University of Pennsylvania

Annual Progress Report: 2009 Formula Grant

Reporting Period

July 1, 2010 – June 30, 2011

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.

<u>Research Infrastructure Project 1: Project Title and Purpose</u>

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.

Anticipated Duration of Project

1/1/2010 - 12/31/2013

Project Overview

Penn faculty members in the Department of Physics and Astronomy are increasingly engaged in research with biomedical implications. At present the School of Arts and Sciences does not have the requisite research space to house research being conducted at the interface between the physical and biological sciences. Specifically, we will undertake the following renovations and specialized equipment installations in the 3rd and 4th floors of the Laboratory for Research on the Structure of Matter (LRSM) building:

1) Renovate approximately 3065 sf on the 3rd floor of the LRSM to provide state-of-the-art chemistry preparation facilities, including new fume hoods, partitioned spaces for microscopy including temperature control and dust suppression, and addition of dust suppression for existing laser tables. New casework to be provided as appropriate.

Project will also include installation of major new equipment, including special-purpose microscopes, electronics, and associated equipment.

2) Renovate 1692 sf on the 4th floor of the LRSM to provide laboratory space for an incoming faculty member to work on the application of techniques in modern physics to problems in developmental biology. Project to include (a) optics lab requiring precise temperature control, airlock, new walls, floors, and light fixtures (711 sf); (b) wet laboratory including small autoclave, lab-grade dishwasher (604 sf); (c) microscopy facility for work with flies, including casework for four stereomicroscope stations, CO_2 distribution to all stations, repaint walls as required.

Principal Investigator

Richard M Schultz, PhD
Associate Dean School of Arts and Sciences
University of Pennsylvania
204B Carolyn Lynch Laboratory
Department of Biology
University of Pennsylvania
Philadelphia, PA 19104

Other Participating Researchers

None

Expected Research Outcomes and Benefits

The principal outcome of the present infrastructure project will be to improve the quality of the faculty performing research at the scientific frontier where biomedicine intersects the physical sciences. The improved laboratory space will enable the recruitment of new faculty members and the retention of existing ones, who would not consider working at the University of Pennsylvania without the provision of lab space that meets the highest quality standards, including precision control of ambient conditions such as temperature, lighting, etc for research with biomedical implications.

The renovated space will be occupied by: (1) A current School of Arts and Sciences faculty member, Dr. Arjun Yodh. Dr Yodh's work has significant biomedical research significance. Yodh has made seminal contributions to the use of visible and infrared light to probe structure in systems that are normally opaque to visible light, such as human tissue. By detecting the light that has bounced around the tissue before being detected it is possible, through sophisticated mathematical analysis, to reconstruct the properties of the intervening medium, including dynamic time-dependent phenomena such as blood flow. This work has important potential applications in diagnosing and imaging tumors or detecting bleeding in the human brain (stroke). (2) A new faculty recruit whose work involves precision studies of the microscopic mechanism of gene expression in fruit flies – how, precisely, do the genes obtain and use local spatial information to permit the pieces of the organism (body, legs, wings, eyes) to wind up in the right

places? This work has the potential for deepening our understanding of genetic disease syndromes in humans. The research conducted will improve health status by increasing our fundamental understanding of biological phenomena that are important to normal and pathological development, as well as through the development of instrumentation and techniques that have the potential to improve diagnostic and therapeutic procedures.

Summary of Research Completed

Over the past year we have begun but not yet completed the renovation of the remaining three laser labs and one microscopy lab. This will include a total of an additional 1,491 nsf of high quality research space. In each space all new case work will be installed. Rooms will be provided with additional fan coil units to supplement the building HVAC system and improve the trim of temperature and humidity. Heat generated by research instruments will be rejected to the building chilled water loop.

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.

Anticipated Duration of Project

1/1/2011 – 2/15/2012

Project Overview

The overall goal of this project is to renovate laboratory facilities in Stemmler Hall in support of Penn's Musculoskeletal Disorders and Comprehensive Neurosciences Centers. Research in the areas of musculoskeletal disorders and the neurosciences are important components of Penn Medicine's research strategic plan and fit with the school's mission of contributing to improved health outcomes for the citizens of the Commonwealth. These closely related, multidisciplinary research programs address important public health issues such as brain injury and rehabilitation, stroke, neurodegeneration, and tissue injury and repair. The Penn Center for Musculoskeletal Disorders (PCMD) and Comprehensive Neuroscience Center (CNC) were formed to integrate and strengthen the University of Pennsylvania's world class patient care, education, and research contributions in these fields. This project will directly support the research missions of these centers by renovating wet laboratory space on the 4th floor of Stemmler Hall, which will enable the recruitment of 3-5 new investigators, promote interdisciplinary collaboration, and enhance the efficiency of musculoskeletal and neurosciences research. The PCMD and CNC have been

assigned additional laboratory space adjacent to the project area in both Stemmler Hall and the attached Johnson Pavilion, including newly renovated space on the 5th floor of Stemmler which is providing a new home for the PCMD. The move of selected PCMD faculty to this new space creates the opportunity to modernize the Stemmler 4 laboratories from which these programs are moving. Encompassing 4,900 SF, the renovation project will upgrade and enhance currently outmoded wet laboratory space that has remained largely in its original state since Stemmler Hall was built in 1978.

Principal Investigator

Glen N Gaulton, PhD
Executive Vice Dean and Chief Scientific Officer
University of Pennsylvania
School of Medicine
354 BRB II/III
421 Curie Blvd
Philadelphia PA 19104-6160

Other Participating Researchers

None

Expected Research Outcomes and Benefits

The renovations are being completed to allow adjacencies and expansion of the Penn Center for Musculoskeletal Disorders (PCMD) and the Comprehensive Neurosciences Center (CNC). The resulting renovated laboratories will include a modern, open plan laboratory area outfitted with 36 lab bench work stations, two tissue culture rooms, space for freezers and related laboratory equipment, and related storage and other support space. This area is ideally positioned in the midst of the broader neurosciences and musculoskeletal research communities in Stemmler Hall and the attached Johnson Pavilion.

Evaluation of this project will be based on two sets of criteria: (1) the successful construction of research space in support of the mission and goals of the Penn Centers for Musculoskeletal Disorders and Comprehensive Neuroscience Center; and (2) the number of extramural sponsored projects awarded to Center investigators.

Summary of Research Completed

The Penn Center for Musculoskeletal Disorders renovation started with the move of part of three of the groups over to the adjacent Clinical Research Building (CRB). This will permit unobstructed renovation of the 4th floor of Stemmler Hall. The project was out to bid with final bids due on June 30, 2011.

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.

Anticipated Duration of Project

1/1/2010 – 12/31/2012

Project Overview

The broad objectives include the derivation or isolation and characterization of stem cells (for cartilage, tendon and lung) and the use of progenitor cells of the heart in the fabrication of tissue constructs that will promote the maturation and integration of the cells into functional tissues. The Specific Research Aims, including research design and methods are:

Aim 1: Tissue fabrication for regenerated myocardium. Existing techniques that enable the manipulation of biomechanical stress in tissue arrays will be used to evaluate the effects of static and cyclic stress on fabricated myocardial tissues. Co-culturing of human c-kit+ cardiac progenitors cells with neonatal rat cardiac myocytes in fabricated multicellular constructs will be used to determine if this strategy promotes the maturation and integration of fully differentiated myocardiocytes. Also, fabricated multicellular constructs derived exclusively from induced pluripotent stem cells will be evaluated morphologically and functionally.

Aim 2: Building cartilage and tendon from equine induced pluripotent stem (equiPS) cells. Induced pluripotent stem cells will be generated and characterized from horse somatic cells. Attempts will be made to develop protocols to efficiently differentiate equiPS cells into chondrocyctes and tenocytes to be used for engineering replacement cartilage and tendons on appropriate scaffolds. If we can demonstrate success in culture during the time frame of the grant, newly generated cartilage and tendon will be engrafted into horses and evaluated.

Aim 3: Characterizing pulmonary epithelial cells that can affect repair in the mouse lung. The expression of specific genes, such as miR-302/367, will be tested to see if they can expand lung epithelial progenitors in vivo (normal tissue); whether they can expand lung epithelial progenitors after injury; and whether miR-302/367 and Gata6 are required for the effective engraftment of lung epithelial cells in vivo.

Principal Investigator

John D. Gearhart, PhD
James W. Effron Professor
Director, Institute for Regenerative Medicine
University of Pennsylvania
Biomedical Research Building II/III
421 Curie Blvd., Room 1151
Philadelphia, PA 19104-6058

Other Participating Researchers

Jonathan Epstein, MD, Ken Margulies, MD, Christopher Chen, MD, PhD, Dean Richardson, DVM, Russell Addis, PhD, Edward Morrisey, PhD, Karthika Muthukumarswamy, MS – employed by University of Pennsylvania

Expected Research Outcomes and Benefits

The benefit of this research project is that we will gain a much better sense of how to generate complex tissue segments from progenitor and stem cells, an important step in reaching our goal in regenerative medicine of restoring the function of tissues that have been lost as a result of injury, disease, or aging. The information obtained in the studies will be crucial for regenerative repair of diseases and damage to the heart, bone and tendon, and lung. In Aim 1, new ways will be explored to induced cardiac progenitor cells to form heart tissue segments that will then be grafted into a damaged animal heart and these experiments will establish the paradigms for more complex efforts in the future to repair the damaged human heart. Aim 2 proposes to develop new methods for generating pluripotent stem cells from horse somatic cells. The resulting cells will be differentiated into cartilage and tendon progenitors, characterized and tested in the laboratory dish with the ultimate goal of repairing damage in horses. Ultimately this technology could be used in humans. Aim 3 is focused on discerning progenitor cell lineages that will be important in repairing damaged lung tissue. Repairing lung damage has proved difficult and the identification and use of progenitor cells represents a strategy that has proved successful in other organs and should be pursued for the lung. The approaches outlined herein are intended to translate what is known and being learned about stem and progenitor cells for clinical use.

Summary of Research Completed

Aim 1: Tissue fabrication for regenerated myocardium.
1A: Adapt existing techniques to enable manipulation of biomechanical stress in tissue arrays and evaluate the effects of static and cyclic stress on fabricated myocardial tissues.
1B: Determine whether co-culturing of human c-kit+ cardiac progenitors cells with neonatal rat cardiac myocytes in fabricated multicellular constructs promotes their maturation and integration.
1C: Determine the morphological and functional characteristics of fabricated multicellular constructs derived exclusively from iPS cells, fibroblasts and collagen.

To date, we have made significant progress on subaim 1A. Work on subaim 1B was initiated and has consisted of refining techniques for the isolation, purification and expansion of human c-kit+ cardiac-derived progenitor cells using FACS techniques. Work on Aim 1C has not yet started.

Microtissue Arrays. We have completed several studies to optimize culture conditions for cardiomyocytes, cell number/seeding, growth factor concentration, extracellular matrix formulations, and other parameters. Under optimized conditions, approximately 80-95% of the microtissues would form beating constructs that generated measurable forces. We found that electrical pacing as well as mechanical resistance (controlled by the stiffness of the cantilevers) improved the development of these tissues. A manuscript describing this work is in preparation for publication. Currently, we are now turning our attention to the generation of similar constructs using stem cell-derived cardiomyocytes as defined the original proposal, in order to examine how such cells develop to form integrated tissues and generate contractile forces. Previously employed magnetic cell sorting techniques have not produced c-kit cells of sufficient purity, so we have optimized the gating strategy for flow-cytometry-based cell sorting of c-Kit cardiac-derived progenitors with previously demonstrated myogenic potential [Kubo H, et al. Circulation 2008]. Though efforts to expand these cells have met with limited success to date, the small scale of the microtissue arrays will enable seeding exclusively with primary isolates of cardiac-derived c-Kit+ cells if necessary.

Aim 2: Building Cartilage and tendon from equine induced pluripotent stem (EqiPS) cells.
Part 1: Generation and Characterization of EqiPS.
Part 2: Development of protocols to generate EqiPS into chondrocytes and tenocytes.
Part 3: Engraft and evaluation of newly generated cartilage and tendon into horses.

The field of stem cell biology has experienced major advances in recent years with the breakthrough discovery by Yamanaka and colleagues, showing that the introduction of specific factors into somatic cells would revert (or reprogram) these cells into a state similar to that of an embryonic stem cell [Takahashi and Yamanaka, Cell, 2006]. These induced pluripotent stem cells (iPS) show an "unlimited" capacity to self-renew and the ability to generate any cell of the adult body, akin to embryonic stem cells. This finding has opened many new venues of investigation, with an emphasis on the direct usage of these iPS cells in regenerative medicine. In collaboration with researchers from the New Bolton center, Dr. Dean Richardson and Dr. Jeffrey Mason, we are interested in establishing iPS cells from Equine somatic cells for application in horse therapies. Injuries in the horse occur at high frequency in the cartilage and tendon, due to their participation in races, and it accounts for vast research develop at Penn Veterinary School. Moreover, this research should be directly applied to human due to the similarity between these types of injuries in horse and human. The work funded in this Aim is absolutely essential for achieving success in generating stem cells with human tissues for developing therapies for cartilage and tendon repair. Not only the source of the cells is unique but also the use of the cells in transplant paradigm to demonstrate efficacy is important in considering this approac for humans. We need to generate data that indicates proof of concept to gain permission to use this strategy for human therapies. Cells taken from patients will be converted to stem cells and differentiated to cartilage and tendons. It has the added benefit that the results can be used for direct application to a major

heath issue with economically important animals in Pennsylvania that suffer from damage to cartilage and tendons.

Our first approaches for the generation of Equine iPS have shown that embryonic stem cell-like colonies could be obtained by the introduction of the four Yamanaka factors (Oct4, Sox2, Klf4 and c-myc) of mouse origin. These reprogrammed cells expressed high levels of endogenous Oct4, but often did not subsist in cultures for passages higher than 15. Subsequent experiments showed that the number and morphology of the iPS colonies could be improved by adding miRNA302/367 [Anokye-Danso et al., Cell Stem Cell, 2011] to the cocktail of factors and, more importantly, the transduction of this microRNA cluster alone was capable of reprogramming both mesenchymal stem cells and fibroblasts from either fetal or adult horses into iPS cells. Several different lines of EqiPS have been obtained using these different combinations and have been characterized in terms of markers expression, karyotype and in vitro differential potential.

We observed that EqiPS express key pluripotency markers, including Oct4, Nanog, Sox2, Rex1, SSEA1, SSEA3, TRA-1-81 and Lin28 by Immunofluorescence and FACS. We confirmed the correct number of chromosomes for several of these iPS cell lines by preparing chromosome spreads, which showed either 62XX or 62XY karyotypes depending on which cell source was used for reprogramming. In addition, these cells can be kept in culture for periods of at least 3 months, maintaining a stable karyotype and their morphological characteristics, demonstrating true self-renewal capacity in vitro. In order to determine their in vitro pluripotency, we have directed some of these iPS lines to differentiate into different lineages and specific cell types. We were able to obtain Tubulin beta 3 expression when we used protocols for neuronal differentiation and CD34 positive cells when hematopoietic differentiation was induced. We were also able to differentiate these cells into osteoclasts, adipocytes and chondrocytes using standard protocols, as determined by von kossa / Alizarin Red, Oil red O and Alcian Blue stainings, respectively. We are currently performing the same differentiation protocols on newer EqiPS cell lines and developing assays for differentiation of these cells into tenocytes. To determine the pluripotency of EqiPS in vivo we have injected mice with these cells to assess their ability to form teratomas containing cell types derived from all three embryonic germ layers. This ongoing experiment will definitely demonstrate the pluripotency of these EqiPS cells.

When this study was started, there was no report of induced pluripotent stem cells in horse. Recently, a group has showed that it is possible to obtain iPS cell lines derived from equine fibroblasts [Nagy et al., Stem Cell Rev and Rep, 2011]. Our studies corroborate these findings and furthermore demonstrate that the introduction of the single cluster miRNA302/367 is sufficient to efficiently reprogram horse adult somatic cells to pluripotency.

Aim 3: Characterizing pulmonary epithelial cells that can affect repair in the mouse lung.
In the short time frame since this funding period began, we have performed the following studies as well as generated the following reagents:

1. We have now shown that proximal airway epithelial development and regeneration require HDAC1/2 mediated chromatin remodeling complexes. Loss of HDAC1/2 in developing lung endoderm leads to a complete deficiency of Sox2+ proximal airway endoderm progenitors-a phenotype that has not previously been reported. This loss of proximal airway development

leads to a dramatic failure in lung branching morphogenesis and rapid neonatal demise. We have shown that this loss of Sox2+ progenitors is due, in part, to an upregulation in Bmp4 expression and activity. We are currently exploring whether decreased Bmp4 expression can rescue the defects in HDAC1/2 lung mutants.

2. We have now shown that the combined loss of Foxp1/4 in the developing lungs causes a cell differentiation switch from airway secretory cells to a goblet cell phenotype both in the developing lung as well as after lung epithelial regeneration. Moreover, loss of Foxp1/4 in adult lung secretory cells leads to a hyper-reactive airway phenotype which mimics human asthma. These studies suggest that Foxp1/4 could be new causative factors in adult lung airway disease.

3. Previously, we generated both LSL-R26R-miR302/367 targeted ES cells and a floxed allele of the miR302/367 locus. The miR302/367 floxed mouse line and the LSL-R26R-miR302/367 animals are currently being used to assess the necessity and sufficiency of miR302/367 mediated molecular pathways in lung development and regeneration.

4. We have recently shown that the critical miRNA processing enzyme Dicer is essential for lung regeneration in the adult. Postnatal loss of Dicer using a secretory cell specific cre line (CC10-cre) shows that Dicer deficient airway epithelium cannot regenerate after injury. We are currently investigating the molecular pathways acting downstream of Dicer to regulate airway regeneration.

5. We have generated a transgenic reporter line for miRT302/367 and shown that it mimics endogenous expression pattern of this miR cluster. miR302/367-lacZ reporter is expressed in both the endoderm and mesoderm of the developing lung. This reporter is shut off in the adult lung, which correlates with little to no expression of miR302/367 in the adult. However, after naphthalene induced injury and regeneration, the miR302/367 Tg lacZ reporter is dramatically induced as is endogenous expression of this miR cluster. These studies suggest that miR302/367 expression closely tracks the development of lung progenitors and is induced during lung regeneration. We are using gain and loss of expression models as described above to assess the necessity of miR302/367 in lung epithelial regeneration.

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.

Anticipated Duration of Project

1/1/2010 – 6/30/2013

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.

Principal Investigator

David L Porter, MD
Director, Blood and Marrow Transplantation
University of Pennsylvania
PCAM 2 West Pavilion
Hematology Oncology
3400 Civic Center Blvd
Philadelphia, PA 19104

Other Participating Researchers

Ran Reshef, MD – employed by University of Pennsylvania

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

Through June, 2011 we have enrolled a total of 38 subjects on our clinical trial titled *The Role of CCR5 Blockade on the Prevention of Graft-Vs-Host Disease.* This completes initial enrollment to the initial phase II portion. The initial pharmacokinetic analysis portion of the study involved the first 12 patients and this phase of the project was completed and analyzed in the prior year of

the funding period. Based on our results, and since no toxicity was directly attributable to the drug and the lower dose level, we chose 300 mg twice daily for continuation of our phase II study.

Although maraviroc is not associated with an increase in infections or hematologic toxicity in HIV patients, we studied whether CCR5 blockade inhibits effector T-cell responses or hematopoietic activity that might negatively impact non-HIV patients undergoing HSCT. First, we tested whether the presence of maraviroc affected T-cell performance in a series of functional assays and found that pharmacologic doses of maraviroc (0.5-5uM) had no effect on T-cell proliferation and cytokine secretion in response to cognate viral peptide (CMV) stimulation; moreover, there was no diminution of specific T-cell cytotoxicity against CMV peptide-loaded T2 cells.

Second, to investigate the potential effect of maraviroc on hematopoiesis, we plated fresh normal donor PBMC in methylcellulose in the presence of appropriate cytokines and demonstrated that formation of myeloid and erythroid colonies was not significantly affected by the presence of maraviroc compared to untreated cells. These results are in agreement with clinical observations that failure of engraftment was never reported to be associated with SCT from Δ32-CCR5 carriers.

Although I will describe results from all 38 patients enrolled, follow up is quite short and needs to continue out to at least 6 months (and beyond) for all patients.

There were 23 men and 15 women who underwent RIC allogeneic SCT for AML (15), MDS (6), NHL (8), myelofibrosis (4) or aplastic anemia, multiple myeloma, CLL, Hodgkin's disease, and CML (1 each). The median age was 62 (range 21-74) and grafts were from matched siblings in 13 patients and unrelated donors in 25 patients. 6 unrelated donors were 1 antigen HLA-mismatched.

As per the protocol the conditioning regimen was fludarabine and busulfan and in all patients and GVHD prophylaxis includes tacrolimus, methotrexate, and maraviroc.

Maraviroc was well tolerated. The drug was temporarily held in 7 patients due to grade 3 LFT abnormalities (n=2) or grade 3-4 mucositis (n=5). LFT abnormalities did not recur when the drug was restarted in both patients. The adverse event profile was similar to the expected toxicity observed in patients undergoing RIC SCT.

All evaluable patients had adequate neutrophil engraftment (ANC>500/µL) at a median time of 15 days (range 10-27); one subject was not evaluable due to death on day 12 from sepsis. The median time to platelet engraftment (platelet count>20,000/µL without transfusions) was 19 days (range 9-84) and 1 patient failed to achieve independence from platelet transfusions in the setting of early relapse of his T-cell lymphoma.

Among 34 evaluable patients, the cumulative incidences of grade II-IV and grade III-IV acute GVHD at day 100 were 14.7 ± 6.1% (rate ± standard error) (95%CI [5.3, 28.7]) and 2.9 ± 2.9% (95%CI [0.2, 13.2]) respectively (Figure 1a). These rates were 2-3 fold lower than those reported

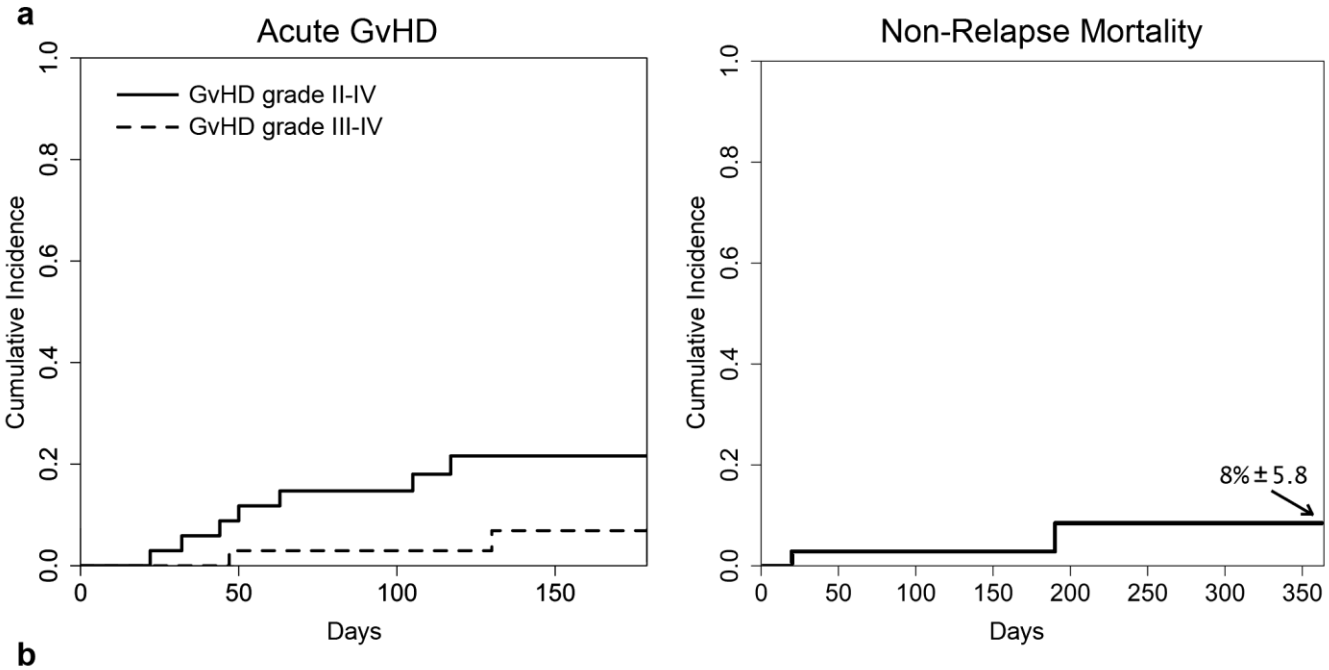
in other studies of RIC SCT. Importantly, in the first 100 days, there were no cases of acute GVHD involving the liver or intestine, strikingly similar to the findings in mouse models in which a CCR5 antibody ameliorated GVHD of the liver and gut. The only cases of acute GVHD that we observed up to day 100 were limited to the skin. At the day-180 benchmark, the rate of acute GVHD was 21.6 ± 7.4% (95%CI [9.3, 37.2]), and organ involvement remained largely confined to the skin with low rates of GVHD in the liver (3.3 ± 3.3%) and gut (8.5 ± 6%) (Figure 1c). In evaluable participants who received a graft from their HLA-matched sibling (n=11), there were no cases of GVHD prior to day-100 and only one case of acute GVHD prior to day-180. Furthermore, non-relapse mortality was quite low as seen in figure 1b.

Follow-up is too short to determine probability of relapse at this time. Continued follow up of these patients will allow us to estimate overall survival and importantly, relapse rates and disease free survival. In addition, we are analyzing samples to identify biological activity of maraviroc in the serum of patients on drug to show this inhibits in-vitro assays of CCR5 activity and migration. A comparison of these outcomes to our historical experience of similar patients is being planned and should be reported in the next cycle. Furthermore, if these data continue to look promising with longer follow-up (current median follow up 200 days), either an extended phase II trial or a randomized phase III study will be planned.

Table 1. Subject Characteristics

		N=38
Dose levels:	150mg bid 300mg bid	7 31
Recipient age, median (range)		62 (21-74)
Recipient age>60		68%
Recipient gender:	M/F (%)	60.5 / 39.5
Comorbidity Index:	Low Intermediate High	21 (55%) 13 (34%) 4 (11%)
Diagnosis:	AML MDS AA/MM/CLL/Hodgkin/CML-BC NHL MF	15 6 1 each 8 4
Donor:	Matched related Matched unrelated Single-antigen mismatched	13 (34%) 19 (50%) 6 (16%)
Donor age, median (range)		38 (20-68)
CMV status:	Recipient or donor positive (or both) Recipient and donor negative	22 (68%) 16 (42%)
CD34+ cells/kg x10^6, median (range)		5.5 (1.1-16.1)
CD3+ cells/kg x10^6, median (range)		188.1 (61.2-543)

Figure 1: Cumulative incidence plots of grade II-IV and grade III-IV acute GVHD in patients undergoing RIC SCT with maraviroc (MVC) added to standard GVHD prophylaxis. N=34 evaluable patients. **B.** The cumulative incidence plot of non-relapse mortality is displayed. **C.** Cumulative incidence rates (± standard errors) of acute GVHD and organ-specific GVHD at day-100 and day-180 after transplant in patients who received MARAVIROC.



b

	Acute GvHD (Gr. 2-4)		Skin GvHD		Liver GvHD		Gut GvHD	
	Day 100	Day 180	Day 100	Day 180	Day 100	Day 180	Day 100	Day 180
Rate ± SE (%)	14.7 ± 6.1	21.6 ± 7.4	14.7 ± 6.1	18 ± 6.8	0	3.3 ± 3.3	0	8.5 ± 6

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.

Anticipated Duration of Project

6/1/2010 - 6/30/2013

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

Caryn Lerman, PhD
Professor and Director, Tobacco Use Research Center
University of Pennsylvania
3535 Market Street, Suite 4100
Philadelphia PA 19104

Other Participating Researchers

James Loughead, PhD, Leah LaPrate, Sean Fleming, Christopher Jepson, PhD – employed by University of Pennsylvania

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

Study accrual was initiated during this period. To date, we have screened 159 smokers for eligibility and 80 of these were enrolled in the project. Thus far, 42 participants have completed both the abstinence and smoking fMRI scans. All smokers are followed for quitting success. Thus far, 41 participants have completed the entire study.

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.

Anticipated 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

Junhyong Kim, PhD
Co-Director, Penn Genome Frontiers Institute
University of Pennsylvania
302 Lynch Laboratories
433 S. University Avenue
Philadelphia PA 19104-6018

Other Participating Researchers

James Eberwine, PhD, John Hogenesch, PhD, Brian Gregory, PhD, Adam Peritz, PhD, Alex Chekholko, MS, Kristin Fields, PhD, Vijay Kumar, PhD – employed by University of Pennsylvania

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

In this reporting period, we have made progress on Specific Aim 1, developed additional proof-of-principle studies for Specific Aim 2, and made additional progress on Specific Aim 3.

Specific Aim 1.

As reported previously, we developed a new Next Generation high-throughput sequencing (HTS) facility within the Penn Genome Frontiers Institute (PGFI). Using partial support from this grant and additional institutional support, we purchased one Solid 4 system and two HiSeq 2000 systems. In this reporting period, we have upgraded the SOLiD 4 system to SOLiD 5500 system, which gained 2-fold additional capacity. In addition, the HiSeq 2000 systems' sequencing chemistry and associated infrastructure was upgraded to version 3 chemistry, which gained another 3-fold throughput. The SOLiD 5500 instrument is capable of generating 15 billion bases of sequence information per day while the HiSeq 2000 instrument is now capable of producing up to 40 billion bases of sequence information per day. Using this facility we have completed nine total SOLiD chemistry sequencing, and 72 HiSeq sequencing during this reporting period. For RNA sequencing, we have carried out 34 different RNA sequencing samples and 2 small RNA sequencing chemistry runs using the HiSeq system. In particular, we have optimized the protocol for large-scale bar-coded SOLiD SAGE RNA profiling process and generated RNA profiles for 64 different yeast transcriptomes with a single sequencing run.

Next-generation sequencing requires considerable computational processing to generate human-usable information from the raw data produced by the sequencing machines. For RNA sequencing application using 100 million sequence reads, we have benchmarked computational loads using the data produced by our HTS facility. For complete mapping using a Penn developed program, RUM (RNA Unified Mapper), 1800 CPU hours (average of 2 Ghz AMD CPUs) using 6 GB of main memory and 250-500 GB of output space is required. The amount of output is data dependent and is a function of sequence redundancy of the genome. To meet these computing demands, we have concentrated on upgrading both our hardware infrastructure and establishing analysis pipelines. During this reporting period, we added 390 TB of a new Isilon network storage device to Penn Genome Frontiers Institute's High-Performance Computing cluster to service the RNA sequencing aims of this grant. To improve data processing efficiency, we have developed a computational analysis pipeline for RNA sequencing data that utilizes both our compute resources and external cloud vendor environments. The core of this pipeline is the RNA Unified Mapper developed at Penn. The main problem solved in this approach is to separate those RNA reads that can be efficiently mapped to the reference genome from those that are more problematic and then applying a variety of exact and heuristic mapping solutions.

In addition to the RUM algorithm, we have been developing a Bayesian integration scheme to quantify RNA expression from RNA sequencing where reads that map to redundant positions are probabilistically integrated. Briefly, one of the problems in quantifying RNA molecules from sequence data is that typically a portion (10-50%) of sequence reads map to multiple locations in

the genome. This leads to a conundrum in how to convert the presence of multiple-mapped reads to the number of RNA molecules transcribed from the mapped location of the genome. Recently, we developed a Bayesian scheme for estimating Single Nucleotide Polymorphism (SNP) from multiply mapped sequences (Simola and Kim, published in Genome Biology doi:10.1186/gb-2011-12-6-r55). The main idea here was to generate a prior model of possible genomic locations for sequence origination and then a conditional probability model for sequence reads dependent on the reference genome. We are working to adopt this model to RNA transcriptome quantification. The main approach is to utilize a transfer function from probabilistic distribution of mapped read densities to output RNA quantities.

Specific Aim 2.

The main idea behind Specific Aim 2 was develop an approach to altering the phenotype of mammalian cells using complex RNA mixtures (i.e., transcriptomes). This idea builds on the previous works in the laboratories of J. Eberwine and J. Kim, where the transcriptome from a donor cell was used to transdifferentiate a mature rat neuron into displaying the phenotype of a rat astrocyte. A component of the current aim was to prototype an in vitro system for generating pools of RNA that can be used for such targeted transdifferentiation experiments. To further this aim, in the current reporting period we have generalized the previous experiments to other cell types and other donor RNA populations, thereby establishing a proof-of-principle for rationale of the proposed in vitro system. The work has been published in PNAS (Kim et al., 2011; doi: 10.1073/pnas.1101223108). Briefly, we showed that the transfer of the adult ventricular myocyte (AVM) transcriptome into either a fibroblast or an astrocyte converts the host cell into a cardiomyocyte. Transcriptome-effected cardiomyocytes (tCardiomyocytes) displayed morphologies, immunocytochemical properties, and expression profiles of postnatal cardiomyocytes. Cell morphology analysis showed that tCardiomyoctes are elongated and have a similar length-to-width ratio as AVMs. These global phenotypic changes occur in a time-dependent manner and confer electroexcitability to the tCardiomyocytes. These data highlight the dominant role of the gene expression profile in developing and maintaining cellular phenotype and therefore the continued development of an in vitro system for generating complex RNA reagents.

Specific Aim 3

In the current reporting period, we have continued to investigate single-cell variability of transcriptomes, developed benchmarks for evaluating single cell transcriptomes, and continued to refine protocols for single-cell RNA sequencing.

We have now generated more than 50 different single cell transcriptome profiles using both microarray-based technologies and RNA sequencing. One of our goals was to understand the multi-dimensional nature of single-cell variability. Using a RNA amplification and microarray analysis, we have previously demonstrated that individual cell's transcriptome profiles show almost as much variation within a given cell type as between two different cell types. In analyzing this variability for rat neurons and astrocytes, we showed that most of the single-cell variation is low-dimensional. That is, the variation is concentrated around a particular direction in the transcriptome space, suggesting that the variation is either subject to regulation or to

stoichiometric constraints. We have now extended this analysis in two ways. First, we have collected and analyzed other species (mouse) and cell types (fibroblasts, cardiomyocytes). A key finding here was that we found evidence that neuronal cell populations are much more variable than other cell populations, potentially related to their inherent function in the brain. Second, we have been developing computational procedures to identify bimodal variable genes—that is, genes that show extreme variation across different cells of the same type. The hypothesis here is that these genes will comprise a set of cell-variable genomic elements that are responsible for heterogeneous responses of cells. One idea is that this kind of inherent heterogeneity is responsible for variable phenotype in degenerative diseases such as Alzheimer's disease. For this work, we have developed a filtering criterion for multi-modal expression and we are carrying out clustering analyses and other analyses to examine if specific set of genes is mostly responsible for cell-to-cell variability.

One of the difficulties of working with single cell measurements is that it is difficult to distinguish between experimental variation and biological variation; once a cell is assayed for its RNA content, there is no other replication to benchmark the measurement. To overcome this problem, we have been generating a set of in vitro benchmark data. We have generated a pool of reference RNA from several different tissues (cortex, hippocampus, liver) from both rats and mice and then used a dilution series to generate a replicate set of RNA sources that have molar quantities similar to mammalian cells (0.1-1 pg). We have then independently amplified the RNA to generate a reference profile. We had previously generated such reference benchmarks using microarray measurement platform. During the previous funding period we have generated a set of RNA sequencing benchmarks. Unfortunately, during this period we also discovered that aRNA sequence libraries generate biased RNA quantification due to 3' bias of the sequence fragments. There were two different problems in existing protocols. First, the quantification of template RNA pools does not follow standard curves established for other kinds of RNA libraries due the different length distributions. Second, mapping the sequenced read back to the reference genome requires additional consideration of both 3'UTR regions and poly-A rich regions. We have refined our aRNA sequencing protocol and computational quantification procedure to account for this bias. We are completing a new set of in vitro dilution benchmarks and also a new set of single cell data from mouse brain cells using this new protocol.

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.

Anticipated 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

Peter O'Dwyer, MD
Professor of Medicine
University of Pennsylvania
Abramson Cancer Center
12 Penn Tower
3400 Spruce Street
Philadelphia, PA 19104

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

A total of 49 patients have been enrolled on the treatment portion of the study. Accrual of 15 patients to each of the breast, colon and germ cell groups has nearly reached the initial target of 15 patients. Based on promising results in breast and germ cell tumors, we hope to increase the sample size to allow for additional patients.

The patient breakdown is as follows:
Breakdown by Gender: Male 22; Female 27
Breakdown by Race: Caucasian 42; African American 3; Hispanic 2; Unknown 2

Breakdown by Age:

10-20yrs	20-30yrs	30-40 yrs	40-50yrs	50-60yrs	60-70yrs	70-80yrs	80-90yrs
1	6	2	9	10	16	3	2

The most frequent and serious side effect seen has been neutropenia requiring dose delays and reductions.

A total of 125 tumor samples were analyzed during this period. Of these, one hundred three (103) patient samples have been testing has been compted for Rb expression. Of the 125 tumor samples thirty nine (39) samples have also been tested for CCND1, 5 of which were positive.

The analysis of the collected samples is continuing and the results will be reported during the next progress report cycle.

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.

Anticipated 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.

Principal Investigator

Don A. Baldwin, PhD
Adjunct Associate Professor
University of Pennsylvania
277 John Morgan Bldg.
3620 Hamilton Walk

Philadelphia, PA 19104

Other Participating Researchers

Michael D. Feldman, MD, PhD, Timothy R. Rebbeck, PhD, Vivianna M. Van Deerlin, MD, PhD, Trevor M. Penning, PhD – employed by University of Pennsylvania

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

<u>Research Team and Materials</u>
Drs. Baldwin, Rebbeck and Feldman hosted six meetings of a Prostate Cancer Molecular Profiling working group to coordinate research in this project, and to invite other Abramson Cancer Center investigators to learn about our work and ways they might collaborate. During the reporting period (the first full year of this project), this grant has spurred two new collaborations that will enhance the project with research that is independently funded and related to, but beyond the scope of, the specific aims described above. Dr. Harold Reithman at the Wistar Institute will use the same cohort of archived prostate cancer cases to perform

telomere length assays, adding a new class of potential biomarkers to the panel of assays to be evaluated. Dr. Priti Lal, Dept. Pathology and Lab Medicine, is a clinical pathologist and colleague of Dr. Feldman who will assist the deep pathology reviews of the archived case cohort and oversee immunohistochemistry assays to screen for the presence of genomic translocations.

The working group reviewed the clinical pathology metrics currently used to assess risk of recurrence, the availability of these metrics for our archived cases, the distribution of formalin-fixed paraffin embedded (FFPE) tumor samples over the range of pathology metrics and outcomes, and the total number of cases available. We created a four-phase research plan that incorporates Kattan score as the clinical predictor of tumor recurrence and prostate specific antigen (PSA) level as the clinical measure of therapeutic failure, Figure 1. The rapid and slow/no recurrence cases for Aims 1 and 2 will be selected from the Phase I cohort. Drs. Feldman and Lal have reviewed about 20% of the archival cases. They have also provided FFPE samples from irrelevant prostate specimens to Dr. Baldwin's lab for development and validation of genomics assays. Dr. Matt Buas was hired as a postdoctoral researcher supervised by Drs. Baldwin and Rebbeck to conduct lab assays and coordinate transfers of FFPE samples.

Aim 1

Four nucleic acid extraction kits were evaluated for ability to recover both RNA and DNA from the same FFPE sample. Tissue sections mounted on glass slides were compared to a reference slide to locate regions containing a large proportion of tumor cells, and these regions were scraped to collect FFPE tissue for extraction. An optimized extraction procedure was established, and several micrograms of DNA and total RNA (including microRNA) can be purified from five serial section slides. RNA from FFPE samples was used for pilot Affymetrix microarray profiling on mRNA and microRNA GeneChips. As expected, formalin fixation degraded the mRNA such that whole-genome profiling patterns are quite different compared to fresh frozen samples of the same tumor, Figure 2. microRNA, however, is apparently much less susceptible to formalin degradation, and the microarray profiling data are very similar to those from matched frozen samples. This justifies the microRNA profiling component of Aim 1 as a means to screen for biomarkers of tumor biology in FFPE material that will translate to RNA from frozen specimens collected by biopsy.

The DNA extracted from pilot samples has been used to test three whole-genome amplification kits. An optimized protocol was established to amplify and label FFPE DNA. Hybridization of this DNA to Affymetrix cytogenetics microarrays is currently in progress. In the time since this project was proposed, Affymetrix has released two new microarrays developed for DNA copy number assays. The CytoScan array is intended for cytogenetics applications, and the OncoScan array provides more probes focused on copy number measurements of known oncogenes while retaining whole-genome coverage. Both incorporate genotyping assays to identify regions with loss of heterozygosity, and development of both has included protocols for use with FFPE DNA. We will complete our evaluation of the original Affymetrix cytogenetics array as described, but have also contracted with Affymetrix for early access to the new microarrays so that this project's FFPE prostate samples can be evaluated.

Copy number + genotyping microarrays are unable to detect balanced translocations. Prostate cancer genomes sometimes contain a balanced rearrangement that fuses the TMPRSS2 and ERG

genes. Recently, the RNA transcripts resulting from such gene fusions were shown to be detectable and discriminated from the normal transcripts of each gene. We attempted to replicate this finding using Affymetrix Human Exon 1.0ST Arrays, which measure the abundance of each exon within all targeted genes. Using cell lines known be positive or negative for TMPRSS2:ERG fusion, we were able to detect the fusion transcript expressed well above normal gene levels, and with an exon abundance pattern that reflects the translocation structure (Figure 3). We will next determine whether this assay can be used for RNA from FFPE samples, and review the literature to assess whether this or related translocations may be relevant to risk of tumor recurrence.

In this reporting period, sample handling, nucleic acid extraction, microRNA microarrays, and whole-genome DNA amplifications have been optimized for Aim 1. We are ready to proceed with profiling of the prostate cancer cohort as soon as the pathology reviews are completed. If justified, we will add exon-level RNA profiling as a biomarker screening assay.

Aim 2

The pilot DNA samples from Aim 1 have been tested in a Sequenom DNA methylation assay. We chose 22 genes previously implicated in prostate cancer, and designed multiplexed PCR reagents to create 46 amplicons from the 5' proximal promoter regions of these genes. Each PCR was individually validated under conditions that will be used for the multiplexed reaction, Figure 4, and the pilot DNA samples were assayed. Figure 5 illustrates methylation data for a region of the GSTP1 gene, and demonstrates differential methylation between normal and tumor tissues for two independent patients.

Detection of cancer-related DNA mutations will be performed using the Sequenom OncoCarta genotyping assay for FFPE samples. We have examined the mutation sites included in the standard OncoCarta panels, and noted that a number of mutations previously found in prostate cancers are not tested. We are now working with Sequenom to create "ProstaCarta" panels for better coverage of our targeted mutation sites.

In this reporting period, a custom DNA methylation assay has been validated and an updated somatic mutation genotyping assay entered the design phase. We are ready to proceed with targeted methylation screening of the prostate cancer cohort when samples are available. The enhanced OncoCarta assay will be validated next, and methods for whole-genome methylation profiling will be evaluated for FFPE specimens.

Aim 3

Funds from this grant were used to purchase a MassARRAY Analyzer 4 instrument that was placed in the Molecular Pathology Laboratory at the Hospital of the University of Pennsylvania. Drs. Baldwin and Van Deerlin (Director of Molecular Pathology) coordinated installation of the instrument and training for the clinical laboratory staff.

Dr. Baldwin initiated a new collaboration with Dr. Jennifer Morrissette, who directs the clinical cytogenetics laboratory. Microarray-based cytogenetics screening is increasingly used to detect DNA copy number aberrations, uniparental disomy and oncogenic loss of heterozygosity. The

prostate cancer samples from this study are being used to evaluate the Affymetrix CytoScan assay for deployment in the clinical practice.

In this reporting period, an instrument was installed in the CLIA/CAP-regulated clinical laboratory that will allow the biomarker assays developed in Aims 1 and 2 to be translated to clinical tests. A new opportunity to use the existing Affymetrix platform for cytogenetics applications may lead to clinical testing of prostate cancer cases.

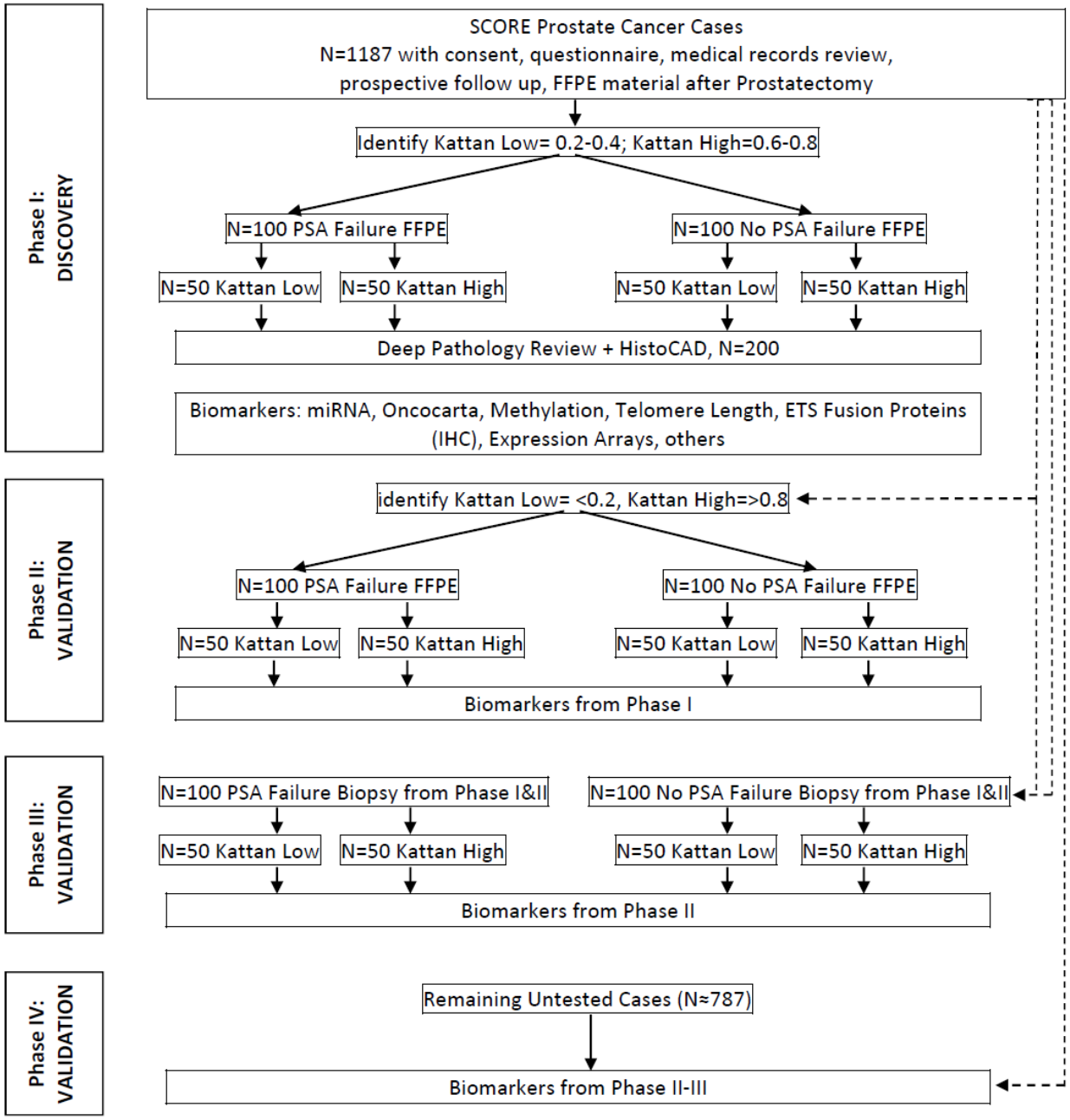


Figure 1. Prostate Cancer Molecular Profiling research plan. The rapid and slow/no recurrence cases for the C.U.R.E. project will be a selected subset of samples from this four-phase, multi-investigator research effort. The plan includes I) targeted and whole-genome screening of a variety of classes of biomarkers to test for correlation with, and improvement over, the clinical metrics of Kattan score and PSA levels; II) validation of candidate biomarker assays in extreme Kattan phenotype cases, III) determination of assay performance in biopsy samples, and IV) biomarker validation in an independent cohort.

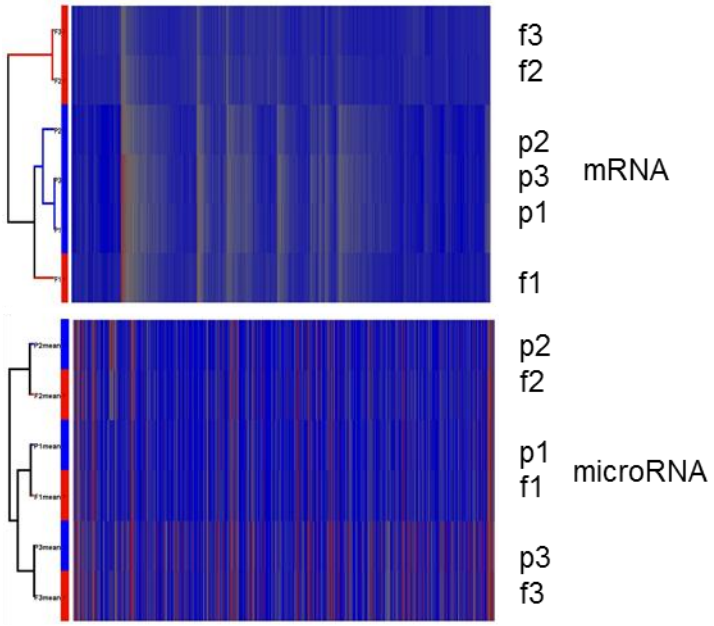


Figure 2. Unsupervised hierarchical clustering of mRNA and microRNA microarray data. Three patient samples were split and either frozen as fresh tissue (f) or embedded in paraffin after formalin fixation (p). Global expression levels of mRNA are more similar by specimen treatment than origin, while microRNA profiles reflect origin (patient) and are less affected by specimen treatment.

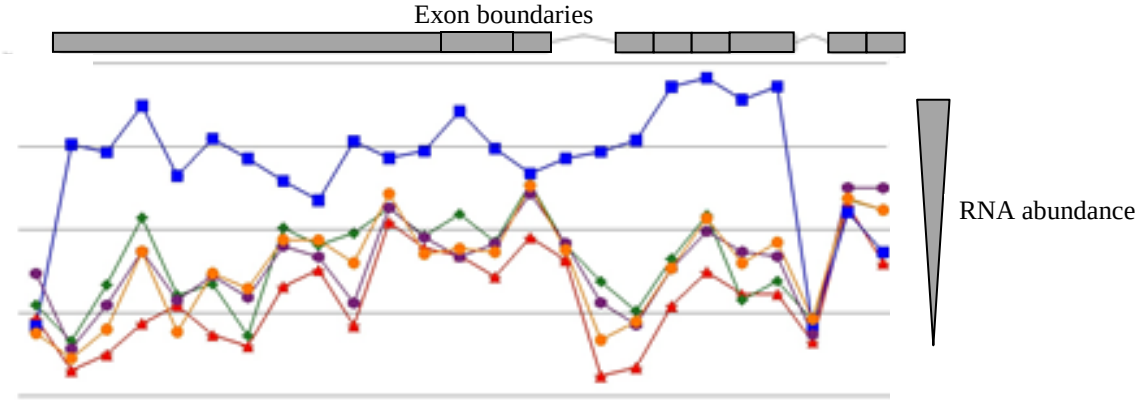


Figure 3. Exon microarray detection of a gene fusion transcript. Exons from the ERG gene are shown as grey boxes along the upper x axis, and relative RNA expression signals from probes to those exons are plotted on the y axis. RNA from a VCaP cell line carrying an ERG translocation (blue squares) was compared to a non-translocation prostate cell line (red triangles), FFPE normal prostate tissue (green diamonds), FFPE tumor 1 (purple hexagons), and FFPE tumor 2 (orange circles). The translocation induces higher expression of only those ERG exons affected by the gene fusion – in the case of VCaP, exons 1 and 2 (the first two on the right) show normal abundance levels while the remaining exons are over-expressed due to inclusion in the fusion transcript.

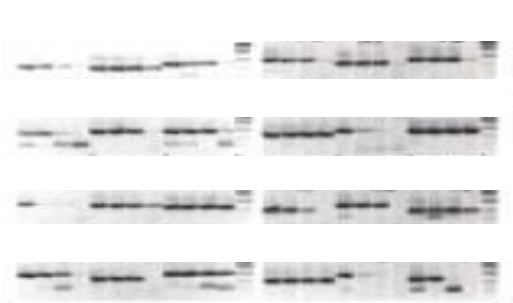


Figure 4. Gradient PCR was used to optimize conditions for PCR amplification of genomic DNA regions targeted for methylation analysis. These example gel pictures demonstrate that this optimization has produced single major amplicons (dark bands) for all targets, with only a few minor background amplicons that should not affect the methylation assay. The 24 amplicons shown have been combined with 22 additional PCRs to target 46 regions of 22 genes and two controls.

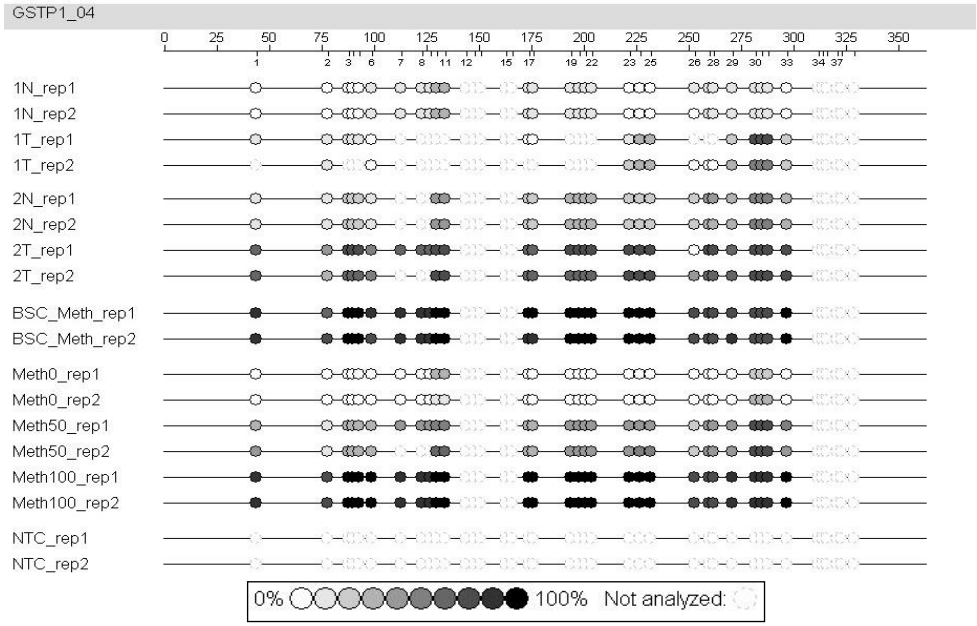


Figure 5. DNA methylation screening of a GSTP1 promoter region. The Sequenom EpiTyper assay was used to detect methylated cytosines within a 350bp region that harbors 39 CpG sites (labeled along upper x axis). The relative percent methylation detected in the amplicon population (derived from bisulfite converted genomic DNA) is indicated by the grey-scale circles for each potentially modified CpG site. The BSC control (fully methylated) and Meth series of dilutions (0, 50 or 100% methylated) demonstrate assay performance. NTC is the no-template negative control. FFPE DNA samples from two patients were assayed using tumor tissue (1T, 2T) or adjacent normal tissue (1N, 2N).

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

Project Overview

Molecular imaging has been identified as a key enabling technology for the development and clinical implementation of targeted treatments in a number of diseases including cancer. By allowing molecular processes to be interrogated in the patient, molecular imaging can identify specific treatment targets in the patient and can confirm that treatment has effectively engaged its target. Currently, positron emission tomography (PET) is the primary molecular imaging technology with human use potential. The strength of PET rests in the development of radiolabeled compounds capable of binding to selected targets. A particular challenge associated with the development of molecular imaging radiopharmaceuticals is that the short half life of the radiopharmaceuticals (approximately 2 hours or less) requires that they be synthesized, assessed for quality and delivered to the patient in a short period of time. The lack of a robust facility on site within the University of Pennsylvania campus to perform the synthesis consistent with the requirements needed for human uses has limited the ability to translate basic discovery into clinical molecular imaging methods.

The University of Pennsylvania has already initiated a project supported by federal ($2 million) and matching institutional funds ($6 million) to install a new cyclotron capable of creating the isotopes needed to synthesize PET radiopharmaceuticals. We propose here to build a facility to allow the actual synthesis of radiopharmaceuticals for human use according to good manufacturing practices (GMP). The facility will support research at Penn and serve as a resource for commercial and academic institutions in the Commonwealth. It will consist of 1000 sq. ft. of pharmaceutical grade, GMP/cGMP laboratory space. The new laboratory space will consist of 7 separate rooms. The four key rooms are clean, production rooms (Class 10,000 or better) for different isotopes: ^{18}F, ^{11}C, ^{124}I (and other solid target produced compounds), and a flexible R & D space. The rooms are sealed, have sealed epoxy floors, clean-room ceilings, HEPA filtration, negative pressure working areas containing hot cells and aseptic shielded cells for the distribution and fractionation of the drug doses. A changing area prior to entering the clean rooms, a wet lab with a fume hood and bio-safety laminar flow hood and workspace for

preparation of necessary solutions, reagents, and drug components, supports the four production rooms.

Principal Investigator

Chaitanya Divgi, MD
Director of Molecular Imaging
University of Pennsylvania
Hospital of the University of Pennsylvania
3400 Spruce Street
Philadelphia, PA 19104

Other Participating Researchers

None

Expected Research Outcomes and Benefits

We expect to be able to dramatically increase our ability to produce radiopharmaceuticals for human use as a result of the proposed GMP facility. This includes doubling our capacity to produce ^{18}F compounds and enabling the production of ^{11}C, which are extremely difficult to produce in the current facility. In addition, all of these compounds will be produced under conditions that will meet the highest regulatory standards for pharmaceutical production. As a consequence, we expect to be able to expand our support for human studies using molecular imaging. This is expected to lead to better techniques to detect and characterize cancer and other diseases. In addition this will accelerate the development of personalized medicine approaches by allowing the detection of specific treatment targets and the impact of targeted therapies. We expect to be able to produce compounds for research performed on the University of Pennsylvania campus and to be able to support research from academic and commercial research organizations across the Commonwealth. Given the importance of the pharmaceutical and biotechnology industry in Pennsylvania, we expect significant commercial partnerships.

Summary of Research Completed

The construction of the GMP facility has been completed. As proposed, the facility consists of a 1000 sq. ft. of pharmacy grade, GMP/cGMP laboratory space adjacent to the new cyclotron facility on Currie Blvd. on the University of Pennsylvania campus. The facility includes 4 GMP rooms, a distribution room and a wet chemistry room as illustrated in figure 1.

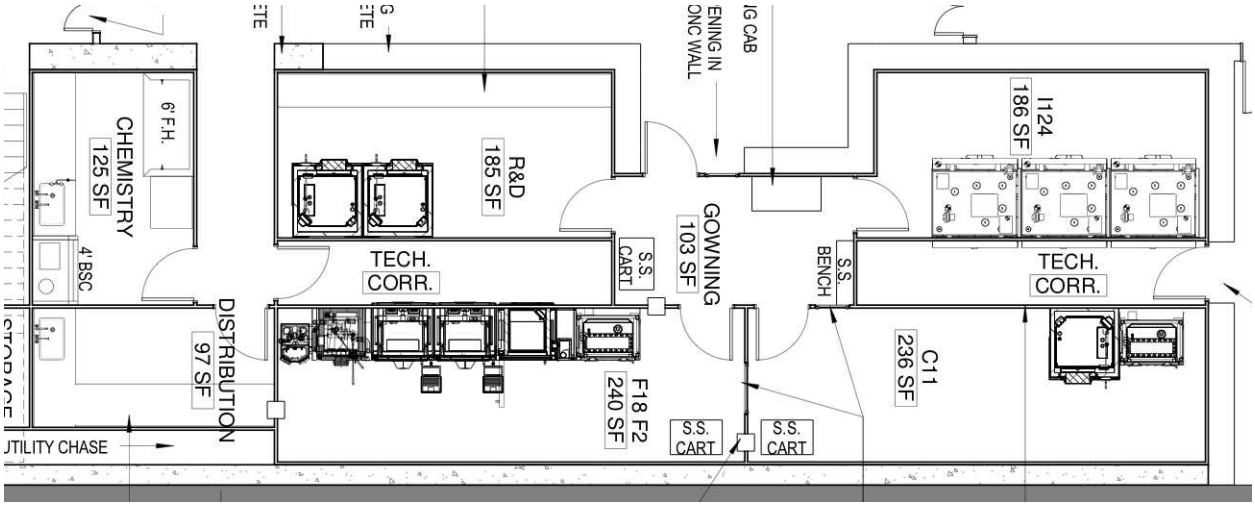


Figure 1: Drawing of the radiochemistry GMP facility

The 4 GMP production rooms are designated for production of 124I, 11C, 18F, and novel research and development to enable a wide range of radiopharmaceuticals to be produced. Each of the four GMP rooms has a sealed epoxy coated floor. The walls are drywall construction, sealed with epoxy paint for ease of cleaning and reduction of particulate matter in the air consistent with the original proposal. The ceilings are gasketed, clean-room ceiling tiles with sealed light fixtures, again to reduce particulate matter in the air. Each room is supplied with HEPA filtered air to achieve a Class 10,000 or better environment consistent with GMP standards for GMP synthesis of radio pharmaceuticals.

The distribution room contains radiation counters for wipe testing and performing the final assays of the drugs before shipping them to the imaging areas. This room also has epoxy floors and walls to maintain cleanliness. Adjacent to this room is a wet chemistry area to be used for making needed chemicals and reagents. This is a clean area, epoxy walls and floor, but will not be classified nor will it have HEPA filters. There is also a gowning/changing area in the vestibule outside of the GMP production rooms to enable compliance with GMP standard.

This facility has a few unique features including distribution of radioactivity from the IBA or JSW cyclotrons, each of which has complementary capabilities. In addition, there are clean-room feed-throughs to reduce entering and exiting production space. Photographs illustrated the completed laboratory are included in figures 2 and 3 below.

Figure 2: GMP room

Figure 3: Gowning area

Having finished construction of the new facility, we are in the process of setting up the GMP synthesis modules and appropriate QC equipment to enable complete operation.

This facility represents the only GMP facility dedicated to the production of novel PET radiopharmaceuticals in the Commonwealth. It will provide the opportunity to pursue clinical trials and ultimate clinical production of advanced molecular diagnostics that will improve the detection and characterization of cancer. It will also provide the opportunity to partner with the region's pharmaceutical industry to develop pharmacodynamic and clinical imaging response markers that will enable personalized approaches to defining appropriate treatment targets and drug doses to optimize treatment outcomes with the minimum toxicity.

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 – 6/30/2012

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.

Principal Investigator

John D. Gearhart, PhD
University of Pennsylvania
Biomedical Research Building
421 Curie Blvd., BRB 1151
Philadelphia, PA 19104-6058

Other Participating Researchers

Russell Addis, PhD; Filipa Pinto, PhD – employed by University of Pennsylvania

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

Twelve transcription factors known to play critical roles in midbrain dopaminergic neuron development and/or maintenance were cloned into the doxycycline-inducible lentiviral vector FU-tetO-Gateway (Figure 1A). Seventy-four unique combinations of these vectors were used to

transduce mouse embryonic fibroblasts. RNA of transduced cells was harvested after 7 days of doxycycline-induced factor expression and assayed via RT-PCR for expression of tyrosine hydroxylase (Th) and DOPA decarboxylase (Ddc), the enzymes of dopamine synthesis. The three-factor combination of ASCL1, LMX1B, and NURR1 resulted in the most robust expression of Th and Ddc. This combination was selected for further analysis.

In order to increase the efficiency of cells receiving all three transcription factors, as well as to reduce variability resulting from different ratios of the factors reaching individual cells, we constructed a polycistronic vector. The open reading frames of ASCL1, LMX1B, and NURR1 were linked via recombinant PCR such that viral 2A peptide sequences separate the three genes, as shown in Figure 1A. A glycine-serine-glycine (GSG) linker was included upstream of each 2A sequence to facilitate protein cleavage. The ASCL1-P2A-LMX1B-T2A-NURR1 cassette (hereafter referred to as ALN) was inserted into FU-tetO-Gateway to produce the tetO-ALN vector. Cleavage at the 2A sites was validated by performing *in vitro* transcription and translation using the T7 promoter present at the start of the Gateway recombination site in the presence of biotinylated lysine. A streptavidin-HRP western blot confirmed complete cleavage at both the T2A and P2A sites (Figure 1B). The tetO-ALN lentiviral vector delivered ALN to both astrocytes and fibroblasts at >99% efficiency, as determined by immunocytochemistry for the C-terminal V5 tag on NURR1.

Astrocytes were transduced with FUdeltaGW-rtTA (the reverse tetracycline transactivator) and tetO-ALN. The following day, doxycycline was added to astrocyte medium to induce ALN expression (Day 0). After four days in astrocyte medium with doxycycline, transduced cells were switched to neuronal medium. Doxycyline was removed on Day 10. After 14 days from the initial ALN induction, 35.1 ± 1.5% of cells expressed type III beta-tubulin (clone TUJ1), a neuronal marker. 50.9 ±3.3% of TUJ1+ cells were also positive for tyrosine hydroxylase, yielding an overall conversion rate of 18.2 ± 1.5%.

To further characterize the induced dopaminergic neurons, we sorted ALN-derived neurons on Day 14 using a MAP2-CD4 reporter vector and magnetic beads conjugated to anti-CD4. RNA was isolated from sorted cells and assayed via RT-PCR for expression of a panel genes shown in Figure 1D. Gene expression in sorted neurons was compared to astrocytes that did not receive ALN as well as mouse embryonic stem cells that had been differentiated to dopaminergic neurons via co-culture with PA6 stromal cells. Sorted neurons displayed robust upregulation of genes expressed in midbrain dopaminergic neurons such as Pitx3, Lmx1a, Engrailed-1, aldehyde dehydrogenase, Foxa2, the vesicular monoamine transporter Vmat2, Msx1, and the dopamine transporter. Expression of the potassium channel Girk2 was seen via immunocytochemistry, and no nuclear Otx2 was detected. Together, these results demonstrate that the induced dopaminergic neurons have an A9/substantia nigra phenotype, rather than an A10/ventral tegmental area phenotype.

To evaluate the electrophysiological phenotype of induced dopaminergic neurons, patch clamping was performed on induced neurons between days 9 and 26 of initial ALN induction. Neurons were identified by screening for GFP driven by the *MAP2* promoter. Current clamp recordings in Day 9 cells showed an immature spiking pattern – generally a single action potential per step current injection. By Day 14-21, neurons demonstrated repetitive firing of

action potentials in individual step current injections (Figure 2A). Of 30 patched cells, 24 fired action potentials (80%). Recorded cells had an average resting membrane potential of -55.4 mV. In voltage clamp, large sodium and potassium currents were seen (Figure 2B), with an average maximum I_{Na} of 1546 pA. Electrophysiological properties of induced dopaminergic neurons are summarized in Table S3. Induced dopaminergic neurons exhibit robust synaptophysin expression (Figure 2C), suggesting the capacity to form synaptic connections. We detected excitatory post-synaptic currents in 6% of recorded neurons, as shown in Figure 2D.

It has been well established that midbrain dopaminergic neurons are pacemaker neurons, spontaneously firing action potentials at a rate between 1 and 9 Hz with an average of 4.5 ± 1.7 Hz. We observed spontaneous firing of action potentials in 43% of recorded *MAP2*-GFP+ induced neurons, a fraction that is consistent with the 50.9 ±3.3% of neurons that were immunoreactive for tyrosine hydroxylase (Figure 2E). The frequency of spontaneous firing ranged from 0.9 to 8.6 Hz, with an average of 5.6 ± 1.2 Hz. The pacemaker activity of dopaminergic neurons is driven by rhythmic oscillations of intracellular calcium ion concentration

To determine whether induced dopaminergic neurons exhibit calcium oscillation, we transduced astrocytes (prior to reprogramming) with a lentiviral vector containing the genetically-encoded calcium indicator GCaMP3 driven by the *MAP2* promoter. We detected robust calcium oscillation, consistent with the established dopaminergic neuron phenotype.

To confirm that induced dopaminergic neurons produce and release dopamine, we stimulated cells with 56mM KCl and measured dopamine release via HPLC. Cells assayed at 17 days post-transduction with ALN released dopamine in response to membrane depolarization while un-transduced cells (0F) did not, as shown in Figure 2F. No significant levels of epinephrine, norepinephrine, or serotonin were detected.

Our study is the first to demonstrate direct reprogramming of astrocytes to dopaminergic neurons. These neurons exhibit gene and protein expression patterns that are consistent with A9 midbrain dopaminergic neurons. It is interesting to note that the average frequency of spontaneous action potential firing in astrocyte-derived dopaminergic neurons (5.6 ± 1.2 Hz) is higher than the firing frequency reported in fibroblast-derived neurons (2.6 Hz), and closer to the 4.5 ± 1.7 Hz average reported for “authentic” dopaminergic neurons. Further comparison will be needed to determine whether this difference can be attributed to the different starting cell populations, or instead is due to differences in neuronal maturity at the day of recording. Global epigenetic profiling will also be informative as to the relative completeness of reprogramming undergone by astrocytes and fibroblasts.

The present study is the first to demonstrate that induced dopaminergic neurons can form synaptic connections, a critical requirement for any future utility in cell-based therapies. Furthermore, by demonstrating that a single vector can deliver all of the factors necessary for reprogramming, this work takes a crucial step toward to ultimate goal of *in situ* reprogramming of astrocytes to dopaminergic neurons.

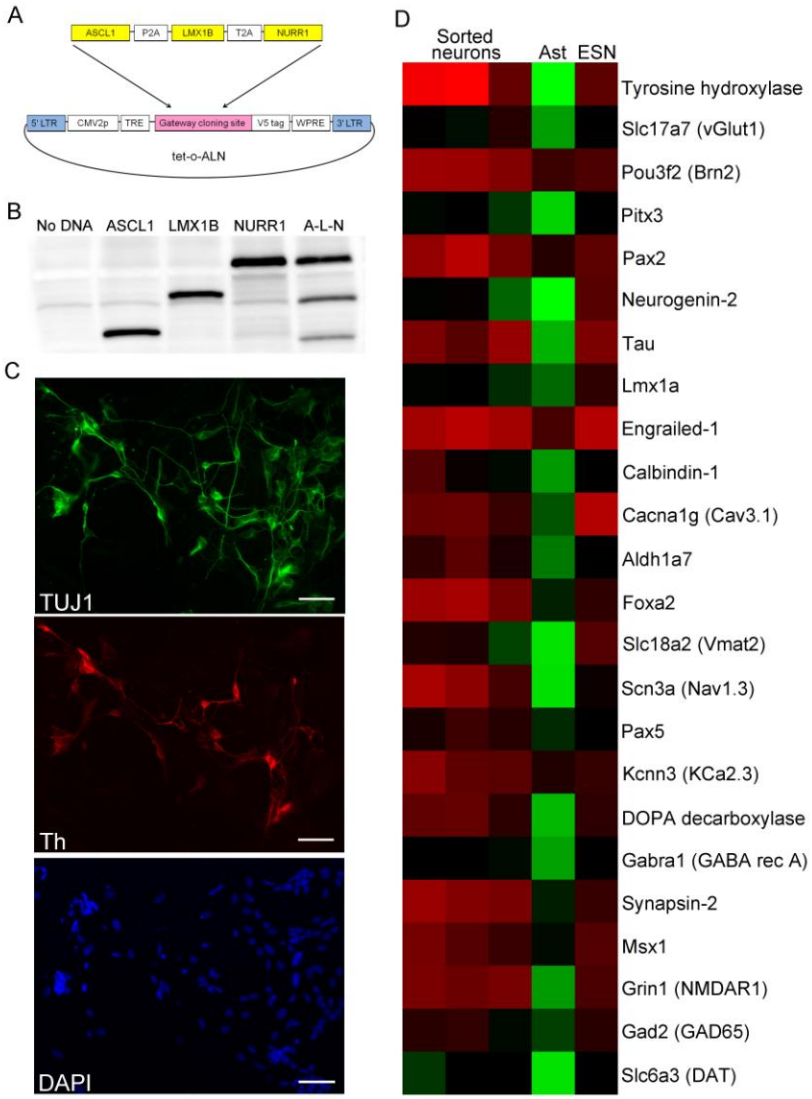


FIGURE 1. Induced dopaminergic neurons derived from astrocytes. (A) Polycistronic vector containing open reading frames of human ASCL1, LMX1B, and NURR1 linked by viral 2A sequences. (B) Complete cleavage at 2A peptide sites confirmed by *in vitro* transcription and translation (TnT) of lentiviral plasmids in the presence of biotinylated lysine. Streptavidin-HRP Western blot shows all newly synthesized protein. Lane 1: No DNA TnT control. Lanes 2-4: TnT performed on original single-factor plasmids. Lane 5: TnT for polycistronic ALN plasmid. Band intensity is proportional to the number of lysine residues present in each protein sequence. (C) Efficient conversion of astrocytes to dopaminergic neurons demonstrated by immunocytochemistry for the neuronal marker TUJ1 (type III beta tubulin), the dopaminergic marker tyrosine hydroxylase (Th), and the pan-nuclear marker DAPI. Scale bars 50 μm. (D) Transcriptional profile of induced dopaminergic neurons. Heat map of quantitative RT-PCR results comparing astrocyte-derived neurons magnetically sorted for a *MAP2*-CD4 reporter. Ast, uninfected astrocytes. ESN, mouse embryonic stem cell-derived neurons, generated via co-culture with PA6 stromal cells. Induced dopaminergic neurons express markers consistent with midbrain dopaminergic neurons.

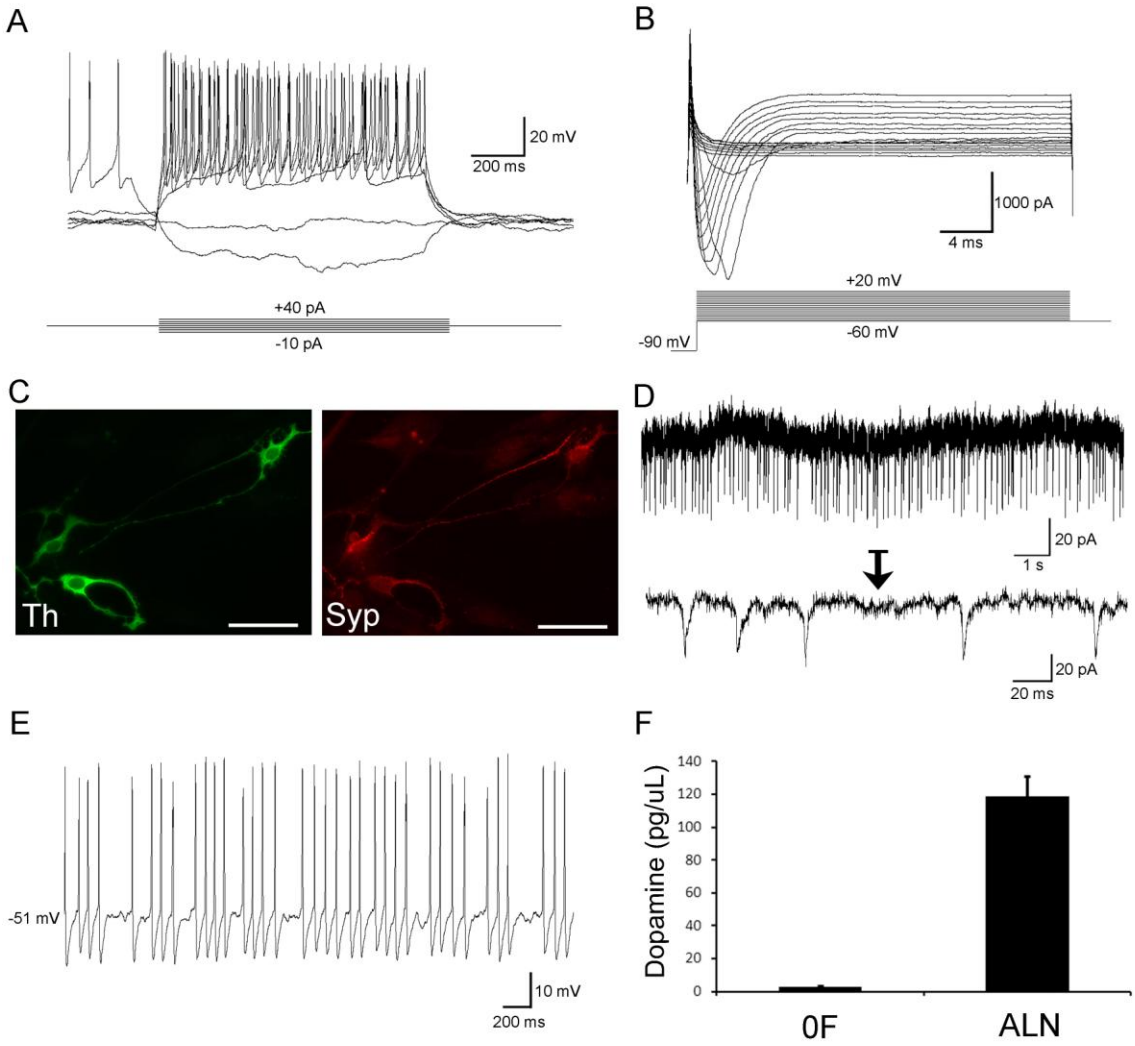


FIGURE 2. Functional characterization of induced dopaminergic neurons. (A) Whole-cell current clamp recordings of a representative neuron, stimulated by 10 pA injections of current. (B) Voltage clamp recordings of currents induced by 10 mV steps of membrane potentials. (C) Punctate synaptophysin (Syp) expression in tyrosine hydroxylase (Th) positive neurons. Scale bars 50 μm. (D) Gap-free voltage clamp recording demonstrating synaptic activity in an induced dopaminergic neuron at -60 mV. (E) Spontaneous firing of action potentials, indicative of the dopaminergic pacemaker phenotype, seen in gap-free current clamp mode with no injected current. (F) Dopamine release quantified by HPLC. Membrane depolarization was induced with 56 mM KCl at 17 days post-infection. 0F: uninfected astrocytes; ALN: induced dopaminergic neurons.

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.

Anticipated Duration of Project

1/1/2011 – 12/31/2011

Project Overview

The overall goal of this project is to improve human health by obtaining a better understanding of how social behavior of one individual can preferentially influence behavior of another individual within a group, e.g., social behavior of alcoholics. The School of Arts and Sciences remains committed to hiring faculty in the life sciences whose basic research has clearly defined clinical implications. Construction of this facility will enable the School to increase the number of faculty whose research has translational implications towards human health. The specific aim of this requested project is to construct two large walk-in rooms (150 nsf each) that will be controlled for temperature, humidity, and light-cycle, and enable investigators to perform a large number of different experiments simultaneously.

Principal Investigator

Richard M. Schultz, PhD
University of Pennsylvania
Office of the Dean
1 College Hall, Room 116
Philadelphia, PA 19104-6377

Other Participating Researchers

None

Expected Research Outcomes and Benefits

Construction of this facility will have positive effects on the School of Arts and Sciences research programs within the Life Sciences by providing a state-of-the-art facility that will house insects that provide disease models that range from neurodegenerative to metabolic to infectious to developmental, as well as social and behavioral. Although targeted for the newly recruited

faculty member, the facility will provide a resource for not only current and future Biology faculty, but also collaborative efforts with faculty from other Schools within the University. Currently, such a state-of-the-art facility doesn't exist within the School of Arts and Sciences or in any of the other Schools. The proposed facility will meet USDA requirements and provide faculty with a facility that will meet the needs for NIH- and NSF-funded research.

Summary of Research Completed

Planning: This project involves renovation of a 558-nsf laboratory space in the basement of Leidy Labs (Rm 009) for the purpose of building an insect growth facility and a small support lab. We went through a planning phase that included a) demolition of the current lab space, b) waterproofing the exterior basement wall, c) design of two (10x7-ft) insect growth chambers, d) design of a small amount of laboratory space associated with the growth chambers. This was all done in collaboration with the new faculty member who is now on site and who will operate this space. The project went out to competitive bid and a contract was awarded. In addition, schematic plans for this facility were submitted to the Animal and Plant Health Inspection Service (APHIS) of the US Department of Agriculture (USDA). These design plans for the growth chambers and associated lab space were approved by APHIS before construction began.

Construction: Demolition of the interior space within the lab is now complete. Earth against the exterior laboratory wall (this is a basement lab) has been excavated and the exterior surface of the building has been waterproofed and a drainage system installed. The insect growth chambers were fabricated off-site as component parts and have now arrived. Assembly of the chambers within the lab is now underway.