University of Pennsylvania

Annual Progress Report: 2008 Formula Grant

Reporting Period

July 1, 2010 – June 30, 2011

Formula Grant Overview

The University of Pennsylvania received $8,975,120 in formula funds for the grant award period January 1, 2009 through December 31, 2012. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Molecular Mechanisms Involved in Reprogramming Cells - The reprogramming of adult cells to a pluripotent state resembling embryonic stem cells is one of the most exciting advances in stem cell biology over the past decade. These induced pluripotential stem (iPS) cells offer the potential for patient-specific regenerative therapies, new sources of cells to understand disease and for drug discovery. This project is fundamental research that will help to elucidate the process by which adult cells are converted to pluripotent cells and provide information that can be used in efforts to make the generation of iPS more efficient and safe for clinical use.

Anticipated Duration of Project

1/1/2009 - 12/31/2012

Project Overview

Induced pluripotent stem cells are the product of reprogramming somatic cells of the adult to an embryonic-like state. As with embryonic stem cells, iPS cells can be used as the starting material for generating specialized cells in regenerative medicine. However, much must be demonstrated before the potential of iPS cells in therapies can be realized. Concerns about iPS cells stem from our lack of information on the fundamental mechanism of the conversion process and how this process can be manipulated safely and effectively, that is, without introducing exogenous genes into the genome, as well as assuring that the iPS cells generated are authentic and will produce normal, specialized cells.

Specific Aim1: Determine the variables and extent of the reprogramming process at the *single* cell level
Specific Aim 2: Determine whether there are limits to the reprogramming process

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Other Participating Researchers

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Expected Research Outcomes and Benefits

Strategies in regenerative medicine to develop therapies for diseased, damaged and worn out body parts will rely heavily on stem cells. Ideally, these stem cells must match the patient or they will be rejected by the body's immune system. Induced Pluripotential Stem (iPS) cells offer the potential for patient-specific regenerative therapies, new sources of cells to understand disease and for drug discovery. Despite their immense promise, iPS cells face several hurdles on the road to clinical applications. If this research is successful, the efficiency for generating patient-specific iPS will be increased, the cells derived from iPS for therapies will be more effective and concerns over the safety of the cells will be alleviated. This project addresses fundamental biologic mechanisms involved in the process by which adult cells are converted to the pluripotent state that must be understood so that we can address the issues of efficiency and safety as well as determine the real utility of the iPS cells in effecting therapies.

Summary of Research Completed

Aim1: Determine the variables and extent of the reprogramming process at the single cell level

No activity during reporting period.

Aim 2: Determine whether there are limits to the reprogramming process

The goal of this research is to determine the capacity of known maternal-effect genes and oocyte-specific maternal genes encoding putative transcription factors to reprogram mouse embryonic stem cells to 2-cell blastomeres.

Research Design: We have identified a transcript, expressed specifically at the 2 – 8 cell stage in mouse pre-implantation embryos to be used as a potential marker for this stage of development. Erv4/MuERV-L is a retrotransposon specifically expressed in pre-implantation embryos. We performed quantitative RT-PCR analysis to determine the relative expression of Erv4 in preimplantation embryos as well as mouse ESCs (Fig 1). Erv4 is highly expressed in 2-cell embryos with reduced expression by the 8-cell stage. Importantly, Erv4 is not detected in the blastocyst or in the mESC lines analyzed, making Erv4 an ideal marker of the 2-cell stage

embryo. We have generated a reporter and selection construct containing a GFP and hygromycin fusion gene flanked by the Erv4 LTRs, which will enable identification and selection of cells with putative blastomere characteristics following treatment with lentiviral vectors containing the candidate reprogramming factors described below. We can utilize the retroviral nature of this construct to easily create lines of stably transfected mESCs.

To induce blastomere-like cells, we will transduce the reporter mESCs described above with combinations of mRNAs of the following factors sub-cloned into the lentiviral vector pLEF (lentiviral EF1a). For these experiments we have chosen to work with maternal-effect genes, i.e., genes that are not required for oocyte development but are required for events following fertilization, are ideal candidate genes for driving the changes of gene expression during the maternal to zygotic transition. The function of these proteins ranges from transcription factors, to chromatin modifiers, to epigenetic modifiers and members of the subcortical maternal complex, this list includes Hsf1, Npm2, Zar1, Pms2, Stella, Dnmt1o, Dnmt3a, Dnmt3l, Ctcf, Sebox, Brg1, Bnc1, Brwd1, Ube2a, Mater, Floped, Padi6, and Tle6. We have sub-cloned cDNAs representing each of these proteins into the lentiviral vector pLEF to transduce combinations into mESCs to induce formation of blastomere-like cells determined by expression of the marker described above. We have chosen lentiviral vectors both because of our experience utilizing them for gene transfer as well as for their ability to integrate into the host genome and thereby enabling prolonged expression in transduced cells.

Progress during reporting period: We have transduced lentivirus bearing cDNAs from the factors listed above in a variety of combinations; however we have not observed either up-regulation of our marker, Erv4, or any phenotypic changes indicative of a change towards the blastomere fate. We have recently learned of new factors found in the egg cytoplasm, which are key players in the reprogramming of nuclei during somatic cell nuclear transfer. Histone H1foo, responsible for the replacement of histones on donor nuclei during reprogramming, and histone H3.3, a variant of the core histone H3, specifically up-regulated in oocytes and related to transcriptional up-regulation of key pluripotency/totipotency genes, are two factors that may prove to be key players in our reprogramming experiments of cells back to a blastomere-like state.

While the primary role of this research is to reprogram a mouse embryonic stem cell back to a 2-cell like blastomere, a process that is putatively controlled by factors in the maternal germ-line from the oocyte cytoplasm, we have also been attempting to reprogram somatic cells to the male germ lineage by using factors that are involved in maintenance and self-renewal of the spermatogonial stem cells (SSCs) located in testis.

As in the experiments described above, we are forcing expression of specific SSC transcription factors and RNA-binding proteins using lentivirus to covert somatic cells to spermatogonial stem cells. The factors chosen were previously defined as important for SSC establishment or maintenance and include Egr2, Egr3, Etv5, Nanos2, Nanos3, Lhx1, Oct4, Lin28, Bcl6b, Plzf, and Sall4. As described above, cDNAs representative of each factor were sub-cloned to lentiviral vectors for transduction to mouse embryonic fibroblasts (MEFs). MEFs were also transduced with a lentivirus bearing a GFP reporter under the control of the Dazl promoter, a gene that has been shown to be up-regulated specifically in the primary spermatogonia of the testis.

Transduced and non-transduced MEFs were maintained in media that supports long-term culture of SSCs and RNA was collected from both transduced cells and non-transduced controls every 7 days for a period of 4 weeks to assess changes in gene expression by real time q-PCR. Transduced MEFs were also monitored for GFP expression indicating up-regulation of the Dazl promoter as well as for morphological changes consistent with the SSC phenotype. At 4 weeks, MEFs transduced with the SSC factors showed an up-regulation in endogenous expression of stem cell specific genes such as Nanog and Pou5f1, as well as in germ cell specific genes including Plzf, Kit, Tex15 and Thy1 compared to non-transduced controls; an expression pattern consistent with that seen in SSCs in vivo. One specific combination of 3 factors significantly induces robust up-regulation of a large number of genes on our panel of germ cell related transcripts (Table 1). Despite this success, we have not yet been able to induce morphological changes in our pMEFs, which would be indicative of a spermatogonial stem cell, however we are continuing to refine our list of factors and testing further combinations.

Gene Symbol	Gene Product	Fold Increase
Etv5	ets variant gene	21
Bcl6b	Bcell Cll/lymphoma	102
Zbtb16	Zinc Finger and BTB domain containing 16	9
Ddx4	DEAD box polypeptide 4	87
Piwil2	piwi-like homolog 2	28
Id4	inhibitor of DNA binding	77
Stra8	Stimulated by Retinoic Acid 8	47
Kit	Kit oncogene	239
Dlll	Delta-like	25
Fas	Fas (TNF receptor superfamily)	40
FoxC2	forkhead box	28
Pou5f1	POU domain	44
Klf4	Kruppel-like factor 4	13
Nanog	Nanog homeobox	213

Table 1. Genes up-regulated at least 9-fold in Combination 6 relative to 0F control. Representative panel of genes expressed in testicular germ cells as well as pluripotency related genes up-regulated ≥ 9-fold in RNA isolated from Combination 6 - treated MEFs after 28 days. Quantities are normalized to 18S RNA and expressed relative to 0F treatment.

Research Infrastructure Project 2: Project Title and Purpose

Nanofabrication Lab for Biosensors and Biomeasurements – Research Infrastructure - This research infrastructure project renovates Penn's Wolf Nanofabrication Facility, expanding the cleanroom (filtered air) space which is required for micron and nanoscale technology and

medical research. This facility addresses key needs that are shared by Penn, the NIH Roadmap, and the Commonwealth of Pennsylvania: it provides tooling that allows test beds to be fabricated to measure properties of cells and to create devices which can change them. Renovation of space in the Moore Building is essential to Penn's efforts to create an interactive environment that both promotes the research initiatives and enables the recruitment of new faculty with expertise in micromedical devices. With these new collaborators and resources, Penn's researchers will create micron and nanoscale biomedical devices for the benefit of the citizens of the Commonwealth.

Duration of Project

1/1/2009 – 10/1/2010

Project Overview

This project enables researchers to build devices for sensing and measuring biological systems from the cellular level down to the molecular level. Building any device at the micron or nanometer scale requires high performance equipment and an environment free of contaminants. Typical electronic devices are not biocompatible and cannot be implanted. Biocompatible devices need special coatings and surface treatments. Adding this capability requires more highly clean space for tools as well as the utilities for operating the tools.

Building one device may require multiple, different fabrication tools. Since the tools are expensive and complicated, they are housed in a shared facility with professional staff. The facility is used by Penn researchers from Engineering, Arts & Sciences and Medicine, with occasional use by researchers from Drexel University, Fox Chase Cancer Center and start-up companies. After the renovation project is over, we plan to help cover the maintenance costs of the lab by offering use of the facility to more non-Penn users for fees which are far less than what it would take for researchers to obtain the tools individually. We will also add more Penn users. This will enable more researchers in the Commonwealth to develop state-of-the-art techniques and patentable devices for biomedical research.

The facility is 3248 sf, of which 1288 sf is the highly clean space required for state-of-the-art fabrication tools. This project converts 670 sf to become highly clean space, which is more than a 50% increase. It also converts 284 sf to a safe and secure storage area for hazardous chemicals and gases, installs scrubbers for safe discharge of exhaust gases and improves lab utilities such as a nitrogen system and hazardous gas monitoring system.

Principal Investigator

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

Nanotechnology includes a wide range of technologies that measure, manipulate or incorporate materials and/or features with at least one dimension between 1 and 100 nanometers (nm). At these scales reside the basic biology, chemistry and physics that govern chemical and biochemical reactions. Understanding what happens inside cells is critical to prevention, detection and treatment of disease. Experiments in nanotechnology are done inside wet laboratories with chemical fume hoods and in spaces designed to accommodate multiple large pieces of complex, sensitive equipment. Such facilities rank among the most expensive structures a university can construct.

Improvements to the Wolf Nanofabrication Facility, including a 50% expansion of the cleanroom space, will enable researchers to do many different types of experiments in the future. One of the first devices which will be built using the Facility will be a prostate cancer detector, built from carbon nanotube transistors. Another device will be a sensor which will be used to map biological and chemical signals in the brain, using nanowires. This will provide a vital platform to understanding the fundamental origins and causes of neurological diseases, ultimately aimed at effective prevention and long-term treatment. Other nanowire devices will be built to detect individual molecules which are indicators of disease. These and many other experiments will be conducted at the University of Pennsylvania, leading to research publications and patents for medical devices of the future.

Summary of Research Completed

As of last year's report, the Nanofabrication Lab for Biosensors and Biomeasurements Research project was complete except for the particle count measuring cleanliness of the lab. In the period July 1, 2010 through June 30, 2011, the only activity was the particle count.

The cleanroom certification took place on 7/9/2010. No equipment was shut down and no users were allowed into the facility; the testing engineer was the only person in the cleanroom during the test.

The Airborne Particle Count Survey was one of the four tests performed. In each room readings were taken in several locations at 48" above the floor. A laser particle counter operating at one-CFM flow rate and a threshold particle size of 0.2 microns was used.

The lithography rooms, deposition room, and the etcher room were rated Class 1,000 (less than 1,000 0.5 micron particles per cubic foot). The gowning area was rated Class 10,000 (less than 10,000 0.5 micron particles per cubic foot).

The last activity on the project was July 9, 2010, and the project was closed. Financial closeout was on October 1, 2010.

Research Project 3: Project Title and Purpose

Effects of Nicotine on Mu Opioid Receptor Binding - We will evaluate whether there are different effects of nicotine on mu opioid receptors in smokers with different genetic backgrounds. This will help us to understand the brain related mechanisms that explain why smokers with a particular gene variant for this brain receptor are at higher risk for smoking relapse.

Anticipated Duration of Project

1/1/2009 - 12/31/2011

Project Overview

Broad Research Objective: To characterize the brain mechanisms that explain the association of the mu opioid receptor (OPRM1) gene Asn40Asp polymorphism association with nicotine dependence.

Specific Aim and Hypotheses: To examine the effects of IV nicotine (vs. placebo) on mu opioid receptor binding potential (MOR BP) in smokers stratified by *OPRM1* genotype.

> Hypothesis 1: Compared to smokers homozygous for the Asn40 allele (high risk), those carrying the Asp40 allele (low risk) will exhibit attenuated effects of nicotine on MOR BP in ventral striatum. Specifically, in the placebo session, the Asn40 group will have higher MOR BP (increased availability of MORs) than the Asp40 group; in the nicotine session, MOR BP should decrease more in the Asn40 group than the Asp40 group (due to greater endogenous MOR neurotransmission).
>
> Hypothesis 2: Asp40 carriers will have attenuated subjective responses to IV nicotine (e.g., "drug liking").

Research Design and Methods : This human positron emission tomography study will assess effects of nicotine on mu opioid receptor (MOR) binding potential in 24 chronic smokers stratified by *OPRM1* A118G genotype (12 AA and 12 AG/GG). In the first experiment, smokers will participate in two PET imaging sessions with [^{11}C]Carfentanil after overnight abstinence: (a) after smoking a nicotine containing cigarette, and (b) after smoking a denicotinized cigarette. The primary outcome variable is MOR binding potential (BP) (reflecting receptor availability) in ventral striatum and additional regions of interest (ROIs). In addition, we will complete a single PET imaging session with 20 nonsmoker controls (10 of each genotype).

In the second experiment, we will follow the identical procedures, except that nicotine will be administered intravenously: (a) IV nicotine 1.0mg/70kg; and (b) IV placebo (saline) (within-subject, double-blind, counter-balanced order). The combination of neuroimaging and genetics in

these two experiments, focusing on different modes of nicotine delivery, will elucidate the functional significance of the Asp40 variant and its role in the neurobiology of nicotine dependence.

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Other Participating Researchers

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Expected Research Outcomes and Benefits

Genetic association studies in psychiatry and addiction are plagued by nonreplications. However, there is a critical mass of positive studies linking the OPRM1 Asn40Asp variant with smoking behavior and relapse. Because nicotine dependence is a complex trait, investigation of in vivo brain imaging data may provide a stronger genetic signal than self-reported behavioral measures used in human genetic association studies. The proposed project will clarify the role of *OPRM1* Asn40Asp in nicotine dependence using in vivo imaging of nicotine effects. This research will provide valuable information to increase our understanding of the neurobiology of nicotine addiction and aid in the development of new medications for this significant public health problem.

Summary of Research Completed

The aim of the original proposal is "to characterize the brain mechanisms that explain the association of the mu opioid receptor (OPRM1) gene Asn40Asp polymorphism association with nicotine dependence. This human positron emission tomography study will assess effects of nicotine on mu opioid receptor (MOR) binding potential in 24 chronic smokers stratified by *OPRM1* Asn40Asp genotype. They will participate in two PET imaging sessions with [^{11}C]Carfentanil after overnight abstinence". The two sessions differ in that one session involves nicotine delivery and the other involves placebo delivery.

There are multiple modes of nicotine delivery that can be utilized to answer this research question using the identical experimental design. One method (which was stated in the original proposal) uses intravenous nicotine delivery and another method uses nicotine delivered via smoking. Prior to intravenous nicotine delivery (which is a higher risk procedure), we tested all study procedures and our hypotheses (using the identical experimental design) with smoked nicotine delivery: a nicotine-containing cigarette versus a denicotinized cigarette. The

hypothesis and study design were identical. However, this formative research was necessary for proof of concept before proceeding to the higher risk intravenous procedure.

The results of this preliminary feasibility experiment were reported in last year's annual report, and a manuscript based on the completed experiment was prepared during the current project period for submission to the Proceedings of the National Academy of Science (PNAS).

Having shown effects of the OPRM1 genotype, which supported our initial hypotheses, we progressed to using intravenous nicotine during the current project period. We enrolled 6 participants into the study using the identical study design. However, due to problems in the cyclotron facility, the production of carfentanil was unreliable and no further scans were completed. However, we are able to perform preliminary analysis of the results with these subjects, and such analysis of nicotine effects on mu opioid receptor binding is underway. These results will be reported in the next project period when analysis is complete

Given the success of our first experiment using nicotine delivered via cigarette smoking, we decided to focus our efforts on further analysis of these data. We are currently completing an analysis of the relationship of MOR binding availability to individual differences in smoking motives and smoking-related psychological comorbidity. These data will be provided in next year's report. These analyses utilize the data from the original proposal and the identical experimental design, and will be reported in the next project period.

Research Project 4: Project Title and Purpose

Validation of Imaging Markers for Use in Cancer Clinical Trials - Biomarkers to identify appropriate patient populations and detect early response represent a significant opportunity to bring cancer drugs to the clinic more expeditiously and at a reduced cost. Advances in medical imaging already can provide quantitative in vivo assays of tumor anatomy and function in cancer patients. Support of quantitative imaging in cancer clinical trials has been limited by the lack of coordinated efforts between clinical imaging specialists and clinical cancer researchers. This project will establish a collaboration between the oncology investigators engaged in clinical trials of cancer therapy and imaging investigators to develop and validate imaging biomarkers to predict and monitor the response to therapy. This will impact our ability to develop and evaluate new therapies for cancer.

Duration of Project

1/1/2009 - 6/30/2011

Project Overview

1. Develop an infrastructure to support standardized analysis of images performed on cancer trials at Penn: The project will develop an operational interface between the cancer center and the radiology department. The approach will employ a dedicated imaging research coordinator with experience in imaging and image management who will coordinate management of imaging data resulting from cancer trials such that they are made available in a centralized 3D imaging

laboratory for analysis and interpretation. A cadre of imaging clinical scientists will oversee the analysis according to standardized protocols. This will have the effect of reducing variability in the determination of such endpoints as progression free survival and improve the performance of cancer trials across the Abramson Comprehensive Cancer center.

2. Explore novel approaches to image analysis to improve imaging markers for cancer trials: The extraction of quantitative data from imaging examinations is a critical component of the imaging clinical trial. This activity may include simple geometric measurements made by radiologists, complex reader assessments, semi automated and automated quantitative analysis which may also include complex modeling. Currently, response and progression on cancer trials is evaluated by measurement of tumor size and assessing growth and shrinking. This analysis does not account for scenarios such as tumor necrosis without significant changes in tumor size, or reduced tumor proliferation. As part of this project, novel approaches to image analysis such as adjusting size for percent necrosis (identified by the absence of contrast enhancement) or dynamic MRI perfusion measurements will be explored. This will involve performing these analyses on patients on trial, and correlating the results against patient outcome (survival, disease free survival). It is anticipated these novel approaches will better correlate with outcome and reduce the number of patients needed to evaluate treatments in future trials.

Principal Investigator

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Other Participating Researchers

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Expected Research Outcomes and Benefits

1. More effective clinical cancer trials at the Abramson Cancer Center: As a result of the coordination and standardization of image analysis, it is anticipated that the data derived from imaging studies will have reduced variability. This will increase the power of trials that utilize imaging endpoints such as progression free survival and response rate. The improvement of effectiveness of the trials utilizing current endpoint will impact a wide range of clinical research performed at the Abramson Cancer Center.
2. Clinical trials based on new imaging marker: The exploration of newer image analysis methods that result in new markers will result in improved endpoint for clinical trials. This is anticipated to result in trial endpoints that will detect response/progression earlier in the course of treatment resulting in quicker trials. This will have a broad impact on the development of newer therapeutics by reducing the clinical trial timeline.

Summary of Research Completed

1. Develop an infrastructure to support standardized analysis of images performed on cancer trials at Penn:

The Clinical Imaging Core (CIC), as a new shared resource supported by the Abramson Cancer Center (ACC), has developed an infrastructure to further the above goal. Work completed in the past year supported by the current funding includes:

- Development of a RECIST tumor response lab led by Dr. Rosen
    - The RECIST lab has undergone several procedural updates to streamline performance. At the time of writing, the lab has undertaken cross-sectional imaging analysis of over 400 imaging exams on over 100 subjects enrolled in clinical studies in the ACC.
- Development of software for on-line request for RECIST analysis by ACC members
    - In collaboration with information technology expertise within both the ACC and the Department of Radiology, the CIC has developed a new service for web-based requesting of RECIST analysis services. This tool allows for archiving of data on specific protocols, including key contact personnel, types and frequency of imaging, and image analysis algorithms (RECIST 1.0, RECIST 1.1, mRECIST).
- Development of on-line software tools for tabulating, storing, and reporting RECIST analyses to ACC members
    - As of this writing, a prototype system for CIC personnel to store RECIST data sets on-line, in anticipation of transfer of RECIST time-point analyses to the ACC clinical trial electronic database tool (VELOS) is under development. Further development of this tool will be supported via funding from the ACC and the Department of Radiology.
- Development of automated imaging analysis tools to streamline the analysis of linear tumor measurements from cross-sectional imaging studies
    - In conjunction with the American College of Radiology Imaging Network (ACRIN), Dr Rosen is currently testing software developed to translate radiologist measurement (annotations) into xml-based data storage to allow for automated retrieval and analysis according to RECIST or other tumor response algorithms.
- Infrastructure to facilitate protocol review regarding technical and regulatory aspects of all imaging in clinical trial work in the ACC.
    - This program was recently initiated in June of 2011, as part of the CIC expansion. To date four protocol review requests have been processed in that time. Currently, the CIC is working with ACC leadership on methods to streamline imaging compliance review at the time of budgetary and feasibility analysis performed in the ACC by the Strategic Planning and Resource Committee (SPARC) analysis.
- Mechanisms for combined reporting of cross-sectional and bone scan results in prostate cancer patients on clinical trials
    - Recognizing the importance of inter-modality correlations for more accurate reporting of tumor response assessment in certain tumor types, especially prostate

cancer, the CIC has initiated procedures to allow for combined CT and bone scan interpretation for tumor response reporting. This endeavor is in corporation of Dr. Naomi Haas from the GU malignancy treatment group and a leader in the Developmental Therapeutics program at the ACC.

2. Explore novel approaches to image analysis to improve imaging markers for cancer trials:

In support of the above goals, current funding for the CIC has provided for the development of the following infrastructure for facilitating novel imaging biomarker studies and image analysis for human subjects undergoing clinical trials at the ACC.

- Infrastructure to facilitate project development teams, including key members of ACC programs and imaging experts in radiology, medical physics, and biomedical engineering
    - Mechanisms for project development to foster new imaging-oncology collaborations have been developed, as part of the CIC portfolio of services. All ACC investigators may request a free project consultation, during which the clinical investigator will be matched with a radiology or bioengineering expert to explore novel methods to develop imaging endpoints in early stage studies of novel therapeutic agents.
- Web-site development and on-line publicity to further advertise the new services of the CIC to ACC members
    - During the past year, a new website has been developed and published within the University of Pennsylvania Health System. This website serves to introduce ACC members to the CIC and includes documents and FAQ pages to address concerns of investigators and administrative personnel on the correct implementation of routine and novel imaging studies in the course of human clinical trials in oncology. The CIC and the website were featured in a recent on-line bulletin distributed to all members of the ACC.
- Establishment of an IND-official and office in the department of radiology to assist in regulatory aspects of protocol development for novel imaging agents
    - Ms. Melissa Myers, a long-time member of the Department of Radiology and experienced clinical coordinator in imaging trials, accepted the new position of IND coordinator in the past year. Her office now performs regulator reviews of all protocols using novel imaging agents under IND mechanisms in order to expedite novel imaging research in cancer patients.
- Identification of key individuals in the Molecular Imaging program in the Department of Radiology to develop methods for improved reporting of standard uptake value (SUVs) for individual tumor lesions in FDG-PET studies
    - Since the departure of Dr. Chaitan Divgi from the University of Pennsylvania, Dr. Rosen has worked with the interim Chair of Nuclear Medicine and Molecular Imaging, Dr. Daniel Pryma, to facilitate the integration of molecular imaging exam performance and interpretation in the CIC. Currently, Dr. Rodolfo Perini of the Molecular Imaging Division of Radiology, and, co-appointed in Hematology-Oncology, is collaborating with Dr. Rosen on mechanisms to improve qualitative and quantitative reporting of PET, SEPCT, and bone scan studies in clinical trial patients.

- Development of novel methods for determining whole tumor glycolytic function from FDG-PET studies
    - New methods for determining whole tumor glycolytic indices, as an alternative to standard individual lesion SUV reporting, are being investigated by researchers in the Department of Radiology.
- Novel methods for quantifying normalized tumor uptake ratios in PET studies using large molecular I-131 labeled radiotracers
    - Dr. Daniel Pryma has developed methods for improved standardization of tumor uptake of novel I-131 radiotracers. This methodology is being used in the current trial of cG250 as a marker of carbonic anhydrase IX (CA-IX) uptake in renal cell carcinoma.
- Implementation of batch processing and analysis of tumor perfusion and permeability data from dynamic contrast-enhanced (DCE-)MRI studies
    - Dr. Rosen has developed methods to improve the volumetric coverage of DCE-MRI perfusional studies for the use in trials involving vascular-targeted agents. Novel software for segmenting tumor and arterial functions is under testing for improved measurement of the effect of these agents on the tumor vascular microenvironment.
- Methods for determining pixel-wise changes in tumor ADC values from diffusion weighted imaging (DWI) in MRI
    - Software to tabulate pixel-by-pixel changes in tumor ADC are currently undergoing testing in a series of studies or targeted agents in phase I and II trials.
- Initiation of quantitative DCE-CT studies and image analysis
    - The CIC is facilitating the use of DCE-CT in anticipation of the opening of the ACRIN-GOG collaborative study on the use of DCE-CT as a biomarker of tumor response to Avastin in ovarian cancer.

Research Project 5: Project Title and Purpose

Understanding the Biology of Residual Neoplastic Disease - A cardinal feature of human breast cancers is the survival and persistence of residual neoplastic cells in a presumed quiescent state following the apparently successful treatment of the initial tumor. Ultimately, these residual cells re-emerge from their dormant state and resume growth, leading to cancer recurrence. Indeed, analyses of bone marrow samples show that disseminated cells are present in 20-40% of primary breast cancer patients without any clinical or histopathological signs of metastasis. As such, residual neoplastic disease is a major obstacle to the successful treatment of breast cancer and other human cancers. Accordingly, the purpose of this project is to investigate the biological properties of residual neoplastic cells, since these cells represent the reservoir from which tumor recurrences invariably arise.

Anticipated Duration of Project

1/1/2009 – 12/31/2012

Project Overview

A particular difficulty in understanding the mechanisms of breast cancer recurrence has been the challenge of identifying and isolating residual neoplastic cells in patients, and the lack of animal models that recapitulate this key feature of breast cancer progression. We have generated conditional transgenic mouse models for breast cancer and have obtained important preliminary evidence indicating that essentially all oncogene-induced mammary tumors that have regressed to a non-palpable state following oncogene down-regulation leave behind residual neoplastic cells. These represent a unique set of genetically engineered models for residual neoplastic disease in breast cancer induced by defined oncogenic pathways. In this project, we propose to use these models to define critical features of residual neoplastic disease relevant to developing improved therapeutic approaches to human cancers.

The specific aims of this project are: Aim I: Identify morphological lesions containing residual neoplastic cells; Aim II: Analyze the cellular components of residual neoplastic lesions; Aim III: Determine if autophagy is a survival mechanism for residual neoplastic cells. These aims will be accomplished by using doxycycline-inducible transgenic mouse models in which oncogenic pathways important in human cancer can be conditionally activated or inactivated. Following tumor development in these models, tumor regression can be induced by oncogene down-regulation in a manner that simulates the treatment of patients with molecularly targeted agents. The tumor cells that survive oncogene down-regulation will first be identified in the mammary glands of these animals by histopathological and cell marking approaches. The cellular components and cellular microenvironment of these residual neoplastic cells will then be defined by co-localization of cell-type specific markers. Finally, the role of autophagy in the survival of residual neoplastic cells will be determined by retroviral transduction approaches. Together, these approaches should permit clinically relevant insights into the biology of residual neoplastic cells and should facilitate the development of more effective therapies targeted against this critical population of cells in patients.

Principal Investigator

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

Breast cancer is typically treated by resection of the primary tumor followed by treatment with some combination of radiation, chemotherapy, and adjuvant hormonal therapy. Depending upon a spectrum of factors including tumor size and grade, lymph node status, and histological

characteristics, patients will either remain disease-free following this course of treatment, or else develop recurrent breast cancer—either locally or at distant sites in the body. While the pathways leading to the initial growth of primary breast cancers have been extensively studied, far less is known about the genes and processes that govern the ability of tumor cells to survive and recur following therapy. This paucity of information is due in large part to the lack of animal models suitable for studying this critically important process.

This research strategy should help to illuminate the biology of the critical population of tumor cells that survives molecularly targeted therapies and that ultimately give rise to tumor recurrence. This project will improve our understanding of breast cancer in several important ways. First, understanding the mechanisms by which tumors recur should aid in the diagnosis and clinical management of breast cancer. Furthermore, the tumor markers and pathways identified will represent potential targets for therapeutic intervention. Drugs targeting pathways that promote survival of residual tumor cells are most likely to extend the survival of patients with dormant, residual disease.

In summary, we have developed a novel set of genetically engineered mouse models to study tumor progression and have coupled these with a unique approach to study the biology of tumor cells that are important for recurrence. This research strategy should provide important insight into the biology of breast cancer recurrence, a process that receives scant attention and remains poorly understood, but that is critically important for the survival of patients with breast cancer.

Summary of Research Completed

To characterize the properties of residual tumor cells present in the mammary glands of previously tumor-bearing mice, we developed an orthotopic model based upon neu-driven epithelial tumor cells labeled with a green fluorescent histone (H2B-GFP) binding protein. Recipient mice maintained on doxycycline were injected with 2x10^5 MMTV-rtTA/TetO-neu cells in the #4 mammary gland. After the formation of tumors, recipient mice were deinduced and the glands examined for evidence of residual lesions. Tumor bearing glands were positive for residual lesions, which were absent in control PBS-injected glands. Residual lesions appeared morphologically identical to the lesions in the intact inducible bitransgenic model previously described by our laboratory. In addition, GFP-labeled cells were located solely within residual lesions and were not observed outside of the lesion or in any other compartment of the gland. This orthotopic model in which tumor cells are labeled with a nuclear H2B-GFP protein, which enables the identification of tumor cells from the stromal and endogenous epithelial cells present in mammary glands bearing residual lesions, was used for the following experiments to characterize residual tumor cells.

Rare GFP-labeled tumor cells are positive for basal epithelial markers

In order to assess and characterize markers expressed by residual GFP-labeled tumor cells in the lesion, a series of immunofluorescence experiments were performed in the above orthotopic model. MTB/TAN primary tumor cells are predominantly positive for the luminal epithelial marker cytokeratin 8 (CK8). There are areas of the primary tumor in which the myoepithelial marker CK14 is also highly expressed, and some CK14-positive cells are also positive for CK8.

Other areas of the primary tumor have rare single CK14 positive cells and these two expression patterns reflect the heterogeneity of MTB/TAN tumors. Recurrent tumors had very rare, low-expressing CK8 positive cells. CK14 was expressed at higher levels than CK8, but again, rare cells were positive. The residual lesion reflects the status of the recurrent tumor more that the primary tumor. There are a small number of CK8 light-positive cells in the residual lesion in the bitransgenic model, but in the orthotopic model no GFP positive cells have been observed as CK8 positive. In the residual lesion there are a higher number of CK14-positive cells and some, though not all, individual cells are also GFP-positive (Fig. 1A). Single, individual GFP positive cells are double-positive for CK5 (Fig. 1B), and a slightly smaller number are positive for CK6 (Fig. 1C).

GFP-labeled residual tumor cells are negative for mesenchymal and stromal markers

The morphology of MTB/TAN primary tumors is epithelial, but this changes to a mesenchymal morphology in recurrent tumors. Therefore, we investigated the following markers for mesenchymal cells. GFP positive cells are negative for PDGFR-alpha (CD140-alpha), although PDGFR-alpha is expressed primarily as a border around the residual cells in the lesion (Fig. 1D). The GFP positive cells are nestled in a region clearly negative for PDGFR-alpha. Nevertheless, GFP-positive tumor cells are seen to express PDGFR-alpha in the recurrent tumor.

In the residual lesion, immunoreactivity for alpha-smooth muscle actin (α-SMA) showed a pattern similar to primary tumors displaying an irregular structure and patterning. However, none of the GFP-positive cells have been observed to co-express α-SMA (Fig. 1E). Fsp-1 positive cells were present in the stromal regions of the primary tumor and were found uniformly throughout the recurrent tumor. In the orthotopic residual lesion, GFP-positive tumor cells do not express Fsp-1. Residual lesions consistently expressed abundant extracellular matrix proteins, including fibronectin and vimentin (Fig. 1F) and residual lesions could thereby be identified by the high and contrasting expression amongst the mammary stroma.

GFP-labeled tumor cells in the residual lesion are non-proliferating

To examine the proliferation status of the tumor cells in the residual lesion, we examined the lesion by immunoreactivity with the antibody Ki-67 (Fig. 2). In the orthotopic primary tumor, where as many as 60% of the cells are GFP positive, Ki-67 positivity accounts for up to 50% of the cells. However, only ~16% of GFP-positive cells were Ki67-positive. In the orthotopic residual lesion, where GFP-positive cells can account for up to 37 % of the cells present, none of the residual tumor cells were positive for Ki-67 (Fig. 2).

To further investigate the cycling status of the residual cells over a longer time period, a 7-day BrdU label of the lesion after full regression of the primary tumor was performed. This revealed that, despite the longer labeling period, no GFP-positive cells were labeled with BrdU. As many as 6% of the surrounding cells in the mammary gland are labeled with BrdU which reflects normal turnover. Consequently, residual lesions could be identified by the lack of BrdU label in the area compared to the surrounding normal mammary gland stroma.

Residual neoplastic disease in intact deinduced MMTV-rtTA/TetO-Ras mice

Given that fully regressed MTB/TRAS tumors recur spontaneously in the same location of the original tumor, this raised the possibility that residual neoplastic cells remained following deinduction that serve as target cells for tumor recurrence that we could visualize in intact animal models. We therefore examined deinduced, fully regressed MTB/TRAS tumors histologically and immunocytochemically for evidence of residual neoplastic disease. We observed that mammary glands bearing tumors that had fully regressed in response to dox withdrawal, lacking any palpable outgrowths, still contained microscopic lesions that may represent residual tumor cells that fail to be cleared upon deinduction (Fig. 3A). These cells retained luminal epithelial character as evident by staining for Cytokeratin 18 (Fig. 3A). Typically, there was a large increase in hemosidarin deposits present by histological staining (Fig. 3A). Changes in myoepithelial content were not evident based upon Ck14 staining (Fig. 3B). By contrast, non-tumor bearing glands harvested from these mice did not display any such lesions by histological staining or by Ck18 immunofluorescence (Fig. 3A)

As both primary and recurrent tumors overexpress the *Ras* transgene relative to MTB/TRAS hyperplastic epithelium (Fig. 3B), we questioned whether these residual lesions overexpressed oncogenic Ras, or whether overexpression of Ras was detectable exclusively in actively growing tumor cells. Bioluminescence *in vivo* suggested that *Ras* transgene levels were completely down-regulated several weeks after deinduction, but were re-activated at a point prior to tumor recurrence. Upon staining for total Ras protein at a time point near the median tumor recurrence latency, we found that these lesions expressed Ras at levels comparable to either primary or recurrent tumors, and significantly higher than surrounding tissue or non-tumor-bearing glands (Fig. 3B). However, despite this overexpression of Ras, the rates of proliferation within lesions were dramatically lower than in either primary or recurrent tumors, as shown by BrdU incorporation (Fig. 3B). This suggested transgene reactivation occurs prior to recurrence, but is insufficient to re-stimulate proliferation within residual disease lesions.

We next questioned whether direct re-activation of the *Ras* transgene could accelerate proliferation of residual lesions. We therefore re-induced mice bearing fully regressed tumors with 0.012 mg/mL dox for 48 hrs. Upon re-induction, we did observe an increase in proliferation in residual lesions containing high Ras expression, similar to levels present in recurrent tumors (Fig. 3B). This could indicate that other cells must re-activate the *Ras* transgene, beyond those observed in fully regressed, non-reinduced glands (Fig. 3B). It is noteworthy that levels of total Ras protein in residual lesions reinduced with doxycycline are comparable to levels in deinduced residual lesions that have reactivated the *Ras* transgene, suggesting that the effects observed are not due to alterations in transgene expression levels in the residual neoplastic cells themselves (Fig. 3B).

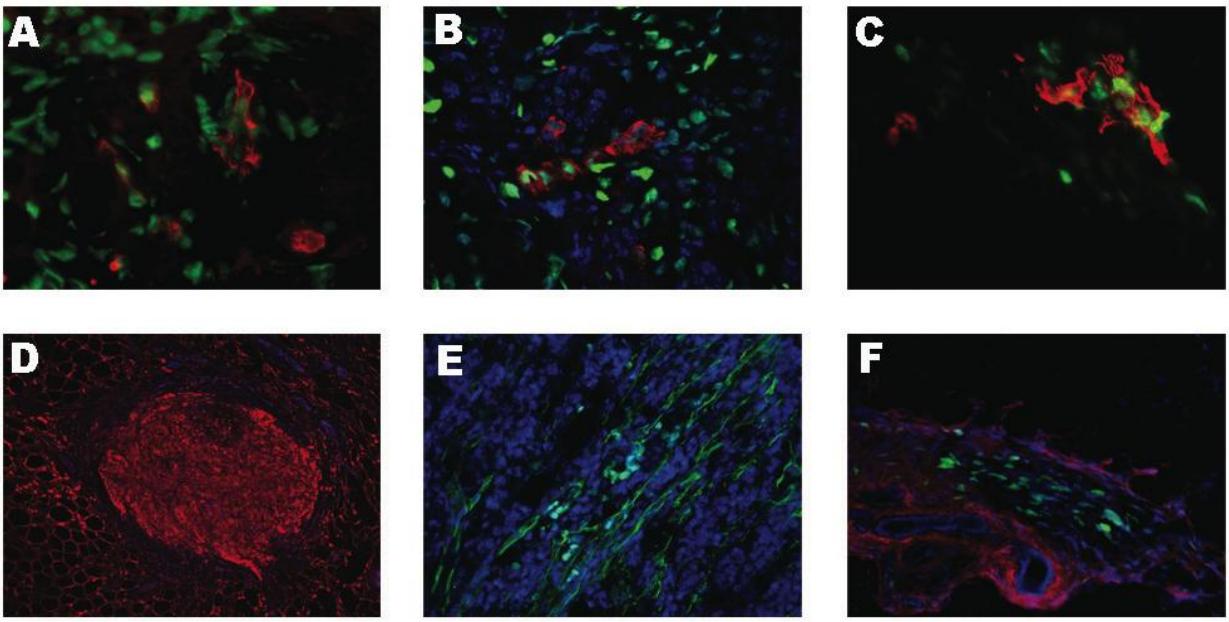

Figure 1 – Immunofluorescence analysis of epithelial and stromal markers in residual lesion. (A) Occasional residual tumor cells (green) express CK14 (red); (B) Occasional residual tumor cells (green) express CK5 (red); (C) Occasional residual tumor cells (green) express CK6 (red); (D) PDGFR-α expression (red) is located around lesion and GFP positive cells are found in center of lesion which is negative for PDGFR-α 200X. (E) α-SMA (green) in residual lesion infiltrates and runs through residual lesion but GFP-positive cells are negative for α-SMA 400X. (F) Residual lesion can be identified by dense fibronectin expression (red). 100X

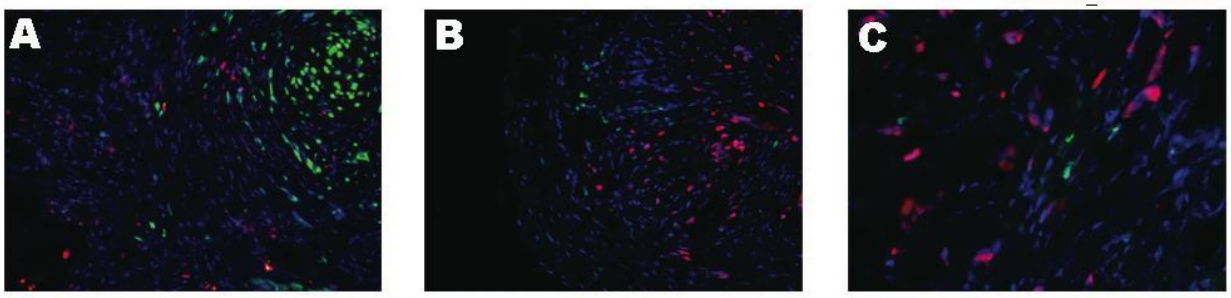

Figure 2 – GFP labeled residual cells are negative for proliferation markers. (A) BrdU labeled cells (red) are predominantly found outside the lesion 200X. (B-C) GFP-labeled cells do not costain with BrdU label (red) 100X.

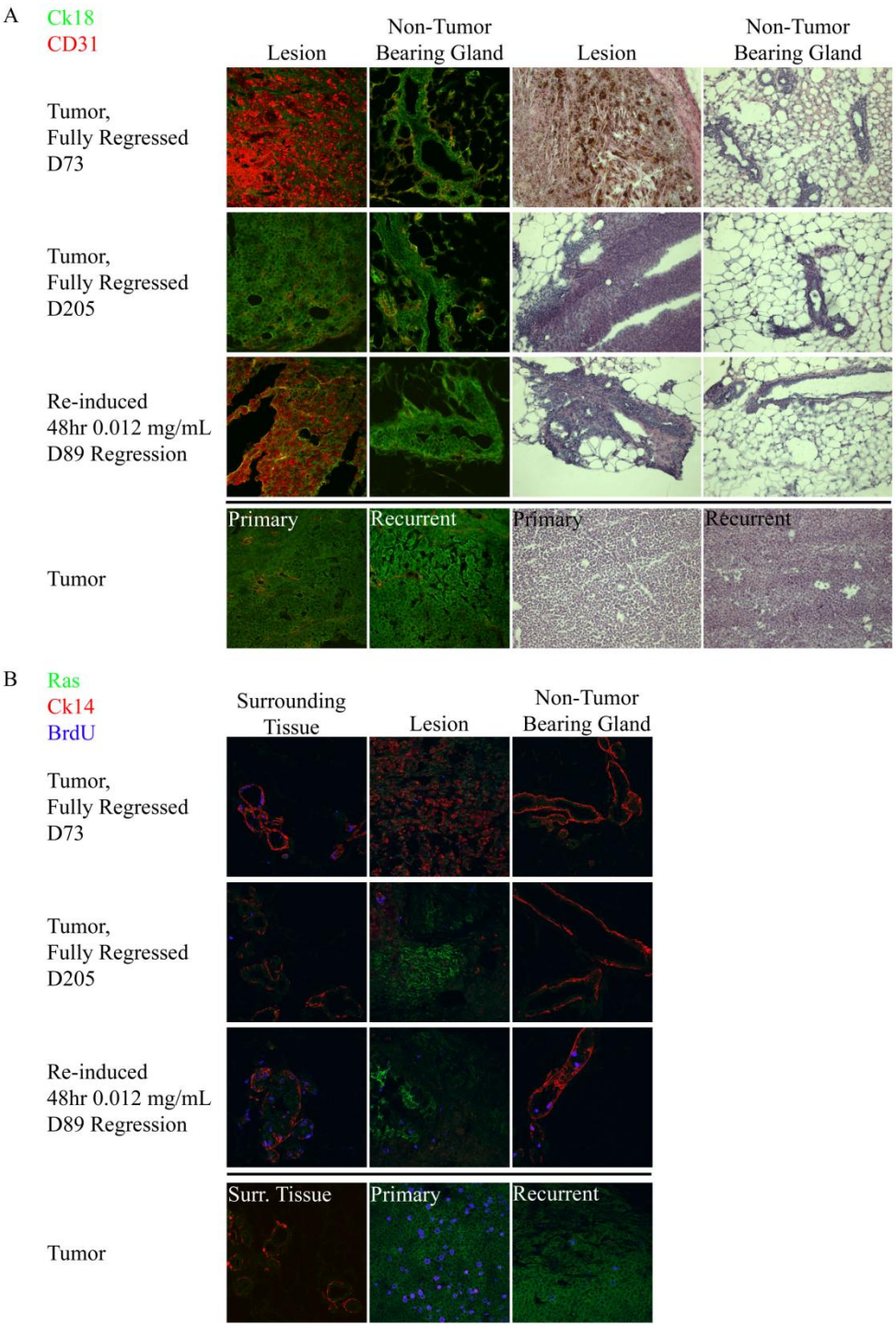

Figure 3 – Residual neoplastic disease in MTB/TRAS mice. (A) Cytokeratin 18 and hematoxylin and eosin staining (10 mm frozen sections) of fully regressed MTB/TRAS tumors deinduced or reinduced with 0.012 mg/mL dox for the indicated times. (B) Immunofluorescent staining for total Ras, Cytokeratin 14, and BrdU in MTB/TRAS mammary glands. Both residual disease lesions and surrounding areas of tumor-bearing glands are pictured.

Research Project 6: Project Title and Purpose

Individualizing Breast Cancer Prevention in Primary Care - The overarching objective of this project is to develop and test a multi-faceted strategy for optimizing the adoption of individualized breast cancer prevention in primary care. Specifically, we will determine whether the use of breast cancer risk reduction strategies among high risk women can be increased by providing the women targeted information and by incorporating breast cancer risk assessment and prevention into the electronic medical record of their primary care providers, and that the combination of these interventions is more effective than either intervention alone.

Anticipated Duration of Project

1/1/2009 - 6/30/2012.

Project Overview

The last ten years have witnessed tremendous advances in breast cancer prevention (with FDA approval of both tamoxifen and raloxifene for primary reduction of breast cancer risk), detection (with the development of breast MRI screening and its inclusion in the American Cancer Society guidelines for screening of high-risk women), and risk assessment (with the availability of improved risk assessment models as well as the availability of genetic testing for *BRCA1* and *BRCA2* mutations). Together, these advances have the potential to substantially reduce breast cancer mortality in the US. However, this potential depends upon their effective translation into clinical practice. Unfortunately, current data suggest that this translation has been less than ideal. Recent data demonstrate that few primary care physicians have prescribed tamoxifen or raloxifene for primary prevention for breast cancer, referred patients for genetic testing, or used breast cancer risk prediction models. This project will complete the development of an integrated tool for risk assessment, pilot this tool, and begin data collection for the mammography portion of the grant.

Aim 1. To determine whether the collection, calculation and provision of individualized breast cancer risk and prevention information at the time of mammography screening increases the uptake of targeted breast cancer risk reduction strategies (chemoprevention, MRI screening, *BRCA1/2* testing) among women at risk.

Aim 2. To determine whether the inclusion of an individualized breast cancer risk assessment and prevention module in the electronic medical record (EMR) increases the uptake of targeted breast cancer risk reduction strategies among women at risk.

Aim 3. To explore whether the effects of these interventions are independent or synergistic.

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Expected Research Outcomes and Benefits

The results of this project will substantially advance progress towards reducing breast cancer incidence and mortality in several ways. First, the project will directly impact a defined population of women, increasing the use of breast cancer risk reduction interventions in this population and thereby decreasing their subsequent breast cancer incidence and mortality. If the interventions lead to an additional 500 women at elevated risk pursuing chemoprevention, MRI screening or *BRCA1/2* testing (a relatively conservative estimate), current data suggest that between 30 and 50 fewer women in the overall population would die from breast cancer simply based upon the study itself. Second, the project will create and evaluate new tools for dissemination of individualized breast cancer prevention that can have a significant impact on the delivery of these advances in primary care and screening settings reaching across the Commonwealth of Pennsylvania. By demonstrating that relatively simple breast cancer risk assessment interventions at the point of care can increase the use of preventive interventions and that this use can be tracked through an EMR, this study has the potential to rapidly advance the dissemination of targeted breast cancer risk reduction strategies to high-risk women across the country. Third, the project will provide new insights into how to influence patient and provider behavior, including the relative impact of patient level and provider level interventions. Fourth, the project will draw attention to the importance of breast cancer risk assessment and prevention more generally, thereby encouraging investment by patients and providers in the development of new strategies for risk prediction and prevention. Although the recent advances have provided important tools for moving forward on individualized breast cancer risk reduction, it is clear that these tools remain imperfect. This project represents an early step in drawing attention to the extraordinary potential for return on investment in this new paradigm.

Summary of Research Completed

Aim 1. To determine whether the collection, calculation and provision of individualized breast cancer risk and prevention information at the time of mammography screening increases the uptake of targeted breast cancer risk reduction strategies (chemoprevention, MRI screening, BRCA1/2 testing) among women at risk.

Progress: In the past year, we have used Commonwealth resources to continue our work on alternative models of breast cancer risk stratification in order to broaden risk stratification into the general population. Although we have a well established Cancer Risk Evaluation Program at the University of Pennsylvania this serves a very limited group of patients and most women in the United States never have any type of breast cancer risk assessment. The goal of this grant is to widen access to such risk assessment specifically to women undergoing routine

mammographic screening for breast cancer. In the past year Commonwealth Funds have been used to support the following:

1. Continued training of staff in risk assessment models and their use

2. Ongoing work with the Information Technology Group to program web-based applications for patient entry of breast cancer risk assessment.
    a. This has been on ongoing goal. After extensive evaluations with our IT group of the different options, we recently we upgraded our Progeny data system to a web-based format (this cost of the actual upgrade was not directly supported by Commonwealth resources). This moves us close to this goal.

3. Continued work with EPIC group on Electronic Medical Records (EMR) modules for breast cancer risk assessment.

4. Trained staff to complete risk assessments on patients undergoing routine screening mammograms under an IRB approved protocol
    a. We were able to use the progress that we made in risk assessment and supplement this funding with a Grand Opportunity Grant from the National Institutes of Health, to an IRB approved protocol. This is a more expansive protocol with not only standard risk assessment, as described and funded here, but also single-nucleotide polymorphism assays. More than 1000 women undergoing routine screening mammograms have been enrolled date. Baseline information has been collected and standard risk assessment performed. Letter with patients' risks are being mailed to participants. Part of this work was presented at the American Society of Clinical Oncology meeting in June 2011.

Aim 2. To determine whether the inclusion of an individualized breast cancer risk assessment and prevention module in the electronic medical record (EMR) increases the uptake of targeted breast cancer risk reduction strategies among women at risk.

Progress: A breast cancer risk assessment questionnaire has been built into the "My Penn Medicine" section of the EPIC electronic medical record. On October 1, 2011 Penn incorporated the Ambulatory Practice Management (APM) into the EPIC system. With this upgrade, there are ongoing initiatives to use the "My Penn Medicine" functionality including the breast cancer risk assessment tool. This will be a major focus for this year.

Aim 3. To explore whether the effects of these interventions are independent or synergistic.

Progress: There has been no progress on this aim as we are implementing aim 2.

Research Project 7: Project Title and Purpose

Clinical and Molecular Predictors of Responsiveness to Angiogenesis Inhibition in Advanced NSCLC - Bevacizumab is a monoclonal antibody targeting VEGF (vascular endothelial growth factor), a humoral protein that stimulates the growth of tumor vasculature (blood vessels), which,

in turn, fuels the growth and spread of cancer. This agent in combination with chemotherapy, has been approved for use in "selected" patients with metastatic non-small cell lung cancer; but it has not been determined *a priori* who actually benefits from this costly, potentially toxic agent. This study will evaluate serum, tissue and radiological correlates to help determine who is likely to benefit from Bevacizumab.

Anticipated Duration of Project

1/1/2009 - 12/31/2012

Project Overview

This project will study a number of tissue and serum markers in patients with advanced, incurable non-small cell lung cancer (NSCLC) (stage IIIB and IV) who are slated to receive bevacizumab in combination with either erlotinib or with novel chemotherapy (pemetrexed and carboplatin). To date, individual investigators and cooperative groups have been unable to identify which patients are likely to benefit from this agent. There are no known molecular or clinical predictors of benefit. While bevacizumab can lead to a survival advantage in combination with standard cytotoxics, it is also costly and potentially toxic. Therefore, it's imperative to eliminate this non-specific "one size fits all" approach. The following correlatives will be assessed:

1. Serum Levels of: E-Selectin (endothelial leukocyte adhesion molecule); ICAM (intracellular adhesion molecules); VEGF (vascular endothelial growth factor), Acidic and basic FGF (fibroblast growth factor); Interleukin-8; Platelet-derived endothelial cell growth factor; transforming growth factor-α, β; Tumor necrosis factor- α; circulating endothelial cells (CECs)
2. Tissue Histology: tissue microvessel density (MVD)
3. Radiographics correlatives with real time DEC-MRI (dynamic contrast-enhanced magnetic resonance imaging), to measure tumor vascularity. FDG-PET/CT (fluorodeoxyglucose positron emission tomography integrated with computed tomography) and FLT-PET/CT (fluor-L-thymidine positron emission tomography), will also be obtained to measure tumor response to therapy and correlate these factors with clinical outcome, including toxicity, response rate (RR%); progression-free survival (PFS) and overall survival (OS).

We anticipate enrollment of 30 subjects over a 3 year period.

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Expected Research Outcomes and Benefits

The following outcomes and benefits are anticipated from this project:

1. Demonstration that molecular, serum, and radiographic correlatives will predict which patients will respond to bevacizumab combinations in patients with advanced NSCLC, for whom empiric therapy has previously been the standard. Correlation of serum, tissue and radiographic correlatives with PFS and OS in advanced NSCLC patients receiving bevacizumab in combination with either erlotinib or carboplatin plus either pemetrexed or taxanes (paclitaxel or docetaxel).
2. Assess the ability of FLT-PET/CT, FDG-PET, DCE-MRI, ADC (Apparent Diffusion Coefficient) mapping to predict response to therapy as determined by conventional restaging with CT RECIST (computed tomography response evaluation criteria in solid tumors).

Summary of Research Completed

Aim 1: Since the last report, we have hired a research nurse, Lynn Werner, who is in charge of consenting patients for biospecimen collection on all newly diagnosed, advanced NSCLC patients, including those receiving bevacizumab. She is charged with maintaining a database on all consented patients detailing basic demographics, treatment choices, and outcomes. Tissue specimens are stored in pathology; and blood is currently being stored under the aegis of Dr. Anil Vachani. Consent for data and biospecimen collection is obtained under the umbrella of Dr. Vachani's IRB-approved, lung cancer specific GEI study, which gives us proper authorization to obtain biospecimens and de-identified demographic information. To date, over 60 patients have been consented, of whom ~ 25 to 30% are receiving angiogenesis inhibitors as part of first-line therapy.

As for bio-correlatives, there is increasing evidence to suggest that genetic polymorphisms may hold the key to identifying which patients are most likely to benefit from angiogenesis inhibition, although we intend to conduct a full battery of tests, including all those listed in the study design. In addition, we anticipate the emergence of new serum bio-signatures that may help us determine which patients are most likely to respond to bevacizumab.

During this reporting period, two key, associated, investigator-initiated IRB-approved studies, one combining bevacizumab with erlotinib in the elderly [UPC 06508], the other pairing bevacizumab with combination pemetrexed and carboplatin [UPC 12508], have completed accrual and closed; and one new study assessing paclitaxel/carboplatin/bevacizumab +/- A12 [ECOG 3508] has been approved. Results for both of the phase II studies that have completed enrollment look promising with respect to tolerability, progression-free and overall survival. A manuscript for this study is in preparation. A poster was presented at a national meeting.

Aim 2: Accrual to the radiographic correlatives has been sluggish.

There is not activity to report for this Aim during this reporting period**.**

Research Project 8: Project Title and Purpose

Reprogramming Cells in Studies of Heart and Lung Development and Repair - Heart and lung disease are the number one and three causes of death and illness in the United States. The ability of the heart and lungs to repair themselves is very limited and poorly understood. Research into how these two organ systems respond to injury as well as try to affect repair will have a dramatic impact on both human and financial costs of these illnesses. Our project will investigate whether non-heart and lung cells can be differentiated into heart and lung cells which would provide a potential source for heart and lung repair. Such discoveries would greatly improve the outcomes of people with cardiac and lung disease.

Duration of Project

1/1/2009 – 6/30/2011

Project Overview

The ability of the heart and lung to effect sufficient repair after chronic or acute injury is poorly understood. Recent advances in regenerative biology offer hope that a combination of stem/progenitor cell therapy and tissue engineering will promote tissue repair and regeneration through the directed activation of endogenous cardiac and pulmonary stem/progenitor cells in vivo and ex vivo or the directed reprogramming of non-cardiopulmonary cells into the cardiac myocyte and lung epithelial lineages. The long-term goal of this project seeks to harness these novel technologies and advance them to explore whether such techniques can be used to promote cardiac and pulmonary tissue regeneration. The aims of this project are to:

1) To directly reprogram adult cardiac fibroblasts to cardiac progenitor cells.
2) Generate and characterize induced pluripotential stem (iPS) cells from congenital heart disease patients to determine the molecular cause of this important category of human congenital disease.
3) Determine whether non-pulmonary cells can be directly reprogrammed into the pulmonary epithelial lineage using known and recently identified regulators of pulmonary epithelial differentiation.

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Expected Research Outcomes and Benefits

This project will define whether non-heart and non-lung cells can be transformed into useable heart and lung cells for future use in regenerative therapies. In addition, the characterization of patient derived iPS cells will greatly enhance our understanding of the causes of congenital heart disease. Although heart and lung disease are important contributors to human illness, very little is understood about their causes. These studies will reveal what genes and molecular pathways are disrupted in specific forms of congenital heart disease. Moreover, from this research we hope to define the basic blueprint of the necessary genetic program required to generate heart and lung cells using adult cell types. The establishment of procedures to generate large numbers of heart and lung cells from readily available adult cell types such as skin cells would greatly improve the chances that heart and lung disease can be treated with these approaches.

Summary of Research Completed

Aim 1: To directly reprogram adult cardiac fibroblasts to cardiac progenitor cells.

During the current year, we have successfully induced expression of a wide transcriptional program in mouse embryonic and cardiac fibroblasts transduced with lentiviral expression vectors for the three cardiac transcription factors MEF2C, GATA4 and TBX5 (MGT). Evaluation of mouse embryonic fibroblasts at 2 weeks and again at 1 month post transduction reveals that cardiac gene expression in induced cells is markedly increased over this time period, as measured by using quantitative PCR arrays containing assays for 30 genes important for heart cell structure and function including ion channels, sarcomeric proteins, transcription factors, etc. (Figure 1). Furthermore, cells collected at 1 month and assayed with these QPCR arrays revealed cardiac transcriptional stability in induced cells as a number of cardiac genes were found to be upregulated in cells treated with doxycycline for the first two weeks only and maintained without for the following 2 weeks (Figure1). Induction of Mef2c and Gata4 form the endogenous loci has been achieved in MGT transduced mouse cardiac fibroblasts by addition of either GATA5 expression or media supplementation with the critical cardiac developmental signaling molecule BMP4 (Figure 2). We have been to demonstrate organization of induced cardiac structural proteins into characteristic sarcomeric structures by histological analysis in MGT-transduced mouse embryonic (Figure 3) and cardiac fibroblasts. MGT-transduction has also successfully induced broad cardiac expression in human dermal fibroblasts, in which alpha-actinin positive immature sarcomeric structure has been thus far detected (Figure 4).

Aim 2: Generate and characterize induced pluripotential stem (iPS) cells from congenital heart disease patients to determine the molecular cause of this important category of human congenital disease.

Our work to date has now expanded beyond the generation of iPS cells from children with congenital heart disease to understanding the mechanisms of CHD formation using iPS cells. Previously, our *in vivo* experiments using fresh source tissue from patients at the Children's Hospital of Philadelphia led to the successful production of iPS cells. We have now carried this work on considerably to completely characterize these cells and differentiate them into all developmental lineages.

1) We have complete the proposed library of CHD patient samples
2) These are fully characterized by IHC and RTqPCR
3) We have expanded this effort to comprehensively expression profile iPS cells from children with CHD and specific mutations to identify early aberrant signaling pathways.

Our focus now is upon modeling discrete phases of human cardiac development *in vitro*.

Aim 3: Determine whether non-pulmonary cells can be directly reprogrammed into the pulmonary epithelial lineage using known and recently identified regulators of pulmonary epithelial differentiation.

Our work has continued to focus on the molecular pathways that regulate lung regeneration. In particular, we have focused on three distinct areas: 1) the role of miRNAs in lung development and regeneration, 2) the role of Wnt signaling in lung development and regeneration, and 3) the role of HDAC mediated chromatin remodeling in lung progenitor development and regeneration. During the past fiscal year our work has lead to several key findings which are summarized below:

1) We have recently demonstrated that Wnt2 is necessary and essential for lung smooth muscle progenitor development through activating both myocardin/Mrtf-b and Fgf10 pathways. These studies illustrate how the Wnt pathway drives smooth muscle development by directly activating myocardin and Mrtf-b expression as well as promoting the activity of the Fgf10 signaling cascade.

2) We recently demonstarted that the microRNA cluster, miR302/367, is directly regulated by Gata6 in the lung endoderm and that this cluster regulates various aspects of lung progenitor biology including cell proliferation, cell polarity and cell differentiation. These studies show that miR302/367 are potent regulators of endoderm cell cycle as well as epithelial cell polarity. We have also generated a lacZ reporter using the promoter/enhancer sequences of miR302/367 and show that these sequences drive expression of lacZ in the developing embryonic lung endoderm. Moreover, this reporter, while silent in the adult uninjured lung, is dramatically activated in a model of injury and regeneration. Thus, miR302/367 may be a new marker of the regenerative state in the lung and experiments are underway to determine whether inhibition or activation of this miRNA cluster will promote or inhibit lung regeneration.

3) We have recently shown that the differentiation and maintenance of Sox2+ proximal airway epithelial progenitors, both in development and in the adult lung, are regulated by an

HDAC1/2 dependent pathway. Loss of HDAC1/2 in development leads to a complete loss of Sox2+ proximal airway progenitor cells. This is mimicked in the adult lung after airway induced injury and regeneration. Given the ability of targeting the HDAC/HAT pathways with small molecules, these findings may have a direct impact on the development of new therapies for upper airways diseases such as asthma and COPD.

4) We have shown that the miR302/367 cluster is able to directly reprogram both mouse and human fibroblasts to an induced pluripotent stem cell state. These findings are very exciting and predict that using advanced techniques of introducing miRNAs into cells without transfection procedures could lead to the establishment of iPS lines simply by introducing these miRNAs.

Figures

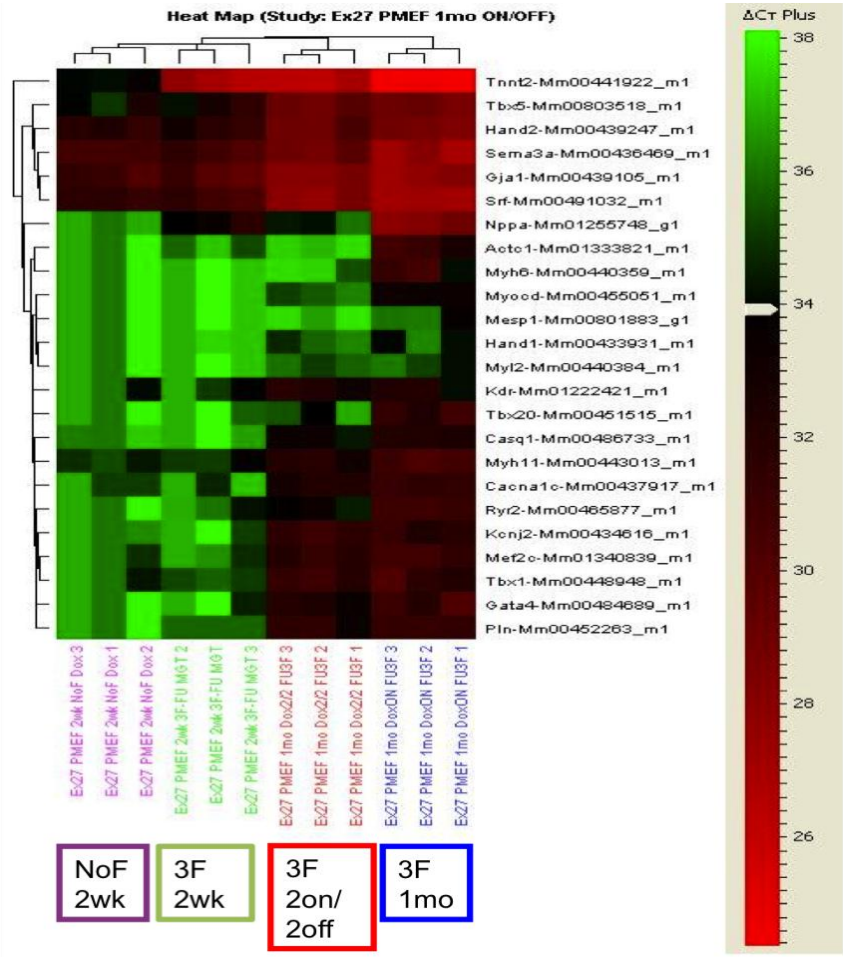

Figure 1. Cardiac gene expression induced in MGT-transduced PMEF's. Cells are treated with doxycycline during the whole culture period with the exception of 3F 2on/off, where cells were cultured without if between 2 weeks and 1 month.

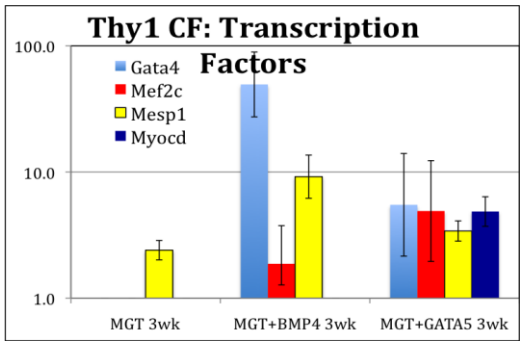

Figure 2. MGT mediated induction of endogenous cardiac transcription factors in Thy1+ mouse cardiac fibroblasts is enhanced by concurrent treatment with GATA5 or BMP4.

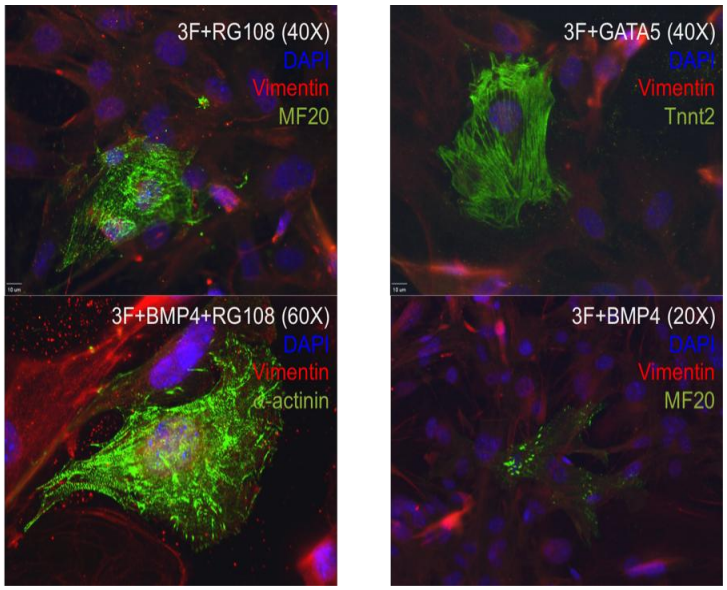

Figure 3. Sarcomeric organization of muscle myosisn (MF20), cardiac troponnin (Tnnt2) and alpha-actinin in MGT-transduced PMEFs after 3 weeks. Vimentin counterstain labels fibroblasts.

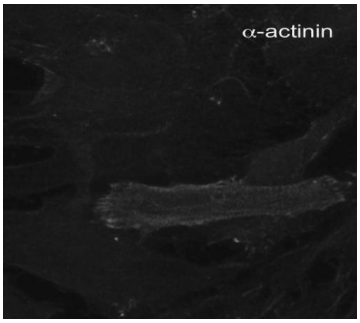

Figure 4. Sarcomeric organization of alpha-actinin in MGT-transduced human dermal fibroblasts at 1 month. Magnification at 40X.

Research Project 9: Project Title and Purpose

Genome-based Bio-marker Discovery and Systems Biology Engineering - Biomarkers allow for convenient diagnosis of particular states of an organism. A robust diagnostic marker is not only important for clinical treatment regimes; it also helps us understand the molecular mechanisms of diseases. For example, many mental diseases like autism are considered psychiatric diseases mainly because neural correlates have not been found. A biomarker, once established, points to possible mechanisms that ultimately might suggest potential therapeutic approaches. Thus, establishment of biomarkers for all organismal normal and abnormal processes is of great significance to both basic and clinical studies. This project aims to develop novel biomarkers using genome-scale data, through the development of novel reagents, computational modeling and analysis, and micro- and nano-engineering.

Anticipated Duration of Project

1/1/2009 – 12/31/2012

Project Overview

Biomarkers are any biological measurements that are indicative of an organism's normal and abnormal states (diseases), as well as other key characteristics of interest. Known genetic markers for disease susceptibility such as Huntington's disease, Cystic Fibrosis, and certain types of breast cancers are classic biomarkers. In recent years, genomics has led to the candidate identification of a whole suite of novel biomarkers. Genome-based biomarker discovery often utilized large-scale sequence data analysis coupled with functional genomics data to suggest that certain gene sequence or gene expression patterns are indicative of organismal states.

More recently, sensitive reporter technologies are being developed to assay in vivo cellular and physiological processes, which in turn can serve as biomarkers. An example is the development of functional dyes that can be imaged in vivo to indicate the progression of protein inclusion bodies in neurodegenerative diseases like Alzheimer's disease. A secondary trend with respect to the discovery and the use of biomarkers is the use of systems-level engineering tools and modeling. An important function of biomarkers is their utility as predictors of future organismal states. For example, in drug development, expensive clinical trials may be considerably shortened if the right combination of biomarkers and predictive models are able to inform the researcher of future toxicity of a candidate compound. This has led to the development of systems biology modeling of entire pathways or even organismal processes to help predict the consequences of perturbation to the processes. Along with systems modeling, another trend is the use of engineering tools to develop fully automated, often miniaturized (e.g., lab-on-a-chip), experimental pipeline to utilize biomarkers for diagnostic devices.

The research group at Penn Genome Frontiers Institute has been developing interdisciplinary teams to identify biomarkers using high-throughput technologies, develop novel sensors that can be used for biomarkers, and develop new models. In this project, our goal is to bring these preliminary studies to larger scale with the following aims:

Aim 1: Develop novel biomarkers for human normal and disease states using high-throughput sequencing, cell-based screening, bio-photonics, and single-cell genomics.

Aim 2: Develop systems models of identified biomarkers and develop single-platform diagnosis devices using identified biomarkers.

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Expected Research Outcomes and Benefits

Biomarkers are efficient diagnosis and prognosis tools for organismal states, including disease states. The main idea is to be able to determine current and future states from a small set of measurements, typically at the molecular level. Typical biomarkers include genetic markers, proteins in the blood, metabolites in body fluid, as well as readouts from biosensors.

The results of this project will generate new biomarkers leveraging information and techniques from genome sciences. Examples include new multi-genic genetic tests, novel biosensors for molecular physiology, and image-based diagnosis. These markers will enable enhanced ability to detect both normal and disease states of humans. Furthermore, this project will include system-level computational models that will help convert biomarker information into prognostic tools. Finally, micro- and nano-scale engineering projects will help develop single-platform tools for converting the discovered biomarkers into clinical tools.

Summary of Research Completed

During the current reporting period, we worked on Aim 1, using newly acquired clinical samples of Retinoblastoma and also the first aspect of Aim 2, developing systems models of biomarkers.

For work under Aim 1, our goal was to develop molecular signatures of 20 matched normal and Retinoblastoma (RB) tumor pairs using the Penn Genome Frontiers Institute High-Throughput Sequencing (HTS) facility. For this work, our approach was to carry out exome sequencing on the matched pairs as well as using RNA sequencing to identify transcriptome variations. Tumor samples were selected based on the presence of classic histo-pathologic markers like state of

differentiation, presence of optic nerve extension or choroidal invasion. The hypothesis was that mutation screening of all exons of known human genes will identify Single Nucleotide Polymorphisms (SNP) that will be diagnostic of high risk RB progression. We used exome capture technology from Agilent service using DNA isolated from blood to differentiate germline changes from somatic changes. Currently, we have completed 21 total sequencing experiments. A total of 15 samples were sequenced on the Illumina platform, including a small RNA 100 cycle read and 14 exome paired-end 100 basepair reads. In addition, we have also carried out a total of 6 ABI SOLiD sequencing slides for exome sequencing. This data is now being analyzed to extract potential genomic biomarkers by associating SNPs to known patient progression indices.

As part of our work under Aim 2, we have been working on tools for systems biology modeling of the circadian rhythm system with the goal of identifying additional key network components, which can become candidates for therapeutics, using statistical patterns (multi-dimensional biomarkers) of gene expression profiles. Circadian rhythms are ~24-hour cycles in physiology and behavior that allow organisms to adjust physiologic process in anticipation of daily environmental changes. It was once believed that these rhythms are generated exclusively from the paired supra-chiasmatic nuclei of the hypothalamus (the central pacemaker in mammals). However, experiments have demonstrated that most peripheral tissues generate cell autonomous rhythms. The importance of these rhythms is implied not only by their conservation throughout phylogeny but also by their ubiquity.

Here, we developed a new Bayesian integration scheme to identify novel "core clock genes" using data from both normal gene expression profiles and knockout/mutation (i.e., perturbation) responsive gene expression profiles along with other expert information including known gene-gene interactions, tissue expression patterns, and phylogenetic distribution of genes. More concretely, the source information set consisted of (1) statistical characterization of whether a gene's expression displays periodic cycling, (2) statistical characterization of magnitude of the expression perturbation response to knock-downs of known clock-related genes, (3) number of known interactions between candidate genes and a set of 18 core clock genes, (4) expression variation across tissue types, and (5) degree of phylogenetic conservation of a given gene in NCBI's Homologene database. Importantly, none of these features appear to be absolute requirements. The canonical core circadian gene CLOCK, for example, does not cycle in the pituitary or SCN while individual knockdown of either RevErb a or RevErb b genes have minimal phenotypic effects. Instead, these features lie on a continuum with each lending some support to a given gene having a core circadian function.

From the data above, we developed a Bayes factor integration scheme using a Naïve Bayesian integration scheme by taking the products of the individual Bayes' factors. Like other naïve Bayesian schemes, our methods do not require ranking the relative importance of each feature. Rather the relative contribution of each feature emerges naturally as features that better discriminate the known core clock genes from non-clock genes are given larger weight in the ultimate classification schemes. However, unlike more common genome-wide integration schemes, this protocol was tailored to include features that are relevant *a priori* to circadian physiology. Rather them simply using co-expression matrixes or Pearson correlations we extracted more specific features of the data particularly appropriate to circadian function. Thus,

while naïve, our integration scheme may be considered "book smart" - preferentially incorporating data sets and features that are relevant to circadian function. Table 1 shows the top 20 genes identified to be potential core circadian rhythm genes. In this table, 9 of the top 15 genes are known clock components. However, we found several novel putative components of the circadian network, which we are now investigating through more direct methods. This procedure demonstrates that by probabilistic modeling of gene regulation system-level response to perturbations and also incorporating expert knowledge, we are able to computationally identify additional components of system regulatory networks. In particular, we demonstrated the utility of this approach by experimentally characterizing one such candidate gene. We confirmed that GM129/C10orf95 directly interacts with other core clock components, oscillates in multiple tissues, and influences the dynamics cellular rhythms in several in vitro tissue models.

Ultimately, it is hoped that this research will facilitate the development of therapeutic agents for sleep, circadian, and psychiatric disorders.

Table 1

Gene (*Mus musculus*)	Gene (*Homo Sapien*)
Clock	CLOCK
Npas2	NPAS2
Arntl	ARNTL
Arntl2	ARNTL1
Per1	PER1
Per2	PER2
Per3	PER3
Cry1	CRY1
Cry2	CRY2
Nr1d1	NR1D1
Nr1d2	NR1D2
Rora	RORA
Rorb	RORB
Rorc	RORC
Fbxl3	FBXL3
Fbxl21	FBXL21
Csnk1d	CSNK1D
Csnk1e	CSNK1E

Research Project 10: Project Title and Purpose

Research Infrastructure: Expansion and Enhancement of Rodent Housing Space - The purpose of this research infrastructure project is to support NIH-funded research in diabetes and other diseases, as well as basic research in genetics through modification of the Clinical Research Building (CRB) animal facility. Renovation of the CRB animal facility is a critical ingredient of an institutional plan to advance Penn's mission to develop new cures through a combination of basic and clinical research. This project will allow us to achieve this goal by: (1) Renovating

space to maximize cage capacity in the CRB animal facility; (2) Upgrading the ventilation system and expanding the "barrier" to reduce the spread of infectious diseases; and (3) Renovating the staff support facilities to modern standards.

Anticipated Duration of Project

1/1/2009 - 12/31/2012

Project Overview

The overall goal of this project is to improve disease prevention and control, and enable increased murine housing in the CRB vivarium. This increased capacity is essential to support critical research being conducted in the area of diabetes and other diseases, and to support basic research in genetics. In working toward this goal, we have launched a multiphase approach to renovation, which began with replacement of the tunnel washer and upgrade of the central ventilation systems, as well as installation of a new autoclave, both of which have been completed. In the next phase of this project, we will renovate former wet lab and behavioral testing space to increase cage-holding capacity, expand the barrier to encompass the entire vivarium, and renovate staff support facilities to modern standards. Subsequent to this, we will upgrade all cage racks/cages in the vivarium to high density models. The specific aim of this requested project is to increase total CRB cage capacity from 7,063 to 15,000, including an increase in barrier capacity from 4,382 to 15,000 cages.

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Expected Research Outcomes and Benefits

Renovation of the CRB vivarium will have positive effects on the school's research programs by providing state-of-the-art animal care husbandry and facilities for Penn's present and future programs, including Genetics/Genomics, Metabolism (diabetes/obesity), Immunology/Transplantation and Neurosciences (behavior/neurodegenerative diseases), among others. It will increase total CRB cage capacity from 7,063 to 15,000, with barrier capacity almost tripling, from 4,382 to 15,000. It will also reduce the potential spread of infectious agents, and improve working conditions for animal care staff. Penn is committed to maintaining full accreditation with AAALAC. This facility will meet AAALAC standards, improve the

quality and quantity of rodent housing, and more adequately meet the needs of NIH-funded research. Lastly, this renovation will provide essential improvements to the working environment for dedicated animal care staff.

Summary of Research Completed

Construction commenced in December 2010. Phase 1 was completed at the end of June 2011. Work completed in this phase consisted of converting empty labs and procedure rooms to new holding and procedure rooms and the construction of new locker rooms/showers/toilets and janitor closet.

Research Project 11: Project Title and Purpose

Development and Validation of a Tool to Assess Perceived Nutrition Environments - Obesity prevalence has reached pandemic proportions in the United States. The availability and distribution of food within a neighborhood has been related to the food intake and obesity prevalence of people living in that neighborhood. To improve our understanding of the relationship between neighborhood food environment and food intake, we need to know more about how people living in the neighborhood perceive what foods are available to them. To address this gap in the literature, the purpose of the current study is to develop a standardized measure of perceived food environment. Development of such a measure will go toward fulfilling one of the research priorities recently identified by a panel of experts for this area of work.

Anticipated Duration of Project

10/1/2009 – 10/14/2011

Project Overview

Research Objective: The study objective is to develop and validate a standardized measure of perceived nutrition environment.

Specific Research Aims:

1) To pilot-test an instrument designed to evaluate perceived nutrition environment in a convenience sample of 16 individuals.
2) To determine the psychometric properties of the instrument developed to measure perceived nutrition environment in a sample of 200 adults: 100 residing in an area of high socioeconomic status and 100 from an area of low socioeconomic status.
3) To explore whether observed nutrition environment and perceived nutrition environment are independent and additive mediators of the relationship between Self-Reported Nutrition Environment and eating behaviors.

Research Design and Methods:
This study uses a multi-phase study design that will enable the research team to develop and validate an instrument to measure perceived food environment. Phase 1 involves piloting the

instrument. A draft measure will undergo internal and external review before being completed by a convenience sample of 16 adults who will also complete an audio-taped cognitive interview where they will be asked to provide feedback about the instrument content. Phase 2 of the study will involve determining the psychometric properties of the instrument. Specifically, 200 adults from geographic areas of high (Chestnut Hill and Wynnewood; N=100) and low (North and West Philadelphia; N=100) socioeconomic status will complete the instrument on two occasions (2-3 weeks apart). Study participants will also complete assessments of background characteristics (demographics, health status), self-reported community, consumer and food nutrition environments and psychosocial factors related to food intake and eating behaviors. Test-retest correlation, internal consistency and criterion validity will be ascertained from these data.

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Expected Research Outcomes and Benefits

This project is expected to yield several important outcomes that will benefit the area of obesity prevention research and programming. The development and validation of an instrument to evaluate perceived food environment will help elucidate the relationship between observed and perceived nutrition environment and the extent to which these variables impact the relationship between food environment and eating behaviors.

Summary of Research Completed

This study is in progress and is in Phase 2, the main measurement phase. This study uses a multi-phase study design to develop and validate an instrument to measure perceived food environments.

<u>Phase 1</u>

Phase 1 was completed during this reporting period. It consisted of developing and pilot testing the initial instrument. Beginning in July 2010, a convenience sample of 15 adults was recruited to fill out the questionnaire and complete an audio-taped cognitive interview. A variety of recruitment methods was used, including: posting flyers in community centers, libraries, train

stations, and other high traffic areas; recruiting in-person at a library or community center; and printing information in a local newspaper.

In order to be eligible to participate in Phase 1, individuals had to: live in one the designated zip codes in the greater Philadelphia area, have lived in his/her neighborhood for at least 6 months and plan to live there for the next month, be between the ages of 18 and 65, and perform some or all of the food shopping in his/her household. Eligible participants should also be able to speak and read English fluently, have a working telephone number, be able to provide informed consent, and be willing to complete the study requirements. Of the 66 adults who were screened for eligibility, 30 were eligible to participate. The main reasons for ineligibility included not living in an eligible zip code (48.5%, n=32) or not being between the ages of 18 and 65 (10.6%, n=7). Fifteen eligible adults were enrolled in Phase 1 of the study and the remaining eligible adults were later contacted to participate in Phase 2.

Fifteen cognitive interviews were conducted between July 28, 2010 and September 17, 2010. After providing informed consent, participants completed the questionnaire followed by the cognitive interview. During the cognitive interview, each participant was asked to provide feedback about the instrument's content and format. The participants took an average of 22 minutes (SD=7) to complete the questionnaire and an average of 40 minutes (SD=9) to complete the cognitive interview. All pilot study participants received a $25 gift card to either CVS or Target to reimburse them for their time and travel related expenses.

The information gathered from the cognitive interviews was compiled and reviewed. The qualitative data from the cognitive interviews was paired with the quantitative data collected from the questionnaire to drive the modifications that were made to the instrument. The majority of the changes made to the questionnaire included rewording items to improve clarity. Other changes included dividing a question into two separate questions and adding guidelines on how to define one's neighborhood. Revising the questionnaire and preparing for Phase 2 of the study took place in October 2010.

Phase 2

Recruitment for Phase 2 began in November 2010 with the goal of having 200 participants complete the questionnaire on two separate occasions (2-3 weeks apart). The 15 eligible adults who were recruited during Phase 1 were contacted first to participate in Phase 2. Similar recruitment strategies that were used in Phase 1 were also used during Phase 2, including: posting flyers in community centers, libraries, train stations, and other high traffic areas; recruiting in-person at a library or community center; and sending information through community email list-servs.

The eligibility criteria for Phase 1 and Phase 2 were the same (see eligibility criteria listed above); however, 5 zip codes in the designated neighborhoods (Chestnut Hill, North and West Philadelphia, and Wynnewood) were added for Phase 2. Of 267 adults who were screened for eligibility, 233 were eligible to participate. The main reasons for ineligibility included not living in an eligible zip code (61.8%, n=21) or not being between the ages of 18 and 65 (23.5%, n=8).

Eligible participants either provided written consent in-person or verbal consent over the telephone. Participants then had the option to schedule a time to complete the questionnaire in-person or receive a copy of the questionnaire in the mail to complete and return in a provided envelope. Within 2-3 weeks of completing the first survey, participants completed the same questionnaire a second time. Participants had the same option as before – either schedule a time to complete the questionnaire in-person or receive a copy of the questionnaire in the mail to complete and return in a provided envelope. Participants received $25 in gift cards for completing both questionnaires, a $10 gift card for completing the first questionnaire and a $15 gift card for completing the second questionnaire. Participants receiving the questionnaire in the mail were given a reminder phone call and/or email if the completed questionnaire was not returned after 10 days of being mailed.

The first questionnaires were completed between December 6, 2010 and June 3, 2011. Of the 233 eligible participants, 94.8% completed the first questionnaire (n=221). The second questionnaires were completed between December 22, 2010 and June 26, 2011. Of the 221 participants who completed the first questionnaire, 97.3% completed the second questionnaire (n=215).

Currently, the questionnaire data are being prepared for data analysis.

Observed Nutrition Environment

The observed nutrition environment is being evaluated using the Nutrition Environment Measures Survey (NEMS). NEMS data provides objective assessments for environmental attributes of both neighborhood food stores (e.g., food availability, food quality and freshness, food price and shelf space, store type, and neighborhood) and restaurants (e.g., food availability, price, and promotion). NEMS data is currently being collected for the areas being targeted by the study.

From October 2010 through January 2011, restaurants and stores in the designated areas (Chestnut Hill, North and West Philadelphia, and Wynnewood) were enumerated. Within the study area, a total of 769 restaurants and 762 stores were enumerated. NEMS data is being collected on a sample of the restaurants (n=121) and stores (n=168). A portion of the stores and restaurants are being rated by two different raters to assess inter-rater reliability.

NEMS restaurant data collection began in mid-February 2011 and currently 105 restaurants have been rated and 16 restaurants are either closed or are have been excluded (e.g., the location only sells alcohol). Sixteen restaurants were double rated for reliability purposes. NEMS store data collection is beginning in mid-July 2011.