

# Temple University

## Annual Progress Report: 2008 Formula Grant

### Reporting Period

July 1, 2010 – June 30, 2011

### Formula Grant Overview

Temple University received \$2,005,437 in formula funds for the grant award period January 1, 2009 through December 31, 2012. Accomplishments for the reporting period are described below.

### **Research Project 1: Project Title and Purpose**

*Resolving Inflammation in Arthritis* - Conventionally, inflammation has been perceived as a progressive reaction that gradually dissipates, so the therapeutic approach has been to block activation of inflammation. COX-2 inhibitors, which inhibit production of the pro-inflammatory prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and reduce pain, have been widely prescribed. However, a new concept that views inflammation as an active, orderly process with phases, induction-progression-resolution, and considers chronic inflammation as “failure to resolve” has emerged. In an animal model of arthritis, collagen-induced arthritis, we found that inhibiting COX-2 during resolution exacerbated inflammation. Thus, the purpose of this project is to investigate how the resolution works, and what molecules control the machinery. This is imperative for safety as well as for designing better therapy.

### **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 2: Project Title and Purpose**

*Identification of the Role of TULA-2 a Novel Phosphatase in Osteoclast Differentiation and Function* - Osteoporosis and other bone-related maladies afflict 150 million Americans and constitute a significant burden to ever-rising health care cost. This project will evaluate the role of TULA-2, a novel phosphatase in bone remodeling in mouse skeleton. In addition, ex vivo experiments will be performed to investigate the role of TULA-2 in differentiation and function of osteoclasts. The research proposed here will help in understanding the basic biology behind

bone resorption and will help identify targets for therapeutic interventions to control bone loss.

### **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 3: Project Title and Purpose**

*C/EBP(s) and the Transcription of Dihydrodiol Dehydrogenase 1 in Human Ovarian Cancer Cells* - Transcription factors (TFs) induce the expression of the target gene by binding regulatory sequences on the target gene's promoter region. CCAAT/enhancer binding proteins (C/EBPs) are a family of TFs that are important for cellular differentiation and proliferation. The expression levels of C/EBPs transcription factors have been found to change during pathophysiological conditions of the cell. Preliminary studies have identified potential C/EBP binding sites on the dihydrodiol dehydrogenase gene 1 (DDH1) promoter in ovarian cancer cells. DDH are a family of aldo-keto reductases involved in the de novo detoxification of xenobiotics. This study aims to clarify the role of C/EBP TFs in regulating the transcription of DDH1 in ovarian cancer cells.

### **Duration of Project**

7/1/2009 - 5/10/2010

### **Summary of Research Completed**

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

### **Research Project 4: Project Title and Purpose**

*Development of a Microvascular Network on a Chip* - Recognition of drastically altered endothelial surfaces in various pathologies (e.g., tumors) has led to the concept of endothelial cell adhesion molecule mediated targeted delivery using nanoparticles as drug carriers. However, currently there are no realistic test beds for preclinical testing and optimization of these nanoparticles in a realistic environment. Development of more complex, realistic in vitro flow chambers will not only advance our knowledge in targeted drug delivery but may also reduce the need for animal experimentation. In this project we will design an anatomically realistic in vitro microfluidic chip for testing and optimization of drug carriers and develop protocols for successful growth of cells on the chips to study drug carrier-cell interaction.

## **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 5: Project Title and Purpose**

*CBI Antagonists as Neuroprotective Drugs* - The purpose of this project is to improve our understanding of how a drug that was designed to block the effect of chemicals produced by the brain called cannabinoids might be used to protect the central nervous system from injury following trauma or an interruption in blood flow.

## **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 6: Project Title and Purpose**

*The Role of GSK3 in Psychostimulant-Induced Behaviors* - Abuse of psychostimulants including cocaine and amphetamine continues to be a major public health problem that leads to loss of employment productivity, breakdown of families and communities, increases in crime and violence, and a variety of health issues not only for the individual but also for their children. Treatments for addiction to psychostimulants are lacking. As such, identification of potential novel targets for treatment of psychostimulant abuse is needed. This project will examine a novel cellular pathway that mediates some of the behavioral effects of cocaine and amphetamine. The role of the Akt-GSK3 pathway in psychostimulant-induced behaviors will be investigated, as will methods to regulate its function in order to reduce the addictive properties of these drugs.

## **Duration of Project**

2/1/2009 - 7/31/2010

## **Project Overview**

Identifying the mechanisms underlying drug-induced reward and plasticity is critical for the

design of therapeutics to treat psychostimulant addiction. One molecule that has received attention recently for its role in psychiatric disorders and the therapeutics used to treat them is glycogen synthase kinase-3 (GSK3). Evidence suggests that GSK3 is uniquely situated to modulate neuronal function and plasticity. Further, our published and preliminary data demonstrate that GSK3 activity is necessary for the manifestation of cocaine-induced behaviors and that the activity of GSK3 is regulated during cocaine exposure in a brain-region specific manner. The central hypothesis that will be investigated is that GSK3 is a critical molecular mediator of psychostimulant-induced actions including drug reward and drug-seeking behaviors. Further, we hypothesize that exposure to psychostimulants results in adaptations in GSK3 signaling and that these adaptations may underlie drug-induced neural plasticity contributing to the perpetuation of addictive disease.

The first research aim will be to determine the role of GSK3 in cocaine- and amphetamine-induced reward, extinction, and reinstatement. The contribution of GSK3 to the rewarding effects of cocaine and amphetamine will be determined using the conditioned place preference procedure in adult male mice. The role of GSK3 in the different phases of drug-induced reward will be evaluated including the development and expression of conditioned reward and the retrieval and reconsolidation of drug-associated contextual memories. The goal of this aim is to establish GSK3 as a target for new therapeutics for the treatment of addictive disease.

The second research aim will be to define the regulation of the GSK3 signaling pathway during cocaine and amphetamine exposure. The activity of GSK3 is regulated by its phosphorylation state. Changes in GSK3 phosphorylation, and hence activity, will be measured following acute and repeated administration of cocaine and amphetamine. Immunohistochemistry with antibodies that are selective for the phosphorylated state of GSK3 will be used to characterize the cellular and subcellular localization of drug-induced changes in GSK3 function. Identification of the cell types showing regulation of GSK3 activity will be determined, as will drug-induced changes in the subcellular distribution of GSK. GSK3 will be identified on tissue sections from mouse brain following administration of cocaine and amphetamine.

### **Principal Investigator**

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### **Other Participating Researchers**

Nicole M. Enman, BS – PhD student in the Department of Pharmacology at Temple University School of Medicine

## Expected Research Outcomes and Benefits

The goal of this project is to provide pre-clinical evidence to support the potential use of modulators of the GSK3 signaling pathway for the treatment of cocaine and/or amphetamine addiction. These studies will determine if modulation of GSK3 can interfere with processes involved in addiction to these psychostimulants. In addition, this project will investigate how exposure to cocaine and amphetamine alters this important intracellular pathway in brain regions critically involved in mediating the rewarding and conditioned stimulus effects of drugs of abuse. Establishment of GSK3 as a novel target for the treatment of psychostimulant addiction could be a major public health advancement and significantly reduce the costs of addiction to the individual and to society.

## Summary of Research Completed

### Materials and Methods

*Aim 2: Regulation of phosphorylation of glycogen synthetase kinase 3 (GSK3) in brains from amphetamine-injected mice.*

This study investigated the regulation of the phosphorylation of GSK3 by amphetamine. Adult male CD-1 mice were pretreated with valproic acid (300 mg/kg, i.p.) or saline followed by acute amphetamine (2 mg/kg, i.p.) or saline 30 minutes later. The caudate putamen, nucleus accumbens, and frontal cortex were removed by gross dissection 60 minutes following amphetamine injections. Brain tissues were sonicated in 1% SDS with 1mM NaF and 1mM NaVO<sub>4</sub>, boiled for 5 minutes, aliquotted, and stored at -80°C until assayed. Protein concentrations were determined by a modified Lowry assay. Protein samples were subjected to SDS-polyacrylamide gel electrophoresis (7.5% Tris-HCl BioRad Ready-gels, Hercules, CA) and transferred to nitrocellulose membranes for 95 minutes. Membranes were blocked for 1 hour in 5% nonfat dry milk and Tween-TBS solution prior to incubation overnight at 4°C in phospho-GSK3 $\alpha/\beta$  (1: 2,000, Cell Signaling, Beverly, MA) and total GSK3 $\alpha/\beta$  (1: 5,000, Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies. Membranes were also incubated in anti-tubulin antibody (1: 300,000, Sigma, St. Louis, MO) at room temperature for 90 minutes to correct for protein loading or transfer differences. Membranes were subsequently washed in Tween-TBS and incubated for 1 hour at room temperature with infrared anti-rabbit or anti-mouse secondary antibodies (1: 10,000, Licor Biosciences, Lincoln, NE). Nitrocellulose membranes were imaged and analyzed using the Odyssey infrared imaging system (Licor Biosciences, Lincoln, NE).

### Results

Mice were pretreated with valproic acid (VPA; 300 mg/kg, i.p.) or vehicle 30 minutes prior to amphetamine (2 mg/kg, i.p.), and brain tissue was obtained 60 minutes later. Protein extracts from the caudate putamen, nucleus accumbens, and frontal cortex were analyzed by immunoblot for levels of phosphorylated and total GSK3 $\alpha$  and  $\beta$  and tubulin (Fig. 1). Analysis by two-way Analysis of Variance (ANOVA) showed that levels of phosphorylated ser<sup>21</sup>-GSK3 $\alpha$  in the caudate putamen were similar among experimental groups (Interaction:  $F(1, 36) = 3.454, p < 0.05$ ; Pretreatment:  $F(1, 36) = 0.895, p > 0.05$ ; Treatment:  $F(1, 36) = 2.164, p > 0.05$ ) (Fig. 1A).

Significant interaction and pretreatment effects were detected for levels of phosphorylated ser<sup>9</sup>-GSK3β in the caudate putamen (Interaction:  $F(1, 36) = 5.225, p < 0.05$ ; Pretreatment:  $F(1, 36) = 3.685, p < 0.05$ ; Treatment:  $F(1, 36) = 6.380, p > 0.05$ ) (Fig. 1B). Post-hoc analysis showed significant elevations in phosphorylated ser<sup>9</sup>-GSK3β in mice injected with VPA-amphetamine compared to vehicle-amphetamine (vehicle-amphetamine vs. VPA-amphetamine,  $p < 0.01$ ) or valproate-saline (vehicle-amphetamine vs. VPA-saline,  $p < 0.05$ ).

In the nucleus accumbens, levels of p-ser<sup>21</sup>-GSK3α (Interaction:  $F(1, 34) = 3.025, p > 0.05$ ; Pretreatment:  $F(1, 34) = 0.542, p > 0.05$ ; Treatment:  $F(1, 34) = 2.636, p > 0.05$ ) (Fig. 1C) and p-ser<sup>9</sup>-GSK3β (Interaction:  $F(1, 34) = 1.286, p > 0.05$ ; Pretreatment:  $F(1, 34) = 0.080, p > 0.05$ ; Treatment:  $F(1, 34) = 0.120, p > 0.05$ ) (Fig. 1D) were not significantly different between groups.

In the frontal cortex, significant interaction effects were detected for levels of phosphorylated ser<sup>21</sup>-GSK3α (Interaction:  $F(1, 36) = 10.14, p < 0.01$ ; Pretreatment:  $F(1, 36) = 1.309, p > 0.05$ ; Treatment:  $F(1, 36) = 0.0001, p > 0.05$ ) (Fig. 2E), and post-hoc analysis showed significantly higher levels of GSK3α in the frontal cortex of mice pretreated with valproate compared to vehicle prior to amphetamine (vehicle-amphetamine vs. VPA-amphetamine,  $p < 0.01$ ). Significant interaction effects were also found for levels of phosphorylated ser<sup>9</sup>-GSK3β in the frontal cortex (Interaction:  $F(1, 36) = 0.021, p < 0.05$ ; Pretreatment:  $F(1, 36) = 0.077, p > 0.05$ ; Treatment:  $F(1, 36) = 0.488, p > 0.05$ ) (Fig. 2F). Post-hoc analysis determined significantly higher levels of phosphorylated ser<sup>9</sup>-GSK3β in the frontal cortex of mice administered valproate-amphetamine compared to vehicle-amphetamine (vehicle-amphetamine vs. VPA-amphetamine,  $p < 0.05$ ).

Total levels of GSK3α and GSK3β in each of the three brain regions tested were similar in all experimental groups (data not shown). Because GSK3 is inactivated by phosphorylation on ser<sup>21</sup> (α) or ser<sup>9</sup> (β), these results demonstrate that valproic acid inhibited GSK3α and GSK3β activity in the frontal cortex, as well as GSK3β activity in the caudate putamen of amphetamine-injected animals.

### Publication

Since the end of the last reporting period (June 2010), we have completed the final evaluation of the resulting data and prepared a manuscript which will be submitted for publication to “Behavioral Brain Research” by the end of July 2011.

“GSK3 modulates amphetamine-induced hyperactivity” by Nicole M. Enman and Ellen M. Unterwald.

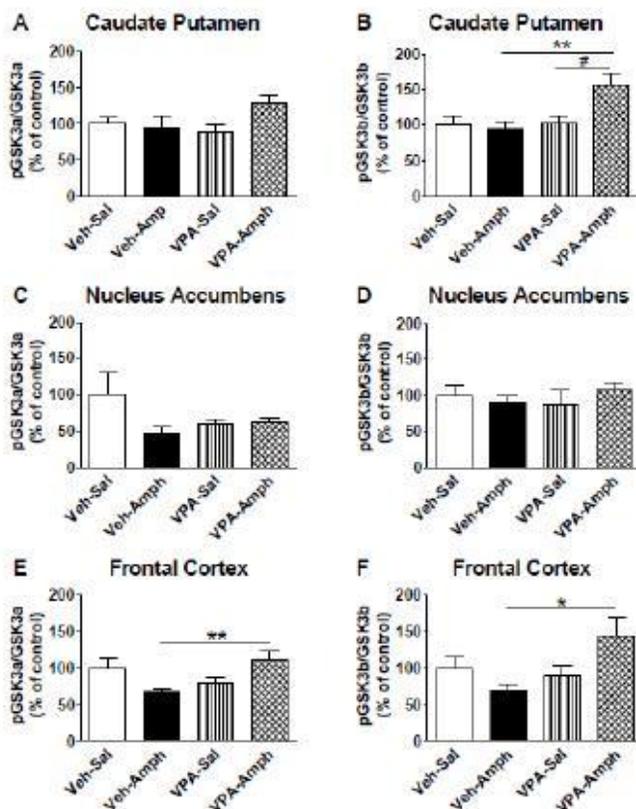


Figure 1. Brain tissue was obtained 60 minutes following amphetamine (2 mg/kg) or saline administration from mice pretreated with valproic acid (300 mg/kg) or vehicle. Western blots of protein extracts obtained from caudate putamen, nucleus accumbens, and frontal cortex were analyzed. (A) Pretreatment with valproic acid did not significantly alter levels of phosphorylated GSK3 $\alpha$  in the caudate putamen. (B) Phosphorylated GSK3 $\beta$  in the caudate putamen was significantly increased in mice pretreated with valproic acid compared to vehicle prior to amphetamine (\*\* $p < 0.01$ ) and saline (# $p < 0.05$ ). (C) Administration of valproic acid prior to amphetamine did not significantly alter phosphorylation of GSK3 $\alpha$  or (D) GSK3 $\beta$  in the nucleus accumbens. (E) Pretreatment with valproic acid significantly increased levels of phosphorylated GSK3 $\alpha$  in amphetamine treated mice compared to pretreatment with vehicle (\*\* $p < 0.01$ ). (F) Levels of phosphorylated GSK3 $\beta$  in the frontal cortex were significantly greater in mice pretreated with valproic acid prior to amphetamine (\* $p < 0.05$ ). Data points represent the mean + SEM (n=9-10/group).

### **Research Project 7: Project Title and Purpose**

*Biomimetic Composite Design* - The purpose of this project is to refine analytical models of novel composites intended for use as dental restorative materials. The novelty of the composites lies in borrowing strengthening and toughening strategies from naturally occurring composites, i.e., seashells. The refined models will be useful for designing the new composites.

## **Duration of Project**

5/1/2009 - 5/31/2010

## **Summary of Research Completed**

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

## **Research Project 8: Project Title and Purpose**

*Interactions Between Cytotoxic and Antiangiogenic Drugs* - Combination anticancer drug therapy is standard for most cancer types, yet the types of drugs that are combined in therapeutic regimens is changing. Rather than combining toxic drugs with severe and overlapping toxicities, new efforts seek to combine drugs not only with different and less severe toxicities, but also drugs with different cell and molecular targets or mechanisms of action. Antiangiogenic drugs are among a new portfolio of agents that may be referred to as targeted drugs that can interfere with the growth and spread of tumors by various mechanisms, and may compliment the action of more traditional cytotoxic drugs. The purpose of this project is to understand how two targeted drugs can be optimally combined with a cytotoxic drug in a preclinical brain tumor model.

## **Duration of Project**

1/1/2009 – 8/30/2009

## **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 9: Project Title and Purpose**

*Control of Cellular and HIV-1 Gene Expression by Cellular CDK9, A Potential Target for HIV Therapeutics* - HIV is the causative agent of AIDS (Acquired Immunodeficiency Syndrome). Highly Active AntiRetroviral Therapy (HAART) is a drug combination currently used to reduce viral loads by neutralizing at least two different HIV proteins. However, because HIV mutates, viral strains resistant to current HAART drug combinations arise. It is believed that anti-HIV drugs directed to host cellular proteins that are essential for HIV replication may be a solution to this problem. Therefore, there is an urgent need for identification and validation of such cellular targets and the development of new high throughput assays to identify drugs against them. This project aims at both further characterizing a cellular kinase complex that is hijacked by an HIV-1 viral protein and to design strategies to identify drugs targeting this essential step on the HIV life cycle.

## **Duration of Project**

1/1/2009 – 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 10: Project Title and Purpose**

*Data Analysis of Cognitive-Behavioral Therapy as an Augmentation Strategy for Social Anxiety Disorder* - We have completed a study of the efficacy of cognitive-behavioral therapy in the augmentation of response to the antidepressant paroxetine for patients with social anxiety disorder. The study was conducted at two sites (Temple University and the New York State Psychiatric Institute). Data files have been constructed at each site but funding expired prior to the initiation of formal data analysis suitable for professional publication. Therefore, the purpose of this project will be the construction of the final merged and cleaned dataset and the conduct of the statistical analysis for this project.

## **Duration of Project**

7/1/2009 – 6/30/2011

## **Project Overview**

Social anxiety disorder is the third most common mental disorder in the US. Its symptoms include anxiety and avoidance of social situations and/or situations in which one might have to perform in front of others. It is associated with significant impairment in social, educational, and occupational functioning, as well as increased risk for other disorders that carry their own significant burdens, e.g., major depression and alcohol and substance abuse disorder. Viable treatments exist, but most patients complete trials of these treatments with significant residual impairment. Therefore, the study of augmentation treatment strategies is of great importance.

The broad objective of this project is to ready the data files necessary for analysis and conduct the analyses of the outcomes of our recently completed study of the efficacy of cognitive-behavioral therapy (CBT) as a method to augment the response of patients with social anxiety disorder who showed less than complete response to treatment with the selective serotonin reuptake inhibitor paroxetine. This research was funded by a grant from the National Institute of Mental Health, but expired before primary data analyses could be conducted. The final dataset has not been compiled and the sophisticated statistical analyses, based on hierarchical linear modeling that is the current standard in our field, have not yet been conducted. These essential analytical steps comprise the specific aims of this project.

## **Principal Investigator**

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## **Other Participating Researchers**

Carlos Blanco, MD, PhD, Henian Chen, PhD, Andrew B. Schmidt, LCSW – employed by New York State Psychiatric Institute

## **Expected Research Outcomes and Benefits**

Final and complete analyses of the data from the study of CBT augmentation of paroxetine treatment of social anxiety disorder will have several important (inter-related) outcomes and benefits. First, and most immediate, is that these analyses will serve as supporting data for a grant application to be submitted in 2009 to the National Institute of Mental Health (NIMH) that will further examine the nature and utility of augmentation treatments for social anxiety disorder. Second, completion of the data analysis will allow us to publish a paper in a top-tier journal and give our findings significant exposure, which should result in an increased interest in/utilization of CBT approaches in general and augmentation strategies in specific.

## **Summary of Research Completed**

One of the specific aims of this project was the completion of a grant application to the NIMH that would further examine the nature and utility of augmentation treatments for social anxiety disorder. This was accomplished in the previous reporting period, and preliminary results from the data analyses referred to above were included in that application (see previous annual report). This was an A1 application, which means that no further revisions of that application would be accepted for review. After consultation with our Program Officer and the Branch Chief, we concluded that further pursuit of this line of research funding would not be advisable, and no further applications in this line have been submitted. However, we have remained active in the pursuit of external funding of research that would examine treatments for social anxiety disorder, especially among those whose distress and impairment is not typically reduced by first-line or standard treatments. We have done so by submitting applications to NIMH and the National Institute of Drug Abuse (NIDA) to fund the development and evaluation treatments for persons with social anxiety disorder who use either alcohol or cannabis to self-medicate their distress and who further complicate their lives in the process. Three such proposals have been submitted, two of which were submitted during the final quarter of the previous reporting period (one at the very end), but which were inadvertently omitted from that report, and are thus included here:

*Integrated MET-CBT for Social Anxiety Disorder with Alcohol Misuse*  
Submitted to NIMH 3/15/10; A1 application, scored but not funded

*Integrated MET-CBT for Social Anxiety and Marijuana Use Disorders*  
Submitted to NIDA 6/15/10; original application, scored but not funded

*Novel Personalized Treatment for Dually Diagnosed Anxious Marijuana Users*  
Submitted to NIDA 3/16/11; A1 application (revision of above with changed title to reflect input from study section), pending initial review

We also submitted an additional application during the current reporting period, based on the premise that many of the biased views of social interactions evident in social anxiety disorder (the modification of which leads to clinically significant change) are also evident among persons with problems with aggression:

*A Computer-Based Cognitive Bias Modification Program to Treat Aggression*  
Submitted to NIMH 2/15/11; original application, unscored

Dr. Heimberg was principal investigator (PI) or one of multiple PI's on all of these applications.

The second specific aim of this project was the development of the final dataset for the original project, the conduct of the final analyses of outcomes of the treatments examined in that study, and the submission of the resultant paper to a top-tier journal in the field of mental health treatment. This goal has not been accomplished at this writing although work continues in that direction, and we hope to be able to complete our movement toward this goal in 2011. Progress toward that goal has been hindered by continuing staff turnover at the sister site of the original project, such that we have been unable to completely enter, clean, and certify the data at all the time points in the grant study. In that study, comprehensive assessment of social anxiety disorder symptoms and related impairment took place at Week 0 (baseline), Week 12 (after open-label treatment with the selective serotonin reuptake inhibitor paroxetine), Week 28 (after a 16 week period of continued treatment with paroxetine with or without cognitive-behavioral therapy, CBT, randomly determined), and at follow-ups at Week 40 and Week 52. Less comprehensive assessments were also conducted at Weeks 4, 8, and 20.

Although we have not been able to complete the work on the full dataset, we have accomplished the bulk of the work for the Week 0 assessment and for the major outcome measures at Weeks 4, 8, and 12. This allowed us to pursue other projects working from this database that are related to the nature of social anxiety disorder or moderation of attrition from or response to treatment with paroxetine, which we see as related to the overarching goals of our research program and consistent with the goals of this specific project. These projects have resulted in two presentations at professional meetings (one in the current reporting period) and three manuscripts which are currently under review for publication. The results of these studies are described in the remainder of this report. The submitted manuscripts are included with the final report.

### Fear of Positive Evaluation in Patients with Social Anxiety Disorder

We designed the Fear of Positive Evaluation Scale (FPES) in previous research to assess *fear of positive evaluation*. Most research on social anxiety disorder has come to the conclusion that social anxiety is related to fear of negative evaluation, and the support for that contention is robust. However, evolutionary theory suggests that fear of positive evaluation (FPE), which we define as the sense of dread associated with being evaluated *favorably* and *publicly*, which begs a direct social comparison of the self to others and therefore causes a person to feel conspicuous and “in the spotlight” is a type of cognition which may be substantially related to social anxiety. Although previous findings on the psychometric properties of the FPES have been highly encouraging, only one previous study has examined the psychometric profile of the FPES in a sample of patients with social anxiety disorder. In the current study, we examined the psychometric profile of the FPES in 226 patients with a principal diagnosis of social anxiety disorder and 42 non-anxious controls. The FPES demonstrated strong internal consistency ( $\alpha = .85$ ) and test-retest reliability ( $r = .80$ ) in the clinical sample, and patients ( $M = 39.60$ ,  $SD = 14.92$ ) scored significantly higher than controls ( $M = 13.07$ ,  $SD = 10.99$ ),  $F(1, 266) = 120.52$ ,  $p < .001$ ,  $d = 2.02$ . Patients’ FPES scores correlated between .27-.50 with measures of symptom severity and impairment (all  $ps < .01$ ). FPES scores were also significantly more strongly related to scores on measures of social anxiety than to scores on the Beck Depression Inventory II. Hierarchical regression analyses revealed that FPES scores accounted for significant variance in social anxiety scores after controlling for scores on a measure of fear of negative evaluation (see Table 1). A comparison of patients who received CBT compared to those randomized to a waiting list revealed greater FPES change in the CBT group ( $M = -17.41$ ,  $SD = 16.36$ ) than the wait list group ( $M = 1.41$ ,  $SD = 10.10$ ),  $F(1, 54) = 26.35$ ,  $p < .001$ ,  $d = 1.38$ . This study provided encouraging support of the psychometric characteristics of the FPES and the clinical validity of the construct of fear of positive evaluation.

### Perfectionism and Social Anxiety Disorder

Perfectionism is a trait with adaptive and maladaptive components that has been linked to several psychological disorders, including social anxiety disorder. Extant assessments of perfectionism are based on different theories, and the extent of convergence across these assessments remains unclear. The present study clarifies the core dimensions assessed by leading perfectionism measures and tests them across groups, including a clinical sample of persons with social anxiety disorder. Multiple perfectionism measures were used for an exploratory factor analysis in an undergraduate sample and a confirmatory factor analysis in a larger sample of individuals with social anxiety disorder. Consistent with prior research, findings suggest that some of the most frequently utilized perfectionism measures converge on two factors: (a) maladaptive and (b) adaptive/orderly perfectionism. We report here a subset of the findings for the patient sample only. Patients completed the Multidimensional Perfectionism Scale (MPS) and the Almost Perfect Scale Revised (APS-R), along with measures of social anxiety, depression, and quality of life. Both perfectionism factors significantly and uniquely predicted depression and quality of life, in opposite directions, as expected. However, the hierarchical regression for depression scores revealed a significant interaction between maladaptive perfectionism and social anxiety, such that when maladaptive perfectionism was high, the relationship between social anxiety and

depression was significantly positive ( $\beta = .57, t = 5.61, p < .001$ ), but this relationship was not significant at low levels of maladaptive perfectionism ( $\beta = .13, t = 1.34, p = .183$ ). See Figure 1.

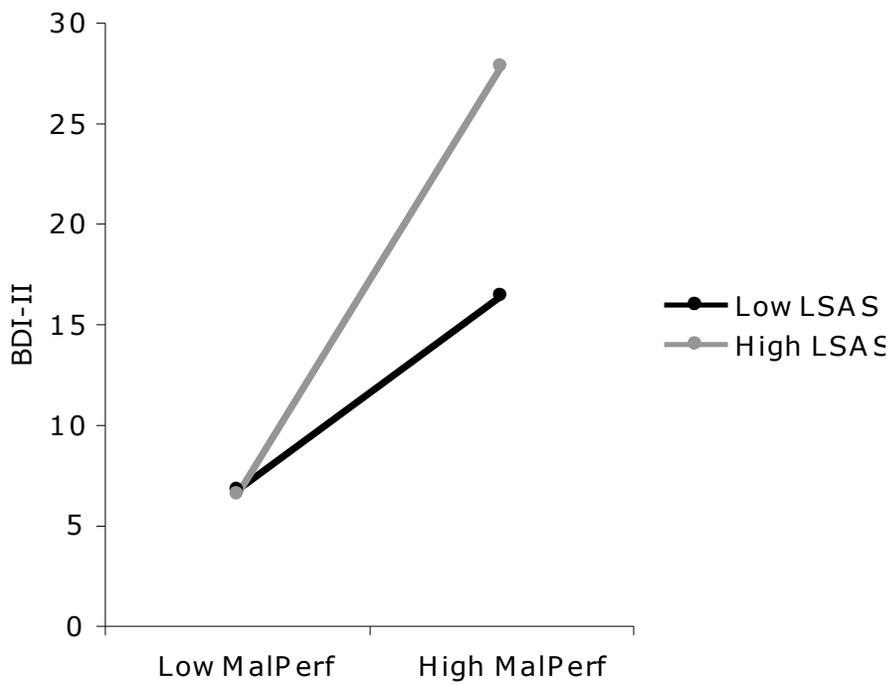
*Childhood Maltreatment: Implications for Symptom Severity and Response to Pharmacotherapy*

Childhood maltreatment has been associated with severity of symptoms, reduced quality of life, impaired functioning, and reduced resilience in individuals with social anxiety disorder. No study has investigated the possible link between specific types of childhood maltreatment and outcome of treatment for social anxiety disorder. However, several studies suggest a link between childhood maltreatment and response to pharmacotherapy or cognitive-behavioral therapy for depression. We replicated previous work on the effects of childhood maltreatment in social anxiety disorder and examined its impact on response to pharmacotherapy. One hundred fifty-six individuals seeking treatment for social anxiety disorder completed the Childhood Trauma Questionnaire, which includes subscales measuring physical abuse and neglect, emotional abuse and neglect, and sexual abuse, along with measures of severity of social anxiety, quality of life, and disability. Data from the subset of 127 patients enrolled in the paroxetine trial were analyzed to gauge the impact of childhood maltreatment on attrition and response. Except for physical and sexual abuse, all types of maltreatment were related to greater symptom severity. Emotional abuse and neglect were related to greater disability, and emotional abuse, emotional neglect, and physical abuse were related to decreased quality of life (Table 2). Attrition from pharmacotherapy was significantly predicted by emotional abuse and physical abuse (Table 3); however, only the effect of emotional abuse remained robust after controlling for severity of social anxiety symptoms. A time by emotional abuse interaction ( $p < .012$ ) suggests that, for those who completed the full trial of paroxetine, the impact of emotional abuse on severity of social anxiety weakened significantly over time. In sum, emotional maltreatment was most strongly linked to dysfunction in social anxiety disorder. Individuals with a history of emotional abuse were more likely to dropout from pharmacotherapy for social anxiety disorder; however, if they stayed the course, their outcomes were similar to those without a history of emotional abuse, findings that may have important implications for treatment providers.

Table 1. Regression weights from hierarchical regression analyses examining the unique variance in social anxiety measures accounted for by measures of fear of positive and negative evaluation.

Variable	<i>B</i>	<i>SE B</i>	Beta
Liebowitz Social Anxiety Scale			
Step 1:			
BFNE-S	1.00	.26	.18
Step 2:			
BFNE-S	.67	.25	.18
FPES	.51	.09	.38
Social Interaction Anxiety Scale – Straightforward			
Step 1:			
BFNE-S	1.09	.12	.45
Step 2:			
BFNE-S	.92	.12	.45
FPES	.23	.04	.31
Social Phobia Scale			
Step 1:			
BFNE-S	1.26	.21	.43
Step 2:			
BFNE-S	1.00	.21	.43
FPES	.30	.09	.31

*Notes:* FPES = Fear of Positive Evaluation Scale; BFNE-S = Brief Fear of Negative Evaluation Scale-Straightforward. All overall adjusted  $R^2$ s > .20, all  $SE$ s < 18.21; all  $R^2 \Delta$ s > .08, all  $F$ s > 11.26, all  $p$ s < .001.



*Figure 1.* Interaction of Maladaptive Perfectionism (MalPerf) and Liebowitz Social Anxiety Scale (LSAS) scores in predicting Beck Depression Inventory-II (BDI-II) scores. *High* = +1 SD; *Low* = -1 SD.

Table 2. Impact of different types of childhood maltreatment (CTQ subscale scores) on social anxiety symptom severity (LSAS), quality of life (QOLI), and disability (LSRDS)

Variable	<u>LSAS</u>	<u>QOLI</u>	<u>LSRDS</u>
	$\beta$ (SE)	$\beta$ (SE)	$\beta$ (SE)
Childhood Physical Neglect	.20 (1.76)*	-.09 (.09)	.12 (.48)
Childhood Physical Abuse	.16 (1.78)	-.21 (.08)*	.06 (.48)
Childhood Sexual Abuse	.07 (1.78)	.01 (.09)	-.01 (.48)
Childhood Emotional Neglect	.20 (1.77)*	-.27 (.08)**	.22 (.48)**
Childhood Emotional Abuse	.24 (1.77)**	-.33 (.08)**	.26 (.46)**

LSAS, Liebowitz Social Anxiety Scale; QOLI, Quality of Life Inventory; LSRDS, Liebowitz Self-Rated Disability Scale.

\*\*  $p > .01$       \*  $p > .05$

Table 3. Univariate logistic regression of maltreatment variables with completer status<sup>a</sup>

Variable	OR	(95% CI)
Childhood Physical Neglect	1.13	(0.83, 1.54)
Childhood Physical Abuse	0.73	(0.57, 0.93)*
Childhood Sexual Abuse	1.03	(0.77, 1.38)
Childhood Emotional Neglect	1.10	(0.82, 1.47)
Childhood Emotional Abuse	0.73	(0.56, 0.96)*

<sup>a</sup> Completer status dichotomized into completer vs. non-completer of 12 weeks of paroxetine treatment

\*  $p > .05$

### **Research Project 11: Project Title and Purpose**

*RAD51 Causes Genomic Instability in Chronic Myeloid Leukemia* - BCR/ABL kinase-positive leukemia cells display genomic instability leading to the resistance to imatinib mesylate and malignant progression of the disease to fatal blast crisis. We reported previously that BCR/ABL enhances homologous recombination repair (HRR) of DNA double strand breaks (DSBs) due to stimulation of expression and tyrosine phosphorylation of RAD51. However, the fidelity of HRR is compromised in leukemia cells. We will investigate in depth the role of unfaithful DNA polymerase beta, BCR/ABL-RAD51 interaction, and constitutive tyrosine phosphorylation of RAD51 in compromising the fidelity of HRR eventually leading to genomic instability in leukemia cells.

## **Duration of Project**

1/1/2009 – 12/31/2010

## **Project Overview**

The unifying hypothesis for this project is that BCR/ABL oncogenic tyrosine kinase causes overexpression of RAD51 protein, directly interacts with RAD51 and phosphorylates RAD51 on Y315 resulting in stimulation of unfaithful homologous recombination repair (HRR). This mechanism may contribute to genomic instability in chronic myeloid leukemia (CML) generating BCR/ABL mutants resistant to small molecule inhibitors such as imatinib, dasatinib and nilotinib, and producing chromosomal aberrations facilitating malignant progression of the disease from chronic phase (CML-CP) to fatal blast crisis (CML-BC).

We are planning to obtain preliminary evidence that RAD51 overexpression, interaction with BCR/ABL and/or tyrosine phosphorylation plays an important role in genomic instability in CML. To achieve our goals we will target BCR/ABL-induced overexpression of RAD51, BCR/ABL-SH3 - RAD51-PP functional interaction, and BCR/ABL-mediated constitutive phosphorylation of RAD51 on Y315 (RAD51-pY315).

RAD51 overexpression will be downregulated by previously validated commercially available RAD51-specific siRNA (Dharmacon); siRNA against non-coding sequence will be used as control. BCR/ABL-RAD51 interaction will be blocked by peptide aptamers containing proline-rich (PP) fragments of RAD51 (Genemed Synthesis, San Antonio, TX). Enhanced activity of RAD51-pY315, which probably depends on facilitated interaction with partner proteins and/or DNA substrates (to be examined in this project), will be abrogated by the aptamers containing phospho-Y315 of RAD51. Then, leukemia cells will be irradiated and chromosomal instability will be evaluated in surviving cells by spectral karyotyping (SKY) and by G-banding analysis to minimize the possibility of a false-positive result in SKY.

In addition, our preliminary data implicated a few polymerases possibly responsible for this effect; the primary suspect is DNA polymerase beta, which is overexpressed in leukemia cells and co-localizes with RAD51 nuclear foci. Therefore, polymerase beta will be downregulated by the expression plasmid carrying the shRNA and fidelity of HRR products will be determined by sequencing.

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## **Expected Research Outcomes and Benefits**

We expect to obtain preliminary data about: (1) the role of unfaithful DNA polymerase beta in resistance to imatinib mesylate, (2) the role of BCR/ABL-RAD51 interactions contributing to chromosomal aberrations in CML. This information may be useful for planning novel strategies preventing/delaying the development of resistance to imatinib mesylate and blast crisis in CML patients.

## **Summary of Research Completed**

We show that BCR/ABL kinase (non-mutated and TKI-resistant mutants) facilitate recombination repair (RR) of DSBs. Although recombination usually represents a faithful mechanism of DSB repair, it may generate chromosomal aberrations when similar (homeologous), but not identical (homologous) templates are employed during the repair. To study unfaithful homeologous recombination repair (HomeoRR) a reporter repair cassette containing I-SceI endonuclease-inducible DSB site and a repair template displaying 1% divergence sequence relative to the DSB site was integrated into the genome of 32Dcl3 murine hematopoietic cells and BCR/ABL-positive counterparts. BCR/ABL kinase caused about 3-fold increase in HomeoRR activity implicating its role in accumulation of chromosomal aberrations in CML cells (Fig. 1).

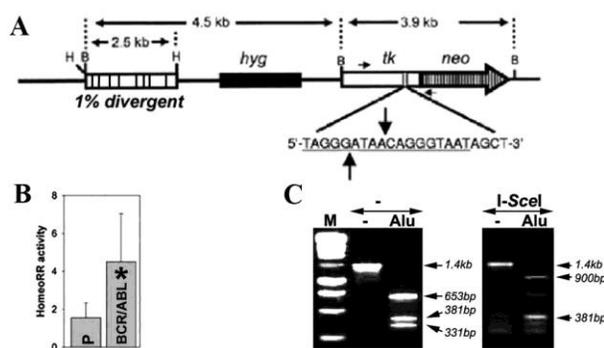


Fig. 1. BCR/ABL kinase promotes HomeoRR. (A) HomeoRR substrate contains a functional hygromycin gene (*hyg*), used to select for stably transfected cells, and a *tk-neo* fusion gene that is disrupted by a 22-bp oligonucleotide containing the 18-bp recognition site for endonuclease I-*SceI* (underlined sequence). Site of staggered cleavage by I-*SceI* is indicated. In addition, a 2.5-kb HindIII fragment (stripped rectangle) containing complete HSV-1 *tk* gene as recombination template (1% divergent in comparison to HSV-1 in *tk-neo* fusion gene) is inserted. Gene conversion of I-*SceI* – indicated DSB restores the expression of *neo* gene. (B) DSB in HomeoRR substrate cassette integrated into the genome of parental 32Dcl3 cells (P) and cells expressing p210BCR/ABL kinase (BCR/ABL) was induced by co-transfection with I-*SceI* – DSB inducer) and GFP (transfection control) expression vectors. HomeoRR activity was measured as % of G418-resistant cells in GFP+ population. (C) HomeoRR was confirmed in the mixture of G418-resistant clones by RT-PCR followed by AluI restriction digest generating predominant 900bp and 311bp bands. \* $p < 0.01$  in comparison to P.

## **Research Project 12: Project Title and Purpose**

*Enhancing Diabetic Foot Education by Viewing Personal Plantar Pressures* - The purpose of this project is to examine the efficacy of a novel patient education strategy, compared to a standard diabetic foot education. The proposed diabetic foot care education uses personal computer-animated plantar pressure data to educate patients on why and how they should care for their feet. The effectiveness of the diabetic foot education will be examined by looking at the following: (1) personal daily foot care as measured by foot-self care behavior scores; (2) subject understanding of peripheral neuropathy as assessed by the Patient Interpretation of Neuropathy Questionnaire; (3) occurrence of foot complications; and (4) peak barefoot plantar pressures.

### **Duration of Project**

1/1/2009 – 6/30/2011

### **Project Overview**

In this project, the efficacy of an enhanced patient education strategy, compared to a standard diabetic foot education, will be investigated in a randomized control trial. The proposed enhanced diabetic foot education uses personal, computer-animated, multicolored, plantar pressure maps. The project will include 30 high-risk diabetic subjects. All subjects will receive a

conventional foot care education and plantar pressure measurements. However, subjects randomized to the test group will receive the additional, enhanced diabetic foot education based on their footprints. A presentation of abnormal barefoot plantar pressure and how that pressure may be alleviated with proper shoes is postulated to motivate high-risk diabetic patients with peripheral neuropathy (loss of feeling in the feet) to take a more active role in caring for their feet. All participants will be monitored prospectively for a year along with quarterly palliative foot care and customary diabetic shoes. However, this project will focus on baseline, 1-month, and 3-month follow up evaluations. If the proposed visual diabetic foot education yields a more effective strategy, the subjects in the test group, as compared to the control group, are anticipated to show (1) better personal daily foot care, (2) greater understanding of peripheral neuropathy, (3) fewer foot complications and (4) greater reductions in peak dynamic plantar pressure at follow up visits. The proposed diabetic foot education has a great potential to be an effective educational tool especially for those underserved minority communities, where health literacy is often a major challenge.

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### **Expected Research Outcomes and Benefits**

This project targets methods of preventing diabetic foot complications. Effective diabetic foot care education is an important component in combating serious consequences of diabetic foot problems. This is an especially critical issue where health literacy is a concern. If the proposed education, which is personal and more visual than the traditional method, yields improved patient understanding and compliance, it can lead to significant reductions in the number of foot complications and health care costs. In addition, it may improve the quality of life of patients and their families. In this project, 30 participants who have diabetes and are at risk of developing foot complications are evaluated for three months to test the feasibility of the novel diabetic foot education. Participants may benefit directly by gaining a greater understanding of diabetic foot problems, improving their daily foot care, having periodic access to podiatric diabetic foot care, and preventing serious diabetic foot complications. In addition, any knowledge that is gained on efficacious educational strategies will be beneficial to others diagnosed with diabetes and will hopefully lead to a reduction in diabetic foot complications.

## Summary of Research Completed

Forty (40) at risk diabetic subjects were enrolled, randomized to either control or intervention group, and followed for 3 months. Characteristics of subjects at baseline are summarized in Table 1.

Subjects in the control and intervention groups were similar in age, educational level, body mass index, and severity of diabetic foot risk category. There were differences in gender and duration of diabetes between the groups. However, such demographic characteristics are expected given the small sample size.

Among 20 subjects in the control group, 19 and 16 subjects completed 1-month and 3-month evaluations, respectively, while all 20 subjects in the intervention completed all three evaluations. Subjects in the control group lost an average of 2.9 pounds (1.4%) and 4.8 pounds (2.3%) at 1-month and 3-month evaluations, respectively, as compared to baseline. Similarly, subjects in the intervention group lost an average of 1.1 pounds (0.5%) and 4.1 pounds (1.8%) at 1-month and 3-month evaluations, respectively, as compared to the baseline.

A sample of foot care behavior findings is presented in Table 2 and illustrated in Figure 1. While all subjects reported improved foot care behavior (that is, daily foot inspection, see Figure 1a) at visit 2 (1-month follow up), an even greater difference was noted for foot inspection response at visit 3 (3-month follow up). *Whereas subjects who received the standard education returned to the response of the baseline, subjects in the treatment group maintained 'correct' foot care behavior at visit 3.* Similarly, subjects who received standard education maintained similar foot care behavior (that is, avoidance of barefoot walking indoors, see Figure 1b) at all visits. However, subjects who received the individualized foot education by viewing their own plantar pressure footprint showed progressively improved behavior (that is, avoidance of barefoot walking) over time – 40% at baseline, 60% at 1-month, and 75% at 3-month.

Preliminary results suggest that the proposed novel foot care patient education may yield improved foot care behavior in at-risk diabetic subjects as compared to the standard patient education method. This study is limited by small sample size, short follow up period, and reliance on self-reported behavior assessments. Additional studies are needed to evaluate the utility of using personal plantar pressure as an educational tool.

Table 1: Baseline characteristics of study participants

	Total	Control	Intervention
N (Male)	40 (25)	20 (9)	20 (16)
Age (years)	53.1	53.0	53.3
Education (years)	14.1	13.6	14.4
Duration of Diabetes	9.8	12.2	7.5
Weight (pounds)	216.9	208.6	225.3
Height (cm)	176.1	174.3	178.1
Body Mass Index	31.9	31.3	32.5
A1C	8.9	9.2	8.5
Number of Subjects in Risk Category			
1 (peripheral neuropathy)	12	5	7
2A (neuropathy+foot deformity)	21	10	11
2B (peripheral arterial disease)	4	3	1
3A (foot ulcer history)	1	1	1
3B (previous amputation)	2	1	1

Table 2: Summary of Self-Reported Foot Care Behavior Questionnaire

% of subjects who answered correctly to:	Visit 1		Visit 2		Visit 3	
	C	T	C	T	C	T
A1. Examine your feet daily?	70.0%	60.0%	78.9%	90.0%	62.5%	90.0%
A7. Walk barefoot indoors?	60.0%	40.0%	57.9%	60.0%	62.5%	75.0%
B7. Treat calluses with a blade?	85.0%	70.0%	89.5%	100.0%	87.5%	95.0%
B8. Wear sandals or slip-ons?	25.0%	50.0%	10.5%	45.0%	43.8%	50.0%

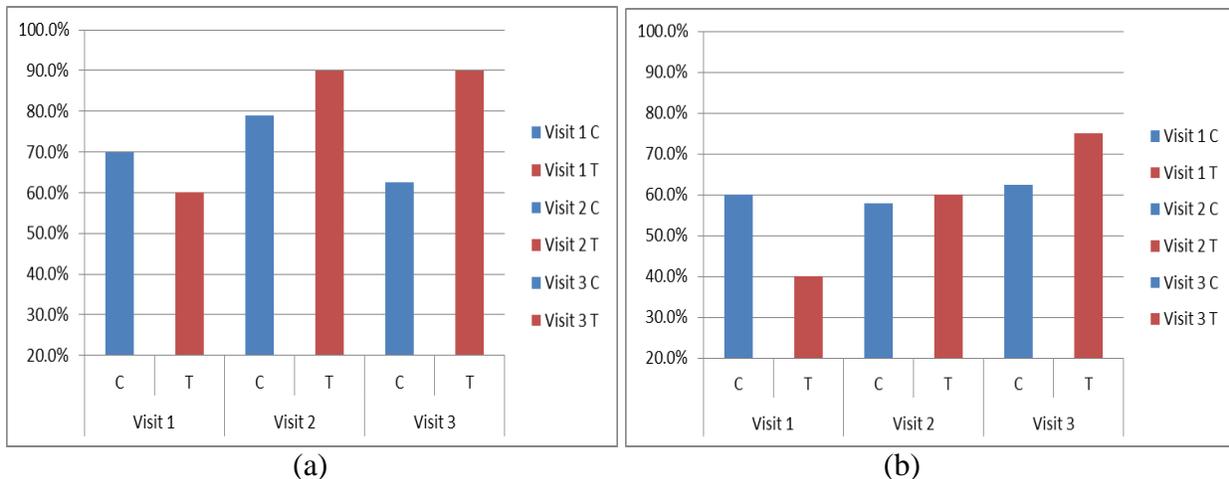


Figure 1: Percentage of subjects in the control (C) or intervention (T) group who reported that they inspected their feet daily (Figure 1a) and never walk barefoot indoors (Figure 1b).

## **Research Project 13: Project Title and Purpose**

*Tactile Imaging Sensor and Hyperspectral Data for Early Breast Cancer Detection* - The purpose of this project is to investigate the feasibility of a novel portable tactile and hyperspectral system for early breast cancer detection. A portable system would dramatically increase the probability of detecting breast cancer early, especially in remote areas. We propose to develop a tactile imaging sensor for breast cancer detection. When an object touches the sensor, the light will be diffused in varying amounts, which can be captured with a camera. Furthermore, we propose to integrate the tactile sensor image with the hyperspectral camera image to generate more accurate diagnosis of the breast cancer. Hyperspectral camera images allow health care providers to see in “different” colors—bands. The material composition of an object can be identified using a hyperspectral camera. This system will allow minimally trained health care providers to detect breast cancer.

### **Duration of Project**

7/1/2009 – 5/31/2011

### **Project Overview**

Objectives and Specific Aims. The broad objective of this research is to investigate the use of a tactile image sensor and hyperspectral images to detect breast cancer. Specific aim one is to develop a tactile imaging sensor that will image the touch sensation of the breast. This will enable the system to detect the palpable breast cancer tumors. Specific aim two is to develop a hyperspectral imaging subsystem with a novel light source to detect the calcification of the breast tissues. This will enable the detection of non-palpable breast cancer detection.

Research Design and Methods. We propose to develop a tactile imaging sensor that will quantify the touch sensation. Tactile sensation (palpation) is one of the most reliable methods of detecting breast tumors. We hypothesize that the healthy tissues will have similar elasticity throughout, and unhealthy tissues will have different elasticity. Thus, we will measure the softness/hardness of the tissues. Utilizing total internal reflection principle, the tactile imaging subsystem will quantify the breast tissues’ elasticity, which in turn can be used to determine breast tumors.

Another subsystem consists of the hyperspectral camera subsystem, lighting subsystem, and processing subsystem. Hyperspectral camera is a combination of charge-coupled-device (CCD) camera with spectral information in each pixel. We propose to use high intensity light emitting diodes and a liquid crystal tunable filter. We will identify which band will be most effective in detecting calcification. We will also test different LEDs to determine the wavelength that will have the largest penetration. The processing subsystem will identify and characterize the calcification of the breast tissues.

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## **Other Participating Researchers**

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## **Expected Research Outcomes and Benefits**

Early and reliable detection of breast cancer is an important issue. Utilizing multiple modalities will improve the reliability of breast cancer detection and decrease false alarms. A novel tactile sensor subsystem will detect tumors by palpation, and a novel hyperspectral subsystem will detect calcification of the breast tissues. We propose to investigate the feasibility of such a system. If successful, a portable system will detect cancer cells with high accuracy in almost real-time. This will allow breast cancer screening to be performed in nonhospital settings. If this preliminary project is successful and a system can be developed, then the impact will be in three areas: breast cancer early detection, detection from a remote location, and intraoperative tool.

One of the most common reasons for irregular breast cancer physical examination is procrastination. People are simply too busy to make an appointment to see a doctor for a potential problem that may or may not be there. One of the impacts of this project is to solve this problem by providing a Tactile and Hyperspectral System near the patients. For example, this system could be checked out from the local library.

Tactile and Hyperspectral system can be used in remote locations, where breast cancer specialists are not available. For example, in a rural area where there are no major hospitals nearby, a small pharmacy may have a Tactile and Hyperspectral System to detect breast cancer.

Tactile and Hyperspectral System provides almost real-time information about the breast cancer cells. So, Tactile and Hyperspectral System can be used as an intraoperative tool. During an operation surgeons can view the progress of their surgery by viewing tactile and hyperspectral images on a screen. After the surgery, the surgeon can verify that all the cancer cells have been removed by scanning for potential problem areas. This intraoperative tool will reduce wrong site surgery, excessive removal of the healthy tissues, and insufficient removal of the cancerous cells.

## **Summary of Research Completed**

Characterizing and locating embedded tumors will greatly enhance the detection and treatment of breast cancer. In this project, a novel tactile imaging sensor, that is capable of detecting and characterizing the sub-surface object, was designed, implemented, and tested. Using the salient features of the captured image, we estimated inclusion characteristics such as size, depth, and

Young's modulus. To test the performance of the proposed system, we use a realistic tissue phantom with embedded stiff inclusions. The experimental results showed that the proposed system can detect inclusions and provide the relative values of inclusion's mechanical properties such as size and elasticity. Using these relative values, we can discern malignant and benign tumors.

Simulations. The imaging principle is numerically simulated using Matlab software. Throughout the numeric simulation, we can obtain the electromagnetic wave pattern in the multi-layer optical waveguide and demonstrate the total internal reflection. We also show that if an optical waveguide is deformed by an external force, the light is scattered and seen from the surface of an optical waveguide. Figure 1(a) represents an optical waveguide before the light injection, as seen from its side. Three Polydimethylsiloxane (PDMS) layers and one glass plate layer are represented by different colors. We assume that the light is injected from the left side of the waveguide. The light injection result is shown in Figure 1(b). Once the light is injected into the waveguide, a small portion of light diffracts away because of the discontinuity of the mediums, air and the waveguide. However, due to Snell's law, we can clearly see the sinusoidal oscillation of the other light, and it continues to propagate in the waveguide. Figure 2(a) shows the deformation caused by an indenter (around 1000mm). Figure 2(b) shows the light scattering due to the contour change by the indenter.

Phantom Experimental Results. We demonstrate the capability of the tactile imaging sensor to characterize an inclusion embedded in the soft tissue. For this experiment, three tissue phantoms with embedded stiff inclusions (simulated tumor) have been manufactured. The tissue phantoms consist of size, depth, and hardness tissue phantoms. Each phantom includes three inclusions. The phantom was made of a silicone composite having Young's modulus of approximately 5 kPa. These phantoms were custom made by the CIRS Inc., Virginia. The inclusion was made using another silicone composite, the stiffness of which was higher than the surrounding tissue phantom. The experimental results of a phantom study are as follows.

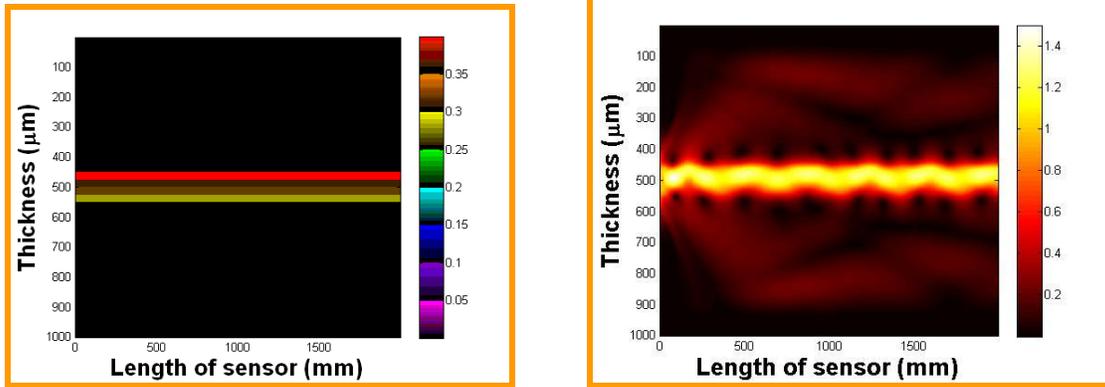
Size Phantom. The size phantom includes three hard inclusions with diameter of 2 mm, 8 mm, and 14 mm. Each inclusion was placed 5 mm below the surface of the phantom. The Young's modulus of each inclusion was 120 kPa, which is for fibrous tissue at 5% pre-compression with loading frequency of 4.0 Hz. The schematic of the size phantom is shown in Figure 3. For each inclusion case, we obtained fifteen tactile sensation images. Then the relative diameter of an inclusion has been estimated and averaged. Figure 4 represents the sample tactile sensation images of each inclusion. The relative diameter estimation results are shown in Figure 5. The plot shows that 14 mm diameter case had the highest mean diameter size of 8.77 mm, and the most variation, with a standard deviation of 1.24 mm. Conversely, 2 mm diameter case had lowest mean diameter of 1.67 mm, and the least variation, with a standard deviation of 0.48 mm. The error is larger for larger size inclusion.

Depth Phantom. The depth phantom has three inclusions with different depths of 4 mm, 8 mm, and 12 mm. The diameter of all inclusion was 7 mm and their Young's modulus was 100 kPa, which is about the invasive ductal carcinoma hardness. Consistent with the size phantom experiment case, we obtained fifteen tactile sensation images of each inclusion. Then, the relative depth of an inclusion was estimated and averaged. The depth estimation result is shown

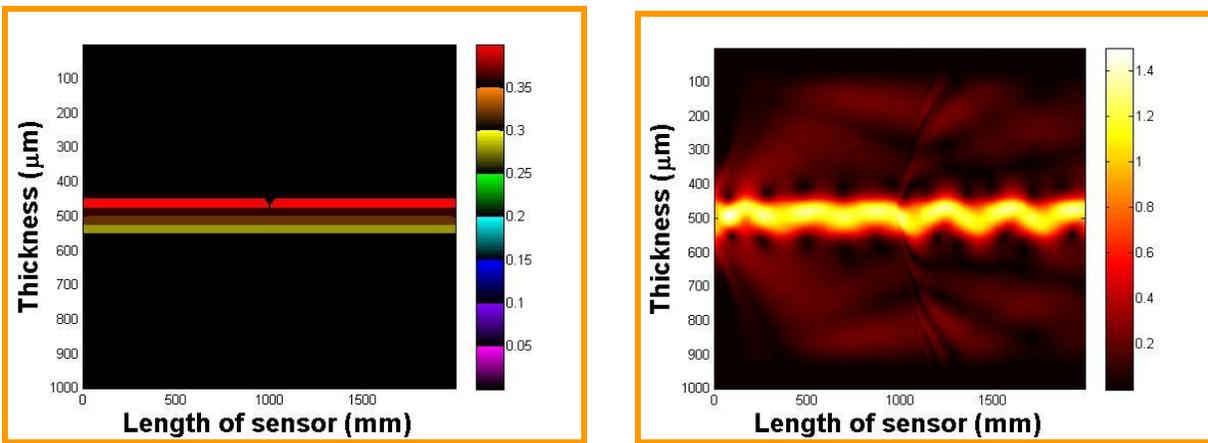
in Figure 6. The plot shows that the 4 mm depth case had the highest mean of 0.59 mm, and the least standard deviation, 0.03 mm, while 12 mm depth case had the lowest mean depth of 0.34 mm with the most variation, 0.08 mm. This shows large error for the depth estimation.

Hardness Phantom. The hardness phantom has three inclusions with different Young's modulus, 40 kPa, 70 kPa, and 100 kPa. The tissue elastic modulus was chosen to represent normal glandular tissue (40 kPa) and invasive ductal carcinoma (100 kPa) at 5% precompression with loading frequency of 4.0 Hz. Then we added one more inclusion with the in between elastic modulus of 70 kPa. The diameter of inclusions was 10 mm, and they were placed 5 mm below the surface of the phantom. Using the multiple compression ratios, we obtained fifteen tactile images of each inclusion. Then, the relative Young's modulus of an inclusion was estimated and averaged. The Young's modulus estimation result is shown in Figure 7. The plot shows that the 40 kPa Young's modulus case had the lowest mean of 82.59 kPa, and the smallest standard deviation, indicating that the observations were close to the mean. On the contrary, 100 kPa Young's modulus case had the more widely spread out Young's modulus, 252.25 kPa mean value with 35.87 standard deviation, as can be seen in its error bar chart. 70 kPa Young's modulus case had an average of 149.93 kPa, and standard deviation of 29.83 kPa. The error was larger for the stiffer inclusion. Nevertheless, we could distinguish relative hardness of the inclusions from the tactile images.

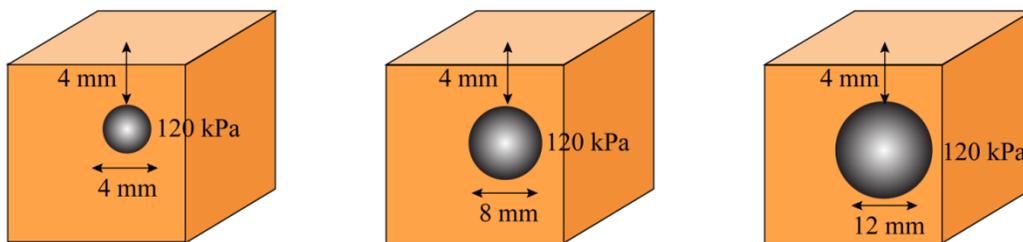
Conclusions. A novel tactile imaging sensor capable of characterizing the inclusion in the tissue was designed and experimentally evaluated. To emulate the human finger layer, a multilayer optical waveguide was fabricated as the main sensing probe. The total internal reflection principle (TIR) was used to obtain the high resolution tactile image. We analyzed the feasibility of the TIR using wave optics, which clearly showed the tactile image when the sensing probe was compressed. Then we utilized geometric optics to analyze the light illumination angles. The performance of the tactile imaging sensor was experimentally verified using tissue phantoms with embedded hard inclusions. The experimental results showed that the relative size and hardness estimation errors were smaller than the relative depth estimation errors. If the inclusion's size was smaller, the estimation error was also smaller. Also the shallower depth inclusion case has smaller depth estimation error than the inclusions embedded deeper. Furthermore, if the Young's modulus of an inclusion was smaller, the estimation error was smaller than the higher Young's modulus case. We conclude, however, that we could distinguish between soft, medium, and hard inclusions, which will allow us to distinguish malignant and benign tumors. This work is the initial step towards achieving a tactile imaging sensor for embedded breast tumor detection and characterization.



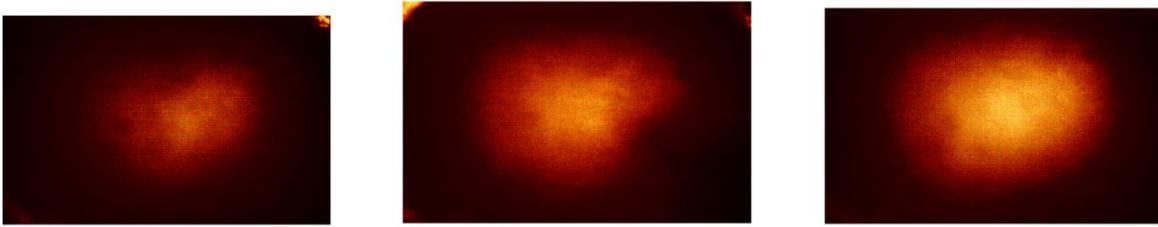
**Figure 1:** (a) The optical waveguide, as seen from its side. (b) The light oscillation in the optical waveguide due to the Snell's law.



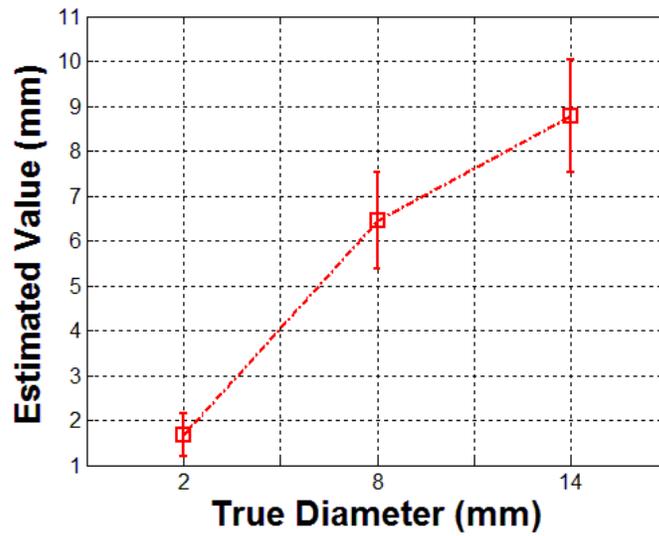
**Figure 2:** (a) The optical waveguide, as seen from its side. (b) The light scattering in the optical waveguide due to the waveguide deformation.



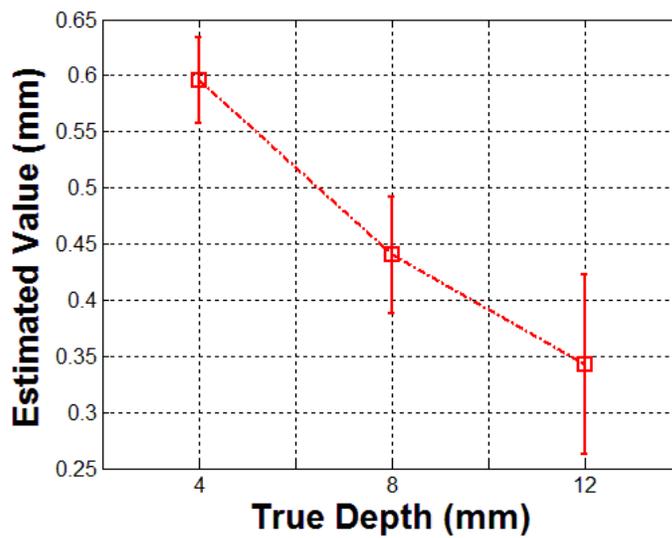
**Figure 3:** The schematic of the size phantom



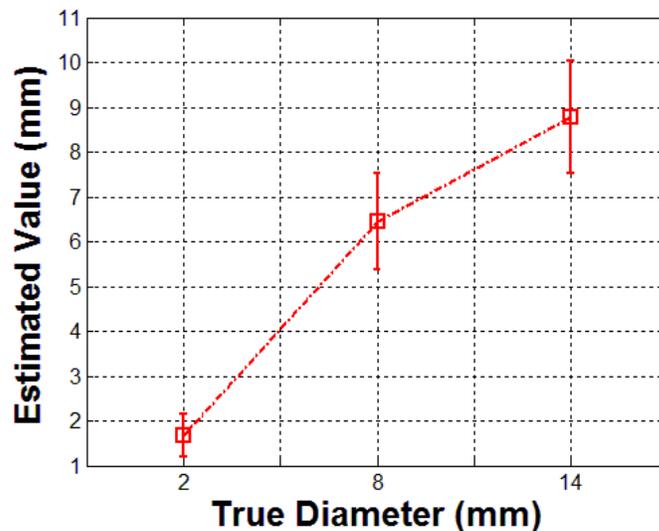
**Figure 4:** The tactile sensation image of three inclusions embedded in the size phantom. (a) 2 mm diameter inclusion, (b) 8 mm diameter inclusion, (c) 14 mm diameter inclusion.



**Figure 5:** Error bar chart of estimated relative diameter of each inclusion



**Figure 6:** Error bar chart of estimated relative depth of each inclusion



**Figure 7:** Error bar chart of estimated relative hardness of each inclusion

### **Research Project 14: Project Title and Purpose**

*Small Animal Model of Medulloblastoma for Translational MR Neuroimaging at 3 Tesla* - This project will support the acquisition of a customized mouse brain coil which will allow us to adapt our brand new state-of-the-art human 3 Tesla MRI scanner for small animal neuroimaging. We will utilize the mouse brain coil for imaging of our transgenic mouse brain tumor model of medulloblastoma. The ability to perform high resolution neuroimaging and longitudinal studies of our mouse model of medulloblastoma on a human 3 Tesla magnet will give us the ability to directly translate our findings to non-invasive diagnostic, molecular, and therapeutic neuroimaging in the clinical setting.

### **Duration of Project**

7/1/2009 – 7/31/2010

### **Project Overview**

Medulloblastomas and primitive neuroectodermal tumors (PNETs) are highly cellular malignant primary brain tumors representing the most common solid tumors arising in children. The major limitations of the current state of the art clinical Magnetic Resonance Imaging and Spectroscopy to diagnose this disease include the inability to clearly define the parenchymal interface between normal and tumor regions; to differentiate tumoral edema from infiltration; to diagnose small primary or metastatic lesions; and to differentiate medulloblastomas or PNETs from other primary central nervous system (CNS) neoplasm.

Currently, to our knowledge, realistic animal models to study these medulloblastoma tumors are lacking. It is therefore necessary to develop and study the imaging characteristics of these CNS tumors using spontaneous brain tumor animal models that mimic human disease, as well as

develop image processing tools to quantitatively analyze these tumors to improve diagnostic accuracy. Spontaneous tumor models in mice are generally thought to be more realistic models of human cancer than xenografts, and the lesions these animals develop present special challenges for imaging.

We will use high field Magnetic Resonance (MR) imaging to develop optimal characteristics to image these spontaneous CNS tumors. Quantitative methods for tissue segmentation based on fuzzy connectedness and medical image data mining techniques will be developed and tested to analyze these tumors. The imaging and image post processing methods will be validated using histological staining and immunohistochemistry for the transgene T-antigen, the viral oncoprotein expressed in these tumors.

Such a unique spontaneous brain tumor model will be useful for validation of the novel quantification methods proposed in this project. The long-term goal of this project will be to use these tools for validation and quantitation of tumor-specific contrast agents and therapeutics for medulloblastoma in children. These methods of imaging and quantification may be useful for in vivo imaging, diagnostics, and monitoring of response to tumor-specific therapy in other human CNS cancers. The magnetic resonance imaging (MRI) coil and accessories acquired through this project will allow us to use a human MRI to scan our small animal model of human medulloblastoma brain tumors at high power. In vivo imaging of this model will allow us to test and validate non-invasive novel diagnostic and therapeutic strategies that can be directly used in the clinic.

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### **Expected Research Outcomes and Benefits**

In addition to its role in chronic central nervous system (CNS) demyelination, JC virus (JCV) induces medulloblastomas in several experimental animal models. Morphologically, the medulloblastomas induced in transgenic mice are indistinguishable from human medulloblastomas. Of particular interest is recent evidence demonstrating the association of JCV with human medulloblastoma and the detection of the viral early protein, T-antigen, in a significant number of these childhood tumors. It is therefore suggested that JCV may serve as a

tool for the development of an animal model to study the molecular mechanisms of tumor formation and to further investigate the molecular mechanisms by which JCV may induce cellular transformation. This information is critical for devising and implementing safe and effective therapeutic protocols for brain tumor treatment. In this project, we will utilize a state-of-the-art 3 Tesla human MRI for translational research to develop and test novel imaging techniques for accurate identification, delineation, and quantification of CNS tumors in a unique spontaneous transgenic mouse medulloblastoma model. The goal of this project is to develop sensitive and specific procedures for in vivo imaging of our transgenic mouse model of human medulloblastoma. The availability of high resolution MR images will assist us with disease diagnosis, detection of recurrent disease prior to clinical signs or symptoms, and evaluating the efficacy of therapeutic modalities during treatment.

### **Summary of Research Completed**

During the period of 07/01/10 to 07/31/10, activities related to equipment training were completed. User training on the instrument was performed on site by the manufacturer's field service specialists. The immediate benefit from validation of the improved image processing techniques is in the ability to perform longitudinal studies on our spontaneous brain tumor mouse model. The long-term goal of this project will be to use these tools for validation and quantitation of tumor-specific contrast agents and therapeutics for medulloblastoma in children. These methods of imaging and quantification may be useful for in vivo imaging, diagnostics, and monitoring of response to tumor-specific therapy in other human CNS disorders.

### **Research Project 15: Project Title and Purpose**

*Developing Radiological Risk Communication for Low-literacy Populations* - Radiological exposure from a terror event has been identified by the Centers for Disease Control and Prevention (CDC) and the Office of Homeland Security as a potential threat worthy of significant national preparedness. Little research has been done, however, to develop risk communication strategies for those with limited literacy. The purpose of this project is to assess whether new approaches to designing risk communication are valid methods to develop emergency preparedness materials for people with low literacy. The project will pilot the use of health literacy measures as well as physiological measure tools (i.e., skin response, eye tracking and respiration) with General Internal Medicine Clinic patients at Temple University Hospital. These pilots will enable us to effectively use these evaluative tools with individuals with low levels of literacy.

### **Anticipated Duration of Project**

9/15/2009 – 12/31/2011

### **Project Overview**

The overall goal of these two pilot studies is to provide data to indicate the feasibility of proposed research in the re-submission of a National Institutes of Health (NIH) grant. PILOT ONE will show the feasibility in securing subjects with low literacy through Temple University's

General Internal Medicine clinic and which literacy tool is easiest to administer. PILOT TWO will show the feasibility of using the psycho-physiological equipment with subjects who are of low literacy. This will allow us to understand the special needs of this population when using these research measures. Specific methodology will be used.

1. **PILOT ONE:** Conduct a pilot with 30 patients of the General Internal Medicine clinic at Temple University Hospital using both the Rapid Estimate of Adult Literacy in Medicine (REALM-R) (N=15) and the Short Test of Functional Literacy in Adults (S-TOFHLA) (N=15) to assess general literacy levels of patients as well as assess the feasibility of using both measures in the clinical setting. Patients who are waiting to see the doctor will be asked if they would be willing to participate in the study. Once consented, the subject will be asked to either read words (REALM-R) or circle the correct answers (S-TOFHLA) in the tool. When the subject is done, the participant will be given a \$10 gift card and the examiner will score the test. This pilot test will allow the investigator to provide data illustrating that she will be able to find sufficient numbers of people with low literacy to take part in the research as well as information about which measure is easiest to use in the setting.

2. **PILOT TWO:** Conduct a pilot (N=10) to assess the feasibility of using psycho-physiological measures with low-literate subjects. Because the equipment tracks eye movements it will be important to be able to demonstrate that this equipment is not too difficult for low-literate subjects to use. Subjects will have sensors attached for skin resistance monitoring and heart-rate monitoring, and a headset for eye-tracking/pupil-dilation measures. Subjects will then be shown a colorectal cancer (CRC) screening decision aid while data from the sensors and eye-tracking measures are being recorded. Sensors and the eye-tracking headset will then be removed and subjects will complete a survey on their experience using the psycho-physiological measures and be given a \$30 gift card. This pilot will utilize an existing low-literacy level tutorial on colorectal cancer that has been developed for another project as a mechanism to evaluate the acceptability of the equipment as well as the validity of results in a low-literate population.

### **Principal Investigator**

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### **Other Participating Researchers**

None

### **Expected Research Outcomes and Benefits**

Individual subjects will benefit in three ways: (1) the knowledge that they have contributed to the

development of more effective health decision aids for those with low literacy; (2) knowing they have helped to develop more effective procedures for testing health decision aids and the development of more effective ways to communicate with the public about health issues; and (3) the knowledge and experience gained from having participated in a scientific study will contribute to their personal understanding of how scientific studies in general are conducted and, specifically, how eye-tracking and psycho-physiological assessments are done.

Society will benefit in two ways: (1) the development of more effective, literacy-appropriate health decision materials will make it easier for the public to learn about health issues and, in the case of the larger NIH proposal, prepare for radiological terror events; and (2) the development of more sophisticated assessment procedures, such as the combined perceptual mapping, eye-tracking, and psycho-physiological measures, which will lead to more valid and reliable research techniques. This information is critical for developing educational materials and communications that assist the public in preparing for or responding to public health situations.

## **Summary of Research Completed**

### Overview

This fiscal year of the project was directed at completing PILOT STUDY TWO of the study, which aims to assess the feasibility of using psycho-physiological measures with low-literate subjects. We encountered a number of technical problems with getting our psycho-physiological equipment ready for use, as well as adapting the existing Colorectal Cancer Screening tutorial we developed previously for use with the equipment, during this time period. These issues have now been resolved and we are currently beginning data collection with subjects.

### Specific Accomplishments

#### *Adaptation of Existing Colorectal Cancer Screening Tutorial*

The Colorectal Cancer (CRC) screening tutorial that will be used for PILOT STUDY TWO was developed for an NIH funded R21. This tutorial was used with patients at a primary care clinic using a touch screen computer monitor and was accessed using a web link to our research partner, a patient education company that develops web-based health tutorials. Thus, the tutorial was “housed” on their website and accessed via a high speed internet connection at the clinic. This was done because of the size of the tutorial, which utilizes graphics, voice-over and video “testimonials.”

When adapting this tutorial to use with our psycho-physiological measures, however, we realized that we would be unable to utilize the web-based link because the eye-tracking and biofeedback equipment require a stable picture on screen that can be coordinated with an “advance” key to distinguish where eye tracking on one image stops and eye tracking of another image begins on a separate page. To analyze eye tracking you also have to have separate .bmp files to superimpose eye tracking data to visually understand where participants were looking. Thus, we had to re-do the tutorial to adapt to these limitations. This required creating a stable powerpoint presentation with text and graphics. We then had to embed audio voice-overs for each slide, as well as video

“testimonials” into the presentation, so that the end result was similar to the original. We worked with our research partner, Patient Education Institute, who provided us with audio and video files. Because there was no monies budgeted to pay them, however, we had to do all the programming ourselves. This required finding a student with the correct computer programming background to do this and then working with him on his timeframe to get the tutorial in the right format. This was completed in the fall semester, 9/2010 through 12/2010. The tutorial is now functional and able to be used for data collection using the psycho-physiological measures.

### *Updating Psycho-Physiological Measure Equipment and Training of Staff*

During the spring semester (1/2011 to current), we worked on updating our current psycho-physiological equipment and moving it to a secure and private location. The equipment was set up in a large room, the Risk Communication Laboratory, which has desks for graduate research assistants and other meeting space. This room originally only had one or two graduate students working in it at any time but with increased grant and research activity, by the spring there were over five undergraduate and graduate students working in the lab and we felt we could no longer provide privacy to those participating in the research. We secured a separate, smaller room to set up the psycho-physiological equipment that would provide privacy and silence during the testing. We then had to move all the equipment into this space and ensure its functionality. During this process we uncovered a number of problems with the equipment and had to work with the company who makes the equipment to update software. In addition, because the tutorial has over 25 separate slides, the eye tracking and biofeedback equipment software had to be coordinated so that when we gathered data we could distinguish when participants started and stopped looking at one slide and went to the next. This required us to be able to start, pause and stop the data collection, as well as start the eye tracking/pupilometer and biofeedback data collection at the same time. The current psycho-physiological software does not do this so we had to create a set of computer programming “macros” that would accomplish this. We again had to enlist the help of a computer programming student to help us since there was no budget to pay a professional or have the psycho-physiological equipment company provide consulting services. We were able to identify a student who was able to develop two macros that now enable us to start, pause and stop the eye tracking, pupil dilation, skin response and heart rate data monitoring software together. We are also able to pause between slides so that in data analysis we will be able to differentiate how they looked at and responded to each slide individually. During this time we also developed a script and study protocol to ensure smooth testing of participants.

As a result, all equipment is now functional and we have spent the last month (June 2011) training staff on how to use the equipment. Staff members are now competent on the equipment and we have begun to recruit low literacy participants to begin data collection. We anticipate this data collection will be done by the end of August and data analysis will then occur.

### Publications or Presentations Resulting from Research

An abstract outlining PILOT STUDY ONE results was submitted to and accepted by the American Public Health Association for presentation at their annual meeting in November, 2010. The specific title and authors for that presentation were:

“Use of REALM-R vs. S-TOFHLA in an urban African American clinic population to assess health literacy: Practical Implications.” SB Bass, CN Wolak, GM Rovito, TF Gordon and L Ward. American Public Health Association Annual Conference. November 2010. Denver, CO.

### **Research Project 16: Project Title and Purpose**

*Using a Telemedicine System to Promote Patient Care Among Underserved Individuals -* Hypertension (high blood pressure) affects more than 65 million people in the US with African Americans disproportionately affected. Untreated hypertension is associated with an increased risk for heart attacks, sudden death, stroke, and kidney failure. Home-measured blood pressure has become an important method of managing hypertension. This study will test the effectiveness of an Internet-based telemedicine system coupled with home-measured blood pressure to strengthen the patient-provider relationship and to educate and empower individuals with hypertension to take a more active role in their own health care.

### **Anticipated Duration of Project**

9/15/2009 – 12/31/2012

### **Project Overview**

Despite the importance of controlling hypertension and available therapy, inadequate blood pressure control remains all too common. For hypertension care, strategies are needed that support a Patient Centered Care (PCC) approach. We believe that telemedicine can provide a PCC model for hypertension care. We have established a telemedicine system for disease management, based on a personal health record and have used this system in diverse disease states (heart failure, cardiovascular disease (CVD) risk reduction, hypertension, diabetes). This research project will support PCC by increasing access, and automating care reminders and feedback for both patients and health care providers. Urban underserved patients with uncontrolled hypertension (BP>140/90 mmHg) will be randomized to either usual care or telemedicine. Blood pressure, weight, blood glucose and lipids, and physical activity will be measured at baseline and at 6 months. The primary endpoint is the proportion of subjects who achieve goal blood pressure.

The specific aim of this Department of Health (DOH) funding is to recruit 82 additional subjects for this study. Our expanded recruiting efforts have required unanticipated personnel commitments that exceed budgeted personnel time. Additional funds of \$30,000 will allow recruiting efforts to complete the study on time.

Successful completion of the project will lead to new Agency for Healthcare Research and Quality (AHRQ) funding, approximately \$1,200,000 of direct funding, and 400,000 of indirect funding. Funding to provide wider dissemination is dependent on showing a positive effect of our telemedicine intervention for hypertension.

## **Principal Investigator**

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## **Other Participating Researchers**

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## **Expected Research Outcomes and Benefits**

DOH funding will support recruiting 82 subjects to extend our findings to a broader population, which includes type 2 diabetes. Effective chronic disease management requires: (1) patient participation in decision making; (2) active patient self-monitoring and self-management; (3) effective patient-provider communication; and (4) adherence to established guidelines and quality indicators. The telemedicine system addresses all these issues and supports patient's engagement in their own care. The patient's knowledge will be increased by the motivational/educational messages (covering all quality measures – advice on sodium, alcohol, exercise, weight loss, DASH diet), by the educational modules on the site, and by links to other websites. The cardiovascular knowledge questionnaire will be utilized to test this hypothesis.

The patient self-management and engagement in their self-care (Patient Centered Care) is supported by the system. The frequency of self-monitoring can be measured. The patient is sent automated reminders to send data and receives feedback and reinforcement when data is sent. Trend lines in blood pressure and physical activity are provided to both patient and physician. The net result will be sooner and more frequent changes in medication; better control of hypertension; and timely office visits.

Thus for the underserved populations, the telemedicine system addresses the suboptimal control of blood pressure, and improves care in a cost effective manner. The telemedicine system can be rapidly updated as new national guidelines are developed, and offers the possibility of rapid dissemination throughout the U.S.

## **Summary of Research Completed**

This study involved recruiting up to 252 patients for evaluation of a home measured blood pressure method of controlling hypertension. The main study is funded by the Agency for Health Care Research and Quality (AHRQ). Formula funds were requested for addition of a second recruiting site (Christiana Medical Center, Delaware) to facilitate recruiting of the total subjects needed for statistical analysis. Recruiting of new subjects ended in September 2010, and the last patient recruited completed the 6 month follow up in March of 2011. No further

recruiting is anticipated, and the remaining study time is committed to completing database analysis and verification, analysis of results and completing of abstracts and papers to report the outcome data.

Study Design and Methods: The primary end-point is the proportion of subjects who achieve goal blood pressure; secondary endpoint is the reduction in blood pressure comparing control to telemedicine groups.

Patient Population: We recruited primarily minority individuals with hypertension from two medical centers. The study population consisted of 81% African-American, 15% Caucasian. The remaining 4% consisted of Hispanic, Asian and Native American subjects.

Patient Recruiting: Subjects with known hypertension were recruited from the primary care practices at Temple University Medical Center, and Christiana Medical Center, local churches and community centers, and also from public service announcements, notices in local newspapers, posters in the Medical Center, and referrals from primary care physicians. Patients were also recruited from Christiana Medical Center to expand our study population.

Patient Selection: Males and females at least 18 years of age were selected based on presence of hypertension (systolic blood pressure >140 mmHg or diastolic blood pressure > 90 mmHg). All patients were required to be literate and have telephone access.

Exclusion Criteria: We did not enter patients with newly diagnosed hypertension or with class 3 or 4 heart failure. We also excluded patients with angina, patients with significant cognitive deficits from stroke or dementia, patients with end stage renal disease on dialysis, patients living in nursing homes or boarding homes, and patients who were unable to use a scale, digital sphygmomanometer or pedometer at home. Patients were excluded who were pregnant, or who were unable, after training, to use the Internet. Patients who were unable to understand the study protocol or not competent to sign the informed consent document were also excluded.

Computer Training: All patients were trained on use of a computer and the Internet and on how to access the Internet. Each patient was instructed on the details of the Telemedicine system on a demonstration terminal in the research center. The patients were provided with a login name and password to gain access to the secure telemedicine site and made data entries with coaching by the research team. Training on use of the interactive voice response (IVR) system (i.e., how to respond to prompts) was incorporated into the patient training program for the Internet.

Sample Size: Our primary endpoint was a doubling (from 25% in the control group to 50% in the Telemedicine group) in the number of subjects at goal blood pressure. A sample size of 70 patients per group provided 90% power and one tail type I error equal to 4% to detect a 25% difference in the primary endpoint. Because we anticipated a number of diabetics in the study for a subset analysis, and to account for a 20% dropout rate, we increased sample size to 250 subjects to allow independent analysis of non-diabetic and diabetic subjects.

Intervention(s): After reviewing eligibility for the study, informed consent to participate was obtained and subjects were randomized to usual care or control using a random number table. All

subjects in the Telemedicine arm, were given a sphygmomanometer, a scale (if needed) and a pedometer to count steps per day and instructions regarding proper use of these devices. All participants received a logbook and were encouraged to record their measurements at least once a week (i.e., blood pressure, weight, average number of steps) over the course of the study. Medical history, physical examination, electrocardiogram, blood studies, urinalysis, height, weight, blood pressure measurement, and waist circumference were obtained on the initial assessment. A fasting blood sample was obtained from each subject to determine fasting serum cholesterol, LDL, HDL, triglycerides, and glucose levels. The subjects completed a cardiovascular knowledge, medication self-efficacy questionnaire, and the Consumer Assessment of Healthcare Providers and Systems (CAHPS®).

Outcomes: We expected a greater proportion of the Telemedicine group were to be at goal blood pressure and experienced a greater reduction in blood pressure than the control group. Effective chronic disease management required: (1) patient participation in decision making; (2) active patient self-monitoring and self management; (3) effective patient-provider communication; and (4) adherence to established guidelines and quality indicators. The Telemedicine system addressed all these issues and supported patient's engagement in their own care. The patient's knowledge was increased by the motivational/educational messages (covering all quality measures - advice on sodium, alcohol, exercise, weight loss, DASH diet), by the educational modules on the site, and by links to other websites. The cardiovascular knowledge questionnaire was utilized to test this hypothesis. The management plan to reduce blood pressure, based on medical guidelines, together with quality measures was built into the system. The plan and quality measures were sent to both the patient and the health care provider. This medication plan showed the suggested drugs from the medical guidelines. It showed an exact match between current drugs and guideline suggested drugs; this placed more responsibility on the patient or might suggest an increase in dose of drug was needed. The net result was shared decision-making in both establishing and modifying the treatment plan. We confirmed these ideas by the CAHPS Clinical and Group survey and by our office visit questionnaire. The frequency of self-monitoring was measured. The patient was sent automated reminders to send data and received feedback and reinforcement when data was sent. Trend lines in blood pressure and physical activity were provided to both patient and physician. The net result was sooner and more frequent changes in medication; better control of hypertension; and timely office visits. Periodic examination of the research database confirmed telemedicine usage and these consistent changes. We saw in the Telemedicine group a better understanding of cardiovascular disease, increased self-efficacy, and an improved patient-provider relationship. Finally, the patients who were more involved in their care – the patients who self-monitor on a regular schedule – had the larger decreases in blood pressure. Thus for the underserved populations, the Telemedicine system addressed the suboptimal control of blood pressure, and improved care in a cost effective manner.

Outcome measures: The primary endpoint for this study was a doubling in % of patients who had reached goal blood pressure from 25% in the control group, to 50% in the telemedicine group at 6 months. Secondary endpoints included comparisons by treatment group for: (1) the difference in number of medications prescribed according to current guidelines; (2) the difference in percent of patients participating in self-monitoring of blood pressure as defined by taking at least bi-monthly measurements over the six-month study period; (3) the difference in mean score on

the Cardiovascular Knowledge Questionnaire; (4) the difference in mean score on the Medication Adherence Self-Efficacy Questionnaire; and (5) the difference in scores on the CAHPS Clinician and Group Survey-Adult Primary Care Questionnaire.

Preliminary Results: Baseline demographics for 140 non-diabetic patients who completed the 6 month protocol included: age -  $57 \pm 11$  years, female - 70%, African American - 81%, White - 15%, smoker - 18%, hyperlipidemia - 42%, Internet at-home - 54%. Income was at or below the poverty level for a family of 4 in 46% of subjects. At 6-months, the Telemedicine group had a greater reduction of systolic BP (figure 1,  $P = 0.037$ ); 59% of Telemedicine and 52% of Control subjects reached systolic BP  $< 140$  mmHg ( $P = 0.26$ ). Of interest is the observation that diabetics had a significant reduction in blood pressure in both control and telemedicine subjects (figure 2) but the two groups had similar reductions ( $p = \text{NS}$ ). There were no effects of sex, race, age or education on attainment of goal BP. Clinical trial No. NCT00644267

Figure 1. Reduction in systolic blood pressure comparing controls to telemedicine in non-diabetics.

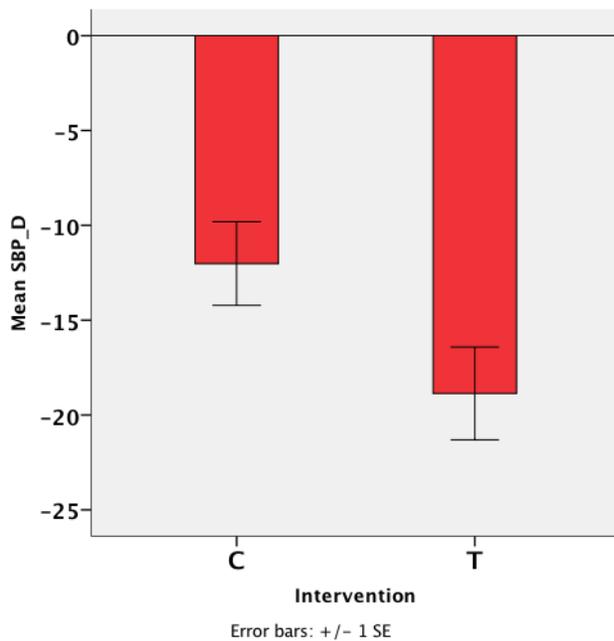
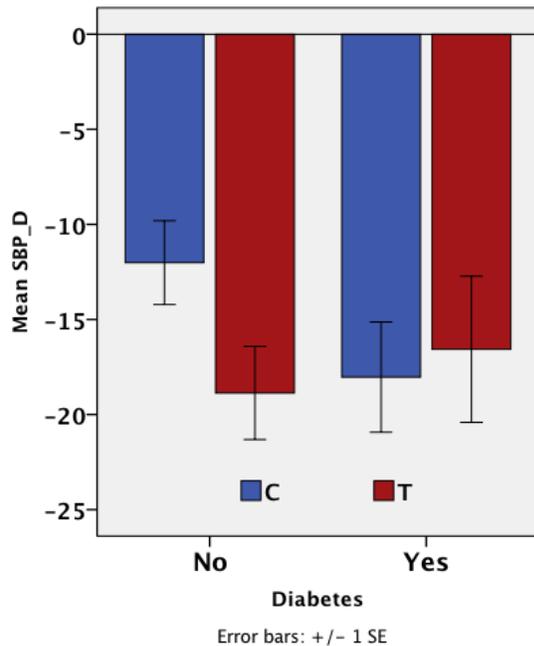


Figure 2. Comparison of systolic blood pressure reduction in non-diabetics and diabetic subgroups. Diabetics did not show a difference between control (C) and telemedicine (T) although both groups had significant reductions in blood pressure after 6 months.



### **Research Project 17: Project Title and Purpose**

*Control of Calcium Entry Mechanisms in Smooth Muscle* - An understanding of the molecular mechanisms of store-operated channels and the coupling interactions between Stim sensors and Orai channels has important implications in vascular SMC function and the development of therapies to treat contractile and growth diseases, including hypertension and atherosclerosis. The purpose of this project is to understand and clarify the basic pharmacology of the activation of these channels which may provide crucial new methods to control  $Ca^{2+}$  in vascular smooth muscle cells.

### **Duration of Project**

9/15/2009 – 5/31/2010

### **Summary of Research Completed**

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

## **Research Project 18: Project Title and Purpose**

*Assays to Identify Inhibitors of Cellular and HIV Transcription in Human Cells* - To develop assays to monitor inhibition of RNA polymerase II (RNAPII) activity and/or phosphorylation or other markers of transcriptional activity in human cells. Identification of compounds with the ability to inhibit particular events during a transcription cycle by RNAPII will generate tools to study the control of gene expression *in vivo* and *in vitro*. Further characterization of the selectivity of these compounds as C-terminal domain (CTD) kinase inhibitors *in vitro* will allow identification of their direct targets. Compounds with enhanced selectivity towards particular cyclin/CDK complexes, or with defined inhibitory potency towards a subset of CDKs may have enormous potential in the clinic for the therapeutic treatment of HIV, cardiac hypertrophy or even certain cancers.

### **Duration of Project**

9/15/2009 – 12/31/2010

### **Project Overview**

HIV-1 transcription is an essential step in the viral life replication cycle and is dependent on viral and host transcription factors. Selective inhibition of HIV-1 transcription, and hence replication, should be feasible if a small compound specifically targets a viral protein essential for productive transcription (i.e., Tat), a required host cofactor non-essential for cellular transcription, or if it interferes with the interactions among viral and cellular cofactors without altering cellular functions. Potential cellular indirect targets include RNA polymerase II (RNAPII) C-terminal domain (CTD) kinases, such as CDK9 or CDK2. However, developing pharmacologic inhibitors based on inhibition of kinase activity *in vitro* has disadvantages, as the compounds might exhibit poor selectivity, toxicity or fail to enter the cell. More robust screenings can be designed by measuring inhibition of HIV transcription or host cofactors in living cells. This project addresses strategies for developing assays for primary and secondary screening of libraries of small pharmacological compounds for selective inhibitors of site-specific kinases targeting the CTD of RNAPII, which might help in the identification and/or characterization of HIV-1 inhibitors. We will use fluorescence-based western blot analysis and/or “in-cell western assays” to measure differential inhibition of Ser-2/Ser-5 phosphorylation on the CTD of endogenous RNAPII. HeLa or other suitable human cell lines will be grown in 3 cm dishes or in 96-well plates and treated with compounds for predetermined periods of time. Subsequently, cells will be either (1) harvested for multiplex fluorescence-based western blot analysis or (2) fixed and permeabilized. Membranes or fixed cells will be incubated with antibodies specific for phosphorylated Ser-2 and Ser-5 followed by fluorescent-coupled secondary antibodies. Fluorescence will be measured and quantitated using a fluorescent imaging system to be acquired for this project. Assays will be developed using compounds known to inhibit CDKs, RNAPII CTD phosphorylation and HIV-1 transcription.

## **Principal Investigator**

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## **Other Participating Researchers**

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## **Expected Research Outcomes and Benefits**

Recent advances in technology have resulted in development of instrumentation that allows acquiring more accurate and more quantitative protein expression and modification data than previously possible with hitherto antiquated detection methods. Through the acquisition of a state-of-the-art imaging equipment scanner, we intend to enhance our research capabilities through increasing the quality and the output of the results gained from current methodologies. Multiplex fluorescence detection will allow simultaneous monitoring of protein expression and phosphorylation data, or phosphorylation of two independent events that can be used as markers of cellular processes, in this case changes in cellular transcription. We plan to develop assay(s) that should be amenable to developing primary and/or secondary screenings of libraries of small chemical compounds for inhibitors of particular processes in cellular transcription that are utilized by the HIV-1 virus. The same assays could be easily adaptable to perform other types of high throughput screening, provided that suitable markers are validated for the identification of compounds with therapeutic and or research applications.

## **Summary of Research Completed**

As previously reported in last year's report, ECL-based western blot approaches were set up quickly and produced data of high quality in a routine basis. In our hands, imaging with the FluorChem Q imager is superior to conventional western blot analysis using X-ray film and more cost effective. This is because the data is linear over more orders of magnitude, because its easiness and speed of quantification, and the rapid generation of publication quality (Jayadeva et al., 2010). We have now completely phased out the use of X-ray film, with significant savings to our lab. Also the data is digital, obviating the time consuming film scanning and the selection of best exposure times to ensure linearity, which sometimes is not even possible with a single set of exposures, requiring repetition of an additional western blot. In addition, elimination of film has also resulted in a decrease in the use of the X-ray film developer and toxic chemicals. Given the results obtained in our laboratory, we expect most other labs at the Fels Institute will take advantage of its CORE availability and like us phase out X-ray film based western blots.

On the other hand, the development of fluorescent based-approaches required further experimentation. This experimentation was carried out with my own grant funds from other

sources. During this reporting period, the set up of the FluorChem Q imager instrument for fluorescent detection and quantitation progressed with only part-time dedication to the project, and remained active to the end of 2010.

The ultimate goal of the project selected for the set up of fluorescent based-approaches was to develop strategies for development of assays for screening of libraries to identify selective inhibitors of RNAPII phospho-specific kinases by examining Ser-2/Ser-5 phosphorylation levels on the CTD of endogenous RNAPII. If CDK9 is truly selective for Ser-2 phosphorylation, identification of small compounds that selectively block Ser-2 phosphorylation in cells could lead to identification of inhibitors of CDK9 that readily enter the cells. Identification of non-toxic CDK9 inhibitors may have enormous potential in the clinic for the therapeutic treatment of HIV, cardiac hypertrophy or even certain cancers (Garriga and Graña, 2004; Marshall and Graña, 2006). With this aim in mind, we developed fluorescent-based western blot analysis to simultaneously measure RNAPII expression and Ser phosphorylation upon treatment of cells with a compound known to inhibit CDK9. Also, the sensitivity, reliability, and linearity of the fluorescent-based analysis were compared to western blot analysis using chemiluminescence detected by either direct imaging or traditional X-ray film.

Human cervical carcinoma HeLa cells and BJ fibroblasts immortalized with human telomerase (BJT fibroblasts) were utilized for these studies. Whole cell protein lysates from HeLa and BJT cells untreated or treated with a pharmacological inhibitor of CDK9, Flavopiridol (FVP) were prepared as described previously (Garriga et al., 2010; Sotillo et al., 2009). Two separate representative experiments are described below using Fluorescence (FL) and Chemiluminescence (ECL) western blot techniques. 10-12 µg of cell lysate were loaded per lane and proteins were resolved via SDS-polyacrylamide gel electrophoresis (PAGE, 8% minigels) The separated proteins were then transferred to a low-fluorescence PVDF membrane (Millipore) in 10 mM CAPS (pH 11.0) containing 10% methanol for 40 minutes. The choice of this membrane is critical to ensure the lowest possible background for fluorescent-based western blot analysis. Membranes were incubated overnight with primary antibodies specific for phosphorylated Ser-2 and Ser-5 (Table 1). Subsequently, the membranes were incubated with either (1) fluorescent-coupled secondary antibodies (Kodak-X-sight) or (2) horseradish peroxidase (HRP)-coupled anti-mouse/anti-rabbit secondary antibodies (ECL), for 1 hour (See Table 1). Fluorescent-coupled secondary antibodies from Kodak were selected upon comparing antibodies from various vendors because they appeared to generate less background signal.

For fluorescent-based western blot analysis, the membranes were incubated with a mixture of Kodak X-sight polyclonal and monoclonal secondary fluorescent-coupled antibodies depending on the source of primary antibodies raised in different host species. Fluorescence allowed successful detection of secondary antibodies specific for each species with a fluorophore in the form of two distinct colored images, Red for anti-mouse (549nm) and Green for anti-rabbit (650nm) (provided by Kodak kit). Upon sequential incubation of the blots with fluorescent-based antibodies, the membrane was washed with phosphate buffered saline (PBS) several times. After the washes, fluorescence was measured and quantitated using a Multiplex Fluorescent imaging system FluorChemQ. For western-blot analysis using ECL, the membranes were incubated with an HRP-polyclonal secondary antibody depending on the source of primary antibody. Upon sequential incubation of the blots with the antibodies, the membranes were washed with 1X TBS

-T wash buffer (20 mM Tris base, 137 mM NaCl, and 0.5% Tween-20 mixed in 1L distilled water). The obtained signal was detected using the luminescent imaging feature of the FluorChemQ imager and on an x-ray film by performing exposures in the dark. The ECL bands on the film were captured with a scanner and quantitated using ImageJ (NIH software).

## Results

In theory, if CDK9 plays a key role in the regulation of transcription by phosphorylating Ser-2 residues on the CTD of endogenous RNAPII, we would expect to see a gradual decrease of Ser-2 phosphorylation in cells that are treated with a compound known to inhibit CDK9 activity (Garriga et al., 2010; Salerno et al., 2007).

In Exp-1a, differential inhibition of Ser-2 phosphorylation on the CTD of RNAPII and simultaneous RNAPII expression was measured in BJT cells that were treated with a potent inhibitor of CDK9, Flavopiridol (FVP-300 nM) using fluorescent-based western blot analysis. For this purpose, the membranes were initially incubated with a mixture of primary antibodies to Ser-2 phosphorylated and total RNAPII, followed by the corresponding fluorescent-coupled secondary antibodies, Kodak X-sight anti-rabbit/anti-mouse. Both, primary and secondary antibodies were prepared at dilutions as listed in Table 1. The data in Fig. 1 is normalized to 100% for the sample treated with FVP at 0 h and the percent ratio for the remaining samples (1, 2, 14, and 30 h) is calculated relative to this 100% ratio. Also, a ratio of red:green signal was calculated by using FluorChemQ data analysis tool. As expected, with an increase in time incubation with FVP (0-30h), there was a gradual decrease in Ser-2 phosphorylation signal (red signal). Also, the total RNAPII expression (green signal) remained fairly constant as expected.

In Exp-1b, signal ratios observed for the differential inhibition of Ser-2 phosphorylation in Exp-1a were compared to that obtained by using ECL. For ECL western blot analysis, the membranes were incubated with a primary antibody to Ser-2 phosphorylated RNAPII followed by the corresponding HRP-coupled secondary antibody, anti-rabbit with dilution as listed in Table 1. The results are shown in Fig. 2A and 2B. For ECL, the data is normalized to 100% for the sample treated with FVP at 0 h and the percent ratio for the remaining samples (1, 2, 14, and 30 h) is calculated relative to this 100% ratio. For ECL-X-ray film, the bands were densitometrically captured with a scanner and the normalized ratios were calculated using ImageJ software. Normalization of the time zero values to 100% allowed a successful comparison of signal data between the two western blot techniques. For example, in case of the sample at 30 h in Fig. 2, the calculated percentage of signal with fluorescence was 77.34 whereas that with ECL was 6.18. Overall, the results of Exp-1 clearly show that under our experimental conditions, Ser-2 phosphorylation drops approximately 20 fold when measured by ECL (FluorChemQ or film), but it drops only modestly when measured using fluorescent-coupled antibodies. This was unexpected. Therefore, we decided to compare both detection systems using 2-fold decreasing protein amounts ranging from 20 to 0.625  $\mu$ g. In these experimental conditions one would expect linear quantitation with each assay unless there was signal saturation or lack of sensitivity.

In Exp-2, simultaneous expression of RNAPII and Ser-2 or Ser-5 phosphorylation along with CDK9 expression was measured using HeLa cell lysates. With the aim of comparing the differences in signal ratios obtained by using fluorescence and ECL, the protein lysates were

diluted in a gradual 2-fold range (20 µg-0.625 µg), resulting in an overall decrease of 32-fold in the total protein concentration. These results are shown in Figs. 3A, 3B and 3C. The quantitation of these data is normalized to 100% for the sample containing the highest protein concentration (20 µg) and the percent ratios for all other protein concentrations are calculated relative to this 100% ratio. Normalization of the highest protein concentration data to 100% allowed comparison of signals between the two western blot techniques. With each two-fold decrease in protein concentration, we expected to see a two-fold decrease in signal strength with both techniques. Also, in theory, we expected a more reliable linear correlation with fluorescence due to its presumed higher sensitivity compared to ECL. However, the quantitation of the data shown in Fig. 4 clearly demonstrates linearity in the signals detected by ECL imaging over a 32-fold decrease in protein concentration (upper left panel). Fluorescence detection demonstrates no such significant linear correlation in detecting a 32-fold decrease in protein concentration (upper right panel). Also, data obtained with ECL-film was comparatively much less linear.

## Discussion

Fluorescent-based western blot analysis was developed to simultaneously measure RNAPII expression and RNAPII Ser-2/Ser-5 phosphorylation upon treatment of cells with a potent inhibitor of CDK9 activity, FVP. Furthermore the linearity, reliability, and sensitivity of fluorescent-based analysis were compared to traditional western blot analysis using chemiluminescence. Overall, our results show that FluorChemQ ECL detection exhibits excellent signal linearity and little background, making it the system of choice for specific protein detection and quantitation. Thus, its use is fully implemented in our lab and has phased out X-ray film based western blot analysis in all our projects. (The first set of western blot images using this system in our lab has been already published in Jayadeva et al., 2010). However, the limitation of ECL to detection of one single form of RNAPII per blot, makes it a less desirable method for screen over large number of samples, that would have to be analyzed in duplicate, each with a different antibody. In contrast, while simultaneous detection of two different forms of RNAPII is possible with dual fluorescence detection, the signals were not proportional to protein amounts presumably due to lack of sensitivity of the fluorescence detection and/or high background. When background signal is high, low specific signals cannot be quantitated accurately. Therefore, it is necessary to subtract the background while quantitating the signals for each assay. Because the primary antibodies used for fluorescence and ECL detection are the same, it is clear that they are not the reason for the poor resolution of the fluorescence detection. High background may be caused by the quality of membrane and solutions, concentration of primary and secondary antibodies, and the quality of secondary antibodies. We were careful to avoid all these problems and used concentrations of primary antibodies that work efficiently by ECL, and concentrations of secondary antibodies and membrane specifications suggested by manufacturers. The signal detection by fluorescent-based western blots could be improved by the usage of a more sensitive photosensor instrument, blocking reagents that lower fluorescence background, a higher concentration of secondary antibodies above that suggested by the manufacturers and/or use of other compatible sets of fluorescence coupled antibodies with less associated background. Several experiments using fluorescent-coupled secondary antibodies manufactured by different companies can be designed to select the antibodies with less background, as new antibodies are being developed. It is clear from these studies that FluorChem Q imager detection of ECL-based signals works reliably as

long as a good primary antibody is selected, as we have successfully used the system with multiple different antibodies with no need to go back to use X-ray film for any antibody since we phased it out. In contrast, fluorescent-based detection with the FluorChem Q imager systems requires a specific set up of conditions for each primary antibody. This is in part illustrated by comparison of the upper right and left panels in Fig. 4. While the slope for the ECL signal obtained with three different primary antibodies is the same, the slopes obtained using fluorescent detection are significantly different.

In summary, while we have developed multiplex western blot assays that allow the simultaneous detection and quantitation of RNAPII and either RNAPII phosphorylated on Ser-2 or Ser-5, these assays remain qualitative at this stage. Further refinements as described above are needed to improve the sensitivity of fluorescence detection to ensure that a screening based on simultaneous detection of two signals does not generate an undesirable number of false positives that will require time-consuming secondary screens to rule them out. Also, lack of sensitivity may also lead to missing precious potential hits. On the other hand, the Alpha Innotech imager is fully implemented for ECL-western blot analysis and it is routinely serving as core equipment in the Fels Institute.

Table 1- List of Primary and Secondary Antibodies in this report.

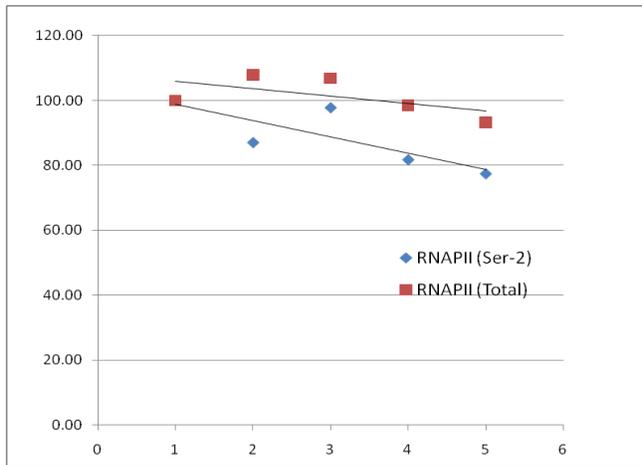
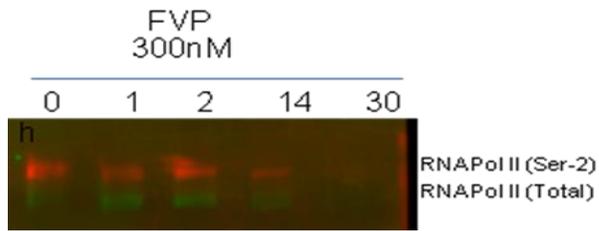
Exp 1- BJT cells treated with FVP at 300 nM:

Mix	Target	Primary Ab	Secondary Ab
1 (ECL)	RNAPII (P-Ser-2)	$\alpha$ -P-Ser-2 (poly 1:2000)	HRP-Anti-rabbit (1:20,00)
2 (FL)	RNAPII (P-Ser-2)	$\alpha$ -P-Ser-2 (poly 1:2000)	Kodak X-sight 650 nm Anti-rabbit (1:2500)
	RNAPII (Total)	$\alpha$ -8WG16 (mono 1:2000)	Kodak X-sight 549 nm Anti-mouse (1:2500)

Exp 2: HeLa cells:

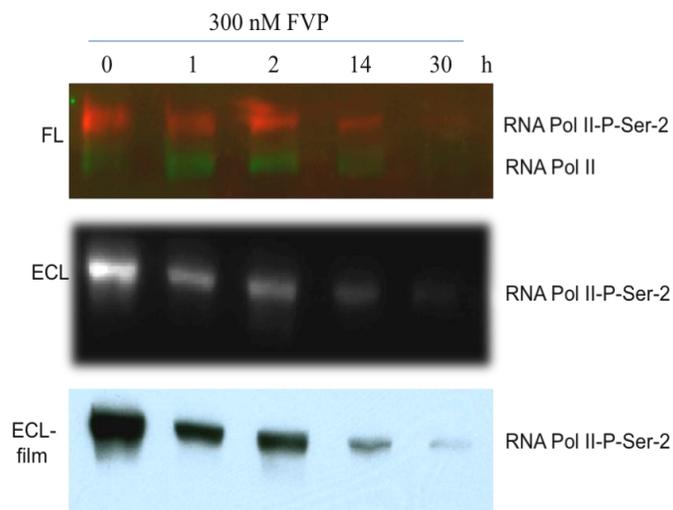
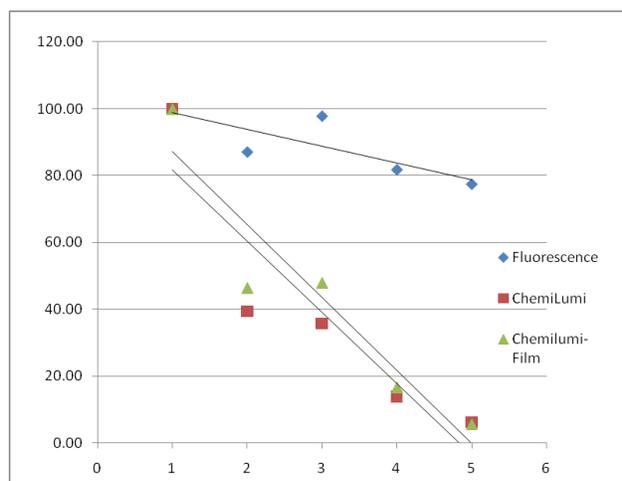
Mix (ECL)	Target	Primary Ab	Secondary Ab
1	RNAPII (P-Ser-2)	$\alpha$ -P-Ser-2 (poly 1:2000)	HRP-Anti-rabbit (1:20,00)
2	RNAPII (P-Ser-5)	$\alpha$ -P-Ser-2 (poly 1:2000)	HRP-Anti-rabbit (1:20,00)
3	RNAPII (CDK9)	$\alpha$ -CDK9 (poly 1:2000)	HRP-Anti-rabbit (1:20,00)

Mix (FL)	Target	Primary Ab	Secondary Ab
1	RNAPII (P-Ser-2)	$\alpha$ -P-Ser-2 (poly 1:2000)	Kodak X-sight 650 nm Anti-rabbit (1:2500)
	RNAPII (Total)	$\alpha$ -8WG16 (mono 1:2000)	Kodak X-sight 549 nm Anti-mouse (1:2500)
2	RNAPII (P-Ser-5)	$\alpha$ -P-Ser-2 (poly 1:2000)	Kodak X-sight 650 nm Anti-rabbit (1:2500)
	RNAPII (Total)	$\alpha$ -8WG16 (mono 1:2000)	Kodak X-sight 549 nm Anti-mouse (1:2500)
3	RNAPII (CDK9)	$\alpha$ -CDK9 (poly 1:2000)	Kodak X-sight 650 nm Anti-rabbit (1:2500)



Band (h)	Ser-2 %	Total %	Ratio R:G
0	100.00	100.00	1.00
1	87.05	107.86	0.81
2	97.84	106.83	0.92
14	81.70	98.52	0.83
30	77.34	93.17	0.83

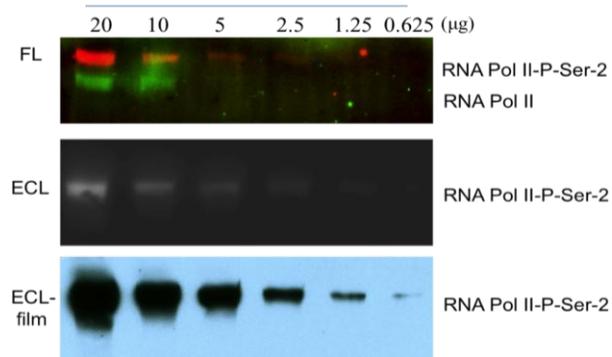
Fig. 1. BJT fibroblasts were incubated with 300 nM FVP for 0, 1, 2, 14, 30 h. Chart comparison (bottom) of normalized fluorescent signal ratios observed for Ser-2 phosphorylated and total-RNAPII expression.

**A****B**

Band (h)	Fluorescence (FL)	ECL	ECL film
0	100.00	100.00	100.00
1	87.05	39.35	46.49
2	97.84	35.71	48.02
14	81.70	13.89	16.72
30	77.34	6.18	5.70

Fig. 2. (A) BJT fibroblasts were incubated with 300 nM FVP for 0, 1, 2, 14, 30 hours. (B) Chart comparison (left) and table (right) containing quantitation data of normalized signal ratios calculated from FL, ECL, and ECL-film images shown in Fig. 1 (BJT-FVP).

**A**



**B**



**C**

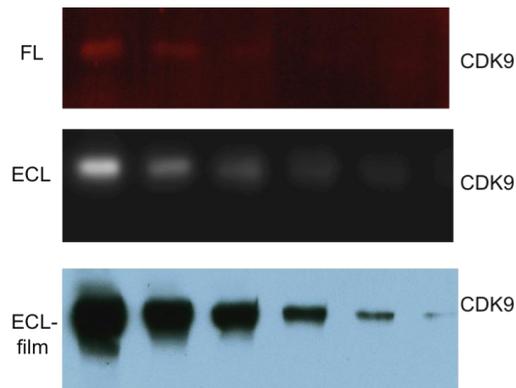


Fig. 3A. Image results from fluorescent (A), chemiluminescence (B) using FluorChemQ and corresponding chemiluminescence-film (C) using ImageJ. Untreated HeLa whole protein lysates (20, 10, 5, 2.5, 1.25, and 0.625 μg) were loaded to obtain a 2-fold dilution in the total protein concentration in each lane.

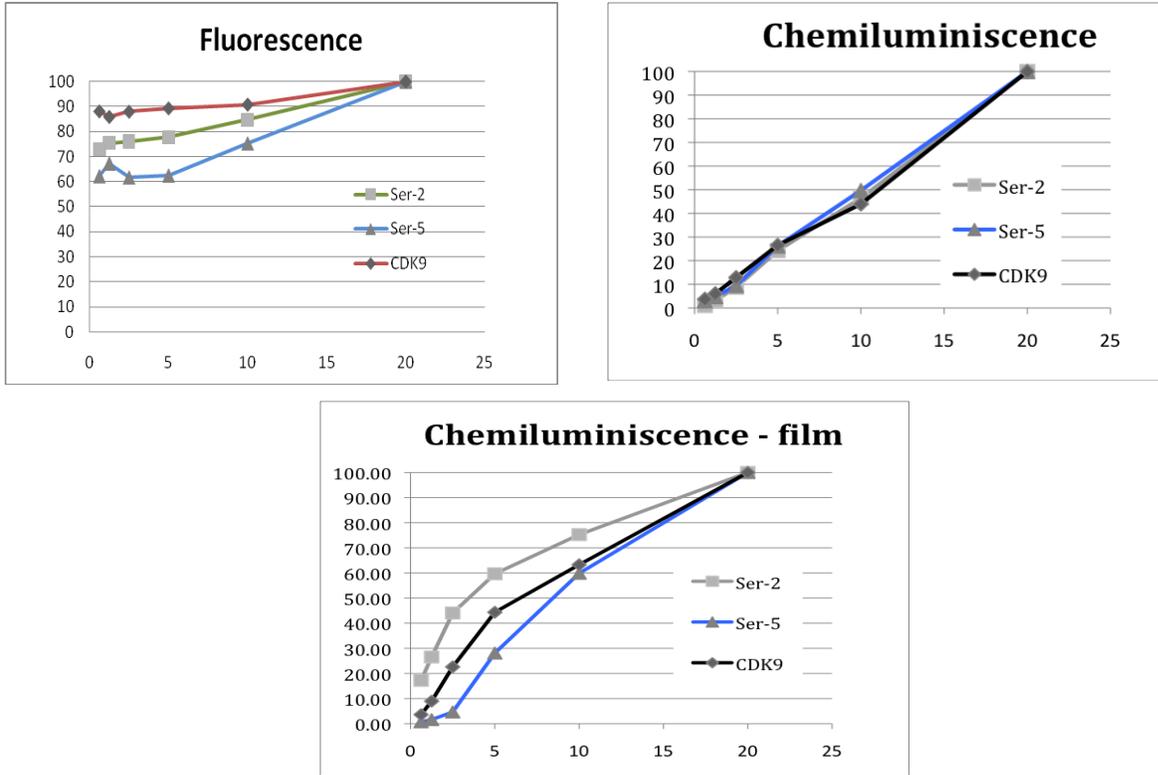


Fig. 4 – Chart representing signal ratios calculated from FL, ECL, and ECL-film images shown in Fig. 3 (HeLa control).

**Research Project 19: Project Title and Purpose**

*Smoking Cessation in Homeless Shelters: Benefits of Physical Activity and Support* - The purpose of this project is to pilot test protocols for a future trial that will provide evidence of the efficacy of delivering a smoking cessation intervention in conjunction with an existing physical activity program vs. cessation alone with the homeless population. Further, this project will examine whether any differences between the two interventions are mediated by social support to inform intervention components for the future trial. To date, this project represents a first attempt at examining the efficacy of smoking cessation intervention combined with physical activity with a sample of homeless individuals. Findings from this project will advance knowledge and understanding of tobacco use behaviors among the homeless, a population that smokes at a rate three times greater than the general population yet is generally overlooked in tobacco related research.

**Duration of Project**

9/15/2009 – 12/1/2010

## **Project Overview**

This project will expand an existing collaboration between Temple University investigators and a community organization, Back on My Feet (BOMF), a non-profit that promotes psychosocial and physical health through an organized running program for residents of homeless shelters. This project is significant in that it will examine the feasibility and efficacy of evidence-based smoking cessation treatment in a non-traditional setting by comparing outcomes between residents who have been physically active in BOMF and non-BOMF residents. By embedding smoking cessation services within a larger existing community-based health promotion program, the BOMF-cessation approach could be a model for increasing the capacity of community programs to promote smoking quit rates and reduce risk of tobacco-related morbidity and mortality. The BOMF-cessation approach should facilitate access and adherence to tobacco intervention services, and it will increase knowledge and understanding of tobacco use behaviors and physical activity – smoking cessation associations. This project will improve our understanding of barriers to substance abuse behavior change and will explore if social facilitation and contingent support for smoking cessation through group physical activity promotes smoking behavior change in an underserved population. Our overarching aim is to test preliminary protocols for recruitment, retention, and intervention delivery to guide future studies. The primary aim is to test the efficacy of smoking cessation treatment in combination with the existing program, BOMF, which promotes psychosocial and physical health. We will recruit homeless individuals from the Ridge Shelter, and enroll consenting, volunteer smokers in cessation treatment that includes 8 weekly behavioral counseling sessions and nicotine gum. All participants will receive the same health and eligibility screening provided by the BOMF program, excluding participants with major medical and psychiatric disorders. All participants will receive counseling and gum, but separate group sessions will be available for smokers who have actively participated in BOMF, and smokers who are not in BOMF. We hypothesize that the BOMF smokers will have higher quit rates, longer abstinence, and higher participant retention at 8-week end of treatment than the no-BOMF group. Our second aim is to improve our understanding of physical activity-smoking cessation associations and the mechanisms that affect smoking behavior change in this population, such as pretreatment nicotine dependence, fitness, and psychological factors.

## **Principal Investigator**

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## **Other Participating Researchers**

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## **Expected Research Outcomes and Benefits**

This project focuses on gaps in substance abuse treatment access by testing the feasibility of implementing evidence-based smoking treatment in homeless shelters— a nontraditional site for substance abuse intervention. Smoking rates in homeless populations triple rates observed in the general population. Homeless smokers rarely choose to quit smoking through traditional channels, although evidence indicates that many individuals want to quit. Thus, examining this project’s innovative implementation of evidence-based intervention is expected to create substantial impact. For example, we expect improved understanding of factors that facilitate smoking behavior change and factors that may improve substance abuse treatment retention in this population. Smoking cessation treatment provides an adaptable model to other substance abuse treatment in this setting. This project also presents the opportunity to examine the relationship between physical activity and substance abuse behavior change while improving understanding of behavioral mechanisms that influence cessation and maintained abstinence. To our knowledge, this would be the first research project to examine the role of physical activity in promoting substance abuse treatment (smoking cessation) in a homeless population. While a full randomized clinical trial (RCT) design is beyond the scope of this project, results obtained from this project will inform us of program feasibility and provide proof of concept for conducting a fully randomized trial.

## **Summary of Research Completed**

Based on the needs assessment information gathered in focus groups, we decided to conduct smoking cessation classes in the evenings on Temple University main campus to allow for clients to take public transportation (e.g., subway). We purchased and used healthy refreshments at each session, reimbursed for public transportation, and provided gift cards at completion of the program. Funding constraints prevented us to provide no cost nicotine replacement products -- something the clients strongly requested (and which would be standard in any sustained, evidence-based smoking cessation intervention).

8-week Smoking Cessation Group Feasibility Study. As part of this project, key personnel (Collins and Nair) provided the smoking cessation intervention that included materials tailored to the populations' needs. Procedures of the weekly smoking cessation group counseling sessions are described in earlier reports.

We successfully recruited smoking men who were BoMF participants via word of mouth -- 7 smokers enrolled. Three participants (43%) completed the full 8 sessions. In broader cross-sections of the U.S. populations of smokers, it is typical to have 30-60% drop-out rates. Given that the target population for this project is among the most challenging to enroll and have the highest recidivism in structured counseling interventions, we rate this enrollment and retention as a moderate success.

Among the three participants who completed the 8-week intervention, all (100%) had quit by the third week and remained ex-smokers through the remainder of the program. A conservative approach to reporting a quit rate for this program would classify those who dropped out as non-quitters. This would result in a quit rate of 43%. While interpretations of this rate should remain

cautious - we want to point out those typical quit rates for smoking cessation counseling programs that do not use nicotine replacement products or withdrawal medications (e.g., Chantix, Bupropion) are typically in the 5-10% range. Given that all participants reported being in recovery for other substance dependence (e.g., cocaine, heroin, etc.), we believe that the quit rates and retention rates were positive reflections of the feasibility of this intervention.

Lessons learned from the feasibility trial include our recognition that transportation, location of groups, and timing around work and shelter schedules was a challenge and a barrier to many who wanted to participate, or who dropped out. An enormous barrier was lack of ability to contact participants (most of whom did not own cell phones, and as homeless individuals, did not have routine contact information.) Furthermore, the BoMF organization continues to test strategies to track participants once they leave the shelter system and their program. This lack of tracking prevented our staff from contacting participants for the 3-month follow-up assessment. Future smoking intervention studies in this population should consider the provision of prepaid cell phones as an incentive to participate -- and as a strategy for participant retention. Also, the lack of nicotine replacement turned some prospective enrollees away.

One of the biggest motivators for smoking cessation and stay quit (relapse prevention) that were repeatedly noted by all participants was that they wanted to quit to improve their physical activity (running races) performance. All participants noted soon after quitting that they observed acute improvements in their respiratory and cardiovascular symptoms associated with smoking -- and that their endurance improved. This experience continued to motivate those who quit smoking. Future programs should capitalize on the mutual benefits of physical activity and smoking cessation, and how positive changes in one behavior can affect positive changes in the other behavior.

Since the project commenced, the project team has expanded into a sustained working group that shares overlapping scientific and treatment interest in bridging physical activity and smoking cessation (and other substance dependence) programs for underserved populations, such as the homeless. This team submitted an internal proposal for funding in 2010 that did not get awarded. The team continues to meet, and has two current proposals in progress with anticipated submission dates in late 2011 - early 2012. One is an NIH R21; the other targets the Pfizer Grand research mechanism.

### **Research Project 20: Project Title and Purpose**

*Cellular Transformation and Transcriptional Regulation of JC Virus in a Human Brain Tumor Model* - Human medulloblastoma is a malignant pediatric brain tumor that is refractory to treatment. Our model of medulloblastoma induced by the human polyomavirus, JC virus, will allow us to study the mechanisms involved in transcriptional regulation of JC virus and investigate a novel splicing pathway which may play a role in cellular transformation and tumor progression.

### **Duration of Project**

9/15/2009 – 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**

*The Role of Mucin in Promoting Survival of CA-MRSA and Streptococcus mutans* - The purpose of these studies is to understand the role of mucin in survival of Community-Associated/Acquired Methicillin Resistant *Staphylococcus aureus* (CA-MRSA) and *Streptococcus mutans*. Mucin is found in saliva and on mucosal surfaces such as the inside of the nose and throat. We found survival of both CA-MRSA and *S. mutans* requires nutrients. *S. mutans* is the major agent of dental caries (cavities) and mucin found in saliva prolongs its survival from days to months. CA-MRSA is the cause of lethal infections in young, healthy student athletes. We found mucin allows for survival of CA-MRSA on artificial turf, suggesting “snotting” by CA-MRSA carriers could deposit CA-MRSA on the artificial turf, where it remains alive for >60 days serving as a reservoir of CA-MRSA for subsequent athlete infections. The goal of these studies is to determine if mucin is providing sugars necessary for the survival of these pathogens.

### **Duration of Project**

9/15/2009 – 11/30/2010

### **Project Overview**

Many Gram-positive pathogens and normal flora survive in the pharynx. Mucin is a major component of the pharyngeal mucosal linings and of saliva. We found mucin prolongs the survival of two major pathogens; *Streptococcus mutans* and Community Associated/Acquired Methicillin resistant *Staphylococcus aureus* (CA-MRSA). *Streptococcus mutans* is a facultative member of the oral plaque community and its production of acid during sugar fermentation is associated with dental decay. In the oral plaque, *S. mutans* is subjected to a “feast or famine” life style, making it necessary for the bacteria to survive short-term starvation. In addition, *S. mutans* located deep in the dental plaque may be subject to longer-term starvation due to sugar limitation and competition. We found survival for longer than 3 days is dependent on nutrients. Mucin can prolong survival to >30 days. In an unrelated study on the survival of CA-MRSA in artificial turf, we found mucin allowed for the survival of significant numbers of CA-MRSA. Much of the evidence linking MRSA to athletes comes from sporadic outbreaks, but it is agreed that CA-MRSA infections among athletes represent a growing public health problem. Artificial turf is generally thought to increase the degree of skin injury following football trauma. This may account for the predisposition of football players to CA-MRSA infection. We determined that CA-MRSA could survive >60 days in high numbers on artificial turf, that survival depended on nutrients, and that mucin could serve as a nutrient source. Bacteria in mucin could be present if an athlete that was a CA-MRSA carrier deposited nasal secretions onto the field. The striking observation that two generally different pharyngeal pathogens could use mucin as a nutrient

source for survival, leads to the hypothesis that mucin may be a source of metabolic sugars for Gram-positive pathogens. This hypothesis will be tested by two specific aims. The first specific aim is to determine if mucin is degraded during incubation with *S. mutans* or CA-MRSA. The second specific aim is designed to determine if mucin can serve as a source of carbohydrates. Mucin contains fucose, mannose and galactose residues. To determine the contribution of these sugars to survival, the enzymes necessary for utilization of these sugars will be mutated and the ability of the mutants to survive in mucin will be compared to the parental strain. These studies will lay the groundwork for future studies on regulation of mucin metabolic pathways in *S. mutans* and for epidemiologic studies on the survival of CA-MRSA in nasal secretions on artificial turf.

### **Principal Investigator**

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### **Other Participating Researchers**

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### **Expected Research Outcomes and Benefits**

The expected outcomes and benefits will fall into three broad categories; (1) general bacteriology, (2) *S. mutans* and dental caries and (3) CA-MRSA survival on artificial turf as a risk factor for student athletes.

(1) General Bacteriology. Recently there has been increasing interest in understanding the basic metabolic properties of pathogens in the host as opposed to grown in laboratory medium. The metabolism of mucin has been studied in only a few organisms and the use of gluconate residues by *E. coli* is one of the only metabolic pathways that has been worked out. If mannose and/or galactose from mucin are being used by two diverse pharyngeal pathogens, this will provide a new metabolic scheme for the utilization of mucin and may have wide implications for both intestinal and naso-oral pathogens.

(2) Production of acids by *S. mutans* results in the dissolution of enamel and the eventual formation of cavities. The proposed metabolism of mannose residues by *S. mutans* results in the production of acetate and may allow for the continued production of acids even deep in the biofilm and in the absence of dietary sugars. These results could have a significant impact on our understanding of dental caries, especially in patients with a good diet that are still prone to cavities.

(3) The presence of CA-MRSA on surfaces has been shown to be a risk factor for infection of

healthy athletes. Artificial turf would be both a potentially abrasive surface and a source for CA-MRSA. The demonstration of mucin as a source of nutrients for survival of CA-MRSA on artificial turf leads to the hypothesis that one potential source for CA-MRSA infections is nasal secretions deposited by carriers. These results would be the basis for epidemiological studies on the survival of CA-MRSA in nasal secretions deposited on artificial turf fields by CA-MRSA carriers.

## Summary of Research Completed

### Specific Aim 1. Degradation of mucin during survival of *S. mutans* and CA-MRSA.

Mucin is a large molecule that forms complexes in excess of millions of Daltons and is present in saliva in high concentrations. We used 0.5%-5% mucin in our in vitro system. Given the high complexity of the molecule, the high concentrations used, and the number of surviving bacteria, especially after 7 days (10,000-100,000), it was not technically feasible to follow the biochemical degradation of mucin. Therefore, this Aim was replaced with determining the effects of sunlight and temperature on MRSA survival on astroturf in the laboratory and experiments to develop methods to detect MRSA and *Staphylococcus aureus* on football playing fields where players deposited oral secretions, which represent a natural source of bacteria in mucin.

*S. aureus* could be detected on areas of a football playing field where players often deposited oral secretions (Fig. 1). Since *S. aureus* has almost identical survival as MRSA, the methods developed can be applied to epidemiologic studies. However, *S. aureus* were detected only when temperatures on the field dropped to less than 37°C. Consistent with these findings high temperatures (44°C) and sunlight (ultraviolet) was found to significantly reduce survival even in the presence of mucin (Fig. 2A and 2B). However, the MRSA still survived 2-3 days at 37°C in sunlight, which would still increase the risk of infection of turf burns on astroturf used everyday for practice. The role of biofilms in prolonging survival in the presence of UV light is currently being examined. A manuscript describing the in vitro results is being prepared.

### Specific Aim 2. Mutation of genes in the mannose and galactose utilization pathways.

Experiments were completed examining the role of the *pdh* operon on survival in *S. mutans* in the presence of mucin. Previous data has shown the *pdh* operon has a significant effect on survival of *S. mutans* during carbohydrate starvation in the presence of mucin. In recent studies, using a *Ppdh*-GFP fusion, we have found that *pdh* is expressed in only a subpopulation of cells (0.1-1.0% of the cells). Early in starvation (~24 hrs), this subpopulation of cells is present as single cells or in short chains. Later in starvation, these cells produce long chains, suggesting the *pdh* subpopulation is surviving and replicating (Busuioc, Buttaro and Piggot *et al.*, J. Bacteriol., 192:4395-4402, 2010). These studies were done in medium containing amino acids and a hexose carbohydrate source that was metabolized to various triose endproducts by the end of exponential growth. Both the amino acids and the sugar endproducts could be used as alternate carbon sources after the primary sugar had been depleted. Therefore, the effect of mucin on survival in CDM lacking amino acids (MCDM) and lacking spent medium was determined. Under these conditions, mucin significantly increased survival of the cells and the survival was dependent on the *pdh* operon (Busuioc, Buttaro and Piggot *et al.*, J. Bacteriol., 192:4395-4402, 2010). Mucin is a glycoprotein that could provide both a fermentable sugar source (e.g.,

galactose) and amino acids, however, the presence of excess free amino acids alone in CDM did not prolong survival, suggesting the contribution to survival may be the sugar residues of mucin. Galactose is the only sugar known to be present in mucin and be metabolized by *S. mutans*. *S. mutans* has two glucose utilization pathways; the Leloir pathway and the Tagatose pathway (Fig. 3). However, an enzyme for the conversion of galactose-6-P to galactose has not been identified in *S. mutans*, so the pathway is incomplete. The tagatose pathway produces glycolytic intermediates that could enter glycolysis and allow for the production of pyruvate. The genes encoding one of the galactose transporters and some of the enzymes in the tagatose pathway were found to be up-regulated in response to mucin in preliminary microarray experiments (*lacFE*, *lacAB*, and *lacD*; Fig. 3). The addition of 28 mM galactose to cultures in place of mucin, prolonged survival in the absence of amino acids (MCDM), but not in the presence of amino acids (CDM). In addition this survival was not dependent on PDH and the *pdh* operon mutants survived with the same kinetics as the parental strain. The concentration of galactose was very specific and results varying from experiment to experiment. Generally, 28 mM galactose could prolong survival, 6 mM galactose was not enough to prolong survival and 100 mM galactose showed no increase in survival, possibly due to the buildup of a toxic tagatose pathway intermediate (Fig. 4). Insertion mutants of *lacAB* were kindly provided by Dr. Burne (University of Florida) and moved into the background of UA159 (the *S. mutans* strain utilized in our studies). The mutants did not grow on galactose as a primary carbon source, however, were also variable in regard to survival so no firm conclusions could be drawn about the contribution of the tagatose pathway to galactose utilization during survival. Overall galactose does not prolong survival in the same way as mucin; it only prolongs survival in MCDM and it is not dependent on *pdh*. Therefore, it was concluded, while galactose may contribute to mucin-induced survival, it is probably not the major molecule in mucin responsible for prolonged survival. A manuscript is being prepared describing these results. Given the difficulty in interpreting these data in a bacterium with a well-defined metabolism, these experiments were not completed with MRSA and the MRSA mucin research was focused on establishing techniques for the epidemiologic studies.

#### Publication:

Waninger, K., Rooney, T.P., Miller, J., Berberian J., Fujimoto, A. and Buttaro, B. A. Pilot Study: CA-MRSA Survival on Artificial Turf Substrates. *Med Sci Sports Exerc.* 43:779-784. 2011.

#### Published Abstract:

M.B. Oxenford, B.A. Buttaro, T.P. Rooney and K.N. Waninger. Pilot Study: CA-MRSA can survive on astroturf under environmental conditions. Chosen for oral presentation and received Best Research Presentation, Student Category. Pennsylvania Academy of Family Physicians Research Day, March, 2011. Collegeville, PA. *Clin J Sports Med.* (abstract) 2011;21:158.

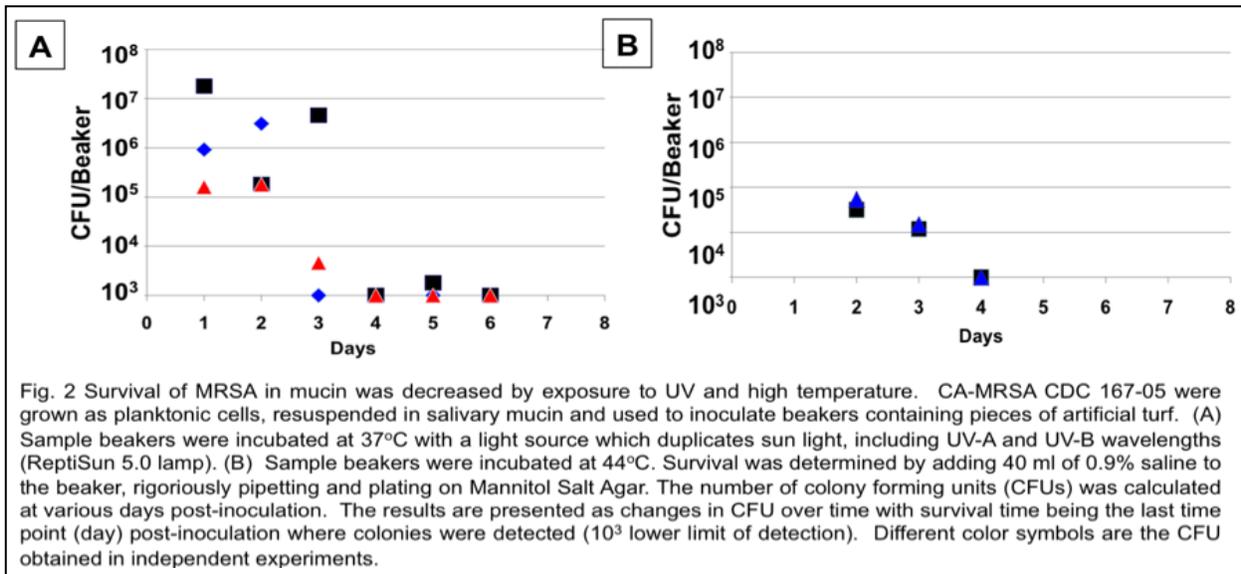
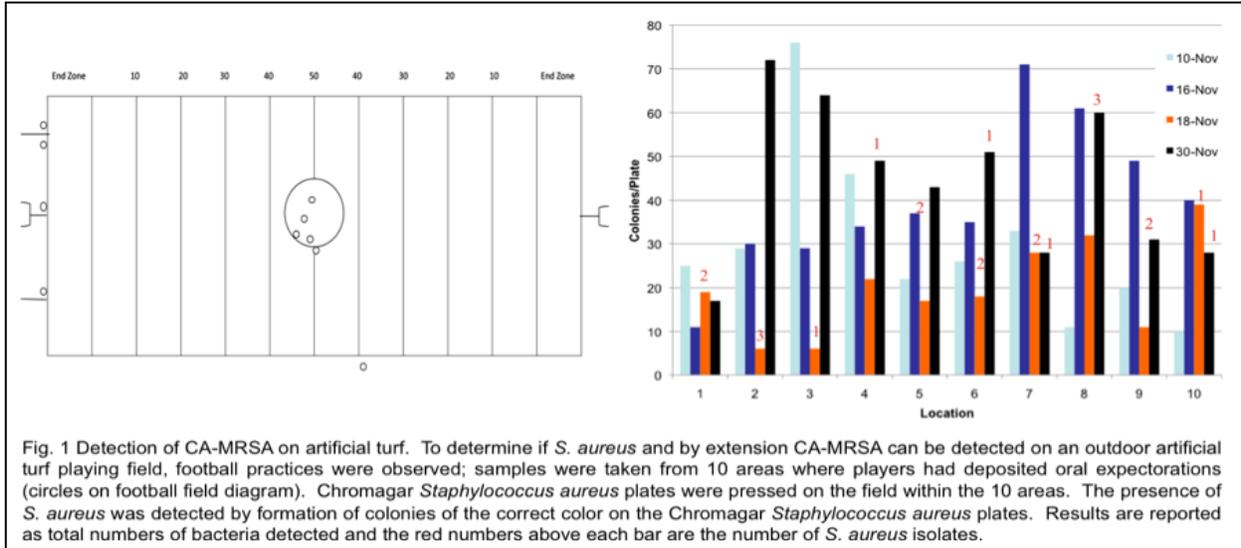
#### Meeting Abstract (from this research):

Oxenford MB, Buttaro BA, Rooney TP, Waninger KN. CA-MRSA can survive on artificial turf surface under environmental conditions. Presented, PA Academy of Family Medicine Research Day, State College, PA, March 12, 2011; American Medical Society of Sports Medicine, Salt Lake City, UT, May 2011.

#### Meeting Abstracts (from collaborations as a direct result of this funding):

Freed M, Vanic K, Hildebrand E, Huffman J, Buttaro BA, Waninger KN. CA-MRSA does not penetrate wrestling mat with applied force. *Clin J Sports Med.* (abstract) 2011;21:155.

Freed M, Vanic K, Hildebrand E, Huffman J, Buttaro BA, Waninger KN. CA-MRSA does not penetrate wrestling mat with applied force. Presented, PA Academy of Family Medicine Research Day, State College, PA, March 12, 2011; American Medical Society of Sports Medicine, Salt Lake City, UT, May 2011.



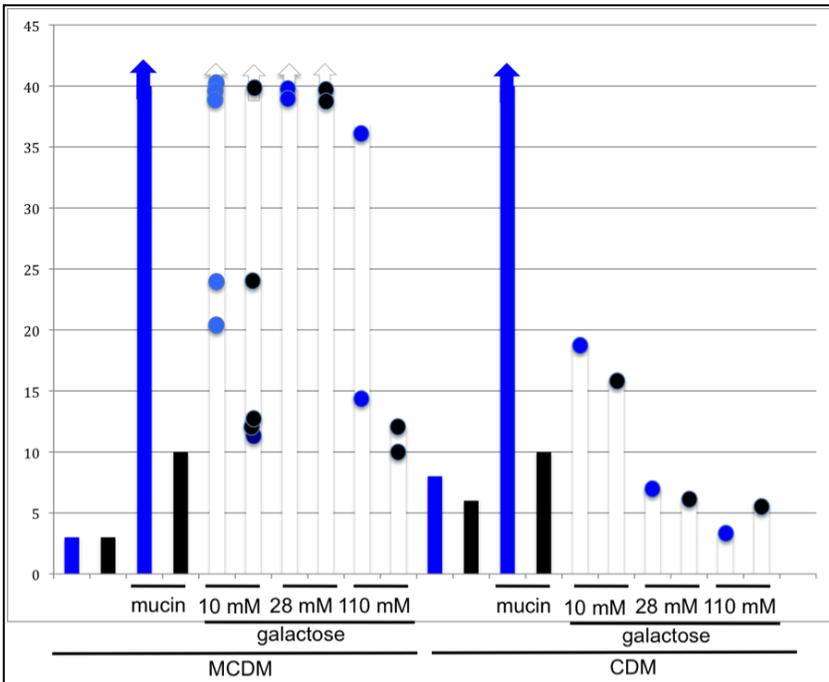
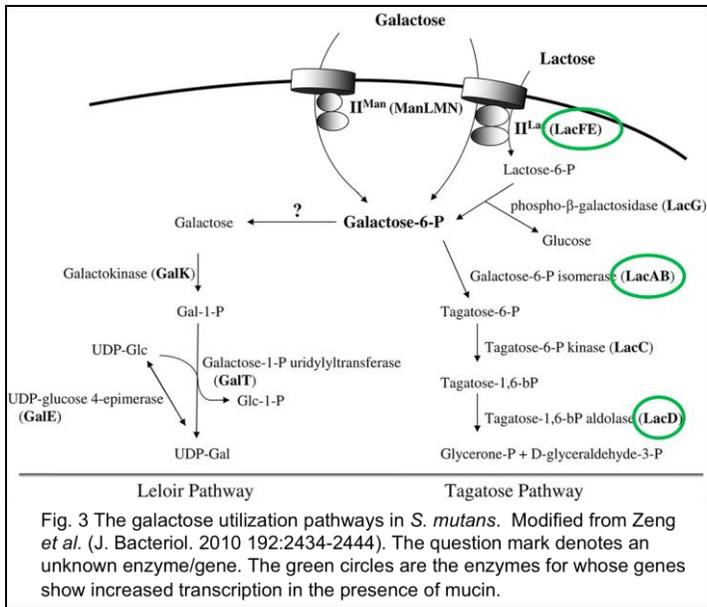


Fig. 4 Survival of *S. mutans* UA159 and  $\Delta pdh$  strains under various conditions. An overnight culture of UA159 (blue symbols) and  $\Delta pdh$  (black symbols) was set up in CDM supplemented with 24mM glucose. After overnight growth, the cells were washed twice in 1X PBS and resuspended in various survival medium. The contents of the medium for any given set of experiments are indicated by lines with the nutrient under the line; MCDM (chemically defined medium without amino acids), CDM (chemically defined medium with amino acids), mucin (0.5% mucin in PBS), galactose at 10, 28 and 110 mM. At various timepoints aliquots were removed and plated on Todd-Hewitt plates to determine survival. The solid bars are representative of multiple experiments yielding the same results. A surviving culture is any culture still culturable after 40 and continued survival is indicated by an arrow at the top of the bar. For the galactose experiments where there was more variability, each individual experiment is denoted by a dot.

## **Research Project 22: Project Title and Purpose**

*Active Owls: Promoting Active Commuting and Physical Activity at Temple University* - The Active Owls project seeks to evaluate a multi-pronged project aimed at promoting physical activity, primarily via cycling. Phase I will assess a social marketing campaign (“Bike Temple”) designed to promote active commuting via cycling among Temple University community members. Phase II will evaluate an intervention aimed at college students using an adapted web-based program that includes both online gaming and social networking through the use of Facebook (“social gaming”). Finally, Phase III will assess child participant and college student perceptions (using focus groups) of a local non-profit neighborhood program designed to promote cycling (“Earn-a-Bike”).

### **Anticipated Duration of Project**

11/18/2009 – 12/31/2011

### **Project Overview**

We seek to evaluate the preliminary efficacy of programs designed to promote physical activity (PA) among members of the Temple University and North Philadelphia communities. The primary objectives of this proposal are to: 1) Determine the efficacy of a social marketing campaign (Bike Temple) designed to increase active commuting via cycling among members of the Temple community. Participants (1000 faculty/staff, 2000 students) will be recruited to complete pre and post questionnaires online, which will assess use of active commuting, perceptions of cycling, and knowledge of the campaign. 2) Design and evaluate the effect of a social networking intervention (using Facebook) for PA and weight gain prevention among college students who are at risk of weight gain. Research participants will be 180 normal and overweight (BMI: 20-30kg/m<sup>2</sup>) college students (ages 18-25) who will be randomly assigned to one of two 6-month conditions (Facebook Intervention or Wait-list Control). Participants will complete measures at baseline and follow-ups (months 3 and 6). Intervention participants will complete PA modules based on the existing iOTA program and compete for prizes by uploading their PA stats to the Facebook group over a 12 week period. 3) Assess the usability of ActiPed technology, a novel monitoring strategy that will automatically transmit physical activity data to the Facebook application via a small device worn on a participant’s shoe. A subsample of intervention participants will wear an ActiPed. Their data will be compared to participants who are instructed to self-monitor and enter data using a standard pedometer. 4) Evaluate participant knowledge of, and interest and engagement in, the Earn-a-Bike program, as well as interest in having Temple University students volunteer with the program. Qualitative data will be obtained via pre/post focus groups held with Earn-a-Bike participants (n = 10) and a single focus group held with Temple University students (n = 12).

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## **Expected Research Outcomes and Benefits**

Hypotheses: 1) A social marketing campaign (Bike Temple) will be successful in increasing knowledge, attitudes, and behavior about cycling to Temple University 2) A Facebook-based social gaming intervention will be successful in increasing time spent in physical activity compared to a control group. 3) By month 6, participants in the Facebook Approach will remain weight stable, while participants in the Wait-list Control will gain 2.0 pounds. 4) The ActiPed will show high rates of concordance with other measures of physical activity outcomes, thus making it a useful tool to implement in other physical activity trials as an objective measure of activity. 5) The Earn-a-Bike focus groups are exploratory in nature. However, based on anecdotal data and Neighborhood Bike Works self-study results, we predict that participant feedback will be generally positive.

Benefits: 1) Bike Temple survey participants may benefit from learning more about their own behavior, as well as from receiving physical activity referrals. Data from this research will help to inform future health promotion campaigns that have the ability to reach broad populations in at-risk groups. 2) Our proposed physical activity intervention study addresses an important public health need (i.e., promoting physical activity and preventing weight gain) among a priority population of young adults using an innovative gaming strategy with high dissemination potential, using the existing platform of Facebook. Although long-term benefits are unknown, it is possible that intervention participants may decrease their chance of gaining weight during study participation. The anticipated benefits to society-at-large are great, insofar as the results will be used to further our understanding of the social and cognitive processes of the game play experiences related to health. 3) There are no known direct benefits attributable to participation in the focus group. However, the anticipated benefits to society-at-large are great, insofar as the results will be used to the further understanding of the factors related to the efficacy of community-based physical activity promotion programs in urban settings.

## **Summary of Research Completed**

### Phase I

Phase I data collection concluded during this reporting cycle, and baseline data analyses have been completed. Results of this study were presented at the 32<sup>nd</sup> Annual Meeting and Scientific Sessions of the Society of Behavioral Medicine in Washington, D.C. (April 2011). A manuscript

based on this research will be submitted by December 2011. Examination of follow-up data will also be completed by December 2011.

Results indicate that second only to walking, cycling is a preferred method of active commuting. Employees (faculty/staff; n=197) and students (n=263) from Temple University participated in an online study related to cycling behavior and perceptions. Perceptions of cycling were assessed using existing measures (Gatersleben & Appleton, 2007; Rissel & Bauman, unpublished). Controlling for distance from home to campus, results of a 2x2 (sex x school affiliation) MANCOVA revealed significant main effects of sex,  $F(6, 450)=7.21$ ,  $p<.0001$ ,  $\eta^2=.088$ , and University affiliation,  $F(6, 450)=4.70$ ,  $p<.0001$ ,  $\eta^2=.059$ . Despite similar rates of cycling behavior between sexes, women endorsed more negative views of cycling (e.g., “It is very frustrating sharing the road with cyclists”;  $p<.0001$ ) and greater concern about cycling-related hygiene (i.e., showering at school/work, appearance after cycling);  $p<.0001$ ). Additionally, men reported greater self-identification with cycling behaviors ( $p<.0001$ ). Employees were more likely to endorse greater safety concern ( $p=.022$ ), but also more pro-cycling attitudes ( $p=.006$ ) than students. Students reported more concern about cycling-related hygiene ( $p=.002$ ) and negative perceptions of cycling ( $p=.012$ ) than employees. These data were reexamined by including only those participants who could reasonably cycle to campus (i.e., living <10 miles from campus). Results supported the previously reported main effect of sex,  $F(6,289)=5.00$ ,  $p<.0001$ ,  $\eta^2=.094$ . Although the main effect of University affiliation was no longer significant in the model ( $p=.121$ ), examination of univariate analyses revealed significant differences related to hygiene concerns ( $p=.017$ ), negative perceptions of cycling ( $p=.023$ ), and pro-cycling attitudes ( $p=.025$ ). Overall, the current results demonstrate a need to tailor cycling promotion campaigns on college campuses according to sex and University affiliation. Results also highlight the importance of targeting employees and students who live within a “bikeable” distance.

## Phase II

During this reporting cycle, data collection for Phase II concluded and preliminary analyses were conducted. Results of this study were submitted during the current reporting cycle and have been accepted for oral and poster presentations at The Obesity Society's 29th Annual Scientific Meeting in Orlando, FL (October 2011).

This pilot study examined the preliminary efficacy, engagement, and acceptability of an 8 week weight loss intervention among college students. Students were recruited in two cohorts (fall 2010 [n=22] and spring 2011 [n=30]). Fifty-two participants (M age=20.47  $\pm$  2.19 years, BMI=31.36 kg/m<sup>2</sup>) were randomly assigned to one of three treatment arms: Facebook (i.e., Facebook used as the platform for delivering handouts and video content; n=17); Facebook Plus (i.e., a multimedia outlet to enhance goal setting, self-monitoring, and social support delivered via cell-phone text messaging; n=18) or Waiting List Control (n=17). The sample was primarily female (86.5%), and racially diverse (57.7% Caucasian, 30.8% African American, 5.8% Hispanic, 1.9% Asian, and 3.8% “other”).

Retention for this pilot study was excellent, with 100% of participants completing the 4 week assessment and 96.2% completing the 8 week assessment. At 8 weeks, weight changes were: -2.41kg  $\pm$  2.54 for Facebook Plus, -0.63kg  $\pm$  2.35 for Facebook, and -0.24  $\pm$  2.59 for Waiting List. High levels of engagement/usability and ratings of acceptability were found: The median number

of reports Facebook Plus participants viewed was 7.5 of 8 reports (50% viewed all reports; 66.7% viewed at least 75% of reports); responded to an average of 69% of self-monitoring texts (M=16.61, SD=6.13; median=17; range=6-24) and 81% of general monitoring texts (M=12.89, SD=3.05; median=15; range= 7-16). 100% of the Facebook Plus and Facebook participants found the program helpful (at least 3 or 4 on a 4 point scale). This shows preliminary efficacy and acceptability for an innovative intervention that leverages technology platforms (Facebook and text messaging) already integrated into the cultural life of college students. Additional data analyses are currently underway examining changes in physical activity and psychosocial measures (e.g., self-efficacy, body image). Additional acceptability data are also being evaluated for the full sample. Manuscript preparation for this study has commenced and one manuscript will be submitted for peer-review by September 2011.

As a secondary analysis, characteristics of college students who were interested in this type of research were examined. Few studies have evaluated weight loss interventions among college students; little is known about the characteristics of treatment-seeking students. Data were examined from students (n=240; M age=20.8±2.9; 85.0% female; M BMI = 27.5±6.7) who responded to recruitment ads. Ads for the weight loss program noted that students should be 18-29 years old and active Facebook/text (unlimited plans) users. Responders completed an online screening survey that included questions about demographics, smoking status, mood, and the Eating Disorder and Diagnostic Scale. More than one-third of responders were *not* overweight (2.1% underweight, 37.3% normal weight, 35.2% overweight, 25.4% obese).

Normal/underweight (9.9%) students were less likely to report binge eating and experience a loss of control compared with overweight/obese (30.2%) students,  $\chi^2(1)=11.6$ ,  $p<.001$ . Additionally, rates of assessed eating disorders did not vary significantly between normal/underweight and overweight/obese students (bulimia nervosa [0.5% vs. 1.4%]; binge eating disorder [1.4% vs. 5.9%]). Groups did not differ on total symptoms of depression, use of compensatory behaviors, or smoking status. Results suggest a surprising number (>1/3) of normal/underweight students would like to lose weight yet are less likely than their overweight/obese counterparts to endorse uncontrolled eating episodes. Although there is a need for weight loss programs on college campuses, interest in weight loss among normal/underweight students might reflect subjective weight and body image dissatisfaction that also warrants specialized intervention programs on campuses. A manuscript based on this work will be submitted for peer review by October 2011.

### Phase III

Data collection for Phase III ended during the current reporting cycle (July 2010). Children (baseline n=31; follow-up n=28) were recruited from and participated at Neighborhood Bike Works (NBW). All focus group recordings were transcribed and triple checked for accuracy by three independent research assistants (this concluded in March 2011), and procedures for qualitative analysis were finalized in June 2011. Qualitative analysis of the data will be conducted by three independent research assistants (these assistants did not transcribe the groups) beginning in July 2011. Data analysis is scheduled to conclude in October 2011, and a manuscript based on this work will be submitted in December 2011. While the data have not been analyzed fully, some preliminary impressions are reported here. First, a majority the participants learned about the program while walking by the NBW location, and about half entered the program for the primary reason of receiving a bike upon completion. All participants

wished that the program was held every day during the week. All of the participants had heard of Temple University and thought it would be a good idea if students were a part of the program to help them learn more about bikes. They all agreed that they would not ride their bicycles to school for fear of parts being stolen. Follow-up data indicated that they enjoyed the program and they would recommend it to their friends. The participants reported learning bike safety rules and the basics to fixing flat tires, chains, and helping others fix bicycles. The majority of boys planned to ride their bicycles every day. A manuscript based on this work will be submitted for peer review by December 2011.

### **Research Project 23: Project Title and Purpose**

*The Role of Osteoactivin (OA) as a Downstream Mediator of Osteoblast Differentiation Induced by BMP2* - Osteoactivin (OA) is a downstream mediator of osteoblast differentiation induced by BMP2. OA expression is regulated by BMP2 through the Smad1 signaling pathway. We hypothesize that OA acts a downstream mediator of BMP2-induced osteoblast differentiation and function, and this effect is mediated by the recruitment of Smad1, homeodomain proteins, and the CBP co-activator to the OA promoter to modulate the induction of its activity during osteoblast differentiation. We will determine whether OA acts as a downstream mediator of BMP2-induced osteoblast differentiation using primary osteoblasts derived from WT, OA KO and OA Tg mice and examine BMP2 responsiveness and Smad signaling. Subsequent analysis to examine the mechanism of activation and regulation of OA transcription by Smad1, Dlx3, Dlx5 and CBP during osteoblastogenesis.

### **Duration of Project**

11/18/2009 – 06/30/2011

### **Project Overview**

#### Specific aims:

1) To examine the role of OA as a downstream mediator of osteoblast differentiation induced by BMP2

Objective A: OA acts as a downstream mediator of BMP2 induced osteoblast differentiation

Objective B: The effects of absence (OA KO) or over-expression (OA Tg) of OA in osteoblasts on Smad signaling induced by BMP2

2) To examine the effect of BMP2 treatment on the recruitment of Smad1, Dlx3, Dlx5 and CBP proteins to the OA gene during osteoblast differentiation

We will first confirm and expand our analysis to determine whether OA acts as a downstream mediator of BMP2-induced osteoblast differentiation using primary osteoblasts derived from WT, OA KO and OA Tg mice and examine BMP2 responsiveness and Smad signaling. Next we will expand our analysis to examine the mechanism of activation and regulation of OA transcription by Smad1, Dlx3, Dlx5 and CBP during osteoblastogenesis.

This project represents an expansion of and next steps in research funded with previous CURE Formula Grants.

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### **Expected Research Outcomes and Benefits**

OA is expected to have a direct and important role in the process of cellular differentiation and in skeletogenesis as a whole. We expect that OA will act as a down-stream mediator of BMP-2 induced osteoblast differentiation.

The work should enhance our understanding of how stem cells differentiate into cartilage and bone. Currently in the United States, 10 million people suffer from osteoporosis and a further 20 million suffer from osteoarthritis. Although current therapies to treat these diseases are met with only limited success, there is much potential in stem cell therapy as a possible cure. To use stem cells for therapy, an intimate understanding of skeletogenesis is required in the hopes to manipulate stem cell differentiation to cure diseases. By understanding the role of factors that regulate the differentiation of stem cells, this work will bring us one step closer to using stem cells to cure skeletal diseases such as osteoarthritis and osteoporosis.

### **Summary of Research Completed**

#### Progress Report:

1) To examine the role of OA as a downstream mediator of osteoblast differentiation induced by BMP2

Objective A: OA acts as a downstream mediator of BMP2 induced osteoblast differentiation

Objective B: The effects of absence (OA KO) or over-expression (OA Tg) of OA in osteoblasts on Smad signaling induced by BMP2

The work proposed above was started but difficulties were encountered with breeding the OA KO and OA Tg mice. Every attempt was made to resolve the issue, but we were not able to

determine the cause within the project period, and therefore this piece of the project was not accomplished.

2) To examine the effect of BMP2 treatment on the recruitment of Smad1, Dlx3, Dlx5 and CBP proteins to the OA gene during osteoblast differentiation

We will first confirm and expand our analysis to determine whether OA acts as a downstream mediator of BMP2-induced osteoblast differentiation using primary osteoblasts derived from WT, OA KO and OA Tg mice and examine BMP2 responsiveness and Smad signaling. Next we will expand our analysis to examine the mechanism of activation and regulation of OA transcription by Smad1, Dlx3, Dlx5 and CBP during osteoblastogenesis.

For this aim, a significant progress has been made that led to publishing one manuscript and one submitted (see below).

#### Summary:

Osteoactivin (OA) is required for the differentiation of osteoblast cells. OA expression is stimulated by bone morphogenetic protein-2 (BMP-2). BMP-2 recruits homeodomain transcription factors Dlx3, Dlx5 and Msx2 to selectively activate or repress transcription of osteogenic genes and hence tightly regulate their transcription during osteoblast differentiation. Considering the key roles of Dlx3, Dlx5 and Msx2 in osteoblast differentiation, here we hypothesize that homeodomain proteins regulate BMP-2-induced OA transcription during osteoblast differentiation. Four classical homeodomain binding sites were identified in the proximal 0.96kb region of rat OA promoter. Deletions and mutagenesis studies of the OA promoter region indicated that all four homeodomain binding sites are crucial for BMP-2-induced OA promoter activity. Simultaneous disruption of homeodomain binding sites at -852 and -843 of the transcription start site of OA gene significantly decreased the BMP-2-induced OA transcription and inhibited binding of Dlx3, Dlx5 and Msx2 proteins to the OA promoter. Dlx3 and Dlx5 proteins were found to activate the OA transcription, whereas, Msx2 suppressed BMP-2-induced OA transcription. Using chromatin immunoprecipitation assays, we demonstrated that the OA promoter is predominantly occupied by Dlx3 and Dlx5 during the proliferation and matrix maturation stages of osteoblast differentiation, respectively. During the matrix mineralization stage, BMP-2 robustly enhanced the recruitment of Dlx5 and to a lesser extent of Dlx3 and Msx2 to the OA promoter region. Collectively, our results show that the BMP-2-induced OA transcription is differentially regulated by Dlx3, Dlx5 and Msx2 during osteoblast differentiation

#### Requirement of homeodomain binding sites for OA promoter activation.

To determine the role of homeodomain transcription factors in OA gene regulation, the proximal 0.96kb rat OA promoter region was analyzed for classical homeodomain binding sites. Four consensus homeodomain transcription factor binding sites were identified in the proximal 0.96kb OA promoter region (Fig 1A). To characterize the role of homeodomain binding sites, a series of 5' deletions of the proximal 0.96kb rat OA promoter region cloned into luciferase reporter vector were performed and tested for OA promoter activity (Fig 1B). The maximal OA promoter (p962)

response was obtained with 100ng/ml of BMP-2 treatment for 24 hours (Fig 1). For subsequent luciferase assays, osteoblasts were treated with BMP-2 (100ng/ml) for 24 hours. The untruncated proximal 0.96kb OA promoter construct, p962 that contained all four homeodomain binding sites showed a maximal promoter activity following BMP-2 treatment (Fig 1B). In contrast to p962, loss of both -852 and -843 homeodomain binding sites in p835, which are five nucleotides apart in the OA promoter region, decreased the BMP-2-induced OA promoter activity by 50%. A 60% reduction in BMP-2-induced OA promoter activity was observed with loss of distal three homeodomain binding sites (-852, -843, -710 homeodomain binding sites) in p685. Finally, deletion of all four homeodomain binding sites reduced the BMP-2-induced OA promoter activity by 80% in p285, indicating that all four homeodomain binding sites are crucial for BMP-2-induced OA transcription in osteoblasts (Fig 1B). The specific role of each homeodomain binding sites was characterized by disrupting homeodomain binding sites (namely H1, H2, H3 and H4 in the cloned p962-OA promoter region, Fig 1C). Since, H1 and H2 sites are in close proximity, we constructed OA promoter construct (pMH1+2) with mutations in both H1 and H2 sites. Mutations in H1, H2, H3 and H4 sites decreased the BMP-2-induced OA promoter activity (Fig 1D). A simultaneous disruption of H1 and H2 sites showed the maximal reduction in BMP-2-induced OA promoter activity, indicating that the OA promoter region requires both H1 and H2 sites for its response. Both deletion and mutation analyses in the homeodomain protein binding sites decreased the responsiveness of proximal 0.96kb OA promoter region to BMP-2 stimulation, indicating that all four homeodomain binding sites are important for BMP-2-induced OA promoter activation in osteoblasts.

### Contribution of homeodomain proteins in OA transcription

To test the specific roles of Dlx3, Dlx5 and Msx2 proteins on OA transcription, we either knocked down or over expressed the homeodomain proteins in MC3T3-E1 osteoblasts and analyzed the effect of knockdown or over expression on the OA promoter activity. Transfection of osteoblasts with Dlx3-siRNA (150nM), Dlx5-siRNA (100nM) and Msx2-siRNA (150nM) resulted in maximal knockdown of Dlx3, Dlx5 and Msx2 protein, respectively (Fig 2A-C). These concentrations of specific siRNAs were used for subsequent luciferase assays (Fig 2D). Transfection with scrambled siRNA did not show any effect on OA promoter response (unpublished data). In comparison to control osteoblasts, knockdown of Dlx3 and Dlx5 proteins resulted in 31% and 49% reduction in BMP-2-stimulated OA promoter activity, respectively (Fig 2D). In contrast, knock down of Msx2 enhanced the BMP-2-induced OA promoter activity by 17% relative to control osteoblast cultures. These results suggest that Dlx3 and Dlx5 proteins up-regulate BMP-2-induced OA promoter activity, however, Msx2 acts as a transcriptional repressor for OA gene expression in osteoblasts (Fig 2D).

The over expression of Dlx3, Dlx5 and Msx2 proteins in MC3T3-E1 osteoblasts was confirmed by Western blotting (Fig 2 E-G). Maximal expression of Dlx3, Dlx5 and Msx2 protein levels were observed at DNA concentrations of 1 $\mu$ g, 0.5 $\mu$ g and 1 $\mu$ g, respectively. For subsequent luciferase experiments, these DNA concentrations were used for Dlx3, Dlx5 and Msx2 over expression in MC3T3-E1 osteoblasts (Fig 2H). Transfection with empty vectors for Dlx3 or Dlx5 or Msx2 did not affect the OA promoter activity (data not shown). The over expression of Dlx3 and Dlx5 proteins increased BMP-2-induced OA promoter activity by 20% and 39%,

respectively (Fig 2H) relative to empty vector transfected osteoblasts. However, Msx2 over expression decreased the OA promoter activity by 24% in comparison to untransfected osteoblasts. These observations are in agreement with the knockdown results (Fig 2D) and suggest that Dlx3 and Dlx5 up-regulate; however Msx2 down-regulates BMP-2-induced OA promoter activity. Notably, OA promoter responded robustly to Dlx5 over expression (15% more) compared to Dlx3 over expression in BMP-2-treated osteoblast cultures. Simultaneous over expression of Msx2 with Dlx3 decreased the Dlx3 enhanced OA promoter activity by 14% in comparison to Dlx3 over expression alone (Fig 2H). Likewise, the co-expression of Msx2 with Dlx5 reduced the Dlx5 enhanced OA promoter activity by 19% in BMP-2 treated osteoblast cultures. In contrast, the co-expression of Dlx3 and Dlx5 proteins synergistically enhanced the BMP-2-induced OA promoter activity relative to Dlx3 or Dlx5 over expression alone (Fig 2H). Taken together, these results indicate that Msx2 antagonizes the stimulatory effects of Dlx3 and Dlx5 on OA promoter activation induced by BMP-2(Fig 2H).

In order to evaluate the responsiveness of homeodomain binding sites to Dlx3, Dlx5 or Msx2 proteins, we co-transfected the OA promoter deletion vectors with Dlx3, Dlx5 or Msx2 over expression vectors and assayed for the OA promoter activity in BMP-2-treated MC3T3-E1 osteoblasts (Fig 3A-C). Dlx3 over expression enhanced the BMP-2-induced promoter activity of untruncated p962 OA promoter vector by 24% relative to empty vector transfected osteoblasts in BMP-2 treated cultures. In comparison to p962, a simultaneous loss of H1 and H2 homeodomain binding sites in p835 showed a 15% reduction in Dlx3-activated promoter activity in BMP-2-treated cultures. Likewise, p685 and p285 OA promoter deletion vectors that lacked three or all of the homeodomain binding sites did not show such a pronounced increase in promoter activity with Dlx3 over expression (Fig 3A). Dlx5 over expression showed a maximal activation (37% increase) in the promoter activity of p962 in comparison to truncated OA promoter vectors, p835 (15% increase), p685 (13% increase) and p285 (10% increase) relative to untransfected osteoblasts in BMP-2-treated osteoblast cultures (Fig 3B). Msx2 over expression decreased the p962 OA promoter activity vector by 24% relative to empty vector transfected osteoblasts in BMP-2- treated cultures. Deletion of homeodomain binding sites showed a gradual decrease in OA promoter activity with Msx2 over expression (Fig 3C). Next, the transcriptional role of specific homeodomain binding sites (namely H1, H2, H3 and H4) for Dlx3, Dlx5 or Msx2 regulated OA promoter activity was evaluated in BMP-2 treated osteoblasts (Fig 3D-F). Dlx3 over expression enhanced the activity of OA promoter mutant constructs in comparison to empty vector transfected osteoblast cultures (Fig 3D). Simultaneous mutation in H1 and H2 homeodomain binding sites showed a minimal increase with Dlx3 over expression, indicating that both H1 and H2 homeodomain binding sites are crucial for Dlx3 upregulated OA promoter response (Fig 3D). Likewise, Dlx5 over expression increased the activity of OA promoter mutants in BMP-2-treated osteoblasts in comparison to empty vector transfected cultures (Fig 3E). Dlx5 up-regulated promoter activity of pMH1+2 vector minimally (Fig 3E). Unlike Dlx3 and Dlx5, Msx2 over expression decreased the activity of OA promoter mutants relative to empty vector transfected osteoblast cultures (Fig 3F). pMH1+2 OA promoter vector with mutations in both H1 and H2 sites responded minimally to Msx2 over expression. Taken together, our results show that all four homeodomain binding sites in the OA promoter region are responsive to Dlx3, Dlx5 and Msx2 regulated OA transcription (Fig 3A-F).

## BMP-2 stimulates the binding of homeodomain proteins to the OA promoter region

The studies with the OA promoter mutants for homeodomain protein binding sites suggest that simultaneous disruption of H1 and H2 binding sites show a minimal BMP-2-induced OA promoter activity (Fig 1D) and significantly reduced the responsiveness of the OA promoter to Dlx3, Dlx5 and Msx2 transcription factors (Fig 3D-F). Thus, *in vitro* binding of the homeodomain proteins to the OA promoter was assayed using wild type (W) and mutant (M) oligonucleotides that contained H1 and H2 sequences (Fig 4A). The M-oligonucleotide contained mutations in both H1 and H2 sites. A gel shift was observed in BMP-2-treated osteoblasts (Fig 4B, Lane 5). Simultaneous disruption of H1 and H2 homeodomain binding sites inhibited interactions between homeodomain proteins and the OA promoter region shown by the absence of gel shift in untreated (Fig 4B, Lane 2) and BMP-2 (Fig 4B, Lane 3) treated nuclear lysates. The specificity of the observed interactions was assayed by competition and supershift assays. Pre-incubation of BMP-2 treated nuclear extracts with 100X (Fig 4B, Lane 6) or 150X (Fig 4B, Lane 7) unlabeled (Cold (H (1+2))) oligonucleotide inhibited the binding of Biotin-H (1+2) oligonucleotide evident from disappearance of the shifted band. Addition of Dlx3 antibody (Fig 4C, Lane 5) or Dlx5 antibody (Fig 4C, Lane 6) to BMP-2-treated nuclear lysates resulted in formation of high molecular weight supershift complex. Incubation of BMP-2-treated nuclear lysates with Msx2 antibody resulted in disappearance of the shifted band (Fig 4D, Lane4), confirming that the shifted band contains Msx2. To further confirm the specificity of the supershifted bands, Biotin-H (1+2)-W oligonucleotide was incubated with Dlx3 (Fig 4C, Lane 2), Dlx5 (Fig 4C, Lane3) or Msx2 antibody (Fig 4D, Lane 2) in the absence of nuclear lysates. These antibodies did not bind to the Biotin-H (1+2)-W oligonucleotide in the absence of nuclear proteins suggesting that supershifted bands were specifically observed with Dlx3 (Fig 4C, Control lane 2), Dlx5 (Fig 4C, Control lane 3) and Msx2 (Fig 4D, Control lane 2) proteins in osteoblast nuclear extracts.

## Occupancy of Dlx3, Dlx5 and Msx2 proteins to the OA promoter during osteoblast Differentiation

Having demonstrated that BMP-2 stimulates the binding of Dlx3, Dlx5 and Msx2 proteins to the OA promoter in osteoblasts *in vitro* (Fig 4 B-D), we next investigated the physiological roles of Dlx3, Dlx5 and Msx2 on the OA promoter regulation during osteoblast differentiation *in vivo* using ChIP assay. The OA promoter region (-871 to -672 of the OA gene) that contains three consensus homeodomain binding sites was amplified (Fig 5A). BMP-2-treated and untreated osteoblast cultures were harvested at day 7, 14 and 21 that represents osteoblasts in proliferation, matrix maturation and matrix mineralization stages, respectively (Fig 5B). OA promoter was predominantly occupied by Dlx3 transcription factor in proliferating osteoblasts, however during matrix maturing and matrix mineralization stages, Dlx3 binding was significantly decreased in untreated osteoblast cultures. Treatment of osteoblast cultures with BMP-2 stimulated the binding of Dlx3 by 7.5-fold and 33-fold at day 7 and day 21, respectively in comparison to untreated osteoblast cultures. At day 14, BMP-2 did not affect the binding of Dlx3 to the OA promoter region in comparison to untreated osteoblast cultures.

Dlx5 showed a differential pattern for association with the OA promoter in comparison to Dlx3 both in untreated and BMP-2-treated cultures. Untreated osteoblast cultures at day 7 showed a

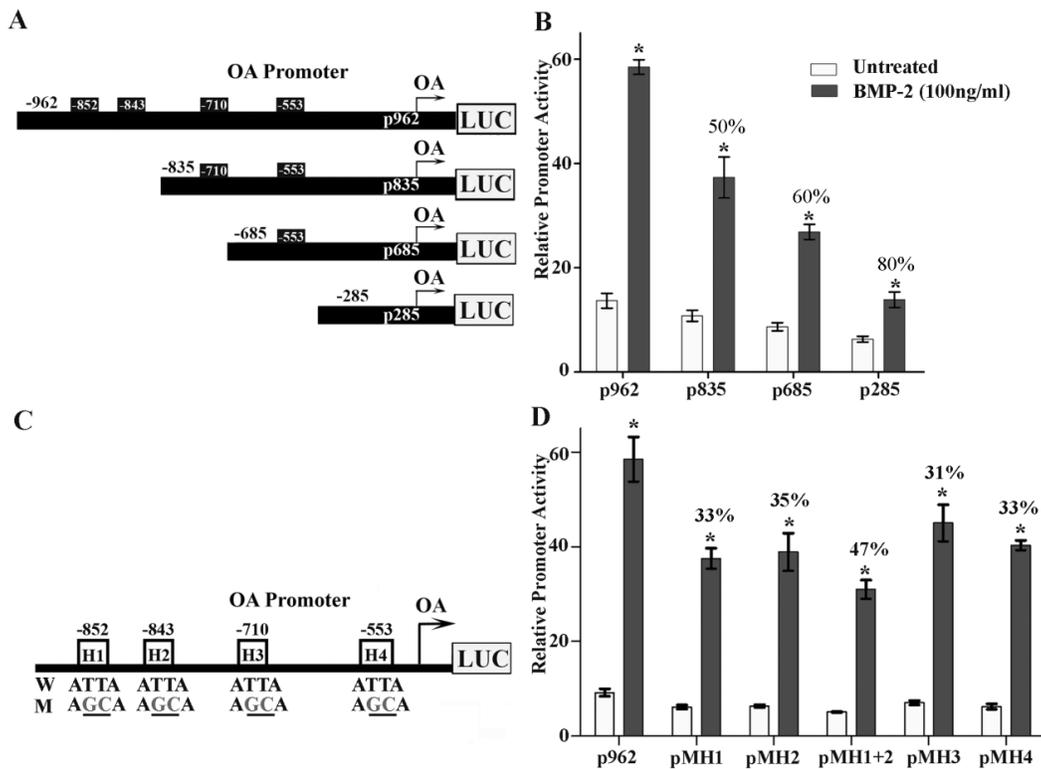
strong association of Dlx5 with the OA promoter, however this binding was significantly reduced in matrix secreting osteoblasts (day 14). Terminally differentiated osteoblast cultures at day 21, displayed a 10-fold increase in Dlx5 binding relative to day 14. In comparison to untreated cultures, BMP-2 stimulated the binding of Dlx5 to the OA promoter region at day 7, 14 and 21 by 7.4-fold, 64.5-fold and 110-fold, respectively.

Under basal conditions, Msx2 showed a similar OA promoter association pattern as described above for Dlx5. As the osteoblast cells progress from proliferation to matrix maturation stages, Msx2 recruitment to the OA promoter was reduced. However, Msx2 was robustly recruited to the OA promoter region during matrix mineralization stage of osteoblast differentiation (day 21). In proliferating osteoblast cultures, BMP-2 enhanced the binding of Msx2 to the OA promoter. However, at day 14 and day 21, BMP-2 had no effect on binding of Msx2 to the OA promoter region. BMP-2 did not show any changes in binding of IgG and Polymerase II to the OA promoter during osteoblast differentiation (Supplemental Fig 2B). Taken together, our ChIP results show that BMP-2 differentially recruits Dlx3, Dlx5 and Msx2 transcription factors to the OA promoter during osteoblast differentiation.

Interestingly, Msx2 is associated with the OA promoter during terminal stages of osteoblast differentiation both in basal and BMP-2 treated osteoblast cultures. BMP-2 did not affect Msx2 binding with the OA promoter region at day 21. Taken together, the OA promoter is regulated differentially by Dlx3, Dlx5 and Msx2 transcription factors during osteoblast differentiation in response to BMP-2 stimulation.

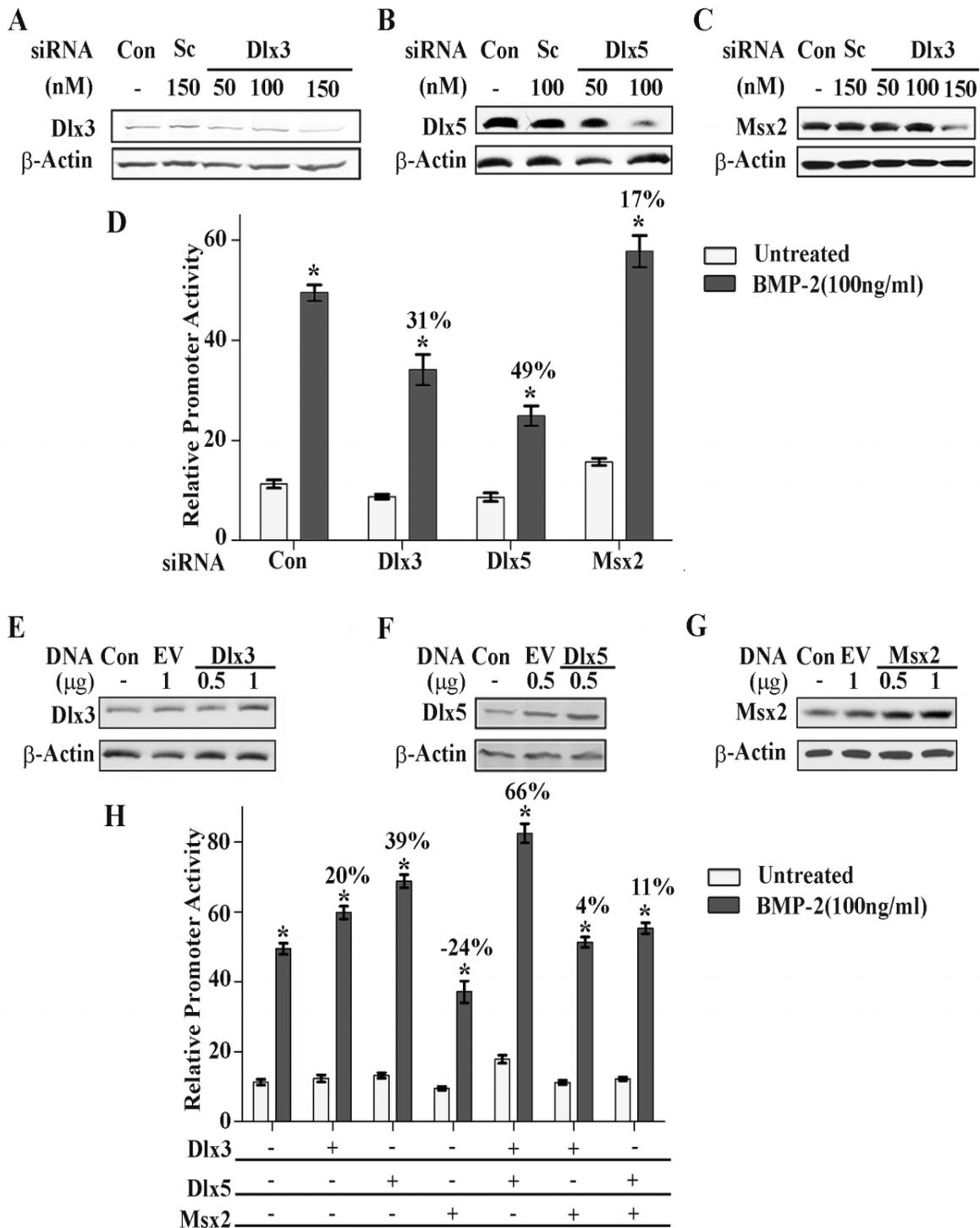
Publications:

[Singh M](#), [Del Carpio-Cano FE](#), [Monroy MA](#), [Popoff SN](#), [Safadi FF](#). Homeodomain transcription factors regulate BMP-2-induced osteoactivin transcription in osteoblasts [J Cell Physiol](#). 2011 Apr 18. doi: 10.1002/jcp.22791. [Epub ahead of print]



**Figure 1. Requirement of homeodomain binding sites for OA promoter activation.**

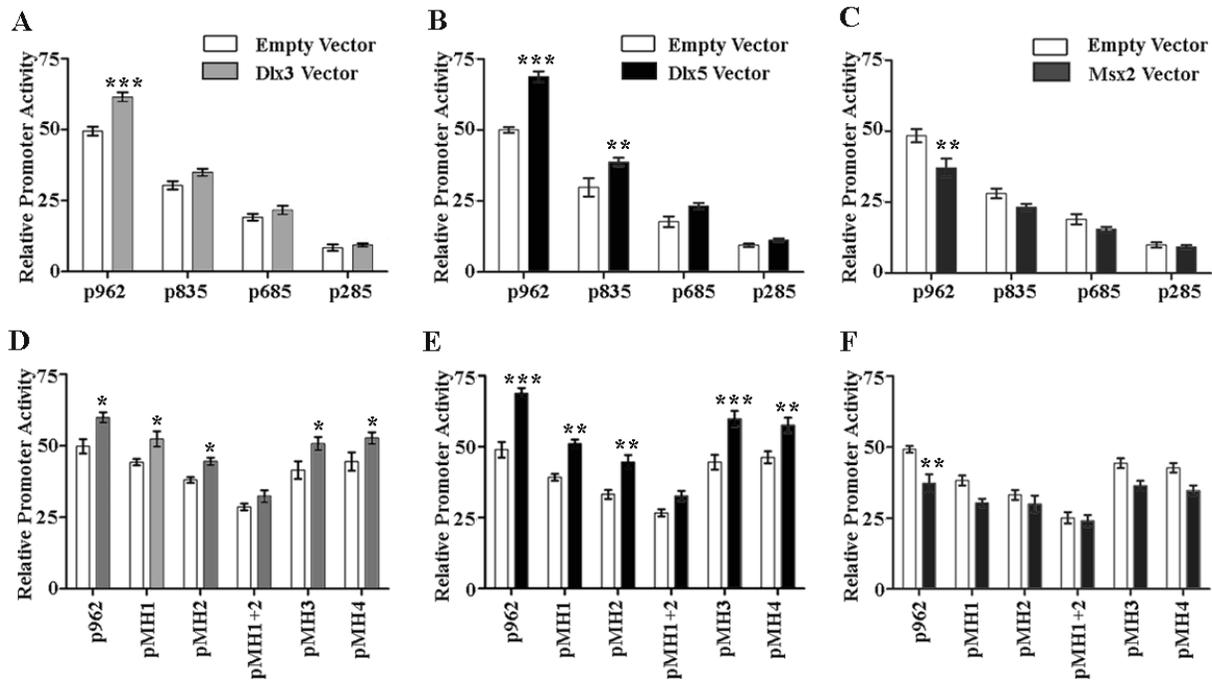
A. Illustration of 5' deletion OA-promoter constructs p962, p835, p685, p285 with nucleotides upstream of the transcription start site (shown with arrow). Position of homeodomain protein binding sites relative to transcription start site in the cloned OA promoter region is shown. Each of the OA promoter construct contains +50 nucleotides of the OA gene. B. Full-length (p962) or deletion mutants (p835, p685, p285) of the OA promoter-LUC vector were co-transfected with renilla vector in primary osteoblasts. Transfected osteoblasts were serum starved over night and then treated with BMP-2 (100ng/ml) for 24 hours. The next day, promoter activity was quantified. The data represents relative luciferase activity (Y-axis) that represents luciferase expression normalized against renilla expression for each OA promoter deletion vector construct. Percentage decrease in activity of OA promoter (p962) relative to OA promoter deletion vectors in BMP-2 treated cultures is shown. C. Schematic of the OA promoter-LUC vector (W) with position of homeodomain binding sites (H1, H2, H3 and H4) relative to the transcription start site is shown. Consensus homeodomain binding sites are indicated in the W-construct. Mutated nucleotides in homeodomain binding sites are underlined in the OA promoter mutant (M) vectors. Arrow indicates the transcriptional start site. D. Full-length OA promoter-LUC vector (W, p962) or OA promoter LUC vector with mutations at homeodomain binding sites (pMH1, pMH2, pMH3 and pMH4) was co-transfected with renilla expression vector. Osteoblasts were treated with BMP-2 (100ng/ml) and relative promoter activity is expressed as described above (B). \*p-value <0.001 represent OA promoter activity in BMP-2 (100ng/ml) treated osteoblasts compared to untreated osteoblast cultures. Percentage decrease in activity of OA promoter (p962) relative to OA promoter mutants in BMP-2 treated cultures is shown.



**Figure 2. Involvement of homeodomain proteins on OA gene expression.**

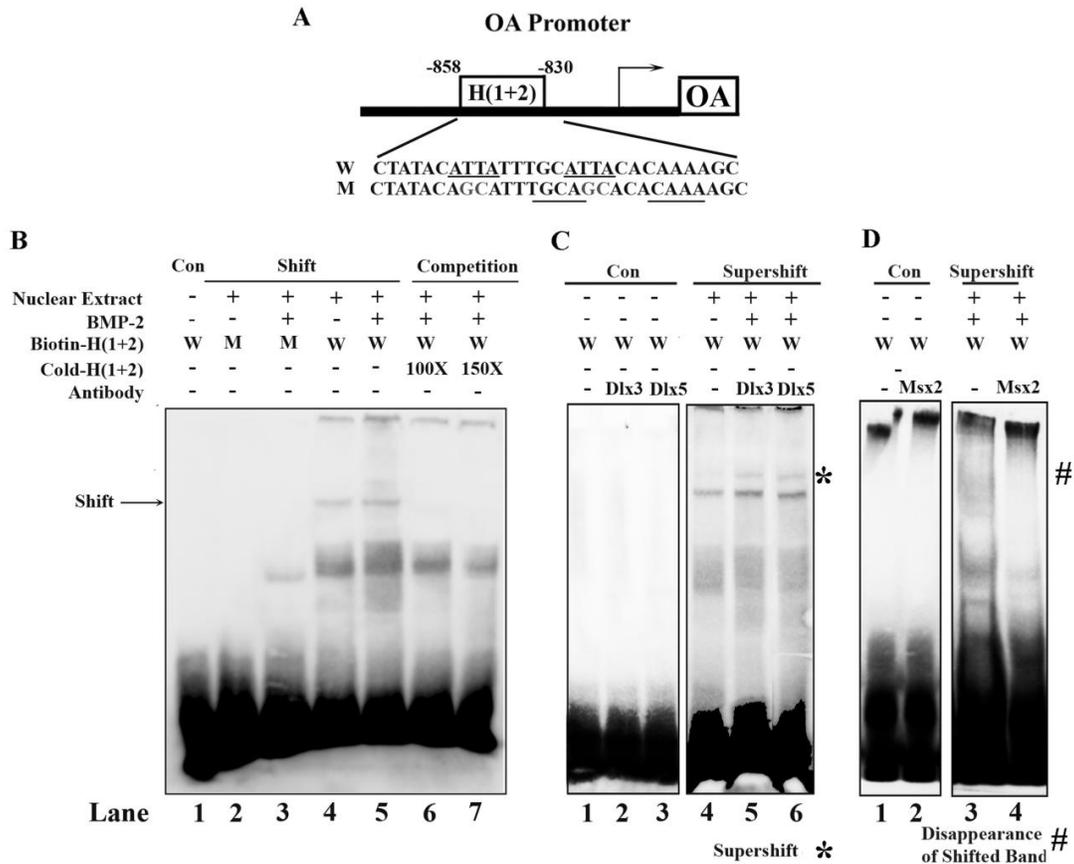
A-C. MC3T3-E1 osteoblasts were transfected with Dlx3-siRNA, Dlx5-siRNA, Msx2-siRNA or their respective Scrambled (Sc)-siRNAs. The cell lysates were collected 48 hours after transfection and analyzed for knock-down of proteins by Western blotting for Dlx3, Dlx5 or

Msx2 proteins and  $\alpha$ -actin. Control (Con) represents untransfected osteoblasts. D. MC3T3-E1 cells were co-transfected with Dlx3-siRNAs (150nM) or Sc-Dlx3-siRNA (150nM), Dlx5-siRNA (100nM) or Sc-Dlx5-siRNA (100nM), Msx2-siRNA (150nM) or Sc-Msx2-siRNA (150nM) and OA-promoter-LUC vector. Renilla vector was co-transfected as a control. Transfected osteoblasts were serum starved overnight and then treated with BMP-2 (100ng/ml) for additional twenty four hours. Luciferase expression was quantified. Data is presented as relative luciferase activity for each condition. Percentage decrease in OA promoter activity with transfection of specific siRNAs in BMP-2 treated conditions is shown. E-G. MC3T3-E1 osteoblasts were transfected with Dlx3, Dlx5, Msx2 over-expression vectors or their empty vectors (EV) pCMV, pCMV5, pET-28c vector, respectively. Cell lysates were collected 48 hours after transfection and Dlx3, Dlx5, Msx2 and  $\beta$ -actin protein expression was analyzed by Western blotting. H. MC3T3-E1 osteoblasts were cotransfected either with Dlx3 (1 $\mu$ g), Dlx5 (0.5 $\mu$ g), Msx2 (1 $\mu$ g) over-expression vectors or combination of any two over-expression vectors or their respective empty vectors and OA promoter-LUC vector. Renilla vector was co-transfected and the transfected cells were treated with BMP-2 (100ng/ml) and analyzed for luciferase expression as described above (D). Renilla normalized luciferase activity is plotted on Y-Axis. Percentage increase in the OA promoter activity with transfection of over-expression vectors relative to empty vector transfected osteoblasts in BMP-2 treated conditions is shown. \*p-value <0.001 represent OA promoter activity in BMP-2 (100ng/ml) treated osteoblasts compared to untreated osteoblast cultures.



**Figure 3. Effect of Dlx3, Dlx5 and Msx2 over expression on OA promoter deletion constructs and OA promoter homeodomain mutant constructs.**

A-C. Full-length OA promoter-LUC vector (p962) or deletion constructs of OA promoter-LUC vector (p835, p685, p285) was co-transfected with Dlx3 (1 $\mu$ g), Dlx5 (0.5 $\mu$ g) or Msx2 (1 $\mu$ g) over expression vector or their respective empty vectors (EV) in MC3T3-E1 osteoblasts. Renilla expression vector was also transfected and osteoblasts cultures were treated with BMP-2 as described in (2D). Y-Axis represents relative promoter activity. D-F. OA promoter LUC vector construct either W (p962) or homeodomain mutants (pMH1, pMH2, pMH1+2, pMH3, pMH4) were co-transfected with Dlx3 (1 $\mu$ g), Dlx5 (0.5 $\mu$ g) or Msx2 (1 $\mu$ g) over expression vector or their respective empty vectors in MC3T3-E1 osteoblasts as described previously in (3A). Transfected osteoblasts were treated with BMP-2 and relative luciferase activity is plotted on Y-Axis as described in (3A). \*p-value <0.05, \*\*pvalue<0.01, \*\*\*p-value <0.001 represent OA promoter activity in BMP-2 (100ng/ml) treated osteoblasts compared to untreated osteoblast cultures.



**Figure 4. Binding of Dlx3, Dlx5 and Msx2 proteins to homeodomain binding sites on OA promoter region.**

A. Schematics of position and sequence of wild type probe (W) used for homeodomain protein mobility shift assay. Consensus homeodomain binding sequence (at -852 and -843) in W-probe is underlined. Nucleotides mutated in -852 and -843 homeodomain binding sites in the mutant probe (M) are shown. Arrow represents the transcription start of the OA gene. B. Mobility shift assay. Controls: Lane 1, Biotin-H (1+2)-W without nuclear extract. Gel-Shift: Lane 2 and lane 3, Biotin-H (1+2)-M was incubated with untreated and BMP-2-treated nuclear extracts, respectively. Lane 4 and lane 5, Biotin-H (1+2)-W probe were incubated with untreated and BMP-2 treated nuclear extracts, respectively. Competition assay: Lane 6 and lane 7, BMP-2 treated nuclear extracts were incubated with 100X or 150X-cold-H (1+2)-W probes, respectively. Position of shift is shown with arrow. C. Dlx3 and Dlx5 Supershift Assay. Controls: Lane 1, Biotin-H (1+2)-W without nuclear extract. Lane 2 and lane 3, Biotin-H (1+2)-W probe (with no nuclear extracts) was incubated with Dlx3 and Dlx5 antibody, respectively. Supershift: Lane 4, Biotin-H (1+2)-W probe incubated with BMP-2-treated nuclear extract. Lane 5 and lane 6, BMP-2-treated nuclear extracts pre-incubated with Dlx3 and Dlx5 antibody, respectively, were further incubated with Biotin-H (1+2)-W probe. D. Msx2 Supershift Assay. Control: Lane 1, Biotin-H (1+2)-W without nuclear extract. Lane 2, Biotin-H (1+2)-W probe (with no nuclear extracts) was incubated with Msx2 antibody. Lane 3 contains BMP-2 treated nuclear extracts incubated with Biotin-W-H (1+2) probe. Lane 4, Msx2 antibody was preincubated with BMP-2-treated nuclear extracts, before addition of Biotin-W-H (1+2) probe.

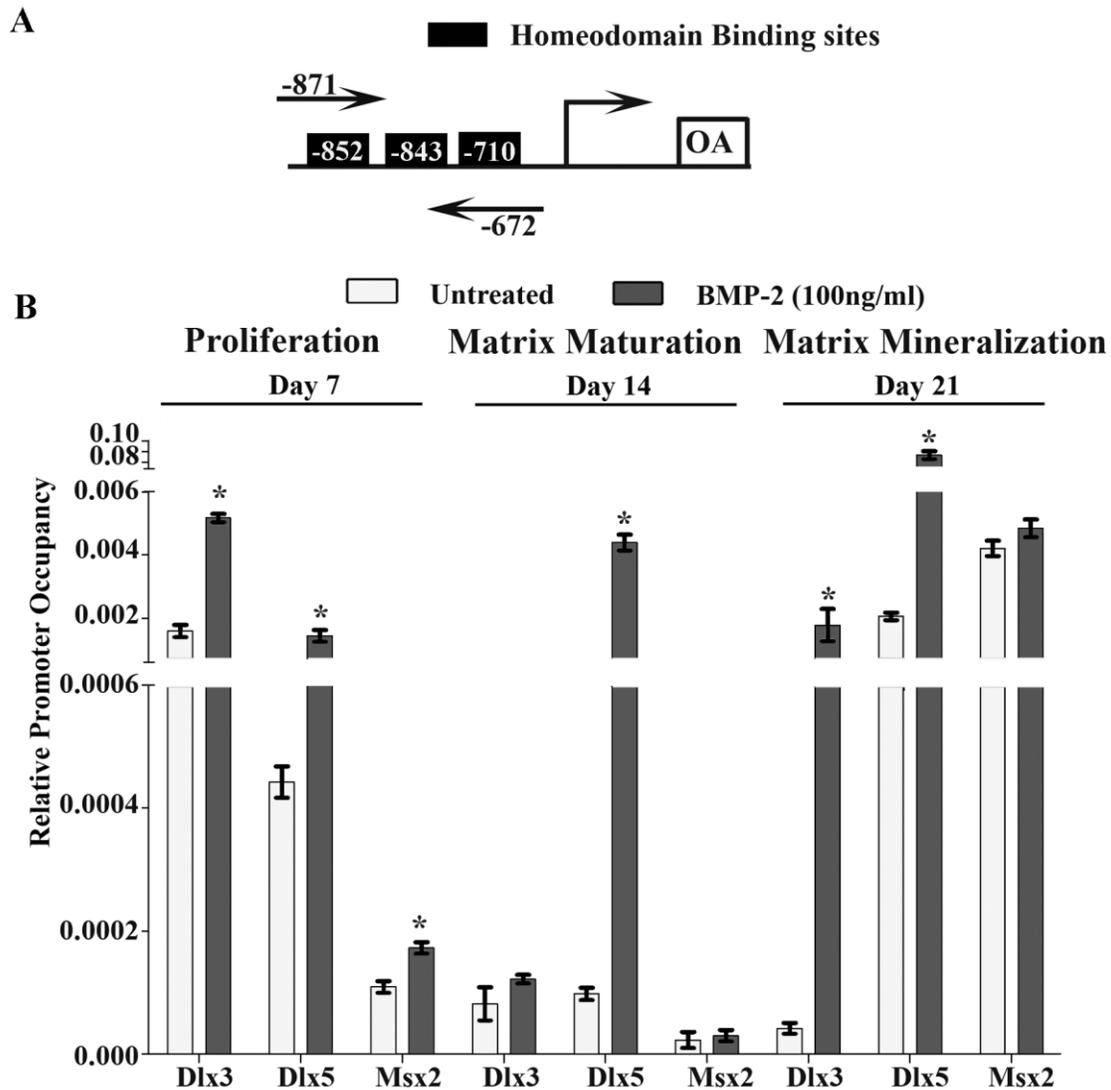


Figure 5. *In vivo* association of Dlx3, Dlx5 and Msx2 proteins with the OA promoter region during osteoblast differentiation.

A. Illustration of OA promoter region amplified for ChIP assay. Arrows mark the location of primers used to amplify OA promoter region. The position of three homeodomain protein binding sites (black boxes) in the OA promoter region is shown. B. Osteoblast cells cultured in differentiating media for 7, 14 and 21 days were untreated or treated with BMP-2 (100ng/ml) and ChIP was performed. \*p-value < 0.001 corresponds to relative promoter occupancy with BMP-2 (100ng/ml) treatment compared with untreated osteoblast cultures.

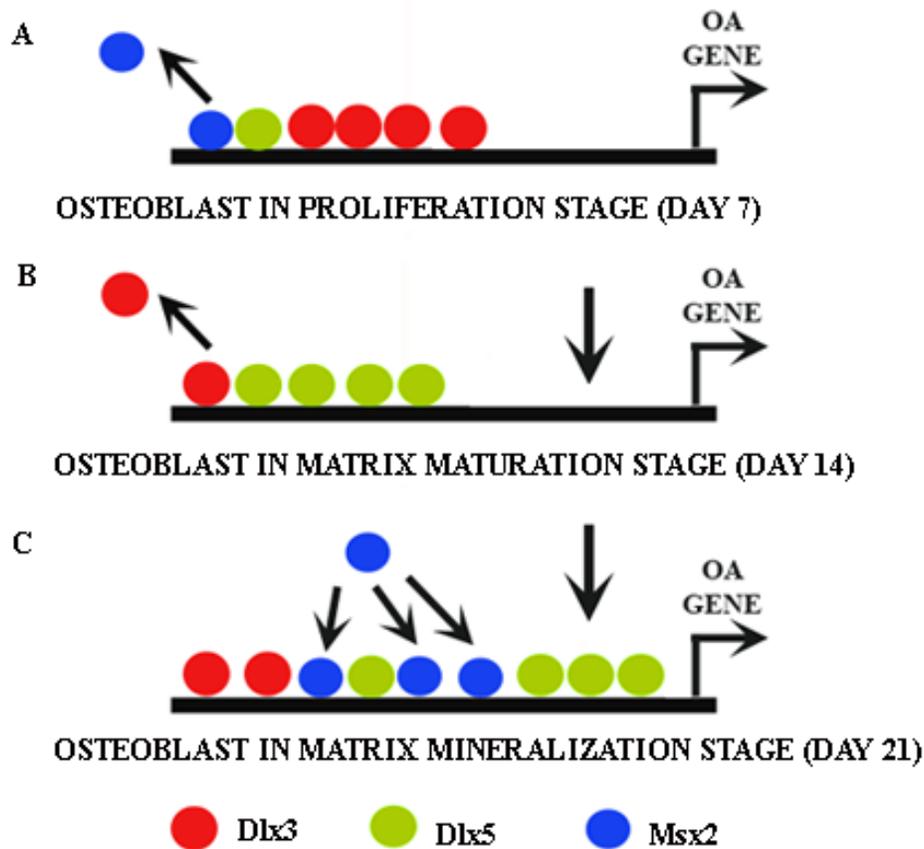


Figure 6. Proposed model for BMP-2-induced OA promoter regulation by homeodomain proteins during osteoblast differentiation.

A. OA promoter occupancy during the proliferation stage of osteoblast differentiation (day 7). BMP-2 treatment stimulated the recruitment of Dlx3, Dlx5 and Msx2 transcription factors to the OA promoter at day 7. The OA promoter was predominantly occupied by Dlx3 transcription factor during osteoblast proliferation stage. B. OA promoter occupancy during the matrix maturing stage of osteoblast differentiation (day 14). As the proliferating osteoblasts differentiate into matrix maturing stage, Msx2 transcription factors is dissociated from the OA promoter and the OA promoter was predominantly associated with Dlx5 transcription factor in response to BMP-2 stimulation. However, BMP-2 treatment did not affect the binding of Dlx3 transcription factor to the OA promoter at day 14. C. OA promoter occupancy during the matrix mineralization stage of osteoblast differentiation (day 21). BMP-2 robustly recruited Dlx5 and Dlx3 to the OA promoter in terminally differentiated osteoblasts.

#### **Research Project 24: Project Title and Purpose**

*Defining a Typology of Low-Literacy African Americans for Colorectal Cancer Screening* - To conduct specialized cluster analyses of data collected under a current NCI R21 grant. The cluster analyses will result in a typology of low-literacy African Americans that will provide the

foundation for development of communication for minority populations as well as expanded future research and grant applications.

### **Duration of Project**

11/18/2009 – 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 25: Project Title and Purpose**

*Understanding Drug Binding to the M2 Channel of the Influenza A Virus* - Funds will be used to support research at the new Institute for Computational Molecular Science (ICMS) in the Temple University College of Science & Technology (CST). The research in question will use computational methods to understand how the so-called M2 proton channel on the surface of the influenza A virus is able to acidify the virus interior as a prelude to replication. The research will also help understand how traditional drugs such as rimantadine act to inhibit the replication cycle of the virus. Importantly, the research will also address the question of how mutations of the flu virus that are involved in the bird flu (so called H5N1) and swine flu (H1N1) enable the flu virus M2 to function in the presence of drugs such as rimantadine. The aim of the computational studies is to inform the rational design of new drugs.

### **Anticipated Duration of Project**

12/1/2009 – 11/30/2012

### **Project Overview**

Novel computer simulation studies will be used to investigate aspects of the way the flu virus functions. The target of the research is the so-called M2 channel, a membrane-bound tetrameric protein bundle that resides in the membrane of the flu virus. This protein bundle serves as a channel for protons that in turn acidify the virion, a process needed to trigger viral replication. The M2 channel has been a target for prophylactic drugs such as amantadine and rimantadine. Unfortunately, mutations in the flu virus have caused these drug molecules to be ineffective against the threat of pandemic flu associated with the H5N1 (Bird Flu) and H1N1 (Swine Flu) mutations of the virus. The recent H1N1 outbreak in the USA makes this work particularly timely.

The computational studies will compare and contrast the structure and dynamical behavior of the membrane-bound M2 protein bundle under different conditions of pH (i.e., different protonation states of channel lining residues). The effect of different mutations will also be studied,

especially with respect to the interaction with drug molecules. The outcome will be a deeper understanding of the function of this key membrane protein associated with the flu.

### **Principal Investigator**

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### **Other Participating Researchers**

Giacomo Fiorin, PhD, Vincenzo Carnevale, PhD – employed by Temple University

### **Expected Research Outcomes and Benefits**

Research in the Institute for Computational Molecular Science (ICMS) in the College of Science and Technology (CST) at Temple University will aim to yield insights into the way the flu virus employs mutations to allow it to render traditional prophylactic drugs ineffective against this target.

The outcomes of this research will be a molecular level understanding of a process that could be key to dealing with the pandemic threat from the flu.

### **Summary of Research Completed**

#### Molecular Dynamic (MD) Simulation Directed Rational Design of Inhibitors Targeting Drug-Resistant Mutants of Influenza A Virus M2.

The progress outlined below has been made through contributions of the PI (one month per year effort) and two postdoctoral research associates: Vincenzo Carnevale (50% effort) and Giacomo Fiorin (50% effort). The research began soon after the ICMS was established at Temple in August 2009. The first year report detailed our initial studies, which have resulted in several publications (e.g., Carnevale *et al*, 2010).

During the past year 2010-2011, we have continued the investigation of the binding mechanism between spiro-piperidine inhibitors and several mutants of the M2 proton channel, in collaboration with the laboratory of Prof. William F. DeGrado at the University of Pennsylvania. This has led to the design of a new potent inhibitor, spiro-adamantane amine, against the drug-resistant V27A and L26F mutants. The simulations summarized below, the molecular synthesis procedure, solid-state NMR experiments and the *in vivo* validation in a plaque reduction assay are fully described in a research paper that we co-authored, currently submitted to *Nature J. Am. Chem. Soc.* (Wang *et al*, 2011).

### *Multiple Hydration Modes of Drug Molecules within the M2 Pore*

Here we performed classical MD simulations of M2 mutants embedded in a hydrated lipid bilayer, in complex with amantadine, spiro-piperidine (bis-cyclohexyl-spiran amine, described in the last year's report), and the more recent bis-cycloheptyl-spiran amine and spiro-adamantane-amine compounds. The recently published 1.65 Å crystal structure (PDB: 3LBW; Acharya *et al.*, 2010) was used as a starting configuration, and each drug was initially placed in the same position as in the solid state NMR structure (PDB: 2KQT) of the amantadine-M2 complex (Cady *et al.*, 2010). Both the wild-type (WT) protein and the V27A mutant were simulated with different drug molecules embedded, each for about 80 ns in total. Figure 1 illustrates three configurations for the WT protein: the drug-free X-ray structure (a), the structure with amantadine bound (b), and with bis-cycloheptyl-spiran amine (d).

To examine the spatial relationship of drug, water, and other atomic groups, we averaged the density of these atoms over snapshots taken between the production phase of the simulations. Two-dimensional density maps were generated by rotational averaging about the pseudo-fourfold symmetry axis of the channel. These density maps allowed us to assess the stability of binding of each drug:M2 mutant complex (Fig 1c,e,f).

We identified several crucial facts. Firstly, drug molecules of the size of amantadine or larger can bind M2 in two configurations, distinguishable by the number of water layers between the His37 side chains and the amino group within a drug's molecule. Amantadine and similar drugs bind with two layers of water molecules to the WT protein (consistently with the solid-state NMR structure by Cady *et al.*, 2010). Instead, the drug-resistant mutants S31N, V27A and L26F exhibit a strong tendency towards displacing one layer of water molecules, and rather hydrating the N-terminal region where proton access the channel pore, thereby neutralizing the binding interactions or the inhibiting power.

A potent and universal inhibitor should be able to bind both the WT protein and these mutants, and also occlude the pore in either the two-layer or the one-layer configurations. We therefore focused on larger molecules than amantadine, which can fill the larger pore of the drug-resistant mutants in a two-layer configuration while binding the WT protein in the one-layer configuration.

### *Rational Design of Spiran-Amine Inhibitors*

The spiro-piperidine compound presented in last year's report presents amantadine-like activity against the wild-type protein, and weak affinity against most drug-resistant mutants (Wang *et al.*, 2009). In collaboration with the DeGrado's lab, we have investigated variants of this molecule by extending either of the two 6-membered rings. One of the most potent compounds obtained is bis-cycloheptyl-spiran amine (7-spiran amine), which binds to the wild-type protein and the V27A mutant with IC50s of 8.7 and 11.3 μM, respectively (to be compared with 16 μM of amantadine against the WT protein).

An explanation for 7-spiran amine's potency can be visualized from Fig. 2, showing the density maps of amantadine and 7-spiran amine against WT and V27A. Amantadine fails to bind a unique site when placed within V27A, and instead switches between the one-layer and the two-layer configurations. Moreover, the apolar adamantane moiety is significantly more hydrated in

the V27A mutant versus WT, explaining the drug's loss in potency. Instead, 7-spiran amine is stably accommodated in a one-layer configuration in the WT, and is shifted towards the N-terminus in V27A, filling the larger cavity created by the mutation at the channel entrance (Fig. 2c). 7-spiran amine is more effective than amantadine at displacing water molecules from the hydrophobic portion of the pore of V27A. However, the diffuse density of the upper ring of 7-spiran amine (Fig. 2d), indicates that, rather than being engaged in a tight complex, the drug molecule experiences a large mobility and does not fully fill the cavity. Thus, we hypothesized that affinity might be increased by further manipulating its apolar group. After several intermediate steps not discussed here (Wang *et al*, 2011), we reached the spiro-adamantane-amine compound (Fig. 2f,g), which has the same binding properties of 7-spiran amine towards WT and V27A. However, unlike 7-spiran amine, spiro-adamantane-amine shows a more clearly defined density at the N-terminal end of the channel, indicating a more stable binding interaction with the protein. Indeed, its IC<sub>50</sub> against V27A is decreased to 0.3 μM. Also, the drug proves effective against WT and L26F as well, with a IC<sub>50</sub>s of 18.7 and 5.6 μM (Wang *et al*, 2010).

### Future Developments

Currently, we are investigating potential inhibitors against the drug-resistant mutant, S31N.

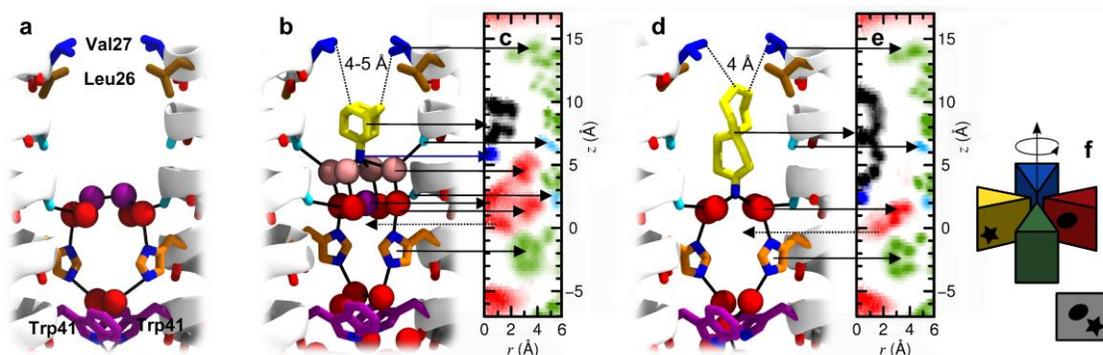


Figure 1 | Layering of water molecules in the M2 channel pore. (a) Structure of the pore of M2 in the 1.65 Å X-ray structure (PDB: 3LBW) at intermediate pH (Acharya *et al*, 2010): the side chains Leu26, Val27, His37 and Trp41 are shown in brown, blue, orange and pink, respectively. Two layers of crystallographically resolved water molecules are shown as red and purple spheres, while backbone carbonyls involved in hydrogen bonds with such waters (Ala30 and Gly34) are in light blue. (b) Representative MD snapshot of amantadine (yellow) within the pore of M2, with water molecules in red, purple and pink. (c) Density map in cylindrical coordinates of the heavy atoms of M2 (green), water (red), and amantadine (black, blue): oxygen atoms of residues 30 and 34 are in light blue. The density is averaged over the four monomers (see (f) for a graphical description of the average over the azimuthal angle shown in the density maps) and over the entire MD trajectory: two spots of the water density are alternatively occupied, and a dashed arrow shows the one not represented in (b). (d, e) MD snapshot and density map of M2 in complex with bis-cycloheptyl-spiran-amine. Note that while amantadine inserts its charged amine group into the outer layer of water molecules (b, c), larger molecules can only be accommodated when such water molecules are displaced (d, e).

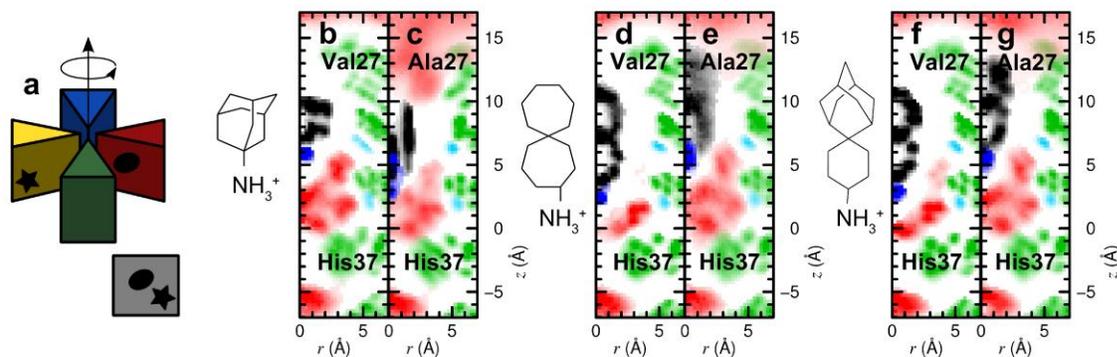


Figure 2 | Density profiles for amantadine, 7-spiran amine and spiro-adamantane-amine. (a) Graphical description of the average over the azimuthal angle shown the density maps. (b-g) Density of protein (green), water (red) and drug (blue, black) heavy atoms, computed from MD simulations, and averaged over the four monomers. Carbonyl groups from Ala30 and Gly34 are shown in light blue. Panels (b) and (c) show densities for the amantadine:WT and amantadine:V27A respectively, (d) and (e) the 7-spiran amine complexes, (f) and (g) the spiro-adamantane-amine complexes.

## **Research Project 26: Project Title and Purpose**

*Improving Cell Therapy for the Damaged Heart* - Loss of cardiomyocytes associated with either longstanding heart failure or acute myocardial infarction could be therapeutically addressed by a treatment that specifically replaces lost myocytes. Currently, a number of clinical trials to test the efficacy of injecting different types of stem cells into the damaged heart are ongoing. Our research is basic in nature and explores the idea that there are already cells within the heart that have the capacity to repair it if it is damaged. The purpose of our research is to modify adult stem cells so that they will repair the damage of the heart after a myocardial infarction.

### **Duration of Project**

7/01/10 – 6/30/2011

### **Project Overview**

We will test the idea that cardiac repair will be enhanced if we use cardiogenic stem cells with a greater potential to survive in the hostile environment of the injured heart and a greater ability to make electrical contact with myocytes in this heart. The first step in the research is to generate genetically modified cardiac stem cells. We have already shown that the cardiac stem cells have the potential to differentiate into a cardiac myocyte when cultured on a feeder layer of neonatal ventricular myocytes. We will use this system to test the idea that we have made new stem cells with an enhanced ability to differentiate into cardiac myocytes and survive hostile environments.

Stem cells will be infected with viruses containing normal and modified gap junctional proteins. We have these reagents in hand. Stem cells will be expanded in vitro, infected with gap

junctional viruses and then plated on neonatal myocytes. We will then measure the percent of these cells that differentiate into cardiac myocytes and over what time course. We have 5 different gap junctional modifications to test. We will determine which of these modifications makes the stem cells best suited for cardiac regeneration. Once these studies are completed we will move on to studies in animal models of cardiac injury.

Cardiac stem cells with documented ability to improve cardiac function will be injected into damaged hearts. These cells will be labeled with molecules that allow us to track these cells. We will determine the ability of these cells to improve cardiac pump function. Noninvasive imaging techniques will be used to quantify cardiac function changes 3, 7 and 28 days after cell injection. At sacrifice we will fix hearts and determine the fate of the injected cells.

These studies have the potential to determine if cardiac stem cells with enhanced engraftment capabilities can increase cardiac regeneration and improve cardiac function.

### **Principal Investigator**

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### **Other Participating Researchers**

Fang Wang, BA – employed by Temple University

### **Expected Research Outcomes and Benefits**

In this study we expect to develop methods to modify adult cardiac stem cell survival and engraftment. Recent studies have shown that when stem cells are injected into the damaged heart, most if not all of these cells die and there is little improvement in cardiac function. Our research will explore novel methods to improve the survival of stem cells and to enhance their coupling to cardiac myocytes in the parent myocardium. Our basic research has shown that in order for a stem cell to differentiate into a cardiac myocyte it must make physical contact with the myocyte. Unfortunately, in the environment of the damaged heart, myocytes uncouple from their neighbors. Our approach will improve stem cell - myocyte interactions that we believe will increase cardiac regeneration. If we are successful we will rapidly translate these techniques to a small scale clinical trial. Our long term plan is to develop more effective therapies for individuals with poor cardiac function secondary to myocardial infarction.

### **Summary of Research Completed**

We have made substantial progress in this research project this year. We have been working in three areas; 1: animal model development and characterization; 2: characterization of modified cardiac precursor (stem) cell lines, and; 3: development of novel reagents to modify stem cell

survival and engraftment.

1: Cardiac injury models: We have now fully established small (mouse and rats) and large (pigs) animal models of cardiac ischemic injury that models the clinical scenario of myocardial infarction, and therapy in humans. Small animal models were developed so that we can develop proof of concept for our ideas. As an example, we are testing the idea that modifying the ability of stem cells to make electrical connections to myocytes on the infarct border zone is essential for cardiac regeneration. We have developed mouse genetic strains that have stem cells with modified gap junctions. These cells will be critical in the early testing that we are about to perform. In addition, we can rapidly test the therapeutic efficacy of multiple cell types in mouse models. These approaches are now fairly high throughput and will give us the ability to define those approaches that might translate into novel therapies. We have performed more than 40 studies in MI mice and have defined the fundamental changes in function during 6 weeks following an ischemic event. These data are essential for comparison as we now test novel therapies. The therapeutic cells that have the most promising effects in mouse MI models will then be tested in a porcine model that we developed this year. Experiments will be performed in adult swine with MI induced by percutaneous balloon catheter placement in the coronary arteries, to induce 60 minutes of ischemia followed by reperfusion. We have shown this year that 80% of the animals survive this procedure, with significant (>25%) decreases in cardiac performance. We are now ready to test novel cells for their ability to improve cardiac function after this injury in this large animal model. If we can show a benefit of these cells in post MI remodeling with improved cardiac function we will begin the process of developing a clinical trial in patients with ischemic heart disease.

2: We have developed techniques to isolate and expand cardiovascular precursor cells from both the bone marrow and the heart. We digest heart tissue and isolate cKit+ cardiac progenitor cells. These cells are then plated in defined media and expanded over the next month. We have developed techniques to infect these cells with lentivirus' containing molecular that can track cell in-vivo (green fluorescent protein). We have performed a series of preliminary studies to show the feasibility of the proposed studies and are about to perform a detailed investigation of the utility of these cells as mediators of enhanced post MI remodeling.

3: We have developed a number of novel reagents that we believe will enhance the survival and engraftment of cardiac and bone marrow derived stem cells in the ischemic heart. We have mutated connexin 43 so that it is resistant to acidosis and ischemia. The theory is that this will allow stem cells expressing this mutant protein to make electrical contacts with myocytes in the infarct border zone, thereby enhancing survival and engraftment.

### **Research Project 27: Project Title and Purpose**

*Determining the Role of c-Abl in Nongenomic Hormone Signaling of Estrogen Receptor Alpha -*  
The purpose of this work will be to determine if the nongenomic signaling of the nuclear hormone receptors contributes to tumorigenesis via activation of both cytoplasmic tyrosine kinases c-Src and c-Abl. The project will deal with the activation of c-Src via nongenomic signaling of nuclear hormone receptors which in turn could cause the activation of c-Abl. This would perhaps define a role for c-Abl in solid tumorigenesis.

## **Duration of Project**

9/01/10 – 6/30/2011

## **Project Overview**

Our goal is to mechanistically derive a singular signaling pathway from cytoplasmic nuclear receptors to Src and Abl as found in solid tumors. To accomplish this we will 1) use mutant constructs of AR, ER, MNAR, Src, and Abl to demonstrate linear signaling; 2) test the role of MNAR, a known scaffold protein as a direct link between c-Abl and the nuclear receptors; and 3) determine the efficacy of both Src and Abl tyrosine kinase inhibitors on hormone-dependent mammary and prostate tumorigenesis. To complete these tasks, MNAR will first have to be cloned to determine its relationship with both Src and Abl. The use of multiple nuclear hormone receptors will also have to be implemented in the study of this signaling cascade. Currently we are focusing on the interaction between ER- $\alpha$  and c-Abl and we plan to biochemically characterize this interaction. This will be executed through a series of experiments including a GST pulldown assay demonstrating the ability of Abl-SH3 to interact with full length human ER- $\alpha$ . In addition to this, we will perform *in vitro* transcription translation binding reactions for both ER- $\alpha$  and c-Abl to determine if the interaction is direct or facilitated by another protein. Using c-Abl P131L in this experiment, we will be capable of verifying the SH3 domain is essential for the interaction of c-Abl and ER- $\alpha$ . To prove the interaction's existence *in vivo* we will transfect various cell lines to transiently express these proteins. At this point, it can be determined whether the interaction is ligand-dependent or independent by treatment with E2. Furthermore, it would be necessary to establish whether this interaction activates the Abl kinase by performing immunoprecipitation experiments with an anti-phospho-Abl antibody and a kinase assay using the GST-Crk substrate. After characterizing the role of Abl, we intend to map the interaction domain of ER- $\alpha$  which we hypothesize is the AF-1 domain. If possible, we would also like to examine the specificity of this ER-Abl reaction and broaden our experiments to include hER- $\beta$ .

## **Principal Investigator**

Scott K. Shore, PhD  
Fels Institute for Cancer Research and Molecular Biology  
Temple University School of Medicine  
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## **Other Participating Researchers**

Meghan E. Jordan – employed by Temple University

## **Expected Research Outcomes and Benefits**

It is expected that we should determine Abl and hER- $\alpha$  interact to initiate the nongenomic hormone receptor signaling cascade, activating both c-Src and PI3K. This interaction may or

may not be E2-dependent. Provided the interaction is E2-dependent, we will elucidate a new role for Abl and a new aspect to a known signaling pathway in cancer. We expect to provide data characterizing a previously unidentified relationship between c-Abl and hER- $\alpha$ , which would progress our knowledge about the pathway, the interaction, and the rationale to inhibit this pathway as a novel approach for treating cancer. By characterizing the interaction of c-Abl and hER- $\alpha$ , we believe we can provide a role for c-Abl in solid tumorigenesis which as of yet has proved to be rather difficult. In doing this, we will provide a singular pathway that can be addressed by a novel therapeutic approach to treat the solid tumors in breast and prostate cancer. Much like Imatinib is used as an Abl inhibitor for Bcr-Abl in patients with chronic myelogenous leukemia, we hope to provide proof-of-principle for a novel therapeutic approach to treat mammary and other steroid hormone-induced solid tumors.

### Summary of Research Completed

A role for ABL in cancer is well established; AMuLV is a murine leukemia virus expressing a truncated c-ABL gene product and the 9,22 translocation gene product BCR-ABL is a human oncogene leading to chronic myelogenous leukemia. Primary breast tumor sections contain phosphorylated c-ABL demonstrating c-ABL signaling is not limited solely to blood cancers. We hypothesize c-ABL activation occurs by an estrogen receptor (ER) dependant mechanism. We propose that protein-protein interaction between ER and c-ABL results in kinase activation and downstream signaling events. The SH3 domain in c-ABL negatively regulates c-ABL enzymatic activity by intramolecular binding. Activation of ABL kinase requires disruption of this association either by sequence deletion (v-ABL) or fusion (BCR-ABL). Given this information, we predicted that the interaction between ER and c-ABL would be SH3-dependent. In our efforts to derive a singular signaling pathway from cytoplasmic nuclear hormone receptors to cytoplasmic non-receptor tyrosine kinases, c-Src and c-ABL we have employed both cell-based and *in vitro* techniques.

In our cell based, *in vivo* experiments, we used both estrogen receptor alpha (ER $\alpha$ ) positive breast cancer cell line, MCF-7, and an ER $\alpha$  positive H1299 cell line that we created via stable transfection (Figure 1). The ER-positive H1299 cell line has increased ER expression as compared to MCF-7 cells. However, this H1299 cell line has less c-ABL expression and approximately even Crk-L expression compared to MCF-7 (Figure 2). Culturing both cell lines in phenol red free media and 10% charcoal stripped fetal bovine serum, we differentially treated the cells with the ER $\alpha$  ligand, estradiol-2 (E2). The cells were serum starved for 48 hours prior to an E2 treatment. To determine the kinetics of ABL activation and possible interaction with ER $\alpha$  in the presence of physiologically relevant E2 concentration, we treated cells with 10 nM E2 over multiple timepoints: 0, 5, 10, 15, 40 and 60 minutes. By examining the total cell lysates of these samples via western blot, we determined that p-Tyr c-ABL levels increased until the 15 minute timepoint which was then followed by a decrease in p-Tyr modification in both the ER+ H1299 cell line (Figure 3) as well as in MCF-7 cells (Figure 4). P-Tyr modification of c-ABL positively correlates with its kinase activity specifically, autophosphorylation of the activation loop stimulates enzymatic activity and substrate phosphorylation. We next sought to determine if following E2 treatment c-ABL was enzymatically active and capable of phosphorylating a downstream substrate, Crk-like (CrkL) protein. Endogenous P-Tyr CrkL was readily observed in cell extracts following E2 treatment by western blotting with a phospho-specific antibody (Figures

3 and 4). To test if the observed CrkL tyrosine phosphorylation was indeed ABL-dependant, we employed two strategies. An *in vitro* kinase assay was performed with immunoprecipitated c-ABL and a purified GST-Crk substrate. Again, maximum phosphorylation of the GST-Crk substrate was at 5 to 15 minutes post-E2 treatment (Figure 5). Secondly we determined P-Tyr CrkL levels in extracts from cells following E2 treatment in the presence of the ABL kinase inhibitor imatinib (Gleevec). Consistent with our other data, E2 treatment increased P-Tyr CrkL levels while pre-treatment with the kinase inhibitor imatinib reduced the levels of P-tyr modified CrkL back to basal levels (Figure 6).

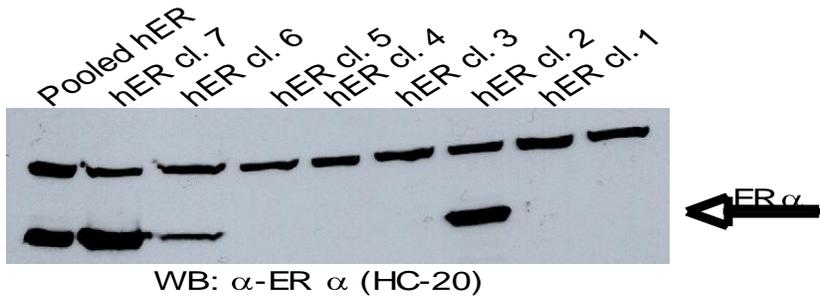
Our results from western blot analysis and the kinase assay supported a ligand-dependant activation of c-ABL by the ER. Both c-ABL and ER are present in the cytoplasm and nucleus, so the *in vivo* determination of both where and when the proteins were in association is an important first step in ultimately understanding the activation mechanism and the biological significance of this interaction.

Cellular fractionation allowed us to separate the nuclear component and extract the cytoplasmic fraction from a large amount of cells. We used immunoprecipitation experiments with cellular fractions prepared from E2 stimulated and unstimulated cells to investigate if there was an interaction between c-ABL and ER $\alpha$  and if this reaction was occurring in the cytoplasm or nucleus. The co-immunoprecipitation (Co-IP) was performed by fractionating cells into cytoplasmic and nuclear fractions using a hypotonic buffer method. Cytoplasmic and nuclear fractions from cells treated with E2 for various times were incubated with anti-c-ABL antibody. Associated ER protein was detected by immunoblotting with ER antibodies. We found that ER $\alpha$  and ABL interact in both the cytoplasm and the nucleus. However, as shown by the immunoprecipitation, the interaction in the nucleus remains intact for a longer period of time than its cytoplasmic counterpart. Immunoprecipitations from both the cytoplasmic and nuclear fractions show a rapid increase in protein-protein interaction in the presence of ligand within approximately 10 minutes. While the cytoplasmic interaction of ABL and ER returns to basal levels within an hour, the interaction of nuclear ABL and ER appears more stable even at the 60-minute timepoint (Figure 7).

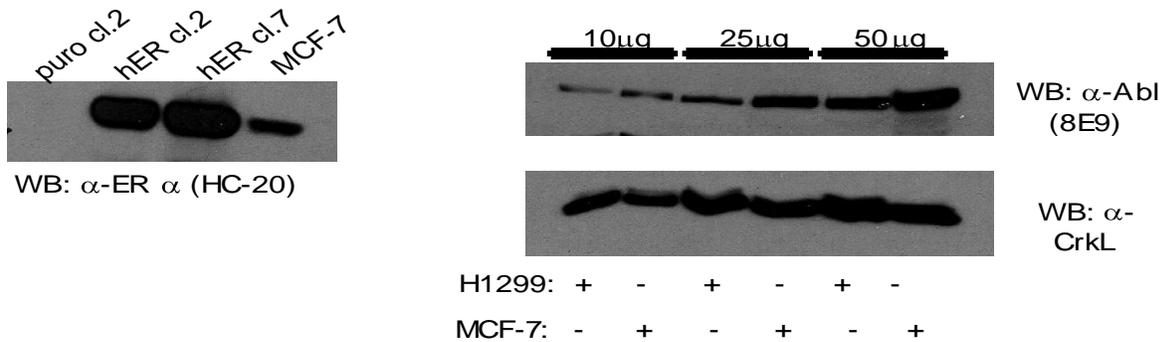
Having shown that c-ABL kinase activity is up regulated in cells treated with E2 when the estrogen receptor is present and ER and c-ABL are present within a dynamic complex in ligand-stimulated cells we tested if the interaction is direct. c-ABL kinase activity is regulated by its own SH3 domain and disruption of the intramolecular SH3 interaction results in activation of c-ABL enzymatic activity. Therefore we tested if there was an interaction between ER and the SH3 domain of c-ABL. Such an interaction could relieve the inhibitory conformation rendering c-ABL an active tyrosine kinase. Glutathione-S-transferase (GST) and GST with the c-ABL SH3 domain cloned in-frame were attached to Glutathione beads. These bead-bound proteins were used to determine if ABL interacts with ER $\alpha$  directly or indirectly in pull-down experiments. Incubation of H1299 ER extracts showed the ER specifically bound to the purified SH3 domain (Figure 8). To investigate the binding further, we again performed pull-downs with extracts from cells this time treated with E2 for either 30 or 60 minutes and used the c-ABL SH3 domain as well as a c-ABL SH3SH2 GST fusion constructs. Again, we find specific binding by ER to the c-ABL SH3 domain in control extracts, but ER binding to SH3SH2 increases in the E2 exposed extracts whereas the SH3 declines (Figure 9). This result suggests that the SH2 domain

of c-ABL may contribute to ER binding, possibly through a P-Tyr in the liganded ER. Using cell extracts cannot allow us to distinguish if the binding is direct or mediated through a scaffolding intermediate. The MNAR protein is a known ER scaffold that has been found to play a significant role in ER c-Src interaction and downstream signaling. We sought to test if the interaction between the c-ABL SH3 and ER was direct by performing a pull-down experiment using in-vitro GST bound c-ABL SH3 was incubated overnight with ER $\alpha$  recombinant protein from rabbit reticulocytes in the GST-binding buffer. The pull downs also allowed us to begin to map the interaction. We found that in vitro translated ER $\alpha$  interacts with the SH3 domain of ABL. In addition, a functional SH3 domain was necessary for the ABL-ER interaction; this was shown by using GST-P131L (mutant) and GST-P131S (wild-type) (Figure 10). These results show that the c-ABL SH3 domain is capable of binding to the ER, most likely directly, but that in vivo binding is more complicated with considerations of liganded ER conformation and structural modification as well as nuclear translocation and additional complexed proteins.

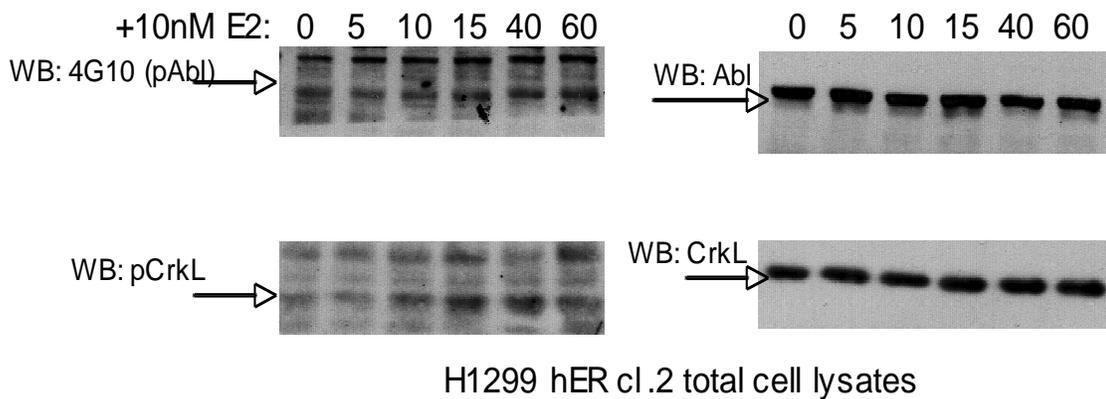
While our results favor a direct binding between c-ABL and ER, involving the SH3 domain, we were curious about a potential role for MNAR. As previously mentioned, MNAR is a scaffold protein that binds to ER in the cytoplasm and interacts with additional signaling protein including Src. MNAR is well expressed in breast epithelial cells but its expression in our H1299 ER<sup>+</sup> cells is very low, if undetectable. To test if MNAR plays a role in c-ABL ER signaling, we performed a transfection experiment whereby MNAR was expressed at super physiological levels from an expression construct. Despite the high level of MNAR expression we could not observe a corresponding increase in P-Tyr CrkL levels after E2 stimulation (Figure 11). Thus we conclude that the activation of c-ABL by liganded ER does not require MNAR and increasing MNAR levels in the cell does not affect the ABL – ER signaling pathway.



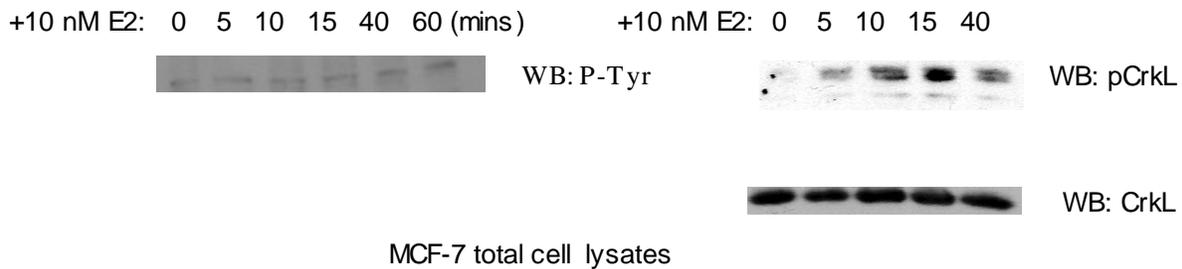
**Figure 1**



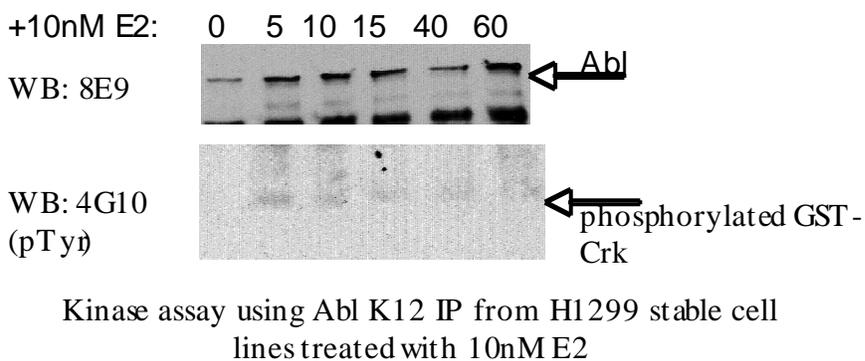
**Figure 2**



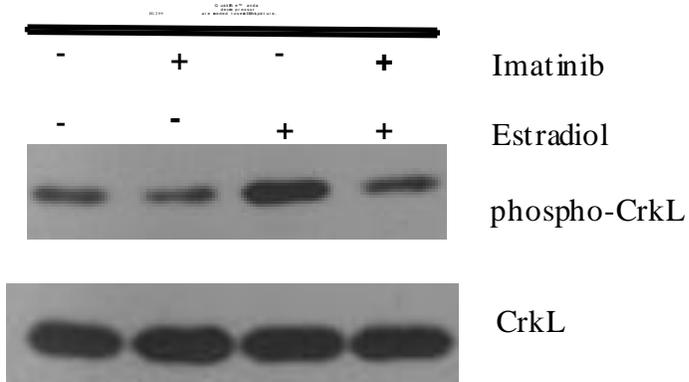
**Figure 3**



**Figure 4**



**Figure 5**



**Figure 6**

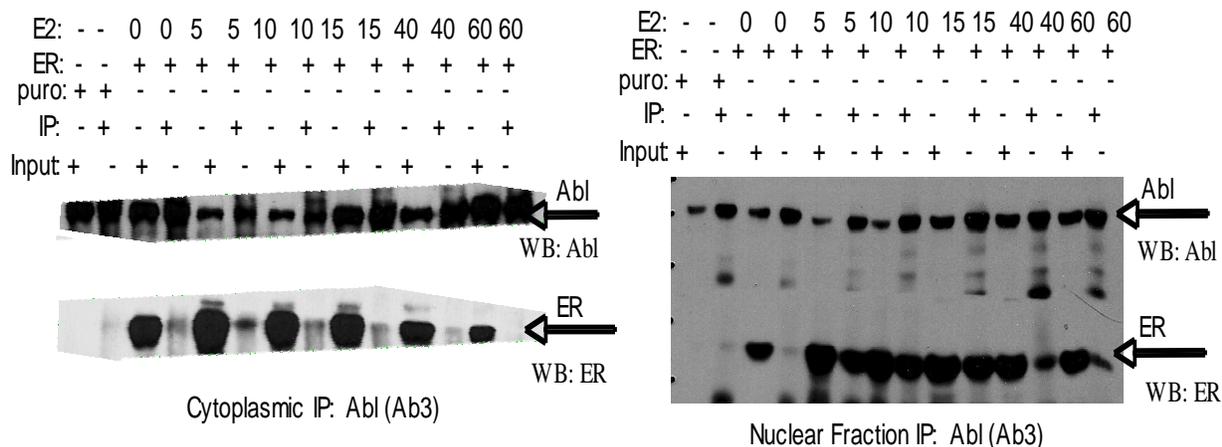


Figure 7

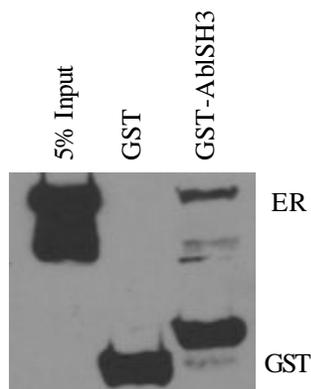


Figure 8

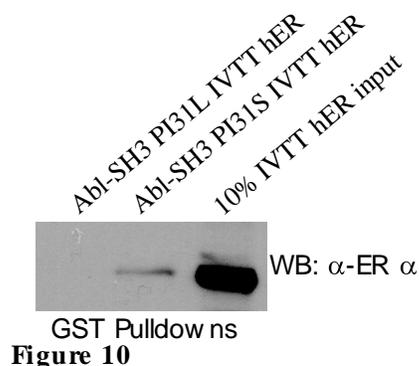


Figure 10

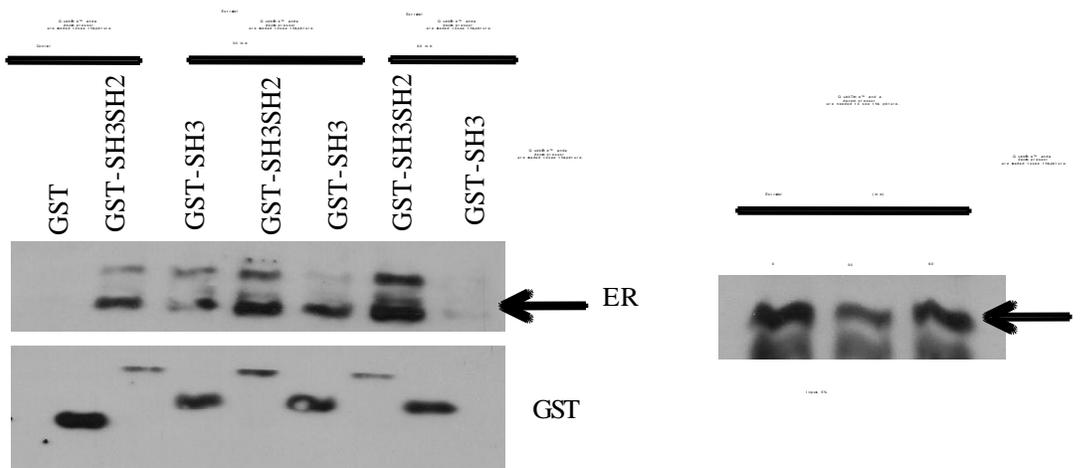
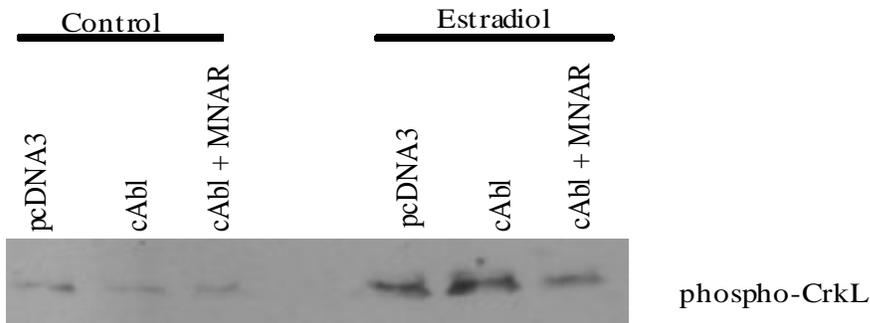


Figure 9



**Figure 11**

### **Research Project 28: Project Title and Purpose**

*Protection from Atherosclerosis in IL-19 Transgenic Mice* - Cardiovascular disease is the number one killer of Americans. Vascular diseases such as atherosclerosis, hypertension, restenosis, and transplant vasculopathy are inflammatory in nature. Very little has been reported on the potential protective effects of anti-inflammatory cytokines on development of vascular disease. We have novel preliminary data which shows that the naturally occurring anti-inflammatory compound, Interleukin-19 (IL-19) can have protective effects on vascular cells grown in culture. The purpose of this project is to determine if IL-19 can reduce atherosclerosis in an important animal model of this disease.

### **Duration of Project**

7/01/2010 – 6/30/2011

### **Project Overview**

Vascular diseases such as atherosclerosis, hypertension, restenosis, and transplant vasculopathy are inflammatory in nature. Although a great deal of attention has been given to the role and effects of pro-inflammatory cytokines, very little has been reported on the potential protective effects of anti-inflammatory cytokines on the vascular response to injury, particularly with respect to direct effects on VSMC pathophysiology. IL-19 is a newly described member of the IL-10 family of anti-inflammatory cytokines. There are three papers which suggest that IL-19 has similar anti-inflammatory effects on immune cells, but we are the only laboratory to publish concerning the expression or presumed function of IL-19 in VSMC pathophysiology or a role in modulation of vascular diseases.

We have preliminary data which indicate that IL-19 is not present in normal vascular smooth muscle cells (VSMC), but can be expressed in response to inflammation and injury. Addition of IL-19 to cultured VSMC reduces their growth, signal transduction in response to inflammatory stimuli, and expression of several inflammatory genes. We have preliminary data in cultured cells indicating that IL-19 modifies post-transcriptional processing of inflammatory gene mRNA. This information has led to the hypothesis that IL-19 can have protective effects on the development of atherosclerosis.

Despite this information NO studies on in vivo effects of IL-19 expression have been reported. We have recently generated an IL-19 VSMC – specific IL-19 transgenic mouse. In this strategy, IL-19 transcription is driven by the SM22 alpha VSMC-specific promoter, and is only over expressed in VSMC. In preliminary experiments, we have shown that the IL-19 knock out mouse has an exacerbated response to a high-fat diet with increased atherosclerosis. These data drive the hypothesis to be tested that the IL-19 VSMC transgenic mouse will have a decreased response to a high fat/high cholesterol diet compared with the control mice, likely as a result of decreased VSMC proliferation.

In Aim 1 we will determine if there will be decreased development of atherosclerosis in VSMC restricted IL-19 transgenic mice fed a high-fat diet. In Aim 2, we will characterize the cellular component of atherosclerotic lesions in the wild-type and transgenic mice by immunohistochemistry.

### **Principal Investigator**

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Associate Professor of Medicine  
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### **Other Participating Researchers**

Stephen Ellison, BS – employed by Temple University

### **Expected Research Outcomes and Benefits**

Vascular restenosis, atherosclerosis, and other vascular diseases are inflammatory in nature, but ultimately depend on vascular smooth muscle cells (VSMC), which make up most of the cellular content of the artery. Accordingly, any compound which may decrease VSMC activation could represent a therapeutic intervention to attenuate most vascular diseases. IL-19 is a compound which occurs naturally in humans and functions to modify inflammatory cells and dampen the immune response. We have previously shown that IL-19 is also turned on in VSMC under inflammatory conditions. We have previously shown that IL-19 has suppressive functions on VSMC which are grown in culture. In this application, we will determine if mice which are genetically modified to always have high levels of IL-19 are protected against atherosclerosis. One outcome we expect is that mice which constitutively express IL-19 will have much less atherosclerosis when fed a high-fat diet. If these experiments are successful, then a major benefit is that IL-19 could represent a new class of naturally occurring therapy to combat multiple vascular diseases.

### **Summary of Research Completed**

AIM 1. Decreased development of atherosclerosis in VSMC restricted IL-19 transgenic mice.

*Rationale and Approach.* In preliminary experiments, we have shown that the IL-19 knock out mouse has an exacerbated response to a high-fat diet with increased atherosclerosis. These data drive the hypothesis to be tested that the IL-19 VSMC transgenic mouse will have a decreased response to a high fat/high cholesterol diet compared with control mice, likely as a result of decreased VSMC proliferation.

*Results.* At 16 weeks feeding a high-fat atherogenic diet, the entire length of the aorta was removed, and a longitudinal cut through the inner curvature of the aortic arch exposes the intimal surface. En face preparations of the aortic surface were stained for lipid with oil red O, and lesion size was quantified by image analysis software. Lesion size is defined by the percent of a standardized area of the intimal surface covered by atherosclerotic lesions.

Figure 1 shows representative aorta from wild-type control mice compared with IL-19 transgenic mice and shows significantly less aortic plaque in the transgenic mice compared with control mice. Thus far, four control mice and seven IL-19 transgenic mice have been fed, aorta processed, and images analyzed and atherosclerosis quantitated. Figure 2 is quantitation of this data, and shows that atherosclerotic burden is significantly decreased in the IL-19 Tg mice compared with control mice (12.88 $\pm$ 2.8 % Vs 3.71 $\pm$ 0.96% for control and IL-19Tg mice, respectively,  $P=0.004$ ). At present, an additional seven mice from each group are being processed and imaged; we should have data within three weeks.

#### AIM 2. Cellular characterization of atherosclerotic lesions in AIF-1 transgenic mice.

*Rationale and Approach.* We will characterize the cellular component of atherosclerotic lesions in the wild-type and transgenic mice by immunohistochemistry to link IL-19 expression with decreased VSMC proliferation, and decrease in inflammatory cell burden in vivo.

*Results.* For inflammatory cells, immunohistochemical staining was performed using antibodies directed against smooth muscle cell  $\alpha$  actin, and anti-leukocyte common antigen CD45. For all experiments, at least 4 cross sections, 100 $\mu$ M apart, were used for immunohistochemistry. Sections were counter stained with hematoxylin and specific protein expression or localization was determined by the number of positively stained cells per high powered field and expressed as a percentage of total cells counted. At least four sections from at least three mice were examined.

Figure 3 shows representative sections from aortic root of control and IL-19 transgenic mice. These were immunostained with SMC actin to identify smooth muscle cells, and also with Oil Red O, to identify foam cells. Figure 4 shows that the IL-19 Transgenic mice have significantly less smooth muscle cell foam cells compared with control mice (37.0 $\pm$ 9.1 Vs 15.1 $\pm$ 5.4 cells per high powered field,  $P<0.01$ ). These same sections were immunostained with anti-CD45 antibody to identify inflammatory cell infiltrate. Figure 5 shows significantly less inflammatory cell infiltrate into aortic root lesions in IL-19 transgenic mice compared with control mice (28.2 $\pm$ 0.9 Vs 17.4 $\pm$ 1.1,  $P <0.001$ ).

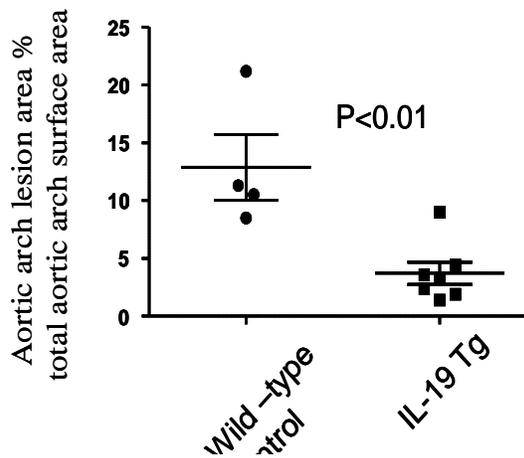
Together, these data indicate that IL-19 has a protective effect against atherosclerosis, quite possibly by reducing SMC foam cell number and inflammatory cell infiltrate. As more mice are

entered into the study we will include that data as well.

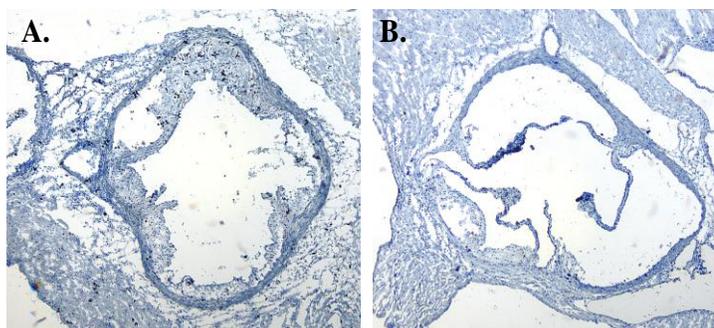
**Figure 1.** representative en face staining for atherosclerotic Plaque in mice. A is wild-type control, B is IL-19 SM22 transgenic. Both mice fed high-fat diet for 16 weeks.



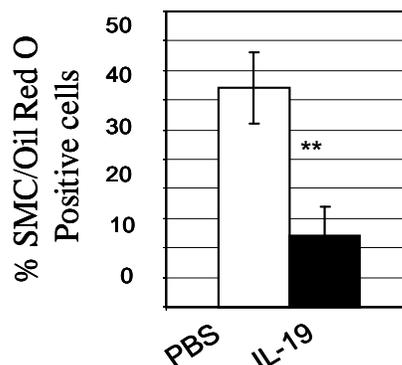
**Figure 2.** quantification of atherosclerotic burden in mice. Atherosclerotic lesions were quantitated by Image-analysis morphology in control (n=4) and IL-19 Transgenic mice (n=7).  $P<0.01$



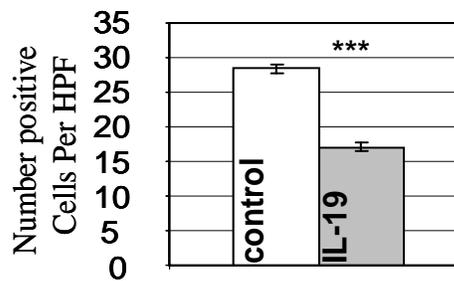
**Figure 3.** representative histological staining for atherosclerotic Plaque in aortic root from mice fed high-fat diet for 16 weeks.. A. is wild-type control, B. is IL-19 SM22 transgenic. Both mice fed high-fat diet for 16 weeks.



**Figure 4.** Reduction in SMC positive foam cells from Figure 3. ( $P<0.01$ )



**Figure 5.** Reduction of CD45 positive Inflammatory cells quantitated from Figure 3.  $P<0.001$ .



## **Research Infrastructure Project 29: Project Title and Purpose**

*Biology Department Core Facility Infrastructure* - Temple University's Department of Biology is undergoing a significant expansion to invigorate its research environment and to improve the education of the next generation of biological scientists. To advance biomedical research, two new animal facilities were built and opened: a Biosafety Level 1 Zebrafish Facility and a Biosafety Level 3 Mouse Facility. While these improvements have strongly advanced Temple's research capabilities, we still require various new instrumentation as well as updates to our current research inventory. The proposed infrastructure project includes the renovations necessary to accommodate new equipment to be used by faculty from the Department as well as the larger College of Science and Technology.

### **Anticipated Duration of Project**

12/1/2010 – 9/30/2011

### **Project Overview**

The objective of the proposed infrastructure project is renovation of space and installation of equipment in the Department of Biology at Temple University. Renovation involves removal of old, outdated equipment, demolition of existing interior walls, removing existing sinks and plumbing, constructing and finishing new interior walls, installing new, additional electrical power capacity, internet drops, distilled and regular water supply lines, drain lines, ventilation, and built-in tables/shelving for equipment. The project includes purchase and installation of a scanning electron microscope (SEM), 40X plan Apo lens, tabletop centrifuge, glassware washer, and ice maker.

### **Principal Investigator**

Joel B. Sheffield, PhD  
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Temple University  
Department of Biology  
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Philadelphia, PA 19122

### **Other Participating Researchers**

None

### **Expected Research Outcomes and Benefits**

The outcome of the proposed project is to provide the physical space and related services (electric, plumbing, ventilation, internet connectivity) necessary to accommodate modern research equipment necessary to enhance the capacity of Temple University researchers to conduct a wide range of biological research involving biochemical analysis. This includes

research related to the cause and treatment of disease, species-specific reproductive innovations, and neural and cardiac development.

### **Summary of Research Completed**

Project 29 was an infrastructure proposal to acquire and install specific pieces of equipment that would be used as part of central facilities for the Department of Biology as well as other departments within the College of Science and Technology. Since there was a long delay between the submission of the proposal and the receipt of the funds, we have had to make certain adjustments in order to remain within the budgetary constraints. Ultimately, we chose to install the following facilities:

1. A Spectrofluorimeter
2. An Ice Machine
3. A Glassware Washer
4. A Desk-Top Scanning Electron microscope.

We have made significant progress in obtaining and installing the instruments. All instruments have been delivered, and all have been installed, except for the Scanning Electron Microscope. That instrument has been delivered, and installation is scheduled for July 19, 2011. The outline below indicates the specific machines and the additional physical modifications that were necessary for their installation.

#### Ice Machine:

*Scotsman Model number AFE325AS1B*

*SN 09051320012485*

Remove old machine; install new plumbing

#### Spectrofluorimeter

*Horiba Fluoromax 4 Spectrofluorometer*

No infrastructure improvements needed

#### Glassware Washer

*Lancer Model number 1300 LX*

*SN 1C033982*

Disconnect and remove old machine

Install new 240 V supply

Install new hot water line, new cold water line, new demineralized water line

#### Scanning Electron Microscope

*SEM - Agilent 8500 SEM*

Removal of sink and cabinet

Removal of air, gas supplies, and pipes

Installation of Electric Receptacles, two connected to the emergency circuit

Installation of desktop

Paint walls