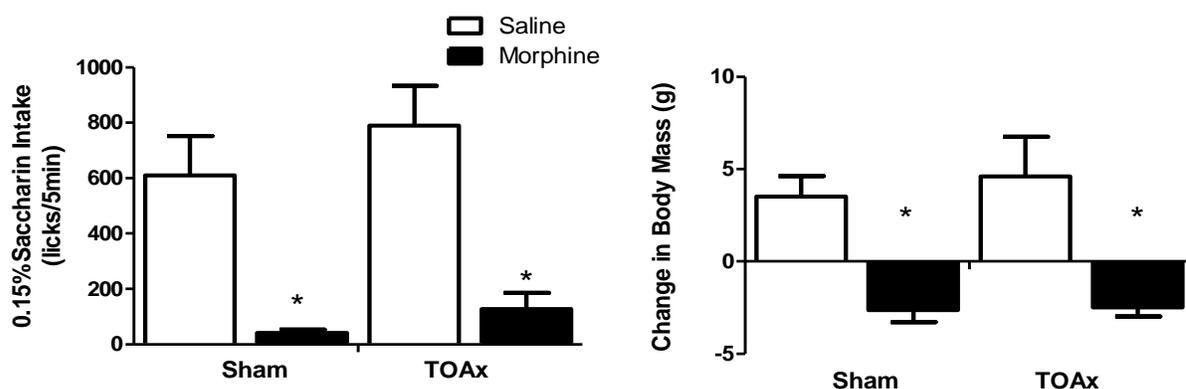


Figure 3. Taste Cue Suppression and Change in Body Mass when Context Predicts Morphine Mean (\pm SEM) 0.15% saccharin intake (left) and change in body mass following Naloxone administration (right) on test day. Both Sham and TOAx rats in the morphine group had lower intake and lower body mass change compared with their respective saline group. * = $p < 0.05$

Research Project 5: Project Title and Purpose

Iron-Induced Changes that Predispose to Malignancy - Hepatocellular carcinoma (HCC) is one of the most common cancers in the world and also one of the most deadly. It is clear that chronic excess iron deposition is implicated in the development of HCC. Understanding the cellular and molecular changes occurring in the liver exposed to chronic excess iron deposition may lead to the future identification of biomarkers for early detection of progression and may also provide improved strategies for early chemopreventive intervention.

Duration of Project

1/1/2008 – 6/30/2009

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 6: Project Title and Purpose

Nutrient Overload as a Causative Factor in Diabetic Retinopathy - The research project focuses on the novel view of nutrients as signaling molecules that act through signal transduction cascades to regulate various cellular functions. The nutrient sensing signaling pathways are not only interconnected at multiple levels but they are also coupled to the insulin receptor signaling pathway. Diabetes is a disease characterized not only by an impaired insulin receptor signaling pathway (due to lack of insulin and/or insulin resistance), but also by elevated blood concentrations of nutrients such as glucose, fatty acids and branched-chain amino acids.

Therefore, the overall purpose of the project is to gain a better understanding of the relative contributions of the impaired insulin receptor and the nutrient-activated signaling cascades to the development of diabetic retinopathy.

Duration of Project

1/1/2008 - 6/30/2009

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

Co-Crystals of Novel Integrase Mutants and Retroviral DNA - The permanent integration of retroviral DNA into cellular DNA leads to immunodeficiency, neurological, and neoplastic diseases. The long-term goal of this project is to benefit human health by developing ways to interfere with retrovirus integration. The viral integrase enzyme causes integration by specifically nicking the ends of viral DNA at a precise location, and then inserting that viral DNA into any site in cellular DNA. However, our understanding of how this one enzyme interacts with, and acts on, two different kinds of DNA is limited. Analyzing the molecular structures of crystals that contain integrase bound to viral DNA could finally reveal how integrase distinguishes between viral and cellular DNA. Thus, this project is directed at making it possible to obtain these long-sought crystals.

Duration of Project

1/1/2008 – 6/30/2009

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 Infrastructure Project 8: Project Title and Purpose

Research Infrastructure - Biological Research Laboratory Construction - The purpose of this project is to design and build an Animal Biosafety Level three (ABSL-3) research laboratory for the study of immunology and infectious diseases requiring high level biocontainment.

Anticipated Duration of Project

1/1/2008 – 12/31/2011

Project Overview

The scope of this project is to design and build an animal biosafety level three (ABSL-3) research laboratory for the study of immunology and infectious diseases requiring high level biocontainment. Even with the knowledge and biotechnology now available, we still face serious threats to human health and well-being from serious and highly transmissible infectious diseases and / or potential agents of bioterrorism such as avian influenza and anthrax. To respond, we must create a research environment and provide the infrastructure necessary to allow investigators to study these pathogens and discover new ways to detect, prevent or cure these diseases.

The project was temporarily put on hold while the Penn State design team put together an expanded design and grant proposal for the National Center for Research Resources (NCRR) Recovery Act Construction Program. The goal of the grant proposal was to expand the ABSL-3 facility to include an insectary, and additional laboratory and animal holding space. In addition the expanded facility will have increased redundancies to meet National Institutes of Health (NIH) construction guidelines. The proposal was successful and an award of \$14.8 million was received to expand the facility. The proposed Biological Research Laboratory will provide space, support, and biocontainment for basic, applied and diagnostic research; national, state and community outreach; and education on important human and / or zoonotic pathogens. It will include state of the art laboratories, an insectary, and animal resources, facilities and services that are recognized within and outside the University as being of the very highest quality consistent with our talents and resources. This facility will be composed of a number of ABSL-3 suites for *in vivo* research using small animal models of human disease (primarily rodents and poultry models). Each suite will have independent air locks to support multiple agent research as well as providing compartmentalization to mitigate cross-contamination concerns. Changing rooms and shower out facilities are included as required. Supporting the ABSL-3 suites will be laboratories for *in vitro* bacteriology, virology, and molecular biology procedures. Outside of the biocontainment area another support laboratory provides preparatory space for the activities within the barrier. A conference/classroom/break room allows for on-site training sessions and staff meetings, and a manager's office and loading dock are also included. This project has been expanded to a \$23,000,000 building project that will encompass 20,000 gross square foot, and will provide laboratories, animal holding space as well as an insectary that will support the critical need for biocontainment research space at the Pennsylvania State University. This facility will be a unique and much needed resource for infectious disease research.

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Biao He, PhD, Girish Kirimanjeswara, PhD, Craig Cameron, PhD, Vivek Kapur, PhD - employed by Pennsylvania State University

Expected Research Outcomes and Benefits

The proposed enhanced Biological Research Laboratory, an Animal Biosafety Level 3 (ABSL-3) facility, presents excellent opportunities for infectious disease research and will greatly enhance the research capabilities on campus. It is not possible to do research with highly infectious agents such as anthrax and avian influenza without proper protection and biocontainment. The new enhanced facility will allow work with vector borne diseases due to the inclusion of an insectary. This facility will provide special air handling capabilities to filter the exhausted air, liquid and solid waste decontamination, high security, and standard operating procedures within the facility to ensure the safe handling of such agents. The facility is organized into a central spine that connects all of the research spaces to the central decontamination and support areas of the project.

Summary of Research Completed

This project is continuing. The environmental assessment was completed and a signed Finding of No Significant Impact (FONSI) was issued. The design team completed final design documents and submitted them to the NIH technical review team. The NIH architects and engineers have reviewed the project in detail and have approved the construction documents. The technical review process is therefore complete and we should receive a revised Notice of Award shortly. This notice will allow the release of the \$14.8 million in federal funds for the project. Penn state is currently preparing a Notice of Federal Intent that will be filed at the time construction begins.

The following is an outline of progress since the previous report.

- June 2010 – approval granted by NIH to continue using Torcon, Inc. construction management (CM) services as Torcon provided constructability and cost estimating services during the design process.
- October 2010 – Torcon prepared a preliminary Guaranteed Maximum Price (GMP) for use by Penn State officials
- November 2010 – Penn State Board of Trustees approved the preliminary GMP and granted approval for the project
- December 2010 – Penn State and Torcon begin preparing sub contractor packages and sub contractor requirements
- January 2011 – sub-contractors begin submitting pre-qualification information to Torcon. Project bidding requirements were posted on the Penn State construction web site.
- February 2011 – Torcon and Penn State hosted a pre-bid conference for sub-contractors. A second pre-bid opportunity was held two weeks later to accommodate subcontractors who could not attend the first pre-bid meeting.
- Bids were received by Penn State and Torcon on subcontractor packages March 23 and 24, 2011

- The design team, Penn State and Torcon conducted package de-scoping meetings with sub contractors following receipt of bids. The final bid packages are in review and the bid package for a CM at risk, including the final GMP as well as a discussion of the pre-qual, pre-bid, and bidding process, will be submitted soon. Once this is reviewed, NIH will issue a revised Notice of Award.
- The contract will be prepared by Penn State and presented to Torcon for signing. Once signed, Penn State issues the Notice to Proceed, the initial job conference will be held and construction begins.

In addition, we have hired a scientific director to oversee the research mission of the facility. Girish Kirimanjeswara is a faculty member with ABSL3 experience who will bring research experience and leadership to the facility. John Henneman has also been hired as the BRL manager. John has 15 years of experience overseeing the construction and operation of BSL3 facilities. He will oversee the construction of the facility, start-up, validation and commissioning, as well as maintenance and operations once the facility is built.

The building has been designed to meet construction and redundancy standards as described in the NIH Design Policy and Guidelines for ABSL-3 and ABSL-3 enhanced research space for high pathogenicity infections (such as avian influenza). The building will feature numerous security features including a perimeter fence, card or proximity reader access, biometric security to access the containment zone, as well as motion detectors, lights and security cameras. Several levels of security will be in place, only limited access to the facility will be allowed, and all entries will be documented. Training and security clearances will be required prior to entry according to a biosafety manual specific to the BRL. Standard Operating Procedures (SOPs) are in development specifically for the BRL to train and certify all personnel working within containment, to ensure safety and security according to Occupational Health and Safety guidelines. Specific policies are under development for practices such as showering, minimal sharps use, and decontamination. SOPs to minimize the production of aerosols are being written, and all animal specific regulations and biosafety procedures will be followed. Biohazardous materials use authorization (Recombinant DNA or Human Pathogen registration, Select Agent use etc.) will be instituted as appropriate.

Research Project 9: Project Title and Purpose

Translational Research in Polycystic Ovary Syndrome - The purpose of this project is to continue studies that will identify genetic contributions to Polycystic Ovary Syndrome (PCOS). PCOS is the most common cause of infrequent menses and excess male hormone in women and affects 5-10% of the female population. We intend to closely study families, where this disorder clusters, to better understand the heritability of its traits, as well as by studying the children of PCOS mothers to better understand how it develops. We will collect and study DNA and human thecal cells from ovarian follicles to better understand how a genetic variant in the Fibrillin 3 gene on chromosome 19 contributes to the etiology of the syndrome.

Duration of Project

1/1/2008 – 6/30/2011

Project Overview

We will study families of women with PCOS (Aim 1) and thecal cells from ovarian follicles (Aim 2)

Specific Aim 1: To phenotype probands with PCOS and their parents and siblings, and to identify children of mothers with PCOS for future study.

Our goal is to phenotype 25 family units (consisting of a proband, her parents, and any sisters) and an estimated 75-100 human subjects. This phenotyping protocol has been in place since 1994, and involves for the proband with suspected PCOS: a questionnaire, blood tests for serum androgens and exclusions of other possible diagnoses (Congenital adrenal hyperplasia, hyperprolactinemia, etc.), blood for DNA extraction, a 2h oral glucose tolerance test, and a transvaginal ultrasound. For the other family members, the oral glucose tolerance test and the transvaginal ultrasound (females only) is optional. Additionally we will identify mothers with PCOS and their young children (Ages 4-16) to participate in a protocol that if funded will study the children longitudinally for reproductive and metabolic abnormalities as they go through puberty.

Specific Aim 2: Find the determinants and identify effects associated with the genetic marker we have already identified (a microsatellite marker within the Fibrillin 3 genet- D19S884). What are the “downstream” effects of variation at D19S884 and neighboring sequences?

We will test the hypothesis that allele 8 of D19S884 “marks” a PCOS risk allele. Experiments will determine whether: (1) FBN3 is a PCOS susceptibility gene, (2) a gene located in the D19S884 region has alleles associated with PCOS, and (3) the D19S884 region regulates expression of a nearby gene that influences risk of PCOS. The goal of these experiments is to investigate the FBN3 locus and surrounding region to determine the underlying mechanism that explains the findings of linkage and association between D19S884 and PCOS. In normal and PCOS theca cells we will investigate: (1) whether allele 8 affects splicing of the FBN3 gene resulting in the formation of a novel transcript (which has unique biological activities that predispose to PCOS), (2) the possibility that D19S884 (and particularly allele 8) is a cis element that regulates expression of the FBN3 or other genes, and (3) if fibrillin 3 influences the function of theca cells.

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Other Participating Researchers

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Expected Research Outcomes and Benefits

Specific Aim 1: Anticipated Results: We expect to continue to find a high prevalence of hyperandrogenism and glucose intolerance among first degree relatives of women with PCOS. Further we expect that most mothers with PCOS and their children will elect to participate in our protocol studying children when it is active.

Specific Aim 2: Anticipated Results: We expect to find alternatively spliced forms of the *FBN3* transcript involving exon 56 that have not been previously reported. We will take CCL25, a secreted chemokine, as an example of how we would approach documenting an association between D19S884 and expression of a candidate gene and the role that gene might play in producing the PCOS phenotype. We expect to be able to utilize the approaches as outlined above for the study of CCL25 to confirm an association between the PCOS risk allele of D19S884 and altered CCL25 levels.

Summary of Research Completed

We have submitted two manuscripts and have been working on additional publications. We have received two extramural NIH grants that were facilitated by this grant that allowed us to continue our recruiting and research.

Research Project 10: Project Title and Purpose

Neural Systems of Ingestive Behavior - The purpose of this project is to investigate the pathways from the central gustatory system to brain structures that mediate reward, such as the nucleus accumbens. These experiments will elucidate where and how the hedonic effects of a taste, its pleasure or aversion, are elaborated from the afferent sensory message.

Duration of Project

1/1/2008 - 6/30/2009

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

Nuclear Trafficking of the Retroviral Gag Protein - This project will support a graduate research assistant who is studying how retroviruses interact with the cells they infect. The project is designed to elucidate the mechanisms by which retroviral Gag proteins, the major viral structural proteins, utilize cellular transport machinery to travel throughout different subcellular compartments. The long-term goal of this work is to identify novel targets for anti-retroviral therapy.

Anticipated Duration of Project

10/1/2008 - 12/31/2011

Project Overview

Our research program focuses on understanding the molecular mechanisms used by retroviruses to commandeer cellular pathways for the assembly of new virus particles. We discovered that RSV Gag is actively transported into and out of the nucleus, which was unexpected, given that retroviral particles are released from the plasma membrane. We identified two independent nuclear localization sequences (NLSs) and a CRM1-dependent nuclear export signal (NES) in Gag. We hypothesize that RSV Gag enters the nucleus to bind to the viral genomic RNA (gRNA), forming a viral RNP complex within the nucleus. Our recent work has garnered substantial support for this hypothesis.

Aim 1. Examine Gag-Gag and Gag-viral RNA interactions in the nucleus and nucleolus. The earliest steps in virus assembly—those immediately following synthesis of Gag on cytosolic ribosomes—are poorly understood. We have preliminary evidence that RSV Gag transiently traffics through the nucleolus, and in this aim we will investigate the kinetics, interactions, and purpose of intranuclear transit of Gag. Of critical importance to this aim are our well characterized RSV Gag trafficking mutants and our newly developed system for imaging Gag protein-protein and protein-RNA interactions in the nucleus. To study nuclear and nucleolar interactions of Gag, we propose to:

- a. Examine Gag-Gag interactions in nuclei of living cells.
- b. Define the mechanism underlying subnuclear and nucleolar trafficking of Gag.
- c. Examine viral RNP complex formation, mobility and kinetics in the nucleus.

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Expected Research Outcomes and Benefits

This project focuses on defining the molecular mechanisms underlying RSV Gag nuclear trafficking and Gag-mediated gRNA encapsidation. The experimental plan is built on a firm

foundation of novel published and preliminary data suggesting that Gag recruits nuclear and cytoplasmic factors to direct viral RNP transport from the nucleus to the site of virus assembly. Our integration of genetic, biochemical, proteomic, and dynamic microscopic imaging form a multi-pronged approach that has the potential to challenge the current dogma regarding how retroviruses select their genomes for incorporation into new virions. Our ultimate goal is to identify new targets for the development of antiretroviral therapy that could be used to treat retroviral diseases like AIDS.

Summary of Research Completed

Subproject 1: Nucleolar interactions of the Gag protein of mouse mammary tumor virus (MMTV) with ribosomal protein L9 (RPL9).

MMTV assembles immature capsids (Type B particles) in the cytoplasm, just outside the nucleus. Capsids are subsequently transported to the plasma membrane for release from the cell. Upon release, the Gag polyprotein precursor is cleaved by the viral protease to produce an infectious virion. The mature products of Gag are the MA, pp21, p3, p8, n, CA, and NC proteins. This assembly pathway characterizes MMTV as a beta retrovirus.

Unlike some oncogenic retroviruses, MMTV does not encode an oncogene. Instead, the mechanism underlying the development of tumors in infected mice results from a complex interplay of virus infection, immune cell transmission, hormonal stimulation of infected tissues, and host genetics. Integration of the viral DNA genome into the host chromosome disrupts normal cellular genes that regulate growth, differentiation and cell cycle control; alteration of these pathways by proviral insertional mutagenesis results in uncontrolled growth and cellular transformation. Additionally, virus-encoded sequences appear to contribute directly to tumorigenesis. For example, the Env protein has transforming activity in cultured cells through the expression of an immunoreceptor tyrosine-based activation motif. Furthermore, comparison of the properties of chimeric viruses derived from the highly tumorigenic MMTV(C3H) strain and the endogenous retrovirus *Mtv1* indicated that the *gag* sequence of C3H imparts an increased incidence of mammary tumors in mice. However, the mechanism by which Gag contributes to tumor formation it is not known. We hypothesized that a cellular factor that interacts with Gag might mediate downstream effects that promote malignant transformation in infected mice.

To identify binding partners of MMTV Gag, our collaborator Tatyana Golvkina, PhD, University of Chicago, and her colleagues performed a yeast two-hybrid (Y2H) screen using MMTV(C3H) Gag as bait and a murine cDNA library from lactating mammary gland as the prey. They found that the ribosomal protein L9 (RPL9), a member of the large ribosomal subunit and a putative tumor suppressor, was an interacting partner of MMTV Gag. In work supported by this grant and performed in the Parent laboratory, overexpression of RPL9 resulted in the localization of a subset of Gag to the nucleolar compartment, suggesting that MMTV Gag transiently traffics through the nucleus. This result was unexpected, because MMTV assembles capsids in the cytoplasm, and MMTV Gag has not previously been reported to localize to the nucleus. Our data indicate that MMTV Gag and RPL9 interact in the nucleolus, a subcellular compartment involved in ribosome biogenesis, cell cycle control, and DNA damage responses, all of which are regulatory processes involved in carcinogenesis. Our findings suggest that the RPL9-Gag

interaction might contribute to the development of tumors in MMTV-infected mice.

To determine whether the nucleolar relocalization of MMTV(C3H) Gag was an exclusive property of RPL9, we examined the effect of cells overexpressing other nucleolar proteins: fibrillarin, nucleolin, and B23. The distribution of MMTV(C3H) Gag was unchanged in these cells (Figure 1A), indicating that general overexpression of nucleolar proteins did not induce Gag localization to nucleoli. We also expressed ribosomal proteins RPL4-GFP and RPS6-GFP to examine whether Gag relocalization was a common feature of ribosomal proteins. MMTV(C3H) Gag did not colocalize with RPL4 or RPS6, indicating that nucleolar retention of Gag was specific to RPL9 (Figure 1B). These results suggest that overexpression of RPL9 specifically “traps” Gag in nucleoli and that the endogenous RPL9 levels are not sufficient to localize Gag to nucleoli at a concentration that allows visualization.

Next, to determine whether the relocalization of Gag by RPL9 was unique to MMTV, we coexpressed murine RPL9-mCherry with the RSV Gag-GFP or HIV Gag-GFP proteins in quail or human cells, respectively. The RSV Gag-GFP and HIV Gag-GFP proteins localize to the cytoplasm and plasma membrane under steady-state conditions, and RPL9 overexpression did not alter their subcellular distributions (Figure 1C). Because RPL9 is highly conserved among higher eukaryotes ($\geq 98\%$ homology between mouse and human or chicken), it is unlikely that differences in RPL9 protein sequences explain the lack of effect. Thus, it appears that the ability of RPL9 to relocalize Gag to nucleoli is specific to MMTV and is not shared by other retroviral Gag proteins.

The tumorigenic effect of MMTV(C3H) Gag in mice was previously mapped to the CA and NC coding regions. To identify the specific region of Gag that was responsible for the interaction with RPL9, we constructed a series of C-terminal truncations of MMTV(C3H) Gag fused to GFP. Expression of the truncated proteins by themselves in NMuMG cells revealed that the GFP-fused proteins were distributed primarily in the cytoplasm with a small amount of fluorescence in the nucleus but complete exclusion of nucleoli. We found that MA-CA.Gag-GFP and subsequent C-terminal deletions were localized diffusely throughout the cytoplasm rather than being concentrated in discrete foci. The diffuse localization was likely due to deletion of the NC region of Gag, which is necessary for Gag-Gag and Gag-RNA interactions that facilitate the formation of capsids in the cytoplasm of transfected cells.

To map the region of RPL9 necessary for the interaction with MMTV Gag, we made N-terminal and C-terminal deletions of mouse RPL9 based on structural studies of prokaryotic RPL6 (Figure 2). Examination of the localizations of the N-terminal (NT) and C-terminal (CT) portions of RPL9 fused to mCherry demonstrated that the two proteins have markedly different distributions (Figure 2C, middle panel). RPL9.NT-mCherry localized primarily to nucleoli whereas RPL9.CT-mCherry excluded the nucleus and formed cytoplasmic aggregates. Interestingly, when expressed with Gag-GFP or in MMTV infected cells (Figure 2D), RPL9.NT did not relocalize Gag to nucleoli, but a subset of the Gag foci colocalized with the cytoplasmic aggregates of RPL9.CT. This observation led us to hypothesize that RPL9 interacts with Gag through its C-terminal region and uses a NLS/NoLS within its N-terminus to traffic to nucleoli. Both the NoLS and the Gag interaction region of RPL9 appear to be necessary to mediate accumulation of Gag within nucleoli. To test this hypothesis, we fused the NoLS of the HIV-1 Rev protein (Figure 2E) to the C-terminus of RPL9.CT in an effort to restore nucleolar

trafficking of RPL9. Addition of the Rev NoLS to RPL9.CT did restore the ability of Gag to accumulate within nucleoli (Figure 2F). As a control, Rev NoLS-mCherry did not alter the distribution of Gag. These results indicate that the C-terminus of RPL9 is sufficient for binding to MMTV Gag.

Our working model is that due to the transient nature of MMTV Gag-RPL9 binding, Gag does not normally appear to accumulate in the nucleolus. Moreover, it is likely that only a small fraction of the total cellular population of Gag traffics through the nucleus and nucleolus, and thus this fraction is not visualized under steady-state conditions. However, with RPL9 overexpression the Gag:RPL9 ratio is altered, tipping the balance in favor of RPL9. Under these conditions, Gag does not disassociate (or disassociates more slowly) from RPL9. Consequently, MMTV Gag appears to be “trapped” in the nucleolus; in other words, Gag continues to enter the nucleolus but it is retained there due to the high expression levels of RPL9. Alternatively, it is possible that a host factor that is limiting in the cell mediates Gag nucleolar/nuclear egress by disrupting Gag:RPL9 binding, displacing RPL9. If RPL9 is present in higher concentrations than the export factor, then Gag would remain bound to RPL9 and unable to leave the nucleus/nucleolus. Lastly, RPL9:Gag interactions could be initiated in the cytoplasm, followed by translocation of the complex into the nucleus. However, the methods used in this study did not detect RPL9:Gag interactions outside of the nucleolus. Further experimentation will be needed to differentiate between these possibilities. In addition, we do not yet know whether the RPL9:Gag interaction plays a direct role in MMTV capsid assembly.

Subproject 2: Optimize conditions to study Gag assembly intermediates in vitro.

We previously discovered that the Rous sarcoma virus (RSV) Gag protein is actively imported into the nucleus where it binds to the viral RNA genome. This Gag:RNA binding event triggers a conformational change in Gag that enhances binding to the host export complex CRM1:RanGTP. The Gag:viral RNA:CRM1:RanGTP complex is exported out of the nucleus and the Gag:viral RNA is transported to the plasma membrane where complete particles are released from the cell. We are determining the mechanisms that control the assembly of Gag:viral RNA into a multimeric ribonucleoprotein complex that is transported to the plasma membrane. We are using biochemical and biophysical approaches to elucidate the molecular architecture of the protein-RNA building blocks used to build a retrovirus particle. Complete immature virus particles are composed of a hexameric lattice of virus-encoded Gag proteins.

In collaboration with John Flanagan, we recently optimized a robust method for synthesizing highly purified Rous sarcoma virus Gag protein from *E. coli*. We are using transmission electron microscopy to examine macromolecular complexes formed by oligomers of Gag bound to nucleic acids. We have synthesized a viral RNA sequence using *in vitro* transcription and will begin optimizing end-labeling with nanogold particles. Using recombinant Gag proteins and viral RNAs, we will be able to perform biophysical analyses of the “building blocks” of retroviral particle.

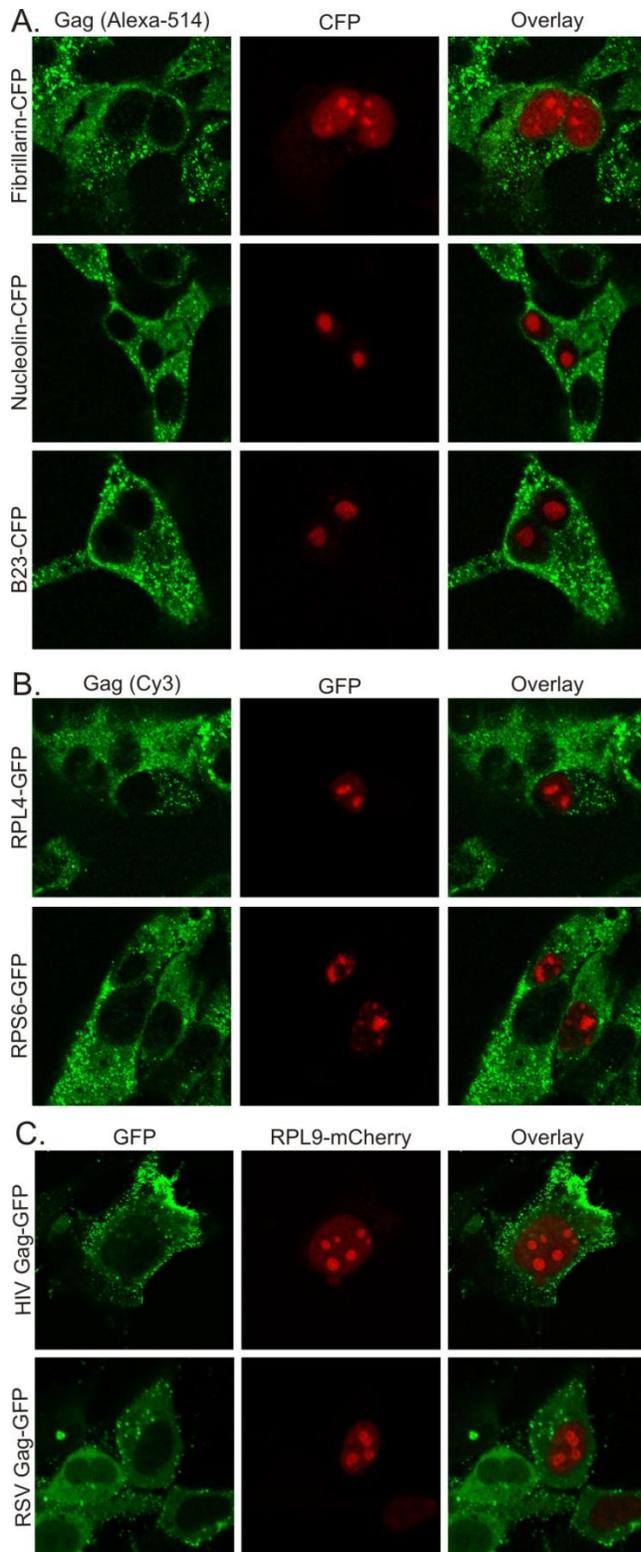


Figure 1. Examination of nucleolar cellular proteins with MMTV(C3H) Gag and other viral Gag proteins with mouse RPL9 in NMuMG cells. A) MMTV(C3H)-infected NMuMG cells were transfected with either fibrillarin-CFP, nucleolin-CFP, or B23-CFP and imaged by confocal

microscopy. Gag proteins were immunofluorescently stained with anti-CA antibodies followed by Alexa-514 conjugated secondary antibodies. Images are false-colored using ImageJ to show Alexa 514 as green and CFP as red. B) GFP-fused ribosomal proteins RPL4 and RPS6 were overexpressed in MMTV(C3H)-infected NMuMG cells. Viral Gag was detected with immunofluorescence using an anti-CA primary antibody and a Cy3-conjugated secondary antibody. Images are false-colored to show Cy3 as green and GFP as red. C) Upper panel: HeLa cells were co-transfected with HIV Gag-GFP and mouse RPL9-mCherry. Lower panel: QT6 quail fibroblasts were co-transfected with RSV Gag-HGP and mouse RPL9-mCherry. Quail cells are shown at a higher magnification than HeLa cells.

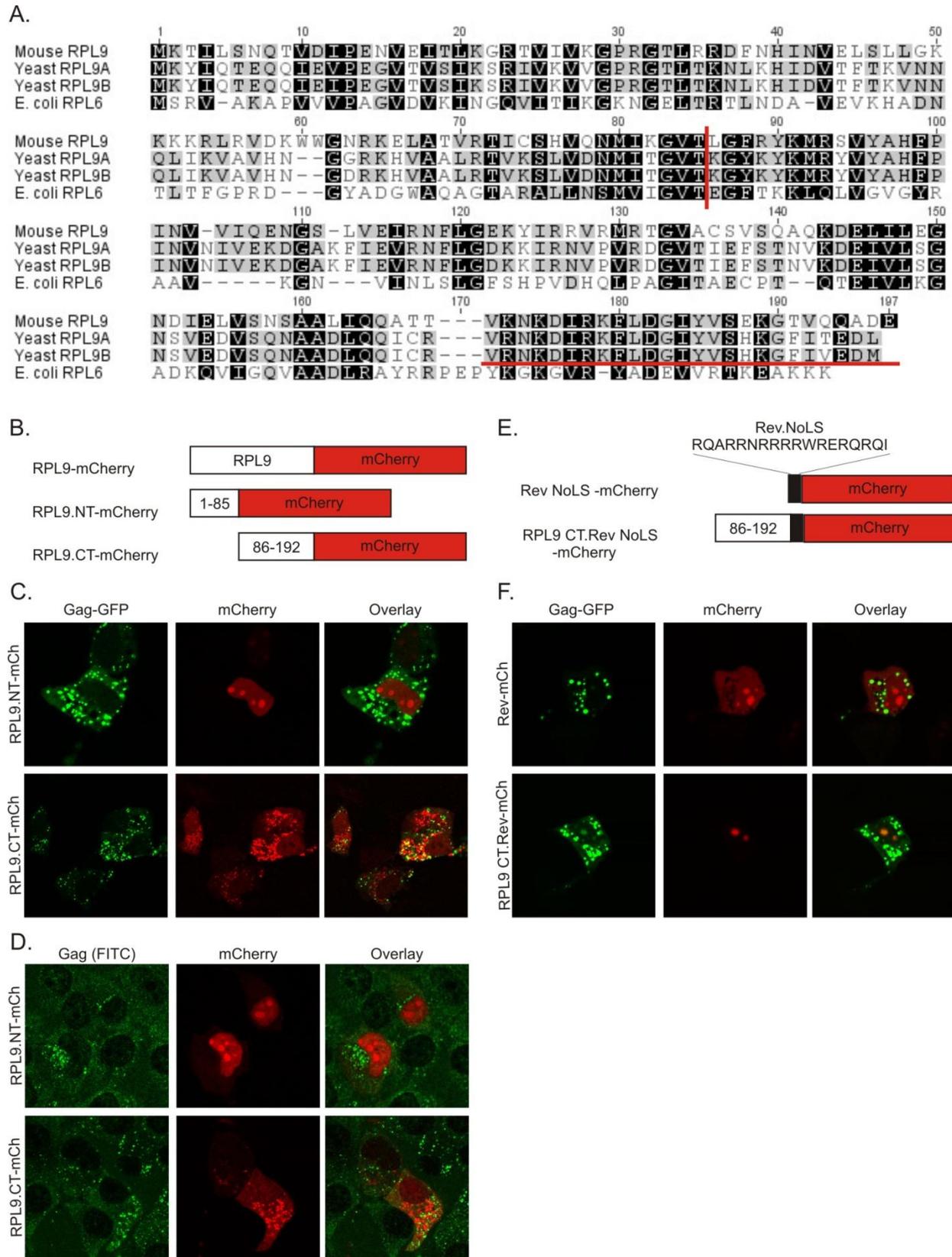


Figure 2. Mapping the Gag interaction domain within RPL9. A) Amino acid comparison of

RPL9 from yeast (NCBI, NP_011368, NP_014332) and mouse (NM_011292), and RPL6 from *E. coli* (BAE77986). Black boxes highlight sequence similarity among all four sequences, gray boxes outline conserved residues among three species. The vertical red line signifies the division between the N-terminal and C-terminal regions of RPL9. The horizontal red line indicates the high sequence homology between mouse and yeast sequences in the C-terminal region (Geneious Pro 5.1.3 software; www.geneious.com). B) Schematic of the N-terminal and C-terminal regions of RPL9 fused to mCherry. The RPL9.NT consists of amino acids 1-85, and the RPL9.CT consists of amino acids 86-192. C) Confocal microscopy of NMuMG cells co-transfected with C3H Gag-GFP and mCherry-fused RPL9 domains. D) Confocal microscopy of MMTV(C3H)-infected NMuMG cells transfected with the N- or C-terminal RPL9-mCherry. Gag was detected with anti-CA primary antibody and FITC-conjugated secondary antibody. E) Schematic diagram of the HIV Rev nucleolar localization signal (NoLS) fused to the N-terminus of mCherry and inserted into the RPL9.CT-mCherry construct between the RPL9 and mCherry sequences. F) Confocal images of Gag-GFP coexpressed with Rev NoLS-containing RPL9 constructs.

Research Project 12: Project Title and Purpose

Chronic Oxidative Stress and the Redox Proteome in Normal Breast Parenchyma - Breast cancer is a disease that is a long time in the making during decades of exposure of breast tissue to pro-carcinogenic conditions. In this project we will test the *hypothesis* that increased levels of reactive oxidants (molecules that disrupt protein function and may be related to carcinogenesis) in human breast tissue are associated with altered levels of specific regulatory molecules in these cells, thereby disrupting normal function. We will use newly developed proteomic techniques to obtain a global picture of the oxidized proteins present in human breast tissue. Demonstrating that these alterations occur would implicate the chronic and progressive exposure to oxidants as a pro-carcinogenic event and potentially provide endpoints that could be assessed during dietary and other therapeutic interventions designed to minimize or reverse these changes.

Duration of Project

1/1/2008 – 12/31/2010

Project Overview

The overall hypothesis addressed in this project is that under conditions of chronic oxidative stress, oxidative modifications of proteins participating in signaling networks important for maintaining cellular homeostasis play an essential role in allowing breast cancer to develop within the stressed mammary epithelium. More specifically, failure to neutralize reactive oxidant species disrupts redox-responsive regulatory proteins and leads to cellular dysfunction, thus providing a milieu for cancer cells to evolve and ‘take over’. The “proof of principle” experiments proposed to test this hypothesis will focus on the cysteine/thiol moiety of proteins because of their known susceptibility to oxidation and evidence of the functional consequences of their state of oxidation. The two interrelated specific aims of the project are: *Aim 1*) to use proteomic methodology to establish the profile and amounts of thiol proteins in their reduced state and in a reversible state of oxidation in mammary epithelium and surrounding stroma of women without breast cancer who are representative of women living in our high breast cancer

risk environment and; *Aim 2*) use cytochemical techniques to localize and assess the levels of markers of chronic oxidative stress in breast parenchyma of the same women.

Protein will be obtained for proteomic analysis from terminal ductal lobular units and interlobular stroma isolated by laser capture microdissection from sections from flash-frozen breast tissue obtained at reduction mammoplasty from cohorts of women of different age groups. Profile of reduced and reversibly oxidized cysteine/thiol proteins in the extracted proteins will be obtained using two-dimensional difference gel electrophoresis. Localization of markers of oxidative stress (including 4-hydroxynonenol, protein carbonyls and 3-nitrotyrosine) and their levels in tissues used for the proteomic studies will be determined using the method multiplex immunoblotting of tissue sections. This recently introduced method can yield data that are more quantitative and on a larger number of proteins from a tissue section than can be obtained using classical immunocytochemistry. Our hypothesis predicts that the levels of specific thiol proteins and their state of oxidation will vary as a function of the age of the tissue donors and the levels of markers of oxidative stress.

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Expected Research Outcomes and Benefits

This project seeks to redefine the definition of what is normal. Our preliminary observations demonstrate that “normal” breast tissue obtained from young women in a population with a high breast cancer incidence is, in fact, already marked by signs of chronic oxidative stress, a potential precursor to carcinogenesis. This should, at a minimum, be considered as providing a permissive environment for the carcinogenic process. In this project we will use state-of-the-art protein analytical methods to identify the proteins that are modified in this tissue and to determine the nature of their modifications. This information will provide us with information about cellular processes and functions that may be altered by the modification of the proteins, potentially providing new insights into the mechanisms of carcinogenesis.

We expect the results we obtain in this project to offer two benefits in the future. One of these is a better understanding of the events that result from subjecting breast tissue to chronic oxidative stress. There are strong indications that this exposure disrupts normal biology and leads to carcinogenesis. We expect that the "discovery proteomics" approach that we will utilize will

help us identify some of the specific proteins and biological processes that are involved in these disruptions, but were not previously linked to breast cancer carcinogenesis. The second benefit that will be derived from this study will be the identification of affected proteins that may serve as early markers of oxidative stress. These proteins may also prove useful in assessing levels of risk and for monitoring the effectiveness of efforts to reduce oxidative stress by dietary, pharmacologic, or other means, thereby contributing in the future to efforts to prevent breast cancer.

Summary of Research Completed

Our emphasis in this project period has been on Specific Aim 2. Our hypothesis predicts that the presence of 4HNE adducts, our lead marker of chronic OxS, is associated with changes in expression of specific genes that can be linked to carcinogenesis. In order to enable us to test this hypothesis critically we sought to bring to this project recently developed cytochemical methods that would allow for co-localizing multiple proteins in a human breast tissue section. The first such multiplex method we tried was developed by investigators at the NIH. It involves the quantitative transfer of protein from a single formalin fixed paraffin embedded (*FFPE*) tissue section to a stack of up to 20 specialized membranes that can then be probed individually, each for a different protein, using antibodies with the requisite specificity. The major limitation of this method proved to be a lack of imaging system with a resolution needed for co-localizing the transferred proteins. Collaboration with researchers at General Electric Global Research (GE-GR) enabled us to apply their recently developed powerful multiplex immunocytochemical technology (Mx-ICC) to this project that greatly increases the potential to obtain a comprehensive *in situ* proteomic profile of individual cells in tissue sections.

Findings using Mx-ICC. The MX-ICC technology developed by GE-GR makes it possible to localize and quantify a virtually unlimited number of proteins in a *single* FFPE tissue section. It depends on a proprietary reagent for inactivating fluorescent properties of Cy-dyes while leaving DNA staining with 4'6-Diamidino-2-Phenylindole (DAPI) and tissue morphology intact, on advanced imaging hardware and on algorithms for mining the large datasets generated. Briefly, FFPE tissue sections are subjected to successive rounds of immunostaining with two antibodies at a time, tagged with Cy-3 and Cy-5, respectively. After each round of immunostaining images are collected and the Cy-dye generated fluorescence eliminated. DAPI-stained nuclei serve to register (superimpose) the images precisely. Immunostaining is assigned to specific cell types by incorporating rounds of immunostaining with cell-type specific antibodies. In pilot studies this technology revealed marked differences in the immunophenotype of 4HNE+ and 4HNE- ME cells in tissue sections from women in their 20s (Figure 1). The findings from this collaboration revealed marked differences in protein expression profile between 4HNE+ and 4HNE- mammary epithelial cells and already in tissue from young women in their 20s (Figure 1). We were encouraged by the marked alterations in expression of proteins associated with the presence of 4HNE adducts to compare the transcriptional profile of 4HNE+ and 4HNE- tissues using a microarray by SABioscience that focuses on genes responsive to OxS.

Collaboration between Dr. Weisz and researchers at GE-GR is continuing. However, progress has been slow largely because of proprietary patent consideration. This has led us to recently establish a collaboration with Dr. Frank Martin, at Lancaster University in the UK, who can

bring to this project the emerging technology of infrared biospectroscopy. Dr. Martin is one of an as yet small cadre of investigators who is advancing the application of this powerful technology to biomedical research.

Findings using infrared biospectroscopy: This technology allows one to derive biomolecular profiles of cells in tissue sections, and to compare these to the biomolecular profile of other cells or cell types. It is based on the fact that when biomolecules absorb IR, signature vibrational or IR spectra, that reflect the chemical constituents of the interrogated sample. The spatial resolution of this is limited by the brilliance of the light source. Currently the most brilliant light source for these experiments is synchrotron-based radiation. Data obtained using this instrument are subjected to a mathematical operation, named Fourier transform, which decomposes a signal into its constituent frequencies. Hence the designation Fourier-Transform Infrared Spectroscopy (FTIR), and S-FTIR when spectra are obtained using synchrotron-based radiation. Several synchrotron-based instruments are available to Dr. Martin one of which, based in Switzerland, was used in the pilot study described below. For these experiments Dr. Martin received from Dr. Weisz tissue sections that contained a number of structures, including terminal ductal lobular units (TDLUs) where cells of origin of the majority of BC reside. From parallel immunostained sections, it was possible to ascertain where the 4HNE⁺ and 4HNE⁻ elements were present. Some of the results from this experiment are shown in figures 2-A, 2-B and 2-C: Figure 2-A shows the unstained area of breast tissue that was mapped by S-FTIR microspectroscopy and two different tissues containing TDLUs with corresponding parallel sections stained with H&E. Figure 2-B shows the typical information-rich average spectra for each category, i.e., 4HNE⁺ ME vs. 4HNE⁻ ME. Figure 2-C shows the findings from a series of experiments in which point spectra were acquired from myoepithelial and luminal cells at single-cell resolution through an aperture of 10 $\mu\text{m} \times 10 \mu\text{m}$. Because each spectrum is acquired at 4 wave-number resolution it contains some 235 data points and, when several spectra are being compared or examined, this generates a complex dataset. Consequently, for comparative purposes, spectra need to be reduced to single points – in simple terms, the closer such points are to each other the more similar the spectra are, the further away the more dissimilar they are. Significant differences ($P < 0.0001$) were noted for spectra of 4HNE⁺ vs. 4HNE⁻ ME (Figure 2-B); Similar comparisons were carried out for intra-lobular and inter-lobular stromal elements. The resultant cluster vectors loading plot highlighted that these differences were mostly associated with protein conformational changes (Amide I) and structural changes in the DNA (symmetric phosphate) (not shown). This is a highly relevant finding because these would be expected to be the molecular targets of OxS mechanisms. Importantly, when an image map of a 4HNE⁺ TDLU was generated that focused on these discriminating wave-numbers, clear evidence was noted of individual myoepithelial cells being most positive for these alterations (2-C). These findings clearly indicate that this biospectroscopy approach is readily translatable to a molecular pathological characterization of individual cell phenotypes associated with 4HNE-induced lesions. Additional experiments are planned using Raman spectroscopy using a 785 nm laser that can generate spectra through a 1 $\mu\text{m} \times 1 \mu\text{m}$ aperture, i.e., sub-cellular resolution. Data obtained from this first experiment is now being analyzed further by Dr. Martin. While data acquisition by this method can be completed in a couple of days, mining the data takes several months.

Expression of genes responsive to OxS or that participate in OxS defense in breast parenchyma with many 4HNE⁺ cells, as compared to breast parenchyma with only few 4HNE⁺ cells (Figure

3): SABioscience RT2 Profiler™ PCR Array was used to quantify transcripts for 84 genes that are responsive to or participate in OxS defense. RNA was extracted from cryosections of flash-frozen (FF) reduction mammoplasty specimens at either end of the spectrum of 4HNE immunostaining from subjects ranging in age from 17-30. Remarkably, only a single transcript was significantly (>2-fold) higher in 4HNE+ (experimental) tissues than in 4HNE- (control) tissues, while transcripts for 26 genes were significantly (>2-fold) lower (Figure 3). Moreover, MGST3, the sole transcript elevated >2 fold in 4HNE+ tissues encodes an enzyme that catalyzes the formation of leukotriene C4 that is linked to inflammatory responses. The findings obtained using this microarray were confirmed for four of the genes by qRT-PCR. This finding highlights the difference between responses to acute and chronic OxS. A pattern, in which gene expression is reduced while novel in the context of carcinogenesis, is becoming identified in a number of pathologies associated with chronic OxS, as well as in the context of “pre-conditioning” when tolerance to stressors is desirable.

The selection of genes represented in SABiosciences OxS microarray was based on responses known to be elicited by *acute* OxS. The expression of at least some of these genes would be expected to be increased in response to OxS. A pattern in which gene expression is reduced, such as found in 4HNE+ breast tissues, while novel in the context of carcinogenesis, is becoming identified in a number of pathologies associated with *chronic* OxS, as well as in the context of “pre-conditioning” when tolerance to stressors is desirable. Within the context of carcinogenesis our findings support the hypothesis that a state of chronic OxS in the ME elicits adaptive response in a subpopulation of cells that can evade mechanisms that have evolved to limit the propagations of cells with oxidatively damaged DNA. By evading defensive responses to OxS and oxidative damage, such as apoptosis, such cells could persist, accumulate mutations, and replicate, which is a recipe for generating transformed cells able to initiate a cancer. To test this hypothesis would require selecting a very different set of genes from those represented in the SABioscience microarray used in our study, such as, genes linked to process of apoptosis, associated with DNA damage-response or the mutator phenotype.

In order to obtain a more comprehensive, unbiased view of differences between 4HNE+ and 4HNE- tissues, aliquots of RNA used in the above studies were subjected to genome-wide transcriptional profiling using Illumina DASL chips. Data from this assay are now being analyzed and will form part of a publication based on the above findings. Preliminary analysis confirms the down-regulation of OxS associated genes in 4HNE+ samples.

Summary and what the funds have accomplished:

The findings provide the first glimpse into what has until now remained a black box, the protracted latent phase of breast carcinogenesis. Evidence of lipid peroxidation in ME of girls as young as 14, and in over half of the teenagers, with all its functional implications, is reminiscent of the finding of atherosclerotic plaques at autopsy in young US soldiers killed in the Korean War. It was this finding that led to the realization of the need to learn about the early, silent stages in the evolution of the cardiovascular (CV) disease and for the need for early intervention if its progression to its potentially deadly ultimate manifestation was to be prevented. While the link between atherosclerotic plaques and CV disease is obvious, the link between foci of chronic OxS in ostensibly normal ME and the emergence of BC decades later is currently only inferential. Demonstration that there are profound changes in molecular structure and gene

expression already in young women will strengthen this link and focus attention on the need for early intervention. Current clinical trials for BC prevention are mostly limited to women considered to be at high risk for developing, i.e., a risk over and above the unacceptably high risk that our environment poses. The fact that it takes decades of exposure to a pro-carcinogenic environment for a BC to emerge, and learning what mechanisms are promoting its emergence, has to be viewed as an opportunity to develop strategies for stopping or even reversing the clock. Hopefully, it will help towards shortening the lag-time between the emergence of evidence linking environmental factors to our BC epidemic and responding to it by developing effective preventive strategies.

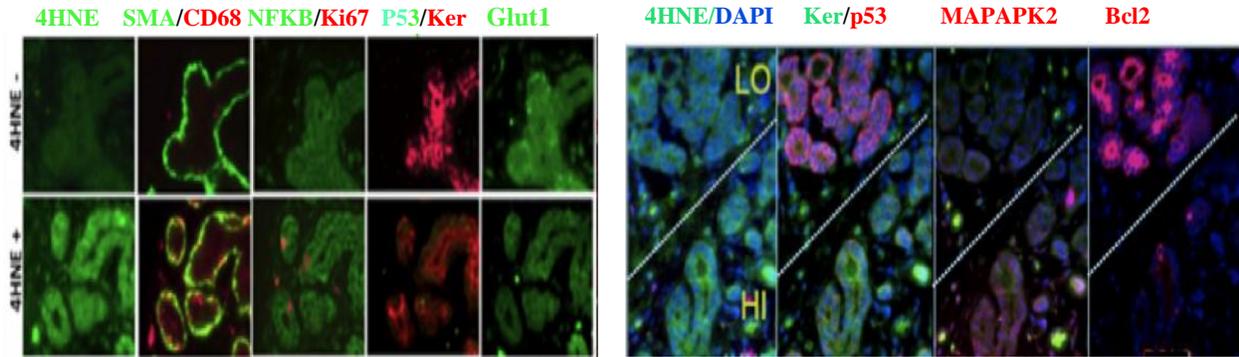


Figure 1. Correlation between 4HNE adducts and proteins (listed above panels) in FFPE tissue section from RM surgical specimens: Immunostaining was carried out by the PI's collaborators at GE-GR using GE-GR's proprietary multiplex immunocytochemical (MX-ICC) technology. *Left ten panels: micrographs from two subjects representative of tissues used for transcriptional profiling (Figure 3): Upper five panels, a section from a 24 year old subject with few 4HNE- TDLUs, lower five panels, a section from a 22 year old subject with many 4HNE+ TDLUs:* Sections were stained sequentially for: 1) 4HNE protein adducts (green) + the nuclear stain DAPI used for registration purposes (blue, not shown); 2) Cy5-CD68, macrophages (red) + Cy3-smooth muscle action (SMA, myoepithelium (green); 3) Cy3-NFkB (green) + Cy5-Ki67, replicating cells (red); 4) a Cy5-a Pancytokeratin antibody (recognizes cytokeratins 8 and 18) a marker of luminal epithelial cells (red) + Cy3-p53, pro-apoptotic (green); 5) Glucose transporter Glut1 (green). Immunostaining for five of the seven proteins in 4HNE+ sections differed from that in 4HNE- sections (CD68, NFkB, Ki67, p53m, cytokeratin marker). *Right four panels:* Photomicrograph of a section from a 25 year old subject showing a difference in immunophenotype of cells in two adjacent areas in the same section: In the area above the diagonal yellow line cells are 4HNE immunonegative or only minimally immunopositive, while in the area below the line cells are 4HNE strongly immunopositive. The section was stained sequentially for: 1) 4HNE (green) + nuclear stain DAPI (blue) used for registration purposes; 2) a pancytokeratin AB against cytokeratins 8 and 18 (green) and p53 (red); 4) + MAPAPK2A a "stress" responsive protein. A point of interest is the strong immunostaining for both p53 and Bcl2, two proteins linked to the balance between pro and anti-apoptotic responses in the upper, 4HNE- cells, but not in the lower 4HNE+ cells. Analysis of the massive amount of data generated by this technology will require using the data mining tools developed by GE-GR.

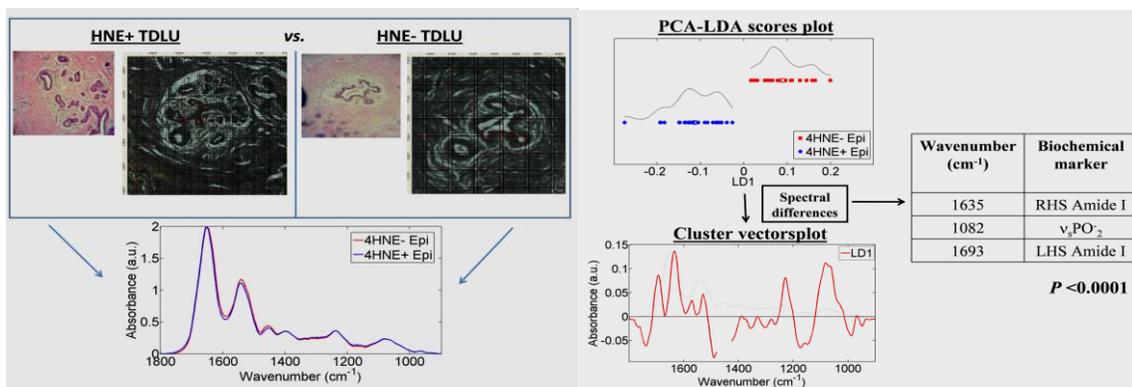
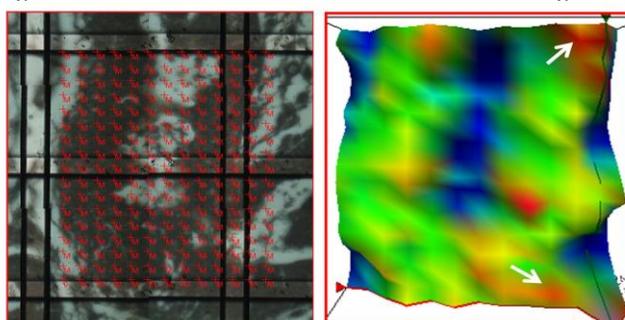


Figure 2-A

Figure 2-B



Red= High; Blue = Low

• The highest changes are seen in the myoepithelial layer (arrow) of the ducts and a couple of luminal epithelial cells

Figure 2-C

Figure 2-A. Spectral interrogation of breast tissue TDLU by S-FTIR microspectroscopy: A 10 μm -thick unstained section was floated onto an IR-transparent 1x1 cm BaF₂ window and de-waxed. A parallel H&E-stained 4 μm sections is shown in the smaller panels. Through a 10 μm \times 10 μm aperture, point spectra were acquired from TDLUs as 4HNE+ or 4HNE- identified based on parallel immunostained sections. From spectral analysis, mean spectra from the biochemical-cell fingerprint region (1800 cm^{-1} to 900 cm^{-1}) were readily derived. Typical wave-number absorbance intensities are observed; to determine differences between 4HNE + vs. 4HNE- TDLUs.

Figure 2-B. Initial data reduction of data from epithelial cells using principal component analysis-linear discriminant (PCA-LDA) algorithms. PCA-LDA analysis takes a spectrum acquired at 4- cm^{-1} resolution containing 235 datapoints and reduces it to a single point in a scores plot, thereby maximizing class segregation whilst minimizing within-category heterogeneity. In the scores plot, the closer spectral points are to each other the more similar they are and the further away the more dissimilar (4HNE positive versus 4HNE negative). The distribution of spectral points is non-uniform. Scores plots allow for a visual representation of the data. From this, one can construct a vector, named cluster vector, through the mean of each cluster, or class (*i.e.*, TDLUs classed as 4HNE positive or negative). This allows one to identify the wave-numbers specific to each class; in this case, because there are only two classes the cluster vector for each class is a mirror image. The spectral differences between epithelial cells designated either 4HNE positive or negative are associated with protein conformational changes highlighted by alterations of the Amide I peak (the centroid of which is at 1650 cm^{-1}) and backbone structural alterations to DNA associated with symmetric phosphate stretching vibrations ($\nu_s\text{PO}_2$).

Figure 2-C. IR spectral image map, which allows tracking the spatial distribution of chemical entities, based on levels of relative absorbance intensity at a chosen wave-number pixel-by-pixel. This generates an image map in which absorbance intensity is proportional to thermal color changes: blue (lowest) < green < yellow < red (highest). PCA-LDA consistently highlighted Amide I alterations (1650 cm^{-1}) as being a major contributor to IR spectral segregation of the different assigned classes, pointing to an important role of alterations in conformation of proteins in the segregation of TDLUs classed as 4HNE positive or negative. Image maps were acquired at a spatial resolution of 10 μm \times 10 μm from 10 μm -thick unstained sections on IR-transparent BaF₂ windows (above left). When the spectral intensity was tuned for Amide I across this IR image map, there was clear evidence of highly positive myoepithelial cells (see white arrows, above right) exhibiting an altered Amide I phenotype along with isolated luminal cells.

Figure 3. Fold difference between breast tissues at either end of the spectrum of 4HNE immunostaining in the level of transcripts of genes responsive to OxS or that participate in OxS defense represented. Assays were carried using SABioscience RT2 Profiler™ PCR 84 gene microarrays with threshold set at Ct. 30. The two “housekeeping” genes used for normalization, HTRP1 and RPLPO, were shown using SABioscience Housekeeping PCR arrays not to differ significantly in their level of expression between 4HNE+ and 4HNE- tissues (shown below, Fig 4). A two-fold difference between 4HNE- (control) and 4HNE+ (experimental) sample was considered significant.

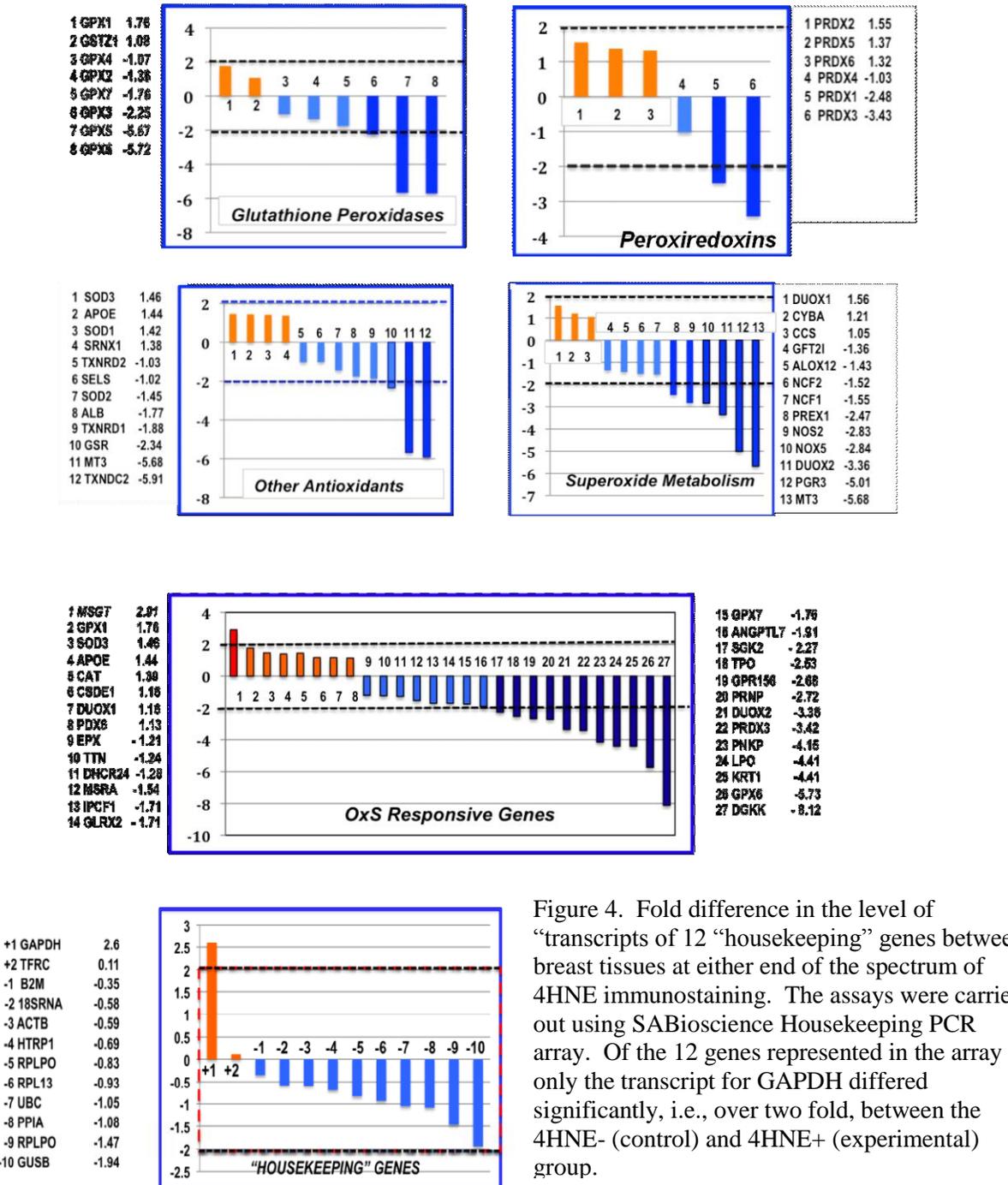


Figure 4. Fold difference in the level of “transcripts of 12 “housekeeping” genes between breast tissues at either end of the spectrum of 4HNE immunostaining. The assays were carried out using SABioscience Housekeeping PCR array. Of the 12 genes represented in the array only the transcript for GAPDH differed significantly, i.e., over two fold, between the 4HNE- (control) and 4HNE+ (experimental) group.

Research Infrastructure Project 13: Project Title and Purpose

Research Infrastructure for New Pesticide Technologies for Control of Insect-Borne Diseases like Malaria - This project is to provide the eight environmental chambers which will form the core of a state of the art Laboratory for the Experimental Analysis of Human Infectious Insects at Penn State. Insects vector some of the globally most important human diseases, including malaria, and the control of mosquitoes continues to be one of the most potent weapons against these diseases. But existing chemical insecticides are failing. The new laboratory will develop novel strategies and technologies for sustainable control of vector-borne diseases. Fungal biopesticides offer considerable promise as a new, sustainable and environmentally friendly approach to protecting human health. In addition to globally significant research and extension outputs, this project has many potential Pennsylvania population based applications for other vector borne diseases, such as, West Nile, Lyme, erratically present diseases such as Eastern Equine Encephalitis and St Louis Encephalitis. All of these diseases have wildlife reservoirs and are carried to humans via insects. History shows that we can expect a variety of other vector-borne diseases which will emerge in future years.

Anticipated Duration of Project

1/1/2008 - 12/31/2011

Project Overview

The scope of this project is to design and build the environmental rooms which provide the light, humidity and temperature necessary to culture, maintain and experiment with the insect vectors which transmit diseases such as malaria, West Nile, dengue, sleeping sickness and elephantiasis. These chambers will form the key infrastructure to develop the Laboratory for the Experimental Analysis of Human Infectious Insects. This year, PSU recruited internationally the core personnel to found this laboratory. This laboratory will be a mosquito experimental facility in which large numbers of several species of the mosquitoes which transmit malaria can be reared. These mosquitoes will be used to assay the malaria transmission-blocking efficacy of natural and genetically engineered fungal isolates. Colonies of different species of mosquitoes will also be reared to investigate the potential of this technology for the control of other vector borne diseases, such as Dengue, West Nile Virus, Lyme Disease, Eastern Equine Encephalitis and St Louis Encephalitis. To this end, a laboratory building will shortly be renovated by PSU to provide a Laboratory for the Experimental Analysis of Human Infectious Insects.

The current grant application is for a key component of the research infrastructure: the eight environmental chambers required for mosquito colony maintenance and experimentation. These chambers will provide state of the art lighting, heat and humidity control required for large-scale production and maintenance of blood feeding mosquitoes which transmit human disease. The new facility will include a secure containment facility which will enable population and behavioral analysis of insects infected with malaria, as well as the facilities to culture human malaria for infecting mosquitoes.

A major use for this facility will be to develop fungal biopesticides against mosquitoes. These

have the potential to provide cost-effective, green, evolution-proof control of malaria. Chemical insecticides like DDT have been – and continue to be – critical components of malaria control programs worldwide. However, their effectiveness is degrading in the face of environmental and health concerns and, most importantly, the evolution of resistance in mosquito populations. New strategies are needed. Fungal biopesticides offer considerable promise. Building on its existing strengths in infectious disease dynamics, in agricultural pest control and in insect ecology, and with the recent strategic recruitments, Penn State seeks to be the leading world Center for developing and applying biopesticide technology to malaria, West Nile, Lyme, and other vector-borne diseases, which will emerge in future years.

Principal Investigator

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Other Participating Researchers

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Expected Research Outcomes and Benefits

The requested infrastructure will enable experimentation which will develop biopesticides for malaria control. There are in excess of a billion clinical cases of malaria annually, with over a million of these ending in death. This burden falls disproportionately on the poorest people on the planet, particularly their children. Increasingly concerns over human and environmental health are constraining the use of chemical insecticides, and the spread of mosquitoes resistant to chemicals like DDT is reducing the efficacy of traditional insecticides when they are deployed.

Fungal biopesticides would overcome these problems, thus generating substantial – and critically, sustainable health gains. In addition, the technology has the potential to be applied to other vector borne diseases of global human and animal health importance, including West Nile, Lyme, Eastern Equine Encephalitis and St Louis Encephalitis, dengue and sleeping sickness.

The science required to generate these improvements in human health could also generate insights into host-pathogen interactions, pathogen-pathogen interactions, and resistance management, which will be applicable in other health contexts, including the development of new chemical insecticides and the identification of novel antimalarial compounds.

Summary of Research Completed

The Infectious Insect Facility has continued to experience a significant set-back during the start-

up and commissioning of two key process systems. The delays caused by these problems were beyond the control of the PI.

The most challenging problem was with the operation of the Reverse Osmosis (RO) water system. This process water system is critical to the operation of the Conviron environmental chambers. The RO water serves the ultra-sonic humidification system that is integral to each chamber. The RO system was manufactured by Pure H₂O Technologies, Inc. (IPure) of New Jersey. The IPure unit was operating marginally upon initial start-up in August 2010. Throughout the next few months, it required frequent service from the manufacturer and parts were not readily available. The manufacturer was not cooperative with scheduling warranty work and demanded payment for any visit prior to arriving. Since their equipment did not appear to be operating properly, the University resisted paying for warranty service. This tension contributed to delays in the repair and operation of the system. By December 2010, the RO water system was still not operating properly. In early January 2011, the problems with IPure were elevated to higher levels within Penn State.

On January 14, 2011 – IPure had a representative on site. They said their unit should have a larger softening unit on it otherwise the membranes will wear out quicker. IPure also indicated a setting was incorrect on the unit for chlorine removal. Filters would plug up in four days and send the unit into alarm and shut it down. It was also discovered that this unit required a manual reset anytime there was a power outage. IPure was to take water samples as part of their scope of work. That did not appear to happen. Revisions were made to the RO system with IPure. Spare parts were ordered to keep in stock.

On January 26, 2011 the Conviron Chambers were operating but not all were producing the required relative humidity (RH). By February 2, 2011, five of the six chambers were operating properly.

On February 10, 2011, the RO system had shut down again. The system was only able to handle 450 gallons of processing. Penn State installed a larger water softener to reduce the hardness. Electricians also discovered a voltage problem with the IPure system. The project team began looking into temporary or replacement RO systems due to the continued failure of the IPure system.

On February 11, 2011, the project team determined that the IPure unit needed to be replaced. Penn State sought out a proven RO unit manufacturer. Siemens Water Technologies Corp was recommended then contacted. Provisions were made to have a unit shipped to the project site. Within a few days, the new equipment was procured. Demolition of the existing unit began on February 22, 2011. The new replacement unit arrived and was installed on February 28, 2011. The system was operating properly serving the Conviron Chambers. Functional testing of the chambers was able to proceed now that the RO water system was replaced.

Unfortunately for the PI, this time of trouble shooting and remediation caused delays of over 6 months.

With the completion of the RO water process system replacement, the environmental chambers

were finally able to be tested. The system was challenged to run through its spectrum of temperature and humidity set-points. A team of technicians and engineers reviewed the performance of the chambers and found discrepancies in the operating parameters. This testing lasted through March and April 2011. Ventilation and make-up air into the chambers was not meeting the performance criteria established by the chamber manufacturer. Through a series of tests, the manufacturer and facility engineers concluded that the ventilation and make-up air needed to be pre-treated prior to entering the chamber. In early May 2011, a humidification system was designed to satisfy this pre-treatment requirement. Materials were ordered and installed in early June 2011. The chamber manufacturer has provided the PI with guaranteed performance parameters once this installation is completed. The research team had cautiously started experiments in the chambers in late May and early June. The units appear to be functioning properly now. Further testing and exercising the equipment ranges will be completed as the research schedule allows the parameters to be changed in each chamber.

In summary, the critical process equipment needed to support the PI's work with infectious insects was not able to properly support the research requirements. The inferior equipment was removed and replaced with appropriate equipment that appears to be functioning properly.

There is cautious optimism that the system is now working. Remaining is the remote alarms and monitoring, and the acid test of whether it will all work in a Pennsylvania winter.

Research Project 14: Project Title and Purpose

Coupling Mechanisms of NOP Receptors and Calcium Channels - There are four opioid receptors that have been described and that are involved in transmitting pain signals within the nervous system. One of these receptors is known as the 'opioid receptor-like 1 receptor' or NOP receptor. When this receptor was discovered, it was found that activating the receptor would either cause pain or inhibit pain. Our laboratory has found that this receptor is expressed in stellate ganglion neurons and that it has the ability to be either silent or active in the absence of any agonist. The purpose of this research project is to study the pharmacology of constitutively active NOP receptors and study the mechanism by which these receptors obtain an 'active' state in the absence of agonists and how sodium ions affect this 'active' receptor.

Duration of Project

1/1/2008 - 6/30/2009

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

Dilatory and Constrictor Control of Coronary Blood Flow Velocity - The purpose of this study is

to better understand mechanisms that control blood flow in the coronary arteries. This is important because the coronary arteries provide all of the oxygen to the heart muscle. The coronary circulation is highly unusual because the heart muscle cannot change the percentage of oxygen removed from blood as the amount of activity increases. Thus blood flow regulation becomes crucial in understanding how the heart works. In this project we will examine what substances make blood vessels enlarge and how and why the nervous system may make them become smaller.

Duration of Project

1/1/2008 - 6/30/2011

Project Overview

Understanding how coronary blood vessels dilate and constrict can provide important clinical information. To date, most data have been published in animal models because human experiments have required invasive methods. Specifically, intracoronary Doppler guidewire techniques have been required to obtain direct coronary artery blood flow velocity data. In recent years reports demonstrate that advanced Transthoracic Duplex Ultrasound (TDU) technology may enable investigators to measure real time coronary blood flow velocity in humans. We propose a series of validation studies followed by a series of experiments to further characterize dilator and constrictor responses in humans.

In the first series of experiments, we will examine the dilatory effects of adenosine as coronary blood flow is measured using an invasive Doppler guidewire technique in patients during coronary angioplasty. These measurements will then be compared to coronary flow determinations obtained non-invasively with the Duplex Ultrasound technique. Studies will be performed in the same patients the morning after catheterization.

We will then begin a series of non-invasive experiments designed to determine how local factors dilate coronary arteries. We will then couple studies of coronary blood flow with non-invasive examination of left ventricular function using tissue Doppler techniques. We will examine the effect of static handgrip exercise on coronary blood flow and systolic and diastolic left ventricular function. Our initial hypothesis is that tissue Doppler indices of systolic ventricular function will increase in response to handgrip. We will also examine the effect of different levels of LDL cholesterol on the resistance responses to handgrip. We postulate that there will be a relationship between LDL levels and the coronary resistance response to handgrip.

Finally, we will compare the coronary blood flow responses during exercise and during hyperoxia in stable heart transplant patients and in normal control subjects. We hypothesize that responses to handgrip will be attenuated in transplant recipients whereas the responses to hyperoxia will not be attenuated.

Principal Investigator

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Expected Research Outcomes and Benefits

It is very important to understand the mechanisms by which the vessels that supply the heart muscle adjust their size as flow requirements change. Research in people on these issues has been dramatically hampered by the invasive nature of the methods needed to accomplish these goals. For this reason the overwhelming majority of science in this area has been performed in animal models. While this massive body of literature has greatly improved our care of patients with heart disease, many crucial questions remain. Indeed many of the most basic issues remain to be determined in humans. For example what substance(s) are responsible for the ability of human blood vessels to dilate as the work of the heart increases? Although many substances have been proposed the relative contribution of each of these substances remains unclear. In an analogous fashion, it is unclear what role the autonomic nervous system plays in regulating blood flow. Animal studies suggest that engagement of the sympathetic nervous system, a branch of the autonomic nervous system causes blood to be effectively redistributed from the outside of the heart to the inner portions of the heart. Studies to determine if such a system is operable in people have been lacking. In this application we will examine a new sound wave method to measure the speed of blood traveling in the large coronary arteries. We will validate this method and then we will examine a series of questions to examine the regulatory mechanisms described above in human subjects.

Summary of Research Completed

Compared with traditional echocardiographic two-dimensional (2D), Doppler and tissue Doppler imaging models, 2D speckle tracking is a relatively novel technique to quantify Left Ventricle (LV) global and regional myocardial function. 2D speckle tracking based-strain, strain rate (SR) and torsion analysis has been used in research and clinical practice.

Previous studies have shown that these parameters may provide more sensitive and acute assessment of the myocardial function. Comparing and combining these myocardial function parameters with coronary blood flow velocity data may provide more detailed information on coronary physiology. However, the impact of cardiac volume loading on LV strain, strain rate and torsion in the healthy human heart has not been previously studied. To determine whether

acute preload reduction affects 2D speckle tracking-derived LV strain, strain rate and torsion, we assessed the influence of a graded reduction of preload, caused by Lower Body Negative Pressure (LBNP), in 12 healthy young subjects (26 ± 1 yrs, 6 females and 6 males). LV strain, strain rate and torsion were measured by 2D speckle tracking technique before and during LBNP at 0, -10, -20, -30, -40 and -50 mmHg.

The primary results included: 1) Compared to baseline, LBNP caused significant decreases in LV end-diastolic volume ($P<0.05$) and stroke volume ($P<0.01$) while heart rate increased ($P<0.01$). LV longitudinal strain and strain rate decreased as LBNP increased; and 2) Multiple indices of LV torsion including LV total torsion, apical and basal rotation and peak early diastolic torsional rate, were unaffected by LBNP. These results indicate that 2D speckle tracking-derived longitudinal strain and strain rate are sensitive to acute preload change. Thus, the effects of preload should be considered when strain and strain rate are used as indices of systolic and diastolic function. LV torsion may provide a relatively load-independent measure of myocardial function.

Two abstracts from this study had been submitted to Scientific Sessions of the American Heart Association 2011.

Abstract #1

Longitudinal Strain and Strain Rate Based on 2D Speckle Tracking Echocardiography (STE) are Preload Dependent. Zhaohui Gao, Rachel Drew, Afsana Momen, Ifesinachi Ndukwu, Matthew Muller, Urs A. Leuenberger, Lawrence I Sinoway

Background: 2D-speckle tracking echocardiography (STE) has emerged as a promising tool for obtaining strain and strain rate. These measurements have been proposed as novel indices of myocardial deformation and function, and may be especially important in characterizing subclinical myocardial dysfunction. However, the effects of left ventricular (LV) loading conditions on 2D-STE derived LV longitudinal strain and strain rate have not been elucidated.

Aim: To determine whether acute preload reduction by Lower Body Negative Pressure (LBNP) affects 2D-STE derived LV longitudinal strain and strain rate.

Methods: The influence of a graded reduction of preload, caused by LBNP (0, -10, -20, -30, -40 and -50 mmHg), on the strain and strain rate using 2D STE were assessed in 12 healthy individuals (26 ± 1 yrs, 6 females and 6 males). Systolic peak strain (%), systolic (S-Sr), early and late diastolic (E-Sr and A-Sr) strain rate (S^{-1}) were measured from standard LV apical views at each LBNP level.

Results: Compared to baseline, LBNP caused significant decreases in LV end-diastolic volume ($P<0.05$) and stroke volume ($P<0.01$) while heart rate increased ($P<0.01$). LV longitudinal strain and strain rate decreased as LBNP increased (Fig 1).

Conclusion: Our results indicate that 2D STE-derived longitudinal strain and strain rate are sensitive to acute preload changes. Thus, the effects of preload should be considered when strain and strain rate are used as indices of systolic and diastolic function.

Abstract #2

Left Ventricular Torsion Based on 2D Speckle Tracking Echocardiography (STE) is Preload Insensitive. Zhaohui Gao, Rachel Drew, Afsana Momen, Ifesinachi Ndukwu, Matthew Muller, Urs Leuenberger, Lawrence I Sinoway.

Background: Left ventricular (LV) torsion plays an important role in myocardial performance. 2D STE has emerged as a promising tool for LV torsion analysis. However, it is unknown if LV torsion is affected by alterations in preload.

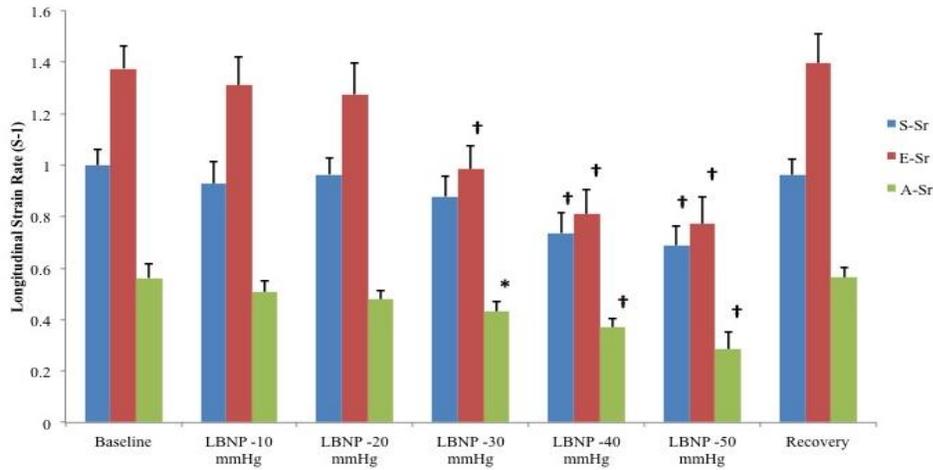
Aim: To determine whether acute preload reduction by Lower Body Negative Pressure (LBNP) affects 2D STE derived LV torsion parameters.

Methods: LV apical and basal rotation, and peak early diastolic torsional rate were measured by 2D STE in 12 healthy subjects (26 ± 1 yrs, 6 females) before and during LBNP at 0, -10, -20, -30, -40 and -50 mmHg. LV total torsion was calculated as the net difference between systolic apical counterclockwise rotation and basal clockwise rotation. Peak early diastolic torsional rate was also measured.

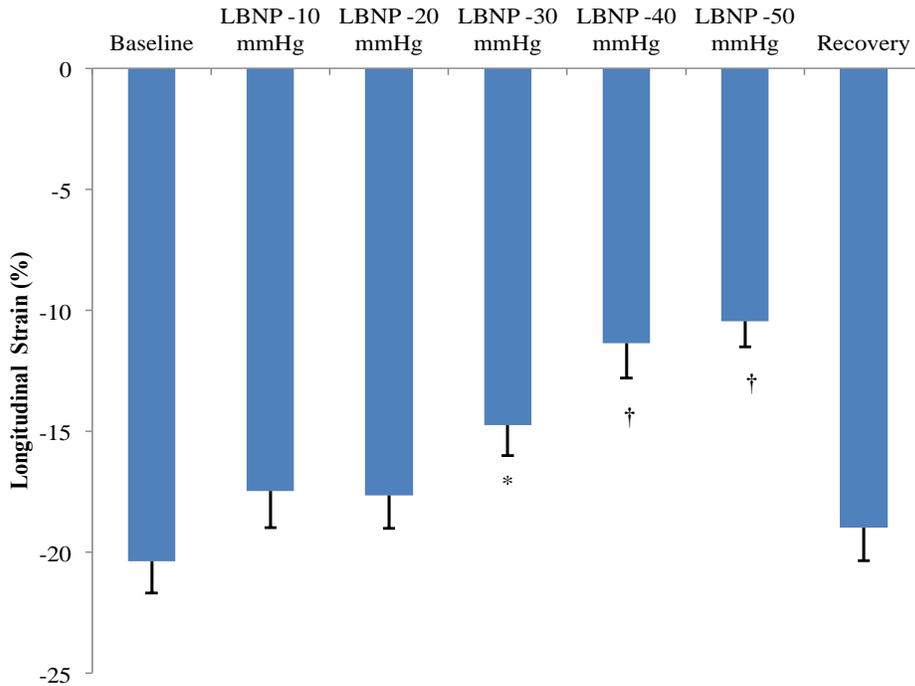
Results: Compared to baseline, LBNP caused significant decreases in LV end-diastolic volume ($P < 0.05$) and stroke volume ($P < 0.01$) while heart rate increased ($P < 0.01$). Interestingly, multiple indices of LV torsion including LV total torsion, apical and basal rotation and peak early diastolic torsional rate, were unaffected by LBNP (See Table 1).

Conclusion: Our results support the concept that LV torsion parameters assessed by 2D STE are preload independent indices of myocardial function. Quantification of LV torsion by 2D STE may, therefore, provide relatively load-independent measure of myocardial performance.

Figure 1.



*, $P < 0.05$; †, $P < 0.01$ vs. Baseline. S-Sr, longitudinal peak systolic strain rate; E-Sr, longitudinal early diastolic strain rate; A-Sr, longitudinal late diastolic strain rate. Statistical analyses were performed by repeated measures ANOVA.



*, $P < 0.05$; †, $P < 0.01$ vs. Baseline.

Table 1.

Variables	Baseline	LBNP					Recovery	P value
		-10 mmHg	-20 mmHg	-30 mmHg	-40 mmHg	-50 mmHg		
SBP (mmHg)	121±4	120±4	119±4	118±4	115±5	113±5	118±2	0.91
DBP (mmHg)	60±3	59±3	59±3	61±3	62±3	64±3	60±3	0.88
MAP (mmHg)	79±3	78±3	78±3	78±3	79±4	79±4	79±3	0.99
HR (bpm)	65±4	64±4	66±4	70±3	77±3 [†]	83±3 [†]	59±3	<0.01
LVEDV (ml)	109±8	109±10	111±10	89±10	79±6 [†]	83±10*	117±7	<0.05
LVESV (ml)	45±4	48±6	47±5	41±6	35±4	38±4	46±3	0.41
SV (ml)	64±6	61±4	64±5	49±5*	44±3 [†]	45±7*	71±5	<0.01
LV Torsion (°)	14±2	12±2	13±2	15±2	13±2	15±4	14±2	0.95
Apical Rotation (°)	11±2	9±2	11±2	13±2	10±3	12±5	12±2	0.92
Basal Rotation (°)	-4±1	-3±1	-4±1	-2±1	-5±1	-3±1	-2±2	0.57
Early diastolic torsional rate (°/s)	110±15	90±21	86±19	86±13	105±13	93±27	87±8	0.87

*, $P < 0.05$; [†], $P < 0.01$ vs. Baseline. Statistical analyses were performed by repeated measures ANOVA.

Research Project 16: Project Title and Purpose

Environmental Heavy Metals, Biomarkers of Susceptibility and Renal Cancer - This research will assist important efforts in cancer prevention by investigating cancer risk in a large population-based study among individuals at higher risk of renal cell cancer (RCC) (i.e., male smokers), by the use of established biomarkers of heavy metal exposure. The overall goal of the proposed study is to identify the risk of heavy metal exposure in the development of RCC at levels of exposure experienced by the general population and to determine whether there are other susceptibility factors (both genotypic and phenotypic) that can help predict cancer risk.

Duration of Project

1/1/2008 – 6/30/2008

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

Molecular Basis of Mechanotransduction in Bone Cells - The purpose of this project is to establish an animal model for studying mechanical loading-induced bone formation, and to identify important proteins involved in the loading-induced bone formation. Understanding of the loading-induced bone formation will lead to novel treatments for osteoporosis.

Duration of Project

1/1/2008 – 6/30/2009

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

Disseminating Effective Habits for Long-term Weight Loss - Most weight-loss interventions are not effective in producing long-term weight loss. The National Weight Control Registry (NWCR) has followed over 4000 individuals who achieved long-term weight loss to try to identify the characteristics of individuals who successfully maintained meaningful weight loss, including their dietary and exercise habits. We proposed to extend this study by developing a website www.achievetogether that will use information gathered from individuals who have successfully lost weight and kept it off to help guide those looking to lose weight. These data will include identification of successful strategies, details of how these strategies were implemented, and how barriers were overcome. We will then determine whether use of the website is effective in producing weight loss, and how patterns of use predict successful weight loss.

Anticipated Duration of Project

1/1/2008 - 12/31/2011

Project Overview

The intent of this project is to test the utility of a website that contains data regarding weight-loss strategies that have been proven successful in producing sustained weight loss (www.achievetogether.com). Overall, the study will be conducted in three phases. In Phase 1, we will conduct in-depth interviews with 50 individuals who have lost weight and kept it off for at least one year. These individuals will be questioned regarding which strategies were used, how the strategies were implemented, and how barriers to using these strategies were overcome. The

transcripts and quantitative data from their interviews will be entered into the www.achievetogether.com website. In Phase 2, we will recruit 100 overweight individuals and conduct a pilot randomized trial of the website, to understand whether use of the website will lead to better weight loss at 3 months. In Phase 3, we will make the website available to employees of the Milton S Hershey Medical Center, publicize its availability, track its use and analyze its use over time in order to better understand how to disseminate this and other web-based health interventions.

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Jennifer Kraschnewski, MD, MPH – employed by Penn State University

Expected Research Outcomes and Benefits

The obesity epidemic is a growing concern in American health care. Thirty percent of the adult population is obese, and nearly two-thirds of the adult population is overweight. Obesity is associated with greater risk of serious diseases such as diabetes, heart disease, stroke, and some types of cancer. Being overweight can also result in social problems such as stigmatization and discrimination. In this project, we will gather detailed information from individuals who have been successful in both losing weight and keeping the weight off for at least a year. In particular, we will focus on collecting diet and exercise information, as well as details on how these strategies were implemented, and how barriers were overcome. We will make this information available through a website, www.achievetogether.com, and test whether having this website available as a resource makes a difference in the ability of these individuals to lose weight.

Individuals accessing the website will be encouraged to enter information about their current exercise and eating habits, and the website will provide automated feedback about each habit. Habits will be categorized, into 3 categories: a) habits that are not working, b) habits that work, but that need to be used more consistently and c) habits that work and are used consistently. Users will be encouraged to change or delete habits that are being used but not helpful, more consistently use habits that are helpful but not being used, and continue to use habits that are helpful and being used consistently.

In the final phase of this project, we will make the website available to employees of the Milton S Hershey Medical Center and its insurance provider, Highmark. Given the interest of most adults in losing weight or maintaining a healthy weight and the fact that excessive weight is a risk factor for many common health conditions in the USA (e.g., diabetes, hypertension, high

cholesterol), we believe that a web-based weight intervention may serve as a perfect offering for an employee health web-based portal.

Summary of Research Completed

Randomized Controlled Trial (RCT) Data Analysis:

We continued to analyze data from this RCT and produced a journal manuscript, which has been accepted. This manuscript reference is below.

Re-Development of Weight Loss Website:

Since the last progress report, the research team has been working on developing a revised version of the weight loss website, www.achievetogether.com, based on data from user and feasibility testing. The new application has been developed and is currently undergoing beta testing by the research team before being able to be rolled out to the Penn State Community, the Phase 3 Aim of this Strategic Plan.

Additional Work. We have been able to expand this project to collect more qualitative data for an R01 resubmission. We completed the following since the last progress report:

Adult Home Visit Pilot:

A cross-sectional study was conducted the fall of 2010 with a purposive sample. We identified 20 participants from two groups: those who were successful with losing and maintaining weight, and those who were not. The Electronic Health Record (EHR) known as EpicCare at Geisinger Medical Center was used for recruitment. Geisinger Medical Center uses an EHR in which an individual's weight is recorded during any office visits. Based on this weight history, Geisinger sent a letter to recruit potential research subjects from Geisinger clinics in Central PA, who met the inclusion criteria. The letter indicated that the study consisted of one visit to the participant's home. The benefit of using EpicCare was the ability to easily identify patients; however, the patients were located approximately 60-100 miles from the research site, which meant considerable travel.

Inclusion for successful participants (SP) age 21-60 were: (1) Had at least one weight recorded each year for at least the past 4 years; (2) Had at least two clinic visits in which weight was recorded in EpicCare in the past 12 months; (3) The most recent weight is at least 10% less than the maximum weight recorded in the past 12 months for short-term weight loss or past 4 years for long-term loss; (4) BMI \leq 25.0; (5) No history of a medical condition based on ICD-9 diagnosis codes. Unsuccessful participants (USP) met all criteria except #3. Interested individuals were instructed to contact Penn State's research study line and were screened over the phone for additional exclusion criteria. If the participant was eligible, he/she was scheduled for a study visit, conducted at their home with a research coordinator and qualitative researcher. From 217 potential participants who met the inclusion criteria, 54 were eligible and 20 completed the study (Figure 2).

Study Design

Participants were contacted one day prior to study visits to confirm their appointment. Upon consent, demographics, Weight History Questionnaire, and two weights taken from a Tanita scale were completed. The participant was told that they could stop the study at any time, or skip over questions that he/she did not wish to answer. The researcher (blinded) conducted an in-depth semi-structured interview to collect data on weight loss and weight loss maintenance practices, as well as barriers to weight. The interview was digitally audio recorded and ranged between 35 and 62 minutes (mean of 46 minutes). The researcher then asked the participants to give a tour of their kitchen and eating areas, the inside of their cupboards, and the areas in which they exercise (if applicable). These sessions were digitally video recorded and ranged between 10 and 31 minutes (mean of 17 minutes). Participants were asked to elaborate on the top three practices they felt contributed to their weight. Lastly, the participant received a handheld video camera (2-hour recording time), and was instructed on its use. The purpose of this self-recorded observational data was to film both meal preparation and meal consumption for three meals during a one-week period (one breakfast, one lunch, and one dinner). To record additional in-situ behaviors, participants were asked to record weight successes and barriers in a written journal. A self addressed, pre-paid mailer was given to return the camera (containing the recordings) and journal.

Each participant's BMI was calculated based on self-reported height and calculated scale measurements. This variable was used to identify the population who were considered successful or unsuccessful at weight loss. The minimal-risk study was approved by the (blinded for review) Medical Center Institutional Review Board. Written, informed consent was obtained from each participant. Participants received a gift card after the initial interview, and after returning the video.

Qualitative Methods

The data analyzed included audio interviews transcriptions, video observation transcription, and field notes of the lead author. These data were imported into qualitative data management software (NVivo 9, QSR International) for the process of analysis. To provide data comparisons, we specifically looked for data that appeared to contradict each other (discrepancy or dissonance). The observations and interviews were focused, in common, on food habits and the food environment; therefore, we delimit the discussion to data in these two domains.

First, an investigator reviewed all data resulting from observations (video transcriptions, field notes) and interviews (audio transcripts, field notes) on six different occasions to identify and describe instances of discrepancies or consistencies between what was discovered via interview and observation. As a result of immersion in the data, reading it line-by-line and comparing across data sources, initial codes were created to describe and reflect salient concepts (e.g., the participant states "I drink only water" in the interview; the observational note indicates "there is sweetened iced tea" in refrigerator). Data comparisons across methods were listed in one document. To enhance the dependability of these comparisons, the data were shared with another qualitative researcher for peer review and debriefing and discussion. Additionally, results were shared with the research coordinator who attended the study visits but was not part of the data analysis process, and she confirmed the findings to accurately reflect the data. Finally, results were shared with a physician external to the study process to determine that no

practices were dangerous to health or welfare.

Family Home Visit Pilot:

The above stated procedures for the home visit pilot in adults were also examined with children and families. Currently, 4 home visits with families have been completed and data is being analyzed.

Recent Publication in Press:

Sciamanna CN, Boan J, Kiernan M, Miller CK, Rolls BJ, Jensen G, Hartman TJ, Loken E, Hwang KO, Williams RJ, Clark MA, Schubart JR, Nezu AR, Lehman E, Dellasega C. Are the practices associated with weight loss different from those associated with weight loss maintenance? Results of a National Survey. In Press, American Journal of Preventive Medicine.

Figure 1. Randomized Controlled Trial of Weight Loss Website CONSORT

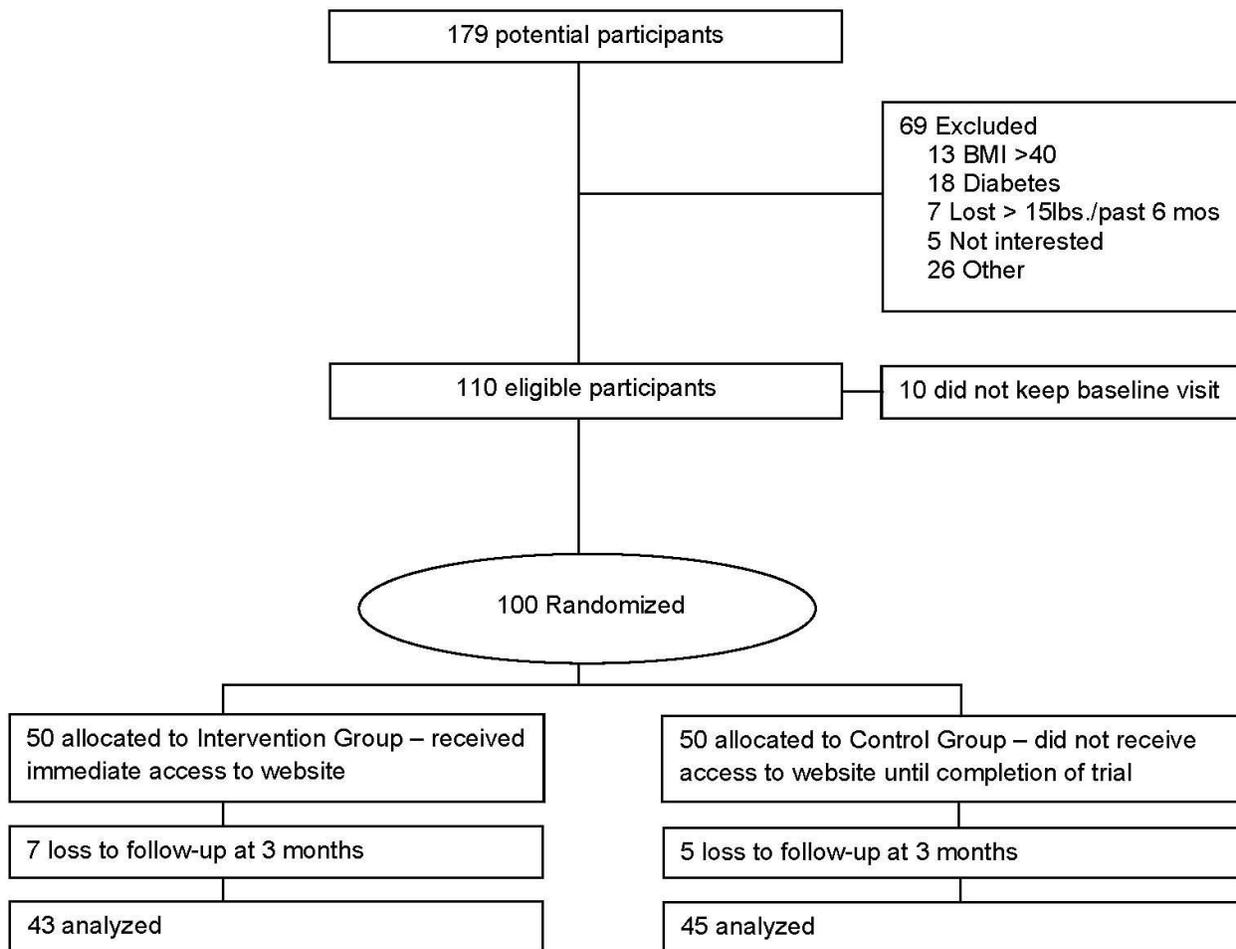


Table 1. Changes^a in Outcomes during the 12-Week Intervention (n=89).

Outcome	Control Group (n = 50)	Intervention Group (n = 50)	Difference Between Groups
Body weight, kg	0.6 (-0.3, 1.4)	-1.4 (-2.2, -0.5)**	2.0**
BMI, kg/m ²	-0.4 (-1.2, 0.5)	- 1.1 (-1.9, -0.3)**	0.7
Systolic BP, mm Hg	-3.6 (-6.7, -0.5)*	-7.1 (-10.2, -3.9)**	3.5
Diastolic BP, mm Hg	2.2 (-0.2, 4.6)	-1.5 (-3.9, 1.0)	3.7*
IWQOL Total Score	0.6 (-2.2, 3.5)	2.6 (-0.2, 5.4)	2.0
Weight Control Practice use	0.0 (-0.3, 0.3)	0.4 (0.2, 0.7)**	0.4*
Total daily caloric intake, kcal	-139.8 (-267.0, -12.5)*	-174.9 (-305.2, -44.6)**	35.8

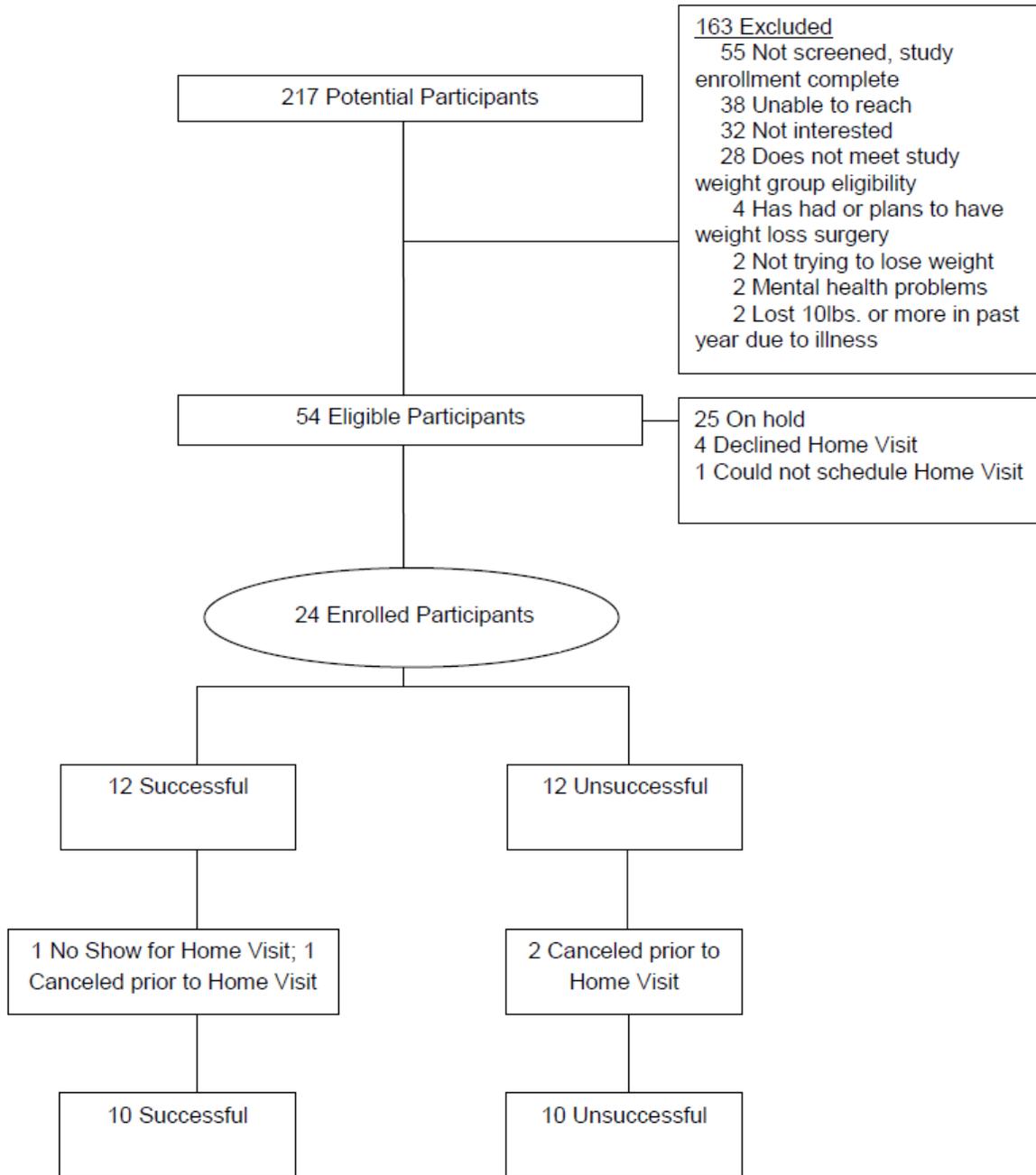
BMI, body mass index; BP, blood pressure; IWQOL, Impact of Weight on Quality of Life questionnaire

^a Changes reported as means (95% CI), unless otherwise stated.

* *P*-value < 0.05

** *P*-value ≤ 0.01

Figure 2. Geisinger Adult Home Visit Pilot CONSORT



Research Project 19: Project Title and Purpose

Selective Autophagy in Yeast and in Mammalian Cells - The objective of this project is to examine whether a selective autophagy pathway that we originally identified in *Saccharomyces cerevisiae* is also present in mammalian systems. Vacuole Import and Degradation (*VID*) genes

were isolated in regards to their role in the vacuole dependent degradation of gluconeogenic enzymes in yeast. Our preliminary results indicate an even broader role for *VID* genes in the survival of cells during chronological aging and during oxidative-stress in yeast. Cells lacking several of the *VID* genes are sensitive to oxidative stress and they have shorter life spans. Thus, *VID* genes protect cells from these stresses. Homologues of *VID* genes are found in mice and humans. It is the goal of this study to examine whether a similar Vid pathway exists in mammalian cells.

Duration of Project

7/1/2008 - 6/30/2009

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 20: Project Title and Purpose

Netrin-1 in Ischemia Reperfusion Injury of the Kidney - The purpose of this project is to gain a better understanding of how netrin-1 prevents ischemic renal injury. We will determine the receptors subtype which mediates netrin-1 protective effect against ischemic kidney injury and pathways through which netrin-1 mediates the protective effects.

Duration of Project

7/1/2009 - 6/30/2010

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 21: Project Title and Purpose

Structures in the Unfolded State Initiate Protein Folding - The central dogma of molecular biology states that DNA is transcribed into a messenger RNA which is translated into the amino acid sequence of a protein. However, we do not understand the means by which that linear sequence of amino acids is transformed into a unique three dimensional structure that is capable of function. This project examines the mechanism by which protein folding is initiated, identifying individual residues and structures that direct the folding of the protein to the correct final structure.

Duration of Project

7/1/2008 - 6/30/2010

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 22: Project Title and Purpose

Mechanisms of Gene Regulation by EBV EBNA-1 Protein - The potential of Epstein-Barr virus (EBV) to cause cancer is dependent on establishment of a life-long latent infection within its human host's B lymphocytes. We have discovered a novel autoregulatory mechanism through which EBV can control expression of its EBNA-1 protein, whose function is critical to EBV persistence in B cells. We hypothesize that this enables the virus to maintain EBNA-1 levels below a threshold to prevent detection and removal of infected cells by the host's immune surveillance. The purpose of this research is to evaluate autoregulation of EBNA-1 expression in the context of infected B cells to permit us to test whether it is capable of allowing infected cells to evade the immune response. Ultimately, this may lead to therapeutic approaches to disrupt autoregulation of EBNA-1 to enhance the anti-EBV immune response.

Duration of Project

7/1/2008 - 6/30/2010

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 23: Project Title and Purpose

Regulation of Mitochondrial Dysfunction and Diet-induced Obesity by ALCAT1 - Cardiolipin is an important lipid required for health and diseases. Like cholesterol, there are "good" and "bad" cardiolipins which are determined by the structure of the lipid. Increased levels of bad cardiolipin play a causative role in diabetes, obesity, hyperthyroidism, and aging. ALCAT1 is a cardiolipin synthetic enzyme recently identified in our lab. We have shown that this enzyme catalyzes the synthesis of bad cardiolipin, and its enzyme activity is elevated in diabetes and obesity. The project will validate ALCAT1 as a drug target for the treatment of diabetes and obesity using sophisticated molecular, cellular, enzymatic, and transgenic approaches.

Duration of Project

7/1/2008 - 6/30/2010

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 24: Project Title and Purpose

Novel Pathway Mediating Peripheral Sensitization of Esophageal Vagal Sensory Afferent Nerves- Abnormal esophageal sensations, such as esophageal-related noncardiac chest pain and heartburn, are common complaints. They are generated from noxious stimuli on esophageal sensory afferent nerves and transmitted to the central nervous system via both spinal and vagal pathways. Esophageal inflammation sensitizes sensory afferents and enhances these abnormal sensations to induce visceral hypersensitivity. This involves both peripheral and central sensitization. The mechanism of peripheral sensitization of esophageal sensory afferents is still unclear. The long-term goal of our research is to study the mechanism of peripheral sensitization in a validated esophageal hypersensitivity model. This project focuses on peripheral sensitization of vagal sensory afferents in our guinea-pig *ex vivo* esophageal-vagal preparation.

Duration of Project

7/1/2008 - 6/30/2009

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 25: Project Title and Purpose

The Role of Microglial Priming in Diabetic Retinopathy - The purpose of this project is to conduct preliminary experiments to test the hypothesis that a systemic inflammatory event will couple with diabetes to cause a pronounced increase in retinal inflammation. We will use animal models to determine if infection or inflammation in other parts of the body may cause excessive inflammation in the diabetic retina. If so, this response may explain why some diabetic patients suffer from extreme retinal damage, while others do not. We will also determine if microglial cells in the diabetic retina are the initiators of this hyper-response. If so, then treatments that control microglial behavior may be useful for preventing the progression of diabetic retinopathy.

Duration of Project

11/24/2008 - 12/31/2009

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 26: Project Title and Purpose

Gag and CA Protein Interactions in Retroviral Capsid Maturation - This project examines the molecular mechanism by which the internal structure of infectious retrovirus particles, such as that of HIV, is formed during the process of virus release from the infected cells. Specifically, this work will test the novel hypothesis that a critical step in the virus assembly pathway is an interaction of the mature capsid protein CA with one or more molecules of its precursor protein Gag. This hypothesis will be tested with proteins derived from both the avian Rous sarcoma virus and HIV. By documenting the molecular mechanisms involved in detail, this project will provide critical insights that may be utilized for the development of new anti-retroviral drugs.

Duration of Project

11/24/2008 - 6/30/2010

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 27: Project Title and Purpose

The Effects of Air Quality on Human Reproduction - Air quality has been identified as a significant risk factor for respiratory and cardiovascular disease and for a variety of cancers. It is likely, based on preliminary data from this project that air quality also adversely affects human reproduction. We will use the model of assisted reproductive technology, i.e., in vitro fertilization (IVF) for our study. This paradigm allows us to determine exact dates of oocyte development, ovulation (i.e., the day of oocyte retrieval), fertilization, implantation, and duration of pregnancy, which allow for more accurate correlation of outcomes with ambient air quality. Our primary goal is to establish the relationship between air quality at the home zip code of the subject or in the IVF lab and fertility outcomes after IVF. Our long range objective of this study is to design a randomized trial to improve air quality both in the lab and in the IVF subject's external environment to improve reproductive outcomes.

Duration of Project

11/24/2008 - 6/30/2010

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 28: Project Title and Purpose

New Methods for Studying Mitochondrial Mutations and Common Chromosome Fragile Sites in Cancer - Mutations are the cause of human genetic diseases. Thus, elucidating the mechanisms and risk factors of mutagenesis is of great significance. We intend to test the utility of new methods for studying mitochondrial mutations, the methods that rely on using the latest sequencing technologies. Additionally, we will investigate alterations in chromosome structure that are a hallmark of solid tumors. The mechanisms by which such alterations arise during cancer are not fully understood. We propose to use a combined computational-biochemical approach to discover DNA sequences in the human genome that are associated with sites of chromosome breakage.

Anticipated Duration of Project

11/24/2008 - 12/31/2011

Project Overview

The first part of this project is focused on mitochondrial mutations causing >200 human genetic diseases. Mitochondrion is an organelle of cellular energy synthesis and, as a result, possesses mutagenic metabolites in high quantities. Our First Specific Aim is: *To investigate the use of novel sequencing technology to the studies of mitochondrial mutations*. We will test how high throughput short-read sequencing can improve the detection of levels of mitochondrial disease-causing mutations. This is critical for precise estimation of mitochondrial DNA (mtDNA) mutation rates and for the prognosis and treatment of mitochondrial diseases as well as for preventing mitochondrial disease transmission. As part of this Specific Aim, we will collect blood and buccal cell samples from 500 mother-child pairs, isolate DNA from these samples, and will investigate mutations that occur in the maternal germline.

The objective of the second part of this project is to elucidate how the structure of an individual genome underlies the risk of developing chromosomal aberrations during cancer. We have discovered that microsatellites (short DNA repeats) pose a significant block to DNA synthesis in a sequence-dependent manner. Microsatellites are present within common fragile sites (CFS), regions of the genome prone to chromosomal instability in tumor cells. Our Second Specific Aim is: *To test a hypothesis that DNA replication inhibition within CFS is due to the density and/or arrangement of specific microsatellite sequences, relative to other areas of the genome*. We

propose to use statistical classification and comparative genomics techniques to identify critical features of the human genome associated with chromosomal fragility. Genome elements identified computationally will be tested directly using biochemical analyses. The experiments outlined in this study will elucidate DNA replication dynamics through CFS and the mechanisms/factors responsible for DNA breakage. The identification of key sequence elements responsible for CFS can lead to elucidation of individual genetic risk factors and environmental exposures that act to increase chromosomal instability during cancer.

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Francesca Chiaromonte, PhD, Laura Carrel, PhD, Kristin Eckert, PhD, Ian Paul, MD- employed by Penn State University

Expected Research Outcomes and Benefits

An improved identification of mutations in mtDNA will be of great clinical and scientific significance. *First*, this will allow for more accurate identification of disease-causing alleles and thus a better diagnosis and earlier treatment of diseases caused by mutations in mtDNA. This will have a major effect on and is expected to improve genetic counseling. *Second*, this project will provide important information on the mechanisms of mutations in mtDNA – the cause of genetic diseases.

This research is highly relevant to unraveling cancer etiology. Understanding DNA replication dynamics through CFS will help to elucidate the mechanisms and endogenous factors responsible for inducing DNA breakage at these chromosomal sites. In addition, current CFSs were identified using cytogenetic techniques, and likely represent only a subset of genomic regions that are prone to rearrangement. Our proposed bioinformatics approach will allow us to understand global mechanisms of chromosomal instability in human cells and can lead to a fine-scale mapping of chromosomal rearrangement sites within the human genome in future studies. The identification of key sequence elements underlying DNA replication inhibition and DNA breakage can lead to the identification of individual genetic risk factors or environmental exposures that increase chromosomal instability during neoplastic progression.

Summary of Research Completed

Specific Aim 1. Here we summarize our progress on the mitochondrial Aim of the project.

A. Purpose

Originally believed to be a rare phenomenon, heteroplasmy is emerging as an important

component of eukaryotic genetic diversity. Heteroplasmies, which are defined as the presence of more than one mitochondrial DNA (mtDNA) variant within a cell, tissue or individual, can be used as genetic markers in applications ranging from forensics to cancer diagnostics. As the frequency of heteroplasmic alleles may vary from generation to generation due to the bottleneck occurring during oogenesis, it is of critical importance to investigate the dynamics of maternal mtDNA transmission.

B. Results

In this study, we sequenced mtDNA at high coverage from blood and buccal tissue of nine individuals from three families with a total of six maternal transmission events. Using simulations and re-sequencing of clonal DNA, we devised a set of criteria for detecting polymorphic sites in heterogeneous genetic samples that is resistant to the noise originating from massively parallel sequencing technologies. Application of these criteria to nine human mtDNA samples revealed four heteroplasmic sites.

C. Conclusions

Our results suggest that the incidence of heteroplasmy may be lower than estimated in some other recent re-sequencing studies, and that mtDNA allelic frequencies differ significantly both between tissues of the same individual and between a mother and her offspring. We designed our study in such a way that the complete analysis can be repeated by anyone either at our Galaxy website (<http://main.g2.bx.psu.edu/>) or directly on the Amazon Cloud. Our computational pipeline can be easily modified to accommodate other applications, such as viral re-sequencing. These results have recently been published in *Genome Biology* (Goto et al. 2011). An additional manuscript describing the use of the Amazon Cloud for analysis of mitochondrial heteroplasmy has been accepted for publication (pending a minor revision) in *Nature Biotechnology* (Afgan et al., in press).

Specific Aim 2. Common fragile sites (CFS) are specific genomic loci prone to formation of breaks and gaps that can be seen on metaphase chromosomes. CFS instability is known to be induced by replication stress, and recent studies have uncovered several cellular proteins necessary for their stability. Due to their propensity for breakage, CFS are also frequently deleted or translocated in cancer. Sequence analyses of many CFS have determined that they contain repetitive DNA sequences with non-B DNA potential such as hairpins, triplex structures and bent DNA. FRA16D, located at 16q23.3, is one of the most commonly expressed and well characterized CFS. Our lab showed that the replicative polymerases δ and α are significantly inhibited during synthesis of FRA16D sequences with non-B DNA structure potential. We hypothesized that the clustering of DNA elements with non-B DNA structure potential in CFS sequences inhibits replicative DNA polymerases, and that specialized DNA polymerases/replicative proteins are required to complete CFS replication.

A. Experimental design.

To test our hypothesis, four new DNA templates were generated that correspond to human CFS regions with non-B DNA potential, including microsatellites, inverted repeats and regions of high DNA flexibility (Table 1).

B. Biochemical Analyses of replication inhibition.

B.1. Modifications of experimental design. We improved our published assay by two modifications. First, we developed a method of normalizing DNA synthesis to a control template. This allows us to make direct comparisons between polymerases and templates, and to analyze the data statistically. Second, we tested various buffers for the capacity to support DNA synthesis by a variety of polymerases. Synthesis by polymerase h (Pol h) was poor in Tris pH 7.5 and BisTris pH6.5 buffers, while synthesis by polymerase delta holoenzyme (Pol d) was poor in Hepes, pH 7.2 buffer. However, we observed good synthesis by all polymerases using a KPO₄ pH 7.6 buffer. Importantly, this modified buffer better reflects the nuclear physiologic conditions of human cells.

B.2. Effects of replication accessory proteins on Pol delta synthesis progression. Synthesis quantification showed significant slowing of Pol delta, particularly at predicted hairpins and mononucleotide repeats. We analyzed the extent to which several replication proteins alleviate Pol delta during CFS DNA synthesis. All of the proteins examined were previously reported to interact with Pol d: PCNA, RF-C and RPA. Results of preliminary experiments using each protein individually and in combinations were negative. We also tested the effect of [dNTP] substrate concentration. We observed a slowing of Pol delta DNA synthesis rate and increased sequence-specific inhibition as the [dNTP] concentration was lowered to near physiologic conditions. Our tentative conclusion is that the presence of accessory factors does not alleviate Pol d pausing within CFS regions.

B.3. Synthesis by specialized polymerases κ and η . Loss of the specialized DNA polymerase η was recently shown to cause increased instability at CFS in cells. Thus, we tested DNA synthesis progression by Pol eta and the related enzyme, Pol kappa through CFS sequences. Using the AT-1 template, Pol delta progression through the microsatellite region is significantly inhibited, relative to the control template, at all times examined (Figure 1A). In contrast, Pol eta progression is not significantly slower on the AT1 template, relative to the control template (after 5 min). Moreover, Pol eta synthesis progression is significantly faster than Pol delta using this template (Figure 1A). A similar result was measured using the IR region 1 template (Figure 1B). However, the differences between the enzymes are sequence-specific, as pol η showed no significant differences in the overall completion of template synthesis relative to Pol δ using the AT Repeat 2 template. Preliminary results demonstrated that Pol κ was the least slowed overall of the three polymerases, and exhibited the greatest ability to completely synthesize FRA16D templates. This suggests that Pols κ and η may both contribute to replication at CFS in order to maintain stability in normal cells.

C. Scientific outcomes

We have identified critical *cis*-acting DNA elements with CFS sequences, as well as *trans*-acting replication proteins that contribute to complete CFS replication. Our findings support a model whereby specific sequences with non-B DNA potential impede replication by Pol delta and contribute to CFS breakage under replication stress by preventing complete replication. Two manuscripts describing our results are in preparation; a new NIH-R01 grant submission is planned for September, 2011.

Cited publications resulting from funding in this period:

Afgan, E., D. Baker, N. Coraor, H. Goto, I. M. Paul, K. D. Makova, A. Nekrutenko, and J.

Taylor. Harnessing cloud-computing for biomedical research with Galaxy Cloud. Nature Biotechnology (accepted).

Goto, H., B. Dickins, E. Afgan, I. M. Paul, J. Taylor, K. D. Makova and A. Nekrutenko. 2011. Dynamics of mitochondrial heteroplasmy in three families investigated via a repeatable re-sequencing study. *Genome Biology* 12: 59.

Table 1. Templates created for CFS Experimental Assay

Region	A + T (%)	Repeat Elements ¹	Predicted Hairpins ²	Flexibility Peak ³	Representative CFS
Control*	75	None	3 (3-4 bp)		FRA 16D
AT Repeat 1*	74	[A/T] ₂₈ , [AT/TA] _{24i}	4 (2-21 bp)	Peak 5	FRA 16D
AT Repeat 2	72	[A/T] ₉ , [AT/TA] _{22i}	2 (19-21 bp)	Peak 4	FRA 16D
AT Repeat 3	80	[A/T] ₂₂ , [AT/TA] ₂₅	3 (3-24 bp)		FRA3B
IR Region 1*	53	[A/T] ₁₉ , IR _{36i}	4 (2-12 bp)		FRA 16D
IR Region 2	51	IR _{37i}	5 (2-17 bp)		FRA 16D
IR Region 3	64	[A ₄ (TA ₃) ₃ TA ₄], IR _{29i}	6 (3-20 bp)		FRA 16D

*Templates previously investigated by Shah et al., 2010. NAR. 38: 11459

¹ Identified with RepeatMasker or non-B DNA database (IR, inverted repeat; i, interrupted repeat; <http://nonb.abcc.ncifcrf.gov/apps/Query-GFF/Features>)

² Predicted by Mfold software under most thermodynamically stable conditions

³ Flexibility determined in Reid et al., 2000.

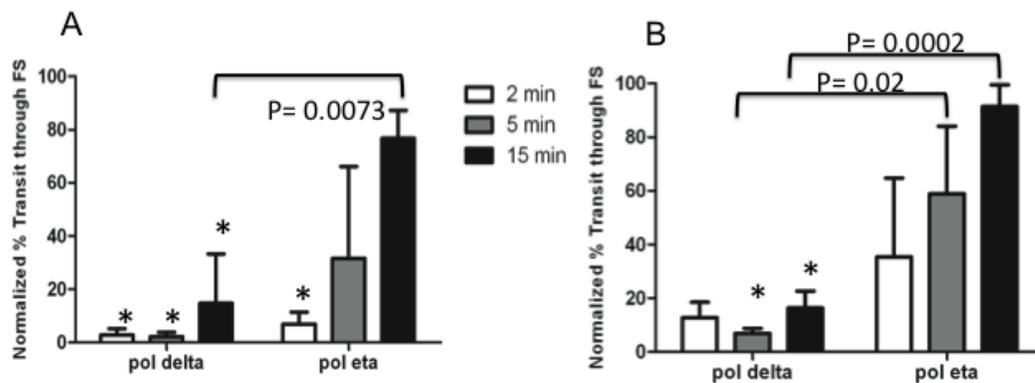


Figure 1. Comparative DNA synthesis by Pol δ and Pol η within FRA16D sequences. DNA synthesis products were quantitated after the indicated times using either (A). AT Repeat 1 template, or (B). IR Region 1 template. The extent of synthesis was normalized to that measured on corresponding control template. Bars indicate the mean and standard deviation of at least three independent determinations. *, significantly different from % synthesis on the control template ($p < 0.01$). Brackets indicate statistical comparisons between polymerases at the indicated times. Statistical analyses performed using the student t-test (unpaired; 2-tailed).

Research Project 29: Project Title and Purpose

Proteomic and Molecular Analysis of Formalin Fixed, Paraffin Embedded Tumors - Every day, benign and malignant tumor tissues are biopsied or removed surgically and evaluated by a pathologist to establish a diagnosis and prognosis (i.e., Is it cancerous? What type of cancer?). However, much information that might aid in making a diagnosis remains unstudied within these collected tissue samples. One difficulty has been that the tissues are preserved in such a way (formalin fixation and paraffin embedding) that makes standard methods of molecular biology difficult to use. Recently, emerging technologies have made it possible to obtain some of this untapped information. Our goal is to couple these techniques in a way that has not yet been done at Penn State University – College of Medicine (PSU-COM) to compare two different types of tumors. If successful, these methods will be important for basic science studies, and could be developed further as powerful clinical diagnostic tools.

Duration of Project

11/24/2008 - 4/30/2011

Project Overview

The molecular diagnosis of cancers has been the subject of intensive research in recent years. The goal of this work is to perform a demonstration project that seeks to diagnose specific tumors using a series of techniques that have not been previously performed at the PSU-COM. Our objective is to perform laser capture microdissection of *formalin fixed, paraffin embedded*

(FFPE) tissues followed by proteomic and molecular analyses. To accomplish this goal, we have assembled a matrix biologist, a proteomics expert, an orthopaedic oncologist and a practicing orthopaedic pathologist with long term experience in sarcoma. We believe that a successful outcome would be of great benefit to the research community at PSU-COM and elsewhere because it would facilitate a wide variety of studies.

Specific Aim 1: Distinguish two sarcoma tumor types by their ECM protein composition.

Hypothesis: Tumors that arise from different source tissues can be distinguished based upon the extracellular matrix proteins they produce.

Rationale: Even closely related tissues such as cartilage and bone have distinct, non-overlapping extracellular matrix (ECM) protein expression. We believe these protein patterns remain after oncogenic transformation, and will allow the distinction of the two tumor types. Laser capture microdissection will allow the selective proteomic analysis of only the ECM proteins, and result in a simpler sample with greater relative protein content.

Specific Aim 2: Distinguish between similar tumors with clinically distinct behaviors.

Hypothesis: Benign and malignant tumors arising from the same tissue can be distinguished by the expression of key genes.

Rationale: Mesenchymal neoplasms that share a common tissue origin can have a similar histopathologic appearance, but radically different clinical behaviors. We will use laser capture microdissection to isolate cells from FFPE tissues, and extract RNA to compare benign and malignant mesenchymal tumors for expression of genes known to affect metastatic potential.

Specific Aim 3: Identify grade-specific extracellular matrix protein biomarkers for cartilage lesions.

Hypothesis: The histological range of benign to high-grade cartilage tumors will be reflected in the extracellular matrix (ECM) proteins produced locally within the tumors. These abundant, stable proteins can serve as definitive biomarkers to distinguish benign from malignant cartilaginous lesions.

Rationale: Tumor grade is the single most important prognostic factor in cartilaginous lesions (Giuffrida et al., 2009). It is also the greatest determinant in selecting a course of clinical treatment. Importantly, determination of cartilage lesion grade is particularly difficult and reliability is low even among experienced radiologists and pathologists. Thus, improvement in grading reliability will have an immediate and positive impact on clinical outcomes. The spectrum of benign to high-grade cartilage tumors is observable by subtle histological differences. These differences will be present at the molecular level in the extracellular matrix (ECM) proteins produced locally within the tumors. Mass spectrometry can be used to identify these differences between grades. Accordingly, these abundant ECM proteins can be used as grade-specific biomarkers for cartilaginous lesions.

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Expected Research Outcomes and Benefits

Specific Aim 1 Expected Outcomes: Because dozens of ECM proteins are produced exclusively by osteoblasts (e.g. osteocalcin) or chondrocytes (e.g. type II collagen), we anticipate the identification of 10-20 proteins that are uniquely produced by either osteosarcoma or chondrosarcoma. Upon successful completion of preliminary samples, work is to continue on patient samples of enchondroma and chondroma tumors. These samples will be de-identified, formalin fixed paraffin embedded archival tissues. Based on our early work, we anticipate the identification of 100-150 proteins per sample quantified by iTRAQ, and that approximately 5% of these will be statistically informative between tumor types.

Specific Aim 2 Expected Outcomes: Due to the hypoxic, anhydrous conditions of tissues in paraffin, we anticipate the successful extraction of RNA from LCM captured cells. Even if the mRNA is slightly degraded, TaqMan primer sets typically amplify only very short amplicons (100-150bp), and so detection may be only slightly affected and will be normalized by GAPDH and beta actin controls. Additionally, we expect to see increased expression of the integrin subunit alpha V, and MT1-MMP (MMP14) in the more metastatically aggressive cancers.

The major benefit of these two Specific Aims will be a new ability to distinguish these two tumor types from one another. This is especially critical. They respond differently to chemotherapy. Therefore, there may eventually be a direct clinical benefit to the patients with these tumors as they may be diagnosed with greater certainty, and treated with greater confidence. Further, these techniques can be applied to many other tumor types. The potential exists that these techniques could aid clinical treatment of cancer patients in several additional ways; for example, improved tumor staging or determination of metastatic potential.

Specific Aim 3 Expected Outcomes: Cartilaginous lesions have observable histopathological differences at the extremes of the cartilage lesion spectrum. These differences are manifested at the molecular level in the local ECM proteins produced within the tumor. *The disparities in ECM protein composition, if identified, could serve as abundant biomarkers that have the potential to diagnostically aid intermediate staging or have prognostic value.*

Summary of Research Completed

In the final year of funding, our efforts to identify biomarkers for cancerous lesions of connective tissue have focused upon markers for metastatic potential. Understanding the mechanisms of osteosarcoma metastatic progression is key for the identification of candidate therapeutic targets, and the development of more effective therapies. The goal of the work over

the last year was to identify proteins that have key roles in the metastatic potential of cells. To this end, differences in protein expression between two osteosarcoma cell lines characterized by opposite metastatic potential were determined. The study employed SAOS-02, a well characterized osteosarcoma cell line with low metastatic potential, and a related highly metastatic cell line which was derived from SAOS-02 cells through serial in vivo panning.

Samples were analyzed using FASP/iTRAQ - LC-MS/MS technique. This approach yielded significant differences in levels of several proteins that have been previously implicated in carcinoma metastatic potential, and this is the first report of potential roles in mesenchymally derived sarcoma metastatic potential. Four differentially expressed proteins were selected for further verification by two independent techniques.

Methods:

Cell Culture: The human osteosarcoma cell lines SAOS-02 and LM7 were generous gifts from Dr. Eugenie S. Kleinerman (Department of Cancer Biology, The University of Texas, MD Anderson Cancer Center). Cells were grown in monolayer culture in tissue culture flasks (Costar), and cultured in Dulbecco's Modification of Eagle's Medium (DMEM cellgro cat # 10-014-CV) with 1 g/L glucose, L-glutamine, and sodium pyruvate, supplemented with 10% fetal bovine serum (FBS, Atlanta biological) 1% of non-essential amino acids (MEM NEAA Cellegro), sodium pyruvate (Cellegro), and penicillin-streptomycin (Invitrogen) and maintained in a humidified incubator at 5% CO₂ atmosphere. All experiments were conducted on cells harvested at 100% confluence.

Sample preparation for Mass Spectrometry:

Protein Harvesting - Three flasks of 150 cm² were washed three times with ice cold Phosphate Buffer Saline (PBS) pH 7.4 to remove the serum contaminant protein. Subsequently the cells were dissociated by incubating for 20 min in ice cold versene solution (0.025 M of EDTA in PBS pH 8) with gently shaking. The cells were centrifuged at 1K x g for 10 min at 4°C, rinsed two times in PBS to remove the excess of EDTA, and counted. After counting the cells were lysated in a nuclei extraction buffer (1X protease inhibitor Complete® ROCHE, 1.2 mM EDTA, 4% glycerol, 70mM of glucose). The plasma membranes were gently lysed by nitrogen decomposition, by incubating in a nitrogen bomb (Parr cell disruptor vessel, cat n.4639) for 20 min at 800 psi, pressure sufficient to break only the plasma membrane. The entire nitrogen lysis was conducted on ice. The lysates were separated from the intact nuclei by centrifugation at 2k g at 4°C. The nuclei were pelleted and separated from the membrane/ cytoplasmic fraction. The proteins in the supernatant were separated by Chloroform for/methanol precipitation. The proteins precipitated were then dialyzed overnight in a 3.5 kDa cut off membrane (Fisherbrand) against CaCl 14mM, Hepes-Tris 10 mM pH 7.4. The dialyzed proteins were pelleted at 18k x g for 20 min, and resuspended in the same lysis buffer(SDS 2%, Tris-HCl 50 mM pH 7.6, urea 4 M, DTT 450 mM) used for the proteomic analysis. The resuspended proteins were denatured by heating at 95°C for 5 min and the concentration measured by reading spectrometrically at 280 nm and A260 and using the following conversion: protein concentration (mg/ml) = (1.1 x A280) - 0.76 x A260). The sample was ready to be prepared for mass spectrometry.

Protein Digestion - For each sample, 1mg of starting protein was used for digestion. Each 1mg sample was divided into four different 10 kDa MWCO cut off spin columns (Amicon® Ultra cat no. UFC501024). The sample was washed three times in urea buffer (4 M urea, Tris HCl 0.1 M pH 8) by centrifugation at 14K x g for 40 minutes. The sample was alkylated by 50mM of iodoacetamide (IAA) and incubated for 20 min in the dark. The IAA was removed by four urea buffer washes and spin filtration. The concentration of urea (that could inhibit the tryptic activity) was reduced by a wash with ammonium bicarbonate buffer (50mM of ammonium bicarbonate). Alkylated proteins were digested in column by trypsin (sequencing grade modified trypsin, Promega cat # V5111). The enzyme was diluted in ammonium bicarbonate buffer to the concentration of 10U / µg of protein, and the working pH range (optimum at pH 7.8), the digestion was performed by incubate overnight at 48°C in humidified chamber. The undigested proteins were separate from the peptides by centrifuge at 14.000 x g for 40 min. The residual peptides not eluted were harvested by a further elution with 0.5 M of NaCl. The eluted peptides solution were desalted by using a C18 column (PALL life science, Nanosep® Centrifugal devices cat # OD010C33). The concentration of the eluted peptide was measured spectrometrically at 280 nm. A yield variable between 37-50% was obtained. The eluted peptide solutions were concentrated by speed vacuum and the concentration adjusted to 5 µg/µl, for iTRAQ tagging.

iTRAQ Labeling - iTRAQ labeling was done in accordance with the manufacturer's protocol (Applied Biosystem Inc., Foster City, CA), with some minor modification. To increase the consistency and ensure the reliability of the analysis, each experimental group was divided in two sub-groups, analyzing in this way each condition twice. A total amount of 125 µg of peptide was used per tag. The tag of 117 and 118 were used to label the sample SAOS-02, the isobaric tag 113 and 114 were used for LM7 sample.

2D-LC separations: SCX Separations were performed on a passivated Waters 600E HPLC system, using a 4.6 X 250 mm PolySULFOETHYL Aspartamide column (PolyLC, Columbia, MD) at a flow rate of 1 ml/min. Buffer A contained 10 mM ammonium formate, pH 2.7, in 20% acetonitrile/80% water. Buffer B contained 666 mM ammonium formate, pH 2.7, in 20% acetonitrile/80% water. The gradient was Buffer A at 100% (0-22 minutes following sample injection), 0%→40% Buffer B (16-48 min), 40%→100% Buffer B (48-49 min), then isocratic 100% Buffer B (49-56 min), then at 56 min switched back to 100% A to re-equilibrate for the next injection. The first 26 ml of eluant (containing all flow-through fractions) was combined into one fraction, and then 14 additional 2-ml fractions were collected. All 15 of these SCX fractions were dried down completely to reduce volume and to remove the volatile ammonium formate salts, then resuspended in 9 µl of 2% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid and filtered prior to reverse phase C18 nanoflow-LC separation. For 2nd dimension separation by reverse phase nanoflow LC, each SCX fraction was autoinjected onto a Chromolith CapRod column (150 X 0.1 mm, Merck) using a 5 µl injector loop on a Tempo LC MALDI Spotting system (ABI-MDS/Sciex). Buffer C was 2% acetonitrile, 0.1% trifluoroacetic acid, and Buffer D was 98% acetonitrile, 0.1% trifluoroacetic acid. The elution gradient was 95% C/ 5% D (2ul per minute flowrate from 0-3 min, then 2.5ul per minute from 3-8.1 min), 5% D→38% D (8.1-40 min), 38% D→80% D (41-44 min), 80% D→5% D (44-49 min) (initial conditions). Flow rate was 2.5 µl/min during the gradient, and an equal flow of MALDI matrix solution was added post-column (7 mg/ml recrystallized CHCA (a-cyano-hydroxycinnamic acid), 2 mg/ml

ammonium phosphate, 0.1% trifluoroacetic acid, 80% acetonitrile). (Gradient used prior to 2008 was 100% C (0-4 min), 0→10% D (4-10 min), 10%→25% D (10-30 min), 25%→40% D (30-35 min), 40%→80% D (35-38 min), 80% D (38-42 min), 80%→0% D (42-43 min), 0% D (43-50 min), with a flow rate of 3.0 ul per minute with an equal flow of MALDI matrix solution.) The combined eluant was automatically spotted onto a stainless steel MALDI target plate every 6 seconds (0.6 µl per spot), for a total of 370 spots per original SCX fraction.

Mass Spectrometry Analysis:

After sample spot drying above, thirteen calibrant spots (ABI 4700 Mix) are added to each plate manually. MALDI target plates (15 per experiment) are analyzed in a data-dependent manner on an ABI 5800 and/or 800 MALDI TOF-TOFs. As each plate is entered into the instrument, a plate calibration/ MS Default calibration update is performed, and then the MS/MS default calibration is updated. MS Spectra are then acquired from each sample spot using the newly updated default calibration, using 500 laser shots per spot, laser intensity 3200 (this can change somewhat with laser age and tuning). A plate-wide interpretation is then automatically performed, choosing the highest peak of each observed m/z value for subsequent MS/MS analysis. Up to 2500 laser shots at laser power 4200 are accumulated for each MS/MS spectrum. When the MS and MS/MS spectra from all 15 plates in a sample set have been acquired, protein identification and quantitation are performed using the Paragon algorithm as implemented in Protein Pilot 4.0 software searching the spectra against either species-specific subsets (plus common contaminants) of the NCBIInr database concatenated with a reversed "decoy" version of itself, or the entire NCBIInr database. Search settings are used, and identifications must have a ProteinPilot Unused Score > 1.3 (>95% Confidence interval) in order to be accepted. In addition, the only protein IDs accepted MUST have a "Local False Discovery Rate" estimation of no higher than 5%, as calculated from the slope of the accumulated Decoy database hits by the PSPEP. Note that this Local or "Instantaneous" FDR estimate is much more stringent than $p < 0.05$ or 95% confidence scores ProteinPilot.

Confirmation of proteomic data by Western Blotting.

To confirm the protein expression differences found by the proteomic assays, we used Western blotting as a complementary technique for a good experimental validation of the proteomic data. Validation was carried out by running three independent western blotting experiments. In each experiment the proteins were extracted as previously described, from three different T25 flasks. Total protein was harvested from cells at 100% confluence. Briefly, cells were rinsed three times in ice cold PBS to remove the contaminant serum proteins, then lysis buffer (50 mM Tris-HCl pH 7.6, 1X protease inhibitor Complete®ROCHE, 50 mM DTT and 2% of SDS) was applied to the cells on ice. The cells were gently scraped, the lysate sonicated to shear DNA and reduce the relative viscosity and clarified by centrifugation at 16,000 x g for 5 min. Thirty-five micrograms of lysate were separated by 10% polyacrylamide gel, electroblotted on Polyvinylidene Fluoride membrane (Biorad) and then blocked with 5% non fat milk powder in PBST (Phosphate Buffer Saline with Tween 0.05%). The detection of the protein marker were carried out by probing at room temperature for 2 hr with monoclonal antibody against α -Tubulin (Santa Cruz Biotechn.sc-8035), and Fascin 1 (Santa Cruz Biotechn. sc-2143) and goat polyclonal antibody against HSP60 (Santa Cruz Biotechn.1052), VMAT (Santa Cruz Biotechn.sc-7718), Creatin Kinase B (Santa Cruz Biotechn.sc-15157). Thereafter the membrane was rinsed three times in PBST, incubated with horseradish peroxidase conjugated anti mouse or anti goat IgG for 1 hr at room temperature,

and rinsed again three times in PBST. The detection of the signal was developed by using luminol-based chemiluminescence system.

Results:

A total of 8,217 unique peptides were identified at the highly stringent confidence levels described above. From these, a total of 1,215 unique proteins were identified. An arbitrary level of a two-fold difference in protein levels is often used in peer reviewed literature as a cutoff for biological importance. Using this cutoff, 24 proteins were found to be down regulated in LM7 cells, and 9 proteins up regulated. These are presented in Table 1 below. The proteins listed in **RED** were chosen for additional validation.

Additional protein validations are ongoing, and a manuscript is in preparation.

Table 1 - proteins significantly different in metastatic and non-metastatic cells

	Protein name	Gene symbol	Fold change	Level
1	Vesicle amine transport protein 1 homolog	gi 60552323	7.48	DOWN
2	Heat shock protein 60	gi 77702086	6.64	DOWN
3	Calreticulin	gi 62897681	6.34	DOWN
4	Protein disulfure isomerase family a member 3	gi 47938352	5.32	DOWN
5	Prolyl 4-hydroxylase, beta polypeptide	gi 48735337	4.79	DOWN
6	Filamin C	gi 7715914	4.23	DOWN
7	Lactate dehydrogenase B	gi 54696396	4.07	DOWN
8	Protein disulfure isomerase A4 precursor	gi 51105825	4.04	DOWN
9	Heat Shock protein 70	gi 5729877	3.75	DOWN
10	Heat shock protein 90 beta	gi 83699651	3.72	DOWN
11	Calumenin	gi 5921197	3.66	DOWN
12	Phosphoribosylglycinamide formyltransferase,	gi 78070756	3.30	DOWN
13	Peptidil prolyl isomerase (cyclophilin B)	gi 61354171	3.10	DOWN
14	adenosylhomocysteinase isoform 1	gi 9951915	2.95	DOWN
15	prolyl 4-hydroxylase, alpha I	gi 63252888	2.84	DOWN
16	ERO1-like precursor	gi 7657069	2.68	DOWN
17	ACLY variant protein	gi 68533125	2.63	DOWN
18	annexin VI isoform 2	gi 71773415	2.57	DOWN
19	filamin B	gi 8100574	2.50	DOWN
20	150 kDa oxygen-regulated protein variant 1	gi 86611373	2.44	DOWN
21	thioredoxin domain containing 5	gi 56204860	2.29	DOWN
22	Protein disulfur isomerase A6	gi 5031973	2.28	DOWN
23	Zyxin	gi 58530845	2.10	DOWN
24	cathepsin D (lysosomal aspartyl protease)	gi 54697170	2.06	DOWN
25	filamin A, alpha (actin binding protein 280)	gi 57284203	1.89	DOWN
26	creatine kinase-B	gi 180555	12.71	UP
27	Fascin 1	gi 4507115	7.52	UP
28	DPYSL3	gi 50417352	3.94	UP
29	Heat shock protein HSP 90-alpha	gi 92090606	3.45	UP
30	Chain C, Form Of Annexin V	gi 809190	2.48	UP
31	Annexin A1	gi 55959292	2.33	UP
33	enolase 1, (alpha)	gi 66268795	2.31	UP
34	arginyl aminopeptidase	gi 40316915	2.27	UP
35	ketratin 1	gi 7331218	1.98	UP
36	tyrosyl-tRNA synthetase variant	gi 62898948	1.98	UP
37	elongation factor 2	gi 60685056	1.97	UP
38	Chain B, Cyclophilin-A Complex With Suc-Agpf-Pna	gi 99031692	1.90	UP

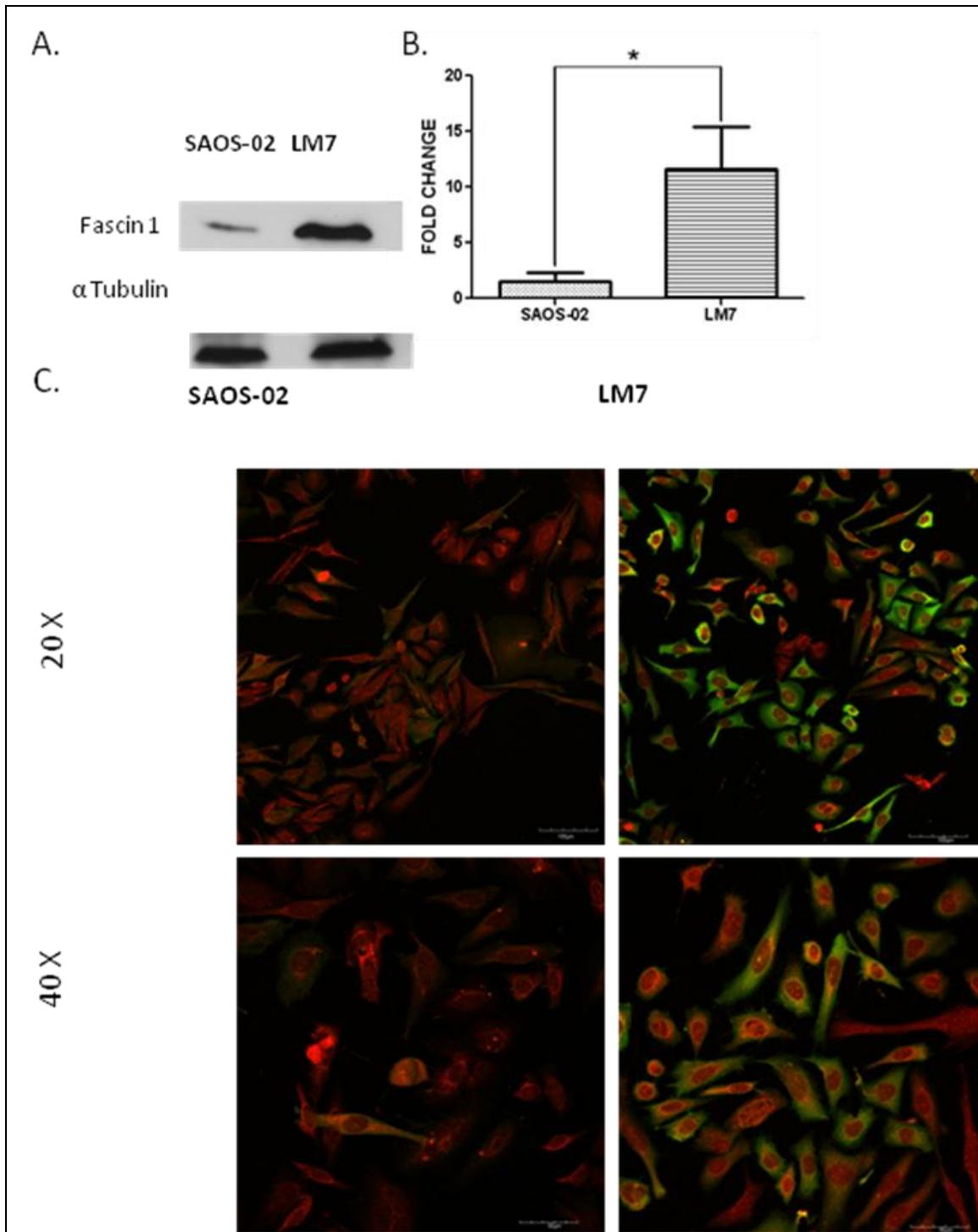


Figure 1: Validation of Mass Spectrometry data for Fascin1.

A. Representative images of immunoblotting against Fascin1; B. Quantification of expression by densitometry using QuantityOne[®] software. The high metastatic cell line LM7 show a higher expression compared with the low metastatic cell line SAOS-02 of about 7.5 folds change. C. Confocal images of representative field of immunocyto-chemistry against Fascin1 (green) counterstained by Alexa 494 phalloidin (red) in SAOS-02 and LM7 osteosarcoma cell line.

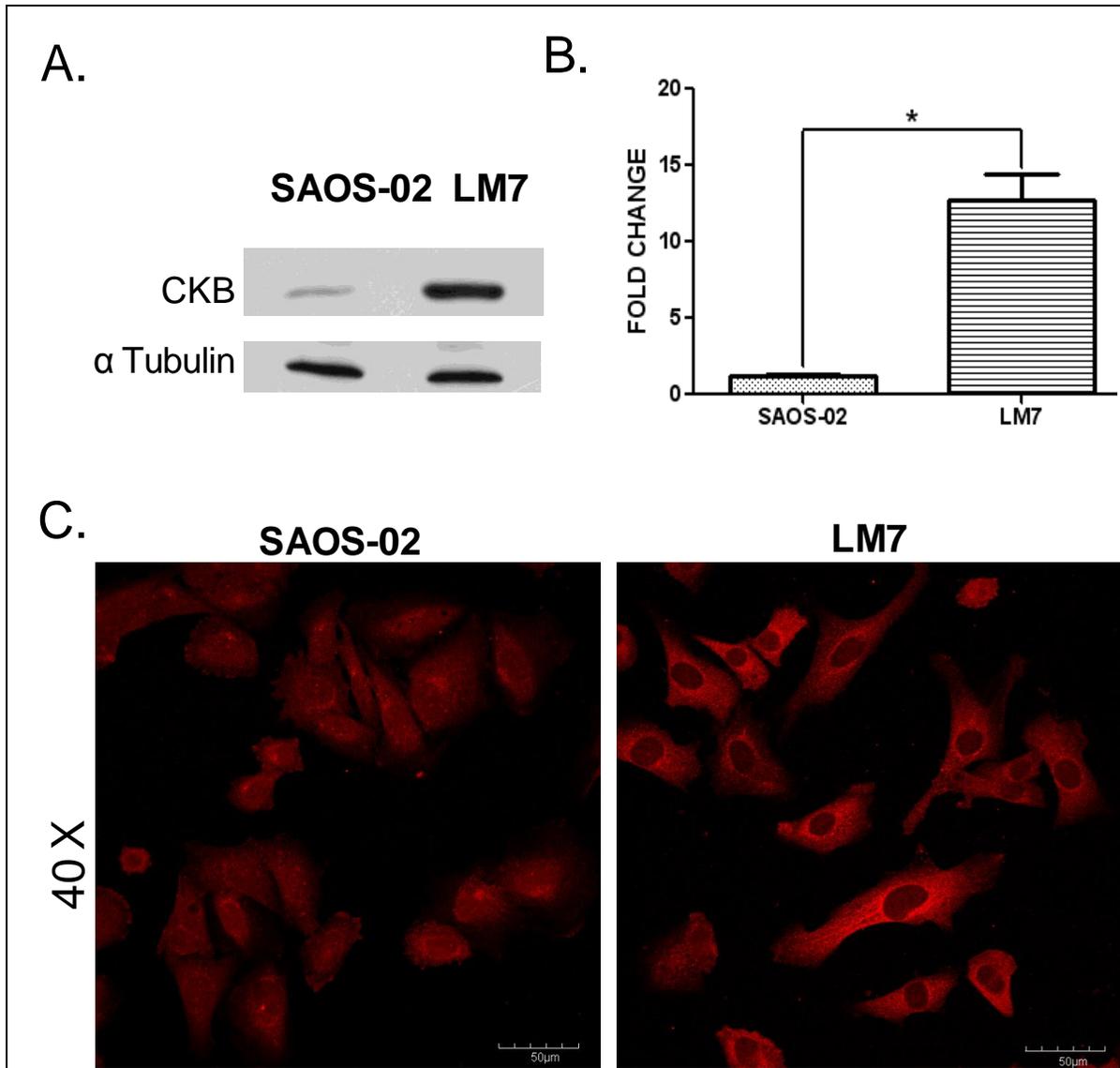


Figure 2: Validation of Mass Spectrometry data for Creatine Kinase B. A. Representative images of immunoblotting against Creatine Kinase B; B. Quantification of expression by densitometry using QuantityOne[®] software. C. Confocal images of representative field of immunocyto-chemistry against V-Mat1 (red) in SAOS-02 and LM7 osteosarcoma cell line.

Research Project 30: Project Title and Purpose

Signaling Pathways in Epidermal Stem Cell Proliferation and Skin Carcinogenesis - Our research will attempt to determine what role the polyamine pathway plays in early skin cancer development. There is strong experimental evidence indicating the location of epidermal stem cells that are modified by carcinogen exposure is in a specific area of the hair follicle. By directing expression of transgenes to this region of the epidermis in mice, we can modify gene

expression in stem cells and ask what effect these genetic alterations have on tumor development. Understanding the pathways that control epidermal stem cell proliferation is important to the area of public health, because these cells are targets for gene therapy approaches in cancer prevention and treatment.

Duration of Project

11/24/2008 - 6/30/2009

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

Genetic and Proteomic Analysis of the UL84 Gene of Human Cytomegalovirus - In immunocompromised individuals, opportunistic human cytomegalovirus (HCMV) infections can produce life-threatening syndromes involving almost every organ, but more commonly pneumonia, hepatitis, diseases of the central nervous system, and secondary immune suppression. In addition, HCMV can cross the placenta and is the most common viral cause of congenital defects. The purpose of this research project is to identify the mechanisms of action of an essential viral protein. Elucidation of these mechanisms could lead to the identification of therapeutic targets for management of HCMV illnesses.

Duration of Project

11/24/2008 - 12/31/2010

Project Overview

Human cytomegalovirus causes mostly sub-clinical or mild primary infections in immune competent hosts but persists in 60-90% of the human population. In immunocompromised individuals, opportunistic infections can produce life-threatening syndromes involving almost every organ, but more commonly pneumonia, hepatitis, CNS diseases, and secondary immune suppression. In addition, HCMV can cross the placenta and is the most common viral cause of congenital defects. Although several anti-virals limit morbidity and mortality, the drugs have significant toxicity and the development of resistance is common. None of these agents has been licensed for use in pregnant women. Accordingly, there remains widespread interest in the further elucidation of the virus-host interaction to reveal potential molecular targets for control. The UL84 gene is essential for viral DNA replication and also participates in the regulation of gene transcription in virus infected cells. Evidence suggests that this gene has other important functions as well. The goal of this project is to identify new UL84 binding partners and develop mutants that will provide a foundation for a more directed grant proposal. The working hypothesis is that multiple activities of UL84 are directed toward modification of the functions of

cellular proteins. The first specific aim will employ recombinant DNA technology to develop mutant viruses with altered UL84 genes. These mutants will be useful reagents for analysis of UL84 gene function. The second specific aim will evaluate cellular proteins that are candidates for participating in functional interactions with the UL84 protein. Candidate cellular proteins identified previously by a global proteomic investigation will be tested individually for their ability to bind to the UL84 protein by standard methods to analyze protein-protein interactions. Ultimately, elucidation of the roles of UL84 protein interactions with cellular proteins should reveal mechanisms used by the virus to reprogram the host cell and could lead to the identification of therapeutic targets for management of HCMV illnesses.

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

We expect to identify one or more cellular proteins that associate with the viral UL84 protein in infected cells and to have learned whether a candidate cellular protein's function is altered in infected cells and whether or not the protein is required for infection. Depending on the nature of the cellular protein, this information will be used to formulate one or more specific hypotheses regarding how UL84 alters host cell function. We also expect to have produced a series of viral mutants with UL84 gene alterations and to have determined which of the gene lesions interferes with viral replication and which ones produce proteins that retain the ability to promote replication. The mutants will be critical reagents for investigating the precise mechanism by which UL84 protein alters the function of a particular cellular protein. Detailed investigation of such mechanisms will be the focus of a future proposal to support the work. The health benefit of particular projects such as this one cannot be predicted. The combined efforts of many investigations of viral gene function are likely to generate information that suggests new therapeutic targets for control of HCMV disease.

Summary of Research Completed

Specific Aim 1a. To develop genetic reagents for analysis of UL84 gene function: to construct a set of structurally and functionally informed set of mutants.

Last year we reported the isolation of four new viable UL84 mutants, in addition to the four reported previously. All are in the same cloned TB40/E genome (BAC4) containing a GFP

marker (TB40BAC4GFP). The first has two point mutations (L228A, L230A; m5) in a sequence shown to function as a nuclear export signal. The second (del536-553, m6) deletes a sequence conserved in primate cytomegaloviruses. The third mutation (L359A, m7) alters a second nuclear export signal, and the fourth mutation (L228A, L330A, L359A); m5/7) affects both nuclear export signals and has been reported to produce a protein whose export cannot be detected. We now report that all produced a UL84 protein product that can be detected by indirect immunofluorescence (Fig. 1). MRC5 cells were infected with either one of the mutants, the wt parent, or a mutant that replaces the UL84 gene with a bacterial gene expression cassette (null). At 48 hours after infection the cells were fixed and stained with anti-UL84 mouse monoclonal antibody followed by a Cy3-conjugated anti-mouse antibody. All the cultures were infected, as demonstrated by immunofluorescence detection of the viral IE2 protein (data not shown).

Although the m5, m7, and m5/7 mutants are predicted to interfere with nuclear export of UL84, the distribution of the predominantly nuclear signal between the nucleus and cytoplasm of these mutant UL84 proteins did not appear to be significantly different from that of the wild type. The intensity of fluorescence with these three mutants was reduced somewhat, suggesting that these mutant proteins accumulate to lower levels than the wt or m6 proteins.

All of the mutant viruses, including the m1- m4 viruses reported last year, grew in human retinal pigmented epithelial (ARPE-19) cells (see Fig. 2). However some of the mutant viruses exhibited infected cell phenotypes in these cells that were very different from that of the wild type. Cultures were infected with wt or a mutant virus at 0.01 to 0.1 pfu/cell and maintained under an agar overlay that was replenished every seven days. Infected cells were visualized easily by the expression of GFP from the viral genomes. Figure 2 shows representative images of 22 day-old cultures. The exposures were adjusted automatically so that differences in GFP intensity are not apparent in this figure. When the same exposure time was used, cells infected with mutants m3, m6, and null all showed reduced intensity of GFP fluorescence (data not shown), suggesting that virus DNA replication and probably virus production (see Aim 1b below) was reduced in these infections.

In the auto-exposure images, the wt virus produced large foci of infection characterized by necrotic centers and surrounding clusters of mostly distinct infected cells. Foci produced by infection with the UL84-null virus also had necrotic centers, but they were usually surrounded by large syncytia. The wt virus also produced syncytia but they were far less common and usually smaller. Mutants m1 and m6 most closely resembled the UL84 null virus, whereas m4 and m7 most closely resembled the wt. The other four mutants displayed a distinct pattern consisting of both small syncytia and clusters of individual infected cells. Notably, the m5/7 pattern resembled that of m5, not m7.

Mutations in the HCMV genes UL20, UL24, UL27 (glycoprotein B), and UL53 (gK) increase syncytium formation. Our data show that UL84, either directly or indirectly, also may influence the extent of syncytium formation. It is tempting to speculate that the cytoplasmic location of some UL84, which is not obviously related to the known function of this gene in viral DNA replication, may be related to its participation in a membrane-related function. Of note, the viability of these UL84 mutations in the TB40BAC4 genetic background provides an opportunity

to study this phenotype that might not be available in a virus where at least some UL84 mutants are non-viable.

Specific Aim 1b. To develop genetic reagents for analysis of UL84 gene function: to produce a UL84-null viral genome and develop a complementation system for its propagation.

Last year we reported that the UL84 gene, which we and others showed is essential for the growth of HCMV strains AD169, Towne and FIX, is not essential for growth of strain TB40/E BAC4. To complete our analysis of TB40BAC4GFP Δ 84, the TB40 strain that has a complete replacement of the UL84 reading frame with a bacterial gene, viral DNA accumulation was assayed over a four-day time course. Human diploid fibroblast MRC5 cells were infected at a multiplicity of 0.5 in 6-well tissue culture plates. The infecting fluid was removed, the cells were washed, and fresh medium was added. GFP fluorescence was monitored throughout the time course to confirm that the same fraction of cells was infected with each virus. At 8 hours after infection, and at four one-day intervals beginning at 24 hpi, DNA was extracted from duplicate wells using the QiaAmp DNA Mini Kit (Qiagen Corp) according to the protocol provided by the supplier. Triplicate samples were analyzed by qPCR using the Opticon 2 detection system (Bio-Rad Corp). HCMV UL99 DNA sequences were amplified with primers 5'-GTGTCCCATTCCTCGACTCG-3' and 5'-TTCACAACGTCCACCCACC-3'. Values obtained were normalized for cell number using GAPDH genomic DNA sequences amplified with primers 5'-GATATTGTTGCCATCAATGAC-3' and 5'-TTGATTTTGGAGGGTCTCG-3'. The data show (Figure 3) that the kinetics of accumulation for both the parent and mutant strains were similar, with a modest lag and about half a log reduction in DNA accumulation in mutant-infected cells. The difference in viral DNA synthesis was consistent with the difference in virus yield reported last year. These results show that viral DNA synthesis in TB40BAC4-infected cells is UL84-independent, as expected from the virus yield data. The data reported last year and here were included in a manuscript that was published (see Publications below).

HCMV strains exhibit significant genetic diversity and the TB40BAC4 genome sequence differs in a number of ways from that of the UL84-dependent strains AD169, FIX, and Towne. The two most likely sources for the UL84-independence of strain TB40GFPBAC4 Δ 84 are that either the TB40BAC4 genome contains a UL84-independent origin of viral DNA replication or that the genome encodes one or more proteins (or protein variants) that can bypass the UL84 requirement. To address the first issue, we performed two experiments. In the first, we deleted the known origin of replication (oriLyt) from TB40BAC4 or TB40BAC4 Δ 84 by insertion of a GalK cassette, using methods reported last year for UL84. Neither oriLyt deletion strain was viable. Proof that the viability of these strains depends on the deleted sequence requires restoring the deletion, but the tentative conclusion is that a previously unrecognized UL84-independent origin is not the reason for UL84-independent replication of TB40BAC4.

In a second experiment, the TB40BAC4 oriLyt was cloned into a pGemT vector and electroporated into MRC5 cells. The electroporated cells were distributed to three tissue culture dishes. Twenty-four hours later, one was infected with TB40BAC4, one with TB40BAC4 Δ 84, and one was mock infected. Four days later the cells were harvested and DNA was extracted. Duplicate DNA samples from each preparation were treated with either DpnI or Sau3A. DpnI digests only dam-methylated plasmid DNA, replicated DNA in MRC5 cells is resistant. Sau3A

recognizes the same sequence as DpnI but digests DNA regardless of its dam-methylation state. Thus the Sau3A-treated sample provided a baseline for quantifying DpnI-resistant (replicated) DNA. The plasmid DNA was analyzed by qPCR using primers that amplify a plasmid backbone sequence that contains multiple DpnI/Sau3A sites. Sample recovery was normalized by qPCR assay of cellular GAPDH DNA. A representative experiment with duplicate electroporations and sets of infections is shown in Figure 4.

Cells infected with either virus accumulated DpnI-resistant DNA to levels substantially higher than those obtained from mock-infected cells, a control that established the limits of DpnI digestion of the plasmid DNA in the samples. When a plasmid that contained no origin of replication was used in a similar protocol, the amounts of DpnI-resistant DNA were similar in infected and uninfected cells (data not shown). Thus increases in DpnI-resistant DNA reflected oriLyt-dependent replication. Wt-virus infection produced two- to three-fold more replicated DNA than infection with the $\Delta 84$ virus.

These data show that oriLyt and proteins expressed by $\Delta 84$ -infected cells were sufficient to obtain UL84-independent plasmid DNA replication. Quantitatively, the results are remarkably similar to the three-fold differences in viral DNA and infectious virus production between wt- and $\Delta 84$ -infected MRC5 cells (see Figure 3 here, and Figure 8 in last year's report). In addition, the results support our interpretation of the failure of TB40BAC4 isolates with oriLyt deletions to replicate, that is, that UL84-independent replication does not depend on an unrecognized origin. Given the high level of conservation between oriLyts of different HCMV strains, we currently favor the hypothesis that UL84-independent replication of TB40BAC4 is a consequence of one or more viral transacting products either not expressed or only expressed in strains that are UL84-dependent.

Specific Aim 2a. To evaluate cellular binding partners of UL84 from a proteomic data set by determining whether the interactions can be confirmed in infected and/or transfected cells.

No further activity on this aim.

Publications

Spector, D. J. and Yetming, K. 2010. UL84-independent replication of human cytomegalovirus strain TB40/E. *Virology* 407:171-177. PMID 20855098

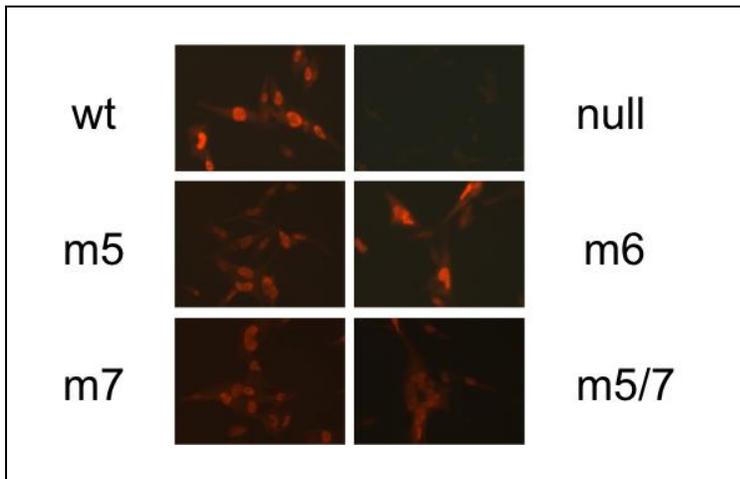


Figure 1. Expression of UL84 mutant proteins in infected MRC5 cells. For details, see the text.

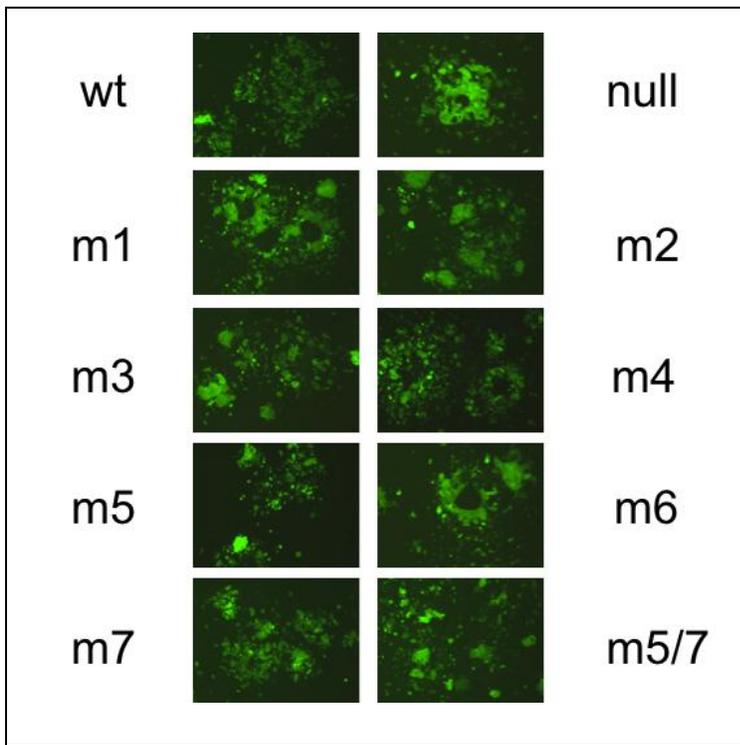


Figure 2. Cell phenotypes of UL84 mutant-infected ARPE19 cells. Cells were infected with GFP viruses containing the UL84 allele indicated and covered with an agar overly. GFP was imaged 22 days after infection.

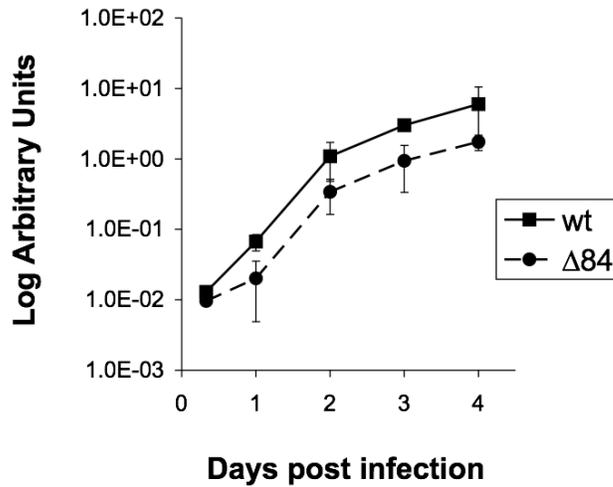


Figure 3. Viral DNA accumulation in cells infected by a UL84-null strain of HCMV TB40/E. Viral DNA accumulation was assayed in infected MRC5 cells and values corrected for cell number. The error bars indicate the standard deviation of determinations from two independent cell samples (each determination an average of triplicate assays). wt: TB40GFPBAC; Δ84: TB40GFPBACΔ84GalK.

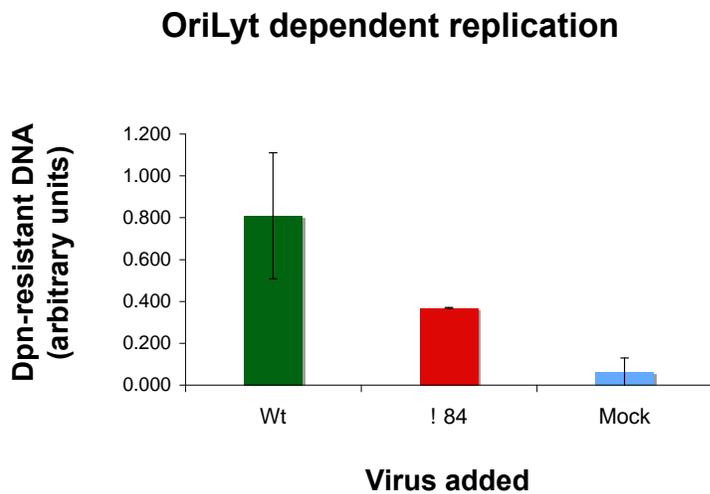


Figure 4. Replication of a plasmid containing the HCMV oriLyt in transfected cells infected with wt or a UL84 deletion mutant of TB40GFPBAC. Virus designations are as in Fig. 3.

Research Project 32: Project Title and Purpose

Development of a Novel FdUMP Prodrug for Treatment of Breast Cancer - 5-Fluorouracil (5-FU) is one of the most active drugs in breast cancer chemotherapy. Studies have shown that the potency of 5-FU is mainly a result of the inhibitory effect by its metabolite FdUMP on the enzyme thymidylate synthase (TS). Conversion of 5-FU to FdUMP is very inefficient and requires multi-step enzymatic activation. This metabolic dependency often causes various resistance or toxicity to 5-FU and limits its clinical use. To offer more efficient and less toxic alternative of 5-FU, a novel agent FdTSP was designed to generate FdUMP in tumor cells through an enzyme-independent pathway and overcome the hydrophilicity of FdUMP that limits its entry into cells. The goal of this project is to establish the use of FdTSP as a superior alternative to 5-FU to treat breast cancer in an *in vitro* cell culture model.

Duration of Project

11/24/2008 - 6/30/2011

Project Overview

The broad objective of this project is to investigate the potential use of a novel FdUMP prodrug, 5-fluoro-2'-deoxyuridin-5'-yl bis[2-(p-tolylsulfonyl)-1-methylethyl] phosphate (FdTSP) as a superior alternative to 5-fluorouracil (5-FU) for the treatment of breast cancer. We currently will focus on the evaluation of growth inhibitory effects of this agent in *in vitro* preclinical models. The *specific aims* of this study are, therefore: (1) to investigate the growth inhibitory effects of FdTSP in 5-FU sensitive and resistant human breast cancer MCF-7 cells; and (2) to investigate the activation of FdTSP in breast cancer cells.

Research Design and Methods:

(1) *Investigate the growth inhibitory effects of FdTSP in both 5-FU sensitive and resistant human breast cancer cells.* (a) Synthesis of FdTSP. We will synthesize FdTSP according to our published procedures which basically utilized a phosphoramidite P(III) chemistry approach. (b) Cell culture and RT-PCR. The human breast cancer MCF-7 will be used to test the growth inhibitory activity of FdTSP. 5-FU resistant cell lines will be prepared by treatment of MCF-7 cells continuously with stepwise-increasing concentrations of 5-FU every 2 weeks up to 200 µg/mL. 5-FU resistant clones will be selected and subcultured. To identify the possible mechanisms of 5-FU resistance, the expressions of genes involved in 5-FU metabolism that might be altered by exposure of the cells to 5-FU will be examined by reverse transcriptase-polymerase chain reaction (RT-PCR). (c) Growth inhibition assay. The growth inhibitory effect of FdTSP will be conducted using MTS assay and the results will be compared to that of 5-FU in 5-FU sensitive and resistant MCF-7 cells.

(2) *Investigate the activation of FdTSP in breast cancer cells.* For FdTSP to serve as a FdUMP prodrug against breast cancer, it needs to enter the tumor cells and then release FdUMP intracellularly. Therefore, we will examine the activation of FdTSP in breast cancer cells by measuring the levels of FdTSP, FdUMP and its decomposing byproduct vinylsulfone. The levels of these compounds will be determined by HPLC and LC-MS/MS methods.

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Expected Research Outcomes and Benefits

Despite advances in chemotherapy, 5-fluorouracil (5-FU) has remained as a standard chemotherapy in the treatment of breast cancer for over four decades. The potency of 5-FU has prompted studies on the mechanism of action of this drug, which is mainly through the inhibition of thymidylate synthase (TS) by its metabolite 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP). The requirements for antitumor activity of 5-FU are (1) the achievement of sufficient concentrations of active metabolite FdUMP in tumor cells and (2) the maximum inhibition of TS. Attempts at improving the efficacy of 5-FU have been achieved through the combination with biochemical modulators and the development of infused 5-FU/LV regimens and oral prodrugs of 5-FU [e.g., capecitabine (Xeloda®)] to increase the bioavailability of 5-FU. However, use of 5-FU is still limited by its toxicity and resistance, which can be mainly attributed to dependency or aberrations in its metabolism.

Direct administration of FdUMP may offer therapeutic advantages over 5-FU or its oral prodrugs with respect to bypassing the multi-step enzymatic activation and possible toxic side effects caused by the incorporation of 5-FU into DNA or RNA. However, due to the presence of phosphate group in the structure, FdUMP does not enter the cells efficiently and is susceptible to be degraded extracellularly by phosphohydrolase, hampering its clinical use. The emergence of resistance to 5-FU, likely as a result of reduced metabolic activation to FdUMP in tumor cells, has prompted efforts to develop prodrug that release FdUMP intracellularly and bypass the requirement for metabolic activation. FdTSP has recently been developed by our group as a potential tumor-selective FdUMP prodrug. We expect that FdTSP will be effective against breast cancer and will be superior to 5-FU. Results of this study are requisite in providing a basis for further investigation of this drug in an *in vivo* preclinical study. Successful completion of this project may facilitate this class of anticancer agents to reach clinical application and provide more effective chemotherapeutics.

Summary of Research Completed

Aim (1): To identify the possible mechanisms of resistance to 5-FU in the 5-FU resistant MCF-7 cells (MF-7/FU1.5, MCF-7/FU2.5 and MCF-7/FU12.5) developed in Aim (1b), initially we proposed to determine the expressions of genes involved in 5-FU metabolism that might be

altered by exposure of the cells to 5-FU by reverse transcriptase-polymerase chain reaction (RT-PCR) (Aim1c). Since we have known that the TS is the direct target of FdTSP and TS expression is regulated at both transcriptional and translational levels, alternatively, we examined the target protein TS level of protein expression in these cells. Western blotting analysis was performed to compare the TS levels in MCF-7 and 5-FU resistant MCF-7 cell lines.

Experimental procedure: Briefly, total cellular proteins of each cell line were homogenized in lysis buffer. Cell debris and particulate fractions were removed by centrifugation at 13,000 x g for 10 min at 4 °C. Protein concentration was measured using Bio-Rad DC protein assay kit. Equal amounts of protein (approximately 35 µg) was diluted with 3 × sample buffer, heated at 100 °C for 5 min and then loaded to 12% SDS-PAGE. After electrophoresis, protein was transferred onto PVDF membranes and subjected to immunoblot analysis using TS106 monoclonal antibody as the primary antibody and horseradish peroxidase goat antimouse IgG as the secondary antibody. The antigen/antibody complexes were visualized using the Enhanced chemiluminescence kits.

Results: We found that TS expressions were increased in all 5-FU resistant cells as compared to parent MCF-7 cells. Fig 1 shows the Western blot analysis of TS expression in MCF-7 and 5-resistant MCF-7 cell lines MCF-7/FU1.5, MCF-7/FU2.5 and MCF-7/FU12.5. Increased expression of TS has been indicated to contribute to the resistance of 5-FU treatment.

Aim (2): Kinetic study was performed at 37°C in (a) growth media and (b) total cell extract to evaluate the stability of FdTSP in media and to mimic the behavior of FdTSP inside the cells.

Experimental procedure: Five million MCF-7 cells were incubated with 10 µM concentration of FdTSP in growth media. At various time intervals (1 min, 5 min, 15 min, 30 min, 1h, 2 h, 4h, 8h), cells were counted using the trypan blue dye exclusion method and were centrifuged to form a cell pallet (1500 rpm for 10 min). The supernatants were collected and stored at -80°C. The cell pallet was treated with ice-cold 70% methanol/30% H₂O. The residue above was set at -80°C and then lyophilized. The dried extract was reconstituted and subjected to HPLC and LC-MS/MS analysis.

HPLC and LC-MS/MS analysis were performed using an Agilent 1200 HPLC and an ABI 3200 LC-MS/MS system. Chromatographic separation is conducted by Extend-C₁₈ column (150 mm × 4.6 mm, 5 µm, Agilent Technology) with mobile phase consisting of 0.1% formic acid (FA) in H₂O and methanol at a flow rate of 200 µL/min. The following program is used: 0-5 min, 10% methanol in H₂O/FA, 5-10 min, linear gradient to 70% methanol, 10-20 min, 70% methanol in H₂O/FA, 20-50 min, linear gradient to 90% methanol in H₂O/FA. An injection of 5 µL is used for standard samples as well as cell extract samples.

The mass spectrometer is operated in the negative ion mode with nitrogen as a nebulizing and drying gas. The total eluent flow is directed to the ESI source. ESI source parameters and MS/MS parameter is optimized for maximum sensitivity during direct infusion of standard. The selective multiple-reaction monitoring (MRM) mode is used for analysis of FdTSP and its metabolites in the samples. The parent ion of analyte (m/z) is trapped and fragmented simultaneously by using an isolation width of 1 mass unit (mu).

Results: A representative HPLC chromatogram of FdTSP extracted from cell media is shown in Figure 2. FdTSP is detected by both MS [top panel and insert, for FdTSP $m/z=719$] and UV (bottom panel). The retention time for FdTSP is 22 min. The results were expressed as the percentage of starting material FdTSP remaining after each incubation period in comparison to the zero time incubation control sample. These results were plotted against the respective incubation times and the half-lives were calculated from rate constant of the decay equation. Under the experimental conditions, the half-life of FdTSP in media is determined to be 14.6 min.

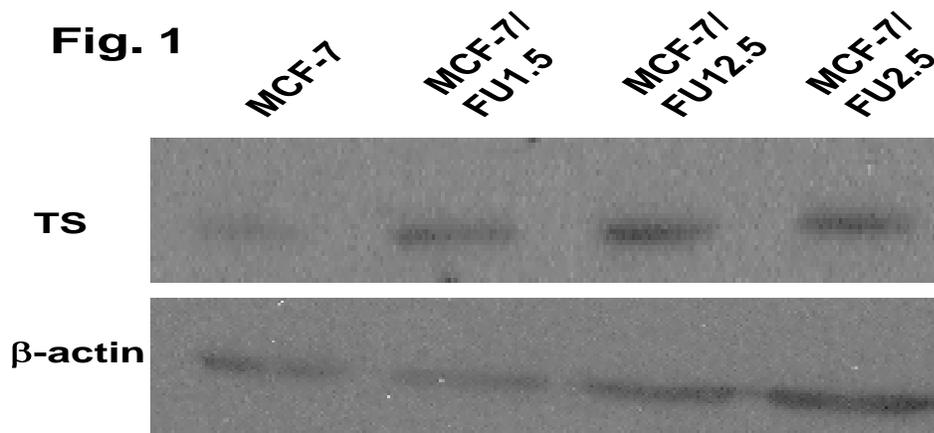
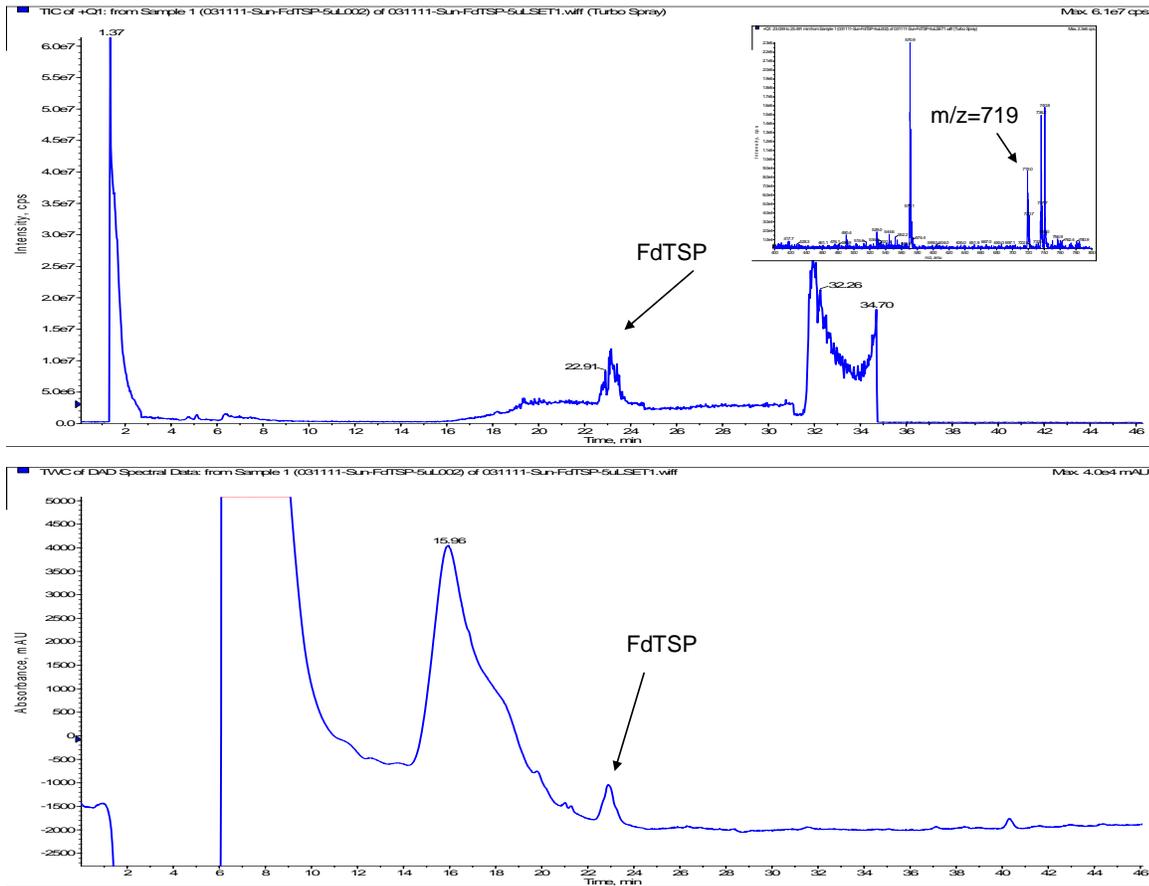


Figure 2.



Research Project 33: Project Title and Purpose

Antihypertensive Effects of Tetanic Baroreceptor Stimulation - The purpose of this project is to investigate the effect of brief high frequency carotid sinus baroreceptor stimulation on chronic blood pressure reduction in dogs.

Duration of Project

11/24/2008 – 6/30/2011

Project Overview

Fifty million Americans suffer from hypertension, of which 66% are not adequately controlled and at least 10% are multi-drug resistant. Stimulation of pressure sensors in blood vessels, the baroreceptors, decreases blood pressure (BP); implantable electrical stimulators have been used to provide continuous baroreceptor activation. These devices effectively lower basal BP, but because constant stimulation disables endogenous mechanisms of BP regulation, subjects using these devices suffer compromised responses to orthostatic and other daily cardiovascular

challenges. The continuous stimulation may also damage tissues. The objective of this project is to test an alternative strategy: long-term potentiation of the endogenous baroreflex mechanisms by occasional, brief high frequency (tetanic) stimulation of the baroafferent nerves.

The aortic depressor (ADN) and carotid sinus (CSN) nerves are the major baroafferent inputs carrying pressure signals from the baroreceptors to the brain. In the brainstem, the nucleus of solitary tract (NTS) is the first relay of the ADN and CSN signals. Recently, using a rat model, we showed that a brief tetanic stimulation of the ADN increased the size of subsequent ADN evoked responses in the NTS for 10-15 hours, which implies that following strong activation of the ADN, baroreflex depressor responses are enhanced; thus, brief baroreceptor nerve stimulation might ameliorate hypertension for many hours. In this project, we will systematically investigate the effects of CSN tetanus on basal BP in normotensive (Specific Aim 1) and obesity-induced hypertensive (Specific Aim 2) dogs. Four dogs will be used. In Aim 1, the protocol consists of 3 phases: Baseline (7 days), Tetanus (7 days), and Recovery (7 days). The tetanus phase consists of one daily bout of tetanus. In Aim 2, obesity hypertension will be induced over several weeks by a high fat diet; and then the protocol of Aim 1 will be repeated. We hypothesize that the seven successive daily tetanus applications will produce increasing basal BP reductions.

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Expected Research Outcomes and Benefits

Expected Research Outcomes: We expect that one daily bout of high frequency (tetanic) carotid sinus baroreceptor afferent stimulation will produce increasing basal blood pressure reductions over the 7 experimental days.

Benefits: Occasional brief tetanic stimulation offers a new and effective clinical method for controlling hypertension, especially multi-drug resistant forms. In contrast to continuous stimulation, the method proposed would produce less tissue damage and enhance, rather than diminish, normal moment-to-moment baroreflex control of blood pressure, and better preserve the normal orthostatic and exercise regulation of blood pressure. The proposed method naturally lends itself to an extremely compact, electrically passive (no battery), implantable baroafferent nerve stimulator, and the project's long-range goal is to develop such a device.

Summary of Research Completed

Throughout the past 12 months, we have continued to make significant progress in this project. The specific aims of this project were to demonstrate the feasibility of (1) continuous blood pressure recording several hours a day for 2-3 weeks; and (2) the chronic implantation for 2-3 weeks of a pair of current stimulation electrodes around the left and right carotid sinus areas, in the canine model. The ultimate goal of our project is to attract extramural funding to systematically test the overall hypothesis that following strong activation of the baroreceptor nerve, baroreflex depressor responses will be enhanced and that brief baroreceptor nerve stimulation will ameliorate hypertension for many hours.

The support from this grant has allowed us to conduct experiments on a total of 4 animals. All studies received prior approval from the Institutional Animal Care and Use Committee (IACUC) of the Pennsylvania State University College of Medicine. Our major accomplishments for the first year include:

- (1) Creating and testing a data acquisition system;
- (2) Protocol design for the acute experiments;
- (3) Training of team members; and
- (4) Completion of pilot experiments on vagal nerve stimulation, which showed that stimulation produced current intensity dependent blood pressure reductions (see figure 2 in last year's progress report).

This year we performed experiments on the left and right carotid sinus nerves, and extended the experiments from acute collection of data to the chronic stage.

We encountered a number challenges in chronic data collection, including chronic blood pressure recording and chronic carotid sinus electrode implantation. Chronic blood pressure can be recorded using a conventional BP recording technique employing silicon tubing implanted in the femoral artery and BP measurement via a pressure transducer attached to the femoral cannula, or by a telemetry blood pressure technique. The latter technique prevents the potential complication of blood clotting within the cannula, but is much more expensive to use than the conventional technique. A panel of experts, including the Chair of the Department of Comparative Medicine of the Penn State College of Medicine and the Chair of the IACUC committee, was convened. This committee decided that the limited scope of these experiments did not justify the substantial additional cost of the telemetry. Dr. Tiffany Whitcomb developed a novel methodology for minimizing clotting, and also designed the aseptic procedure for the daily pressure transducer connection. A novel jacket that safely and securely holds the chronically implanted femoral cannula and the carotid sinus electrodes was also designed by the research team.

With the above procedures in place, we were able to collect chronic data, and the femoral cannula was kept patent for more than a month. During this period we recorded blood pressure for 4-6 hours a day for more than 2 weeks. There was no clotting or infection that occurred in this animal during the period, and the blood pressure signal was clear and reliable. Figure 1(A) demonstrates the representative traces of the hourly blood pressure recording from Days 2, 10 and 15. Figure 1(B) shows the summarized daily mean systolic blood pressure over a 2 week

recording period. The successful chronic pressure recording documented the feasibility of our chronic blood pressure recording technique.

The other challenge for this project was the chronic carotid sinus electrode implantation. We successfully surgically identified the left and right carotid sinus, stimulated the carotid sinus, and generated stimulation intensity-dependant blood pressure reductions. Figure 2 illustrates the relationship between intensity of the current applied to the carotid sinus and the magnitude of the reduction in the blood pressure. The left panel shows individual arterial blood pressure traces (green) during carotid sinus stimulation at different current intensities (i.e., at 2, 5, 7, 9 and 10 mA), while the right panel summarizes the relationship between the magnitude and duration of the blood pressure reduction with the current intensity (i.e., the transduction curve of the amplitude of carotid sinus current stimulation and the blood pressure reduction and the transduction curve of the amplitude of carotid sinus current stimulation and the duration of the blood pressure reduction). Between the threshold and saturation levels, the magnitude of blood pressure reduction exhibits a linear relationship with the stimulation current applied to the carotid sinus. These data confirm that we were able to identify the anatomic location of carotid sinus baroreceptor regions and elicit stimulation current dependent blood pressure regulation.

In order to more effectively elicit blood pressure response, we continued to improve our electrode design for the carotid sinus stimulation. During the first year period, we designed and tested several versions of the electrode (see Figure 3 in the first year progress report); and the electrode in Figure 3(A) above was chosen as the most desirable design. In Figure 3(A) electrode, the direction of the stimulating current is perpendicular to the nerve conduction direction. To test whether the electrode was more effective when the direction of the stimulating current (applied from the electrode) was parallel with the nerve conduction direction, we designed the electrode in Figure 3(B) in this year. Electrodes in Figure 3(A) and (B) were implanted, respectively, around the left and right carotid sinus of the dog used in the chronic experiments.

However, despite our new electrode design (Figure 3) and our success in the acute preparation in eliciting current amplitude-dependent blood pressure reduction in carotid sinus stimulation (Figure 2), we have thus far not achieved a desirable blood pressure reduction effect from a chronic preparation.

In sum, in the past year we elicited current amplitude-dependent blood pressure reduction by stimulating the carotid sinus in acute experiments, extended the preparation from the acute stage to the chronic stage, and successfully recorded blood pressure for more than 2 weeks. In addition, we designed new carotid sinus stimulating electrode and chronically implanted this newly designed electrode. Finally, with the support of these CURE funds, we have achieved our goal of securing an NIH R21 grant, which will enable us to continue to systematically test our hypothesis.

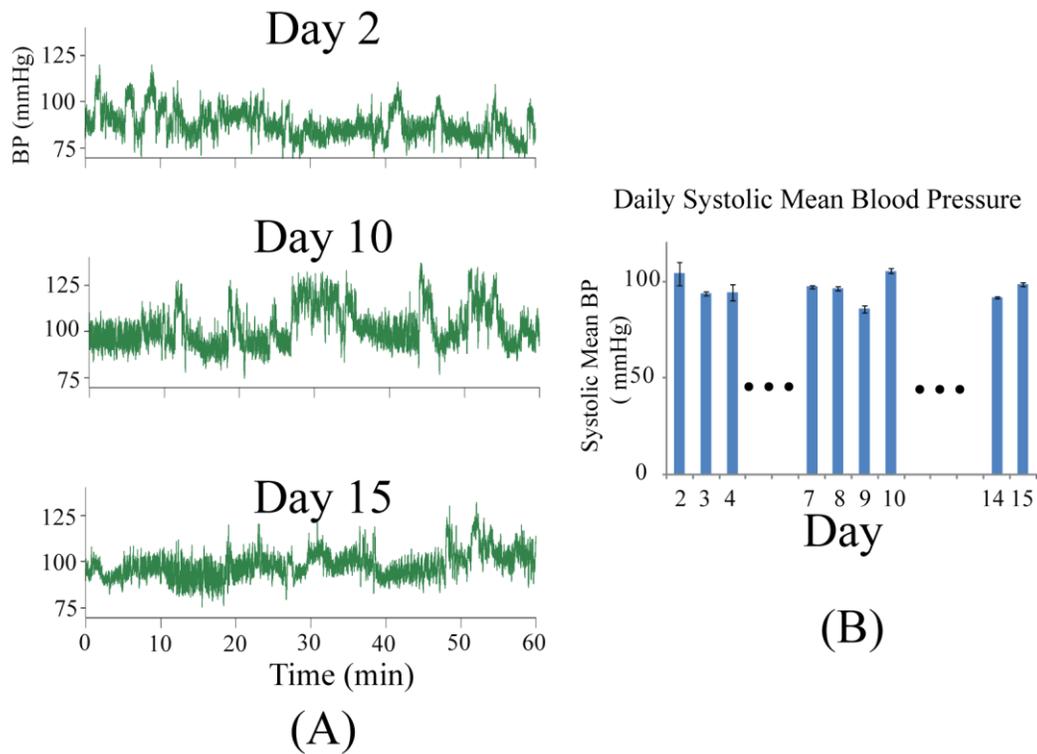


Figure 1. Chronic blood pressure recording from canine #4. (A) Representative traces of the hourly blood pressure recordings from Days 2, 10 and 15. (B) Daily mean systolic blood pressure over a 15 day recording period. Data are presented as mean \pm SE.

Dog AC Left Carotid Sinus Stimulation Responses

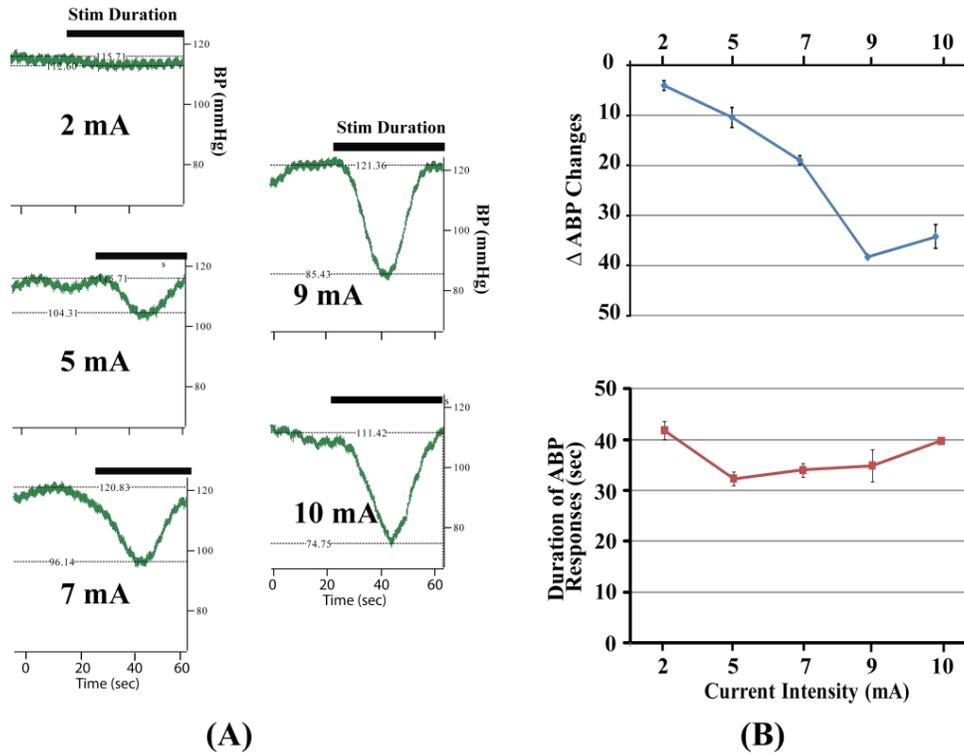
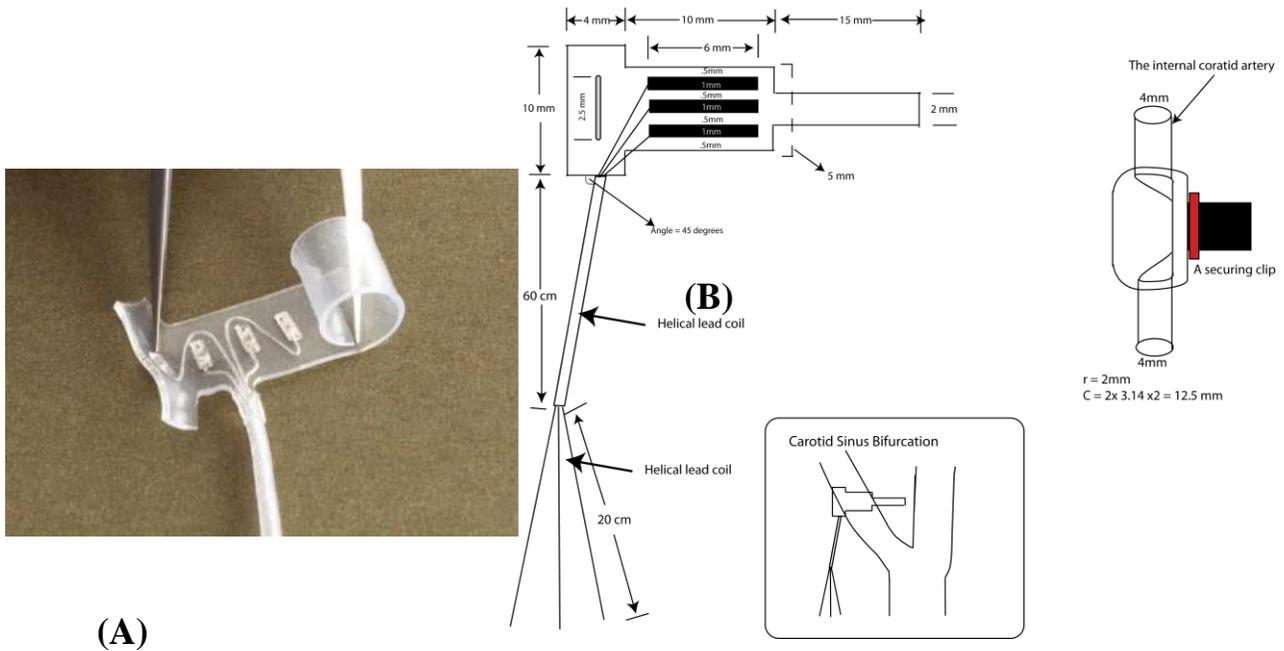


Figure 2: The magnitude of arterial pressure reduction linearly correlates with the current intensity of the carotid sinus stimulation. Panel A provides example trials of arterial blood pressure responses to the carotid sinus stimulation at different current intensities. Panel B is the summarized effects of arterial blood pressure reduction (the upper panel B) and the duration of arterial blood pressure reduction (the lower panel B) for the carotid sinus stimulation. Data are presented as mean \pm SE.



(A)

Figure 3 (A) is the design from the last year, where the stimulation current from the electrode is perpendicular to the nerve conduction direction. (B) A new design from this year, where the stimulation current from the electrode is parallel to the nerve conduction direction.

Research Project 34: Project Title and Purpose

The Role of STAT1 Inactivation in the Development of Inflammatory Bowel Disease - The overall goal of this project is to elucidate the role of STAT1 and STAT3 signaling in inflammatory bowel disease (IBD) development. Our specific hypothesis for this project is that neutralization of interferon-gamma in a STAT3 deficient IBD model will reduce STAT1 activation and prevent IBD development. Our STAT3KO and STAT1/3KO double deficient model will provide a unique system to explore the downstream target genes for future exploration as novel therapeutics.

Duration of Project

7/1/2009 - 6/30/2010

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 35: Project Title and Purpose

Effects of Early Diabetes on the Microvasculature: A Link between the Eye, Brain, and Heart -

The purpose of this project is to examine the relationship between blood vessel responses from the eye, brain, and heart to stimuli, which cause the vessel to get bigger (i.e., dilate) or smaller (i.e., constrict) in individuals with pre-diabetes and diabetes. Dysfunction of the eye's retinal blood vessels to dilate or constrict may prove to be a valid surrogate marker of stroke and heart disease. This project will use non-invasive Ultrasound Doppler to examine heart and brain blood flow and will use a novel tool known as the Dynamic Vessel Analyzer that allows direct visualization of the retinal blood vessels in healthy individuals and individuals with pre-diabetes and diabetes.

Anticipated Duration of Project

1/1/2009 - 12/31/2011

Project Overview

Diabetes causes functional impairment and structural damage of large and small vessels in multiple organ systems leading to vision loss, stroke, and myocardial infarction. Chronic hyperglycemia is associated with macrovascular dysfunction, but it is less well understood how the spectrum of glucose intolerance affects microvascular function of the eye, brain, and heart. Previous studies suggest that pre-diabetics may be at risk for vascular dysfunction, but this has not been well examined. It is also unclear whether or not the opposite spectrum of disease, diabetics with vascular complications, will result in a more severe vascular impairment. Therefore, the purpose of the study is to examine the relationship between changes in retinal, cerebral, and coronary vasoreactivity in patients with pre-diabetes and diabetes with and without vascular complications compared to age- and gender-matched healthy controls.

Specific Aim. To characterize microcirculatory vascular function (i.e., vasoconstriction and vasodilation) non-invasively in the cerebral, retinal, and coronary vascular beds along the spectrum of glucose dysregulation (healthy controls, impaired fasting glucose (IFG), type 2 diabetics without vascular complications, and type 2 diabetics with vascular complications) before and after hypocapnia and hypercapnia and flicker-light induced retinal vasodilation. We hypothesize that cerebral, coronary, and retinal vasoconstrictor and vasodilator responses during hypocapnia and hypercapnia, and flicker-induced retinal vasodilation will be attenuated in both pre-diabetic and diabetic groups compared to controls and that the degree of dysautoregulation worsens with disease progression.

Secondary Aim. It has been suggested that retinal blood vessels may be an index to the cerebral and coronary beds; however, examination of all three vascular beds simultaneously in the same subjects has not been explored. Thus, we seek to compare the degree of vascular regulation between these critical vascular beds for IFG and type 2 diabetics. We hypothesize that the degree of reactivity will be similar between the three vascular beds and between the groups, and the impairment of retinal vasoreactivity can predict the degree of vascular function of the brain and heart. We will use Doppler Ultrasound, and the Dynamic Vessel Analyzer (DVA), which is a

relatively new diagnostic tool that allows direct visualization of the microcirculation of the retina. Retinal microvascular dysfunction may prove to be a valid surrogate marker of cerebrovascular and cardiovascular disease in chronic hyperglycemia, and could be a valuable tool to estimate the risk of stroke and myocardial infarction in this population.

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Expected Research Outcomes and Benefits

Individuals with diabetes are at an increased risk of stroke and developing heart and vascular disease. Chronic hyperglycemia (i.e., high glucose) is associated with vascular dysfunction and is suggested to be an early event in the atherosclerotic process. It is not clear when during the spectrum of glucose impairments the brain, eye, and heart vascular beds (i.e., cerebral, retinal, and coronary) become impaired and whether or not these changes are reversible by early treatment. Our expected outcomes are that blood vessel responses to dilator and constrictor stimuli will be impaired in early diabetic and diabetic individuals and that the degree of impairment will worsen with disease progression. This information is important to study so that interventions may be started early to delay or prevent strokes and heart attacks. Thus, the benefits of this study include providing: 1) non-invasive insight into retinal, cerebral, and coronary vascular function and their relationship to each other; and 2) potential valuable information regarding health and further advance our understanding of the impact of the spectrum of early diabetes on vascular reactivity in critical human blood vessels.

Summary of Research Completed

Methods. The studies are conducted in the General Clinical Research Center at Penn State College of Medicine. The methods of recruitment and informed consent procedures, and study group assignment have been reported in the previous progress report.

Measurements performed in this study have been previously described in detail.

Additionally, the following *laboratory measurements* are obtained in all study participants: Total cholesterol, HDL, LDL, HDL/LDL ratio, triglycerides, fasting glucose, fasting insulin, HbA_{1c}, hsCRP.

Experimental interventions and study protocols have been described in detail in our previous progress report:

In addition to the measurements we have reported in our last progress report we are now also acquiring data on retinal blood vessel oxygenation at baseline and during hyperoxia. For these measurements we are using a novel noninvasive method that has been developed by Imedos, Germany. Data analysis is currently in progress.

Analysis, Statistics, and Sample Size have been previously described.

Milestones/Results

Continuing the project since January 2009 we have completed the following research using the Dynamic Retinal Vessel Analyzer:

We have enrolled 71 subjects and completed measurements in 62 subjects (13 controls, 18 subjects with prediabetes, 31 diabetics). Eighteen additional subjects will be enrolled during the next few months to complete the study as planned. Extensive recruitment efforts in collaboration with the Office of Research will allow us to successfully meet target enrollment and to finish data analysis as planned.

We have continued to examine the relationship between changes in retinal, cerebral, and coronary vasoreactivity in patients with pre-diabetes and diabetes with and without vascular complications compared to age and gender matched healthy controls. Detailed statistical analysis will be completed at completion of the study. The provided figures summarize current trend analyses. Demographics for the data analyzed and presented at the ARVO meeting in April 2011 are summarized in Table 1.

1. Retinal Vessel Measurements

Results of the retinal vasoreactivity in response to hyperoxia are provided in Figure 1. Retinal artery and venous responses to 100% oxygen are attenuated in pre-diabetics and in diabetics compared to healthy controls. The strongest attenuation occurs in diabetics, but changes in vasoconstriction responses are already present at early disease stages indicating high sensitivity of the DVA method to detect endothelial dysfunction. Consistent with our previous findings we see an attenuated response in retinal arterial and venous vasoreactivity to light-flicker stimulation and hypercapnia in prediabetics and diabetics compared to healthy controls (see Figure 2). Analysis of Arterial/Venous Ratios as an indicator of vascular risk shows a linear decrease with disease progression (see Figure 3). Retinal vessel oxygenation data are currently being analyzed.

2. Coronary and Cerebral Blood Flow Data

Our coronary data are currently being analyzed.

Analysis of cerebral blood flow data (based on 45 individuals) suggests that prediabetics and diabetics have decreased cerebral vasoreactivity following hyperventilation/breathhold

stimulation compared to controls (see Figure 4).

As previously reported, we have confirmed in additional data analysis that duration and poor disease control are associated with a greater degree of impairment of cerebral vasoreactivity (see Figures 5 and 6). The relation between cerebral and retinal blood vessel reactivity and cerebral vasoreactivity following hypercapnia, hyperoxia as well as additional ultrasound parameters will be explored following completion of data collection.

Publications and Presentations

Slocomb J, Lott ME, Patel S, Shivkumar V, Camacho F, Bettermann K. The influence of diabetes on retinal arteriovenous ratios and cerebral blood vessel behavior. The 4th Annual Thematic Research Forum on Diabetes and Obesity, November 10, 2010.

Bettermann K, Slocomb J, Shivkumar V, Camacho F, Lott M. Retinal vasoreactivity as a marker of cerebral vessel disease in type-2 diabetes. The 4th Annual Thematic Research Forum on Diabetes and Obesity, November 10, 2010.

Bettermann K, Slocomb J, Shivkumar V, Camacho F, Lott M. "Retinal Vasoreactivity as a Marker for Cerebral Vessel Disease in Type II Diabetes?" 9th International Congress on Treatment and Therapeutic Perspectives in Alzheimer disease, Parkinson Disease, MS and Epilepsy, Athens, Greece. January 29th, 2011.

Lott M, Slocomb J, Shivkumar V, Bettermann K. Impaired Retinal Reactivity in Pre-Diabetes and Diabetes. The 2011 Diabetes and Obesity Research Spring Summit, State College, PA, March 14, 2011

Lott M, Slocomb J, Shivkumar V, Bettermann K. Impaired Retinal Reactivity in Pre-Diabetes and Diabetes. ARVO (Association for Research in Vision and Ophthalmology) meeting, Fort Lauderdale, FL, May 5, 2011.

Grants submitted

Based on the preliminary data of this study the Co-PIs have submitted proposals to the following funding agencies:

- 1) NIH/NIDDKD/NCCAM 6/6/2011 deadline.
- 2) Internal grant to Barsumian Trust June 25, 2011.

Summary of Research Completed as of 6/18/11

Table 1: Subject Demographics (data completely analyzed as of April 2011)

	Controls	Pre-Diabetes	Type 2 Diabetes	P value
Number of subjects	12	6	18	
Women/Men (no.)	6/6	5/1	10/8	
Age (yrs)	58 ± 3	54 ± 4	56 ± 2	.73
Body mass index (m/kg²)	28.9 ± 1.1	31.8 ± 2.4	31.3 ± 1.3	.37
Intraocular pressure (mmHg)	13.1±0.7	14.5±1.0	16.3±0.6*	.005
Artery to vein ratio	.90±.02	.85±.03	.82.02*	.02
Resting vital signs				
Heart rate (bpm)	60±2	63±4	68±2*	.02
Mean artery blood pressure (mmHg)	95±2	97±4	100±2	.30
Systolic blood pressure (mmHg)	129±5	136±7	142±4	.13
Diastolic blood pressure (mmHg)	79±3	80±4	82±2	.65
Plasma fasting blood levels				
Glucose (mg/dl)	88±2	94±7	121±14	.12
Hemoglobin _{A1c} (%)	5.5 ± 0.1	63 ± 0.2*	7.5 ± 0.5*	.003
Insulin (IU/ml)	6 ± 1	6 ± 2	22 ± 8	.17
Total cholesterol (mg/dl)	207±6	208±16	178±9*	.05
Low density lipoprotein (mg/dl)	133±4	138±16	104±8*	.03
High density lipoprotein (mg/dl)	52±4	53±8	49±2	.68
Ratio	4.2±.30	4.2±.97	3.8±.19	.59
Triglycerides (mg/dl)	113±20	127±28	127±11	.80
High sensitive C-reactive protein (mg/L)	2.81±0.68	3.38±2.62	2.09±.60	.67

* Significantly different than healthy controls (P<0.05).

Figure 1: Retinal vessel vasoreactivity following hyperoxia. A) Arterial retinal vasoconstriction response, B) venous retinal response.

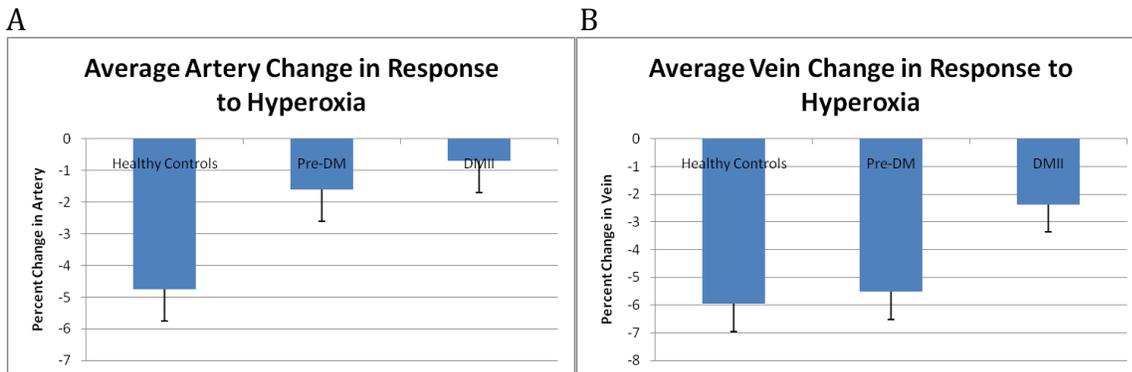


Figure 2: Retinal vessel vasoreactivity following light flicker stimulation. A) Arterial retinal vasoconstriction response, B) Venous retinal response.

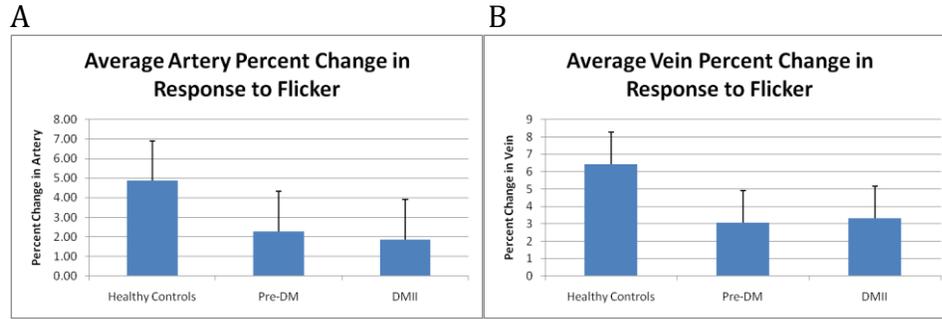


Figure 3: Arterio-venous ratios (AVRs) among different study groups.

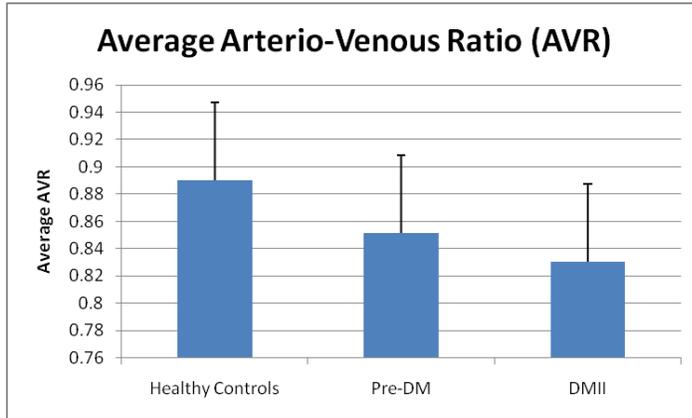


Figure 4: Cerebral vasoreactivity data (measured by mean flow velocities of the medial cerebral artery) following hyperventilation/breathhold among study populations.

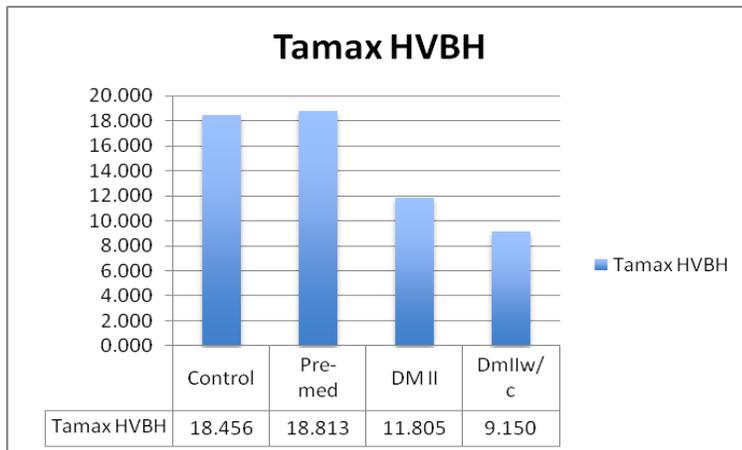


Figure 5: Cerebral blood vessel reactivity (measured by mean flow velocities of the medial cerebral artery following hyperventilation/breathhold) and its association with duration of diabetes.

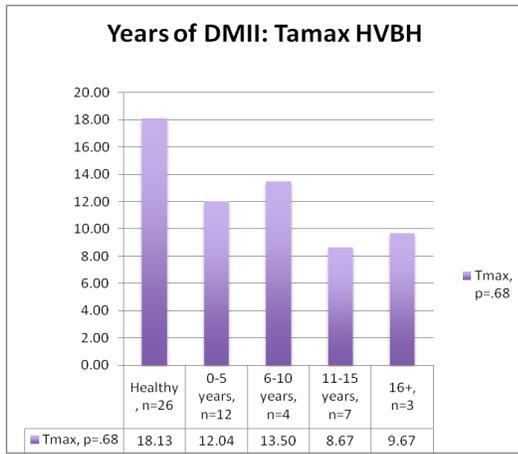
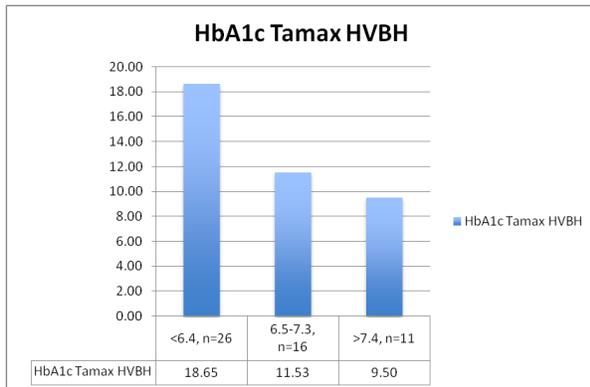


Figure 6: Cerebral blood vessel reactivity (measured by mean flow velocities of the medial cerebral artery following hyperventilation/breathhold) and its association with severity of diabetes.



Research Project 36: Project Title and Purpose

Adapting an RNA Sensor Platform to Protein Detection Using Aptamers - We are developing a chip-based RNA sensor, with the initial application for detection of circulating tumor cells (CTCs) in the blood of patients. This device utilizes antisense oligonucleotides (ASOs) targeted to selected cancer-related RNA molecules. Nanowires (NWs) are functionalized with an ASO, and the selected marker RNAs are hybridized to the functionalized NWs. A second ASO, targeted to another site on the marker, is attached to Au particles, and in turn hybridized to the marker RNA bound to the NWs, forming a “hybridization sandwich”. This binding causes a shift in the resonance frequency of the NWs, which can easily be detected. We have shown that derivatized NWs remain functional throughout conditions necessary for “bottom-up” assembly, allowing multiplexing for many different markers. While this platform is being developed, we

have initiated a Clinical Trial with melanoma patients, where CTCs are harvested; melanoma marker RNAs are initially being detected using real-time PCR, and the balance of the samples are being banked for subsequent detection when the chip-based RNA sensor is ready.

Duration of Project

11/24/2008 - 6/30/2010

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 37: Project Title and Purpose

Development of Novel Molecular Subtyping Methods for Identifying Pathways of Transmission of MRSA - This project will build on existing, unique research expertise at both University of Pennsylvania (UP) and Hershey Medical Center (HMC) to create a critical mass focused on preventing the transmission of methicillin-resistant *Staphylococcus aureus* (MRSA) in Pennsylvania communities and healthcare settings. It will translate basic scientific advancements in the fields of genomics, epidemiology, molecular epidemiology, infectious diseases, pathology and clinical medicine to prevent the transmission of MRSA to susceptible hosts. Combining these novel molecular subtyping methods with conventional epidemiologic analysis will allow us to determine the pathways by which specific endemic and epidemic clones are being transmitted in both Pennsylvania communities and at HMC. Once these pathways have been identified then targeted intervention strategies can be implemented to prevent MRSA transmission.

Duration of Project

11/24/2008 - 12/31/2009

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 38: Project Title and Purpose

Sequencing of LGL Leukemia Retrovirus Genome - The broad long-term objective of this project is to understand the etiology of large granular lymphocyte (LGL) leukemia and related autoimmune diseases, including rheumatoid arthritis (RA). Preliminary serologic and molecular data indicate a high likelihood that patients are infected with a novel retrovirus with similarities to both human T-cell leukemia viruses (HTLV) and human immunodeficiency viruses (HIV). In

particular, we have demonstrated transmission of an infectious retrovirus from LGL leukemia cells to co-cultured HOS target cells. Evidence for retroviral infection in this LGL-HOS cell line includes morphologic signs of retroviral infection such as formation of syncytia and other cytopathic effects, demonstration of high levels of reverse transcriptase activity, and detection of 100nm retroviral-like particles of type C morphology using electron microscopy. So far, however, we have been unsuccessful in characterizing this virus at the genomic level. Recent advances in sequencing technology and bioinformatics capability pioneered by investigators at Huck Institute have led to characterization of ancient and environmental genomes. This project represents, then, a cross-campus collaboration aimed at characterizing the LGL virus. Specifically, we plan to determine the retroviral genome of the LGL virus utilizing pyrosequencing of LGL-HOS mRNA.

Anticipated Duration of Project

11/24/2008 - 12/31/2011

Project Overview

Broad Objectives and Specific Aim: The broad long-term objective of this project is to understand the etiology of large granular lymphocyte (LGL) leukemia and related autoimmune diseases, including rheumatoid arthritis. LGL leukemia patients have high titer antibodies cross reactive to common structural regions (gag and env) of HTLV and HIV, as demonstrated using a functional peptide array. We have shown transmission of a cytopathic infectious retrovirus from LGL cell lines to target cells. Microarray characterization using a custom-designed chip containing 14,000 oligonucleotide sequences from all known plant, animal, and human retroviruses detected several “hits” which corresponded closely to regions of serologic reactivity. Taken together, these results show that patients with LGL leukemia most likely are infected with a new retrovirus with some homology to both HTLV and HIV. The specific aim of this project is to characterize the LGL leukemia retrovirus.

Research Design: We plan on using a metagenomics approach pioneered by the Schuster laboratory to identify the retroviral genome. Samples from LGL leukemia cell lines or co-cultured cell lines will be subjected to large scale sequencing using the 454 platform. The Metagenomics Analyzer (MEGAN) bioinformatics program will be utilized to identify the low frequency viral sequences. We have validated this approach by demonstrating detection of retroviral sequences in samples obtained from feline lymph nodes.

Principal Investigator

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Other Participating Researchers

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Expected Research Outcomes and Benefits

Discovery of a new retrovirus associated with human disease has obvious health implications. The practical need for retroviral screening assays to prevent transmission by blood transfusion has been well established over the past 25 years. Characterization of a new virus will allow us to determine if the new virus is linked to disease causation in both LGL leukemia and rheumatoid arthritis. New retroviral therapeutics as well as preventive vaccines for such diseases could result from such work.

Summary of Research Completed

Four milestones were in progress for this period:

Milestone 1: Complete Pyrosequencing Experiments:

This milestone is complete. Pyrosequencing and Illumina sequencing was completed on the originally proposed specimens as well as on additional specimens which were proposed in the previous progress report. Additional specimens from feline blood cells infected with feline immunodeficiency virus (FIV), from Human T cell leukemia virus type 1 (HTLV-1) and with human immunodeficiency virus type 1 (HIV-1).

Milestone 2: Analyze Sequencing Data:

This milestone is still in progress. There was a loss of two highly-trained bioinformatics personnel at the sequencing facility. We are providing training of additional personnel at no extra cost to this project for the purpose of expediting the analysis phase of this project. Analysis is therefore ongoing.

Milestone 3: (10/01/2009-03/31/2010) Perform Primary Validation Experiments:

This milestone will be initiated when the sequence analysis is completed.

Milestone 4: (04/01/2010-06/30/2010) Validation of Data:

This milestone will be initiated when the primary validation experiments are completed.

Work Performed:

PBMC Isolation – As mentioned, work on additional specimens was performed. FIV-infected PBMCs were collected from archived specimens and cultured for RNA extraction.

Spleen tissue – Archived anonymized spleen tissues from a patient with LGL leukemia and from two normal donors were homogenized and processed to extract total RNA. Specimens were classified as “T-LGL Leukemia”, “Normal 1” and “Normal 2”.

RNA Extraction – RNA was extracted from cell cultures and homogenized tissue using Trizol. RNA was then reverse-transcribed to make cDNA for emulsion PCR.

Illumina Sequencing –For Illumina bridge amplification sequencing, cDNA was sheared and then ligated with end-specific adaptors (adaptors A and B) and were further processed to create single-stranded 150-200 bp-sized fragments. Illumina sequencing uses a special type of channeled water-tight microscope slide called a flow cell. The flow cell was heavily coated with complementary adaptor sequences. Then, the single-stranded fragments were hybridized by their adaptors to the complementary adaptor sequences which were already immobilized on the surface of the flow cell. Bridge amplification was initiated with the addition of PCR reaction mix and nucleotides. The amplicons consisted of double-stranded DNA bound at both ends (by adaptor sequence hybridization) to the flow cell surface. The ds-DNA was denatured to produce bound single stranded template DNA. This in turn was reacted with labeled nucleotides from the surface up, to produce CCD sequence data. The sequence data is currently undergoing analysis.

Results and Conclusions

The accomplishments planned for this period were completion of Illumina sequencing and pyrosequencing protocols, and also initiation of additional analyses. Specifically:

The additional 454 sequencing data was compared against BLASTx-nr and the resulting data analyzed with the software MEGAN.

We again found sequences with matches to a portion of the HERV HCML-ARV envelope gene, with 70% to 86% sequence homology. Some HCML-ARV sequences were also found in the normal donor specimens, but were from different parts of this HERV and were better matches to the known genomes of HCML-ARV.

We completed additional Illumina sequencing as previously proposed, and have not yet analyzed enough of the data to compare with the 454 data.

The 454 data continues to suggest that the LGL leukemia virus (LGLV) may be an uncharacterized endogenous retrovirus. Again, this will be determined by completion of the ongoing sequence analysis. If an uncharacterized HERV was inappropriately expressed in the leukemia patients, then different validation methods are needed to confirm this finding.

We again found a large number of uncharacterized (not assigned) reads. As previously explained, these represent reads for which there is still no known match in the general NCBI database. Because uncharacterized sequences could contain very novel or unique retrovirus sequences, the uncharacterized reads are still being analyzed specifically against the NCBI viral genome database.

In summary, we have concluded the wet-work phase of the sequencing experiments, and performed additional Illumina sequencing experiments without incurring additional costs. We are now in the analysis phase of the project.

Research Project 39: Project Title and Purpose

Mechanisms of Unexpected Drug Side Effects Related to Obesity and Diabetes - The long-term goals of the project are to elucidate the mechanisms underlying drug side effects related to obesity and diabetes. We believe that investigating the mechanisms underlying unexplained drug side effects is an innovative approach that may reveal new targets for the treatment of obesity and diabetes. Alternatively, it may yield new drugs with fewer side effects. Obesity and diabetes are epidemics facing our state and country. Understandably, our institution has committed to this as one of the research focus areas, along with cardiovascular disease and cancer.

Duration of Project

11/24/2008 - 12/31/2009

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 40: Project Title and Purpose

Dissecting the Interaction between Radiofrequency Ablation and Tumor Antigen-Specific Immune Response in Hepatocellular Cancer: A Murine Model and a Human Protocol - Hepatocellular cancer can be a difficult disease to treat, with radiofrequency ablation (RFA) being a critical component of treatment for patients with inoperable cancer. The risk of recurrence associated with RFA makes it imperative that we understand the precise mechanism of RFA and methods to increase its efficacy. The proposed murine and human studies will help us understand the anti-tumor immune responses to RFA. We hope to build a foundation with our basic science findings and implement this foundation in clinical practice. The insights gained in our research and clinical practice will be used to raise new questions and guide further research. Our experimental endeavors strive to combine surgical modalities with potential immunotherapy that will enable us to harness one's own immune system to fight the cancer.

Anticipated Duration of Project

11/24/2008 - 12/31/2011

Project Overview

Hepatocellular carcinoma (HCC) is the most common primary liver cancer. It has a worldwide distribution with the highest prevalence in Africa and Southeast Asia and a rising incidence in last two decades in Europe and the United States due to a wide exposure to hepatitis C virus (HCV) in the 1960s and 1970s. Therefore, occurrence of HCC may still continue to rise for a long time because of the large pool of subjects infected by HCV. Current treatment modalities,

including surgery and liver transplantation, offer limited survival benefits, with the number of deaths from the disease in the U.S. (16,780) nearly equaling the number of newly diagnosed cases (19,160). New therapies are desperately needed.

Radio frequency ablation (RFA) creates a local necrosis destroying tumoral tissues and this is followed by a marked inflammatory response with dense T-cell infiltrate. Recent studies suggest a significant increase in both the tumor specific T-cell response and memory T-cell responses after RFA treatment. We hypothesize that the RFA-induced necrotic cell death enhances antigen presentation and T cell activation inducing HCC-specific T cell responses. In this pilot study we will evaluate the immunologic effects following RFA in a murine model of HCC and simultaneously determine if RFA will induce T cell responses specific for HCC-associated antigens in humans. The overall goal of this project is to build on this data, gaining new understanding of the effects of RFA on tumor growth and tumor antigen-presentation in HCC.

Specific Aim 1: To study if RFA reverses antigen-specific CD8⁺ T-cell tolerance in a murine model of HCC and demonstrate the possible changes in the function and phenotype of the infiltrating T cells.

Specific Aim 2: To study the T cell responses specific for HCC-associated antigens following RFA in HCC patients.

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Expected Research Outcomes and Benefits

Radio frequency ablation (RFA) is a critical component of treatment for patients with inoperable HCC. Unfortunately, the recurrence rates are high and there is much that remains a mystery regarding the mechanism of RFA. Therefore, the proposed studies are unique and critically needed to expand our understanding of potential anti-tumor immune responses generated by RFA. The results gathered from the project will lead to novel clinical applications improving the efficacy of RFA and further expand our knowledge leading to unique therapeutic approaches by combining RFA and immunotherapy for treatment of patients with HCC.

Summary of Research Completed

An important step for the development of potent cancer therapy is the identification of tumor antigens specific immune responses for tumor tissue. Cytotoxic T lymphocytes (CTLs) are thought to be potent effector cells against cancers. Most of the tumor associated antigens are expressed only in the tumor tissues and testis, and are not present in the normal tissues and are thought to be attractive targets for cancer immunotherapy. CTLs derived from the induction of tumor-associated antigen (TAA) are an attractive procedure for immunotherapy. On the basis of previous reports, it is assumed that most HCCs would express at least one of the three TAAs. Therefore, monitoring immune responses against these TAAs might help in the development of HCC immunotherapy, such as TAA-based vaccination. In this study, we investigated the magnitude of CD8+ T-cell responses against these TAAs determined by an IFN-g enzyme-linked immunospot (ELISpot) assay. This study would be helpful in exploring the clinical application of such a TAA-based immunotherapy for the treatment of Hepatocellular carcinoma.

Patient:

The peripheral blood was drawn from 7 HCC patients before and after radio frequency ablation. The blood drawn from the patients enrolled for the study is from Milton S. Hershey Medical Center, Hershey, PA under the informed consent and approval from Institutional Review Board. Local tumor ablations were performed in all these patients and were assessed by CT scan and/or MRI and ultrasound. Blood (50ml/patient) was processed immediately after drawing the blood. Radiofrequency ablation is necessary for the treatment of liver cancers in

1. Unresectable primary liver tumors (hepatocellular carcinoma)
2. Hepatic metastases from colorectal tumors when all of the following criteria are a) The metastatic tumor(s) is 5 cm or less in diameter; and b) The tumor(s) is unresectable due to comorbidities such as lesion location (i.e., adjacent to a major vein), or an estimate of inadequate liver volume following resection; and c) No extra hepatic metastatic disease is present; and d) When the goal of treatment is curative, defined as complete resection/ablation of all tumor foci.
3. As a bridge to liver transplantation when the intent is to prevent tumor progression or decrease tumor size to achieve or maintain a patient's candidacy for liver transplant.

Due to complexity of the recruitment criteria described above, we had difficulty in reaching the proposed recruitment number of 20 patients. We may not be able to make up the number as proposed by the end of the grant date 12/31/11. However, we will make every effort to at least add a couple of more patients to the studies, as feasible.

Synthetic Peptide for tumor associated antigens:

Some of the potential tumor associated antigens selected for the present study are as follows. They are GPC3A2 144-152 (FVGFFTDV), hTERT 845 (CYGDMENKL), MAGE-3 271-279 (FLWGPRALV), hAFP158-166(FMNKFIYEI), hTERT1088(TYVPLLGSL), hAFP137-145 (PLFQVPEPV), were used for the present study and were synthesized at the core facility, Penn State College of Medicine.

Isolation of PBMC's:

Human peripheral blood mononuclear cells (PBMC's) were isolated from heparinized peripheral blood by gradient centrifugation using lymphocyte separation media (Mediatech Inc., VA). The

buffy layers are collected, washed twice with PBS. Cell number is determined with a hemocytometer.

Generation of monocytes derived human DC:

The isolated PBMC 2×10^7 were plated in a 6 well plate in Cell Genix™ serum free DC media (Cell Genix) for 90 min. The non-adherent cells were frozen down for further use. The recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF) (50 ng/ml) and recombinant human interleukin-4 (IL-4) (10 ng/ml) (R&D) were added to the adherent cells. The adherent cells were cultured for 5 days with serum free DC media containing GMCSF and IL-4 to obtain immature DC (iDC). The co-expression of HLA-DR with CD11c. The purity was determined in the DC populations obtained from the 5-day cultures of adherent cells in the presence of GM-CSF and IL-4 and it was observed that approximately 90% of the cells obtained from these cultures were double-positive for CD11c and HLA-DR, providing the evidence of generation of a DC population in the cultures.

Maturation of monocytes derived human DC:

For the maturation of DC's, the iDC were cultured in DC media for 48 hours with 10ng/ml TNF- α , 10ng/ml of IL-1 β , 10ng/ml of IL-6 (R&D) and 1 μ g/ml of PGE2 (Sigma). Maturation was confirmed by assessing surface markers CD40, CD80, CD83, CD86 and human leukocyte antigen-DR (HLA-DR), as well as variations in CD1a. Flow cytometric analysis for immature and mature DC was performed at day 7, using a FACSCalibur Flow Cytometer (Becton Dickinson, San Diego, CA, USA).

Results:

1. Determining the percentage of subset of DC in the whole blood:

Two subsets of DC's are originally identified in the peripheral blood. They are myeloid DC and plasmacytoid DC's. They differ widely in many aspects like cytokine production, response to microbial action, capacity to migrate and induction of immune responses. We utilized a technique in which 300 ul of blood was used to determine the number of DC subsets in the whole blood of the patient. It is based on the dendritic cell-specific surface antigens CD303 (BDCA-2), CD141 (BDCA-3), and CD1c (BDCA-1). The markers allow easy identification of three distinct dendritic cell subsets in blood: plasmacytoid dendritic cells (PDCs) are identified by CD303 (BDCA-2), type-1 myeloid dendritic cells (MDC1s) are identified by CD1c (BDCA-1), and type-2 myeloid dendritic cells (MDC2s) are identified by CD141 (BDCA-3) expression. The distinct dendritic cell subsets are analyzed within one sample by three-color (PDCs and MDC1s) or four-color (PDCs, MDC1s and MDC2s) flow cytometry. Dendritic cell subset frequency was determined before and after the radio frequency ablation to determine whether radio frequency ablation plays a role in alteration of the DC subset. In Figure 1 we show how the subset population was gated to determine the number of PDC and MDC1 and MDC2 compared to the isotype control for each sample.

In order to demonstrate the adjuvant effect of immune responses to HCC following radio frequency tumor ablation we, analyzed DCs in the peripheral blood of 7 HCC patients. We demonstrated for the first time, that local HCC ablation results in the activation of myeloid DC (MDC). RFA induces hyperthermia within the tumor lesion, which may lead to immunologic and

biologic effects, including accelerated emigration and migration of peripheral blood mononuclear cells, activation of effector cells, induction and secretion of cytokines, expression of heat shock proteins, and apoptosis (Hager ED, 1999). The phenotyping of the blood DC was calculated from the following formula, which gives the absolute number of DC /ml of blood. This is only possible to calculate if we have an absolute number of leukocytes count in the blood (Table 1). In some cases where the absolute numbers of the leukocytes were not able to be obtained, we used a second option of calculating the number of DC subsets (Table 2).

$$\text{Absolute number of DC of each subset per ml of blood} = \frac{\left(\begin{array}{c} \% \text{ gated} \\ \text{sample} \end{array} - \begin{array}{c} \% \text{ gated} \\ \text{control} \end{array} \right) \times \text{Absolute number of Leukocytes}}{100}$$

2. HLA-A2 typing of the HCC patients for the study material:

The potential patients with HCC undergoing RFA were tested for their HLA types. Approximately 100ul of blood from the patient blood was lysed with AKT lysis buffer, then washed with facs buffer and stained with FITC labeled A2 antibody (ebioscience). FITC labeled Rat IgG was used as an isotype control for comparison. Patients 1, 2, 4, 6, 7 are positive for HLA-A2 typing and patients 3 and 5 are negative for HLA-A2. An example of positive HLA-A2 has been shown in the Figure 2 compared to isotype control.

3. Intracellular staining of IFN-γ producing CD8+T cells specific for TAA:

The interferon gamma production was determined in an individual patient (P007) with the peptide TAA. The patient showed 1.74% of CD8+ T cells showing interferon gamma production after radio frequency ablation compared to 0.19% in the pre RFA for hTERT 845 (Figure 3a). Telomerase is a specialized ribonucleic catalytic protein, and telomerase reverse transcriptase (*hTERT*), stabilizes the telomeres of linear chromosomes [Blackburn, 2005]. Although telomerase activity is present during human embryonic development, its expression and activity are repressed in most normal adult tissues. In contrast, most human tumors display high levels of telomerase. Such an expression in cancer cells might be a necessary and essential step for tumor development and progression. On the other hand, other findings indicate that telomerase expression might not be an obligate requirement in some settings for initial tumor growth, but plays an important role for long-term maintenance. Further we analyzed the IFN-γ production by different cell types as shown in the Figure 3b. A scattered diagram of the values of IFN-γ in all the patients is shown against all the six TAA (Figure 4).

4. IFN-γ ELISPOT assay to determine the efficacy of the TAA specific CD8+ T cells:

The ELISPOT assay was performed using an IFN-γ ELISPOT assay kit (BD Biosciences) according to the manufacturer’s instructions. Briefly the mature DC’s were pulsed with above six TAA peptides at 1mg/ml concentration at 37 ° C and irradiated at 10,000rad. Washed and mixed with 1x10⁶ PBL in the ratio of 1:10. The cells were grown for 1 week, restimulated and transferred to IFN-γ antibody precoated ELISPOT plate. (BD Biosciences). After 24 hrs of incubation at 37 ° C, the cells were removed by washing the plate 8 times with PBS. Next, 100 μl of biotin-conjugated monoclonal antibody were added to each well, and the plates were incubated further for 2 hr. at room temperature. Wells were washed 3 times with PBS and

incubated with 100µl streptavidin–HRP conjugate for 1 hr. Unbound antibodies were removed by washing 4 times with PBS. Then, 100 µl of AEC substrate solution was added to each well and incubated until dark spots emerged. Color development was stopped by washing 3 times with water, and the plates were allowed to dry. Using an ELISpot reader (KS ELISPOT compact; Carl Zeiss, Oberkochen, Germany), the number of spot-forming cells (SFCs) per well was counted. TAA-specific CD8+ T-cells were detected by ELISpot assay before and after HCC treatment in most HCC Patients. Literature suggests that strong TAA-specific CD8+T-cell responses suppress the recurrence of HCC. Immunotherapy to induce TAA-specific cytotoxic T lymphocytes by means such as the use of peptide vaccines should be considered for clinical application in patients with HCC after local therapy. The ELISpot of one patient P007 positive for htert 845 is shown below (Figure 3c). The ELISpot assay was performed to detect CD8+ T-cell responses to TAAs before and after treatment.

5. *Peripheral blood lymphocytes before and after radiofrequency ablation:* Fresh heparinized blood was collected before and after radiofrequency ablation. Blood was stained with fluorochrome conjugated monoclonal antibodies anti-CD3, anti-CD8, anti-CD4 and NKp46. In general the CD3+T cells increased in all patients. However it increases at a higher levels in patients 1, 2, 6 and 7 but moderately increased in patients 3, 4, and 5. The CD8+ Tells decreased in patients 3 and 5 with increase in patients 1,2,4,6 and 7. The CD4+Tcells slightly increased in all patients. The NKT cells, patient 1 showed a marginal increase, two patients (3 and 4) the levels of NKT decreased, four patients (2, 5, 6, 7) the levels are significantly increased. (Figure 5).

IFN-γ production by intracellular staining:

Figure 1: DC subsets

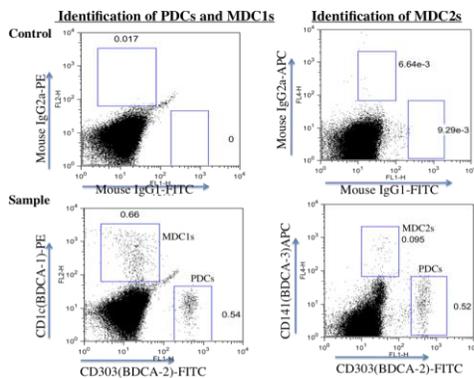


Figure 1: Determination of the subset of DC in the whole blood. The assay is performed on the whole blood. For one test, two samples of 300ul of whole blood is used. One sample is used as a test sample to detect the PDC (plasmacytoid stained for CD303-FITC), MDC1 (myeloid1 stained for CD1c-PE) and MDC2 (myeloid 2 stained for CD141-APC). The other sample is used for as an isotype control. Gated cells determine the number of MDC1, and PDC’s in the first graph and the MDC2 and same PDC’s in the second graph.

Table 1

Mean DC	
Pre	Post
33039.69±1977.1	190393.6±12789
Mean PDC	
Pre	Post
1569.94±1086.9	823.965±350.13
Mean MDC1	
Pre	Post
2129.08±1346.09	7598.47±3526.786
Mean MDC2	
Pre	Post
100.32±44.00	305.65±176.35

Table 1: In this table we demonstrate that the absolute number of DC and DC subset per ml of blood with the formula is demonstrated: Increased in Dc number from 60.8±10.19 to 80.8±4.80 during the process of RFA was demonstrated. The RFA also demonstrated the increase in MDC1 from 0.806±0.315 to 0.9070±0.463 where as the PDC is reduced from 0.25 ±0.25 to 0.131±0.050.

Table 2

Mean DC	
Pre	Post
60.8±10.19	80.8±4.80
Mean PDC	
Pre	Post
0.25±0.25	0.1314±0.050
Mean MDC1	
Pre	Post
0.806±0.315	0.9070±0.463
Mean MDC2	
Pre	Post
0.0483±0.015	0.323±0.287

Table 2: Demonstrated the DC and DC subset as % gated population from the flow cytometry: Increase in the number from 60.8±10.19 to 80.8±4.80 was demonstrated. Also the MDC1 was increased from 0.806±0.315 to 0.907±0.463 with subsequent reduction in the PDC subset from 0.25±0.25 to 0.1314±0.050 was demonstrated.

Figure 2: HLA-A2 Typing

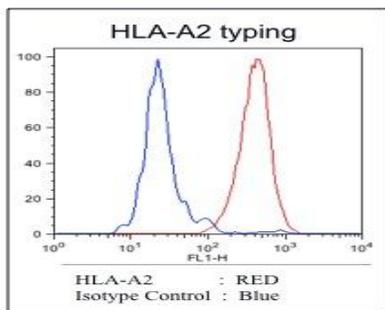


Figure 2: HLA-A2 typing: Cells obtained from 100ul of blood are lysed and stained with FITC-HLA-A2 labeled antibody and FITC labeled isotype control for 30 min on ice. In the figure, histogram is shown. The lines in red represents positive for HLA-A2 and blue represents isotype control.

Figure 3

Figure 3a : IFN-gamma production of PBLs from RFA patient showing stimulation against Htert 845 Tumor antigen detected by Intracellular staining

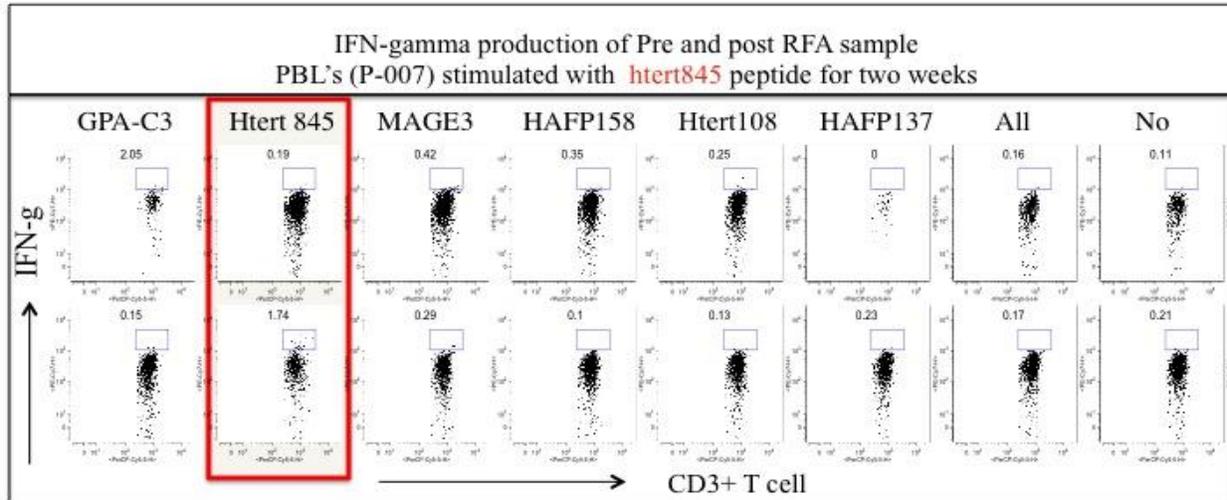


Figure 3a: Peripheral blood lymphocytes from HCC patient were obtained from pre and post RFA procedure. The autologous DC's were pulsed with all the six TAA peptide individually as shown above and irradiated at 10,000 rads. Then mixed with peripheral blood lymphocytes and cultured for a week. Restimulated again with the same peptide and culture for the 2nd week. IFN-gamma production was analyzed by intracellular staining. The IFN-gamma production (1.74%) was demonstrated against htert845 in the post RFA.

Figure 3b: IFN-gamma production against htert 845 Tumor antigen in RFA patient with reference to different cell types.

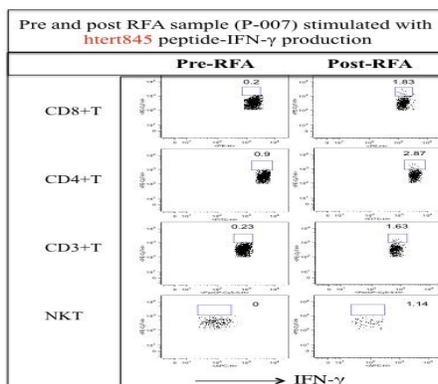


Figure 3b: The figure showing the immune modulatory effect of radio frequency ablation and increased production of IFN-gamma against htert845 TAA antigen in HCC patients with reference to CD8+ T cells (1.83%), CD4+ T cells (2.87) CD3+ T cells (1.63%) and NKT cells (1.14%).

Figure 3c: Positive IFN- γ ELISPOT for htert 845 tumor antigen in the post RFA patient

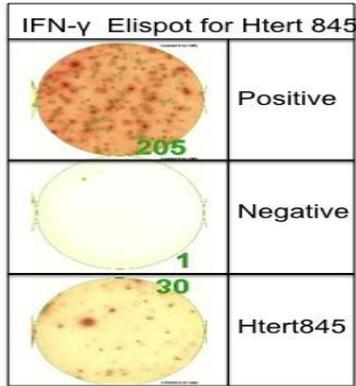


Figure 3c: Example of the positive IFN- γ Elispot for Htert 845 in post RFA patient P-007. Positive and negative controls are shown for IFN- γ Elispot. For positive control the cells are stimulated with Ionomycin (1 μ g/ml) and PMA (50ng/ml).

Figure 4

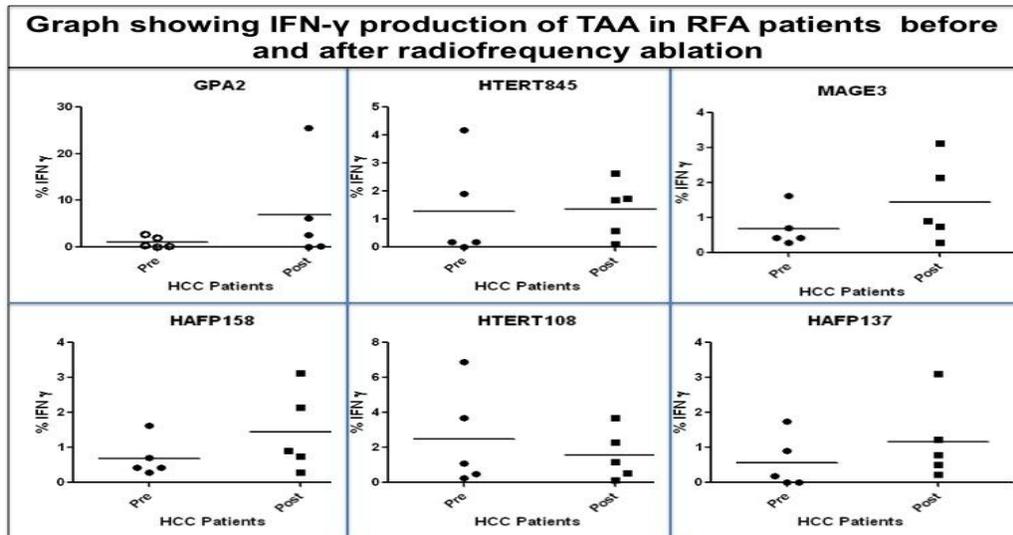


Figure 4. Scattered graph of IFN- γ production in RFA patients before and after radiofrequency ablation against different TAA. The horizontal line in the graphs represents the mean.

Figure 5: Peripheral blood cell types in pre and post RFA patients

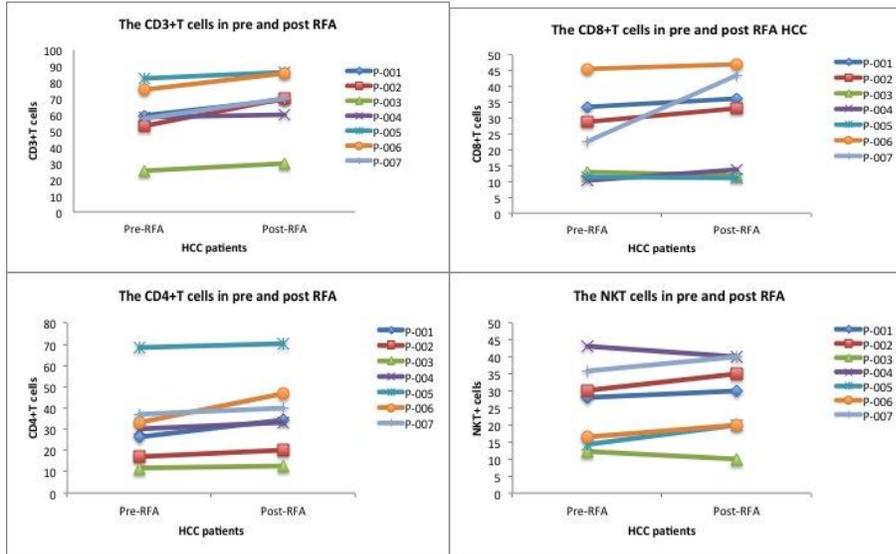


Figure 5. Variation in the levels of T cell subpopulation before and after radio frequency ablation of HCC patient.

Research Project 41: Project Title and Purpose

Central Pennsylvania Women’s Health Study (CePAWHS): Extending the Strong Healthy Women Behavioral Change Intervention to Urban Areas - This project builds on the *Strong Healthy Women* intervention, developed and tested as part of the Central PA Women’s Health Study (CePAWHS). This behavior change intervention was designed to modify risk factors for adverse pregnancy outcomes in non-pregnant pre- and interconceptional women. Behavior change focused on nutrition, physical activity, alcohol/drug/ tobacco use, stress management, and prevention of gynecological infections. A randomized trial in low-income rural communities found significant intervention effects in the areas of nutrient, physical activity, and perceived control of birth outcomes. The proposed research seeks to modify the *Strong Healthy Women* intervention for racially and ethnically diverse urban women in Harrisburg, Lancaster, and York. We hope that this will lead to opportunities to test and disseminate the intervention in more diverse populations with the long-term goal of reducing disparities in adverse pregnancy outcomes across geographic and race/ethnic groups.

Duration of Project

11/24/2008 - 6/30/2011

Project Overview

The project builds on initial findings of the effectiveness of the *Strong Healthy Women*

intervention, developed at Penn State as part of the CePAWHS. The intervention was designed in a 6-session, small-group format, targeting risk-related attitudes, knowledge, and behavior through a mix of presentations, discussion, incremental goal setting, and group- and home-based assignments related to nutrition, physical activity, alcohol/drug/tobacco use, stress management, and prevention of gynecologic infections. Our team conducted a preliminary test of *Strong Healthy Women* in a randomized trial involving 692 pre- and interconceptional women, ages 18-35, living in low-income rural communities in Central PA. There was significant improvement in risk factors for adverse pregnancy outcomes including attitudes, intentions, and behaviors related to nutrition, physical activity, and stress management among intervention participants compared to controls. In analysis of 2-year follow-up data, the intervention was found to impact women's weight and BMI by 2 years and also to reduce gestational weight gain for those who delivered full-term singletons during the follow-up period.

Our experience with a largely white, rural population revealed intervention curriculum and implementation strategies that could be adapted for a more diverse population. We expect that *Strong Healthy Women* could be more broadly disseminated if the content were adapted for urban as well as rural women and for African Americans, who have a two-fold elevated risk of adverse pregnancy outcomes compared with white women. The main objective of the proposed project is to expand the *Strong Healthy Women* intervention to optimally engage urban women. The specific aims of the research proposed here are: (1) To conduct focus groups with race/ethnically diverse pre- and interconceptional women in three urban low-income communities, to guide modifications of the *Strong Healthy Women* intervention; and (2) To pilot test the modified aspects of the *Strong Healthy Women* intervention in race/ethnically diverse groups of pre- and interconceptional women in Harrisburg, Lancaster, and York. Participants will be recruited from community settings in partnership with community organizations. Three focus groups (two composed of African American women and one of white women) will be conducted in each of the three urban areas, for a total of nine focus groups. Focus group data will be used to modify the intervention content to ensure that the content is accessible and motivating to urban women. Once the content modifications have been made, pilot testing of the modified content will be carried out in three additional focus groups in each urban area, with participants selected from the previously conducted focus groups.

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Expected Research Outcomes and Benefits

Recent recommendations to improve preconception health and health care in the United States have called for innovative approaches to reduce adverse pregnancy outcomes, including strategies for improving women's health *before* they become pregnant. More research studying pre-pregnancy health promotion is needed to inform both clinical and public health interventions to improve women's health and pregnancy outcomes. Interventions that contribute to a reduction in adverse pregnancy outcomes, and to the elimination of disparities in these outcomes across geographic and race/ethnic groups, are of great public health importance and will have substantial impact on the overall health status of these women, as well as their children and families.

Summary of Research Completed

During this reporting year, the project was completed. The work related to Specific Aim 2, which began in the previous reporting year, was completed, as described below.

Specific Aim 2: To pilot test the modified aspects of the Strong Healthy Women intervention in race/ethnically diverse groups of pre- and interconceptional women in Harrisburg, Lancaster, and York.

The content modifications to the *Strong Healthy Women* intervention, based on focus groups conducted previously, were less extensive than expected. This was because the focus groups suggested some specific targeted modifications, but not broader or conceptually complex modifications. The main content modifications made were summarized in our previous progress report. These modifications, which affected 4 of the 6 intervention sessions, required some reductions in content in these or other sessions in order to offset increases in the time demands for group participants.

Dry-runs of the modified sessions were conducted during this reporting year with a combination of (1) project staff who role-played the modified sessions, and (2) focus participants from the original focus groups convened for this project. Based on the staff dry-runs, minor alterations were made in session materials. Following this step, two dry-runs with focus participants were conducted. These dry-runs indicated that the materials were user-friendly and that the time burden of the sessions was not increased. Therefore, no additional modifications to the intervention were needed.

Once these steps had been completed, we finalized the protocol for the modified *Strong Healthy Women* intervention, including content, instructions for facilitators, and handouts. As a result of presentations on the study (including a DHHS-sponsored webinar), we developed a users agreement and shared intervention materials with other investigators and states interested in implementing the *Strong Healthy Women* intervention as part of maternal and child health activities or as part of preconception initiatives. Most notably, Indiana and Georgia have expressed interest in adopting *Strong Healthy Women*, and Indiana has completed a user's agreement and received the materials.