University of Pittsburgh

Annual Progress Report: 2012 Formula Grant

Reporting Period

July 1, 2013 – June 30, 2014

Formula Grant Overview

The University of Pittsburgh received $7,752,646 in formula funds for the grant award period January 1, 2013 through December 31, 2016. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Understanding the Role of Inflammation in Chronic Obstructive Pulmonary Disease – Chronic Obstructive Pulmonary Disease (COPD) exhibits significant variability in disease pattern and rate of progression. At this time, no clinical attribute or biomarker can determine disease prognosis in an individual patient. This project will offer the most robust analysis to date defining the natural progression of tobacco-related lung disease, which should lead to a reclassification of COPD into more meaningful biological phenotypes. We further aim to discover biomarkers that identify patients at greatest risk for disease progression. Discovery of new markers associated with unique subclasses of disease will lead to more effective personalized treatment algorithms and monitoring of a therapeutic response.

Anticipated Duration of Project

1/1/2013 – 12/31/2016

Project Overview

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death in the United States. Clinical presentation and clinical progression can vary dramatically between individuals with respect to the traditional measure of impairment in forced expiration and the potentially independent attributes of parenchymal emphysema, airway narrowing, symptoms, exercise impairment, or associated comorbidities. Remarkably, no clinical attribute or biomarker can predict progression rates of these parameters in an individual patient.

Our first aim is to classify COPD into subphenotypes representing unique disease pattern and prognosis using both cross-sectional baseline and six-year longitudinal measures of change in physiologic, radiographic, and clinical/demographic features reflecting the activity of various independent pulmonary and comorbid conditions. Five hundred subjects from the Pittsburgh Specialized Center in Clinically Oriented Research (SCCOR) cohort will return for six-year follow-up measures, including visual and quantitative computed tomography (CT) analysis of

airway remodeling and parenchymal emphysema, physiologic measures of air-flow obstruction and air trapping, incremental shuttle walk exercise, symptom/demographic and quality of life surveys, and measures of vascular and bone comorbidity. We will perform both supervised and unsupervised clustering approaches to accomplish the reclassification. Successful completion of this aim will offer the most robust analysis to date defining the natural progression of tobacco-related lung disease and allow significant progress toward reclassification of COPD into more meaningful biological phenotypes.

Our second aim will be to define peripheral blood molecular and cellular biomarkers that identify progression of independent clinically relevant measures of COPD progression, as well as the unique clinical and prognostic phenotypes defined in Aim 1. We have identified nearly 30 novel blood molecular and cellular biomarkers, many of which have never been linked with lung pathogenesis, that associate with unique cross-sectional COPD subphenotypes and/or short-term longitudinal progression of these phenotypes.

Principal Investigator

Frank C. Sciurba, MD
Associate Professor of Medicine
University of Pittsburgh
3471 5th Avenue, Suite 1211
Pittsburgh, PA 15261

Other Participating Researchers

Yingze Zhang, PhD; Steven Duncan, MD; Joseph Leader, PhD – employed by University of Pittsburgh

Expected Research Outcomes and Benefits

We have developed a longitudinal design wherein we will identify patterns of disease progression over time in patients with tobacco-related lung disease and further define blood markers that are predictive of these patterns. This analysis offers a significant advance beyond the single point cross-sectional methodology available in the published literature. We believe that, given the likelihood that disease activity varies within an individual in the course of chronic disease, cross-sectional characteristics may not always reflect the consequences of molecular and cellular events that drive disease progression. We, therefore, believe that biomarkers associated with disease activity represented by changes in radiographic and/or physiologic indices over the course of a six-year study are more likely to reflect the pathogenic events most relevant to disease subclassification. Novel biomarkers that are predictive of more clearly elucidated subcategories of COPD disease activity, and which will emerge following successful completion of our aims, are desperately needed in the clinical and scientific arenas. Such markers will enable progress with personalized therapeutics that alter disease progression by targeting disease-specific pathways and will distinguish patients with active, progressive processes from those with stable, apparently quiescent, chronic processes that do not warrant expensive or potentially detrimental therapies.

Summary of Research Completed

Specific Aim 1

We continue to maintain Institutional Review Board (IRB) documents and maintain adherence to good clinical practice (GCP) policy as we proceed with participant recruitment and assessment. We continue to successfully recruit participants from the Pittsburgh SCCOR cohort, consisting originally of former smokers (>10 pack-years), age >40, with no significant non-COPD related comorbidities. Patient accrual for the CV3 (six-year +/- six month follow-up) cohort is proceeding per Figure 1. Thus far, 126 patients have given consent, enrolled, and received radiographic, physiologic, vascular, and DEXA body composition testing. Of these, 105 have completed testing in the past year.

Those who have had computational analysis of their completed chest computed tomography (CT) tests are represented in the following figures and tables. These chest CTs have been analyzed using a semi-quantitative visual scoring, as well as quantitative density histogram analysis under the direction of Joseph Leader, PhD.

Blood was drawn on all enrolled patients and T-cell studies were completed on the day of blood collection (laboratory of Steven Duncan, MD). Peripheral blood mononuclear cells (PBMC) were isolated from fresh venous phlebotomy specimens and were characterized for selected cell surface receptors. Blood specimens have been processed and frozen in Yingze Zhang, PhD's laboratory for use in future protein, RNA, and DNA genetic analyses.

Progression in sub-phenotypes over time: COPD not only varies cross-sectionally in phenotypic pattern but has varying levels of activity within an individual and between individuals, resulting in significant variation in progression of the various phenotypic attributes over time (Table 1 and Figures 2-3). The value of the extended longitudinal characterization made possible by this funding has become increasingly clear as we proceed with our six-year (CV3) recruitment, compared to the prior two-year (CV2) analyses. We used a distribution-based method to determine a minimal clinically important difference (MCID) in the change in selected phenotypic parameters over time between CV1 and CV3 visits. Threshold values based on this analysis, which reflect clinically important changes, include a change in forced expiratory volume 1 (FEV1) of 4 percent predicted, F-950 fraction of 0.003, and St. George's Respiratory Questionnaire (SGRQ) of four points. Of the 106 participants completing baseline (CV1) and both CV2 and CV3 follow-up visits, including quantitative CT parenchymal scoring of emphysema, 41 (38.7 percent) demonstrated a clinically important increase in the F-950 quantitative emphysema score, suggesting progression of emphysema at CV2, whereas 47 (44.3 percent) demonstrated progression at CV3. Forty-two (39.6 percent) had clinically important progression, defined by the physiologic parameter FEV1 percentage predicted at CV2, whereas 61 (57.5 percent) demonstrated progression in this parameter at CV3. Furthermore, 38 (35.8 percent) patients demonstrated clinically important progression in the symptom SGRQ quality of life survey at CV2; 45 (42.5 percent) demonstrated a change at CV3. Notably, individual patients demonstrated progression in different sub-phenotypes, defined by these three parameters, such that only 6.1 percent of patients at CV2 and 15.1 percent at CV3 demonstrated progression in all parameters. Importantly, more participants (81.1 percent), with the longer follow-up, demonstrated progression in at least one phenotype at CV3, whereas only 70.8 percent

demonstrated progression in any parameter at the shorter CV2 follow up. This finding validates the critical importance of the longer follow-up supported by this study in potentiating our ability to determine molecular markers of disease activity and progression, which is a focus of this project. In fact, we have observed that, because the variance in the measurements are relatively large in the context of the very slow progression represented in this disease, progression demonstrated at the two-year time point is only a modest indicator of six-year progression (R=0.41). Furthermore, because of regression to the mean, the relationship between (CV2-CV1) progression versus (CV3-CV2) period progression is, in fact, mildly inversely correlated (R=-0.060).

While supervised and unsupervised clustering analyses on the longitudinal CV3 data will require a complete set of data collected on patients over the several-year duration of the project, we have completed statistical analysis with respect to the computational aspects of the CV1 and CV2 data sets, which is being prepared for publication. A peer-reviewed manuscript has been published describing methodologic issues we have discovered inherent to classification of COPD patients. (Bon J, Liao S, Tseng G, Sciurba FC. Considerations and Pitfalls in Phenotyping and Reclassification of Chronic Obstructive Pulmonary Disease. Translational Research. 2013; 162(4):252-7.)

Specific Aim 2

Selected protein biomarker analyses have been performed on the specimens collected from our overall cross-sectional cohort, and these measurements will be used to assess the important component of sub-phenotype disease progression once our CV3 longitudinal data set has matured. Based on our prior pilot studies, as well as peripheral blood mononuclear cell (PBMC) gene expression analyses, we have selected and analyzed 14 markers during this progress period in the entire cross-sectional cohort. Preliminary analysis demonstrates positive associations of several markers with the severity of obstruction, emphysema, and physical impairments. (Table 2).

Additionally, we observed an inverse association with disease progression at CV2 (two-year follow-up) for a subset of these markers (Fig. 4). Two of the markers that are associated with two-year progression in emphysema are sFAS (soluble FAS receptor) and TRAIL (TNF-related apoptosis-inducing ligand). Lower plasma levels of TRAIL and sFAS at baseline are associated with emphysema progression measured by visual score and F -950, respectively. Both proteins are important regulators of apoptosis. Apoptosis of alveolar epithelial cells and endothelial cells has been proposed to be one of the mechanisms leading to the loss of alveoli (emphysema), as well as the pulmonary vascular structure resulting in the pulmonary hypertension comorbidity often seen in advanced COPD patients. The FAS receptor is a death receptor on the surface of cells that leads to apoptosis. Similarly, TRAIL binds to the death receptors DR4 (TRAIL-RI) and DR5 (TRAIL-RII) and triggers the apoptotic process. Additionally, TRAIL plays a role in antagonizing the death-signaling pathway by binding to the decoy receptors DcR1 and DcR2 and activates NFkB, which results in the transcription of genes known to antagonize the death-signaling pathway and/or to promote inflammation.

A manuscript describing the data presented in the 2012 progress report related to Pentraxin 3 (PTX3) as a biomarker of emphysema severity is in final preparation.

Assessment of T-cell surface markers is ongoing; however, analyses with phenotype progression await a more mature CV3 data set.

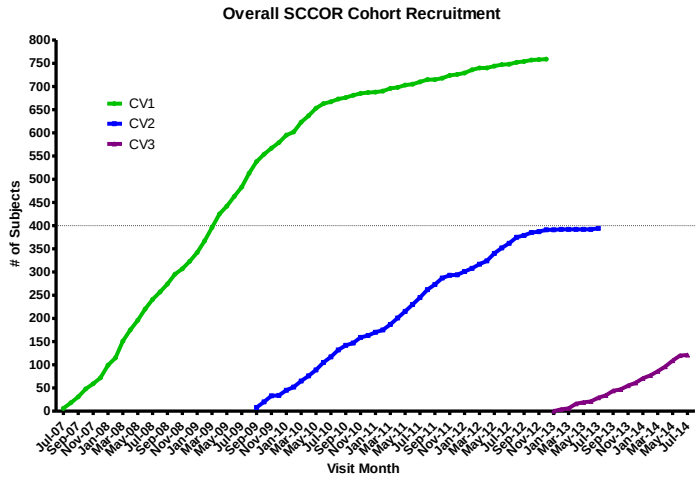

Figure 1. Recruitment of the CV3 extension relative to the overall cohort enrollment.

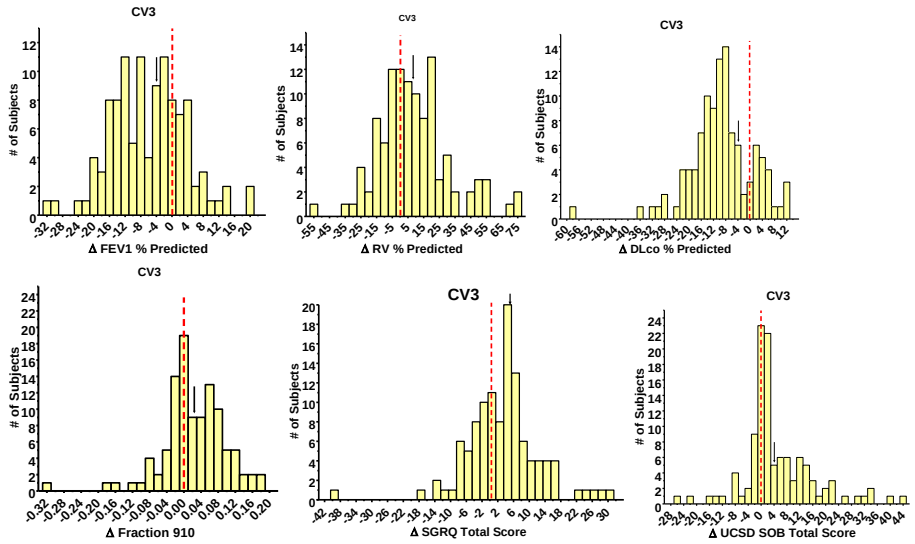

Figure 2. Distribution of change in outcome measures at CV3 visit compared to baseline. Note overall deterioration in each parameter over time; however, great variation exists in individual patient progression. The vertical red line reflects a change of "0." The small black arrow reflects a change determined to be clinically important for each measure. (MCID)

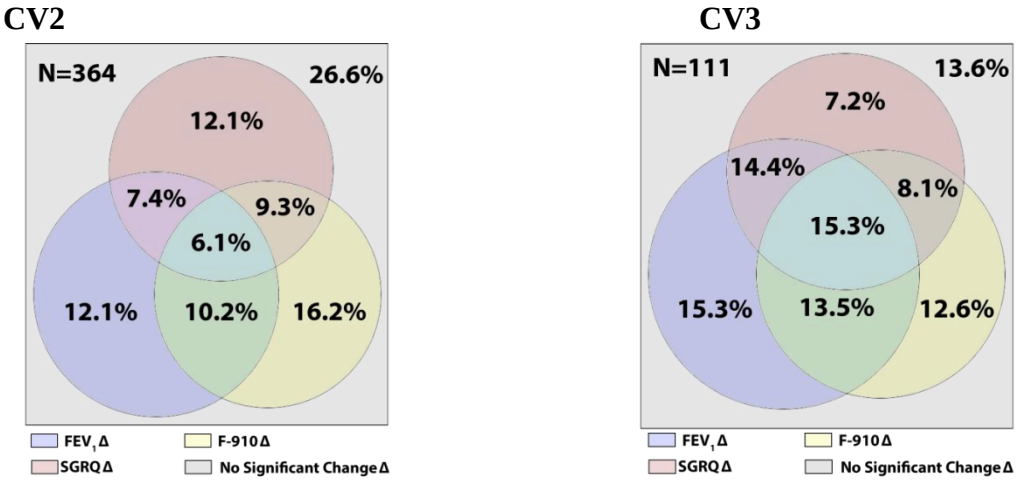

Figure 3. The proportional venn diagrams represent the number of patients demonstrating significant disease progression within each sub-phenotype representing a physiologic (FEV1), radiologic (F-910), and symptom (SGRQ) category at the 2-year CV2 (left panel) and the 6-year CV3 (right panel) points. Notably, a significantly greater proportion of patients have demonstrated progression in each phenotype by the CV3 evaluation, compared to CV2. While only 13.6 percent of patients show no progression in any parameter at 6 years, by contrast only 15.3 percent of them have progressed in every parameter at that time.

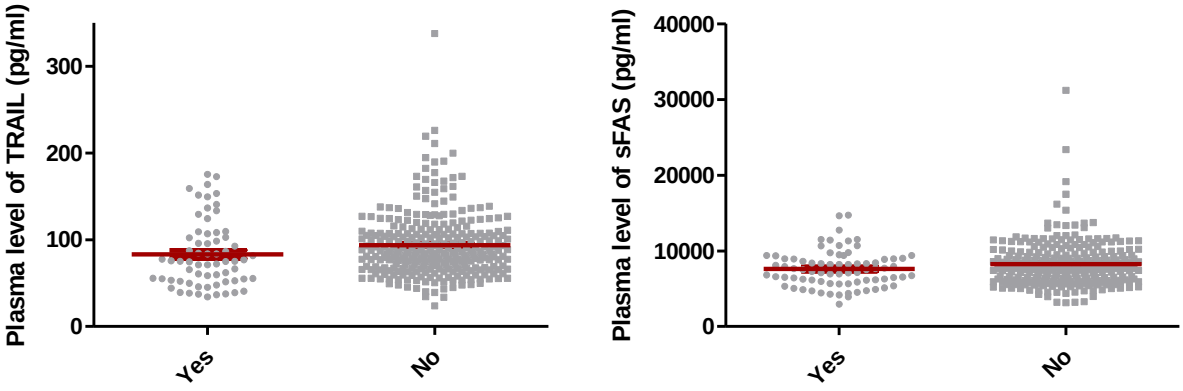

Figure 4. Association of plasma levels of TRAIL and sFAS, both markers of apoptosis regulation, in patients exhibiting clinically important progression of either radiologic emphysema or physiologic obstruction "Yes" versus those with no progression "No" at the CV2 time-point.

	Δ CV2	Δ CV3
FEV_1 % Predicted	-1.6 ± 7.7	-5.8 ± 9.8
RV % Predicted	2.3 ± 18.7	8.6 ± 23.8
DLco % Predicted	-1.2 ± 10.0	-9.5 ± 10.8
SGRQ - Total	1.0 ± 9.5	2.7 ± 9.3
SGRQ - Symptom	0.1 ± 20.0	1.0 ± 16.9
SGRQ - Activity	1.9 ± 15.2	3.8 ± 15.8
UCSD SOB Total	2.0 ± 10.0	5.0 ± 11.4
Shuttle Walk Distance (m)	-7.9 ± 84.6	-69.5 ± 75.2
MLVS	0.2 ± 0.5	0.3 ± 0.7
F-950	0.003 ± 0.02	0.011 ± 0.03
F-910	0.011 ± 0.07	0.022 ± 0.07
MeanHU	-4.1 ± 20.4	-9.5 ± 18.3

Table 1. Changes from baseline at 2 years (CV2) and 6 years (CV3) of the 105 patients enrolled. {FEV1 % Predicted - percentage of the predicted forced expiratory volume at 1 second; RV% Predicted - percentage of the predicted residual volume representing air trapping at the end of forced expiration; DLco % Predicted - percentage of the predicted diffusing capacity of the lung; SGRQ – St. George Respiratory Questionnaire representing disease specific quality of life tool, including the total score and the domains of activity and symptoms ; UCSD SOB - The University of California, San Diego Shortness of Breath Questionnaire; MLVS – representing a semi-quantitative visual emphysema score ; F-950 & F-910 - fraction of lung voxels below -950 and -910 Hounsfield units, respectively, representing quantitatively measured emphysema; Mean HU - mean Hounsfield unit representing mean lung density.}

Table 2. Association of blood biomarkers with clinical phenotype#

Biomarkers*	FEV1%	FEV1/FVC	DLCO_pp	SGRQ	F -950	MLVS
sFas	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**
TRAIL	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**
SOST	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**
TNFRI	0.2487	**0.0275**	0.4848	0.247	**0.0279**	0.0519
TNFRII	0.1943	0.0511	0.9191	0.7993	**0.0195**	0.1013
CRP	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**0.0655**	**<.0001**
SAA	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**
sICAM1	0.7261	0.952	0.1752	0.1881	0.2614	0.4078
sVCAM1	0.1391	0.4177	**0.0192**	0.0526	0.8484	0.5521
MMP1	**0.0343**	**0.0371**	**0.002**	**0.0024**	0.2634	**0.0203**
MMP7	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**0.0103**	**<.0001**
TIMP1	0.8446	0.4379	0.5666	0.6319	0.0844	0.4385
TIMP2	**0.0297**	**0.0029**	0.0653	0.0707	**0.04**	**0.0035**
TIMP4	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**

* all biomarkers are log transforms for analysis.
# age, gender & smoking status adjusted p-value shown.

Research Project 2: Project Title and Purpose

The Application of Adult Stem Cells and Native Tissue Matrices for Tissue Regeneration – Population incidence of degenerative joint diseases, including osteoarthritis, the leading cause of disability, is increasing, particularly in the elderly. The purpose of this research project is to develop novel tissue regenerative approaches to treat such diseases. We will investigate the regenerative activities of the recently identified induced mesenchymal progenitor cells (iMPCs), to use native tissue and cell matrices to engineer scaffolds with the optimal physical and biological microenvironment to support stem cell activities and differentiation, and to apply these novel technologies to enable point-of-care repair of joint defects and other musculoskeletal injuries in the future.

Duration of Project

1/1/2013 – 1/7/2014

Summary of Research Completed

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

Research Project 3: Project Title and Purpose

Dissecting the Molecular Mechanisms of Stress-Response Proteins in Cancer – This project leverages modern biocomputation and experiments to study the molecular mechanisms of stress-response proteins in cancer. We have selected two key stress response proteins, Y box binding protein 1 (YB-1) and heat shock protein 27 (Hsp-27)], that are not only highly conserved but also involved in cancer drug resistance. Our approach will: (i) define, establish, and further study the YB-1 and Hsp27 interaction networks by understanding these networks' composition and evolution and by determining the phenotypic consequences of their perturbations (Aim 1); and (ii) determine the physical principles of protein-protein and protein-ligand interactions in the Hsp27 and YB-1 networks (Aim 2).

Anticipated Duration of Project

1/1/2013 – 12/31/2016

Project Overview

Stress response proteins play a critical role in the establishment, progression, and metastasis of tumors. As such, they serve as important biomarkers for diagnostic strategies as well as targets for therapeutics. Y box binding protein 1 (YB-1) and heat shock protein 27 (Hsp27) are two highly conserved stress response proteins that function in critical aspects of cancer progression and multidrug resistance. YB-1 is a promising breast metastasis marker and is upregulated in prostate cancers. YB-1 also associates with Argonaute proteins, which are central to small RNA-mediated silencing events. Increased levels of Hsp27 expression often correlate with poor prognoses of many types of cancers, likely due to the cellular protective effects of Hsp27. This effect can be due to one or more of the many functions of Hsp27, which include protein chaperoning, redox homeostasis maintenance, and remodeling of the actin cytoskeleton. A more detailed examination of these important genes, their homologs, and their interaction networks will provide important insights into essential aspects of cancer biology.

This project will take a multi-pronged approach to examine the structural, network, and functional aspects of YB-1 and Hsp27 using both computations and experiments to achieve the following two aims:
Aim 1: To establish and characterize the molecular interaction networks of YB-1 and Hsp27
Aim 2: To structurally characterize YB-1 and Hsp27 macromolecular complexes.

Computational approaches such as efficient variants of molecular dynamics simulations, coarse-grained modeling, sequence analysis, network methods, and phylogenetic modeling will yield new insights into the structural determinants of these proteins as well as refining and revealing their interaction networks. *In vitro* and *in vivo* experimental methods, along with next-generation sequencing, will probe the mechanistic bases of YB-1 and Hsp27 function in a variety of settings. Together, these studies provide a comprehensive and integrated approach that will elucidate basic mechanisms underlying the functions of stress response proteins in cancer.

Principal Investigator

Daniel M. Zuckerman, PhD
Associate Professor
University of Pittsburgh
3501 Fifth Avenue
Pittsburgh, PA 15213-2203

Other Participating Researchers

Joseph Ayoob, PhD; Carlos Camacho, PhD; Nathaniel Clark, PhD; Maria Chikina, PhD; Timothy Lezon, PhD – employed by University of Pittsburgh

Expected Research Outcomes and Benefits

The following outcomes are anticipated:

- Description of the functionally important residues in Hsp27 multimerization by structural and evolutionary techniques
- Identification and characterization of YB-1 macromolecular interactions by structural, evolutionary, and sequence-based methods
- Predictions of small-molecule lead-compounds that disrupt Hsp27 multimerization and YB-1 interactions with proteins and RNAs
- Identification and validation of novel short RNAs that bind to YB-1
- Functional characterization of the molecular mechanisms of Hsp27 in cell elimination processes during development.

Summary of Research Completed

To probe the mechanisms of stress-response proteins, we are applying experimental and computational techniques to investigate the structural underpinnings, sequence determinants, evolutionary history, and functional networks of the small heat shock protein Hsp27.

Cell-system to study sequence determinants, expression, localization, oligomerization, and function of Hsp27. Hsp27 phosphorylation, oligomerization, and sub-cellular localization are all linked, though these associations are not clearly defined. To embark on a focused study of these aspects and relationships in cell culture, we first examined endogenous Hsp27 protein levels and subcellular localization in *Drosophila* S2 cells at normal temperature (25°C) and under heat-stress (37°C). Hsp27 expression is barely detectable under control conditions but is significantly up-regulated and easily detected after mild heat shock at 37°C (Figure 1A). The lack of detectable expression under normal conditions, which differs from what is seen in mammalian cultures, provides a good system to investigate the various properties of wild-type and mutant Hsp27 proteins without any potential confounding effects caused by interactions with endogenous wild-type protein, which may exist in various phosphorylation states. Fluorescent immunocytochemistry with anti-Hsp27 antisera on fixed cells corroborated these findings. Heat-stressed cells revealed a distinct increase in Hsp27 levels primarily in nuclei (Figure 1B).

Subcellular localization of exogenously expressed Hsp27. To test whether the nuclear localization of *Drosophila* Hsp27 in S2 cells is dependent upon phosphorylation, we examined the subcellular localization of exogenously-expressed *Drosophila* Hsp27 phospho-isoforms in S2 cells with and without stress and found differences between wild type (wt), unphosphorylatable (3SA), and phosphomimetic forms (3SD) of Hsp27. While Hsp27 is always nuclear, 33 percent of the time it is also found in the cytoplasm (n=88 cells). This phenomenon decreases to 13 percent under heat stress (n=45). The 3SA form of Hsp27 exhibits similar behavior, but under heat stress it has not been observed at all in the cytoplasm (n=12). On the contrary, the 3SD form displays a high degree of cytoplasmic localization (over 70 percent at 25°C and over 80 percent at 37°C). Together, these data suggest that Hsp27 nuclear localization is sensitive to both stress and phosphorylation state in *Drosophila* cells.

Comparative modeling of monomeric and dimeric forms of Hsp27. *Drosophila* Hsp27 has several homologs in the Protein Data Bank (PDB). Based on these templates, we built potential 3D models of Hsp27 in its monomeric and oligomeric forms. These models predict key interactions amenable for experimental testing to better understand self-assembly of Hsp27 and related family members. Monomeric models were built from the *Drosophila* Hsp27 sequence using I-TASSER (Iterative Threading ASSEmbly Refinement). The resulting structures aligned with truncated and full length versions of the highly related human alpha-B crystallin (PDB: 2WJ7 and 2YGD, respectively, Figure 2A-B), which has an overall sequence identity of 30.5 percent, and with a family member of lesser sequence identity: wheat Hsp16.9 (1GME, Figure 2C); 16 percent identity to Hsp27. This low identity reduces confidence of the modeling using the latter protein. A dimer of *Drosophila* Hsp27 was also created by aligning modeled monomers to the dimer structure of human alpha-B crystallin (Figure 2D). Key dimer contacts are mostly nonspecific backbone contacts between the antiparallel beta sheets. Two side chain salt bridges between D46 and R57 are observed. These residues are conserved in *Drosophila*. The presumed dimer structure of *Drosophila* Hsp27 using the wheat Hsp16.9 as a template shows a different interface between monomers, reducing confidence of the model (Figure 2E).

Modeling inter-subunit interactions of the small heat shock protein (sHSP) family. We pursued molecular modeling studies of the role of specific interactions and dynamical fluctuations in the oligomers of the Hsp27 family. The 24-mer structure of HSP16.5 from *methanococcus jannaschii* was our starting point for studying how local structural interactions affect large-scale behavior using a mixed-resolution Monte Carlo (MC) modeling approach designed to study localized interactions within the context of large structures or complexes.

Our modeling focused on the C-terminal tail of one subunit, which acts as "molecular tape" in attaching to a subsequent subunit. A conserved amino acid (aa) stretch of Isoleucine–any aa–Isoleucine (IXI) in the C-terminus was reported to be crucial for this oligomerization. We focused initially on just two HSP16.5 subunits, extracting coordinates from the PDB file 1SHS. The C-terminus of the second subunit ("mol2") interacts directly with the first subunit ("mol1"). We also constructed a mutant model by changing the IXI sequence to three glycines (GGG) to reduce the interaction strength. Finally, we made a truncated construct by removing the IXI sequence and all following residues in the C-terminus. These three constructs were simulated using our Monte Carlo software (Figure 3). Based on 20 million MC steps, substantial rigid-body motions occur between the subunits, and it also appears that specific interactions between the C-

terminus of mol2 and the core of mol1 become weakened. However, no substantial differences emerged among the three constructs: the range of fluctuations for the five independent simulations for each construct overlaps the others. Moreover, since the simulation data did not level off, additional sampling is still required.

Predicting molecular interactions with Hsp27. With the goal of predicting molecular interactions for Hsp27, we have used our genome-wide data set of evolutionary correlations between *Drosophila* proteins to identify genes with similar evolutionary histories in terms of rates of sequence evolution, a statistic we term evolutionary rate covariation (ERC). To demonstrate the predictive power of ERC, we predicted a set of molecular interactions for a *Drosophila* genetic pathway by searching for genes with evolutionary histories correlated with genes previously known to influence the pathway. We made 16 predictions, of which six were confirmed experimentally, leading to our publication (Findlay *et al*. PLOS Genetics. 2014). Notably, other genomic and proteomic techniques had failed to identify new genes in this pathway. Given this successful application in *Drosophila*, we next applied ERC to find novel interactors for Hsp27. We generated a list of the top 100 genes with evolutionary histories correlated with Hsp27. These top hits were significantly enriched for function in gene expression and RNA metabolism (Table 1). Gene enrichment was performed using the Gene Ontology Term Finder application.

In a parallel effort, we also generated an ERC data set using 33 mammalian genome sequences to explore candidate molecular interactions with HSPB1, the human gene encoding the ortholog of *Drosophila* Hsp27. Using this powerful new data set we predicted top ERC hits as potentially interacting with HSPB1. We also examined the genetic contribution of HSPB1 to human disease. Mutations in HSPB1 lead to general neuropathy and to Charcot-Marie-Tooth neuropathy. Using a large data set of disease genes from the Online Mendelian Inheritance in Man catalog (OMIM), we found a statistically significant evolutionary correlation between neuropathy genes, including HSPB1, and genes involved in seizures. This finding suggests that the genes underlying these diseases are functionally related, as confirmed by their shared pathology in neurons.

HSP Network Analyses. Based on multiple alignments, the alpha crystallin HSPB family appears to have undergone several independent duplications in the *Drosophila* and mammalian lineages. Nevertheless, based on phosphorylation patterns and complementation ability, the fly protein Hsp27 has been historically treated as the analog of mammalian HSPB1. However, Hsp27 is most likely not an ortholog of HSPB1 in the strict evolutionary sense, only a paralog, leaving the possibility that some HSPB1 functions are performed by other fly proteins. To investigate the functional correspondence among the entire group of paralogous fly and mammalian HSPB family genes, we make use of high-throughput data and cross-species network alignment to compare these genes with respect to their network similarity. Our computational approach quantifies how much homologous proteins from different organisms share functional partners (based on co-expression and protein-protein interactions) that are themselves homologous.

Clustering based on our network similarity score (Figure 4) produces three distinct clusters: one mammalian (highlighted blue) and two *Drosophila* (highlighted red and green). One of the Drosophila clusters has high network similarity to the mammalian homologs; however, this cluster does not contain Hsp27 but, instead, contains three other less well characterized genes (Hsp67Ba, CG7409, and CG13133). This clustering is driven by global similarity in expression

patterns. These mammalian HSPB proteins are highly regulated, expressing at different levels in different tissues, a pattern also observed for fly genes in the green cluster. On the other hand, the fly genes Hsp27, Hsp26, and Hsp23 (red cluster) are more stably expressed and are mostly regulated by heat shock. Interestingly, this pattern of similarity mirrors the sequence tree based on alignments restricted to the family-defining alpha crystallin domain.

The global clustering pattern may, however, obscure more subtle and context-dependent functional similarities. Using a guilt-by-association approach, we compare the Gene Ontology term enrichments for neighborhoods of the homologous HSPB genes. We find that the mammalian proteins share more functional enrichments with fly proteins in the green cluster. Importantly, however, we also find that they share a "cell death" enrichment term with the fly Hsp27 gene. Specifically, this observation implies that the mammalian HSPB1 and fly Hsp27 share homologous functional interaction partners involved in cell death.

Modeling stress protein signaling pathways. Toward revealing key molecular interactions involved in the YB-1 and Hsp27 pathways (Aim 1a), we are developing generalizable methods for incorporating data from multiple sources to reverse-engineer pathways in cancer. During method development, we are focusing on the insulin (Ins) and insulin-like growth factor I (IGF1) pathways using reverse phase protein array (RPPA) data from cultured breast cancer cells provided by a collaborator at Magee-Womens Research Institute. In tumors, both IGF1 and Ins act as proliferative and anti-apoptotic agents. We have constructed a mathematical model of these pathways to help identify the source of their aberrant behavior in cancer (Figure 5A).

To further clarify the activity of these pathways, we have conducted a statistical analysis of RPPA data for 21 cultured breast cancer cell lines stimulated with Ins and IGF1. The results of our statistical analysis reveal potentially important regulators of the time progression of IGF1 stimulation, including Caveolin, COX2, and β-catenin, none of which are included in the previous mechanistic model (Figure 5B). Our multiscale modeling approaches are still ongoing and will directly translate to uncovering the details of YB-1 and Hsp27 pathways.

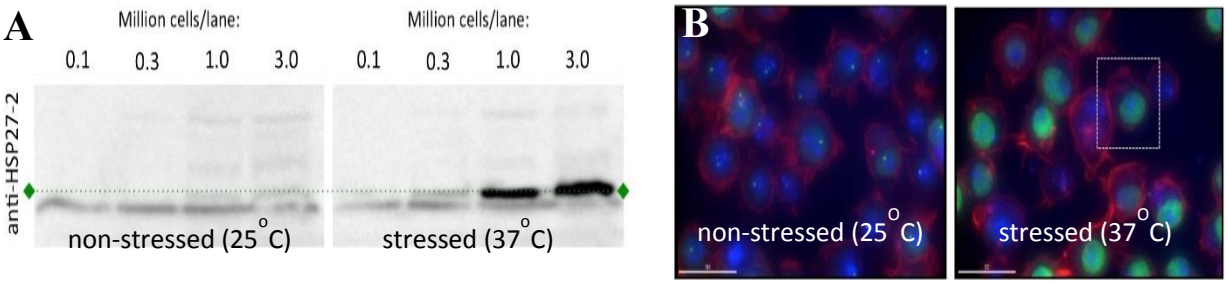

Figure 1. A) Hsp27 immunoblot with and without an hour of heat-stress to 37°C. Hsp27 protein is evident only in stressed cells (green line). B) Immunoflourescent staining of S2 cells fixed after same stress exposure; rhodamine-phalloidin (actin)–red, Hsp27–green, and DAPI (nuclei)–blue.

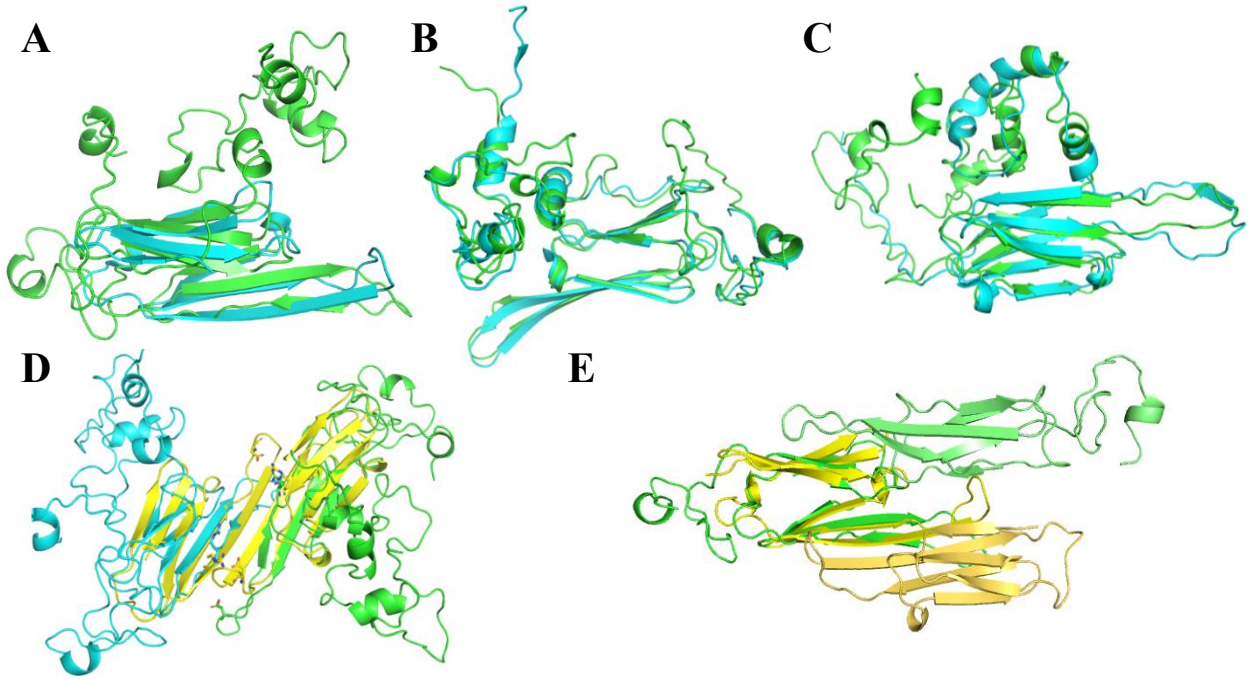

Figure 2. A-C) Hsp27 monomer models (green) created from other sHSP family members (blue): truncated alpha-B crystallin (A), full length alpha-B crystallin (B), wheat Hsp16.9 (C). D-E) Hsp27 models (green/blue) created from other sHSP family members (yellow): truncated alpha-B crystallin (A), wheat Hsp16.9 (B). Amino acids forming salt bridges in A are shown as stick forms.

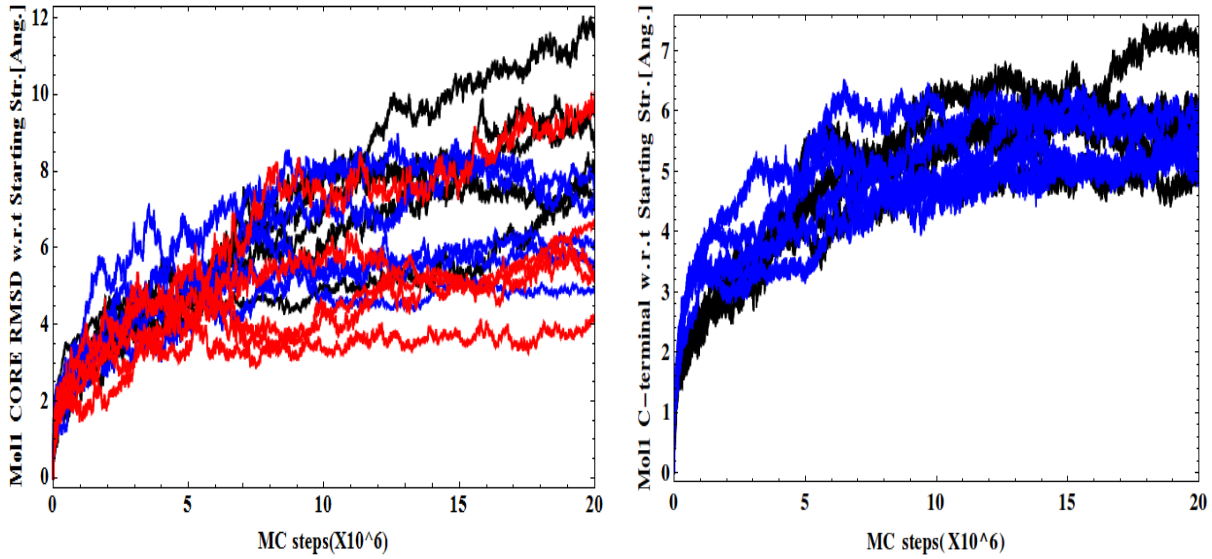

Figure 3. Fluctuations in Monte Carlo simulations of two subunits of HSP16.5 from *methanococcus jannaschii*. (A) RMSD of rigid-body motions of mol1 core with reference to mol2 core. (B) RMSD of structural fluctuations of the mol2 C-terminus with reference to the mol2 core. Five simulations; wild type – black, mutant – blue, truncation – red.

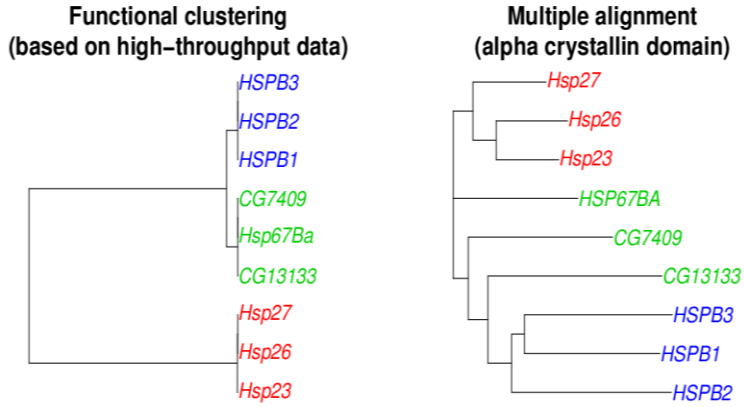

Figure 4. Clustering of HSP family members using functional clustering based on high-throughput data (left) and multiple alignment of family members using only the highly-conserved and family-defining alpha crystallin domain. Mammalian proteins in blue; *Drosophila* proteins in green and red.

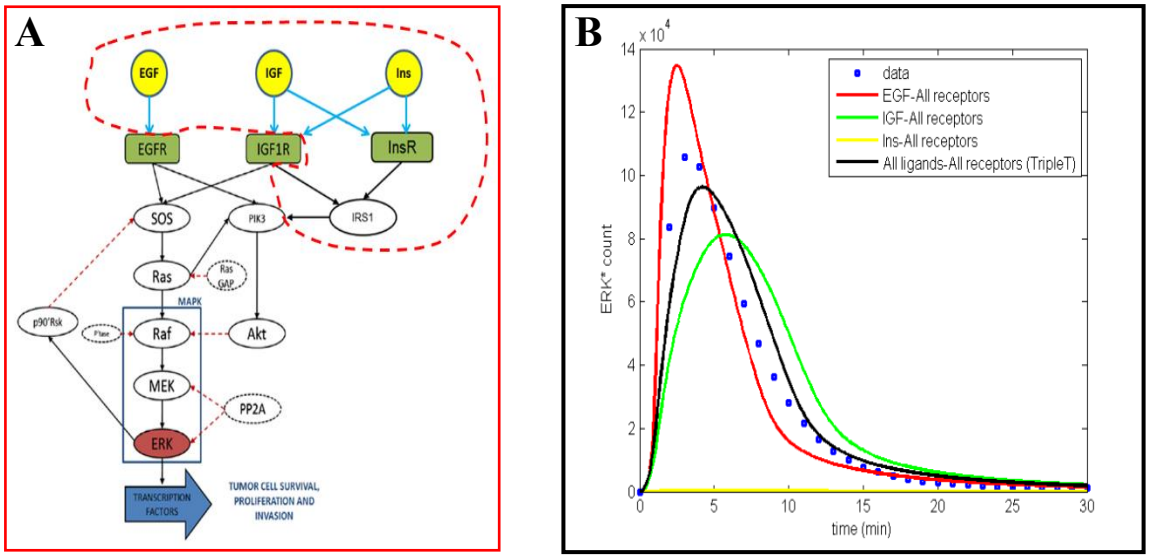

Figure 5. A) The mechanistic pathway model based on Bianconi et al., 2012 (Biotech Adv 30:142-153). Important features in this model that are not present in previous models include: (1) explicit inclusion of Ins, IGF1, and epidermal growth factor and their receptors; (2) inclusion of the insulin receptor substrate 1 (IRS1) adaptor protein; (3) explicit handling of dimer formation and activation, including the possibility of IGF1R/InsR heterodimers. B) Model results indicate the behavior of activated ERK in response to stimulation with each of the three ligands in isolation.

Table 1. Functional enrichment for Hsp27 correlated genes.

Gene Ontology Term	Cluster Frequency	Genome Frequency	Corrected P-value
nucleic acid metabolism	36.6%	12.6%	7.83E-05
RNA metabolic process	32.4%	10.9%	0.00037
nucleobase-containing compound metabolic process	39.4%	15.6%	0.00038
Gene expression	35.2%	14.5%	0.00412

Research Project 4: Project Title and Purpose

Mitochondrial Biology in Cancer: A New Therapeutic Target for Killing Tumor Cells – The purpose of this study is to develop novel strategies that target mitochondrial biology to kill tumor cells. Two complementary approaches will be examined. We have recently discovered that inhibition of mitochondrial fatty acid oxidation (FAO) causes diminished oxidative phosphorylation, loss of adenosine triphosphate (ATP), and cell growth inhibition. FAO inhibitors alone and in combination will be examined for tumor cell growth inhibition, and novel *in vivo* imaging will be used to monitor fatty acid uptake into tumors. The second aim of this project is to inhibit mitochondrial dynamics in combination with cisplatin to initiate apoptotic cell death. Both *in vitro* and *in vivo* models will be examined.

Anticipated Duration of Project

1/1/2013 – 12/31/2014

Project Overview

Tumor cells have increased energy demands and alterations in their cellular metabolism in order to undergo rapid proliferation. Mitochondria are important organelles that provide energy in the form of ATP and help to convert key intermediates into cellular building blocks such as nucleotides for DNA, fatty acids for lipids, and amino acids for proteins. These organelles also have the potential to kill the cell through the release of pro-apoptotic factors. This project investigates mitochondria as a therapeutic target for the treatment of several types of cancer, including breast, ovarian, melanoma, and head and neck, among others. Since mitochondria are shared by all cells in the body, the therapeutic window of inhibiting this organelle's function might be very small, and monotherapy will have to be augmented with combinations of chemotherapies. This project will develop strategies to combine mitochondrial poisons with standard anticancer drugs to achieve efficient killing of tumor cells.

Specific Aim 1: We will test the novel hypothesis that tumor cells generate a large portion of energy through the process of fatty acid beta-oxidation. This aim will develop innovative approaches using fluorescence microscopy and positron emission tomography (PET) to visualize fatty acid beta-oxidation in living tumor cells. This aim will also test the idea that inhibitors of fatty acid beta-oxidation alone and in combination with a standard chemotherapy (cisplatin) will inhibit tumor cell growth.

Specific Aim 2: We will exploit a novel finding from our laboratory, that inhibition of an important mitochondrial protein, Drp1, in combination with administration of cisplatin causes rapid initiation of apoptosis. We will explore the effects of combination treatment with mdivi-1, a novel thio-quinazolinone derivative that targets Drp1, and cisplatin in a panel of cell lines representing multiple cancer types. We will also explore structure-function relationships of this novel inhibitor to increase treatment efficacy and examine efficacy and toxicity in a xenograft model. Finally, we will analyze the effectiveness of this treatment against ovarian cancer cells isolated from patients.

Principal Investigator

Bennett Van Houten, PhD
Richard M. Cyert Professor of Molecular Oncology, Pharmacology and Chemical Biology
University of Pittsburgh
5117 Centre Avenue
Pittsburgh, PA 15213-1863

Other Participating Researchers

Sufi Thomas, PhD; Robert Edwards, MD; Carolyn Anderson, PhD; Eric Gotzeman, PhD; Lee McDermott, PhD – employed by University of Pittsburgh

Expected Research Outcomes and Benefits

This year, one in four Americans who die will die of cancer. This project will investigate new ways to kill tumor cells. Specifically, this study will target the mitochondria of tumor cells, which provide energy and important metabolites that support cell growth. One limitation of current treatment strategies is that cancer cells have inherent or acquired resistance to the killing effects of specific chemotherapeutic agents. This project seeks to develop new strategies to overcome drug resistance by combining novel mitochondrial toxins with highly used anticancer agents such as cisplatin. Our first approach involves eliminating the ability of tumor cells to burn fatty acids in the mitochondria. Under this aim, we will visualize cellular uptake of fatty acids using fluorescent and PET tracers; and, for the first time, we will be able to track fatty acid uptake into tumor cells. We will also ask whether inhibition of fatty acid beta-oxidation in combination with standard anti-cancer therapy (cisplatin) will inhibit tumor cell growth. Our second approach is to inhibit normal mitochondrial dynamics in combination with cisplatin. We have found and patented that mdivi-1, a novel thio-quinazolinone derivative that targets a key enzyme controlling mitochondrial fission (Drp1), given in combination with cisplatin, activates apoptotic death in cells that are inherently resistant to cisplatin. This aim will test the hypothesis that cisplatin plus mdivi-1will increase the effectiveness of cisplatin and overcome both inherent and acquired resistance to cisplatin. Cisplatin resistance in ovarian cancer is a common problem that limits patient outcomes. This aim will also explore novel structure-function relationships of the parent compound (mdivi-1) to improve delivery and efficacy. Finally, this aim will include pre-clinical xenograft studies that will generate information essential to the conduct of a future phase I clinical trial. Completion of this project is expected to result in new therapeutic targets for the treatment of breast, ovarian, melanoma, and head and neck tumors.

Summary of Research Completed

Examine the effect of orlisat, an inhibitor of fatty acid synthase, on the growth of melanoma cell lines (WM-983A and WM-983B) (Aim 1)
We have made good progress on this milestone and found that orlistat is more effective on the metastatic melanoma cell line WM-983B with an IC50 of 36.5 $\pm$ 23.9, whereas the IC50 of the primary melanoma cell line, WM-983A, was 106.23 $\pm$ 25.9. Briefly, cell proliferation was determined using a CyQUANT Direct Cell Proliferation Assay kit (Invitrogen). The survival

fractions were calculated after setting untreated control cells at 100 percent. The data were plotted and curve fitted using GraphPad Prism software.

Examine the effects of etomoxir on the growth of four breast cancer cell lines (Aim 1)
Cells were seeded in a 96-well plate at a density of $2x10^3$ cells per well. Twenty-four hours later, cells were treated with etomoxir, and cell numbers were determined using the CyQUANT assay. The IC50 for etomoxir was determined 72 hours after treatment using the GraphPad Prism program. We have finished this aspect of the project for four breast cancer cell lines that differ in their tumor type, as well as a normal epithelial cell line (MCF10A). Results are shown in Table 1. These data indicate that breast cancer cell lines obtain a significant amount of energy from mitochondrial respiration fed by fatty acid beta-oxidation.

Examine the effects of etomoxir on levels of oxidative phosphorylation in these breast cancer and head and neck cancer cell lines (Aim 1)
The bioenergetic profiles of breast cancer cell lines were obtained using the Seahorse XF24-3 Extracellular Flux Analyzer. After baseline measurements, etomoxir (Eto; 50 or 100 μM) was added to the cells twice to check the effect of blocking fatty acid (FA) β-oxidation on oxygen consumption rate (OCR). We have found that etomoxir treatment results in a 50 percent reduction in OXPHOS in MDA-MB-231 triple negative breast cancer cells and a 75 percent decrease in HER2-enriched SK-BR 3 breast cancer cells (Figure 1).

Examine 18-18F-fluoro-labeled 4-thia-oleate uptake in a human xenograft tumor model of head and neck cancer cells, Cal33 (Aim 1)
We found that uptake of this tracer was not very efficient and we have stopped working on this aspect of the project.

Complete our survey of several cell lines for their sensitivity to the combination of cisplatin and mdivi-1 (Aim 2)
Overcoming platinum drug resistance represents a major clinical challenge in cancer treatment. We discovered a novel drug combination using cisplatin and a class of thioquinazolinone derivatives including mdivi-1 (mitochondrial division inhibitor-1) that induces synergistic apoptosis in platinum resistant tumor cells, including those from cisplatin-refractory end-stage ovarian cancer patients. Surprisingly, using Drp -/- SV-40 transformed mouse embryonic fibroblasts, we have found that Drp1, the supposed target of mdivi-1, is not necessary for this effect. Mdivi-1 impairs DNA replication; and its combination with cisplatin induces a synergistic increase of replication stress and DNA damage, causing a preferential upregulation of a BH3-only protein, Noxa. Mdivi-1 also represses mitochondrial respiration independent of Drp1, and the combination of mdivi-1 and cisplatin triggers substantial mitochondrial uncoupling and swelling. Upregulation of Noxa and simultaneous mitochondrial swelling causes synergistic induction of mitochondrial outer membrane permeabilization (MOMP), preceding robust mitochondrial apoptotic signaling independent of Bax/Bak. Thus, the novel mode of MOMP induction by the combination through the “dual-targeting” potential of mdivi-1 on DNA replication and mitochondrial respiration suggests a novel class of compounds for a platinum-based combination option in the treatment of platinum-, as well as, multidrug-resistant tumors.

We have completed this survey and recently published this work (Qian et al., *Oncotarget* 2014,

in press). We have found that cisplatin and midivi-1 work synergistically to kill every type of tumor cell we have examined, including ovarian cancer cells, which are resistant to cisplatin (Figure 2). MTS colorimetric survival assay was performed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI), according to the manufacturer's instructions. The survival fractions were calculated after setting untreated control cells at 100 percent after 72 hours of continuous exposure. The data were plotted and curve fitted using GraphPad Prism software.

In addition, we found that mdivi-1 alone is sufficient to cause a G2/M arrest in tumor cells but not normal cells.

Begin synthesis and screening of new thioquinazolinone compounds for synergistic killing of tumor cells with cisplatin (Aim 2)
In collaboration with Peter Wipf, PhD, (Department of Chemistry, University of Pittsburgh) we have screened 69 new compounds, whose structures resemble mdivi-1, and found 11 compounds to work synergistically with cisplatin to kill tumor cells; four of these compounds had higher efficacy (worked at lower doses) than midivi-1. We are continuing to examine how these compounds work to synergize cisplatin-induced cell killing.

Initiate our analysis of combination of mdvi-1 and cisplatin on ascites fluids from ovarian cancer patients (Aim 2)
In collaboration with Karyn Hansen, MD, and Robert Edwards, MD, from Magee-Womens Research Institute, we have found that high serous ovarian cancer cells cultured from a cisplatin–resistant patient's ascites fluid are highly sensitive to the combination effects of cisplatin and midivi-1 (Figure 3).

Examine the toxicity of mdivi-1 and cisplatin in an MDA-MB-231 human xenograft model (Aim 2)
Due to our success in treating primary ovarian cancer cells from ascites fluid, we have initiated xenograft studies with A2780 and A2780-cisplatin resistant cell lines.

<u>Related PI publications (7/1/13-6/30/14):</u>
1. de Moura MB, Van Houten B. Bioenergetic analysis of intact mammalian cells using the Seahorse XF24 Extracellular Flux analyzer and a luciferase ATP assay. *Methods in Molecular Biology* (Clifton, NJ). 2014;1105:589-602.

2. Qian W, Wang J, Van Houten B. The role of dynamin-related protein 1 in cancer growth: a promising therapeutic target? *Expert Opinion on Therapeutic Targets*. 2013;17(9):997-1001.

3. Shaw PG, Chaerkady R, Wang T, Vasilatos S, Huang Y, Van Houten B, et al. Integrated proteomic and metabolic analysis of breast cancer progression. *PloS One.* 2013;8(9):e76220.

4. Qian W, Wang J, Roginskaya V, McDermott L, Edwards R, Stolz DB, Llambi F, Green DR, Van Houten B. Novel combination of platinum drugs and mdivi-1 overcomes cisplatin resistance by synergistically inducing MOMP and apoptosis independent of Drp1. *Oncotarget* (in press, 2014)

Cell line	Tumor Type	$EC_{50} \pm LogEC_{50}$ SE
MDA-MB 231	Claudin-low	134.2 ± 2.13 μM
BT20	Basal	99.61 ± 1.20 μM
SK-BR 3	HER2-enriched	158.8 ± 2.20 μM
ZR-75 1	Luminal	113.1 ± 2.05 μM
MCF10A	"Normal" breast epithelial cells	22.6 ± 1.36 μM

Table 1. Effects of etomoxir on the growth of four breast cancer cell lines.

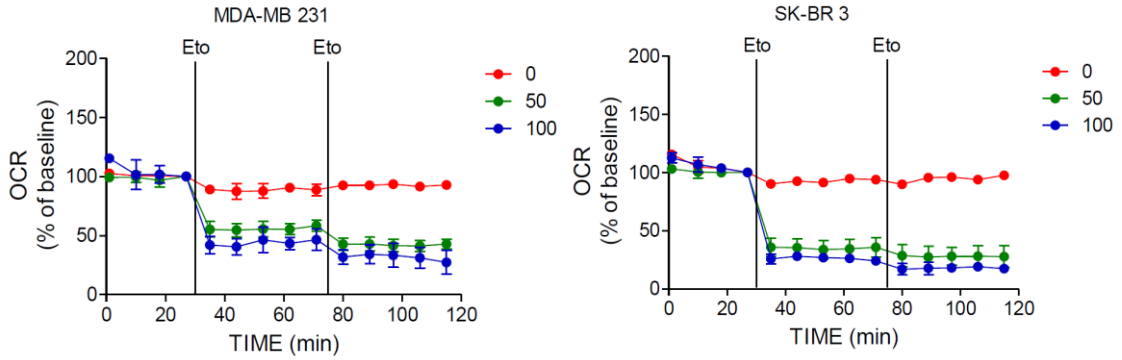

Figure 1. Etomoxir inhibits a large portion of oxidative phosphorylation (OXPHOS) in two breast cancer cell lines. Cells were placed in a Seahorse XF24-3 Extracellular Flux Analyzer; and the oxygen consumption rate (OCR), a measure of oxidative phosphorylation, was measured before and after injection of etomoxir (Eto) in the cell culture media.

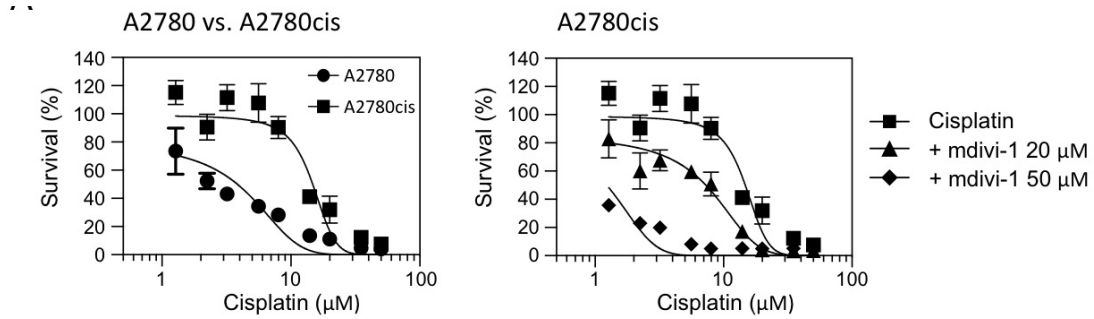

Figure 2. The combination of cisplatin and mdivi-1 efficiently overcomes acquired cisplatin resistance in human ovarian cancer cells. Left panel, the comparison of cisplatin sensitivity in ovarian cancer cells A2780 and their derivative cisplatin-resistant A2780cis cells. Right panel, mdivi-1 treatment in combination with cisplatin induces cell death of cisplatin-resistant ovarian cancer cells.

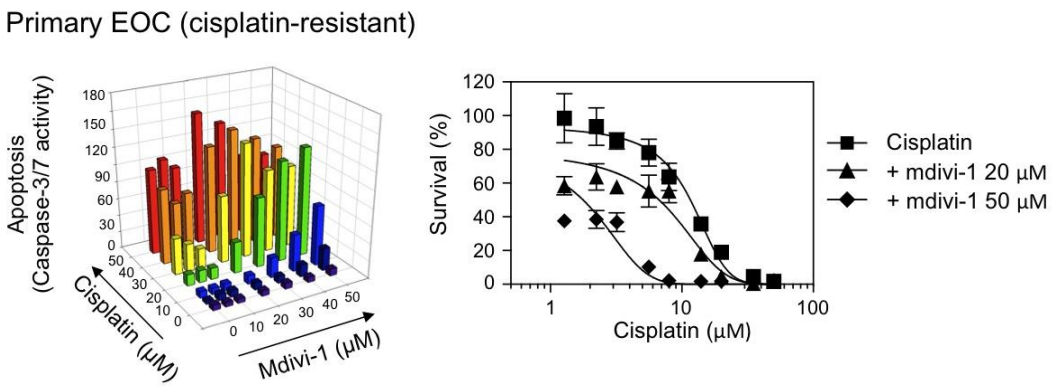

Figure 3. Left panel: Primary ovarian cancer cells derived from a cisplatin–resistant patient's ascites fluid were treated with the cisplatin/mdivi-1 drug combination for 20 hours, and a luminescent caspase 3/7 assay from Promega was used to measure apoptosis. Right panel: The same cells were treated for 72 hours, and an MTS assay was used to monitor survival.

Research Project 5: Project Title and Purpose

A Pilot Study Evaluation of Sulforaphane in Atypical Nevi – This pilot trial is designed to determine the tolerance and pharmacokinetics of broccoli sprout extract-derived sulforaphane as a melanoma preventive agent in patients with multiple atypical nevi and a history of prior melanoma.

Anticipated Duration of Project

1/1/2013 – 12/31/2014

Project Overview

Sulforaphane is a bioactive compound derived from precursor molecules found in high concentration in Brassica vegetables like broccoli and broccoli sprouts. In relation to melanoma, application of broccoli sprout extract rich in sulforaphane (BSE-SFN) decreased ultraviolet radiation (UVR) erythema response in human skin and may thus protect against the DNA-damaging effects of UVR. We are conducting a pilot trial of daily oral BSE-SFN as a non-toxic, natural chemopreventive agent for melanoma, which will be administered in groups of six patients at three dosages of 50 mcg/d, 100 mcg/d, and 200 mcg/d for 28 days each, with the goal of evaluating two objectives:

1. The primary objective is to determine the safety and clinical as well as pathological effects of BSE-SFN for patients with multiple atypical nevi and a history of melanoma.

2. The secondary objective is to document the pharmacokinetics and pharmacodynamics of BSE-SFN and the ability of oral BSE-SFN to modulate the level of signaling molecules in tissue biopsies of atypical nevi and skin in patients with multiple atypical nevi and a history of melanoma.

Principal Investigator

John M. Kirkwood, MD
Professor of Medicine, Dermatology and Translational Science
University of Pittsburgh
5115 Centre Avenue
Pittsburgh PA 15213 2584

Other Participating Researchers

Shivendra Singh, PhD; Jan Beumer, PharmD, PhD; Yan Lin, PhD; Uma Rao, MD; Jonhan Ho, MD – employed by University of Pittsburgh

Expected Research Outcomes and Benefits

The safety and tolerability of sulforaphane at the three dosages to be tested is anticipated to be proven, and the pharmacological features of this agent administered orally in cohorts of 6 patients over a period of 28 days is expected to be determined. The laboratory corollary studies proposed will determine the pharmacokinetics in blood and skin as well as atypical nevi, which are to be biopsied prior to and following the 28-day treatment interval. Evaluation of the impact of sulforaphane on clinical, pathological and molecular features will inform future phase II cooperative group studies of this compound as a chemopreventive for melanoma.

Summary of Research Completed

Accrual of participants on this study continues. As of June 2014, 12 have been screened, with six screen failures and six enrolled; the final 12 patients are anticipated to be enrolled by the end

of the year. The initial immunohistological studies of the pre- and post-treatment nevus biopsies have been piloted and reviewed with Uma Rao, MD, in the Department of Pathology. These studies, as well as the pharmacokinetic/pharmacodynamic (PK/PD) analyses of the initial six patients, are ongoing.

We have recently modified the protocol to remove the exclusion of tobacco users on this study, as there are no documented influences of tobacco usage on the catabolic and anabolic systems that pertain to sulforaphane metabolism. This exclusion was a major issue for some candidates. In addition, sub-samples of the sulforaphane-rich broccoli sprout powder were retested for potency, certified, and relabeled for another year of shelf life.

Research Project 6: Project Title and Purpose

A Phase III Study of Adjuvant Ipilimumab vs. High-Dose Interferon α-2b for Resected High-Risk Melanoma – In this clinical trial, patients with stage III or stage IV melanoma that has been completely resected and who are at high risk for recurrence and death after surgery will be randomly assigned to receive adjuvant (post-surgical) treatment with either ipilimumab (investigational agent) or high-dose interferon α-2b (HDI, the current standard of care). This study has the potential to change the management of patients with high-risk resected melanoma, if ipilimumab is shown to be superior to HDI as hypothesized.

Anticipated Duration of Project

1/1/2013 – 12/31/2014

Project Overview

This randomized phase III trial will test adjuvant therapy with the anti-CTLA4 monoclonal antibody (mAb) ipilimumab at high-dose (hIpi) and standard dose (sIpi) versus the standard high dose interferon (IFN)-α2b regimen (HDI) in patients with surgically resected stage IIIB/C and M1a/b melanoma at high risk for recurrence and mortality. This ongoing trial plans to enroll 1600 patients nationally with up to 60 patients expected to be enrolled at the University of Pittsburgh Cancer Institute (UPCI).

Specific aims:
(1) Primary Endpoints:

1. To evaluate recurrence-free survival (RFS) between melanoma patients randomized to receive post-operative adjuvant ipilimumab given at either 10 mg/kg (high dose ipilimumab; HIP) or 3 mg/kg (low dose ipilimumab; LIP) versus those randomized to receive HDI using a hierarchical design assessing HIP versus HDI first and LIP versus HDI second, if the first comparison is significant.
2. To evaluate overall survival (OS) between melanoma patients randomized to receive post-operative adjuvant ipilimumab given at either 10 mg/kg (HIP) or 3 mg/kg (LIP) versus those randomized to receive HDI using a hierarchical design assessing HIP versus HDI first and LIP versus HDI second (if the first comparison is significant).

(2) Secondary Endpoints:

1. To evaluate safety and tolerability of post-operative adjuvant ipilimumab therapy given at either 10 mg/kg (HIP) or 3 mg/kg (LIP).
2. Among patients enrolled by Community Clinical Oncology Programs (CCOPs), to compare the global quality-of-life (QOL) between the ipilimumab arms versus HDI and to evaluate the effect of treatment-related side effects that may affect the health-related domains of QOL.

Principal Investigator

Ahmad A Tarhini, MD, PhD
Assistant Professor of Medicine and of Clinical and Translational Science
University of Pittsburgh
5115 Centre Avenue
Pittsburgh PA 15213 2584

Other Participating Researchers

John Kirkwood, MD; Uma Rao, MD; Lisa Butterfield, PhD – employed by University of Pittsburgh

Expected Research Outcomes and Benefits

The current standard of care for high-risk melanoma, high dose IFN-α2b (HDI), has limited efficacy as an adjuvant therapy. Ipilimumab offers a potentially superior adjuvant therapy, and its superiority compared to HDI has already been proven in the treatment of more advanced inoperable melanoma leading to regulatory approval. In addition, this study is unique in its aggressive and systematic investment in the banking of tumor and blood specimens for use in future predictive biomarker studies. Being able to predict which patients will benefit would radically improve our approach to adjuvant therapy and allow us to avoid toxicity in the absence of expected benefit.

Summary of Research Completed

This study is still ongoing at the University of Pittsburgh Cancer Institute (UPCI). As of June 30, 2014, 54 participants have been screened at UPCI, and 33 have met study eligibility criteria and have been treated and followed on the study. Currently, 23 participants are in active treatment or follow-up. Nationally, as of July 1, 2014, 1,556 participants have been enrolled on this study.

The research completed during this period involved the identification of participants for this study, the discussion of the study procedures, and the process of securing informed consent. Participants underwent screening, and those who met eligibility criteria were registered and randomized to one of the three study treatment arms. Patients were followed per study protocol, including the coordination of all required study procedures (office visits, laboratory studies, and staging imaging studies). This is in addition to the standard clinical follow up and the management, documentation, and reporting of adverse events. In addition, the study involves

tissue and blood banking for research in preparation for the evaluation of blood, genetic, chemokine, and immunological therapeutic predictive biomarkers that may serve as predictors of benefit for ipilimumab as well as interferon alpha. The ultimate goal of such biomarker studies is to predict which patients are likely to benefit from therapy and, therefore, should be treated while saving others from unwanted toxicities and cost due to lack of predicted benefit from this form of treatment. An NIH-funded translational biomarker project was awarded during this period.

Research Project 7: Project Title and Purpose

A Phase I Study of ABT-888 in Combination with Carboplatin and Paclitaxel in Advanced Solid Tumors – The overall purpose of this trial is to determine the dose limiting toxicities and recommended phase II dose of the combination of weekly administered carboplatin, paclitaxel, and the poly (ADP-ribose) polymerase (PARP) inhibitor, veliparib (ABT-888). This proposed portion of the trial is a mandatory biopsy expansion at the recommended phase II dose in patients with triple negative breast cancer, which is postulated to have particular efficacy to this combination. Pre- and post-treatment biopsies will be analyzed for a molecular signature of faulty DNA repair mechanisms that may underlie sensitivity to this treatment.

Duration of Project

1/1/2013 – 1/7/2014

Summary of Research Completed

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

Research Project 8: Project Title and Purpose

Phase II Study of Azacitidine and Entinostat (SNDX-275) in Patients with Advanced Breast Cancer – The purpose of this trial is to determine the objective response rate, safety, tolerability, progression-free survival, overall survival, and clinical benefit rate of the combination of the DNA methyltransferase inhibitor azacitidine (5-AZA) and the histone deacetylase inhibitor entinostat in women with advanced breast cancer (triple-negative and hormone-refractory). In addition, an optional continuation stage will evaluate whether the addition of hormonal therapy to 5-AZA and entinostat provides benefit after progression while receiving 5-AZA and entinostat.

Anticipated Duration of Project

1/1/2013 – 12/31/2014

Project Overview

In preclinical breast cancer models, epigenetic modifiers lead to re-expression of aberrantly silenced genes and proteins that represent important therapeutic targets (e.g., estrogen receptor alpha, or ER). Combination therapy with a DNA methyltransferase inhibitor (DNMTI) and a histone

deacetylase inhibitor (HDACI) yields superior ER re-expression and greater restoration of tamoxifen responsiveness than either agent alone. The proposed trial is a multicenter, phase II clinical trial to evaluate the efficacy, safety and surrogate biomarkers of response to the DNMTI 5-azacitidine (5-AZA) and the HDACI entinostat in women with advanced breast cancer. Women with advanced triple-negative (ER/progesterone receptor [PR]/HER2-negative, Cohort A) or hormone-resistant (Cohort B) breast cancer receive 5-AZA at 40 mg/m^2 subcutaneously (days 1-5, 8-10) and entinostat at 7 mg orally (days 3, 10) every 28 days. The primary endpoint is objective response rate (ORR) in each cohort. Secondary endpoints include safety, tolerability, survival, and clinical benefit rate. Exploratory endpoints include pharmacokinetics, pharmacogenetics, and change from baseline of candidate gene re-expression/methylation in circulating DNA and mandatory tumor samples. Because of the potential for restoration of hormone sensitivity, patients are offered continuation of study therapy at progression with addition of hormonal therapy (an optional continuation phase). This trial has a Simon two-stage design, with interim analysis being performed after 13 patients are enrolled per cohort (1st stage). If ≥ 1 response is observed, patient accrual will continue for a total of 27 per cohort (2nd stage). The null hypothesis is that ORR is at most 5%, against the alternative hypothesis that ORR is at least 20%, with type I error of 4% and power of 90%.

Specific Aim 1: *Clinical trial*
Accrual is ongoing at our institution for this phase II study of 5-AZA and entinostat in patients with advanced breast cancer.

Specific Aim 2: *Evaluation of biopsy samples*
Exploratory endpoints for this aim include pharmacokinetics, pharmacogenetics, and change from baseline of candidate gene re-expression/methylation in circulating DNA and mandatory tumor samples.

Specific Aim 3: *Methylation analysis of primary tumors*
Perform methylation analysis on primary tumors and determine whether the primary and metastatic lesions have a similar profile.

Principal Investigator

Rachel C. Jankowitz, MD
Assistant Professor of Medicine
University of Pittsburgh
300 Halket Street, Room 3526
Pittsburgh, PA 15213

Other Participating Researchers

Vered Stearns, MD – employed by Johns Hopkins University, Baltimore, MD

Expected Research Outcomes and Benefits

The expected outcome of this trial is to establish the objective response rate (ORR) in each cohort of patients with advanced breast cancer who are treated with 5-AZA and entinostat. The

secondary endpoints will be to examine the safety, tolerability, survival, and clinical benefit rate of the combined therapy. The unique feature of this trial is the mandatory biopsies of metastatic sites at baseline and after cycle 2, which will allow for exploratory scientific endpoints of change from baseline of candidate gene re-expression/methylation in the tumor samples. By taking advantage of the synergy between HDAC and DNMT inhibitors, we hypothesize that clinically tolerable doses may translate not only into changes in gene methylation, but also disease responses.

Summary of Research Completed

This multicenter, phase II clinical trial evaluating the efficacy, safety, and surrogate biomarkers of response to the DNA methyltransferase inhibitor (DNMTI) 5-azacytidine (5-AZA) and the histone deacetylase inhibitor (HDACI) entinostat in women with advanced triple negative or hormone resistant breast cancer completed enrollment and closed to accrual on September 30, 2013. A total of 40 evaluable patients were enrolled. Three patients at our institution are still being followed, with data being collected every three months. Results from the triple-negative cohort A (13 evaluable patients) were reported last year and were presented at the 2013 annual meeting of the American Association for Cancer Research (AACR), Abstract #4666.

The hormone resistant cohort B enrolled 27 patients, as one partial response was observed in the first stage, allowing for the cohort to be expanded to the second stage. Results from this cohort were recently presented in poster format at the 2014 American Society of Clinical Oncology (ASCO) annual meeting (Abstract #569, "Phase II study investigating the safety, efficacy, and surrogate biomarkers of response to 5-azacitidine (5-AZA) and entinostat in advanced breast cancer.") Patient characteristics are shown in Table 1.

For the 27 patients enrolled in the hormone resistant cohort, the median follow-up is 10.4 months (range 0.5-28.2 months). In the optional continuation phase, 12 patients received a median of 2.5 additional cycles (range 1-9), with one partial response after progression in the primary phase. The response rate was 4 percent (0-19 percent) in this cohort. The median progression-free survival (PFS) was 1.8 months (1.7-1.9 months). In the 12 patients who went on to the optional continuation phase, the response rate was 8 percent, with a median PFS of 1.9 months (1.7-3.7 months) and a median time to death (TTD) of 13.9 months since the time of their progression. In the 14 patients who went on to the event monitoring cohort (who did not proceed to the optional continuation phase), the median PFS was 1.8 months (0.8-1.9), with a median TTD of 7.5 months.

Of the 95 percent of baseline and mandatory biopsies collected; 60 percent of two-month mandatory biopsies were collected and 58 percent of matched biopsies were obtained. Pharmacokinetics analysis was completed in 24 patients. Exposure to 5-AZA (Cmax= 634 ± 286 ng/mL; AUCinf= 730 ± 248 ng*h/mL) and entinostat (Cmin= 0.53 ± 0.42 ng/mL) was similar to previous studies. The most notable adverse events observed on study in cohort A (triple-negative cohort) and cohort B (hormone-resistant cohort) are summarized in Table 2.

Conclusions:
This trial demonstrated successful accrual to a study that mandated tumor biopsies. The

combination of 5-AZA and entinostat was well tolerated; but the primary endpoint, response rate, was not met. Results suggest that some women benefit from epigenetic therapy and/or reintroduction of endocrine therapy beyond progression.

The median time to death observed for the optional continuation phase may reflect selection bias. Ongoing efforts in future studies are planned to determine whether this epigenetic approach may lead to estrogen receptor re-expression or sensitization to subsequent treatments.

Table 1: Patient Characteristics

Characteristic	Hormone Resistant	Triple Negative*
Number enrolled	27	13
Median Age (range)	55 (35-70)	47 (31-67)
Race		
White Black Other	21 (78%) 3 (11%) 3 (11%)	10 (77%) 3 (23%) 0 (0%)
Median no. prior therapies (all settings)		
Hormonal Cytotoxic	3 3	0 3
Location of disease		
Visceral Non-visceral Both	3 (11%) 3 (11%) 21 (78%)	3 (23%) 3 (23%) 7 (54%)
Optional Continuation Phase		
Number (n%)	12 (44%)	4 (31%)

*AACR 2013, Abstract 4666

Table 2: Safety Analysis				
Adverse Event	Cohorts A and B		Optional Continuation	
	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4
Anemia	32 (97%)	1 (3%)	13 (100%)	--
Leukopenia	20 (71%)	8 (29%)	10 (83%)	2 (17%)
Neutropenia	11 (55%)	9 (45%)	4 (57%)	3 (43%)
Thrombocytopenia	10 (100%)	--	2 (100%)	--
Injection site reaction	29 (100%)	--	12 (100%)	--
Fatigue	28 (93%)	2 (7%)	8 (100%)	--
Nausea	24 (96%)	1 (4%)	6 (1%)	--
Anorexia	10 (100%)	--	4 (100%)	--
Hypophosphatemia	7 (78%)	2 (22%)	3 (75%)	1 (25%)

Research Project 9: Project Title and Purpose

Bevacizumab, Metronomic Chemotherapy, Diet and Exercise after Preoperative Chemotherapy for Breast Cancer – The purpose of this phase II trial is to explore the role of anti-angiogenic therapy using bevacizumab combined with metronomic chemotherapy in patients with residual disease after neoadjuvant chemotherapy to determine the efficacy of these treatments. All patients will also be randomized to one of two lifestyle interventions to explore the impact of changes in diet and exercise on relevant biomarkers in a breast cancer survivor population. Given the lack of standard options in this clinical setting, it is hoped that the proposed novel biologic therapy will improve breast cancer outcomes in this high-risk patient population.

Duration of Project

1/1/2013 – 1/7/2014

Summary of Research Completed

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

Research Project 10: Project Title and Purpose

Antigen-Engineered Dendritic Cell Vaccine +/- IFNα-2b Boost for Melanoma – This project tests an improved dendritic cell (DC) vaccine for melanoma that is designed to promote *in vivo* cross-presentation and determinant spreading. We are engineering the DC with three full-length, defined, tumor antigens to activate multiple $CD8^+$ and $CD4^+$ T cell clones; providing antigen presentation for the life of the DC; providing $CD4^+$ T cell help to the $CD8^+$ T cells; using a matured DC; activating innate immunity via natural killer (NK) cell migration and activation; and boosting with systemic interferon alpha (IFNα). Together, this vaccine strategy should more potently activate a polyclonal anti-tumor response, which we predict will lead to more patients who develop determinant spreading and a significant clinical response.

Anticipated Duration of Project

1/7/2014 – 12/31/2015

Project Overview

Improved approaches for treating metastatic melanoma are needed. The newly-approved antibody immunotherapy, ipilimumab, yields durable responses in only up to 10 percent of patients. The small molecule BRAF inhibitor, vemurafenib, induces dramatic responses of BRAF-mutant melanoma that endure only 5.3 months. Dendritic cell (DC) vaccines produce durable responses in a minority of patients (4.2-7.1 percent). More recently, cancer vaccines have shown statistically significant improvements in survival in cancer patients. Immunologic vaccine interventions are ideal given their specificity and potential applicability at earlier stages of disease, but improved vaccine designs and combination approaches are needed to move the field forward and improve the frequency of the durable responders observed. We have created a novel genetically engineered DC vaccine that is being tested in a phase I clinical trial.

Aim 1: Completion of the AdVTMM2/DC +/- interferon (IFN) α clinical trial. We will test: 1a) clinical outcomes in the stage IV patients; 1b) immunologic outcomes (from blood and tumor infiltrating lymphocytes [TIL] at baseline, post-vaccine, and post-IFNα); and 1c) adenovirus (AdV)-specific cellular and humoral responses.

Aim 2: Mechanism and biomarkers of clinical response. We will perform: 2a) DC vaccine transcriptional profiling (+/- maturation, +/- AdVTMM2); 2b) tumor transcriptional profiling (baseline, post-vaccine, and post-IFNα); 2c) peripheral blood signaling induced by IFNα (signal transducers and activators of transcription); 2d) serum profiling (autoimmunity antibodies, lactate dehydrogenase, C-reactive protein cytokines); 2e) molecular mimicry with mycoplasma (impact of baseline memory to mycoplasma); and 2f) germline DNA SNP analysis.

Aim 3: Mechanism of natural killer (NK) cell "activation" in anti-melanoma immunity. We will perform *in vitro* studies to expand our recent findings in AdV/DC-NK cell cross-talk with banked melanoma patient blood and tumor cells by examining: 3a) direct NK cell interactions with melanoma tumor cells (cytotoxicity, cytokines); 3b) helper/type 1 skewing role in shaping adaptive $CD8^+$ and $CD4^+$ T cell responses; and 3c) melanoma tumor impact on NK cell function.

Principal Investigator

Lisa H. Butterfield, PhD
Professor of Medicine, Surgery, and Immunology
University of Pittsburgh
5117 Center Avenue, Suite 1.27
Pittsburgh, PA 15213

Other Participating Researchers

Mary Jo Buffo, MBA; Veronica Sutherland, BS – employed by the University of Pittsburgh Medical Center

Expected Research Outcomes and Benefits

Our new immunotherapy vaccine clinical trial and three research aims have the potential to change clinical practice for melanoma. First, we will take full advantage of state-of-the-art assays to examine genetic, transcriptional, cell surface, and secreted molecules that may identify critical biologic attributes of patient tumors, autologous dendritic cell (DC) vaccines, and their ability to respond to interferon (IFN)α-2b that correlate with clinical outcomes at baseline and/or directly after treatment. Second, we are well-positioned to dissect the role of *in vivo* cross-presentation in anti-tumor immunity by combining assays of vaccine-encoded antigen, non-vaccine shared antigen, and tumor cell immunity with clinical autoimmunity measures and unbiased screens of tumor gene expression. Third, after completion of these new studies, we will have established whether IFNα-2b is valuable as a DC vaccine adjuvant and whether we have improved immunologic and clinical responses over our previous DC vaccines. Either result will support phase II testing of the superior vaccine regimen. We are centrally involved in vaccine immunotherapy trials across the cooperative groups and can expand in that direction. Last and most important, we hope to directly reduce or eliminate melanoma disease progression in vaccinated patients.

Summary of Research Completed

During the period of 1/7/14-6/30/14, we made the following progress on the clinical trial: Melanoma patients #9 through #20 were screened and enrolled into the trial. All enrolled patients were scheduled for their blood draws and vaccines. During this time, 25 vaccines were scheduled, and 19 of 25 were successfully completed (manufactured and delivered). In addition, blood samples for analysis (from 14 scheduled time points) were obtained, processed for serum and peripheral blood mononuclear cells (PBMC) and banked cryopreserved, in liquid nitrogen (PBMC) or at -80°C (serum). Fresh, whole blood flow cytometric analysis was performed to test for absolute counts and percentages of $CD3^+$ T, $CD19^+/CD20^+$ B, and $CD56^+$/CD16 NK cells, $CD14^+$ monocytes, and both monocytic and plasmacytoid dendritic cells, suppressive $CD3^+/CD4^+/CD25^{hi}/FOXP3^+$ regulatory T cells, and myeloid-derived suppressor cells and the activation states (CD69) of these immune cells.

Each DC vaccine has been tested for the planned phenotypic assessments and sterility to confirm the safety and identity of the vaccines. They express MHC class II, CD80, CD86, CD40, CD11c, CD83 and CCR7. They are also tested for production of IL-12p70 and IL-10. We have also tested the percentage transduction efficiency to confirm that sufficiently high levels of each viral vector-encoded melanoma antigen (tyrosinase, MART-1, MAGE-A6) are expressed. Because these levels have been lower than desired, the laboratory is testing optimization steps in the manufacture process, including reduced transduction volume.

The budgeted funds for the new equipment were spent as proposed. The new 10-color flow cytometer, the BD Fortessa, was delivered and set up; and the laboratory flow cytometry technologist, Jennifer Muzzio, has had her BD flow cytometer user training. She and Ms. Buffo are moving our existing flow cytometry panels (in 3-4 colors) to new, more efficient 8-10 color panels. This process will require several months but will result in more information from fewer patient cells. The whole blood staining machine, the BD "SPA" (Sample Prep Assistant), was also delivered, the lab staff has been trained, and this updated piece of equipment is now in use. Work on Aims 2 and 3 for the clinical trial has not yet begun.

Research Project 11: Project Title and Purpose

Research Infrastructure: Biomedical Science Tower 10th Floor East Laboratory Renovation – The purpose of this project is to renovate approximately 25,000 square feet of laboratory space on the 10th floor of the Thomas E. Starzl Biomedical Science Tower (BST). The BST, a biomedical research facility providing nine floors of dedicated laboratory space, is located in the heart of the University of Pittsburgh campus. The proposed design will renovate the eastern half of the 10th floor. The renovated space will be used to create and expand critical research facilities for the Department of Immunology. The proposed design will create a consolidated, modern, flexible, open laboratory space with associated support spaces.

Duration of Project

1/7/2014 – 6/30/2014

Project Overview

The Thomas E. Starzl Biomedical Science Tower (BST), a biomedical research facility providing nine floors of dedicated laboratory space, is located in the heart of the University of Pittsburgh campus. Constructed in 1989, this 20-year-old facility suffers from aging infrastructure, deteriorating interior construction, and an outmoded layout of cloistered laboratories with no natural lighting, limited opportunities to foster collaboration through proximity of research and shared resources, and no flexibility to respond to changing research priorities and instrumentation needs.

The specific aim of this project is to modernize and expand existing laboratory space for the Department of Immunology to promote collaboration and open resource sharing.

Principal Investigator

Arthur S. Levine, MD
Senior Vice Chancellor for the Health Sciences,
John and Gertrude Petersen Dean of Medicine, and
Professor of Medicine and Molecular Genetics
University of Pittsburgh
3550 Terrace Street
Pittsburgh, PA 15261

Other Participating Researchers

None

Expected Research Outcomes and Benefits

The proposed renovations will reconfigure approximately 23,000 gross square feet (GSF) of space on the 10th floor of the BST to create a consolidated, modern, flexible, open laboratory space with associated support space and chemical fume hood alcoves. These changes will increase the efficiency of space usage, incorporate natural lighting, modernize utility distribution systems, improve overall workflow, and create a dynamic environment for open resource sharing, which will foster scientific collaboration and communication and facilitate faculty recruitment.

The renovated space will be used to modernize, consolidate and expand critical research activities of the Department of Immunology under the new leadership of Mark Shlomchik, MD, PhD.

Summary of Research Completed

We completed renovations to 23,000 GSF of laboratory space. The renovation revamped the east side of the floor by creating contiguous swaths of flexible, open biomedical research laboratories that annex the perimeter corridor while maintaining support laboratory functions that require segregation in an adjacent, easily accessible zone. The renovation modernized utility distribution systems with updated, energy-efficient components and replaced 20-year-old fixed casework systems with flexible systems that can be easily altered to accommodate changing needs for space and workers with physical disabilities. This renovation allowed the University to reassign and expand research programs to enhance collaboration, resource sharing, and proximity of compatible functions. The renovation increased space efficiency on the floor plate and enhanced the recruitment of new faculty and staff.

Recruitment details are listed below:

Mark J. Shomchik, MD, PhD, recruited from Yale University, was named chair of the Department of Immunology in July 2013. Dr. Shlomchik's research interests focus on autoimmunity, blood transfusion and bone marrow transplantation, B cell immune responses, and

transgenic animal models for human disease. Dr. Shlomchik is perhaps best known for his work on systemic autoimmune diseases such as lupus.

He currently holds four NIH R01 grants, and he has two pending NIH grants. His research is also supported by Merck, Roche, the Lupus Research Institute, a program project grant, a P30 grant, and additional co-investigator support.

Louise D' Cruz, PhD, studied in the laboratory of Ludger Klein, PhD, at the Institute of Molecular Pathology (IMP), Vienna, Austria, where she focused on the factors required for CD4+ regulatory T (Treg) cell development and survival. Dr. D'Cruz is currently the recipient of a prestigious K99/R00 Transition to Independence Award from the National Institute of Allergy and Infectious Diseases, which runs through 2016. Dr. D'Cruz will have progressive involvement in the teaching of graduate and medical students.

Dario Vignali, PhD, was recruited from St. Jude Children's Hospital because of his outstanding and well-funded work on the negative regulatory mechanisms of immune control, expertise that was lacking in our immunology community. We expect that he will invigorate this area of research and stimulate numerous collaborations with and among faculty in several departments and the University of Pittsburgh Cancer Institute (UPCI). He will be coleader of UPCI's Cancer Immunology Program. He will also codirect a newly expanded Tumor Microenvironment Center.