University of Pennsylvania

Annual Progress Report: 2009 Formula Grant

Reporting Period

July 1, 2013 – December 31, 2013

Formula Grant Overview

The University of Pennsylvania received $9,897,440 in formula funds for the grant award period January 1, 2010 through December 31, 2013. Accomplishments for the reporting period are described below.

Research Infrastructure Project 1: Project Title and Purpose

Renovation for Lab Space for the Fundamental Physics Techniques to Biomedicine - The purpose of this project is to upgrade and renovate 4750 square feet of laboratory space to standards appropriate for a 21st century laboratory performing research at the interface between the physical and biological sciences. The renovations include outfitting the laboratory space with sophisticated instrumentation, including a two-photon microscope and a high-powered laser light source. The renovated space will be occupied by investigators from the Department of Physics & Astronomy, whose research has implications for biomedicine, including work at the frontiers of diagnostic imaging using infrared light as well as the use of innovative techniques to make measurements of unprecedented precision aimed at understanding how genes guide basic process of development of an organism.

Duration of Project

1/1/2010 – 8/30/2012

Summary of Research Completed

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

Research Infrastructure Project 2: Project Title and Purpose

Development of Laboratory Space at Penn Medicine – Research Infrastructure - The purpose of this research infrastructure project is to renovate space in Stemmler Hall in support of Penn Medicine's Musculoskeletal and Neurosciences research programs. These closely related research programs are critical components of Penn Medicine's research strategic plan and address key needs common to Penn, the NIH road map, and the Commonwealth of Pennsylvania. The project will provide 4,900 SF of renovated laboratory space, creating an interactive

environment that enables the recruitment of new faculty, promoting the study of musculoskeletal and neurosciences issues, and ultimately leading to the translation of health advances for the benefit the citizens of the Commonwealth.

Duration of Project

1/1/2011 – 3/1/2013

Summary of Research Completed

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

Research Project 3: Project Title and Purpose

Generating Tissues from Stem Cells in the Pathways to Therapy - New technologies in stem cell biology, genetic manipulations and tissue engineering are enabling us to move closer to the dream of humankind of repairing tissues and organs that have been lost as a result of injury, disease or aging. In this application, we propose studies that will increase our knowledge of stem cells that are involved in heart, lung, cartilage and tendon development and/or maintenance that will be utilized to direct, promote or enhance the repair of these organs and tissues. The challenges in regenerative medicine are enormous and we need to gain more knowledge of the cells involved in these processes so that effective and safe therapies can be developed.

Duration of Project

1/1/2010 – 12/31/2012

Summary of Research Completed

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

Research Project 4: Project Title and Purpose

The Role of CCR5 Blockade on the Prevention of Graft-Vs-Host Disease - Allogeneic stem cell transplantation (SCT) is the only cure for many patients with hematologic malignancies, but success is limited in large part by graft-vs-host disease (GVHD). There is an immediate need for more effective methods of GVHD prevention. Experimental data suggest a role for the chemokine receptor CCR5 in the pathogenesis of GVHD. Inhibition of lymphocyte trafficking to prevent GVHD explores a new paradigm in the pathophysiology of GVHD. The purpose of this project is to test the hypothesis that blocking lymphocyte trafficking using Maraviroc, an inhibitor of the chemokine receptor CCR5, after allogeneic SCT is feasible, safe and effective in

preventing GVHD. Our long term goals are to understand the role of lymphocyte trafficking in the pathogenesis of GVHD.

Duration of Project

1/1/2010 – 12/31/2013

Project Overview

Based on compelling clinical observations and our preliminary data, we will pursue a clinical trial to determine the feasibility, efficacy and safety of Maraviroc, a CCR5 inhibitor, as part of GVHD prophylaxis in patients undergoing reduced-intensity conditioned (RIC) allogeneic SCT. We will also explore correlative immunologic studies to assess immune reconstitution after transplant. This project is designed to test the role of CCR5 inhibition on GVHD prophylaxis, and to enhance the understanding of the role of lymphocyte trafficking in clinical transplantation. The specific aims of the current project are:

Aim 1: Determine the dosing, safety, and feasibility of the oral CCR5 inhibitor Maraviroc in combination with conventional tacrolimus and methotrexate (tac/mtx) as GVHD prophylaxis in patients undergoing reduced intensity (RIC) allogeneic SCT. This will be a prospective phase I/II, single arm, single-center trial.

Patients will receive Maraviroc beginning after the last dose of the conditioning regimen (day-2) until day +30 in addition to a standard GVHD prophylaxis regimen of tac/mtx. Patients will undergo a 7 time point detailed pharmacokinetic analysis. Toxicity will be monitored and recorded using the NCI CTCAE 3.0 criteria.

Aim 2: To assess the efficacy of the CCR5 inhibitor Maraviroc as GVHD prevention in combination with conventional tac/mtx during RIC allogeneic SCT. This aim tests the hypothesis that inhibition of lymphocyte trafficking through CCR5 inhibition early after allogeneic SCT will decrease the incidence of acute GVHD.

Aim 3: To explore the effect of CCR5 inhibition on post-transplant immune reconstitution and on biological surrogates for GVT activity, and identify additional candidate chemokine receptors that can be used as therapeutic targets in GVHD prevention.

<u>Subaim 3a</u>: Determine the effect of the CCR5 inhibition on immune reconstitution after RIC SCT to test the hypothesis that the CCR5 inhibitor Maraviroc promotes immune reconstitution following RIC SCT.

<u>Subaim 3b</u>: Determine the effect of Maraviroc on in vitro surrogates for graft-vs-tumor (GVT) activity. We hypothesize that CCR5 inhibition preserves the GVT effect after allogeneic SCT. We will identify and compare induction of tumor-specific immunity in patients undergoing RIC SCT with and without the CCR5 inhibitor Maraviroc using: 1) antigen-specific T-cell assays; 2) functional T-cell studies; 3) tumor-specific T-cell assays.

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Other Participating Researchers

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Expected Research Outcomes and Benefits

Allogeneic stem cell transplant (SCT) is the only curative therapy for many patients with hematologic malignancies, but success remains limited in large part by GVHD. GVHD occurs in approximately 50% of matched related donor transplants and 50-70% of unrelated donor transplants. It is a major cause of mortality from either direct organ toxicity or because the intensive immune suppression used to prevent and treat GVHD results in extensive mortality from infectious and other complications.

Universally, the most effective methods of GVHD prevention also inhibit graft-vs-tumor (GVT) activity and increase relapse rates. Because of increased relapse rates and complications related to poor immune function, there has been little improvement in outcome after allogeneic SCT by intensifying GVHD prophylaxis or by just using different drugs that still primarily kill T cells. The paradigm of preventing GVHD through extensive killing of donor T cells has largely been exhausted and must shift to allow for preservation of GVT activity and immune reconstitution.

Methods that inhibit GVHD initiation without killing T cells, permit rapid immune reconstitution, and preserve GVT activity would represent a novel and extremely important advance in transplant therapy and will have many implications for future allogeneic SCT. Toxicity from GVHD prophylaxis, and risks of morbidity and mortality from GVHD after SCT can be minimized. Furthermore, GVT activity may be preserved. In addition to improving outcome for current transplant candidates, development of more effective and safer therapies to prevent and treat GVHD is imperative since this would open up application of potentially curative therapy to many more patients presently unable to withstand toxicities associated with transplant, even with a RIC approach.

Summary of Research Completed

Completion of Aims 1 and 2 has been previously reported.

Aim 3 has been completed as well as we have now been able to study immune reconstitution and begin preliminary studies involving other chemokine receptors that may be used as targets for GVHD prevention. In aim 3a we studied immune reconstitution by looking at lymphocyte counts and recent thymic emigrants. We were able to compare these to a similar group of concurrent

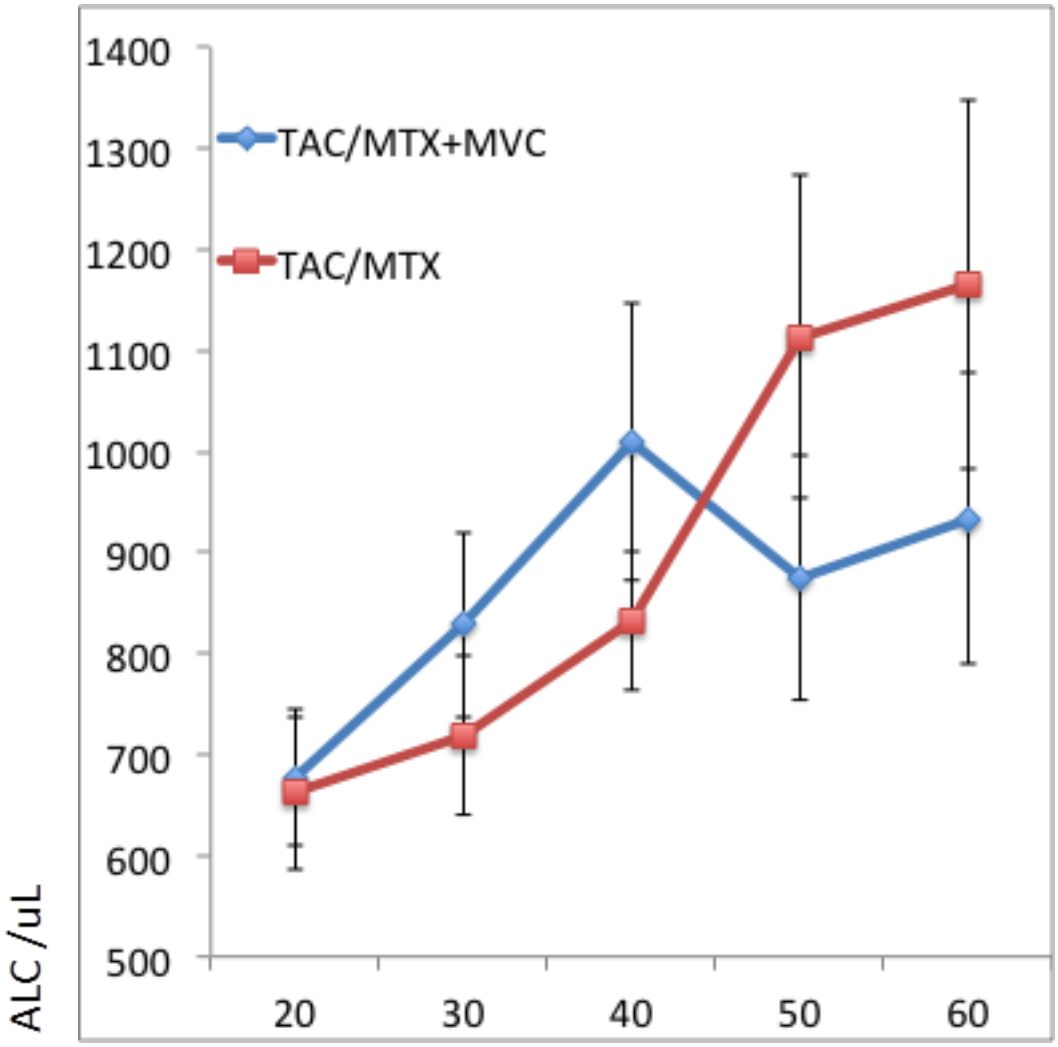
control patients treated in a similar way but without the addition of maraviroc for GVHD prophylaxis. Figures 1 and 2 suggest higher lymphocyte counts (Y axis) at day 30 and day 40 (X axis). Available data suggests that rapid lymphocyte recovery is associated with lower risk of subsequent relapse.

Furthermore there was a higher percentage of recent thymic emigrants (as tested by studying the percentage of CD31 positive T cells at day 30) in recipients of maraviroc as shown in Figure 2. This is logical since data suggests that CCR 5 prevents trafficking to the sinus. CCR 5 blockade might promote T cell trafficking to the thymus and enhance the immune recovery.

For aim 3B we studied recovery of T cell chimerism as a surrogate for graft versus tumor activity. Maraviroc had no impact on recovery of donor T cells when compared to our control population. We were also able to show that T cells exposed to maraviroc retained functional activity using cytokine release assays and in vitro cell killing. We were not able to compare tumor-specific immunity however. This is been very difficult to do in this or other settings.

To complete aim 3 we have begun to study other potential candidate chemokine receptors that may be used as therapeutic targets in GVHD prevention. Using microarray analysis for gene expression profiling, we studied samples collected and banked from this trial from a small number of patients who had no GVHD at the time of sampling. Four of these patients went on to develop GVHD within 14 days and four of these patients had no GVHD for at least 2 months after sampling. We identified 879 differentially expressed genes. Among the highest differentially expressed genes in patients destined to develop GVHD were CCR 5 as well as CCR 2. CCR 2 is another important chemokine receptor necessary for lymphocyte trafficking. The differential expression profiles are shown in Figure 3.

Based on these results, we performed hematopoietic stem cell transplant in mice using donors that were deficient in CCR2. Preliminary data confirmed older data in the literature showing that T cells that do not express CCR 2 resulted in less GVHD than control T cells. There is an available dual CCR 2/CCR 5 antagonist currently in phase II trials for the treatment of HIV. The drug is called cenicriviroc. We have been in discussions with Tobira Therapeutics, the company that has rights to this compound, about potential funding for future studies looking at dual inhibition of CCR 2/CCR 5. These discussions are ongoing at the time of this report.


1400
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ALC /uL
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Figure 1.

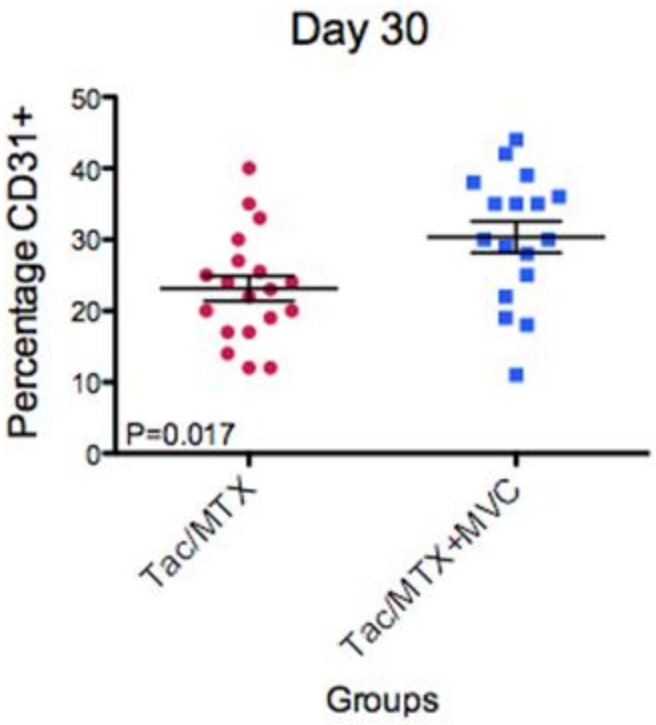

Figure 2.

IMPORTANT LYMPHOCYTE TRAFFICKING GENES:

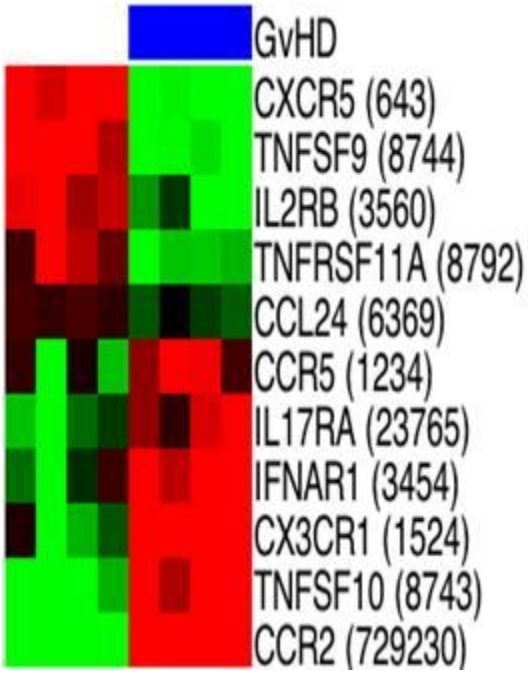

Figure 3.

Research Project 5: Project Title and Purpose

Does Neural Activation During Nicotine Abstinence Predict Smoking Relapse? - The purpose of this research is to determine whether patterns of brain activity, assessed using functional MRI at rest and during performance of neurobehavioral tasks, predict smoking relapse over an 8-week observation period among smokers who wish to quit smoking. If the results of this study are positive, this work would lay the foundation for the refinement and validation of functional neuroimaging as a bio-marker of smoking relapse. Such novel approaches can be used to identify smokers at greatest risk for relapse and for screening novel medications under development to treat nicotine addiction.

Duration of Project

6/1/2010 – 12/31/2013

Project Overview

Our previous work demonstrated that, among non-treatment seeking smokers, regional cerebral blood flow (rCBF) is increased following 14 hours of overnight abstinence, as compared to smoking as usual. Specifically, increased rCBF was observed in the anterior cingulate, medial and left orbitofrontal cortex. Two smoking urges items ("cravings for a cigarette" and "urges to smoke at this time") that predict relapse were strongly correlated with CBF increases in several regions that comprise the brain's reward and visual circuitry. In the current study, we propose to extend these findings by: (1) testing whether the magnitude of rCBF after overnight abstinence in the reward circuitry predicts smoking relapse after 8 weeks, and (2) whether BOLD fMRI during performance of validated neurocognitive tasks predicts smoking relapse. One hundred participants enrolled in this trial will be invited to complete two MRI scans (one smoking as usual and one after 14 hours of abstinence from nicotine; order counterbalanced). Following the fMRI scans, they will receive smoking cessation counseling and be followed for 8 weeks to confirm smoking status. Identification of the neural substrates of relapse following a quit attempt could inform the development of novel medications. Further, the identification of a "brain signature" that predicts relapse may allow for the use of neuroimaging to screen novel medications and identify those that reverse the liability profile.

Principal Investigator

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Other Participating Researchers

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Expected Research Outcomes and Benefits

Tobacco use is the greatest preventable cause of morbidity and mortality in the U.S. Despite decades of research to develop better treatments, only one in four smokers is able to quit smoking and remain abstinent. The current study will determine whether resting cerebral blood flow and regional brain activation during performance of cognitive tasks during nicotine abstinence predicts relapse in smoking cessation treatment. A positive result would provide initial support for neuroimaging as a tool to predict smoking relapse liability and possibly for early screening of novel medications for nicotine dependence. Given the need for improving the outcomes of smoking cessation treatment, this approach could have a significant health benefit by: (1) identifying high risk smokers who require more intensive treatment; and (2) using neuroimaging to tailor therapy to individual smokers' needs, thereby improving outcomes.

Summary of Research Completed

A total of 82 smokers completed two fMRI scans (total of 164 fMRI scans). Given the data showing the expected low quit rates for counseling only (no medication), we focused our analyses on early quitting success (first 7 days). This measure has been shown to be highly predictive of quitting success at 6 months.

<u>Large-Scale Brain Network Coupling Predicts Acute Nicotine Abstinence Effects on Craving and Cognitive Function</u>: We tested the hypothesis that the strength of coupling among 3 large-scale resting state networks – salience, executive control, and default mode – will reflect the state of withdrawal and predict abstinence-induced cravings and cognitive function. For this analysis, we developed and tested a composite measure of connectivity between these networks, examined abstinence effects, and used this to predict clinical outcomes.

As shown in Figure 1, four networks were generated from group Independent Component Analysis (gICA) of the resting state data identified as the Salience Network (SN), Default Mode Network (DMN), and left/right Executive Control Network (ECN). Spatial maps were converted to *z*-score images and then thresholded at $Z = 3.5$ via a mixture model fit. Network maps are displayed in red-yellow overlaid onto the Talairach standard brain based on neurological (left = left) convention. The key SN regions included dorsal ACC/paracingulate gyrus (Talairach coordinates: 3, 21, 33), bilateral anterior insula (L: -39, 9, 3; R: 51, 15, 0), DLPFC (L: -30, 45, 30; R: 30, 48, 30), supramarginal gyrus (L: -57, -45, 30; R: 57, -42, 33), and precuneus (12, -69, 36). Together the LECN and RECN covered bilateral DLPFC (L: -42, 45, 6; R: 45, 33, 25), parietal cortices (L: -39, -51, 42; R: 39, -57, 48), middle temporal gyrus (L: -51, -54, -9; R: 51, -57, -9), and dorsal medial frontal gyrus (L: -6, 27, 45; R: 6, 30, 45). The DMN included PCC (6, -57, 27), bilateral angular gyrus (L: -42, -69, 33; R: 48, -63, 30), vmPFC/rostral ACC (3, 54, 3), and temporal gyrus (L: -57, -9, -15; R: 60, -18, -15).

In Figure 2, rendered brain figures illustrate network components identified by group ICA. Compared with smoking, the Resource Allocation Index (RAI) m_L decreased in abstinence ($p = 0.002$); m_R showed a decreasing trend ($p = 0.04$). The thinner width of the path connecting Salience Network and Executive Control Network (SN-ECN), as well as the path connecting Salience Network and Default Mode Network (SN-DMN), in abstinence (vs. smoking) illustrates

decreased coupling between the networks.

As shown in Figure 3, there was a significant reduction in correlation between Salience Network and Default Mode Network (SN,DMN) (p = 0.02) in abstinence versus in the smoking condition.

Figure 4 shows partial regression plots of the difference of QSU-brief urge score ΔQSU_{a-s} against the difference of the Resource Allocation Index (RAI) $\Delta m_{R,a-s}$ between the abstinence and smoking sessions. As the RAI measure of connectivity differences in abstinence (vs. smoking) was reduced, craving symptoms in abstinence (vs. smoking) were increased. In addition, there was a significant negative correlation between the RAI change and change in BOLD signal during subsequent performance of a working memory task in the vmPFC and PCC; these results suggest that as connectivity decreased in abstinence versus smoking at rest, there was less suppression of these default mode network regions during the working memory task. Thus, as hypothesized, we have linked resting state brain activity with clinical symptoms that promote smoking relapse.

The findings of this study (*JAMA Psychiatry*, in press) provide novel evidence that resting state functional connectivity between the salience network and the executive control and default mode networks is decreased during abstinence. Importantly, the greater the reduction in coupling, the more severe the cravings to smoke (an important clinical symptom that predicts relapse). Further, resting state measures predicted working memory-related BOLD signal, specifically the ability to suppress regions in the default mode network in which activity is associated with task-irrelevant thoughts. In conclusion these data support the clinical predictive value of a novel resting state neuroimaging biomarker.

We conducted further analysis from this study to test the hypothesis that increased working memory related BOLD signal in task positive regions and suppression of activity in default mode network regions would predict short term smoking relapse. Seventy-two treatment seeking smokers completed two fMRI sessions (smoking as usual vs. 24hr abstinent) during performance of a visual N-back task, followed by brief counseling and a short-term quit attempt. Relapse during the first 7 days was biochemically confirmed by the presence of the nicotine metabolite cotinine. Mean percent signal change was extracted from *a priori* regions of interest: bilateral DLPFC, MF/CG, PCC, and vmPFC. Signal from these brain regions and additional clinical measures were used to model outcome status, which was then tested with resampling techniques for validation.

As shown in Figure 5, relapse to smoking was predicted by increased withdrawal symptoms, decreased DLPFC and increased PCC activation (abstinence minus smoking). ROC analysis demonstrated 81% correct classification of relapses for the full model, a significant improvement over the model with clinical variables only.

We conclude that abstinence-induced decreases in left DLPFC activation and reductions in suppression of left PCC activation is a marker for smoking relapse. Mechanisms identified are potential targets for development of neuroscience-based interventions for smoking behavior change. A manuscript has been submitted for peer review at *Biological Psychiatry*.

Figure 1. Map of resting state networks identified by group ICA

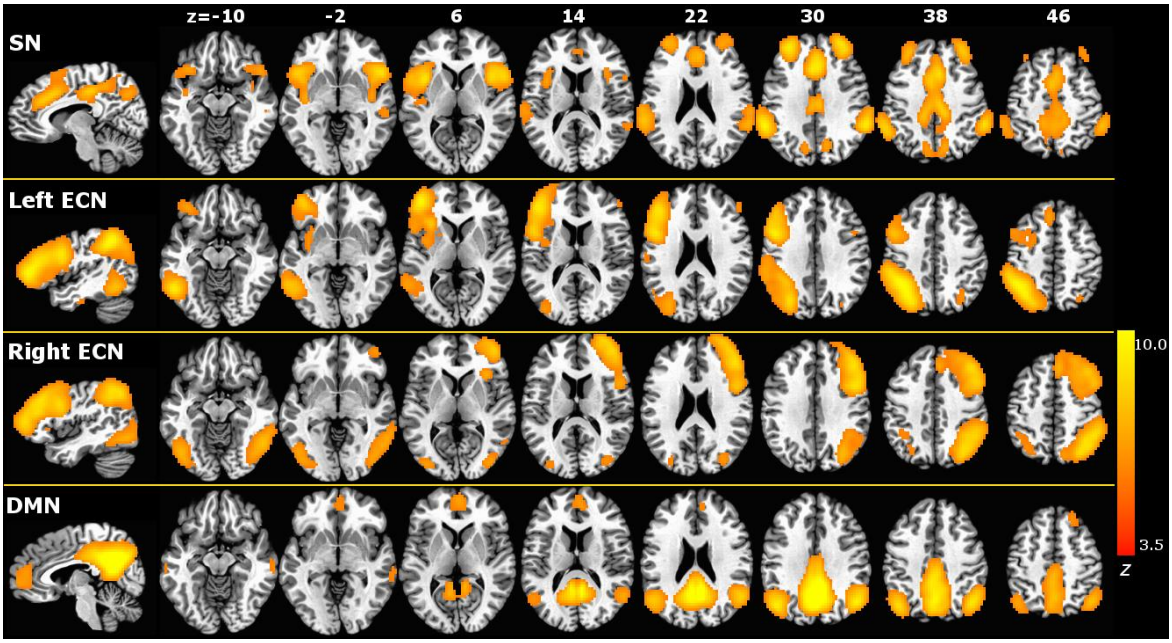


Figure 2. Network components identified by group ICA

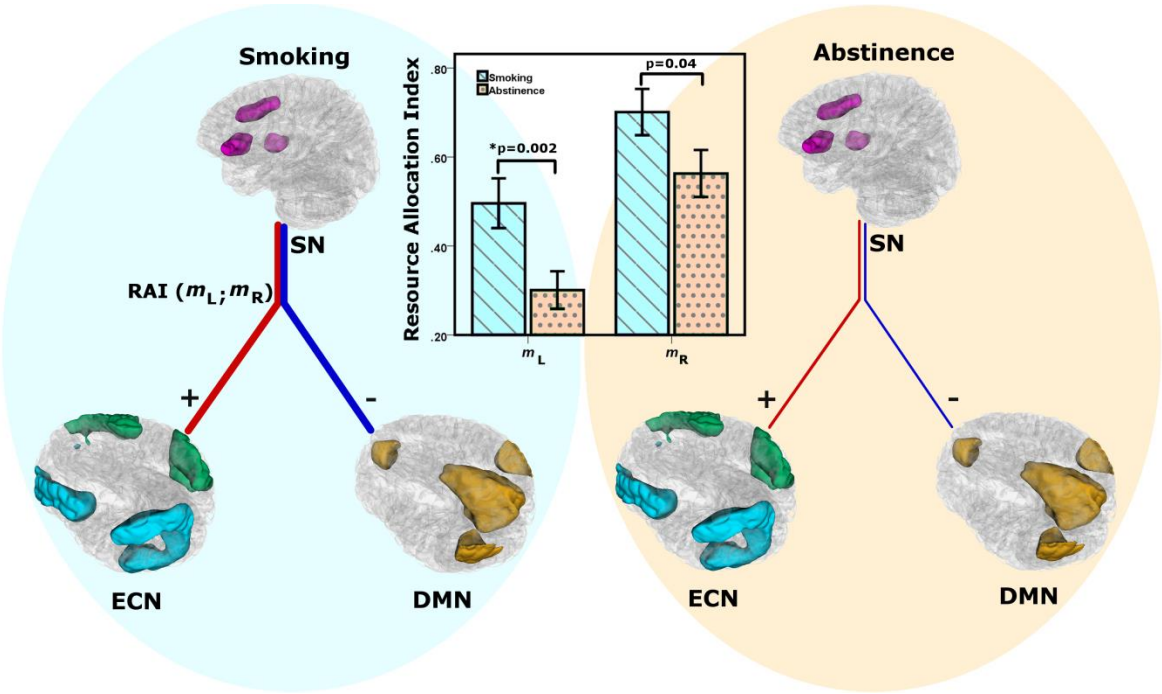


Figure 3. Network coupling during abstinence versus smoking sessions

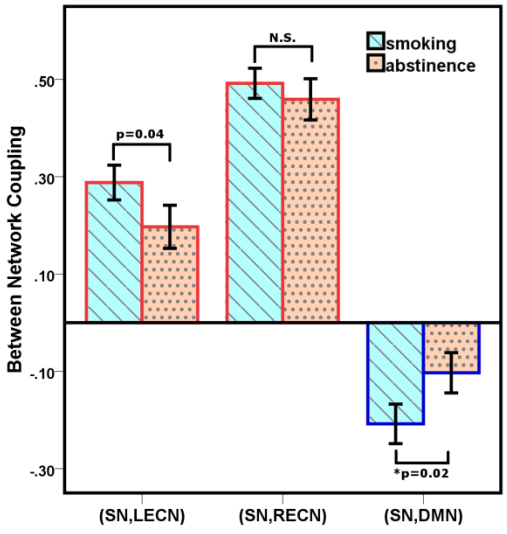


Figure 4. Correlation between change in RAI score and change in craving between sessions

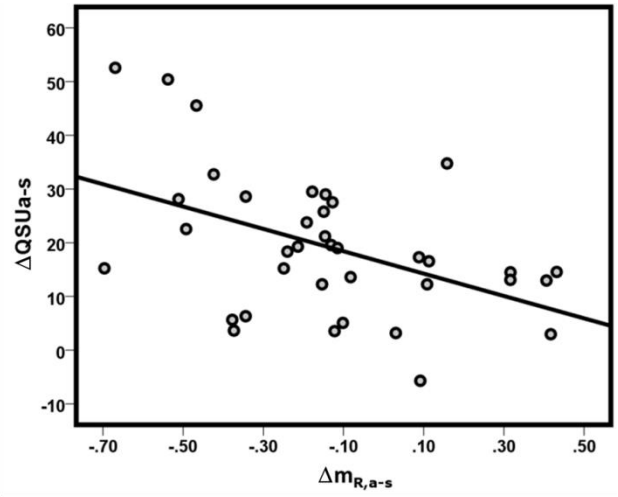
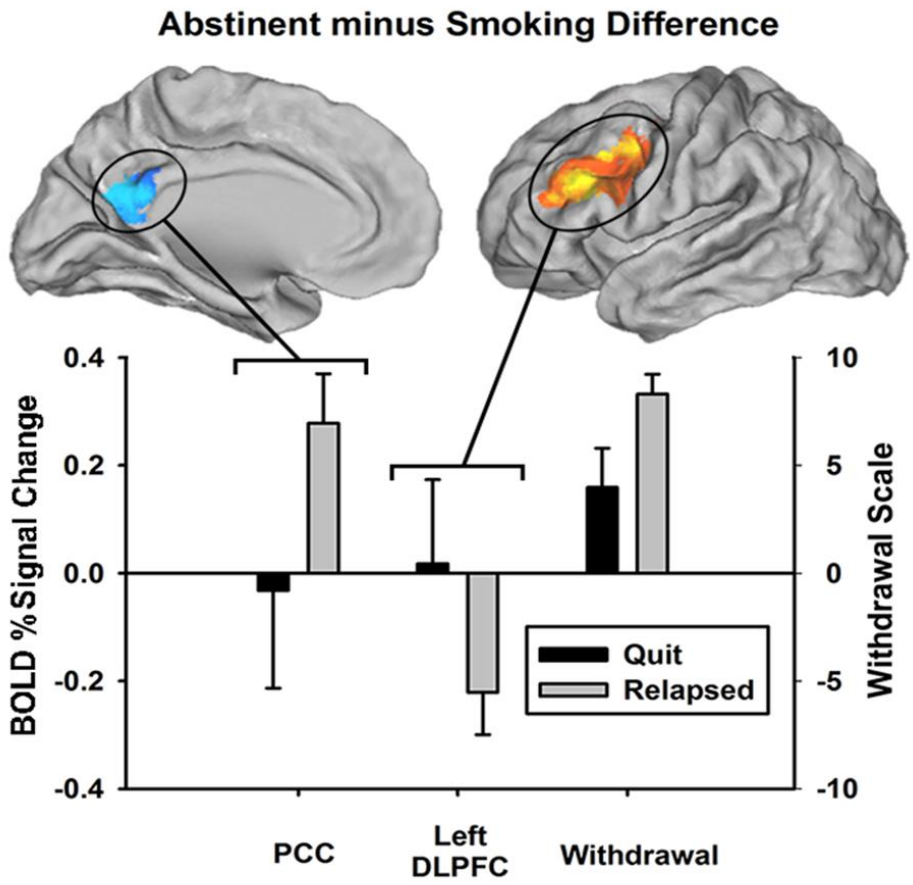


Figure 5. Changes in working memory-related BOLD signal predict smoking relapse



Research Project 6: Project Title and Purpose

High-Throughput High-Resolution Tools for RNA-Based Functional Genomics - The purpose of this project is to develop new high-throughput tools that will allow high-resolution assays of RNA pools of cells and to quantitatively manipulate RNA levels in cells. In addition, these tools will be applied to a survey of cellular response to perturbations as well as normal development. The results of this study will generate new research capacities and form the basis for new instruments and procedures relevant to RNA-based therapeutics. The use of quantitative RNA manipulations of a cell and assays at the single-cell resolution will help researchers understand the heterogeneity of functional responses of cells and their consequences for therapeutics.

Duration of Project

1/1/2010 - 12/31/2013

Project Overview

Genomics has impacted every area of biological and biomedical science and its concepts and

technologies have become fundamental to understanding human diseases. A key aspect of the genome science is that it has been powered by the development of new technologies that allow efficient measurement and manipulation of biomolecules in ever larger numbers, at ever greater throughput, and at ever finer resolutions. In particular, the measurement and manipulation of RNA populations have led to the development of functional genomics and to the proposals for RNA-based therapeutics. While the study of genome-wide RNA populations is already more than 15 years old, continued discovery of novel functional RNA elements is still opening up entirely new areas of biology. Quantitative assessment and manipulation of RNA at high-resolution is just starting. The goal of this proposal is to develop new high-throughput tools to manipulate and measure the broad functional RNA populations of cells at high resolution, including at the level of single cells, and to examine the potential for quantitative RNA manipulations for therapeutics. The specific aims of this project are:

1. Establish a facility for next generation RNA sequencing at high resolution including up to single-cell RNA sequencing.
2. Prototype a new tool for generating a quantitative library of RNA that can be used for transfection and other quantitative cell manipulation studies.
3. Use the facilities and the tools established in Aims 1 and 2 to understand RNA population variability at multiple levels in mammalian systems and to investigate the roles of noncoding expressed RNA.

Principal Investigator

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Other Participating Researchers

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Expected Research Outcomes and Benefits

The completion of this research will result in the following outcomes and benefits:

1. Establishment of a single RNA sequencing facility with the capacity to amplify and sequence RNA populations from individual cells. The facility will integrate current Nextgen sequencing technologies with high-flow process for RNA amplification and bioinformatics processing for RNA sequence reads. This facility will allow Penn genomics researchers to greatly extend the resolution of functional genomics, both for the RNA populations and for the biomaterial.
2. Prototype of a novel instrument for rapidly generating quantitative mixtures of RNA

libraries. The results will allow us to develop in the future a full instrument, which will generate combinatorial mixtures of RNA-based reagents. We expect the prototype to form the basis for a novel instrumentation grant to federal agencies.

3. The understanding of RNA population variability at multiple resolutions, which can be used to understand the heterogeneity of cellular responses to perturbation treatments (e.g., small molecule drugs). The understanding of novel RNA pathways in cellular function that can be used to develop RNA-based therapeutic targets.

Summary of Research Completed

During this reporting period, we carried out final work related to all three of our aims.

For specific Aim 1, we continued to refine computational infrastructure for RNAseq quantification that is specific to the single cell level. We created a data repository for single cell transcriptome experiments that now contains more than 500 single cell RNA sequencing data. We also refined preprocessing steps for single cell RNA libraries. These preprocessing steps include filtering for specific single cell amplification artifacts, checking for possible contamination through a subsampling of the sequence reads and comparing to Genbank, and for generating a searchable BLAST library of the reads. We also switched the main alignment algorithm from RUM (Grant et al., Bioinformatics 27:2518) to the STAR aligner (Dobin et al. Bioinformatics 29:15). This led to a speed increase of more than 100-fold in our overall pipeline.

For specific Aim 2, we carried out three different works. First, we characterized in vitro transcribed (IVT) RNA library for various dilutions and mixtures to assess the ability of the RNA sequencing procedures to accurately quantify the RNA. This work helped validate RNA sequencing methods as well as helped ground-truth the library quantification required for use of the IVT RNA. The work describing this ground-truthing study was submitted to Genome Biology in December 2013. Second, we quantified RNA present in five different mouse cell types at the single cell level. This involved single cell RNA isolation and characterization for over 100 total samples. We then developed a novel procedure to find the minimal number of transcription factors whose expression statuses separate each cell type. We used this information to generate a transcription factor minimal change network for the five different cell types. This work was submitted to Genome Research in October 2013 and the paper is currently being revised. In the third part of our work, we used the information from the single cell transcription factor network to select a small set of transcription factors that might be used to effect a transdifferentiation cell remodeling from an astrocyte to brown adipose tissue cells. We selected 7 different transcription factors and we generated expression RNA using IVT, and then verified and quantified the RNA. We then used the combinatorial library to photo-transfect astrocyte primary cell cultures following the procedures we described in Sul et al. (PNAS 106:7624). These cells are currently being monitored for changes in their phenotype.

For specific Aim 3, we submitted and published our study of retained introns in mouse and rat neurons:

Mugdha Khaladkar, Pete T. Buckley, Miler T. Lee, Chantal Francis, et al.: Subcellular RNA Sequencing Reveals Broad Presence of Cytoplasmic Intron-sequence Retaining Transcripts in

Mouse and Rat Neurons. PLoS ONE 8(10): e76194, 2013.

In the above work, we comprehensively characterize the set of cytoplasmic introns at the genome-wide level and found surprising levels of retained introns in each of these species. To analyze these retained introns and other functional non-coding RNAs, we developed a new algorithm to characterize functional RNA structures without folding or aligning the RNA structures. The algorithm called NoFold, utilizes a set of stochastic models for RNA structure to score each candidate RNA sequence against a library of RNA models. The resulting quantitative data is used to formulate a feature space and then novel sets of RNA are clustered within this space. Using this algorithm, we characterized the cytoplasmic retained introns found in neurons and delineated several possible motifs that might be involved in RNA localization. We submitted this manuscript to RNA in September 2013. The paper was provisionally accepted pending revisions which we are currently carrying out.

Research Project 7: Project Title and Purpose

Phase II Trial of Cyclin-Dependent Kinase Inhibitor PD 0332991 in Patients with Cancer - A targeted approach to cancer treatment is proposed. Most tumors have abnormalities in the control of the cell cycle, permitting unregulated proliferation of cancer cells. We have established the safety of an oral agent targeted to the cell cycle proteins cdk4 and cdk6, and have characterized its pharmacology. In this proposal we are selecting patients whose tumors have molecular features that suggest they may respond to a cell cycle inhibitor, and in four different diseases perform clinical, biological, and imaging studies to identify the characteristics of responders, to better perform future trials in populations who may benefit.

Duration of Project

5/1/2010 - 12/31/2013

Project Overview

Our broad research objective is to bring to the clinic, and evaluate critically, interventions directed to inhibition of the cell cycle within tumor cells. We have performed pilot studies with the reagent we will now evaluate in such a way as to first, ascertain that it is doing what we expect; and second, to identify the characteristics of responding patients, in order to better direct successful therapy. Our specific aim may be expressed as:

1. To investigate cell cycle inhibition by PD 0332991 in patients with Rb-positive melanoma, colon, breast and germ-cell tumors, and its association with clinical benefit.

This aim is supported by our early phase studies of this approach. In the initial phase I human study of PD 0332991, we demonstrated appropriate doses at which this oral compound was tolerable in cancer patients, on two different schedules of administration. We demonstrated patient benefit in a number of diseases, including in three patients with resistant germ cell malignancy, as we recently reported in the New England Journal of Medicine. In this project, we will target patients whose tumors are shown to have altered regulation of the cell cycle in a

number of ways, and will perform molecular, pharmacological, and imaging analyses to expand our understanding of which patients will benefit from treatment, and how best to apply it. We believe that the intensive study of relatively small numbers of patients can contribute greater understanding of how best to use promising novel drugs such as these, and how to select the populations who will derive the greatest benefit from their use.

Principal Investigator

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

1. We will define the activity of PD 0332991 in the treatment of four tumors: melanoma, colon, breast and germ-cell tumors. The results will either lead to the further development of the compound in these diseases, or to a focus elsewhere. Based on our initial studies in which several patients had long-term control of the disease, we hope to alter the trajectory of disease growth and patient deterioration, to allow longer survival and improved quality of life with a single daily pill.
2. We hope to identify specific patient populations with a high probability of responding to the treatment.
3. This project will provide an additional treatment option for three of the most common cancer diagnoses that afflict Pennsylvanians.

Summary of Research Completed

Of the total 114 patients, there were 47 males and 67 females. The racial/ethnic breakdown of the patients was:

African American: 8	Asian: 2	Unknown: 2
Hispanic: 3	Caucasian: 99	

Accrual

Disease	Screened	RB+	CCND1+	Entered
Breast	128	111	25	52
Colon, Kras mutated	39	35		18
Germ Cell	61	60		30
Gastric/Esophageal	30	28		11
CCND1 amplified*	132		35	21(16 BR, 4GE)
Thymic	1	1	1	1
Renal Cell	1	1	1	1

*Note that all breast and gastroesophageal patients were tested for CCND1 amplification

Toxicity

The expected profile of neutropenia, thrombopenia, and fatigue is observed without any unexpected events. This is a remarkably well-tolerated drug.

We have developed, and are presenting next month, an algorithm for patient treatment that is a first step to a safer and more effective dosing strategy.

Translational Research

We have performed detailed pathological analyses of the tissues of treated patients. The results, still preliminary, suggest that the degree of RB staining and its intracellular location may determine responsiveness to therapy.
We performed a critical proof-of-principle trial in colorectal cancer showing through FLT-PET imaging (a novel approach to analysis of cell cycle inhibitors) that the drug accomplished in the tumor cells what it was designed to do, and that the magnitude of the effect was proportional to that in bone marrow.

Research Project 8: Project Title and Purpose

Genomic Profiling for Molecular Diagnostics of Prostate Cancer - This project is designed to identify nucleic acid patterns in prostate tumors that predict the likelihood and time frame of cancer recurrence following radical prostatectomy. The patterns discovered in DNA or microRNA will be evaluated in the context of candidate markers from other genomic profiling techniques, different from those to be used in this project, that have been recently identified by researchers at the University of Pennsylvania and other institutions. The combined data sets will inform the design of focused panels of molecular tests that can be clinically evaluated for their potential to help guide decisions about cancer therapy. The molecular diagnostics developed during this project will be transferred to the Hospital of the University of Pennsylvania for clinical practice, and will serve as a model for translational genomics in the Abramson Cancer Center.

Duration of Project

1/1/2010 - 12/31/2013

Project Overview

This project's two objectives are genomic profiling of prostate cancer, and translational genomics to develop new molecular diagnostics for oncology. While the project is designed with numerous ties between objectives, each is flexible and success in one is not dependent on the other. Genomic profiling will use a new high-resolution assay for DNA copy number across the genome, and also will measure the levels of microRNAs. DNA amplification or deletion alters the local genetic copy number and can change the activity levels of affected genes, a phenomenon known to sometimes cause cancer. Gene activity can also be altered by microRNAs, normally and in cases of aberrant oncogenic microRNA over- or under-expression.

The translational genomics work will use previously identified changes associated with prostate and other cancers, combined with any new markers identified in this project, to design multiplexed diagnostic tests. Multiplexing measures multiple targets in a single sample of RNA or DNA. Cancer usually requires changes at several genes to occur, so multiplexed tests are a powerful approach for screening a tumor sample to determine what genes have been affected and make corresponding diagnoses, prognostic estimates, and therapy choices.

The three specific aims will use 1) whole-genome molecular profiling, 2) targeted molecular profiling, and 3) diagnostic molecular profiling to study archived prostate cancer samples. This existing collection includes cases of slow or rapid progression in patients of Caucasian (Cau) or African American (AA) ethnicity. The profiling research will therefore compare differences in gene activities to identify aggressive tumors, and determine if these patterns can be applied diagnostically within or across ethnic populations.

Aim 1 will compare tumor samples from 50 slow vs. 50 rapid recurrence cases. These 100 samples will be profiled using Affymetrix microRNA and Cytogenetics GeneChips to measure microRNA levels and DNA copy number. In Aim 2, the samples will be tested for DNA

methylation (another mechanism for regulating gene activity) at a targeted subset of genes recently shown to be altered in prostate cancer. A set of known oncogenic mutations will be tested for association with the tumor types in this project using the Sequenom OncoCarta assay. For Aim 3, a set of individual DNA or RNA tests relevant to oncology will be combined with diagnostic markers from Aims 1 and 2 to design multiplexed panels for the Sequenom system. These panels will be tested for precision, sensitivity and specificity appropriate for deployment as clinically useful tests.

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Expected Research Outcomes and Benefits

Aim 1 will produce lists of microRNAs and genomic DNA regions that have altered abundance in rapid recurrence prostate tumors compared to slow progressors. Examination of these microRNAs, their predicted gene targets, and the genes in regions of altered copy number will illustrate the molecular genetic mechanisms that may be operating during tumor expansion. Such genes and mechanisms benefit oncology research by providing candidate drug targets or suggesting therapeutic strategies. Even if microRNAs or genes cannot be assigned to an oncogenic mechanism, their alterations may be indicative of a good or poor prognosis and thus guide treatment strategy just by testing for what patterns are present.

Aim 2 will advance previous research by determining whether gene activities altered in prostate cancer are due to DNA methylation, thereby uncovering additional parts of the mechanisms that cause prostate cancer pathology. Similarly, DNA mutations previously known to be oncogenic in other cancers will be tested in this cohort to see if these mutations are associated with prostate cancer; the resulting compilation of relevant DNA mutation or methylation patterns will provide candidate prognostic markers.

Aim 3 will create new molecular diagnostic tests in multiplex panels that increase efficiency and provide a broader view of molecular parameters important for treating cancer. These panels start with genetic tests not currently available at the Hospital of the University of Pennsylvania that are used in oncology research but have not yet been deployed clinically, or are used elsewhere as

single-marker clinical tests, and then incorporate additional markers from Aims 1 and 2. Evaluation of their performance in the clinical Molecular Pathology Laboratory, using the prostate cancer cohort, will provide the first example of translational genomics for molecular diagnostics in the Abramson Cancer Center. The project will thus advance the local oncology testing options available to patients and physicians and establish a platform for adding new tests as they are developed.

Summary of Research Completed

We have previously reported on the development of biomarker assays for miRNA and methylation as proposed in Aims 1 and 2. In the most recent funding period, we have completed the evaluation of biomarkers as predictors of risk and outcomes in prostate cancer cases. We have evaluated the accuracy and precision of two existing nomograms for risk of biochemical failure following prostate treatment in both Caucasian and African American men developed by Kattan (Kattan, Wheeler et al., "Postoperative nomogram for disease recurrence after radical prostatectomy for prostate cancer." 1999) and Stephenson (Stephenson, Mori et al., "Treatment of erectile dysfunction following therapy for clinically localized prostate cancer: patient reported use and outcomes from the Surveillance, Epidemiology, and End Results Prostate Cancer Outcomes Study." 2005). Both nomograms assign risks based on diagnostic PSA, Gleason sum, surgical margin status, and presence or absence of extracapsular extension, lymph node involvement, and seminal vesicle invasion. Neither model includes racial or ethnic ancestry as a covariate or a stratifying factor.

As shown in Figure 1, we have generated Kattan score data and correlated these data with race and clinical (PSA) failure. Among 109 African Americans and 937 European Americans, we find significant association betwee Kattan Score and probability of failure in both races ($p<0.001$). However, there was no difference in the prediction by race.

Using the Kattan nomogram as a base, we have begun todevelop an expanded nomogram that includes important new predictors of 5-year biochemical failure after prostate cancer treatment. We have begun to use the selected covariates and biomarkers with strong correlation with aggressive PCa disease, as shown in Table 1, to develop the new nomogram, as described in the article titled “The addition of interleukin-6 soluble receptor and transforming growth factor beta1 improves a preoperative nomogram for predicting biochemical progression in patients with clinically localized prostate cancer” (Kattan, Shariat et al. 2003, Kattan, Vickers et al. 2009).

These data provide the basis for a variety of additional studies outside the scope of this project. We will be able to generate a comprehensive mdodel based on both clinical featurs and biomarkers based on the research undertaken in this project. In particular, we will be able to generate model results that are applicable to African American men.

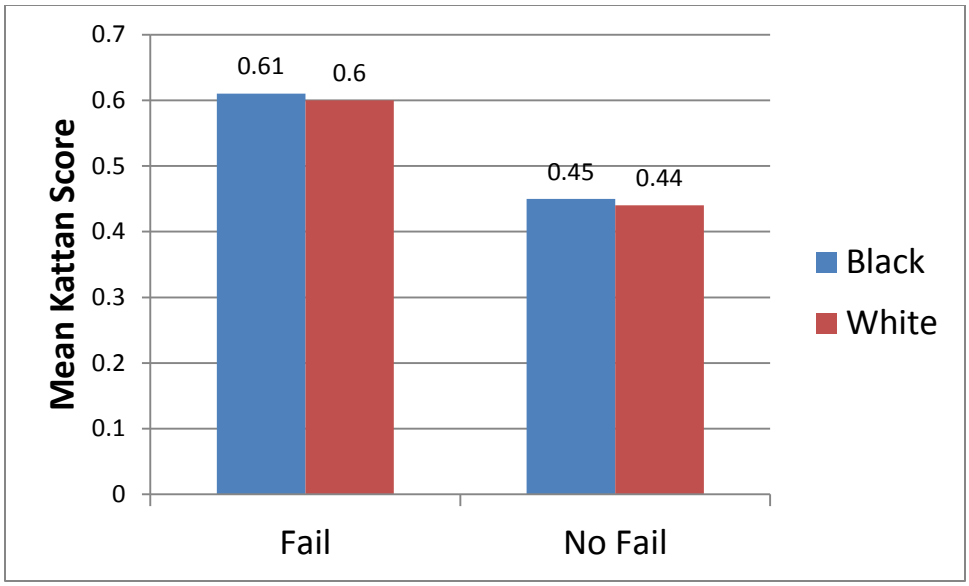

Figure 1: *Mean Kattan Score by PSA Failure and Race*

Table 1: Selected biomarkers being generated in the SCORE and MADCaP databases

Biomarkers	**Description**	**Assay***	**Function**
PCa-associated factors:			
TMPRSS2-ERG	Transcription factor gene fusion	FISH	Found in 36-78% samples; associated with aggressive disease
Ki 67	Tumor proliferation rate protein	IHC	Correlates with cancer-specific and overall survival
GSTP1	glutathione S-transferase P1 gene	qRT-PCR	Hypermethylated in 60-80% Pca; in serum, urine, biopsy tissue
OncoCarta (ProstaCarta)	A pre-designed panel for mutation profiling	qRT-PCR	Identify other potential biomarkers important in PCa
Prostate Specific proteins:			
MSMB	Prostate specific protein (10q11.2)	Microarray	Independent predictor of recurrence
PSMA	Cell surface membrane protein	IHC	Associated with PSA recurrence in high risk cohort
Androgen Pathway:			
AR	Intracellular receptor protein	IHC	Predictor of decreased biochemical recurrence-free survival
CYP3A gene	CY3A4*1B/ CY3A43*3 enzymes (7q21)	qRT-PCR	Associated with PCa occurrence and severity
SRD5A2	5-alpha reductase II	qRT-PCR	A49T, V89L variant correlates with extracaspular disease

Assays*: FISH-Flourescence In-Situ Hibridization; IHC- Immunohistochemistry; qRT-PCR- Quantitative reverse transcriptase polymerase chain reaction

Status**: Wet lab analysis of gene expression profile either ongoing or to be done in Dr. Rebbeck's lab in patient samples from the SCORE and MADCaP databases

Research Infrastructure Project 9: Project Title and Purpose

Infrastructure for a GMP Facility for Radiopharmaceutical Production - The research infrastructure planned here will develop a facility for the production of experimental radiopharmaceutical under Good Manufacturing Conditions (GMP). This project leverages the ongoing instillation of a new cyclotron facility to develop an infrastructure to support the development and translation of new radiopharmaceuticals for imaging and targeted therapy. The addition of this facility will enhance our ability to produce new radiopharmaceuticals for human use. The development of targeted imaging agents will accelerate research efforts in

neurodegenerative disease, psychiatry, addiction, cardiology and cancer. In addition, the facility will be unique in the region and be available to support research originating in other universities, biotech companies or pharmaceutical companies in the Commonwealth.

Duration of Project

7/1/2010 - 6/30/2011

Summary of Research Completed

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

Research Project 10: Project Title and Purpose

Direct Reprogramming of Fibroblasts and Astrocytes to Dopaminergic Neurons – We aim to directly reprogram fibroblasts and astrocytes to dopaminergic neurons using defined factors. This will provide a novel source of these neurons for use in pharmacological studies, cell-based therapies, and ultimately studies involving *in vivo* generation of dopaminergic neurons in patients with Parkinson's Disease.

Duration of Project

11/23/2010 – 12/31/2013

Project Overview

Advances in reprogramming have revealed that differentiated mammalian cells possess remarkable plasticity – from the induction of pluripotency *in vitro* to the conversion of pancreatic exocrine cells to endocrine cells *in vivo*. This pancreatic "lineage reprogramming" raises the intriguing possibility that other cell types might be capable of *in vivo* reprogramming. If this could be applied to the mammalian brain, it is conceivable that dopaminergic neurons (DNs), whose degeneration is the hallmark of Parkinson's Disease (PD), could be generated by direct reprogramming. As a first step, we propose to directly reprogram mouse embryonic fibroblasts (MEFs) to dopaminergic neurons. Earlier this year, a report demonstrated the conversion of fibroblasts to glutamatergic and GABA-ergic neurons. We aim to take a similar approach, directly converting MEFs to DNs by ectopically expressing a core set of key transcription factors. We have selected eleven transcription factors that have been previously shown to play important roles in the normal embryonic development of DNs. We will generate drug-inducible lentiviral vectors to deliver each of these factors to MEFs. We will then evaluate the ability of these factors to reprogram MEFs into DNs by monitoring the up-regulation of DN-specific genes, assaying for the presence of dopamine in the cell culture medium, and the ability of reprogrammed cells to generate action potentials in patch-clamp studies. Upon optimization of this protocol, we will proceed to test the same set of transcription factors in astrocytes, ultimately moving toward *in vivo* reprogramming studies in animal models of PD.

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Expected Research Outcomes and Benefits

We expect that the direct reprogramming of fibroblasts and astrocytes to dopaminergic neurons (DNs) will provide a novel source of DNs for use in pharmacological studies and cell-based treatments for Parkinson's Disease. This approach will allow for the direct generation of DNs without the use of stem cells.

Summary of Research Completed

In the last six months of 2013, we continued our studies of three transcription factors – ASCL1, LMX1B, and NURR1 – and evaluated their ability to directly convert adult monkey astrocytes into dopaminergic neurons. We constructed a lentiviral vector in which the astrocyte-specific Vimentin promoter (*Vim*) drives expression of a polycistronic cassette consisting of ASCL1, LMX1B, and NURR1 (abbreviated ALN). As a control vector, we also built *Vim*-GFP. This *Vim*-GFP vector was validated to drive robust transgene expression in adult African green monkey astrocytes *in vitro*. However, the *Vim* promoter did not drive expression of the ALN polycistronic cassette at a high enough level to permit effective conversion of astrocytes to dopaminergic neurons. Repeated experiments showed that although low levels of ALN could be detected, no dopaminergic neurons were generated. Accordingly, we have discontinued the use of the *Vim* promoter lentiviral vectors. In their place, we have returned to the adeno-associated viral (AAV) vectors that we used in the past. Due to size limitations of AAV versus lentiviral vectors, the polycistronic ALN cassette cannot be packaged into an AAV backbone. We therefore have generated AAV vectors expressing each transcription factor individually. While this adds an extra layer of complication that is not found when using a polycistronic vector, it permits us to use the clinically-applicable AAV system. Given our earlier results that AAV serotype 5 can effectively transduce African green monkey astrocytes *in vivo*, we are confident that this is the best method as we move forward.

In the previous annual report, we indicated that we had engineered versions of the transcription factors ASCL1, LMX1B, and NURR1 that were fused to the VP16 transactivation domain. VP16, originally isolated from herpes simplex virus, is a powerful activator of gene transcription. It has been widely used in molecular and cellular biology in applications such as two-hybrid screens when potent transcriptional activation is desired. Recently, the VP16 transactivator was shown to significantly enhance the generation of induced pluripotent stem (iPS) cells when fused

to the reprogramming factors Oct4, Sox2, and Nanog. To generate VP16 fusions, we engineered a drug-inducible lentiviral vector with a Gateway (Invitrogen) site that permits the cloning of an open reading frame (ORF) and places an N-terminal VP16 sequence on each gene. Using this vector, we generated transactivated fusions of ASCL1, LMX1B, and NURR1. By testing combinations of these VP16 fusions with the native forms of each factor, we identified VP16-ASCL1 as being the key factor to mediate transdifferentiation of adult primate astrocytes into neurons. When VP16-ASCL1 is expressed with native LMX1B and NURR1, monkey dopaminergic neurons were readily produced. However, subsequent experiments have called into question the notion that VP16 is required. We built a polycistronic lentiviral vector in which the VP16-ASCL1 fusion was expressed along with LMX1B and NURR1: VP16-ASCL1-LMX1B-NURR1. Surprisingly, this construct did not drive effective conversion of adult monkey astrocytes to dopaminergic neurons. Closer examination of the individual transgenes via Western blotting and immunocytochemistry revealed that VP16-ASCL1 was not being properly expressed. This may have been due to the 2A peptide sequence that remained on the C-terminus of ASCL1 following 2A cleavage. When we switched from the polycistronic cassette to individually expressed transcription factors, we found that ordinary ASCL1 (i.e. without the VP16 fusion) was capable of driving conversion of astrocytes to dopaminergic neurons (see Figure 1, below). Previous failures of the original ALN cassette (published in PLoS One in 2011) in monkey cells may not have been due to a lack of VP16, as we hypothesized in our most recent annual report, but rather due to improper folding of ASCL1-2A.

We have now engineered AAVs to express ASCL1 with and without VP16, along with separate AAVs for LMX1B, its homolog LMX1A, and NURR1. These were submitted to the Penn Vector Core for packaging in December of 2013. Although the experiments using these new vectors will be completed after the end of this grant funding period, we have made substantial progress toward understanding the requirements for *in vivo* conversion of adult monkey astrocytes into dopaminergic neurons. The results of these studies should prove invaluable toward the development of novel regenerative medicine-based therapies for Parkinson's Disease.

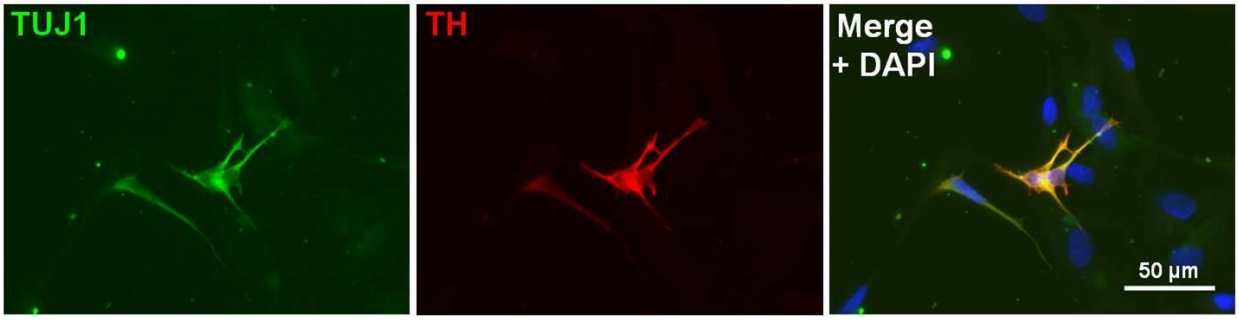

Figure 1. Dopaminergic neurons (iDNs) generated by direct reprogramming of adult monkey astrocytes. TUJ1: type III beta-tubulin, a pan-neuronal marker; TH: tyrosine hydroxylase. Immunocytochemistry was performed three days following induction of reprogramming factors ASCL1, LMX1B, and NURR1.

Research Project 11: Project Title and Purpose

Construction of Insect Growth Chambers - Research Infrastructure – The purpose of this research infrastructure project is to provide a state-of-the-art facility for a newly recruited faculty member of the Department of Biology, as well as six other Biology faculty, that will enable them to conduct research that focuses on reciprocal interactions between social behavior, reproduction, and aging using insect models, as well as neurodegenerative and infectious diseases. Moreover, its construction will enable the Department of Biology to expand its research scope using insect models to study human diseases. The facility will be comprised of two independent walk-in temperature- and humidity-controlled insect growth chambers that will be housed within a laboratory located in Leidy Laboratory.

Duration of Project

1/1/2011 – 3/30/2012

Summary of Research Completed

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

Research Project 12: Project Title and Purpose

Development of High Throughput Methods for Generation of Induced Pluripotent Stem Cells – Current methods for generating human induced pluripotent stem cells (iPSC) are labor intensive and inefficient. Recent publications have demonstrated improved efficiency of generating iPSCs using human adipose stem cells (ASCs). We also demonstrate an important advance in iPSC reprogramming using miRNA-mediated approaches, which may in the near future allow reprogramming to be accomplished without the need for transfection or even recombinant DNA techniques. The purpose of this project is to optimize methods using current and the novel miRNA approaches to generate iPSCs that improve throughput and efficiency. These methods will allow large-scale studies requiring the generation of iPSCs from a large number of patients.

Duration of Project

7/1/2011 - 6/30/2013

Summary of Research Completed

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

Research Project 13: Project Title and Purpose

Ocular Surface Limbal Stem-Cell Therapy for Corneal Regeneration – A clear cornea is essential for good vision and depends on epithelial integrity and stromal avascularity. Corneal renewal and repair are mediated continuously throughout our life by adult stem cells of the limbus, the narrow zone between the cornea and the conjunctiva. Ocular burns and many other conditions may destroy the limbus, causing limbal stem cell deficiency with painful recurrent epithelial erosions, light sensitivity, scarring and permanent visual loss. Transplantation of limbal stem cells is the only definite treatment. The purpose of this project is to develop a reliable and reproducible technique for culturing human limbal stem cells in order to use them for transplantation and cornea regenaration in patients with limbal stem cell deficiency.

Duration of Project

4/10/2012 – 12/31/2013

Project Overview

Limbal stem cells (LSCs) are responsible for the constant renewal of the cornea epithelium that is necessary for clear vision. Damage to LSCs can cause limbal stem cell deficiency (LSCD) with painful light sensitivity and blindness. The only definite treatment for LSCD is restoration of the LSC function by limbal transplantation. Unilateral cases of LSCD may benefit from limbal autograft from the fellow healthy eye of the same patient. The main risk of this procedure is removal of too many LSCs causing LSCD in the healthy eye. In order to minimize this risk, a small area of 1-2mm^2 can be cultured and expanded before transplantation. The success rate of limbal transplantation depends on the amount of LSCs preserved in the transplant. Under usual cell culture conditions, stem cells tend to differentiate and lose their potential for lifelong regeneration. Different research groups worldwide have used different culture conditions in order to maintain LSCs in culture. There is no consensus and no direct head to head comparison of different culture conditions of LSCs for preservation of their regeneration potential.

The objective of this project is to develop and directly compare culture conditions of LSCs for use in future clinical transplantation in patients with LSCD.

Specific Aim 1: Determine the most successful and consistent method of enzymatic or combined enzymatic/mechanical isolation of LSCs from limbal tissue for efficient and successful cultivation, based on yield, viability, colony forming efficiency (CFE) and expression of potential LSC markers.

Specific Aim 2: Explore ways to avoid the use of animal products in the feeder supportive cells (mouse fibroblasts) and the media (bovine serum) for successful cultivation of LSCs for future clinical transplantation that is xeno-free, while supporting growth of LSCs measured with CFE, percentage of holoclones, preservation of LSC markers, and subcultivation.

Specific Aim 3: Determine optimum oxygen levels for successful culture of LSCs, comparing traditional culture at 20% O_2 versus lower amounts of O_2 (14%, 5% and 2%), based on CFE,

percentage of holoclones, preservation of LSC markers, and ability to subcultivate them.

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Expected Research Outcomes and Benefits

The expected outcome of this project is the development of a reliable and reproductive technique for culturing human limbal adult stem cells (LSC) in order to use them for transplantation and cornea regeneration in patients with limbal stem cell deficiency (LSCD). Several research groups worldwide have performed successful clinical transplantations outside the United States, using different ways to culture LSC. Clinical trials have never been started in the United States because these methods do not meet our FDA guidelines. The methodology of LSC cultures needs to be optimized in order to obtain FDA approval for future clinical trials on patients with LSCD and cornea blindness. Additionally, knowledge gained from establishing ways for clinical use of cultured adult stem cells can also be useful for future applications in other organs, since many human organs harbor adult stem cells that can be potentially expanded for organ regeneration in the future.

Summary of Research Completed

Limbal epithelial cells were isolated from 139 donors using 15 variations of 3 dissociation solutions. All culture conditions were compared to the baseline condition of murine 3T3-J3 feeders with xenobiotic (Xeno) Keratinocyte Growth Medium at 20% O_2. Five Xeno and Xeno-free media with increasing concentrations of calcium and Epidermal Growth Factor (EGF) were evaluated at 5%, 14% and 20% O_2. Human MRC-5, dermal (fetal, neonatal or adult) and limbal stromal fibroblasts were compared. Statistical analysis was performed on number of maximum serial weekly passages, percentage of aborted colonies, Colony Forming Efficiency (CFE), $p63\alpha^{bright}$ cells and RT-PCR ratio of p63α/K12. Immunocytochemistry and RT-PCR for p63α, ABCG2, Bmi1, C/EBPδ, K12 and MUC1 were performed to evaluate phenotype.

<u>Results:</u> Dispase/TrypLE was the isolation method that consistently showed the best yield, viability and CFE. On 3T3-J2 feeders, Xeno-free medium with calcium 0.1mM and EGF 10ng/ml at 20% O_2 supported more passages with equivalent percentage of aborted colonies, $p63\alpha^{bright}$ cells and p63α/K12 RT-PCR ratio compared to baseline Xeno-media. With this Xeno-free medium, MRC-5 feeders, showed the best performance, followed by fetal, neonatal, adult HDF, and limbal fibroblasts. MRC-5 feeders supported serial passages with sustained high

expression of progenitor cell markers at levels as robust as the baseline condition without significant difference between 20% and 5% O_2.

Conclusions: LSC function can be maintained *in vitro* under appropriate Xeno-free conditions.

During this period we prepared and submitted a manuscript for publication which was accepted and published in the journal of Investigative Ophthalmology and Visual Science.