University of Pennsylvania

Annual Progress Report: 2007 Formula Grant

Reporting Period

July 1, 2009 – June 30, 2010

Formula Grant Overview

The University of Pennsylvania received $8,868,580 in formula funds for the grant award period January 1, 2008 through December 31, 2011. Accomplishments for the reporting period are described below.

Research Infrastructure Project 1: Project Title and Purpose

Research Infrastructure: Construction of Life Sciences Building, Phase II - The purpose of this infrastructure project is to provide state-of-the-art laboratory space for investigators in the Departments of Psychology and Biology in the School of Arts and Sciences. Most importantly, it will enable realization of one of the key elements of the strategic plan of the School of Arts and Sciences, entitled "Genes to Brains to Behavior." This thrust emphasizes the growing importance of the biological underpinning, including the genetic basis, of even the most complex behaviors. This vitally important space will help Penn to attract the high-quality faculty, students, and postdoctoral researchers who are the essential critical success factor in modern research in the life sciences. These researchers, from a wide variety of fields, will stimulate each other and their students to greater research success in this cutting-edge field.

Summary of Research Completed

This project was dropped prior to the expenditure of any grant funds.

Research Infrastructure Project 2: Project Title and Purpose

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

Summary of Research Completed

This project was dropped prior to the expenditure of any grant funds.

Research Infrastructure Project 3: Project Title and Purpose

SVM Research Infrastructure—Behavioral Testing Laboratory - The purpose of this infrastructure project is to upgrade and renovate approximately 225 net square feet of laboratory space in the Hill Pavilion Vivarium Barrier Facility to provide necessary mouse behavior testing space for conducting extramurally funded research projects related to understanding the role stress plays in the development of obesity and depression. The renovated space will allow researchers to produce high quality, reproducible and consistent results. As rodent behavior is highly dependent on the testing environment, it is imperative for these studies that we have designated testing space that is soundproof and not utilized for any invasive procedures. The space will be temperature controlled and free from outside disruption and noise, and able to be controlled by testing investigators.

Duration of Project

1/1/2008 - 6/2/2008

Summary of Research Completed

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

Research Project 4: Project Title and Purpose

Financial Incentives for Smoking Cessation - Despite progress in recent decades in helping people to quit smoking, about 65 million Americans still smoke cigarettes. Tobacco addiction is the leading cause of preventable mortality in the United States, and even though 70% of smokers report wanting to quit, only about 3% of smokers succeed annually.The purpose of this proposal is to provide supplemental funding to complete a CDC-funded trial of financial incentives for smoking cessation. In this randomized controlled trial, we have completed follow-up of subjects at 12 months with highly significant differences in quit rates for subjects in the incentive (14.7%) and control groups (5.0). At 18 months post enrollment incentive group subjects still had significantly higher rates smoking cessation (9.4% vs. 3.6%, $p<0.001$). Funding is requested to complete analyses of cost effectiveness and process evaluation.

Anticipated Duration of Project

7/1/2008 - 12/31/2010

Project Overview

The objective of this project is to complete long-term follow-ups on a randomized controlled trial of financial incentives for smoking cessation. Previous efforts to test the effectiveness of financial incentives for smoking cessation in the workplace have generally used incentives of small magnitude and have been statistically underpowered. In partnership with General Electric (GE), we designed a scalable financial incentives intervention that could be broadly utilized by employers if effective in increasing smoking cessation rates.

878 GE employees including subjects from Erie and Grove City (GE Rail), Trevose (GE Water) and Hatfield (GE Energy), were enrolled in a randomized controlled trial, in which subjects were randomized to receive either information about smoking cessation programs within 20 miles of their workplace or the same information plus a package of financial incentives worth $750. The randomization was stratified by worksite, income, and degree of nicotine dependence. Intervention arm participants were eligible to receive $100 for completing a smoking cessation program, $250 for quitting smoking anytime in the first 6 months of study enrollment, and $400 for continuous abstinence between the 6 month and 12 month follow-up visits. Self-reported cessation was regarded as valid only if confirmed by a negative cotinine test. Quit and relapse rates were compared between groups using a two-sided chi-square test. Results were analyzed using intention to treat, with any subjects lost to follow-up assumed to have resumed smoking.

Due to greater than expected effectiveness of financial incentives in achieving both short- and long-term quit rates which induced budget shortfalls, supplemental funding was requested in 2008 to fulfill the following specific aims:

1.) Complete 18 month follow-ups on all subjects to determine long-term effectiveness without incentives;
2.) Examine cost effectiveness of this incentive program in achieving long-term tobacco cessation.

All subjects have now completed 12-month and 18-month follow-up visits. During the first 6 months following study enrollment, 14.9% of the incentive group completed a smoking cessation program vs. 6.8% of the control group (p-value<0.001). Quit rates in the first 6 months were significantly higher in the incentive group compared to the control group 20.9% vs 11.8%, p-value=0.001). At 12 months, long-term quit rates were 14.7% in the incentive group vs. 5.0% in the control group (p-value<0.001), and at 18 months, long-term quit rates were 9.4% in the incentive group vs. 3.6% in the control group (p-value <0.001).

The relative differences in quit rates observed at 9/12 months were largely sustained 6 months later after cessation of the incentives, as the ratio of 2.6 of quit rates between incentive and control groups at 15/18 months was only slightly lower than the ratio of 2.9 observed at 9/12 months.

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

This study is one of the only studies to have shown a large and significant difference in long-term tobacco cessation rates in an employer setting. Optimizing the design of such an intervention and determining the long-term cost effectiveness is essential to realize the full potential benefits of this intervention in reducing the burden of smoking-related illnesses such as cardiovascular disease and cancer among the millions of Pennsylvanians and other Americans who smoke. Based on the results of this study, GE has publicly committed to offering this program or one resembling it to all 152,000 employees domestically starting in January of 2010.

Summary of Research Completed

The study proposal called for performing cost-effectiveness analyses covering 2 time horizons: 1) a within-trial analysis that evaluated what was observed during the year of follow-up during the trial and 2) a long-term projection of the short-term effects of the trial to include longer term outcomes such as morbidity and mortality avoided. The strength of the within-trial analysis is that we directly observed the measured changes in benefits and costs assessed. The strength of the long-term projection is that it projects benefits such as cancers or heart attacks avoided (as well as positive or negative costs) that are unlikely to be observed within the relatively short timeframe of the trial.

During the last 6 months, we completed the within-trial analysis. This analysis incorporated both within-trial costs, comparing costs across treatment arms at the 3/6 month and 12/15 month follow-ups. The costs included in the analysis were self-reported health care costs and smoking cessation costs, costs associated with administering the study and the incentives, and work-loss costs. During each follow-up visit, patients were asked to self-report costs of nicotine replacement therapy, prescription drugs for smoking cessation, smoking cessation programs, and other health care use. The cost of nicotine replacement therapy and prescription drugs for smoking cessation programs were based on wholesale costs. Smoking cessation program costs were based on self-reported costs as well as prices reported by each specific program. Medical costs were based on the Agency for Healthcare Research and Quality (AHRQ) data for physician, emergency room, and hospital visits. Using this, we compared the cost per successful quitter and cost per quality adjusted life year (QALYs) saved ratios.

We have now completed the within-trial analysis but cannot report details on the results here because we cannot publicly disseminate this on the State's website since that would preclude publication in many academic journals. In brief, the magnitude of the cost-effectiveness ratios were calculated by first measuring incremental costs for those in the incentive group vs controls. Then we compared the percentage point reduction in smoking between groups and the resulting gains in quality-adjusted life years. From this, cost-effectiveness ratios were calculated. These were significantly less than $50,000 per QALY, indicating that this intervention would be considered highly cost effective.

We are actively working on the long term projection analysis. This will use potential relapse rates across the treatment arms and the distributions around each specific cost for the groups to predict future cost-effectiveness ratios. These long-term projections will allow for the cost-analysis to go beyond the within-trial cost-effectiveness to determine how financial incentives may impact future costs with regards to smoking cessation.

Update on process evaluation analyses from main trial

The purpose of the process evaluation was two-fold: 1) to better understand why incentives did not influence some smokers to quit; and 2) to examine for whom incentives were most effective and whether incentives influenced some positive changes in attitudes or behaviors among non-quitters.

To date, we have completed analyses and are preparing the following two manuscripts for publication:

1) "Why are financial incentives not effective at influencing some smokers to quit? Results of a Process Evaluation of a Worksite Trial Assessing the Efficacy of Financial Incentives for Smoking Cessation."

In this paper, we examine why financial incentives are not effective at influencing some smokers to quit. We explored participants' awareness and attitude toward program components, their perceptions about the value and structure of the incentives, and the optimal amount of incentives needed to motivate successful quit attempt. Closed-ended process evaluation measures were developed and added to the short-term and long-term follow-up surveys and administered to all participants. Questions regarding awareness of and perceptions about financial incentives were only asked of participants in the incentive arm. We conducted descriptive statistics for all close-ended measures and used chi-square tests to assess statistically significant differences by quit status. A series of open-ended questions exploring why and how incentives were helpful in cessation efforts was administered to a subset of these participants (n=36) until thematic convergence was reached. These open ended items were administered at the end of the survey and responses were recorded and transcribed. Qualitative responses were coded for emergent themes and analyzed with the quantitative responses to triangulate findings.

Our results suggest that despite high rates of program awareness, financial incentives are not effective at motivating some smokers to quit. It appears that internal motivation to quit needs to be sufficiently high for incentives to be effective. The majority of quitters in the incentive group

were already motivated to quit and reported they would have quit for less money. Non-quitters reported lack of self-motivation to quit and benefits of smoking as reasons why financial incentives were not effective. Even a hypothetical doubling of the incentive amount was not perceived by non-quitters as adequate compensation for cessation stressors.

This is the first study that we are aware of which examines how participants perceive and utilize financial incentives for health behavior change. These results can help inform optimal design of future incentive-based programs. The paper is being submitted to the Journal of Occupational and Environmental Medicine for publication.

2) "For whom are financial incentives most effective at motivating smoking cessation? Multivariate analysis of national worksite trial assessing the efficacy of financial incentives for smoking cessation."

In this paper, we conducted secondary analyses to examine what factors predict successful long-term quit status and whether incentives influence some positive changes in smoking-related attitudes and behaviors among non-quitters. We conducted bivariate and multivariate analyses using baseline and long-term follow-up data from the main trial. The main outcome was long-term quit status. We examined the association between quit status and the following baseline characteristics: a) socio-demographic characteristics (e.g. gender, race, etc); b) self-reported health status; c) smoking and cessation related behaviors including cigarettes smoked per day, nicotine dependence, participants' readiness to quit smoking, and previous quit attempts; d) social and environmental cues to smoking including presence of other smokers in household; e) participants' motivational orientation and time-preferences. Multivariate logistic regression analysis was conducted to determine which baseline characteristics significantly predicted long-term quit status.

Bivariate results suggest that long-term quit status is significantly associated with contemplating quitting at baseline, quit attempts in the past year, low nicotine dependence, and delayed discounting. However, in multivariate analyses, only extrinsic motivation and delayed discounting significantly predicted successful quit attempts. Financial incentives did not influence non-quitters to make positive changes toward quitting (e.g. the number of cigarettes smoked by non-quitters were no different in the incentive and control groups). The results of this analysis can help inform optimal targeting of future incentive-based programs. We are in the process of writing up these results and intend to submit to the American Journal of Health Promotion.

<u>Update on RE-AIM analyses</u>

To date, we have completed data collection and analyses for the RE-AIM component of the process evaluation. RE-AIM is a commonly used framework for program evaluation that assesses Reach, Efficacy, Adoption, Implementation, and Maintenance. The main objective of our RE-AIM analyses is to examine factors at both the individual (employee) and organizational (worksite) levels to evaluate the overall impact and generalizability of the financial incentive intervention. We developed study instruments based on the RE-AIM constructs and conducted in-depth telephone interviews with program administrators at 20 worksites and focus groups with

employees at 10 worksites. In our analyses we examine: the proportion and representativeness of target population reached; type of recruitment strategies used; variation in retention and quit rates across worksites sites; barriers and facilitators to participation and program administration; and factors that may maximize maintenance of similar programs in the future. We are in the process of drafting a manuscript of findings and intend to submit these to a peer-reviewed journal in September 2010.

Research Infrastructure Project 5: Project Title and Purpose

Center for Genetics and Complex Traits – Research Infrastructure - The purpose of this research infrastructure project is to renovate space on the second floor of Blockley Hall in support of the Center for Genetics and Complex Traits. This new center is a major initiative of the University of Pennsylvania School of Medicine's Research Strategic Plan and addresses three key needs that are shared by Penn, the NIH Roadmap, and the Commonwealth of Pennsylvania: Advancing new pathways of discovery (bioinformatics); Building research teams of the future (molecular epidemiology-statistical genetics-bioinformatics); and Re-engineering the clinical research enterprise (enhancing translational research). Renovation of space in Blockley Hall is essential to Penn's efforts to create an interactive environment that both promotes the Center's translational outreach initiatives and enables the recruitment of new faculty with expertise in statistical genetics and informatics. With these new collaborators and resources in a position to succeed, Penn's Center will create new studies that lead genetics/genomics research into the future for the benefit of the citizens of the Commonwealth.

Anticipated Duration of Project

7/1/2008 - 06/30/2010

Project Overview

The goal of this project is to develop support for an integrated institutional program on the genetics of disease and complex traits. To achieve our goal we propose to unite critical interdisciplinary expertise in clinical and molecular informatics, with the methodologies of epidemiology and statistical genetics through the Center for Genetics and Complex Traits. The primary missions of the Center are to apply this integrated approach across disease-based themes, and to train new scientists with cross-disciplinary expertise.

The specific aim of this project is to renovate approximately 10,724 gsf of space on the second floor of Blockley Hall which will be designed specifically for, and used exclusively in support of, the mission and goals of the Center for Genetics and Complex Traits.

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None

Expected Research Outcomes and Benefits

The renovations are being completed to allow adjacencies and expansion of the Center for Genetics and Complex Traits (CGACT) which is a Type I Center of the Center for Clinical Epidemiology and Biostatistics. The theme of CGACT is to develop and apply epidemiological and statistical approaches that integrate knowledge from the human genome, basic molecular and cellular biology, biomedical informatics, behavioral science, bioethics and the clinical sciences to understand complex human disease traits. These methods will be applied to study etiology and outcomes associated with cancer, sleep disorders, psychiatric disorders including addiction, obesity, diabetes, autoimmune disorders, asthma, and cardiovascular disease, all of which are major disease burdens in the Commonwealth, leading to lost income and reduced quality of life for our citizens.

Summary of Research Completed

The new space contains private offices, shared offices, large and small conference rooms, administrative support space and a centrally located break room. The flexible layout allows for reallocation of spaces among the core faculty. Enclosed offices allow for "heads-down" research while the open areas of the plan encourage interaction between the faculty and their staff.

The design and layout of the new space has met the objective of providing a co-location for the core faculty of the Center. This shared space allows for an integrated approach for the exploration of the genetic basis of human disease and complex traits. We are pleased that this project optimally advances the mission and goals of the Center for Human Genetics and Complex Traits. The space will provide critical support and interaction between researchers thereby facilitating the development of opportunities in translational research.

Since the project completion and occupancy, the Center for Genetics and Complex Traits has accomplished the following research initiatives:

1) Established a core faculty of Center Scholars with combined expertise in molecular and cellular genetics, epidemiology, statistical genetics and clinical informatics. The center supports membership of 10 core faculty and 43 affiliate faculty members. These faculty have expertise in all areas of molecular epidemiology and statistical genetics, including the primary areas listed above. Faculty also cover a variety of disease and content areas, including most major common diseases.

2) Developed collaborative outreach initiatives and Associate Center Scholars that apply these

interdisciplinary skill sets to major clinical disciplines in Penn Medicine. While study of the genetic basis of human disease and disease traits can theoretically be applied to any clinical area, to ensure focus of the Center in achieving this goal, programs were focused in the areas of Cancer, Cardiovascular Medicine, Behavior/Addiction Sciences, and Pharmacogenetics. Additional outreach initiatives are being instituted in the areas of Pulmonary Disease, Renal Disease and Transplantation, and in Sleep Disorders.

3) Developed and coordinated core resources for faculty and trainees in Methods Development and Study Design, Clinical Informatics, Biostatistics and Statistical Genetics, Molecular Diagnostics and Genotyping, as well as Cell and Tissue Distribution Banks.

4) Developed educational programs including courses, thesis level projects, seminar series, and a visiting scholar series in concert with existing degree granting programs of Penn Medicine. We have developed educational programs, including courses, seminar series, and visiting scholar series. Degree granting educational programs is a key area for CGACT activities. These activities include Human Genetics Concentration and coursework in the Human Genetics concentration in the MSCE program.

Research Project 6: Project Title and Purpose

Integrated Approaches to Genome-based Therapeutics - It is now widely appreciated that the genotype plays a significant role in outcomes of disease treatment. For example, the toxicity of a particular drug can be modulated by the patient's genome-wide genotype—leading to the recent idea of "Personalized Medicine." Furthermore, about 2000 human diseases such as Parkinson's, diabetes, and others are thought to arise from defects of genomic function, suggesting new therapeutic strategies that might use RNA/proteins/small molecules to affect system-level function. The purpose of this project is to identify strategies for genome-based therapeutics, develop an understanding of the interaction between individual genomes and intervention, RNA/protein based therapeutics, delivery systems for new therapeutics, and computational analyses to aid therapeutic design and system-level understanding of genomic dysfunction.

Anticipated Duration of Project

1/1/2008 - 12/31/2011

Project Overview

Therapeutic drug treatment strategies for human diseases have relied on two assumptions: (1) that the disease can be treated by targeting a single molecule that is the keystone to the dysfunction that led to the disease; and (2) that all treated patients will respond in a similar manner (except for gross physiological factors such as body weight). Despite more than fifteen years of development, only recently has genome-scale data been studied in relation to human diseases and therapeutic strategies. The nascent data has revealed that an individual's response to particular therapeutics, in terms of efficacy and toxicity, may depend to a surprising degree on various genomic factors. Thus, a treatment that might not be effective in one group of individuals might be effective in another group that has a different genomic composition; similar genome-

dependence might be found for toxic side effects. An important direction for the future of therapeutic strategies is to develop an understanding of the interaction between individual genomes and specific interventions.

Another recent development has been the realization that many of these diseases arise from 'systems level' dysfunction, where more than one defect leads to the same or similar observable phenotype. For example, spectrum disorders often manifest in the same clinical outcomes, e.g. dementia, and causes, neuronal cell death, but the path to generating these symptoms is different As noted above, the classic model is to seek a key target whose inhibition or induction might lead to amelioration or even reversal of the dysfunction. However it is becoming increasingly clear that therapeutic strategies for more complex diseases will require multi-parameter manipulation of genomic function through RNA or protein-based agents.

Given the above background, the aims of this project are the following:

Aim 1: Develop an understanding between therapeutic strategies and human diseases through the use of genomic information and clinical/intervention outcome data in humans and model organisms.

Aim 2: Develop novel therapeutic strategies including RNA and protein agents that modulate whole-genome function.

Aim 3: Develop computational analysis tools for system-level modeling of genomic dysfunction and manipulation by therapeutic agents.

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Expected Research Outcomes and Benefits

The first expected outcome of this research is increased ability to better match therapeutic strategies to individual patients, increasing drug efficacy and ameliorating or predicting adverse side effects. The second expected outcome and benefit is to obtain new tools and basic knowledge to develop new classes of therapeutic agents, RNA and protein molecules, which target whole genomic function manipulation. The expected knowledge to be generated includes technologies for RNA/protein delivery, genomic effects monitoring including technologies for in

vivo assays, and computational models for system-level genomic function vis-à-vis genomic interventions. The new research from this project is expected to pioneer a whole new class of therapeutics as well as develop a better understanding of the interactions of the genome with intervention agents.

Summary of Research Completed

We have continued to make progress on Aims 2 and 3 of the project.

Aim 2: Develop novel therapeutic strategies including RNA and protein agents that modulate whole-genome function.

Aim 3: Develop computational analysis tools for system-level modeling of genomic dysfunction and manipulation by therapeutic agents.

In the previous period, we described developing novel techniques for using next-generation sequencing to identify RNA-RNA interactions that might be involved in genomic function and dysfunction. In this period, we developed novel PH-sensitive probes that are membrane impermeable and therefore can serve as read outs for vescile trafficking. The rationale for developing this new sensor is to use it as a high-throughput readout for high-content imaging cell-based screening of therapeutic agents that target vesicle turnover pathways. Such pathways may be critical in cell-degenerative diseases.

In this work we focused on porphyrin-based dendritic pH nanoprobes. Porphyrin chromophore is characterized by strong absorption throughout the UV-vis region and fluoresces with quantum yield ~0.1. Upon protonation of two core imine nitrogens (shown bold in Fig. 1), optical spectra of porphyrins undergo bathochromic shifts, making these chromophores suitable for ratiometric pH sensing, either by absorbance or by fluorescence. By stabilizing the protonated form (porphyrin dication), pK of the macrocycle can be modulated and brought into the desired interval. Stabilization of the porphyrin dication can be affected by way of dendritic encapsulation. Dendritic porphyrins are highly soluble in water, do not aggregate and/or form complexes with biological macromolecules and do not diffuse across phospholipid membranes. Importantly, multiple groups on the dendrimer periphery can be functionalized, providing an opportunity to synthetically manipulate the degree and the rate of cellular uptake.

To develop pH sensitive porphyrin-based nanoprobes, we performed: a) synthetic construction of a dendritic porphyrin with pK in the physiological pH range; b) spectroscopic and acid/base characterization of the probe in bulk solutions and in micro-compartmentalized systems (phospholipid vesicles); c) modification of the dendrimer periphery with the purpose to modulate cellular uptake. We carried out calibration experiments for a synthesized prophyrin-based nanoprobe. The probe's pK was measured in bulk solutions as well as inside large unilamellar vesicles. To test whether the synthesized probes can be internalized *via* receptor-mediated endocytosis, we performed live imaging of immortalized mouse embryonic fibroblasts (MEF) incubated in the presence of probes Glu^3, Glu^3-PEG, and Glu^3-Arg. We also carried out pH calibration by imaging probe solutions, also of known pH, encapsulated in liposomes and/or polymersomes attached to coverslip surface. In summary, we have developed new porphyrin-

based dendritic pH nanoprobes that can be incorporated into the intracellular vesicles via endocytosis and undergo pH changes in the physiological range that can be detected using fluorescence microscopy. Thus, we have developed a new tool and a new method for intracellular pH measurements and pH-based imaging.

In the previous period we developed two computational procedures for system-level modeling of genomic dysfunction. First, we developed a new technique for using next-generation sequencing for detecting genomic elements with variant 3' ends—thereby expanding the catalog of functional genomic RNA elements. Briefly, the template preparation for Illumina sequencing results in excess of reads in the anti-sense direction at 3' ends. Therefore, a differential of sense to anti-sense read coverage statistic can be computed and a peak detection algorithm used to delineate the 3' terminals of templates, allowing us to map the 3'UTR variants. Fig 1shows an example for the Snap25 gene where we see a peak at the canonical 3'UTR (thick bar) and then at least three other peaks downstream indicating three additional 3'UTR isoforms. We have used additional sequence mapping to verify these terminals (by extension into poly-A tracks).

Second, we have begun work on developing new computational procedures for using large-scale siRNA perturbation data to identify whether a perturbation agent directly affects a known systems network and if so, which component is the target of the agent. The rationale for this is that therapeutic agents have broad effects not only on target physiological elements but also on broad genomic regulatory networks. Such effects on regulatory networks might reveal potential for additional targets or suggest ways to mediate off-target effects. We have used the mammalian circadian rhythm as the target network system, leveraging a large set of genomic data collected in the laboratory of J. Hogenesch (Associate Director of PGFI; see also Baggs et al. 2009, PLoS Biology 7(3): e1000052. doi:10.1371/journal.pbio.1000052). We assume that there is a set N of n variables representing the known network components; a set of P of k perturbation conditions; and a set O of monitored output variables. We assume that O contains N and the variables are expression levels of genes.

We are developing two strategies. First, for each gene in the target network (i.e., the set N) we obtain the collection of response vectors to each of the perturbations. We assume that these response vectors form a convex set of possible response regions. Using linear dimension estimation algorithms, we identify the subspace associated with each variable in N. For an unknown perturbation and the response vector, we find the closest projection to the response subspace of the network components. In the second approach, we obtain a non-linear multiple regression of the network variables as a function of levels of all other variables in O over the set of perturbation conditions. The main idea is that the network interactions work for a non-linear subset of possible responses of any network variable that can be estimated as a "surface" in relation to other variables. Then for an unknown perturbation and its resulting response vector we find the closest surface as an estimate of the target network component. Both of these techniques involve the idea that the network interactions delineate a subset of possible "actions" of any gene expression (i.e., network components) and this subset can be estimated by either direct geometic configuration or in relation to other variables. We are testing this model using the data in Baggs et al. (2009). In the next period, we will also build a network regulation simulation model to test this idea.

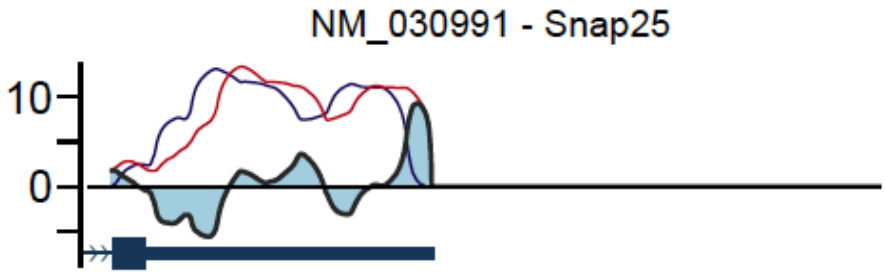

Fig 1. Detection of template 3' ends by comparing sense and anti-sense read depth. Illumina sequencing yields excess of anti-sense reads at the 3' ends of templates. The figure shows sense reads (blue) and anti-sense reads (red) and their differential (filled line) for the Snap25 gene. Thick bar below the plot annotates canonical 3'UTR. The thin bar annotates extended 3'UTR region. The peaks of the differential plot indicate positions of 3' terminals for UTR variants. For this gene, we predict three extra 3'UTR variants.

Research Project 7: Project Title and Purpose

Isolation and Characterization of Lung Cancer Stem Cells - Evidence from leukemia researchers has shown that the capacity of the tumor to grow, propagate, and resist therapy may be dependent on a small subset of cells, termed "cancer stem cells." Although rare, they could regenerate a tumor identical in appearance to the parent cancer. The identification of cancer stem cells in solid tumors was first shown in 2003 in primary human breast cancer. If such cancer stem cells exist in other cancer types, like lung cancer, isolating them could have numerous implications in our understanding of tumor biology (i.e. interactions with the microenvironment) and in designing new therapies (biochemical, molecular, and immunologic). Our goal is to isolate lung cancer stem cells.

Anticipated Duration of Project

7/1/2008 - 12/31/2010

Project Overview

Our long-term objective is to develop new therapies for lung cancer that are based on the unique biological characteristics of this malignancy. Our working hypothesis is that lung cancers contain tumor initiating stem cell populations that self-renew and give rise to the heterogeneous populations making this tumor so exceptionally therapy resistant.

The goals of this project are to establish operating procedures to isolate cancer stem cells using lung cancer stem cells as a paradigm. Once identified, these cells will then be analyzed using genomic and proteomic approaches to identify potential therapeutic targets

for biochemical or immunologic strategies. To accomplish this, we propose the following specific aims.

1. Establish the infrastructure to collect lung specimens in a sterile fashion suitable for cell isolation.
2. Use a variety of approaches (established by our collaborator, Dr. Meenhard Herlyn) to isolate lung cancer stem cells from these tumor specimens and/or primary lung cancer cell lines.
3. Compare the genomic and proteomic characteristics of lung cancer stem cells to "traditionally isolated" lung cancer cell lines or lung cancer cells within tumors with a focus on new therapeutic targets.

In addition to obtaining cancer stem cells, we should also be able to transfer this expertise to a Penn Cancer Stem Cell Core lab so that it can be extended to other tumor types within the cancer center.

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Expected Research Outcomes and Benefits

Lung cancer is the most common cancer killer. Treatment remains very poor, with only 15% of patients cured (all those by surgery). Chemotherapy can extend survival by only a few months and is not curative. We need new ideas and approaches. Based on studies in leukemia, there is a new idea that only a very small number of very resistant cancer cells (called cancer stem cells) are responsible for keeping cancers going, even after >95% of "regular" cancer cells have been killed by chemotherapy. Investigators think they have identified such stem cells in brain tumors and breast cancer. This has not yet been done for lung cancer. If we can identify lung cancer stem cells, we could analyze them carefully to look for specific ways to attack them. This might give us a way to use existing drugs or design new targeted chemotherapy drugs or activate the immune system to specifically kill those cells.

Summary of Research Completed

We have made substantial progress on our project, although in directions that we had not

anticipated.

Our approach to isolating lung stem cells had focused on taking advantage of the purported resistance of these cells to chemotherapy. As reported last year, by exposing lung cancer cell lines to moderate doses of chemotherapy (esp. doxorubicin), we were able to find marked increases in the message levels (by RT-PCR) of known key stem cell genes, such as Oct4a, Nanog, and Sox 2. Over the last year, we have pursued these findings.

1. Expression of Oct4a was increased in chemotherapy-treated lung cancer cells, but in an unusual cytoplasmic distribution. The increase in Oct-4a mRNA was accompanied by increases in Oct-4a protein as measured by immunoblotting (data not shown) and in immunostaining (Fig. 1) An important observation was that the staining for Oct-4a was cytoplasmic, NOT nuclear, as has been previously described.

Thinking we had an enriched stem cell population, we next conducted microarray studies with control and Doxorubin (dox)-treated A549 cells. There were a large number of changes. Among the most striking were a very high enrichment in cell cycle genes, with most, like Cyclin E or the Ki67 proliferation antigen, being highly downregulated (see Table 1). We also saw marked decreases in many kinesin-like proteins (KIFs) that function in chromosome positioning, centrosome separation and establishing a bipolar spindle during cell mitosis. We also saw a 4-fold increase in the cell cycle inhibitor p21.

We also saw increases in many proteases (like the cathepsins), protease inhibitors (cytstatins), a number of HLA class 1 molecules, insulin growth factor 2 and Insulin growth factor binding protein 6. Molecules that would be expected to decrease in epithelial to mesenchymal transition, like E-cadherin, were not changed, however.

Given these array results, it appeared that these cells were not stem cells, but cells in growth arrest. We confirmed this by conducting cell cycle analysis with propidium iodine staining showing G2 growth arrest (note the large peak in the 4x DNA region- arrow) in A549 Dox-treated cells (Fig. 2).

In reviewing the literature, we found that these findings were highly similar to those described in the seminal description of a "senescence like phenotype after exposure to anti-cancer agents" (Chang, 1999a). It thus seemed clear that these cells were in senescence! We confirmed this idea by showing marked increase in blue β-gal (at pH 6.0) staining (Fig. 3), which is the most widely used marker of senescence. We saw the same results with the L55 lung cancer cell line.

Interim Summary: We had developed conditions that put lung cancer lines, A549 (and a second line that we developed ourselves, L55) into DNA damage-induced senescence. Although this phenomenon has been well described in other cancer lines, we made two novel observations:

1. We had observed marked upregulation of the stem cell marker Oct-4a in a cytoplasmic location (pursued over the past year).
2. We had identified a number of novel genes, never associated with senecence before, including HLA class 1 molecules and the cysteine protease inhibitors cystatin SA and SN.

Cellular senescence (along with apoptosis, autophagy, or mitotic catastrophe) is an increasingly recognized outcome of DNA damage due to radiation or chemotherapy. Although senescent tumor cells do not divide, they do appear to remain viable and metabolically active, and thus influence their surrounding cells by secreting factors that are angiogenic, anti-apoptotic and mitogenic. These paracrine tumor-promoting effects suggest a need to eradicate these chemotherapy- induced senescing cells to improve clinical outcome.

We decided to explore the role of Oct4a as a functionally important molecule in lung cancer senescence. Although not our original goal, we viewed the understanding how premalignant or malignant lung epithelial cells respond to DNA damage of great significance in lung cancer. Understanding this process, may be a key to optimizing effects of radiation and chemotherapy.

2. Role of Oct4a in Senescent Lung Cancer Cells: Since no study has ever reported (to our knowledge) a role for Oct-4a (especially cytoplasmic Oct-4a) in senenscence, we investigated further by knocking down its expression with siRNA. We transfected senescent, doxo-treated A549 cells with either scrambled or Oct-4a siRNA, and showed that the cytoplasmic Oct-4a was markedly reduced in Oct-4a-siRNA transfected cells 3 days after initial transfection (Figure 4A).

Interestingly, the Oct-4a-siRNA-transfected cells looked unhealthy under the microscope compared to cells with untransfected cells or transfected with control siRNA (Fig. 4B). We trypsinized the cells and counted live cells (i.e. trypan blue-negative cells). Using our untreated cells as a denominator, we found that 65.4% of the Oct-4a siRNA-transfected cells were dead compared with only a 5-10% reduction in the scrambled siRNA-transfected cells.

We also found that when we transfected A549 or L55 cells with Oct4a siRNA, and then treated with doxorubicin, the cells with low Oct4 were more easily killed (i.e. 50% more cells died. (Figure 5).

Thus, knockdown of cytoplasmic Oct-4a in senescent cells induces cell death and knockdown of Oct4a makes cells more susceptible to chemotherapy-induced cell death. These data support our hypothesis that cytoplasmic Oct-4a may play an important role in supporting chemotherapy-induced senescence by inhibiting apoptosis.

Finally, we are exploring why this form of Oct4a goes into the cytoplasm. One hypothesis was that there was something in chemotherapy-treated cells that bound Oct4a and kept it in the cytoplasm. To test this, we transfected untreated and doxorubicin-treated A549 cells with a commercially available RFP-tagged Oct4a plasmid. The transfected (red) protein was strictly nuclear in its expression in both types of cells, while the dox- treated cells showed endogenous Oct4a in the cytoplasm and the transfected Oct4a in the nucleus (Fig. 6).

We are currently looking at the molecular identity of this "Oct4a-like" molecule and hypothesize it is an isoform that lacks a nuclear localization signal.

Table 1. Genes changed in Doxorubicin-treated A549 cells

Gene	Fold change	Gene	Fold change	Gene	Fold change
Cyclin B1	-13	**P21**	+4.3	**Cystatin SN**	+58
Cyclin D1	1.6	**KIF4A**	-14.3	**Cystatin SA**	+13
Cyclin E1	-2	**KIF11**	-27.4	**MHC Class 1B**	+13
Cyclin A2	-14	**KIF14**	-26.2	**Cathsepsin B**	+4.1
CDC2	-16.8	**KIF15**	-15.5	**Cathepsin D**	+3.4
CDC6	-14.9	**KIF18A**	-14.8	**IGF2**	+28
CDC45	-11.7	**KIF20B**	-11.1	**E-cadherin**	+1.4
CDK inhib 3	-24	**Ag. identified by mAb Ki67**	-24	**IGFBP6**	+6.7

Figure 1. Immunohistochemical staining of Oct4a in A549 (A) and L55 (B) cells following doxorubicin treatment.

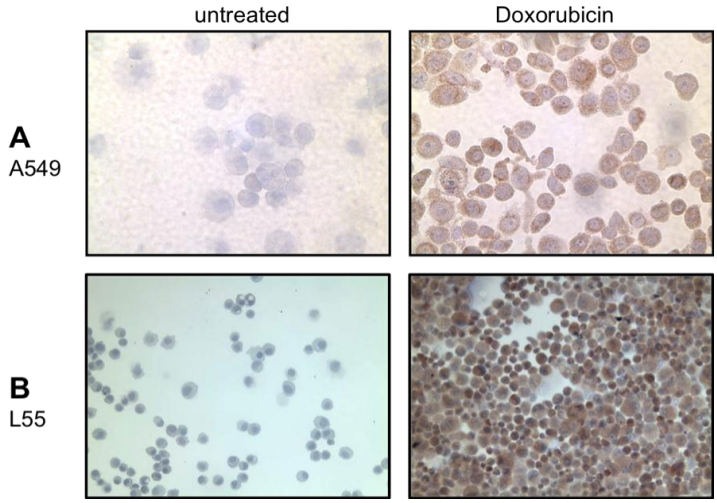


Figure 2. Cell Cycle Analysis

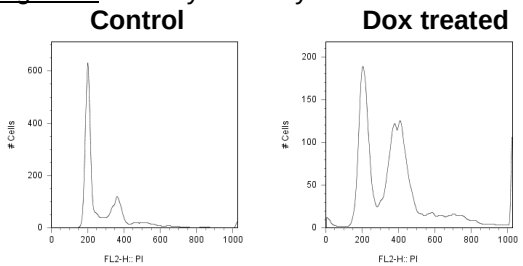


Figure 3. β gal staining of A549 cells

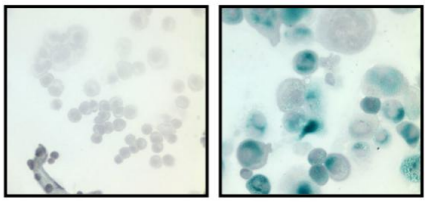


Figure 4. A. Immunostaining for Oct4a was performed on cytospins of cells 3 days after the initial transfection. B. Appearance days after transfection.

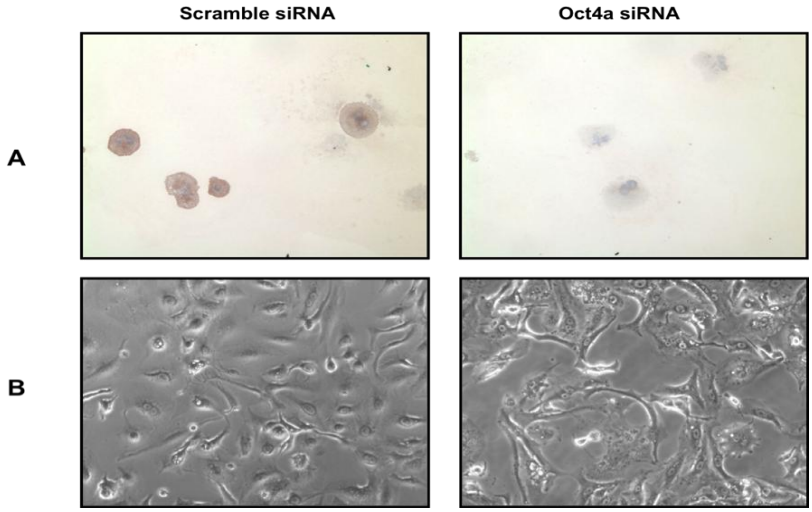


Figure 5. Transfection with Oct4a siRNA before treatment with Doxorubicin increases cell killing of the drug

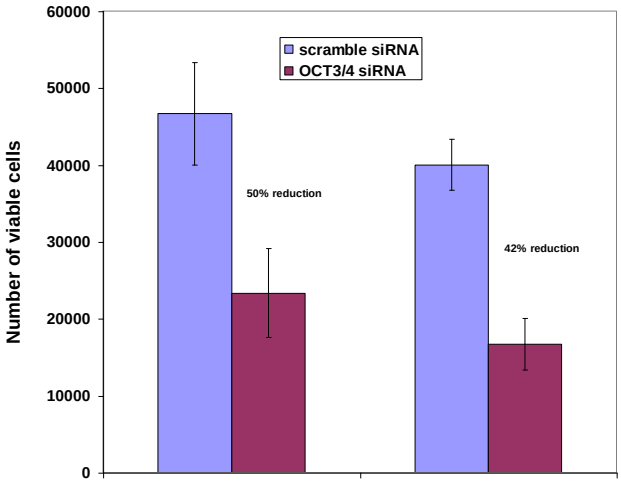
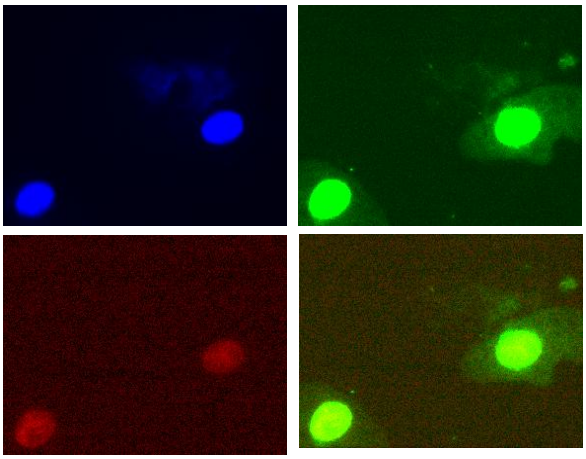


Figure 6. Transfection of Doxo-treated A549 cells with an RFP-tagged Oct4a. upper left- Blue= dapi; upper right- green= staining with an Oct4 monoclonal Ab, lower left- red=RFP. lower right- merge of RFP and anti-Oct4a mAb



Research Project 8: Project Title and Purpose

Phenotyping the PI3 Kinase-AKT Pathway in Human Breast Cancers by Immunohistology - We propose to develop an accurate and convenient method for determining the activity of a critical signaling pathway in breast cancer cells, the phosphatidylinositol-3-kinase (PI3K)-AKT pathway. The PI3K-AKT pathway is activated in many human breast cancers by mutations affecting a number of different genes. These mutations and activation of the PI3K-AKT pathway influence clinical behavior of breast cancers and response to drug treatment. Detection of these mutations currently requires performance of multiple genetic tests, most of which are available only on a research basis. Development of a test based on antibody staining of tumor tissue obtained at the time of initial diagnosis or surgery will allow PI3K-AKT pathway activity to be determined routinely and factored into the care of patients with breast cancer.

Duration of Project

1/1/2008 - 12/31/2009

Project Overview

Histopathology has a central role in cancer diagnosis, classification and management but currently retrieves only a fraction of the information preserved in fixed tumor tissues. With suitable technical and analytical advances, immunohistology should be uniquely able to reveal in vivo dynamic biological events and molecular processes to become a cutting-edge tool for

biomedical research and advancing clinical care. We have developed a prototype computer-assisted image analysis system based on multispectral microscopy that segments nuclei in histologic sections, references multiparameter immunostaining to these nuclei and analyzes multiplex nuclear antigen staining on a nucleus-by-nucleus basis. On this foundation, we are developing an advanced analytical platform that delineates, counts and analyzes whole cells and their nuclear, cytoplasmic and plasma membrane compartments in stained histologic sections. Multiplex immunostaining is referenced to these cells and their major subcellular compartments to enable cytometric and subcytometric multiplex antigen analysis. We refer to this proposed analysis as "multiplex histocytometry" and will develop it into a tool for analyzing traditional and new biomarkers in human breast cancer.

AKT signaling regulates cell growth, proliferation and survival, and activation of the PI3K-AKT pathway is important in breast cancer pathogenesis and influences response to therapy. We will develop an immunohistological approach for assessing activity of this pathway that uses breast cancer samples available for most patients – formalin-fixed, paraffin-embedded (FFPE) tumor from initial biopsy or surgical resection - coupled with quantitative analysis of the immunostaining using the multiplex histocytometry analytical system.

1. Develop multiplex immunostaining for the activated forms of AKT (phosphorylated AKT, p-AKT) and total AKT in cells, along with immunostaining for tumor cell membrane antigens, E-cadherin and HER2/neu. Use histocytometry algorithms and multiplex histocytometry to quantitatively assess AKT activation by determining p-AKT:AKT ratios in the cancer cells of immunostained breast cancer specimens.

2. Assess AKT activation in breast cancers previously characterized as having or not having *PIK3CA* mutations and/or HER2/neu overexpression to determine whether AKT activity determined by multiplex histocytometry reflects the presence of these genetic abnormalities.

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

AKT activation has been shown to promote cell growth; proliferation and survival, and PI3K-AKT pathway activity is important in breast cancer pathogenesis and influences response to therapy. Yet, no quantitative assay of AKT activation or activity of the PI3K-AKT pathway is available for use on clinical samples. Our proposed method for measuring AKT activation (p-

AKT:AKT ratio on a cytometric basis) will be performed on samples that should be available for all breast cancer patients using procedures routinely used in clinical histopathology labs. The results of the AKT activation assay are likely to reflect the presence of certain gene mutations and other genetic alterations in breast cancers, so that the assay becomes an easily performed alternative for several genetic tests. Furthermore, the presence of AKT activation may correlate with breast cancer resistance to certain forms of anti-cancer therapy, so that the assay may be useful for determining appropriate therapy for breast cancer patients. This is an important step towards tailoring therapy to fit the molecular characteristics of patients' cancers. After this assay is successfully developed for determining AKT activation in breast cancers, it should be readily adapted for determining AKT activation in a variety of other human cancers where AKT activation is suspected to play a role in tumor growth and resistance to therapy.

Summary of Research Completed

Aim 1 of this project is to develop robust multiplex immunostaining for the activated forms of AKT (phosphorylated AKT, p-AKT) and total AKT in cells along with immunostaining for tumor cell antigens on the same slide. We have to image these stained slides using a multispectral microscope to capture spectral stain information so that we can spectrally "unmix" the image into individual channels, each representing a component stain in the multiplex combination. Finally, we have to develop the FARSIGHT image analysis software so that it will (i) automatically segment individual nuclei and cells in images, (ii) classify the segmented nuclei/cells into cell types of interest (e.g. breast cancer cells) based on their association with structural/cell-type antigen staining, and (iii) quantify analyte expression on a cellular basis by associating analyte staining with the segmented nuclei/cells. This will allow quantification of p-AKT and AKT analyte antigens in histology images on a tumor cell-by-tumor cell basis, i.e. cytometrically.

Immunostaining for p-AKT showed that this phospho-epitope had to be stained by immunohistochemistry (IHC) using DAB (diaminobenzidine) chromogen. Staining for p-AKT using fluorescent reporters (IF) yielded much fewer positively staining cells and was clearly insufficiently sensitive. Staining for AKT could be accomplished by IHC or IF, but because p-AKT was being stained by IHC, we elected to stain for AKT also by IHC using a different chromogen (SG blue). The antigens (CK, cytokeratin; E-cad, E-cadherin) used for classifying segmented nuclei/cells as breast carcinoma cells and for segmenting whole breast cancer cells are abundant and equally well revealed by IHC or IF, so we immunostained for these using fluorescent reporters. We have put these individual antigen stains together in a robust multiplex immunostaining protocol in which we stain each slide for p-AKT by IHC (DAB), AKT by IHC (SG blue), CK by IF (AlexaFluor 488) and E-cad by IF (AlexaFluor 594). Finally, all slides are counterstained with hematoxylin to reveal nuclei and general tissue integrity and structure. Multispectral microscopy performed in brightfield and fluorescent modes followed by spectral unmixing easily separates staining by the two chromogens, hematoxylin and the two fluorophores into individual channels, even when they are co-localized.

These channels of staining data were entered into FARSIGHT software, which uses the hematoxylin channel to "segment" (delineate) all nuclei in images. It uses immunostaining for cytosolic epithelial cytokeratin (CK) to type segmented nuclei as belonging to breast cancer cells

and immunostaining for plasma membrane antigens (E-cadherin) to segment whole tumor cells. Farsight then associates staining in the p-AKT and AKT analyte channels with the segmented tumor cells and their nuclei to determine which tumor cells express analyte and to quantify expression on a cell by cell basis.

We were unable to make progress with Aim 2 (see below), so developed additional immunostaining protocols and FARSIGHT analytical capabilities for evaluating signaling pathways downstream from AKT that might be relevant for predicting response to mTOR inhibitors. For immunostaining, we developed staining for the activated form of ribosomal protein S6 (phospho-S6), which appears when AKT signaling through mTOR is active and mRNA translation is enhanced. Because of its abundance, p-S6 staining can be performed either by IHC or IF. To enhance the analysis of p-S6 immunostaining, which is cytoplasmic, as well as p-AKT immunostaining, we enhanced FARSIGHT so that it can analyze analyte staining in nuclear and extranuclear (cytosol + plasma membrane) compartments of tumor cells separately. This allowed quantification of p-AKT in the nuclei and cytoplasm of cells, which might be important given that AKT phosphorylates different molecular targets in the two subcellular compartments, and we have noted that breast cancers can have very different nuclear-cytoplasmic distribution of p-AKT staining. Figure 1 demonstrates the ability to separate each cell into nuclear and extranuclear compartments and to analyzing staining in the two compartments separately. The breast tumor was stained with antibodies to p-S6, CK and EMA (epithelial membrane antigen), all by immunofluorescence, and counterstained with hematoxylin. Panels D and E show cell segmentation and classification results with yellow contours outlining the cytoplasmic boundaries of CK+ cells determined using the CK and EMA channels jointly and red contours outlining the nuclear boundaries within tumor cells. The sub-population of CK+ cells that are p-S6+ is in the minority (11%) in this tumor. Visual examination of the p-S6+ cells shows that p-S6 staining, as expected, was predominantly cytoplasmic. This was confirmed by plotting a histogram of the extranuclear to nuclear ratio of p-S6 signal in cells that expressed this antigen (Panel F), which showed that only 10% of p-S6 signal was nuclear. This small amount of "nuclear" p-S6 may be explained by the fact that the image represents a planar projection of a tumor section that is 5µm thick; p-S6 staining in cell cytoplasm situated above or below nuclei in these sections would register as nuclear.

Aim 2 of this project was to use tumors previously characterized as having PIK3CA activating mutations or *PTEN* inactivating mutations (both predicted to result in activation of PI3K-AKT signaling and AKT activation) to determine whether quantification of p-AKT and AKT immunostaining by multiplex histocytometry can predict the presence of these genetic abnormalities. We were unable to procure blocks/slides from genetically characterized human breast cancers in time to perform these validation studies within the funding period of this grant. Instead, we developed additional breast cancer immunostaining and FARSIGHT analytical capabilities to allow analysis of mTOR signaling pathway activity, which might be useful for predicting response to therapy with mTOR inhibitors (see Aim 1).

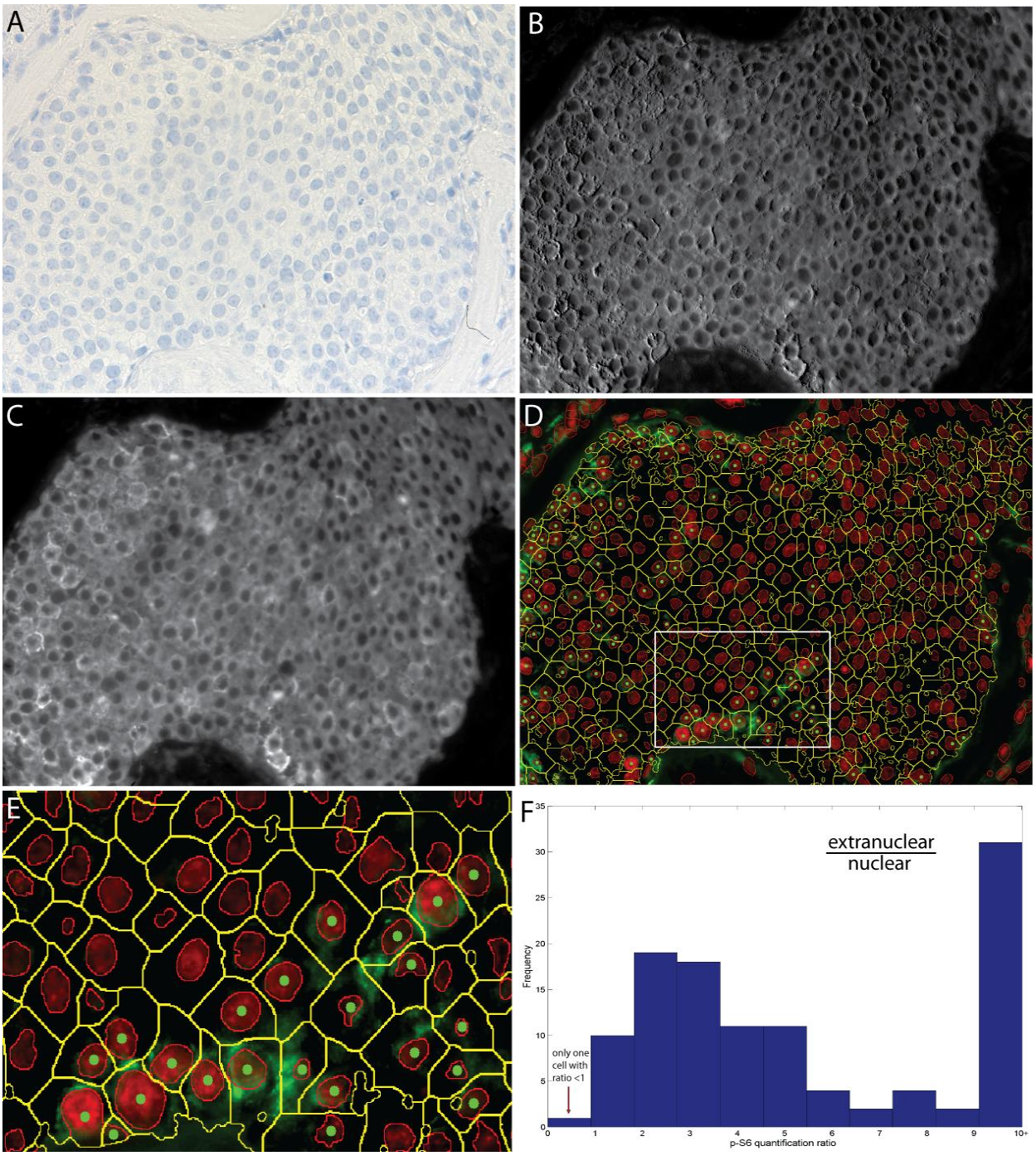

Figure 1: *Analysis of phospho-S6 immunostaining in a human breast cancer.* A section of a breast tumor was stained with anti-p-S6 (AlexaFluor-488), anti-EMA (AlexaFluor-594) and anti-CK (AlexaFluor-555), followed by hematoxylin staining, multispectral imaging (400X) and cytometric analysis. The brightfield image is shown (A) along with unmixed channels for Alexa-555 (CK) (B) and Alexa-594 (EMA) (C). Composite images of p-S6 analyte staining (green fluorescence) along with segmented whole tumor cells are shown (D; E shows an enlargement of the boxed area in D). In each cell, analyte in the nuclear and extranuclear compartment was quantified. Ratios of extranuclear to nuclear analyte were calculated for each positive tumor cell and their distribution is shown (F).

Research Project 9: Project Title and Purpose

Neural Substrates of Varenicline (Chantix®) Efficacy for Smoking Cessation - This project aims to identify the neural mechanisms that underlie varenicline effects on early nicotine abstinence symptoms, specifically, smoking urges and deficits in emotional and cognitive (working memory) processing.

Anticipated Duration of Project

7/1/2008 – 8/31/2010

Project Overview

The study is a within-subject double-blind cross-over neuroimaging investigation of the effects of varenicline (vs. placebo) on early abstinence symptoms. One-hundred treatment-seeking smokers will be scanned on 3 occasions: (1) pretreatment/smoking as usual, (2) after 13 days of varenicline (2mg/day) and 3 days of monitored abstinence, and (3) after 13 days of placebo and 3 days of monitored abstinence (order of varenicline vs. placebo phases counterbalanced). As in our prior research, to simulate the clinical situation, participants will be offered self-help smoking cessation materials and brief counseling; abstinence will be confirmed daily using carbon monoxide assessment. Imaging sessions (at baseline, and on day 13 of each medication phase) will include a resting ASL-perfusion scan (with smoking urge assessments), and blood oxygen level dependent (BOLD) fMRI assessments during performance of validated tasks probing working memory (Fractal N-back Task) and emotional processing (Facial Emotions for Brain Activation Task). The primary outcomes are medication effects (varenicline vs. smoking) on resting and task-related regional brain activation in hypothesized regions of interest. Secondary outcomes include subjective symptoms and behavioral performance on scanning days and during the 3 day abstinence periods. In addition, after the day 13 scan in each medication phase, participants will have a double-blind exposure to a nicotine and denicotinized cigarette and subjective hedonic effects will be assessed. Following the primary investigation, all participants will be enrolled into a 13-week open-label varenicline trial, since we have an obligation to provide therapy to these treatment seeking smokers. The target quit date will occur following one week of treatment, and smoking status will be assessed and biochemically confirmed over the 12 week treatment period. This open-label phase of the study will afford an opportunity for exploratory analyses of the predictive clinical value of imaging data.

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Other Participating Researchers

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Expected Research Outcomes and Benefits

Varenicline is the newest, FDA-approved medication for the treatment of smoking cessation. Although it is the most effective treatment to date, scientists are still trying to identify the neurobiological mechanisms through which it works. By better characterizing these mechanisms, we hope to (1) identify people that will respond best to this pharmacotherapy and are able to successfully quit smoking as well (2) potentially identifying novel targets for the development of new medications for smoking cessation.

Summary of Research Completed

During the prior project period we completed the primary analysis of the neural substrates of varenicline effects on working memory-related brain activation, as detailed in the prior report. This year, we completed the manuscript describing the results of the study (Effects of the alpha4beta2 partial agonist varenicline on brain activity and working memory in abstinent smokers. Loughead J, Ray R, Wileyto EP, Ruparel K, Sanborn P, Siegel S, Gur RC, Lerman C. Biol Psychiatry. 2010 Apr 15;67(8):715-21).

During the current project period, we also completed the analysis of medication effects on brain activity and performance during the emotion identification task. Short-term treatment with the α4β2 nAChR partial agonist varenicline versus placebo significantly altered BOLD signal change in the right middle temporal gyrus (MTG) during performance of a face emotion processing task in abstinent smokers. Differential effects were identified for two clusters within the MTG (BA21). In one cluster (coordinates 61, -43, 1), varenicline increased task-related activity with no significant change during placebo; however, in the other cluster (coordinates 5, -36, -1), placebo treatment was associated with deactivation with no change during varenicline treatment. Because the MTG was not identified in a whole brain analysis of varenicline effects during performance of the N-back task (Loughead et al., 2010), these effects appear to be specific to face emotional processing. Varenicline also improved correct response time on the task overall; however, correct response time did not differ by emotion type and was not associated with BOLD signal change. Thus, task performance effects may be attributable to beneficial effects of varenicline on sustained attention rather than an enhancement of emotional processing.

Effects of varenicline on MTG activity during face emotional processing are biologically plausible and consistent with prior research. A meta-analysis of fMRI emotional face paradigms indicates robust activation of MTG. Autoradiography studies show that the temporal cortex has a high concentration of α4β2 nAChRs, the molecular target of varenicline. Differential distribution of nAChRs in different regions within the MTG might contribute to the different drug effects observed in the two MTG clusters.

Our hypothesis that varenicline would reduce task-related amygdala activity was not supported. Prior studies indicate that amygdala activity during emotional processing is attenuated by anti-depressant treatment. The absence of such effects in the current study may be attributable to relatively weaker effects of varenicline on mood symptoms. Thus, varenicline effects on emotional processing may be more subtle than could be detected with this task and sample size. Together with the analysis of working memory, these results suggest that attenuation of cognitive deficits by varenicline may be a more important neural mechanism of efficacy than are effects on emotional processing. Future studies should evaluate varenicline effects on brain activity associated with smoking cue reactivity and stress response.

Research Project 10: Project Title and Purpose

Combination Immunotherapy Targeting K-ras in Adenocarcinoma of the Pancreas - Pancreatic cancer is the fourth leading cause of cancer mortality in both men and women. Despite a deeper understanding of cause of pancreatic cancer, extensive clinical research has yielded only modest improvements in the outcome of this devastating malignancy. Pancreatic cancer cells are naturally resistant to current chemotherapy and radiation therapy. Based on recent evidence that vaccines have promise in patients with surgically incurable pancreatic cancer, this research will test a new therapy involving a customized patient-specific vaccine in combination with chemotherapy, radiation therapy and when possible, surgery.

Anticipated Duration of Project

1/1/2008 - 12/31/2011

Project Overview

The broad objectives of this research are to 1) assemble the infrastructure and expertise required to evaluate new therapies such as biologic therapies and targeted therapies for advanced pancreatic cancer, and 2) conduct a pilot test of a novel tumor specific K-ras vaccine in combination with chemotherapy and radiation therapy to determine if lymphocytes can kill tumor cells, and whether this impacts on K-ras expression and signaling in the tumor microenvironment.

The three specific aims are (1) Assemble a multidisciplinary team of scientists and physicians to conduct novel trials of biologics and targeted therapeutics for pancreatic cancer; (2) Conduct a pilot study of safety and feasibility of an inactivated recombinant *Saccharomyces cerevisiae* vaccine expressing mutant K-ras protein combined with adoptive T cell transfer and chemoradiotherapy in locally advanced pancreatic cancer and (3) To analyze the specimens from the pilot study to determine the effects on host immune reconstitution, ras tumor immunity and the tumor microenvironment.

The research design and methods are designed to test the hypothesis that combining a patient and tumor specific *Saccharomyces cerevisiae* based K-ras vaccination with adoptive transfer of ex vivo-activated and vaccine-primed autologous T cells will overcome immune tolerance induced by pancreatic cancer, thereby restoring immune surveillance, as demonstrated by induction of T

cell infiltrates in the tumor microenvironment and decreased expression of K-ras in resected tumor specimens. A pilot study to test the susceptibility of pancreatic tumor cells to killing by T cells to establish the feasibility of this approach will be carried out at the Abramson Cancer Center of the University of Pennsylvania.

Principal Investigator

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Expected Research Outcomes and Benefits

This translational project will determine whether a potent cancer vaccine that is customized to the patient's tumor generates a tumor immune response in patients with locally advanced pancreatic cancer. In addition, the study will determine if it is safe and feasible to integrate the cancer vaccine into the standard chemotherapy and radiation therapy that is currently given to patients with pancreatic cancer. This approach is a form of 'personalized medicine' that in theory should have less toxicity, while providing more specific benefit than currently available treatments for this presently incurable form of cancer.

In addition to determining the safety and feasibility of this new approach, the research will assemble a team of experienced physicians, surgeons and basic scientists that will be available to carry out translational research on other new biologic and targeted therapies that are being developed by bench scientists engaged in pancreatic cancer research in the field. Related to the above, this project will serve as a training vehicle for junior physician scientists who are interested in careers of translational medicine in the area of pancreatic cancer, which remains as a significant unmet medical need. Finally, research in pancreatic cancer is under funded by the NIH relative to other cancer sites. Thus, there are a range of scientific and society health benefits that can be expected from the successful completion of this study, from new insights into cancer biology to the possibility of new drugs being developed as a result of this project.

Summary of Research Completed

Since pancreatic cancer is known to be resistant to both radiation and chemotherapy, the goal of this project is to evaluate the resistance of pancreatic cancer to immunotherapy. Specifically, we are examining the resistance of pancreatic tumors to cytotoxic T lymphocyte (CTL)-mediated killing. To circumvent issues of T cell recognition of tumor by T cells due to tolerance, leading

to restricted T cell receptor recognition of tumor cells or to impaired HLA expression by tumor cells, we have generated redirected CTLs that are potent and specific killers of tumors expressing the tumor antigen mesothelin. Mesothelin is a cell-surface protein expressed on virtually all pancreatic cancers, as well as a variety of other tumors. We have generated mesothelin-specific redirected CTLs by introducing mesothelin-specific chimeric immunoreceptors (CIRs) into primary human $CD8^+$ T cells. CIRs consist of an extracellular domain specific for the cell surface target of interest fused to intracellular T cell activation domains. A major advantage of this approach is that it does not require HLA expression by the tumor cells, and therefore permits an assessment of tumor cell susceptibility to killing independent of restricted T cell receptor repertoires or limited HLA expression by the tumor cells. The mesothelin-specific CIR we have generated is termed SS1.BBz. The extracellular domain, SS1, is a mesothelin-specific antibody single chain variable fragment (scFv) fused to the intracellular T cell activation domain from CD137 (4-1BB) in tandem with the T Cell Receptor zeta (TCR-ζ) signal transduction domain (see Figure 1). Introduction of SS1.BBz into primary human $CD8^+$ T cells by lentiviral vector-mediated transduction results in a potent, MHC-independent, mesothelin-specific "serial killer" of mesothelin-expressing tumor cells. It should be noted that we have also generated CIR-expressing CTLs by transient transfection with CIR-encoding RNA. RNA transfection results in high level but transient (36-72 hour) CIR expression. From a clinical perspective, transient CIR expression may be preferred in the event that infused CIR-expressing T cells exert deleterious effects. We have demonstrated that SS1.BBz-expressing T cells, prepared by either lentiviral vector-mediated transduction or transient RNA transfection, efficiently eradicate mesothelin-positive mesotheliomas, both in vitro and in vivo, in a mouse tumor xenograft model. These cells effectively eradicate large tumors in vivo at low effector: target ratios, and often at ratios considerably less than 1:1.

We initially screened a number of pancreatic cell lines for mesothelin expression by flow cytometry. Surface mesothelin levels were variable, ranging from high to undetectable (Figure 2). This data indicates that most pancreatic cell lines and primary pancreatic tumors (not shown) express mesothelin, and thus, in principle, can be targetd by SS1.BBz. Also shown in Figure 2 is mesothelin expression on L55, a lung adenocarcinoma cell line generated and characterized in our laboratory. L55 expresses high levels of mesothelin, and is readily killed by SS1.BBz-expressing T cells. Next, we evaluated the ability of SS1.BBZ-expressing CTLs to kill pancreatic cancer lines, using the traditional ^{51}Cr release assay, an example of which is shown in Figure 3. SS1.BBz-expressing T cells readily killed the mesothelin-positive lung cancer line L55, but they failed to kill the mesothelin-positive pancreatic cancer cell line ASPC-1. This suggested to us that some pancreatic cancer cell lines may be resistant to CTL killing.

From our perspective, however, the ^{51}Cr release assay has multiple drawbacks. One of the principle drawbacks is the inability to identify and recover killing-resistant cells for subsequent analysis. Therefore, we devised a flow cytometry-based killing assay that would allow us to identify and recover, by cell sorting, CTL-resistant tumor cells. This assay incorporates a live/dead discriminator dye, T cell-specific antibodies, tumor-specific antibodies anti-EpCAM, also known as anti-CD326, and calibrated beads. The beads provide an internal control to enable precise quantitation of surviving tumor cells.

After validating our flow cytometry-based assay, we evaluated the ability of SS1.BBZ-

expressing CTLs to kill a panel of pancreatic cancer lines. We used RNA transfection to generate CIR-expressing CTLs, and we extended the length of the assay to 18 hours, based on pilot kinetic experiments performed in the laboratory. A representative experiment is shown in Figure 4. The pancreatic tumor line used in this case, BxPC-3, appeared partially resistant to killing by SS1.BBz-expressing CTLs, as approximately 30% of the tumor cells were killed by tbe T cells at a 1:1 E:T ratio. This is shown in Figure 5, where even as the E:T ratio is increased, some of the cells remain resistant to killing.

We have evaluated the sensitivity of our panel of pancreatic cancer cell lines to CIR-mediated CTL killing (Figure 5). BxP3 and Panc are the most sensitive lines to CTL killing, however, none of the pancreatic lines tested were highly sensitive to SS1.BBz-mediated killing. Currently, we are establishing conditions by which, by exerting continuous selective pressure, we will generate completely resistant sub-lines from partially resistant parental cultures. Comparison of gene expression patterns between derived resistant and parental lines will identify gene products involved in induction of resistance to CTL killing. However, our first assay was complicated by non-specific target killing; we use CD19 CIRs as controls for the specificity of the killing. Therefore, we are adjusting several of the important variables, such as E:T ratio and time of coculture, in order to minimize non-specific killing events.

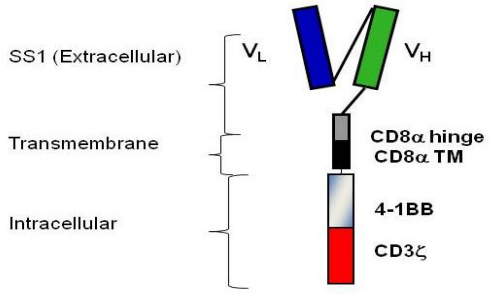

Figure 1. Structure of the CIR SS1.BBz. The extracellular portion consists of a mesothelin-specific scFv, while the intracellular portion contains the 4-1BB (CD137) activation domain fused to the TCRζ signal transduction domain.

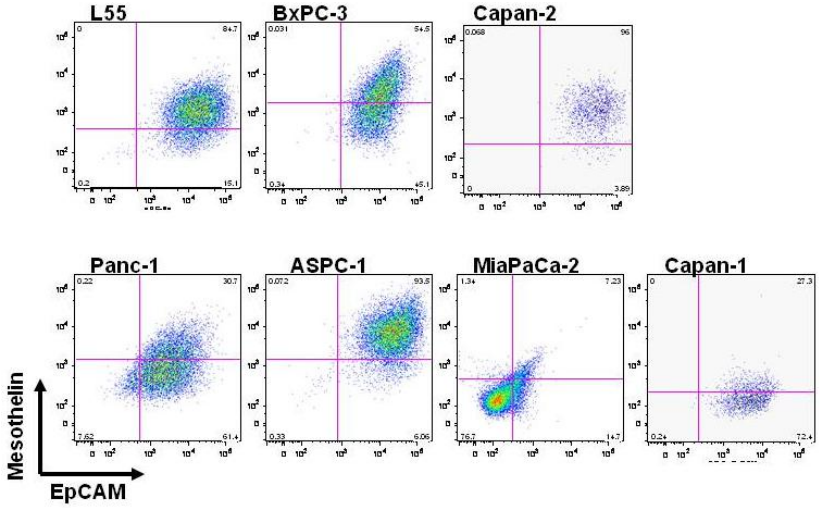

Figure 2. Mesothelin Expression in Pancreatic Cell Lines. Pancreatic cancer cell lines (as well as the control lung cancer cell line L55) were stained with antibodies specific for mesothelin and EpCam, a surface molecule that is also widely expressed on carcinomas. The cells were then analyzed by flow cytometry.

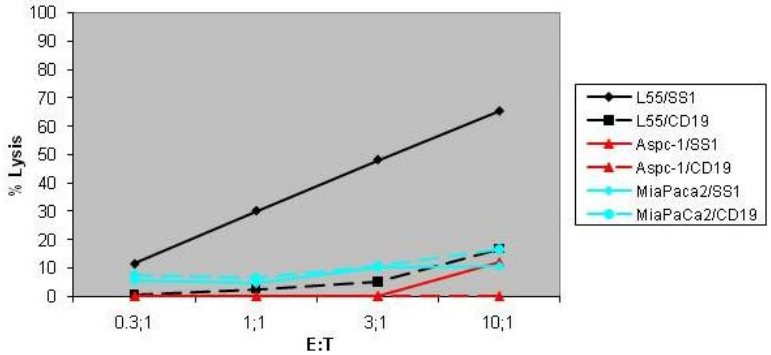

Figure 3. Specific Lysis of Mesothelin-Expressing Tumor Lines. The indicated tumor lines were loaded with ^{51}Cr and cocultured at the indicated Effector: Target (E:T) ratios with SS1-BBz-expressing CTLs or CD19-BBz (a CIR recognizing CD19, a surface antigen absent from these cells)-expressing CTLs. Released ^{51}Cr was measured and used to calculate specific lysis. L55 is a mesothelin-positive lung cancer line included as a positive control. ASPC-1 is a mesothelin-positive pancreatic cancer cell line. MiaPaCa-2 is a mesothelin-negative pancreatic cancer cell line, included as a negative control.

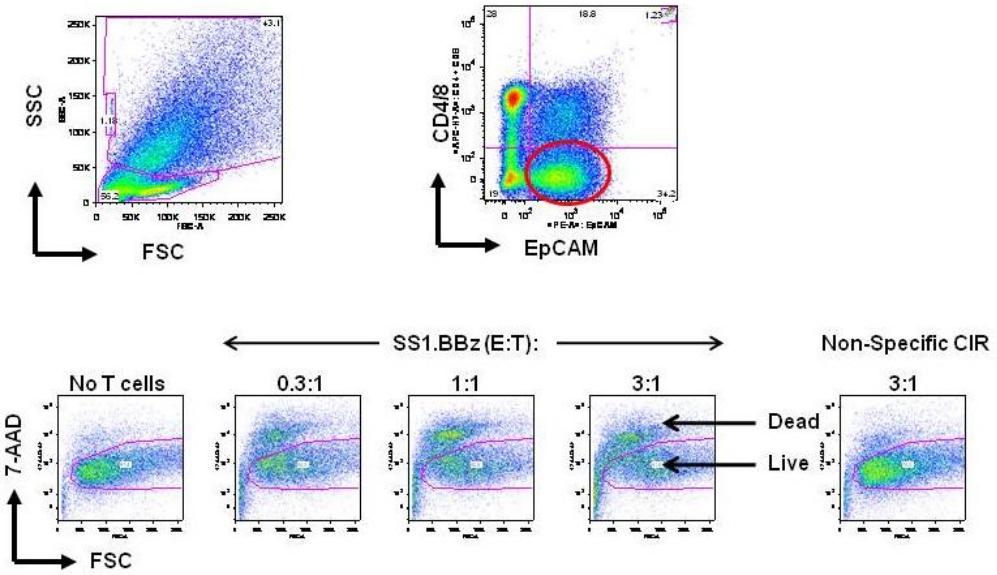

Figure 4. Flow Cytometric Based Killing Assay. Pancreatic tumors (in this case, BxPc3) were incubated with different Effector: Target (E:T) ratios of SS1.BBz-expressing T cells, or T cells expressing a non-specific CIR. After 18 hours, the cells were stained with antibodies to CD4, CD8, and EpCam. They were then stained with the Live/Dead dye 7-AAD. The EpCam positive population (circled in red) was analyzed for 7-AAD uptake. Arrows are used to designate the live and dead cell populations.

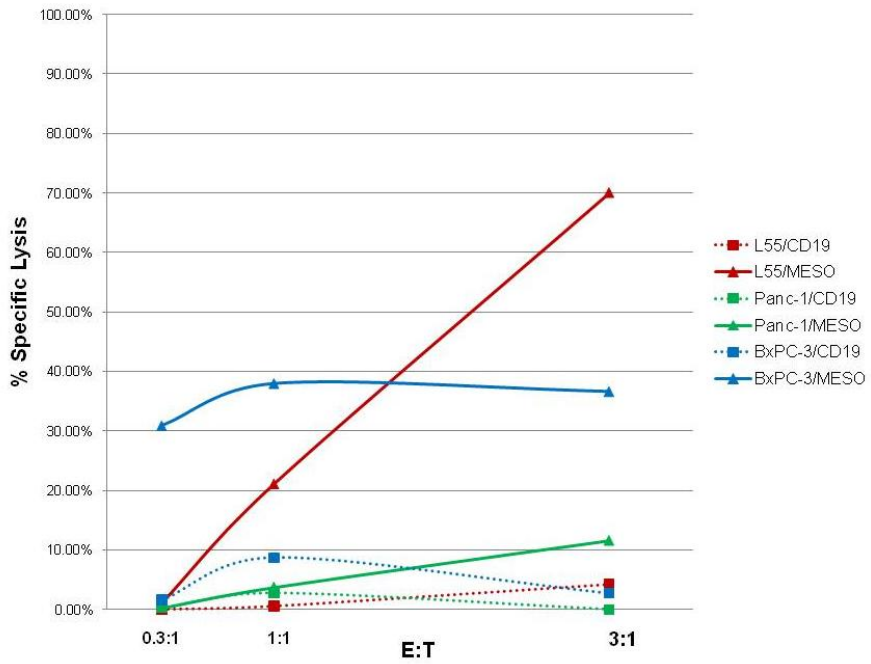

Figure 5. Partial Resistance of Pancreatic Tumor Lines to Killing by SS1.BBz CIR-expressing T cells. A flow-based killing assay (described in Figure 4) was set up, and the percent killing of different tumor lines at different E:T ratios was plotted. Solid lines designate tumor cells cocultured with SS1.BBz-expressing T cells; dotted lines designate tumor cells incubated with non-specific CIRs. Panc-1 (green line) was almost completely resistant to killing; the positive control L55 (red) was highly sensitive to killing, while the pancreatic cancer line BxPC-3 (blue) was partially resistant to SS1.BBz-mediated killing.

Research Project 11: Project Title and Purpose

WISER Sister Pilot Study - The WISER Sister pilot study will recruit 10 women aged 18-35 with elevated breast cancer risk to build to 60 minutes of daily exercise over 5 months and assess the effects of this intervention on factors that affect or reflect breast mitotic activity (including estrogens, MRI breast imaging, other sex steroid hormones, adipokines, and body composition). An ongoing parallel trial with 320 low risk women (the WISER study) will allow for novel comparisons of levels of commonly accepted risk factors for breast cancer.

Duration of Project

1/1/2008 – 5/28/2010

Project Overview

Not all women at elevated risk develop breast cancer and it is acknowledged that there are non-genetic, environmental aspects to neoplastic development among those with elevated risk. The strongest basis for breast cancer prevention among women with elevated risk is through hormonal intervention. However, both surgical and drug based prevention have serious negative long term consequences that may not be acceptable to many women. We propose a preventive

exercise intervention for women with increased risk that might address the same hormonal issues, yet have fewer serious side effects. These women are highly motivated to reduce their risk. Therefore, even if the alteration in risk is marginal (e.g., consistent with smaller tumor size, delayed onset of breast cancer, or reduced breast density that would improve the success of early screening), women at increased risk deserve to know what they can reasonably expect from regular exercise with regard to reducing breast cancer risk. The goal of the proposed pilot study is to establish the effects of 5 months of exercise on variables known to be associated with breast mitotic activity, among a cohort of 10 women with a $\geq$ 18% lifetime risk for breast cancer. The proposed study extends an ongoing study in 320 lower risk women. Combined, these studies will enable novel comparisons of risk factors across low versus higher risk women.

The primary aim is to conduct a pilot study of the effects of exercise over 5 menstrual cycles, with no concomitant dietary changes. The major outcome will be overall estrogen exposure, measured by the area under the curve for estrogen levels (estrone-1-glucoronide, E1G) in daily first morning urine samples across two menstrual cycles. The target group for the study will be premenopausal women (ages 18-35) at elevated risk for breast cancer. It is hypothesized that exercise training will result in a decrease in estrogen exposure.

The secondary aims are to assess changes in other physiologic parameters associated with breast mitotic activity, including: urinary progesterone (PdG); breast density from Magnetic Resonance Imaging; estrogen metabolites (2-$OHE_{1,2}$, 4-$OHE_{1,2}$ 16-OHE_1 & ratios); adipokines (adiponectin and leptin); and body composition (% body fat, lean mass, fat mass, total mass). It is hypothesized that exercise will alter each of these outcomes in a manner consistent with reduction of breast cancer risk.

We also plan to compare levels of urinary estrogens, estrogen metabolites, circulating hormones, adipokines, and body size across two groups of women who differ as to breast cancer risk.

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Nancy Williams, PhD - employed by Penn State University
Mindy Kurzer, PhD – employed by University of Minnesota

Expected Research Outcomes and Benefits

Women at increased risk for breast cancer currently have limited options for reducing their risk. Prophalyactic mastectomy, oophrectomy, or hormonal therapy drugs are the medically proven approaches to substantively reduce risk among BRCA1 or BRCA2 mutation carriers. Most young women of childbearing potential do not choose these options, but would like to know whether there is anything they can do to meaningfully reduce their risk. Exercise has been shown to reduce breast cancer risk in large population based studies. However, the mechanisms for this effect are unclear and the dose of exercise that would be required to substantively reduce risk is unclear. The proposed WISER Sister pilot study will assess the effects of an exercise intervention on risk factors among women with significantly elevated risk. In addition, there is a similar ongoing exercise study in low risk women, though the amount of exercise in that study is about half of what is proposed in this project. Baseline levels of breast cancer risk factors can be compared across these two studies. The results of this pilot study will be used to support a resubmission of a larger randomized controlled trial to assess the dose response effects of exercise on women at increased risk for breast cancer. The pilot work proposed will allow each of these issues to be addressed and will lead to a program of research to assess the effects of exercise interventions on breast cancer risk reduction among women whose risk is elevated due to genetic mutations and/or family history. The risk factors to be measured include overall estrogen and progesterone exposure, estrogen metabolism, breast density from magnetic resonance imaging, circulating hormone levels, markers of metabolic activity called adipokines, and body weight and fat.

Summary of Research Completed

During the last reporting period, research activities were limited to shipping urine samples to Penn State, running assays to measure urine levels of hormones, data analysis, and writing a draft of the final paper. All of these activities are now complete.

Urine samples were stored in a -80°C freezer prior to shipping to Pennsylvania State University, then stored at -20°C until analysis. Microtiter plate competitive enzyme immunoassays (EIA) were used to measure the urinary metabolites of estrogen (estrone-1-glucuronide, E1G) and progesterone (pregnanediol-3-glucuronide, PdG). The secretion of these urinary metabolites parallels serum concentrations of the parent hormones; daily urine collection is more practical than daily blood draws and favored over salivary samples because it is easier with urine samples to collect sufficient biospecimens and adjust for hydration status. Each assay uses a polyclonal capture antibody from Coralie Monroe at the University of California (R522-2 for E1G, R12904 for PdG). The competitors for each assay are each urinary metabolite conjugated to horseradish peroxidase (E1G-HRP, PdG-HRP). An endpoint substrate color reaction is developed with azino-bis-ethylbenzthiazoline sulfonic acid and peroxidase. E1G and PdG standards from Sigma are used for the standard curve, and high and low internal controls are in-house samples. The inter-assay coefficients of variation for high and low internal controls, respectively, are 14.7% and 13.1% for E1G and 15.6% and 12.9% for PdG. All urine samples were corrected for specific gravity using a hand refractometer (NSG Precision Cells, Inc., Farmingdale, NY) to account for hydration status. The average values across the 2 menstrual cycles at each measurement (Pre and Post) were used in the analysis. The data were quantified using the area

under the curve (AUC) method (trapezoidal rule) and by comparing average levels over 2 cycles before the start of the exercise intervention and the last 2 cycles of the 5-cycle exercise intervention.

The exercise intervention resulted in no changes in menstrual cycle length or luteal phase length, but did significantly reduce estrogen AUC and progesterone AUC, as noted in table 1 below. These changes may be relevant to reduction of breast cancer risk.

Table 1. Menstrual and hormonal changes

	Average Pre *(n = 7)*	Average Post *(n = 7)*	*P*-value
Menstrual cycle length (days)	28.9	29.4	$P = 0.51$
Luteal phase length (days)	11.3	11.1	$P = 0.83$
Estrogen AUC (E1G ng/mL)	1120.1	908.2	$P = .033$
Progesterone AUC (PdG ng/mL)	88.2	67.3	$P = .049$

Pre, Cycles 1 and 2; Post, Cycles 6 and 7; AUC, area under the curve; E1G, estrone-1-glucuronide; PdG, pregnanediol-3-glucuronide

Research Project 12: Project Title and Purpose

Stem Cell Niche and Epithelial Tumorigenesis - Stem cells rely on the surrounding microenviroment for their extensive proliferation and pluripotency. Recent evidence suggests that these distinct properties of stem cells are not autonomously achieved but are regulated by a level of external control. Transcription factor p63, a homolog of tumor suppressor p53, has been shown to serve as a master determinant of epithelial stemness in an epithelial-cell autonomous manner. However, the microenvironment controlling these stem cells has not been identified. In this project, factors provided by stem cell microenvironment will be explored. In addition, these factors will be analyzed in the context of epithelial tumorigenesis.

Anticipated Duration of Project

1/1/2008 - 12/31/2010

Project Overview

In general, stem cells require specialized culture conditions such as growth factors and a feeder layer for their long-term proliferation. Mouse embryonic stem (ES) cells, for instance, require a layer of mouse embryonic fibroblasts (MEFs) as well as lymphocyte inhibitory factor (LIF). Likewise, human ES cells also require fibroblasts and certain growth factors. Although researchers do not fully understand the importance of fibroblasts in these systems, these cells are absolutely required to maintain the integrity of stem cells. Therefore, it is important to identify

the factors provided by these fibroblasts to better understand the microenvironment surrounding the stem cells. Recently, researchers found that insulin-like growth factor (IGF)-II is expressed from the surrounding fibroblasts and alone is sufficient to maintain the pluripotency of human ES cells. This demonstration strongly argues that distinctive properties of stem cells are not autonomously achieved but are maintained by the factors produced by the stem cell environment.

Similar to ES cells, epithelial stem cells can also be cultured on specialized fibroblasts called 3T3-J2. These 3T3-J2 cells were established from normal MEFs based on their natural ability to support long term proliferation of epithelial stem cells. In this project, we will identify the factors provided by these 3T3-J2 cells to maintain the integrity of epithelial stem cells by using microarray and proteomics approaches. It is widely believed that abrogation of stem cell's environment might trigger tumorigenesis due to overproduction of cells with high proliferative potential. This project will also demonstrate this point by overexpressing the identified factors in fibroblasts to see whether uncontrolled epithelial stem cells can generate cancers. In addition these engineered mice will be crossed with well-characterized epithelial tumor mouse models to see if tumor grade and onset of tumorigenesis will be altered. Overall, this project is anticipated to bring important information about the stem cell microenvironment and their contribution to epithelial cancers.

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Expected Research Outcomes and Benefits

Recent advances in stem cell research suggest that distinctive properties of stem cells are not autonomously achieved but are maintained by the stem cell environment called "stem cell niche". In the case of epithelial stem cells, the master regulator of stemness is a transcription factor p63, a homolog of the tumor suppressor p53. However, essential factors provided by epithelial stem cell microenvironment are not fully investigated. Thus, this project will lead us to better understand the cellular society of stem cell biology.

Stem cells are tightly controlled for their number, differentiation status and proliferation. Abrogation of these controls might cause cancers because stem cells have extraordinary

proliferative potential. This project will also address this point by overexpressing the stem cell environment factors in mice in a tissue-specific manner in fibroblasts. These mouse models will be unique tools to study the tumorigenesis with altered stem cell microenvironment.

In all, this project will bring significant information about the regulation of epithelial stem cell niche and its contribution to cancers.

Summary of Research Completed

Recent evidence in embryonic stem cell and hematopoietic stem cell fields suggests that properties of stem cells are not autonomously achieved but are regulated by a level of external control by their microenvironment called the "stem cell niche." However, the factors produced by the epithelial stem cell niche that maintain the properties of epithelial stem cells have not been identified. In this project, we hypothesize that epithelial stem cells are controlled by extrinsic factors produced by their unique stem cell niche.

In order to analyze the epithelial stem cell niche, we used 3T3-J2 fibroblasts as epithelial stem cell microenvironment. These cells have been extensively used in epithelial stem cell culture and for therapeutic transplantation. 3T3-J2 cells are fibroblasts that were originally derived from mouse embryonic fibroblasts (MEFs) but these two types of fibroblasts are distinct in their potential to maintain epithelial stem cells (Figure 1). For instance, while MEFs do not support epithelial stem cell growth, 3T3-J2 cells can maintain epithelial stem cells indefinitely and epithelial cells grown on 3T3-cells express high levels of p63, a transcription factor essential for epithelial stem cells. Indeed, epithelial stem cells grown on MEFs show signs of terminal differentiation within one week, but growing epithelial cells on 3T3-J2 cells maintain their immaturity as evidenced by cellular morphology and high levels of p63 expression over multiple passages (Figure 2).

By comparing 3T3-J2 and MEFs by DNA microarray analysis, we identified approximately 500 genes that were more highly expressed in 3T3-J2 cells than MEFs. Among these 500 genes, we are primarily focusing on 10 growth factors that appeared to be significantly more highly expressed in 3T3-J2 cells than MEFs. We hypothesize that these growth factors can directly control the biology of epithelial stem cells. Thus far, we have evaluated the loss-of-function of these 10 selected growth factor genes by shRNA-mediated gene silencing in 3T3-J2 cells. The role of these growth factors in stem cell biology is unknown. Currently, we are investigating how these growth factors function in the maintenance of epithelial stem cells in terms of proliferation, differentiation and stem cell self-renewal.

We also aim to identify the "modifiers" of p63 in epithelial cells at a protein level. We hypothesize that the stability of p63 is crucial for the maintenance of epithelial stem cells because knock-down of p63 by shRNA leads to epithelial cell differentiation in vitro, and loss of p63 expression is highly associated with epithelial cell differentiation in vivo. To identify the p63 modifier gene(s), we performed two separate experiments: (1) identification of direct binding partners of p63 in epithelial cells by mass-spectrometry and (2) identification of genes that show inverse expression profile compared to p63 during epithelial cell differentiation. We

are primarily focusing on ubiquitin ligase and protein kinase genes from our lists as ubiquitination and phosphorylation are the most prominent modifiers of the protein stability.

We reported results from our mass-spectrometry analysis last year and we will now describe the results from the second half of this aim, namely showing which genes are expressed in a manner that is the reverse of p63 expression during keratinocyte differentiation process. First, RNAs were isolated from immature and differentiated keratinocytes using the TRIzol reagent (Invitrogen), and two micrograms of total RNA were amplified using the Affymetrix Small Sample Target Labeling Protocol (Affymetrix Inc.). Biotin-labeled, amplified RNAs were fragmented and hybridized on the Affymetrix Mouse Genome Array at the University of Pennsylvania Core Facility. Data were GC-RMA normalized and genes differentially expressed were identified with GeneSpring software (Silicon Genetics, Agilent Technologies).

In this DNA microarray analysis, we identified approximately 10 kinase genes and 5 ubiquitin-related genes whose expression was significantly upregulated in differentiated keratinocytes as compared to the immature compartment. Among these 15 selected genes that show inverse expression to p63 during keratinocyte differentiation, protein products from 5 genes were also identified as p63 binding partners by mass-spectrometry. These results suggest that gene products in our lists which are both upregulated in differentiated keratinocytes and binding to p63, directly control epithelial stem cells and their differentiation processes by altering the stability of p63. Currently, we are investigating how these gene products contribute to the regulation of self-renewal, proliferation and differentiation of epithelial stem cells. These three features of epithelial stem cells need to be thoroughly understood to better understand the control of epithelial stem cells by their niche in the progression of tumorigenesis and for therapeutic purposes.

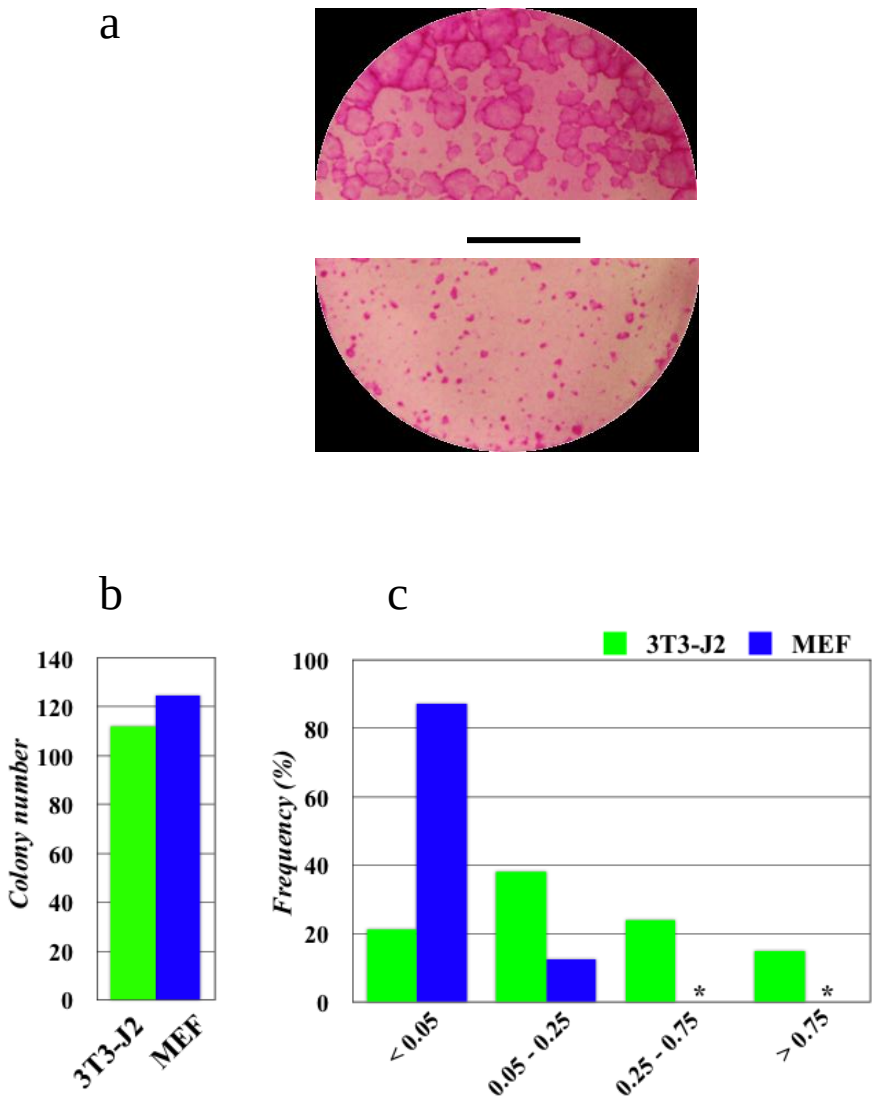

Figure 1. Clonogenic growth of epithelial cells on 3T3-J2 cells and MEFs.
(a) Human primary epidermal keratinocytes were grown on 3T3-J2 cells (top) and MEFs (bottom) for 10 days, followed by Rhodamine-B staining. Scale bar = 1cm.
(b) Total colony numbers per plate. (c) Distribution of clone size (cm^2). Asterisks indicate the absence of clones observed.

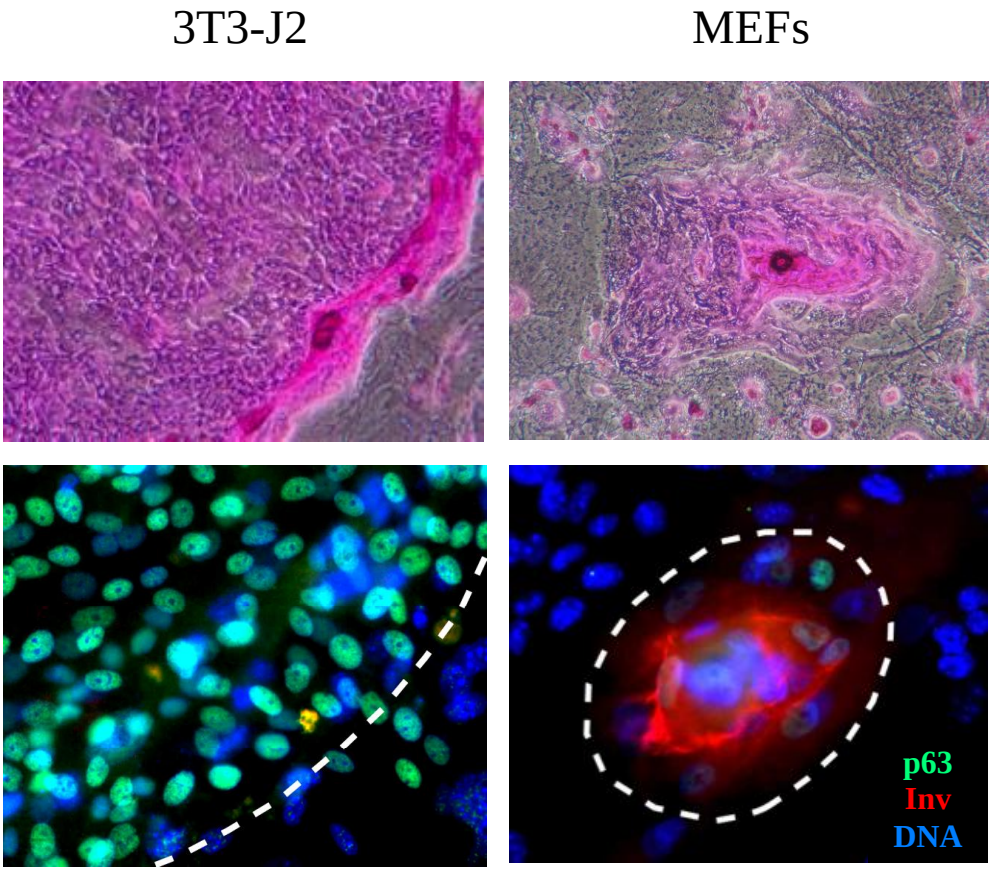

Figure 2. Morphology and differentiation status of epithelial cells grown on 3T3-J2 and MEFs. Phase-contrast and fluorescence imaging of epidermal keratinocytes grown on 3T3-J2 cells and MEFs for 14 days. Top: Rhodamine-B staining; bottom: immunofluorescecnce staining with anti-p63 antbody (Green) and anti-Involucrin antibody (Red), counterstained with Hoechst33258 (Blue). Dotted lines indicate the clone border.

Research Project 13: Project Title and Purpose

Targeting Aberrant BRCA-1 in Breast and Ovarian Carcinomas - The purpose of this study is to determine whether a process called methylation contributes to the inactivation of the BRCA-1 gene in breast and ovarian cancers, even in those patients who do not have mutations of the gene. Our goal is to characterize patients and tumors in which methylation of the BRCA-1 gene occurs, in order to identify patients whose tumors might respond to a new class of drugs called PARP inhibitors. In addition, we will examine whether methylation is associated with an inflammatory response that might be used as a basis for immune-based therapies in this patient population. These strategies are aimed at developing personalized treatment approaches for patients with breast and ovarian cancer.

Anticipated Duration of Project

12/15/2008 - 12/31/2011

Project Overview

As our understanding of the genetic basis for breast cancer behavior and susceptibility have increased, the opportunity for individualized, or personalized therapy has become a reality. It is now possible to identify patients at risk for developing breast cancer based upon *BRCA-1* germline mutation status, as well as test tumors for genetic alterations likely to result in sensitivity to specific therapies, including Her2-based and hormonal therapies for tumors overexpressing Her2/neu or expressing ER and/or PR receptors, respectively. Similarly, advances in understanding the biologic basis of *BRCA-1* mutation-induced tumorigenesis have resulted in specific therapy for *BRCA-1* mutated tumors, based upon inhibition of Poly (ADP-ribose) polymerase-1 (PARP-1). The purpose of this study is to determine whether an epigenetic phenomenon, hypermethylation of *BRCA-1*, contributes to the inactivation of the *BRCA-1* gene in breast and ovarian cancers, even in those patients who do not have mutations of the gene. Our goal is to characterize patients and tumors in which this occurs, in order to identify an additional group of patients whose tumors might respond to PARP inhibitors. In addition, we will examine whether methylation is associated with an inflammatory response that might be used as a basis for immune-based therapies in this patient population. These strategies are aimed at developing personalized treatment approaches for patients with breast and ovarian cancer in whom aberrant BRCA-1 is the major mechanism of growth and progression.

Specific Aim 1: To perform a cohort study to determine the status of *BRCA-1* gene mutation, *BRCA-1* promoter hypermethylation and BRCA-1 protein expression in breast and ovarian cancer patients treated at the Abramson Cancer Center.

Specific Aim 2: To compare the clinico-pathologic characteristics and outcomes in breast and ovarian cancer patients with *BRCA-1* mutations vs. promoter hypermethylation.

Specific Aim 3: To determine whether presence of T-cell infiltration (TIL) is associated with *BRCA-1* mutation or promoter hypermethylation in this cohort of patients.

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Expected Research Outcomes and Benefits

This project is expected to yield several important outcomes that will specifically benefit patients with breast and ovarian cancer. First, we will identify a group of patients who have tumors harboring a specific abnormality (methylation of BRCA-1) that has previously not been well-characterized or understood. We will screen for this abnormality in ovarian cancer patients, as well as in breast cancer patients with one of three characteristics associated with aggressive tumors, 1) young women (<40), 2) African Americans, and 3) those with triple negative (ER-negative, PR-negative, Her2-negative) tumors, for whom better treatments are clearly needed. We will determine whether patients with BRCA-1 methylation also have inflammation associated with their tumors. Ultimately, identifying, characterizing and understanding the biological mechanism of cancer growth and progression in these patients will lead to the personalized therapy of these tumors using both PARP inhibition and immunotherapeutic approaches.

Summary of Research Completed

To accomplish the specific aims, we have amended an existing cohort study, UPCC 19102, "Molecular and Genetic Determinants of Bone Loss in Premenopausal Breast Cancer Survivors Who Received Adjuvant Chemotherapy ("BMD")" a previously-funded study through Commonwealth funds (PI: Angela DeMichele). This study enrolled 128 patients from the practices of the University of Pennsylvania who were (1) premenopausal at the time of diagnosis (2) of sufficiently high risk of recurrence to receive adjuvant chemotherapy and (3) consented to provide a blood specimen (buffy coat) for extraction of germline DNA. Patients were originally enrolled in this cohort from 2003- 2006 and have undergone periodic follow-up since that time. Patients enrolled in this cohort were diagnosed with stage I – III breast cancer at a mean age of 43 (range 27 – 57), and 10% are minorities (including 5% African American). Receptor status of these patients' tumors demonstrates that 23% are ER-negative, 26% are PR-negative, 73% are Her2 negative and 13% are triple negative. Patients have been followed for a mean duration of 4 years from diagnosis for relapse and survival.

The amended protocol for the current study, "*Targeting Aberrant BRCA-1 in Breast and Ovarian Carcinomas ("REPAIR"),* received IRB approval and a Research Coordinator, Sara Komrokian, was hired in June 2009, who is responsible for enrolling patients from the BMD study into the REPAIR study. The process of enrolling patients has been ongoing since IRB approval. Of the original 128 patients from the BMD study, a total of 108 patients are alive and were deemed eligible to approach by their oncologists. To date, 55 patients have been enrolled. The following table summarizes the enrollment activity thus far.

BMD Study Participants	Total Eligible For REPAIR	Enrolled to REPAIR	Agreed/Pending Return of Consent/Questionnaire	No response	Declined
128	108	55	28	24	1

The 55 enrolled patients have signed the consent and completed the study questionnaire. The 28 who are in the "agreed/pending" category were contacted via phone and/or e-mail. They have verbally agreed to do the study and are currently working on completing the study questionnaire. The 26 patients in the "No response" category have been very difficult to reach via phone and/or e-mail and are now being tracked for their next visit to the hospital to approach in person.

Our recruitment strategy in the beginning of enrollment consisted of contacting all of the patients via phone and/or e-mail. The options given for completing the study questionnaire were over the phone, by e-mail, by regular mail, or in person at their next doctor's visit. All but one of the 84 patients contacted agreed to join the study, however the most luck was had with those who completed the consent and questionnaire in person. After a minimum of 3 follow-up calls and e-mails to those who had originally agreed to join the study, a decision was made to track all of the patients' next appointments to the the hospital and complete the questionnaire in person. To date 100% of the patients approached in person at HUP have been enrolled into the study. The following table is a summary schedule of the next scheduled appointments to HUP for the outstanding 52 patients for the remainder of 2010. Our current plan is to stop enrollment 12/31/10 to enable us to begin the laboratory component of the study.

Next Visit	Status: Agreed/Pending (n=28)	Status: No Response (n=24)
August	4	5
September	2	4
October	4	4
November	4	1
December		2
Total	14	16

Since the previous continuing review and approval by the IRB on 7/1/09, there have been many small modifications that were submitted and approved by the expedited review process. The most recent continuing review was granted on July 22, 2010.

For the ovarian cancer component of this trial, patients are being identified from the University of Pennsylvania Ovarian Cancer Tissue Bank, which captures clinical data, follow up, blood samples and tumor tissue on all patients diagnosed with ovarian cancer at the University of Pennsylvania. Dr. George Coukos (Co-Investigator on this project) is leading the effort to identify appropriate study candidates from this database for the current study and collect the appropriate biospecimens.

We have developed a collaboration with two basic scientists who will carry out the critical assays necessary for this study. Dr. Paul Cairns, at Fox Chase Cancer Center, will perform all

methylation studies on the tumors of patients enrolled in the study. Dr. Cairns is a member of the joint Penn-Fox Chase Ovarian Cancer SPORE team, and is currently performing the assays of interest in ovarian cancer patients identified at Penn. He is currently refining this assay for breast cancer specimens in this study. Second, we have established a collaboration with Dr. Franco Marincola, at the NIH, who will perform high-throughput genotyping for the BRCA-1 mutations of interest. Dr. Marincola has optimized this assay for use with the current project specimens. He will perform these assays free of charge.

Research Project 14: Project Title and Purpose

Understanding the Role of Autophagy Inhibition in Cancer Therapy - Many existing cancer therapies can induce cancer cell death in laboratory cell lines but have modest effects when used in cancer patients. Autophagy is a process of "self-eating" that has been observed in human cancer cell lines deficient in the ability to undergo cell death in response to a variety of cancer therapies. Recent evidence suggests therapy-induced autophagy promotes tumor cell survival and resistance to existing therapies by allowing cancer cells to clear the damage caused by these therapies. These results have established autophagy as a therapeutic target in cancer. The proposed clinical trials will determine if high doses of the autophagy inhibitor hydroxychloroquine (HCQ) can be safely combined with existing cancer treatments and if autophagy inhibition can be detected in patients receiving this treatment. In each of these clinical trials, blood and tumor tissue will be collected from patients in order to characterize the relationship between HCQ concentration and changes in autophagy. The results of these studies will provide knowledge that will inform the design of future phase II and phase III trials that will test the hypothesis that autophagy inhibition combined with standard therapies can improve outcomes in a wide variety of cancers.

Anticipated Duration of Project

12/15/2008 - 12/31/2011

Project Overview

Background: While the induction of apoptosis has been the focus of preclinical drug development, the majority of human cancers harbor defects in the pathways that control apoptosis. Autophagy is an intracellular process by which damaged mitochondria and proteins are sequestered in autophagic vesicles and destroyed through fusion with lysosomes. Autophagy has been observed in cancer cell lines deficient in the ability to undergo apoptosis in response to a variety of cancer therapies. Recent evidence suggests that therapy-induced autophagy promotes tumor cell survival and resistance to a number of existing therapies. Preclinical studies have demonstrated that hydroxychloroquine (HCQ) inhibits autophagy by deacidifying lysosomes and therefore promotes the death of cancer cells using autophagy as a survival strategy.

Objective/hypothesis: This project describes specific aims to test the hypothesis that combining autophagy inhibitors with existing therapies is safe, and that autophagy inhibition can be achieved and measured in patients.

Specific Aims: 1) Conduct pharmacokinetic (PK), pharmacodynamic (PD), and genetic studies designed to characterize autophagy in blood and tissue samples from a phase I/II trial of HCQ with radiation therapy with concomitant and adjuvant temozolomide in patients with newly diagnosed glioblasoma multiforme (GBM), 2) Conduct additional phase I and disease-specific phase II trials of HCQ in combination with autophagy inducers in patients with refractory malignancies.

Study Design: The phase I/II trial in patients with GBM will be used to pilot the PK and PD analysis for the clinical trials outlined in specific aim 2. First, a population PK model for HCQ in combination regimens will be developed. A PD assay for autophagy inhibition in peripheral blood mononuclear cells will be used on patient samples. Genotypes of specific genes that regulate autophagy will be obtained in patients with GBM that may predict benefit from HCQ therapy. While this study is accruing patients and preliminary results are obtained from correlative studies, additional phase I trials incorporating the PK and PD endpoints outlined in specific aim 2 will be launched in patients with refractory solid tumor malignancies.

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

This proposal describes the first phase I clinical trials that will establish the safety of combining escalating doses of HCQ with other cancer therapies. These trials will characterize the relationship between HCQ concentration and autophagy inhibition. Characterizing tumor responses in these clinical trials will also identify malignancies in which launching phase II trials may yield promising results. Finally, this proposal also describes assays to identify newer more potent and specific autophagy inhibitors that can be tested in future clinical trials. The long-term goals of these studies are to improve the life span of cancer patients with advanced disease, and to identify promising new regimens that could be tested as adjuvant therapy to improve cure rates in patients with early disease.

Summary of Research Completed

During this reporting period significant progress has been made. Three clinical trials have been actively accruing patients and correlative studies have been conducted on samples from these

patients during this time period (Table 1).

Specific aim 1: Conduct pharmacokinetic (PK), pharmacodynamic (PD), and genetic studies designed to characterize autophagy in blood and tissue samples from a phase I/II trial of hydroxychloroquine (HCQ) with radiation therapy (RT) with concomitant and adjuvant temozolomide (TMZ) in patients with newly diagnosed glioblasoma multiforme (GBM).

The phase I clinical trial of HCQ/TMZ/RT in patients with newly diagnosed GBM (Figure 1) was completed in December 2009. A maximal tolerated dose of HCQ 600 mg po qd when given in combination with TMZ and RT was established. A total of 16 patients have accrued thus far. At 800 mg po qd, all three patients experienced dose-limiting myelosuppresion, and, in one patient, sepsis. In February of 2010, phase II opened in 15 centers through the ABTC CNS consortium and as of 6/30/10, 57/76 patients have been accrued.

Samples collected at multiple institutions have been routed to our lab, catalogued in a database and distributed to collaborators for PK and genetic analysis. PK analysis indicates that patients with the highest estimated peak serum concentrations of HCQ were the patients that had a PD response in PBMC (Figure 1). The genetic studies have identified patients with mutations in *PTEN* and *TP53* in expected proportions. The data from the phase I portion of this trial was presented at the ASCO annual meeting 2010 as a poster. A manuscript describing these data is currently being written for anticipated publication during the next year of funding.

Specific aim 2: Conduct additional phase I and disease-specific phase II trials of HCQ in combination with autophagy inducers in patients with refractory malignancies. The following IRB-approved studies actively accrued patients during this time period:

The phase I/II clinical trial of bortezomib and HCQ in patients with refractory multiple myeloma has accrued to its sixth dose level. Preliminary data are available for the first three dose levels (Figure 2). Thus far prolonged stable disease and one PR have been observed. Toxicities have been as expected with single agent velcade alone and no dose limiting toxicity attributable to HCQ or the combination with bortezomib have been observed. The current dose level is the final dose level for dose escalation, and if no DLTs are observed this dose of HCQ in combination with bortezomib will the phase II dose. In this study serial bone marrow biopsies and parallel PBMC were collected prior to treatment, after two weeks of HCQ as a single agent and after 3 weeks of bortezomib and HCQ. The bone marrow cells were sorted on a column and purified CD138+ myeloma cells were isolated and subsequently analyzed by EM. A striking therapy-associated accumulation of autophagic vesicles has been observed in individual patients, but not yet across dose cohorts, indicating autophagy inhibition has not been reliably achieved at the doses of HCQ studied. PK analysisof HCQ in these patients are ongoing. After careful analysis of these data we have amended the protocol to obtain PD samples at earlier timepoints as dynamic autophagy changes may occur earlier than previously anticipated and may be transient.

The phase I/II trial of temozolomide and hydroxychloroquine in patients with advanced solid tumors has accrued a total of 16 patients (Table 2). There were multiple patients enrolled with brain metastases. At the 800 mg dose level there was one DLT of prolonged grade 4 neutropenia without fever which led to dose expansion. There was one patient with unrelated complete heart

block in this expansion and therefore a decision was made to amend the protocol and expand this cohort further and include serial EKG's to ensure that this event was not drug related. A total of 10 patients have been accrued to this dose cohort and no additional dose limiting toxicities have been observed. At the 800 mg dose level we observed one complete response (Figure 3), one partial response and a number of patients with stable disease, indicating preliminary evidence of activity for this regimen. A 4th cohort of HCQ 1000 mg daily will open in July 2010. PBMC were collected from patients before treatment, 2 weeks after HCQ and 4 weeks after combination therapy. At 400 mg, a significant accumulation of AV was observed in two patients. In one patient with melanoma, treatment with temozolomide and 400 mg HCQ po qd resulted in stable disease, but evidence of new lesions on CT scan resulted in study termination after 4 months of treatment. In characterizing these new lesions, a PET/CT and upper GI with small bowel follow-through imaging, revealed FDG-avid tumors with photopenic centers and evidence of central necrosis. This pattern of tumor effect has not been observed with temozolomide alone.

HCQ trials					
Trial	Therapeutic agents	Disease	Sites	Progress	N
1	Phase I/II Temozolomide (TMZ)/ Radiation + Hydroxychloroquine (HCQ)	GBM	ABTC (15)	Phase I completed. Phase II 75% accrued	77-85
2	Phase I/II bortezomib + HCQ	Myeloma	Penn	6th dose cohort	40-60
3	Phase I TMZ + HCQ	Solid tumors	Penn	4th dose cohort	12-24

Table 1. HCQ clinical trials. GBM: Glioblastoma multiforme NABTT: New Approaches to Brian Tumor Therapy

Phase I/II trial of temozolomide 150 mg/m2 po 7/14 days (dose intense) with hydroxychloroquine in patients with advanced solid tumors

Cohort	HCQ qd dose	No. Evaluable Patients	DLT/ SAE	Best Response
1	200	3	None	3/3 PD
2	400	3	None	1/3 SD
3	800	10	1) DLT: Gr 4 neutropenia 2) SAE: heart block Likely unrelated But lead to cohort expansion	1 CR 1PR 1 SD 5 PD 2 NA
4	1000	Open 7/10		

Table 2. Enrollment on the phase I trial of temozolomide + HCQ. NSCL: non small cell lung cancer; H& Neck: Head and Neck; PD: progressive disease; SD: Stable disease NA: not available

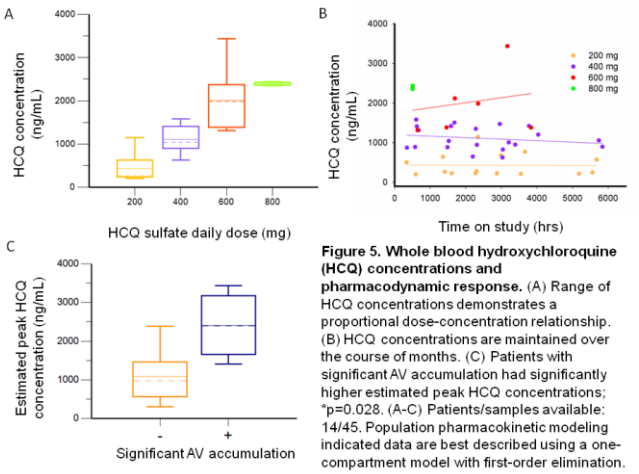


Figure 5. Whole blood hydroxychloroquine (HCQ) concentrations and pharmacodynamic response. (A) Range of HCQ concentrations demonstrates a proportional dose-concentration relationship. (B) HCQ concentrations are maintained over the course of months. (C) Patients with significant AV accumulation had significantly higher estimated peak HCQ concentrations; *p=0.028. (A-C) Patients/samples available: 14/45. Population pharmacokinetic modeling indicated data are best described using a one-compartment model with first-order elimination.

Figure 1. PK of HCQ/TMZ/RT

Bortezomib/HCQ: clinical results

Dose Cohort	*Prior response to Velcade*	*Best response on study*	*Weeks on study*	*Reason for discontinuation*
HCQ 200 mg qod	PD	Progression	11	Progression
+ Bz 1.0 mg/m²	SD	Minor response	77	Progression
	-	Stable disease	18	Lack of response
HCQ 200 mg qod	SD→PD	Stable disease	14	Lack of response
+ Bz 1.3 mg/m²	PR→PD	Minor response	14	Adverse event (unrelated)
	-	Stable disease	24	Progression
HCQ 200 mg qd	-	Stable disease	15	Lack of response
+ Bz 1.3 mg/m²	SD→PD	Stable disease	9	Progression
	PR→PD	Progression	3	Progression
	PR→PD	Stable disease	17	Progression
HCQ 200 mg bid	PR	Stable disease	20	Lack of response
+ Bz 1.3 mg/m²	MR	Minor response	14	Lack of response
	-	Near complete response	11	Fatigue
HCQ 400 mg bid	-	Progression	5	Progression
+ Bz 1.3 mg/m²	-	Progression	0.7	Progression
	-	Stable Disease	15+	-
	-	-	5+	-
HCQ 600 mg bid		Progression	1	Progression

Figure 2. Update for phase I trial of velcade and hydroxychloroquine in relapsed refractory myeloma

Complete response in a patient with melanoma and brain metastases treated with temozolomide and hydroxychloroquine 800 mg qd

Pre-treatment | 2 months TMZ+ HCQ

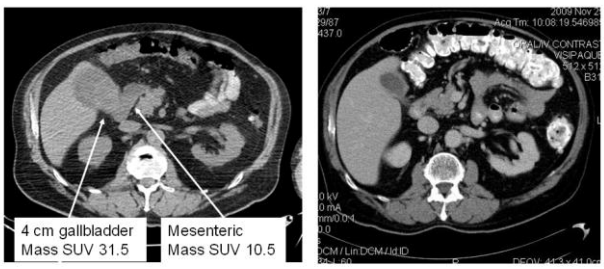


Figure 3. Complete response in patient treated with temozolomide and hydroxychloroquine

Research Project 15: Project Title and Purpose

Pilot Study: Imaging and Modifying Hypoxia in Head and Neck Squamous Cell Tumors -
Head and neck cancer is a devastating disease that kills about 8,000 Americans each year. Many of these cancers are poorly oxygenated (hypoxic) and therefore are harder to kill with radiation therapy and more likely to metastasize. Higher doses of radiation can be given to hypoxic tumors, but this approach results in more toxicity and side effects. Our purpose is to (1) determine the presence and amount of hypoxia in recurrent head and neck cancer using ^{18}F-EF5 PET scanning and using this method, (2) determine whether Nelfinavir, which has safely been used in many HIV patients in the USA, can make tumors more oxic. The knowledge gained in this project may allow patients with oxic tumors to avoid high dose radiation and those with hypoxic tumors to receive a safe drug treatment to make standard radiation therapy more effective.

Anticipated Duration of Project

12/15/2008 - 12/31/2011

Project Overview

Our broad objective is to test a rationally designed, clinically relevant adjuvant drug regime to overcome hypoxia-based radioresistance in recurrent head and neck cancer (rHNSCC). We will study the presence, patterns and extent of hypoxia in rHNSCC, test whether nelfinavir (NFV) modifies pO_2 in these tumors, explore whether ^{18}F-EF5 signal is associated with patient outcome (time to recurrence of disease; TTR) and examine other endpoints that are important in the prognosis and treatment of this disease (blood vessels, apoptosis, proliferation, OPN, pAkt).

Specific Aim (SA) 1a: Describe and compare the ^{18}F-EF5 PET signals from patients with rHNSCC tumors before and after NFV treatment. We hypothesize that hypoxic primary tumors and/or lymph nodes (based on initial ^{18}F-EF5 PET scan) in patients treated with NFV will show decreased PET signal, e.g., primary tumors and/or lymph nodes will become better oxygenated. Each patient will serve as his/her own control.

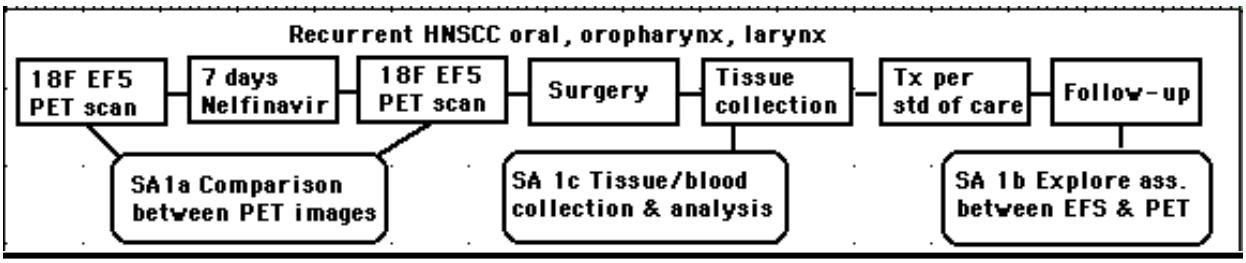
SA 1b: Explore whether the ^{18}F-EF5 PET signals from the primary tumors and/or lymph nodes are associated with patient outcome (time to recurrence of disease, TTR). We hypothesize that improvement in tissue oxygenation in the primary tumor and/or the cervical lymph nodes, as measured by ^{18}F-EF5 signal in the pre- and/or post treatment PET scan will be associated with increased TTR.

SA 1c: Explore the relationship between ^{18}F-EF5 signal and other measures of hypoxia [serum OPN; HIF1α protein expression, proliferation (Ki67), blood vessels (CD31), apoptosis (Apotag), and/or radioresistance (pAkt expression)].

We have performed all, and published most of the techniques necessary to accomplish the proposed specific aims other than serum OPN level analysis, which will be performed by Quyn Le, Stanford University. ^{18}F-EF5 has been safely given to approximately 20 cancer patients (HNSCC in Finland, gliomas at PENN). We have recently published findings that immunohistochemical (IHC) analysis of EF5, the non-radioactive form of ^{18}F-EF5, predict event

free survival (EFS) in HNSCC patients; this observation forms the scientific basis of this project.

Figure 1: Treatment Schema and Specific Aims:



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Expected Research Outcomes and Benefits

More than 50% of US patients with HNSCC died of their disease despite improvements in surgery, radiation and medical therapies in the last decade. Currently, a new approach to cancer care is being developed: to improve patient outcome by prescribing "individualized" therapy where specific critical characteristic(s) of a patient's tumor is/are characterized and targeted. Hypoxia is one of the factors that is being emphasized in HNSCC. It is well accepted that patients with hypoxic HNSCCs have a greater probability of treatment (radiation, chemotherapy, surgery) failure (local recurrence, metastasis) compared to those with oxic tumors. In a recent study, heterogeneous levels of hypoxia were detected in the lymph nodes and/or primary tumor of 78% of 74 patients using hypoxia PET imaging. We expect similar (if not higher) numbers using ^{18}F-EF5 binding in this study of recurrent HNSCC, and we will investigate whether this negative prognostic factor can be modulated with NFV, e.g., can NFV cause tumor reoxygenation? Of substantial importance is that the knowledge and experience that will be gained in this trial is not limited to HNSCC; any cancer type or location can be probed with ^{18}F EF5 and appropriate therapies prescribed.

Summary of Research Completed

This project has not begun and no funds were expended during this reporting period.

Research Project 16: Project Title and Purpose

Research Recruitment, HPV, and Cervical Cancer Prevention in Asian American Women - Cervical cancer is an important public health concern, among minority populations, including Asian American women, who are disproportionately affected by this disease. At the same time, participation by Asian Americans in clinical trials and other biomedical research is low. In this three-step project, we propose to: (Step 1) assess barriers and promoters to research participation among Chinese- and Vietnamese-American women; (Step 2) test different communication and outreach strategies to maximize recruitment of these women into a study using two methods of data collection: a brief questionnaire and biosampling; and (Step 3) perform a preliminary study of the epidemiology of Human Papillomavirus (HPV) among these women.

Anticipated Duration of Project

01/01/2009 - 12/31/2010

Project Overview

Objective: To identify effective recruitment strategies to engage Asian American women in cervical cancer prevention research and to broaden the understanding of Human Papillomavirus (HPV) epidemiology in this population.

Specific Aims: The primary aims are (1) to identify effective culturally-appropriate communication strategies to recruit Chinese- and Vietnamese-American women into cancer prevention studies involving biosampling. This will involve randomization of patients to one of two different recruitment strategies, based upon results of elicitation research; (2) to assess if subtypes of HPV in Chinese- and Vietnamese-American women are similar to subtypes seen in the general population and the same as those targeted by HPV vaccines that are currently available and those under development; and (3) to describe knowledge, attitudes, and behaviors with primary prevention of cervical cancer (including HPV vaccination) among Chinese- and Vietnamese-American women.

Design and Methods: The proposed study will require a three-step, qualitative-quantitative design. Step 1 will employ elicitation research methodology and use language-appropriate focus groups with Chinese- and Vietnamese-American women to assess barriers and promoters to participation in biomedical research involving biosampling (pap and blood), as well as knowledge and beliefs about HPV and HPV vaccination. Step 2 will involve creation and testing of two alternative methods of study recruitment with the target populations, using different message-framing techniques based upon results of elicitation research. The outcome of this step will be the collection of biobehavioral data including: responses to a brief questionnaire and collection of biosamples. In Step 3 biosamples will be tested for evidence of cervical dysplasia and the presence of specific high-risk HPV types. Behavioral data will be analyzed to correlate knowledge, attitudes and behaviors with sociodemographic factors and HPV infection.

This project will produce data for a culturally and language appropriate, population-based study of HPV and cervical cancer prevention among the growing Pennsylvania and U.S. population of

Asian American women.

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Expected Research Outcomes and Benefits

The outcomes of this project will be a clearer understanding of recruitment strategies to engage the understudied population of Asian American women into research involving biosampling. In addition, the investigators will begin to understand the epidemiology of high-risk Human Papillomavirus (HPV) infection within this population, as well as knowledge, attitudes, and behaviors related to cervical cancer risk and prevention. The identification of successful study recruitment strategies will be useful for the development of larger epidemiologic studies that will follow.

This project takes a novel approach to the comprehensive study of a population that is at high-risk, understudied and therefore, underserved. While there is a body of published work related to Asian American women and behavioral factors associated with Pap testing, there is limited data on behavioral issues pertaining to HPV and HPV vaccine uptake specifically in this population. Moreover, no large studies have combined such behavioral research with biosampling among this cohort.

With ever-increasing numbers of Asian Americans in the United States (US) and Pennsylvania (PA) in particular, this study comes at an important time with respect to its potential impact on public health. The ultimate goal is to understand the epidemiology of HPV and to reduce cervical cancer rates in this high-risk population. This project will apply strategies proven effective in enhancing human subject recruitment to enhancing health promoting behaviors, such as HPV vaccine uptake. This project will produce a model intervention to promote cancer prevention in underserved populations.

Summary of Research Completed

<u>Qualitative Phase (Step 1):</u>
Recruitment and data collection for the qualitative phase of the study (Step 1) was completed during this project period. Recruitment took place in the waiting area of Philadelphia District

Health Center #2. The recruitment team consisted of 4 female staff members from the Penn Asian Health Initiatives (PAHI), under the direction of the PI, Dr. Giang Nguyen. Each recruitment team member was bilingual in English and either Vietnamese or Mandarin Chinese. Over a period of 20 weeks, a total of 231 Asian women were approached and invited to participate. Among these, 69 women, ages 18+ agreed to participate in the qualitative study (29.9%) and responded to detailed intake questionnaires. These data were input into an electronic spreadsheet application, using direct double-entry and reconciliation as necessary. Analysis of quantitative data was performed using Stata for Windows.

All women who agreed to participate were invited to return at a later date for focus group/interview sessions; 30 women (9 Vietnamese-speaking, 12 Chinese-speaking, 9 English-speaking) returned for the full focus group/interview sessions. These women provided informed consented to have these sessions audio recorded. Although our goal was to have several participants for each focus group, there were several occasions when only one person was able to attend; in such cases, the focus group proceeded with a single participant. All focus group/interview sessions were recorded and transcribed by PAHI staff. Transcripts were de-identified and assigned a unique identifier code. No adverse events were observed or reported to us. All focus group/interview participants were given copies of the consent documents, in their language of choice (English, Vietnamese or Chinese), and they received monetary compensation ($25) for time and travel. Non-English focus group/interview sessions were transcribed in English to facilitate collaborative review by investigators who were not proficient in Chinese and Vietnamese. Qualitative coding was performed on NVivo software.

Among women who agreed to participate, the mean age was 36 (range 18-59). A small number (6%) were born in the U.S., and fairly equal proportions originated from Vietnam (27%), China/Taiwan (33%), and Other (33%). Mean age of immigration 26 years (range 2-52). Ethnic background was 35% Vietnamese and 65% Chinese. Only 12% reported speaking English very well; 38% spoke English well, 42% spoke English not well, and 8% did not speak English at all. More than half (58%) had received some schooling in the US, and overall 78% had finish 12 years of school, whether in the US or elsewhere. Half were employed, but only 22% had health insurance. About two thirds of the women (63%) were married, and 69% had a daughter.

Table 1 summarizes the awareness and prior experiences that these women reported with regard to Pap testing, HPV, and biomedical research. Overall, a substantial proportion of the women had never had a Pap test and never heard of HPV, and most had not received or heard of the HPV vaccine. The majority had never participated in research of any kind, and most who had participated in research only did survey research rather than clinical trials or sampling. Prior research participation was statistically significantly higher among: younger women, women who immigrated at younger ages, and women with a preference for completing their interviews in English. Awareness of HPV vaccine was statistically higher among younger women, women who immigrated at younger ages, women with better self-reported English proficiency, and women with a preference for completing interviews in English.

Among the women who participated in the focus group/interview sessions, 30% completed the session in Vietnamese, 40% in Mandarin, and 30% in English. Transcripts from the focus

group/interview sessions were reviewed by the research team, including our qualitative expert, Dr. Ellen Giarelli. Our qualitative analysis suggested several findings:

1. Awareness of HPV and HPV vaccination is low.
2. Prior study participation is low. Awareness of the distinction between testing for research and testing for clinical care is low.
3. There is an expectation that results will be provided even when testing is done for the purpose of research.
4. The idea that blood represents the "life force" and is limited in quantity can be a barrier to participation in bio-sampling.
5. The role of family members in deciding about study participation is lower than expected, but can be important for some women.

Further information about the themes identified from the focus group/interview sessions is provided in Table 2 at the end of this section. One academic paper has been written based on the qualitative data and has been submitted for peer review. A second paper based on the quantitative questionnaire data is in the draft stage.

Biosampling and Testing Phase (Step 2 and 3):
The findings from the focus group/interview sessions were used to inform the Bio-sampling Phase of the study. The direct experiences of the recruitment staff were also used as a basis for the next phase. A biosampling study recruitment script was generated incorporating standard recruitment text that was further enhanced by the knowledge gained from our elicitation research. This was used to supplement the standard recruitment and consent process, which includes general information about the study rationale, details about what participants are asked to do, and discussion of the costs and benefits of participation. The creation of the enhanced recruitment message was an iterative process involving all lead behavioral investigators, including communication expert Dr. Joseph Cappella. Examples of components of the enhanced recruitment message but not in the standard message include the following:

1. Greeting: Before assessing language needs, Asian women are approached with a simple "hello" greeting that incorporates English, Mandarin, and Vietnamese ("Hello, Ni hao, Xin chao").
2. References to racial/ethnic group: Rationale for the study emphasizes the fact that Asian women are specifically important to this research, and that the health issue has relevance to Asian women in particular
3. Exemplars: Quotes from women similar to the recruitees (based on our focus group data) were used to show opinions of some about research participation.
4. Clarity about blood sample collection: Given the voiced concern about blood sample collection and the feeling that blood is one's "life force", the amount of blood to be collected is clearly described, and we also specifically include the fact that this is a safe amount that is quickly replenished by the body.

The standard and enhanced recruitment messages were translated into Vietnamese and Chinese with quality check by multilingual staff. The multilingual recruitment team then completed training with mock recruitment sessions to ensure strict adherence to these carefully crafted

messages. Plans for randomization of study recruitment messages for the Bio-sampling Phase were discussed with and approved by our Biostatistics expert, Dr. Wei-Ting Hwang.

All consent forms for the Biosampling phase were translated into Chinese and Vietnamese and were approved by necessary Institutional Review Boards. Protocols for biosample collection in the health center setting were discussed with and agreed upon by administrative and clinical staff at the community health clinic, and logistical details were also discussed with laboratory staff from the University of Pennsylvania Department of Pathology, which will process the samples after collection. A detailed, illustrated 11-page protocol training booklet was created so that all recruitment staff for the biosampling phase could be consistent in their recruitment and sample/data collection activities. Recruitment for the Biosampling and Testing Phase (Step 2 and 3 of the Project) began on July 9, 2010.

Table 1 – Prior experience with Pap, HPV, and biomedical Research

Experience with Pap and HPV	
Ever had pelvic exam	63%
Never had Pap	34%
Ever rec'd an injection	73%
Heard of HPV vaccine	35%
Ever rec'd HPV vaccine (if age 26 or less)	9%
Experience with Research	
Prior research participation	6 (9%)
# who had done research surveys	5
# who had done blood sampling	1
# who had done other biosampling	2

Table 2 - Thematic Findings from Elicitation Research Phase:

Barriers to participation include	Lack of time; loss of privacy; traditional values, particularly in older women; mistrust of institutions; non-English language; confusion about the difference between research and medical care; sexuality as a topic
Concepts about bio-sampling	Blood is too precious to give; there must be a clear reason why the sample is needed
Enhancers to participation	Personalized approach; compensation; more highly educated participant; presence of a more knowledgeable other person; convenient place and time
Recruitment recommendations	Appeal to the person's interest in learning; use language appropriate to the participant's linguistic background; recruitment at the time of a medical appointment; appeal to their personal choice; appeal to the greater good
Other themes related to research participation	Must explain the value of the research; there should be some personal gain (increased knowledge about one's own health or about health in general); endorsement by husband can be helpful for some

Research Project 17: Project Title and Purpose

Individualized Therapy for Advanced Non-small Cell Lung Cancer Based on Clinical and Molecular Typing - The main purpose is to show that *non-empiric* therapy scientifically chosen on the basis of existing clinical and molecular markers can result in improved progression-free and overall survival (PFS and OS) in good performance status patients with advanced Non-small Cell Lung Cancer (NSCLC), compared to historic controls and contemporaneous subjects who are treated empirically. Subsidiary objectives include (1) the collection of tumor tissue and serum to further elucidate the molecular typing of patients with advanced NSCLC; (2) the development of a broad-based, interactive, collaborational database of advanced NSCLC patients treated at the University of Pennsylvania; and (3) parity or improvement with respect to expected toxicities and adverse events (AEs).

Anticipated Duration of Project

12/15/2008 - 12/31/2011

Project Overview

In this project, we will study how to best implement various diagnostic and molecular pathologic studies to allow the optimal selection of customized therapies or enrollment into specific therapeutic protocols for patients with newly diagnosed advanced (stage IIIB and IV) NSCLC. Our goals include:

1) Determining the feasibility of performing immunohistology and mutational analysis (K-ras, EGFR, etc.) in specimens (paraffin embedded and fresh) obtained by current standard of care methodologies employed for minimally invasive diagnosis. ("Run in")
2) Performing a prospective study to evaluate the impact of the program on various outcomes including: a) feasibility, b) the degree to which therapies are changed by "customization", c) health care utilization and cost, d) patient acceptance, and e) clinical outcome, including overall survival (OS), progression-free survival (PFS).

After the initial run-in, we will determine patients' preferences for standard vs customized or protocol therapy and whether or not they have a sufficient biopsy material available for histologic and mutational testing. This project includes the following IRB-approved protocols: UPCC 09506 (phase II trial of erlotinib +/- PF-3512676), UPCC 02508 (randomized phase II of combination docetaxel and carboplatin +/- vandetanib); and UPCC 06508 (phase II study of bevacizumab and erlotinib in elderly patients).

We anticipate enrollment of 210 subjects into this three-year effort; we estimate that 62.5% of enrolled subjects will have sufficient prior tissue available from which histologic or mutational analysis can be performed, and that approximately 80% of these will choose "customized therapy" or go on other treatment protocols. Our goal is to show that customized therapy will result in a 50% increase in overall median survival vs standard therapy (15 vs 10 mos). Assuming 5% are not evaluable, accrual of 200 patients should assure that that there is adequate power (>80%) and type I error (<5%) to demonstrate this difference.

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Expected Research Outcomes and Benefits

We anticipate the following:

1. Demonstration that therapy based on molecular correlates and clinical factors is feasible and can be rationally delivered to patients with advanced NSCLC, for whom empiric therapy has previously been the standard
2. In comparison to standard, empiric therapy, customized therapy and/or formal therapeutic protocol therapy will result in improved outcome, including
    a. Higher response rates to treatment
    b. Delays in tumor progression
    c. Increased survival
    d. Equal or less toxicity
3. The therapeutic platform we develop will enable us to further test tissue and blood with the goal of developing new markers to predict therapeutic benefit as our standard treatments evolve, growing increasingly complex and diverse.

Summary of Research Completed

During the period of this project, the first two of these trials have closed, and we have since dovetailed this project with more recent IRB studies in advanced NSCLC: UPCC 03508 (a phase III trial of paclitaxel/carboplatin +/- figitumumab in advanced non-adenocarcinoma of the lung); UPCC 03508 (an investigator-initiated phase II study of carboplatin, pemetrexed, and bevacizumab);. UPCC 20508 (a randomized phase II trial of carboplatin and paclitaxel +/- ABT869; and several ECOG trials, including E2508 (randomized phase II study in never smokers assigning patients to either standard treatment [paclitaxel/carboplatin +/- bevacizumab] or standard treatment plus erlotinib; E3508 (a randomized phase II trial of paclitaxel/carboplatin/bevacizumab +/- IMC-A12); and E4508 (testing paclitaxel/carboplatin + either IMC-A12, C225 or both in bevacizumab-ineligible patients). Each of these clinical trials has been IRB approved and open during the period of the project.

<u>Goal 1</u>) Determining the feasibility of performing immunohistology and mutational analysis (K-ras, EGFR, etc.) in specimens (paraffin embedded and fresh) obtained by

current standard of care methodologies employed for minimally invasive diagnosis. ("Run in")

Accomplishments during the reporting period:
To date, we have had a number of organizational meetings between thoracic oncology and pulmonology, led by Drs. Langer, Albelda and Vachani, and have enlisted the help and participation of molecular pathology, as well as the input of Drs. Dara Aisner, who has since left Penn following completion of her fellowship, Christopher Watt, Leslie Litzky, and Vivianna Van Deerlin, all in pathology. These discussions have led to the purchase of a pyrosequencer critical for KRAS determination, out of funds extraneous to this project, which have not been expensed to this specific project. These discussions also led to the identification of Dara Aisner, a pathologist and Senior Fellow in Molecular Pathology, who became the point person for both EGFR and KRAS determination. As of 5/17/10, Dr. Aisner has identified over 215 lung cancer specimens from primary lung cancers as well as metastases, both during this project period and from specimens obtained prior to this project period, with adequate tissue for EGFR testing. At least 40 of these specimens have proven (+) for the mutation. Dr. Aisner and Binu Malhotra, one of the residents has who worked under our aegis, are currently compiling an analysis of these patients, assessing baseline demographics, including smoking status, and correlating EGFR status with responses to EGFR TKIs, and final outcome.

Until the purchase of the pyrosequencer, specimens for KRAS were sent to outside labs for assessment. They will now be done within the institution. The results of EGFR and KRAS are reported in tandem, and are now documented in pathology notes in our EPIC medical management system. This change in reporting would not have happened without the impetus of this project, and has proven integral to the viability of the project as we both retrospectively and prospectively identify subjects whose treatment can be tracked. In addition, as a result of efforts stemming from this project, Dr. Aisner has been able to show a similar rate of EGFR mutations in cytology specimens compared to non-cytology specimens [biopsy tissue, etc]. Formerly it was believed that EGFR mutation testing could only be accomplished on tissue samples. Her work has been submitted to national pathology meetings. [Aisner DL, Deshpande C, Baloch Z, et al. Are cytology blocks adequate for EGFR mutational testing? An institutional experience. *Mod Pathol*. 2010;23(suppl 1):396A. Poster presented at: United States and Canadian Academy of Pathology Annual Meeting; March 20-26, 2010; Washington, DC]. Our work has also led to an organizational process within the Department of Molecular Pathology focusing on how best to streamline tissue acquisition, testing and reporting. Corey Langer, the PI of this protocol, serves as an advisor to this ad hoc committee.

Goal 2) Performing a prospective study to evaluate the impact of the program on various outcomes including: a) feasibility, b) the degree to which therapies are changed by "customization", c) health care utilization and cost, d) patient acceptance, and e) clinical outcome, including overall survival (OS), progression-free survival (PFS).

Goal two is more long range, although we have made major headway on goal 2c. Anil Vachani and Corey Langer are now working in collaboration with the The Center for Comparative Effectiveness in Genomic Medicine [CEGeM], led by Katrina Armstrong of the Department of Internal Medicine and the ACC, whose grant proposal evaluating cost efficacy and resource

utilization, was approved by the NCI during the past year. We have meshed our work with hers; it is now integrated into one of four program projects within the CEGeM . We are working with Elizabeth Hansdorf, David Asch, and Sean McGellicot, as well as Dr. Armstrong, and are actively creating modeling for cost effectiveness and quality-adjusted survival based on expected response rates, progression-free survival, overall survival and anticipated costs. We are meeting one to two times a month, and are actively mentoring the CEGeM team on the clinical course of NSCLC; in addition, as part of this meshed project, Drs. Vachani and Langer have discussed "The Emerging role of Personalized Therapy in Advanced NSCLC" during their Advisory Board session on 5/21/10. Initial data from this project should be available in the fall of 2010; we will then go on to determine if clinical reality matches the modeling proposed.

Regarding feasibility, in June 2009, we enlisted the services of a nursing student in the medical oncology clinic during office hours; she consented 15 new patients with treatment-naïve, advanced NSCLC for release of tissue, as well as blood and urine for analysis; her services ended as of September. She has been rehired this summer. More importantly, after a lengthy search, we have now hired Lynn Werner RN, an experienced research nurse/coordinator who will superivise our project, obtaining consents and biospecimens for the prospective component of the project, and actively retrieving retrospective data on newly diagnosed NSCLC patients. She will be physically present during clinic hours to facilitate our accrual.

Regarding consents, we are using existing ICFs established by Dr. Anil Vachani for blood, serum and tissue collection to facilitate molecular analysis. These forms are now part of the new patient package when patients first arrive at Penn for assessment in the Thoracic clinic. We have also determined that a separate consent form will be needed for patients with insufficient tissue available for molecular analysis. Since a repeat biopsy poses some risk and such patients theoretically can be treated empirically without further tissue evaluation, we believe strongly that a formal consent will need to be obtained from these patients before we obtain more tissue.

With respect to data collection, Dr. Vachani has developed a web-based database and case report form (CRF) (available on request) that will be used to collect data for this project. This web-based CRF will be accessible to all members of the project team and continually updated, based on data entered into EPIC. These case report forms will track all patients screened and consented for this project and will include baseline, de-identified demographic data, including co-morbidities, as well as follow-up information regarding these patients. This form will also detail treatment administered, as well as response status, and information on progression-free survival (PFS) and overall survival (OS). Lynn Werner has made critical changes to the CRFs to streamline data collection.

The development of a broad-based, interactive, collaborational database of advanced NSCLC patients treated at the University of Pennsylvania, though a subsidiary effort, is crucial to the success of this project; such a database, heretofore, did not exist.

During the period of 12/08 through 7/01/09, two additional, IRB-approved clinical trials have opened, both targeting specific histologies.. These include UPCC 03508 a randomized phase III trial of CP-751,871 (Figitumumab) in combination with Paclitaxel and Carboplatin vs Paclitaxel and Carboplatin in patients with advanced *non-adenoca* NSCLC; and UPCC 12508, a phase II

trial of Bevacizumab plus Pemetrexed and Caroplatin in Previously Untreated Advanced NSCLC; the latter trial explicitly excludes patients with squamous histology. Patients enrolled on these trials will be recruited for this effort, and their data and outcomes entered onto the in-house database. From 7/01/09 through 7/01/10, four additional IRB-approved clinical trials have opened, with treatment choices based on histology and clinical parameters:

1. UPCC 20508: a randomized phase II trial of carboplatin and paclitaxel +/- ABT869
2. ECOG 2508: randomized phase II study in never smokers assigning patients to either standard treatment [paclitaxel/carboplatin +/- bevacizumab] or standard treatment plus erlotinib;
3. E3508: a randomized phase II trial of paclitaxel/carboplatin/bevacizumab +/- IMC-A12);
4. E4508: testing paclitaxel/carboplatin + either IMC-A12, C225, or both in bevacizumab-ineligible patients

Research Project 18: Project Title and Purpose

Research Infrastructure: Renovation of Space for the Center for Cognitive Neuroscience - The purpose of this project is to provide office and laboratory space for the Center for Cognitive Neuroscience, whose member faculty are currently scattered across the campus in different locations despite their need for close collaboration, and whose faculty are also currently working in poor-quality facilities. The new space will facilitate the research productivity of the group by enabling them to work more collaboratively and to carry out their research programs with adequate office and dry lab facilities.

Anticipated Duration of Project

5/27/2009 – 12/31/2011

Project Overview

The Center for Cognitive Neuroscience is an interdisciplinary community dedicated to understanding the neural bases of the human mind. Its 13 faculty work in a variety of different aspects of human psychology and its brain mechanisms, including vision, attention, language, creativity, social-emotional functions and the development of these psychological abilities in childhood. Seven of these faculty members, with primary appointments in Neurology and Psychology, will move into the renovated space, along with their students, staff and dry lab equipment (mainly PCs), enabling an unprecedented level of collaboration and resource sharing among these labs.

Hence, the broad objective of this infrastructure project is to enable more and better research to be done in this intensely interdisciplinary field, which addresses questions of fundamental importance for understanding the human mind and whose potential applications touch many areas of health and human capital.

The specific aims are to renovate the 3rd and 5th floors of the Goddard Laboratories building, which are currently in poor condition. This space has not undergone any significant renovation

since the building was erected in 1964, and is currently unsuited for laboratory research and, in some locations, not up to current building code. Existing offices and labs will be demolished to the perimeter walls and the entire space will be reconfigured into state-of-the-art dry lab, office and meeting space. The layout of the building is two towers with each floor divided into nine areas by structural concrete beams that support the floor above. The new layout of the space provides for 4 corner offices in each of the two towers per floor with administrative and support places in between the corner offices and a kitchenette area in the glass walkway between the two towers. This renovation also intends to provide ADA compliant restrooms on each floor.

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

One of the 21st century's most exciting scientific frontiers is cognitive neuroscience, the science of understanding how a human mind can arise from the biological processing of the human brain. In addition to the enormous intellectual value of research in cognitive neuroscience, this research is also crucial to understanding a number of vitally important issues facing society, including the diagnosis and treatment of mental and brain disorders and the protection and encouragement of human potential. In the first category are mental illnesses, most notably Attention Deficit Hyperactivity Disorder, Dyslexia, Autism, Depression, Schizophrenia and Drug Addiction and neurological disorders, most notably aphasia, amnesia, Alzheimer's disease and fronto-temporal dementia. In the second are educational issues including child development and prevention/intervention with at-risk children.

Summary of Research Completed

During the past year we have completed the following prelimary steps:

- Completed design documents
- Completed in-house administrative project review and value engineering
- Requested bids from contractors and are currently under contract with the low qualified bidder
- Completed all pre-construction activites in the building.

We intend to begin project abatement in mid-August.