University of Pennsylvania

Annual Progress Report: 2012 Formula Grant

Reporting Period

July 1, 2013 – June 30, 2014

Formula Grant Overview

The University of Pennsylvania 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

HLTF Silencing: A Novel Determinant of Sensitivity to Autophagy Inhibition – Autophagy is a new therapeutic target in cancer, and we have identified HLTF as a potential marker of sensitivity to autophagy inhibitors. The overall goal of this proposal is to understand the mechanistic underpinnings that link HLTF and autophagy, and develop a new predictive assay for HLTF gene silencing that will potentially have a major impact on the development of clinical trials and the treatment of diseases such as colon cancer, lung cancer, gastric cancer, melanoma, and pancreas cancer, for which HLTF is known to be or is likely to be silenced in a high proportion of patients.

Anticipated Duration of Project

1/1/2013 – 12/31/2013

Project Overview

Autophagy is a lysosome-dependent degradative process that protects cancer cells from metabolic and therapeutic stress. Autophagy is upregulated in most advanced cancers and is a new target for cancer therapy. Autophagy inhibition with chloroquine (CQ) derivatives augments the efficacy of many anticancer therapies in preclinical models. Clinical trials are testing the combination of anticancer agents with hydroxychloroquine (HCQ), but there currently is no biomarker that can identify patient subsets that will most likely benefit from this strategy.

A microarray analysis of differentially expressed genes in HCQ-sensitive and HCQ-resistant human cancer cell lines found that the most down regulated gene in HCQ-sensitive cells was helicase-like transcription factor (HLTF), a tumor suppressor gene involved in multiple aspects of maintaining genomic integrity during replication stress. HLTF expression is silenced by promoter methylation in 20-40% of lung, colon, and gastric carcinomas. HLTF gene silencing was found almost exclusively in cell lines that were sensitive to HCQ. Forced expression of HLTF in HLTF silenced cells conferred resistance to HCQ.

Specific aim 1: Characterize the molecular pathway that links autophagy inhibition and HLTF.
Specific aim 2: Optimize an MSP assay that predicts loss of expression of HLTF
Specific aim 3: Determine if HLTF silencing predicts response to therapy in 3 HCQ trials.

This proposal will test the hypotheses that a) HLTF gene silencing confers sensitivity to autophagy inhibitors by allowing HCQ-induced oxidative DNA damage to go unrepaired (aim1); b) An assay for HLTF promoter methylation can be a sensitive and specific assay for HLTF gene silencing (aim2) c) HLTF gene silencing is common in a number of malignancies and predicts clinical response in patients treated with HCQ (aim 3).

Knowledge gained from this work will establish a new mechanistic framework that links autophagy inhibition to the DNA damage response. As novel autophagy inhibitors are currently being developed for clinical trials in cancer patients, completion of these aims will provide the supporting data necessary for future development of a CLIA approved predictive assay that could be used to enroll the patients most likely to benefit from autophagy inhibitors.

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

There are over 100 publications that support the notion that autophagy is a therapeutic resistance mechanism in cancer. There are over 15 in vivo studies spanning many malignancies that found augmented antitumor activity by combining existing cancer therapies with HCQ. Based on this large body of evidence, our group and others have launched clinical trials combining cancer therapies with HCQ (which is safer to dose escalate than CQ). Preliminary results indicate these combinations have activity, and meanwhile there have been a number of autophagy inhibitor programs launched by pharmaceutical companies including Millenium, Lilly, and Roche. Novel autophagy inhibitors will be entering clinical trials in 3-5 years. We ourselves have synthesized a more potent lysosomal autophagy inhibitor, Lys05, which has single agent activity in a number a xenograft models. Lys05 is currently being reviewed by a number of pharmaceutical companies for development potential.

Unfortunately there are no biomarkers available to guide development of clinical trials involving autophagy inhibitors. The current strategy of conducting clinical trials without preselecting patients may be reasonable for phase I trials, but the conduct of a limited number of phase II

trials in an unselected patient population has a low likelihood of finding the tumors that are most sensitive to autophagy inhibitors. A predictive biomarker will help preselect patients most likely to benefit from autophagy inhibition. This proposal will have a *significant impact* because identification of a biomarker such as HLTF methylation, that is present in high fractions of cancer patients, has a reliable non- invasive assay, and provides mechanistic insight into the effects of HCQ would represent a major step in targeting autophagy.

Summary of Research Completed

Work was performed towards the completion of Aim 1, stated above. For Aims 2 and 3, alternative funding has been secured and no State funds will be expended to accomplish them. A summary of research completed towards Aim 1 is below.

Aim 1: Characterize the molecular pathway that links autophagy inhibition and HLTF.

There is a wide spectrum of sensitivity to single agent HCQ in human cancer cell lines. To determine markers of sensitivity to HCQ, we performed an mRNA microarray analysis to compare differentially expressed genes in the following human cancer cell lines: HCQ-S (IC50 <20 µM) HT29, PC9 versus HCQ-R (IC50 > 20 µM) HCT15, HCC827. Principle component analysis demonstrated that gene expression signatures for lung (HCC827, PC9) and colon (HT29, HCT15) cancer, and HCQ sensitivity or resistance were distinct. The gene expression differences between HCQ-S and HCQ-R cells were more marked in the colon cancer cells compared to the lung cancer cells. Our algorithm for analysis first determined which genes were differentially expressed in both HCQ-S cells (HT29 and PC9) versus HCQ-R (HCT15 and HCC827) to generate list A. To generate list B, we enriched list A for genes that were up- or down-regulated compared to HCQ-R cells to a larger degree in the HCQ-S colon compared to the HCQ-S lung cancer cells. Finally, we enriched list B for genes with the largest fold difference in expression between HT29 and HCT15 colon cancer cells (list C). This algorithm identified *HLTF* as a gene that was downregulated up to 46-fold in HCQ-S compared to HCQ-R cells. HLTF (also known as Smarca3) is a member of the SWI/SNF family and is the human homologue of RAD5. Like its sister enzyme SHPRH, HLTF contains a RING finger domain, snf2 family helicase domain, and a DNA binding HIRAN domain. HLTF could maintain genomic integrity by serving as a: 1) DNA helicase, 2) recruiter of DNA polymerases involved in TLS, 3) ubiquitin ligase for PCNA, and 4) a transcription factor. The 3 pieces of evidence that implicate HLTF as a tumor suppressor gene are 1) HLTF protein has multiple functionalities that protect the genome, 2) it is frequently methylated and silenced in malignancy, and 3) HLTF gene silencing has been correlated with higher risk of recurrence and poor overall survival in multiple malignancies. For instance, between 34-55% of colon and gastric adenocarcinomas harbor silenced HLTF and this inactivation occurs early in carcinogenesis. HLTF promoter methylation is never present in normal tissues. In colon cancer, HLTF methylation overlaps with, but is a distinct phenotype from the CpG island methylator phenotype.

We then determined the promoter methylation status in colon cancer cells that were either HCQ-S or HCQ-R. Using methylation specific PCR (MSP) we identified cell lines with methylated (BE, WiDR, HT29, KM12) and unmethylated (HCT15, HCT116) HLTF promoter (Figure 1A). Promoter methylation status correlated with HLTF protein expression (Figure 1B). HLTF

promoter methylation and expression correlated with IC50 (Figure 1C). In a larger panel of human cancer cell lines quantification of HLTF protein expression correlated significantly with HCQ sensitivity (Figure 1D).

Next we isolated stable HT29 transfectants expressing either vector control or HLTF cDNA. HT29-HLTF and PC9-HLTF cells were significantly more resistant to HCQ than HT29-vector and PC9-vector cells (Figure 2A). Treatment of HT29 cells with the demethylating agent 5-azacytidine also converted an HCQ-S into HCQ-R cells (Figure 2B). We found that knockdown of HLTF using shRNA in HCQ-R cells conferred sensitivity to HCQ. HLTF gene silencing predicted sensitivity to a number of other lysosomal autophagy inhibitors including Lys05 (Mcafee, PNAS 2012), and bafilomycin, but not to the proximal autophagy inhibitor spautin.

HLTF silencing, either in native lung cancer cell lines, or in paired HT29-vec and HT29-HLTF colon cancer cells predicted sensitivity to autophagy inhibition even when HCQ was combined with multiple clinical grade Pfizer targeted therapies (PI3K/mTORi, FAKi, IGFRmAb) that each targeted different signaling pathways (Figure 3).

Autophagy inhibition produces ROS-dependent DNA damage

Autophagy inhibition produced ROS equivalently in HLTF-e and HLTF-si cells treated with HCQ (Figure 4A). In HT29 cells HCQ treatment resulted in increased levels of phospho-H2AX, a marker of DNA DSBs, at 100-fold lower doses than in HCT15 cells (Figure 4B). DNA fragmentation was observed in HLTF-si but not HLTF-e cells treated with HCQ (Figure 4C). Knockdown of the low fidelity TLS DNA polymerase pol η completely abrogated the protective effect of HLTF overexpression on HCQ-induced cell death (Figure 4D). These data indicate that HLTF's role in recruiting TLS and preserving genomic integrity is the critical mechanism of resistance to HCQ-associated DNA damage- induced cell death. Next, the effects of caspase inhibition with and without HLTF overexpression on HCQ- associated DNA damage and growth impairment was tested. In HT29-vector cells caspase inhibition blunted HCQ-induced phosphoH2AX and reduced HCQ associated growth impairment (Figure 4D, E). However overexpression of HLTF produced more significant reduction of phospho-H2AX and growth impairment than caspase inhibition. Combined overexpression of HLTF and caspase inhibition completely abrogated HCQ-associated DNA damage. These results indicate that lysosomal autophagy inhibition produces ROS induced DNA damage, which can be repaired in the presence of HLTF and support our molecular model (Figure 5).

HLTF expression promotes resistance to HCQ in vivo

During the latter half of 2013 we performed an in vivo experiment by growing HT29 vector and HT29 HLTF xenograft tumors in the flanks of nude mice and treating these mice with HCQ or vehicle. In HT29vector tumors there was dramatic tumor shrinkage whereas in HT29HLTF tumors HCQ had no effect, providing in vivo confirmation that HLTF expression predicts resistance to HCQ (Figure 6).

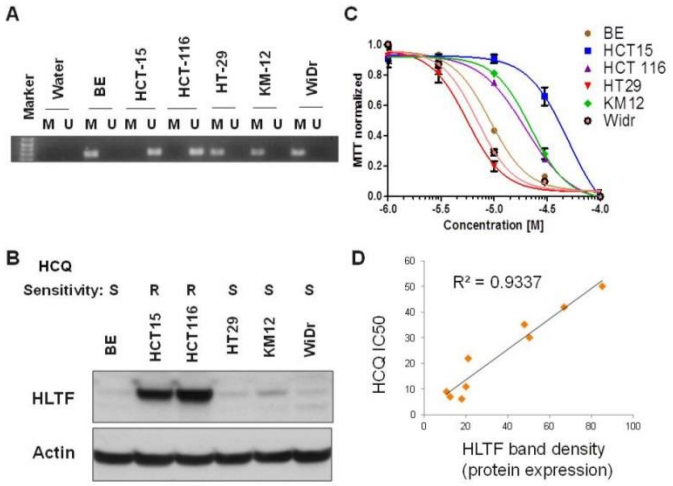

Figure 1. Promoter methylation is associated with low expression of HLTF and sensitivity to HCQ. (A) Methylation specific PCR for HLTF promoter region in a panel of colon cancer cell lines (B) Immunoblotting against HLTF in colon cancer cells lines (C) MTT assay for a range of HCQ doses at 72 hours (D) HCQ IC50 correlates significantly with HLTF protein expression

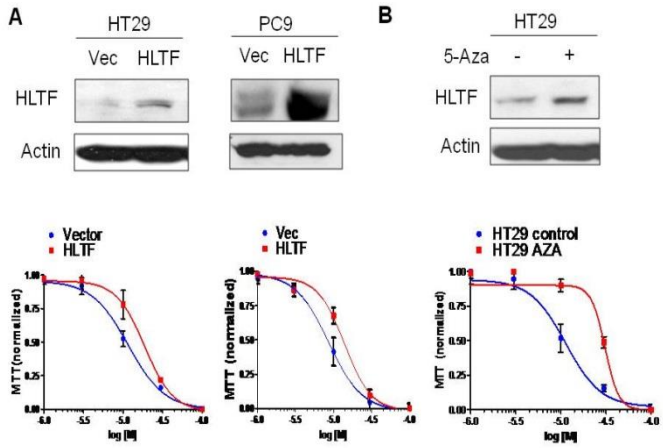

Figure 2. Forced expression of HLTF in HLTF-si cell lines confers resistance to HCQ treatment. (A) immunoblotting and MTT assay of stable transfectants (B) HCQ treatment +/- 5 azacytadine

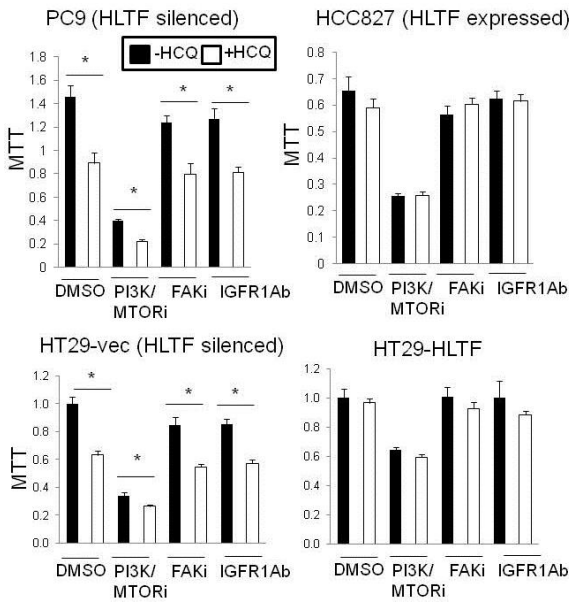

Figure 3. HLTF predicts sensitivity to combinations of targeted therapy and autophagy inhibition. 72 hr MTT assay of indicated cell lines. Cells were treated with Pfizer Inhibitors (i) (all 500 nM) PI3K/mTORi: PF4691502 ; FAKi: PF562271 ; IGFRAb: CP751871 +/- HCQ 5 µM (white). * p<0.05.

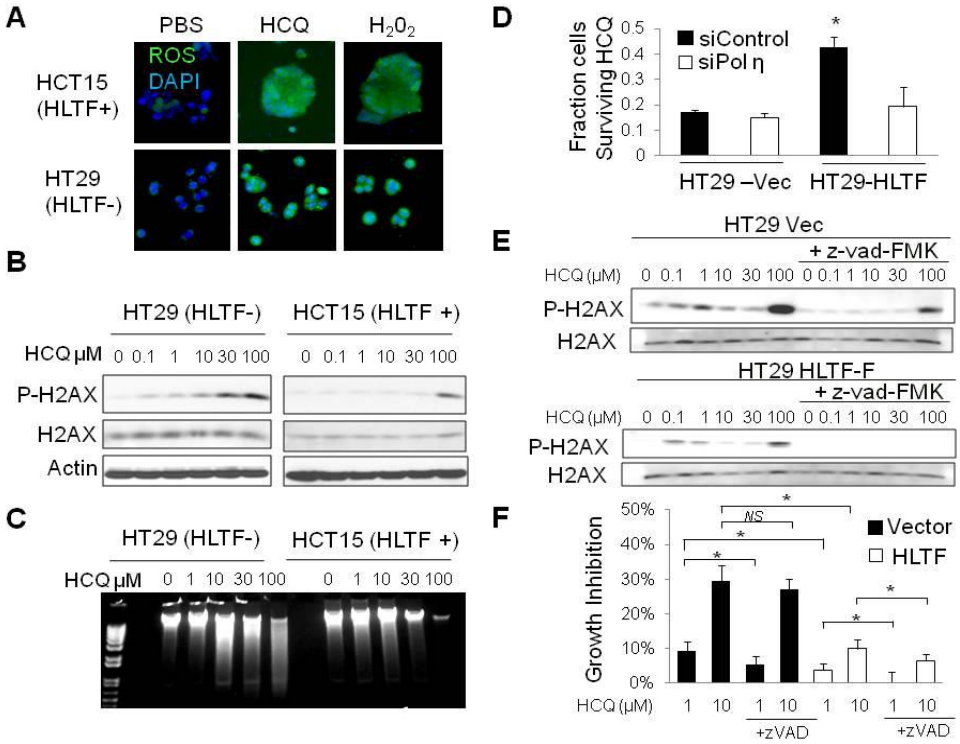

Figure 4. HCQ-associated ROS produces DNA damage that is either repaired by HLTF-pol η, or that results in DSBs and apoptosis. (A) ROS is produced by HCQ irrespective of HLTF status. Phosphorylation of H2AX (B) and DNA fragmentation (C) is more striking in HLTF- than HLTF+ cells. (D) Knockdown of pol η abrogates HCQ resistance achieved by overexpression of HLTF. (E) HCQ-induced phosphorylation of H2AX is blunted with caspase inhibitor z-VAD-FMK (top) or expression of HLTF (bottom left), and is completely absent with combined HLTF expression and caspase inhibition (bottom right) (E) HCQ-induced growth impairment is reduced with zVADFMK, but more significantly by overexpression of HLTF (white); *p<0.05.

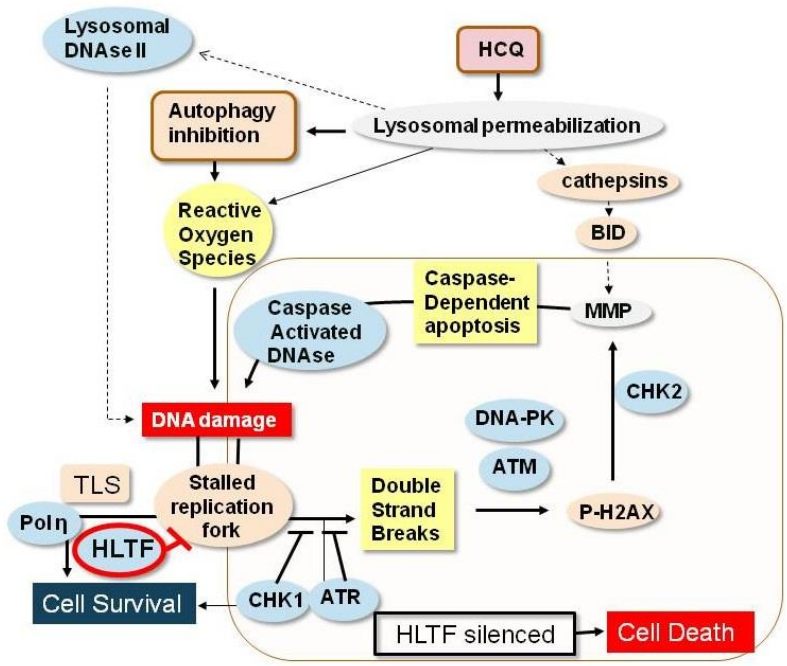

Figure 5. Potential molecular pathways connecting lysosomal autophagy inhibition, DNA damage and HLTF. Solid lines indicate the proposed model. Dashed lines indicate alternative models. MMP: mitochondrial membrane permeabilization. TLS: translesion synthesis

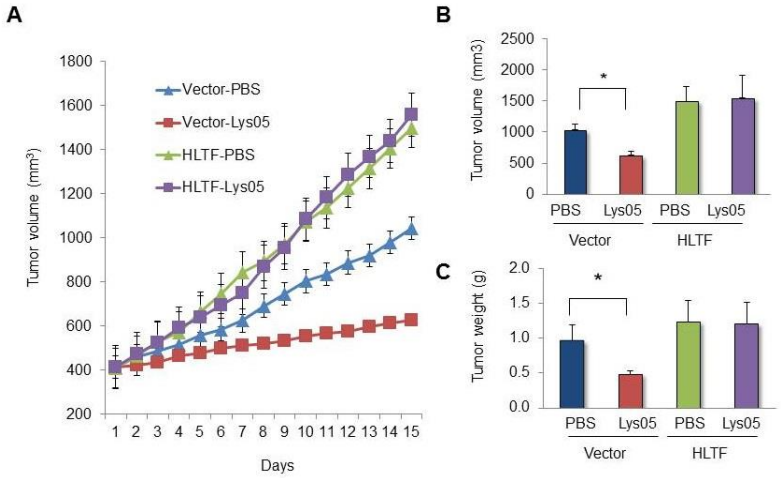

Figure 6. In vivo evidence that HLTF silencing predicts sensitivity to chloroquine derivatives. (A) HLT29 vector and HT29 xenografts were generated in the flanks of nude mice and treated with Lys05 20 mg/kg/day ip for 15 days or PBS. (B) Tumor volume on day 15 (C) Tumor weight on day 15. *P<0.05

Research Project 2: Project Title and Purpose

Molecular Pathways of T Lineage Lymphomas – The proposed research project will provide novel insights and greater understanding of molecular mechanisms through which tumor suppressor proteins cooperate to prevent development of T cell acute lymphoblastic leukemia (T-ALL). Elucidating mechanisms of T-ALL initiation and progression is critical to the

development of treatments for patients with therapy-resistant or relapsed disease. The knowledge and mouse models gained through our studies could lead to more effective clinical management of an aggressive subset of lymphoid malignancies.

Anticipated Duration of Project

11/10/2014 – 12/31/2016

Project Overview

The goal of this research project is to elucidate how DNA damage response (DDR) mechanisms prevent cancer. The ATM tumor suppressor gene controls the DDR by coordinating DNA repair with proliferation and survival to maintain genomic stability and suppress cancer. In humans and mice, ATM deficiency causes T-cell acute lymphoblastic leukemia (T-ALL), which arises from developing T-cells and includes tumor sub-types with TCR translocations. We found that ATM-deficient ($Atm^{-/-}$) mice develop T-ALLs containing *Tcr*δ translocations that delete *Bcl11b*. Since BCL11B is a haploinsufficient tumor suppressor that exhibits ATM-independent DDR functions, we hypothesize that *ATM* inactivation combined with reduced *BCL11B* expression creates a mutant DDR phenotype that drives thymocyte transformation. Yet, we hypothesize that this DDR phenotype can be exploited to develop novel T-ALL therapies that target cancer cells while minimally affecting normal cells.

Aim 1: Investigate Effects of Mono-Allelic *Bcl11b* Deletion on Transformation of ATM Deficient T Cells. In Aim 1, we will test our hypothesis that BCL11B haploinsufficiency cooperates with ATM deficiency to transform developing T cells. To this aim, we will determine the T-ALL predisposition of $Atm^{-/-}$ mice with conditional mono-allelic deletion of *Bcl11b* in thymocytes.

Aim 2: Determine Contribution of Cellular Proliferation upon Transformation of ATM Deficient Thymocytes. In Aim 2, we will test our hypothesis that antigen independent proliferation of ATM-deficient thymocytes causes *Bcl11b* deletions that drive T-ALL. To this aim, we will analyze $Atm^{-/-}$ mice lacking the Cyclin D3 (Ccnd3) protein and incapable of antigen-independent thymocyte proliferation.

Aim 3: Elucidate Precise Mechanisms through which Bcl11b Suppresses T-ALL. In Aim 3, we will test our hypothesis that Bcl11b suppresses T-ALL by preventing the formation of genomic instability in proliferating cells. To this aim, we will generate $Bcl11b^{+/+}$, $Bcl11b^{+/-}$, and $Bcl11b^{-/-}$ lymphoid cell lines and assay their ability to prevent chromatid breaks and translocations in response to DNA replication stress.

Our findings will advance knowledge of how cells coordinate DNA repair with survival and proliferation to maintain genomic stability and suppress transformation. Since these DDR mechanisms are often inactivated in a wide range and high percentage of human tumors, our findings should have important clinical implications with significant impact upon the broad fields of cancer etiology and treatment.

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

Despite advances and promise in patient-tailored cancer therapies that inhibit oncogenes, selection for drug-resistant mutations and/or outgrowth of therapy-induced malignancies are and will remain major clinical problems, especially for cancers like T-ALL with subtypes and molecular heterogeneity caused by genomic instability. Other barriers to such therapies include the time, effort, and money required to design, develop, and test them, as well as the challenge to get these drugs into all tumor cells. Thus, use of chemotherapy involving systemically administered DNA-damaging agents will remain for years a common and widespread clinical practice to treat human cancers. Genomic instability caused by aberrant repair of DSBs is a hallmark of many human cancers, including ~50% of T-ALLs. Mutations in humans and gene-targeted analyses in mice have revealed that DDR mechanisms coordinate DNA repair with cellular proliferation and survival to maintain genomic stability and suppress cancer. Paradoxically, tumor cell intrinsic DDR defects and inhibition of complementary DDR mechanisms can be exploited to destroy tumor cells while minimally affecting normal cells. Current T-ALL treatments involve chemotherapy using DNA-damaging agents chosen without any knowledge or consideration of these cancer cell DDR phenotypes . We expect that our studies could change clinical management of T-ALL through introduction of patient-tailored cancer therapies involving combinations of DDR inhibitors and genotoxic agents that inactivate non-redundant DSB repair mechanisms and also overwhelm compensatory DNA repair pathways of tumor cells, while minimally affecting normal cells.

Summary of Research Completed

There is no activity to report for this reporting period and no expenses were incurred. The lack of activity is due to an unanticipated turnover of personnel to conduct the proposed experiments. A new postdoctoral fellow will start on this project when he joins the lab 11/10/14.

<u>Research Project 3: Project Title and Purpose</u>

Tumor Antigen-specific T-cells and Hepatocellular Carcinoma – This proposal encompasses work designed to demonstrate that a cell-based tumor vaccine can induce potentially protective immunity in cirrhotic patients against tumor-associated antigens relevant to hepatocellular

carcinoma (HCC). We propose to define the mechanism for tumor-induced suppression of these T-cells in patients with HCC, to prove that highly functional effector tumor-specific T-cells can be expanded from cirrhotic patients, and to investigate a cell-based vaccine platform for immunizing cirrhotic patients against tumor-associated antigens. If ultimately successful, a preventive vaccine could have a major impact on survival and reduction of the need for liver transplantation in well-compensated cirrhotic patients.

Anticipated Duration of Project

1/1/2013 – 12/31/2016

Project Overview

The medical burden of hepatocellular carcinoma continues to increase as a consequence of the progressing age, duration of infection, and thus disease stage of hepatitis C-infected individuals. Hepatocellular carcinoma has been shown to express potentially immunogenic proteins such as alpha-fetoprotein and glypican-3. However, few if any T-cells can be detected in or around HCC pathologically, suggesting that the antigen recognition, expansion, homing and/or effector function of T-cells specific for these antigens have been suppressed *in vivo*. We hypothesize that tumor antigen-specific CD8+ T cells with intact capacity to proliferate and exert multiple effector functions exist in cirrhotic patients but in the natural progression to hepatocellular carcinoma develop profound functional impairment due to the suppressive tumor microenvironment. Our goal is to demonstrate that we can efficiently expand polyfunctional tumor antigen-specific CD8+ T-cells from peripheral blood lymphocytes from non-tumor-bearing cirrhotic patients with the intent of ultimately proving that priming and expanding these T-cells prior to the onset of HCC may retard or prevent the onset of hepatocellular carcinoma. We will approach this hypothesis first by examining the role of tumor ablation on tumor antigen-specific CD8+ T-cells from HCC patients. By studying the effects of inhibitory co-stimulation blockade on tumor-infiltrating T-cells and the longitudinal effects of intratumoral ablation on tumor-specific T-cells, we will precisely define the critical mechanisms involved in tumor-induced T-cell suppression in HCC. Secondly, we will attempt to demonstrate that highly functional cytolytic CD8+ effector T-cells targeting tumor antigens can be expanded from peripheral blood lymphocytes of cirrhotic patients at risk for future hepatocellular carcinoma but in whom cancer has yet to develop. Lastly, we will evaluate a cell-based vaccine platform to stimulate hepatoma-specific T-cells, as a platform toward developing a novel, preventive vaccine. The ultimate goal of these studies is to develop pre-clinical validation to support translation of cell-based vaccination into human clinical trials to prevent or delay the development of hepatocellular carcinoma in cirrhosis.

<u>Specific Aims</u>:
AIM 1. To determine the potentially reversible inhibitory influences on tumor antigen-specific $CD8^+$ T-cells that evolve during HCC progression.
AIM 2. To demonstrate that polyfunctional and cytolytic $CD8^+$ effector T-cells targeting tumor antigens, specifically glypican-3 and alpha-fetoprotein, relevant to HCC can be expanded from peripheral blood lymphocytes of cirrhotic patients at risk for future hepatocellular carcinoma.
AIM 3: To compare CD40-activated B-cells with monocyte-derived dendritic cells as a platform

for cell-based vaccination in patients with cirrhosis as a step toward the translational development of a novel preventive vaccine approach.

Principal Investigator

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

Hepatocellular carcinomas have been shown to express potentially immunogenic proteins raising the hope that immune-based therapy could play an important role for future prevention or therapy. We hypothesize that functional tolerance to liver tumor-related antigens is difficult if not impossible to reverse by the time HCC reaches clinical attention. However, we hypothesize that tolerance to liver tumor-related antigens has not yet been established in cirrhotic patients who are at high risk for future HCC development. If true, this population would be an optimal target population for the study of a tumor preventive vaccine. One promising platform for such a vaccine is CD40-activated B-cells. Work on this vaccine platform for other malignancies is highly active at Penn and the PI's laboratory has already begun early study of CD40-activated B-cells in cirrhosis with promising early findings. Following completion of the proposed project, the PI will be ready to proceed with early clinical trials, translating *in vitro* findings into a cost-efficient, effective cell-based cancer prevention vaccine.

Summary of Research Completed

Aim 1: No work was completed on this aim during the reporting period

Aim 2:

In the first year, we primarily focused on completing work on Aim 2. Dendritic cells (DCs) are critical antigen-presenting cells (APCs) for the priming of naive T lymphocytes. While it is difficult to isolate native DCs owing to low numbers of circulating cells, it is possible to differentiate peripheral blood monocytes into DCs, which show similarity to those present in peripheral tissue. Several studies have reported the impaired function of DC in patients with liver cirrhosis related to hepatitis C virus (HCV) infection. However, some recent reports show that monocyte-derived DCs (MoDCs) from chronic HCV patients retain their function. In this study, we demonstrated that MoDCs isolated from cirrhotic individuals retain similar capacity for activation, maturation and antigen-presentation as those from healthy donors and that AFP- and GPC3-antigen specific T-cells could be expanded in more than half of cirrhotic patients.

Subjects and controls were recruited from the Gastroenterology Clinics at the Philadelphia Veterans Affairs Medical Center following informed consent on an institutional review board-approved protocol. Cirrhotic non-tumor patients who were enrolled were routinely screened by sonography every 6 months to exclude interim development of HCC; data from any cirrhotic subject who developed HCC within 12 months of enrollment were excluded. In all patients, HLA-A2.1 expression was determined using flow cytometry with anti-HLA-A2.1 (clone BB7.2, BD Biosciences, Franklin Lakes NJ). **Table 1** shows the study cohort comprising 22 subjects, 15 patients with liver cirrhosis and 7 healthy donors. Cirrhotic patients included patients with chronic HCV infection as well as cirrhosis unrelated to HCV, including hemochromatosis (HFE, n=2), hepatitis B (n=1), alcoholic cirrhosis (n=4), NASH (n=3). There was no significant difference in the age of HD and CIR groups or divergence of the gender and ethnicity distributions among these groups.

We isolated monocytes from $40x10^6$ PBMC using CD14 positive selection and harvested MoDC after following the FAST-DC maturation procedure. Briefly, CD14+ monocytes were cultured in 24 well plates in X-vivo15 medium supplemented with 800IU/ml GM-CSF (BioLegend) and 1000IU/ml IL-4 (BioLegend) for 24hr. The cells were incubated for another 24hr in X-vivo15 medium with a maturation cocktail of proinflammatory mediators [10ng/ml TNF-α (Cell Signaling Technology), 10 ng/ml IL-1β (Cell Signaling Technology), 10 ng/ml IL-6 (Cell Signaling Technology), and 1μg/ml PGE2 (Sigma), 1600IU/ml GM-CSF and 1000IU/ml IL-4]. We observed a significantly higher yield number of $CD14^+$ monocytes and MoDCs in cirrhotic patients than healthy donors ($CD14^+$ monocytes; 7.0±0.8 vs 3.7±0.4, p=0.0036, MoDCs 3.2±0.4 vs. 1.9±0.3, p=0.0207, median±SD respectively). HCV-related cirrhotic patients had the highest cell yield numbers ($CD14^+$ monocytes 7.9±3.0 vs. 3.7±0.9, p=0.004 compared to HD; MoDCs 3.4±1.7 vs. 1.9±0.6, p=0.0369). MoDCs from cirrhotic patients upregulated surface maturation and co-stimulatory markers such as CD86, CD137, CD70, CD40 and CD83 similarly to healthy donors (**Figure 2**). CD70 and HLA-DR expression of immature $CD14^+CD11c^{hi}$ cells were significantly higher in patients with cirrhosis (CIR) than those of healthy donors (HD) (p=0.048, p=0.0394). All maturation markers and co-stimulatory markers were up-regulated in MoDC (Day0 vs. Day2 p-values CD86: <0.0001, CD83: 0.0007, CD40: <0.0001, HLA-DR: 0.0128, OX40L: 0.021, CD70: <0.0001, CD137L: 0.0066). There were no significant differences between CIR (n=15) and HD (n=7), and between HCV related CIR (n=9) and HCV non-related CIR (n=6) and HD (n=7) in terms of the magnitude of upregulation of each of these markers.

We next analyzed whether MoDCs from cirrhotic donors had similar capacity to expand antigen specific T-cell responses. Briefly, T-cells were co-cultured with MoDC that were irradiated and pulsed with 15mer peptides for AFP or glypican-3 (2 liver cancer-related antigens) and CMV/EBV/Flu 9-10mer optimal peptides (positive control) with supplemental IL-2 (50U/ml), IL-15 (10ng/ml) and IL-21 (25ng/ml) added every 2-3 days. On days 13 and 20, T-cells were harvested and restimulated with autologous irradiated and peptide-pulsed MoDC (or PBMC $1.0x10^6$/well if insufficient MoDC remained). On day 27, *in vitro* expanded cells were restimulated for 6hr with AFP, GPC3 or CEF peptide pools, media (negative control) or PMA/ionomycin (positive control) in the presence of anti-CD107a PE and monensin, fixed and permeabilized using BD Cytoperm/Cytofix (BD), then stained intracellularly for IFNγ PE-Cy7 and TNFα APC (BD). Dead cells (Aqua+) were excluded from analysis. On day 27, antigen-expanded T-cells/well were also tested for antigen-specific responses by IFNγ ELISPOT. We

found that there was no significant differences in the expansion of multifunctional CEF-specific CD8+ T-cells between CIR and HD (16.18±24.78 vs. 19.75±17.50, p=0.6919, median±SD respectively). AFP- and GPC3-specific multifunctional T-cells were less frequently detected but did not show significant differences between CIR and HD. IFNγ ELISPOT against AFP and GPC3 were not frequently detected in cirrhotic or healthy donors. Multifunctional CD4 and CD8 responses were observed in more than half of HD and CIR using a cutoff of 0.25% indicating that although these cells circulate a low precursor frequency, after expansion they retain multifunctional effector capacity.

We conclude that using adequate conditions for Mo-DC maturation and T-cell priming, Mo-DCs from cirrhotic patients retain similar capacity for activation, maturation and antigen-presentation as those from healthy donors. Moreover, these findings support the use of autologous DCs as DC based vaccination in cirrhotic patients might be investigated as a means to reduce liver cancer risk among patients with cirrhosis.

Table 1. Baseline characteristics

A.

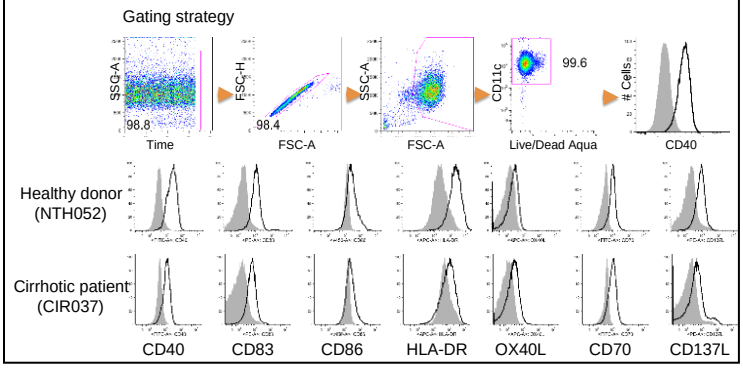
ID	Ethnicity	Gender	ALT	Albumin	Tbili	INR	Platelet	Etiology
CIR004	H	M	34	4	0.6	1.2	169	HCV
CIR018	W	M	19	4.4	1.1	1.1	109	EtOH
CIR019	B	M	23	3.7	0.5	1.2	160	HBV
CIR026	B	M	63	3.4	0.7	1.0	147	HCV
CIR029	W	M	77	4.3	0.9	1.1	151	HCV
CIR030	W	M	52	4.4	0.7	1.0	145	NASH/EtOH
CIR031	H	M	28	3.8	0.6	1.1	151	HCV
CIR034	W	M	73	3.8	1.0	1.1	53	HCV
CIR035	W	M	98	4.5	0.7	1.0	175	NASH
CIR036	W	M	36	4.5	0.9	1.0	198	HFE
CIR037	B	M	51	3.5	0.6	1.0	122	HCV
CIR038	W	M	15	3.7	0.6	1.1	146	HFE
CIR041	W	M	27	3.9	0.6	1.0	206	EtOH
CIR042	W	M	28	4.3	0.7	1.1	87	EtOH
CIR043	W	M	31	3.7	1.4	1.2	52	NASH

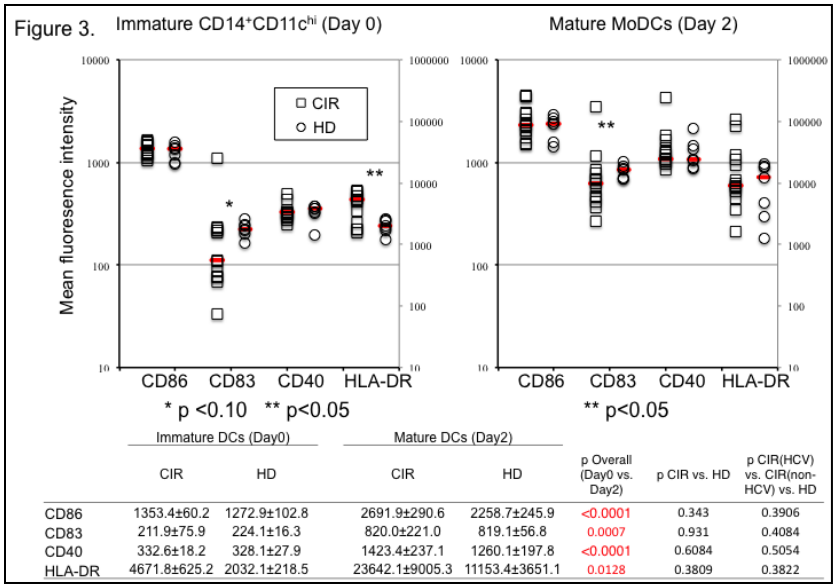
B.

	Healthy donor	Cirrhotic patients	P value
Number of patients	7	15	
Median Age (years), (IQR)	53 (38-56)	62 (56-65)	0.02
Ethnicity (W/B/H)	6/1/0	10/3/2	n.s
Gender (M/F)	5/2	15/0	n.s
Median Alanine Aminotransferase (IU/L) (IQR)	20 (17-31)	34 (27-63)	0.04
Median Albumin (g/dl) (IQR)	4.2 (4.1-4.3)	3.9 (3.7-4.4)	n.s
Median Total Bilirubin (mg/dl) (IQR)	0.7 (0.5-1.2)	0.7 (0.6–0.9)	n.s
Median INR (IQR)	1.1 (1.0-1.2)	1.1 (1.0-1.1)	n.s
Median Platelet Count ($\times 10^3$/μl) (IQR)	196 (176-271)	147 (109-169)	0.004
Etiology (HCV/non-HCV)	–	6/9	

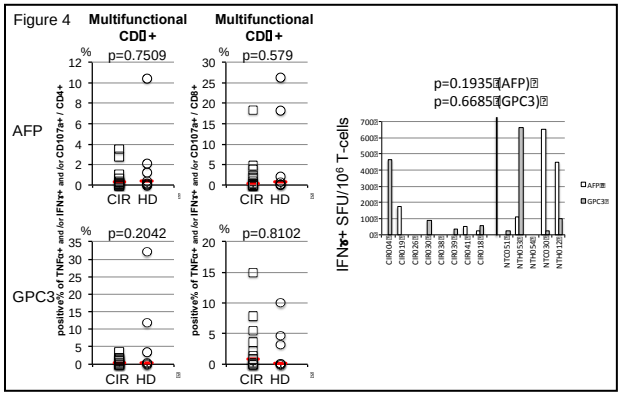
n.s: not significant

Figure 2.





	Immature DCs (Day0)		Mature DCs (Day2)				
	CIR	HD	CIR	HD	p Overall (Day0 vs. Day2)	p CIR vs. HD	p CIR(HCV) vs. CIR(non-HCV) vs. HD
CD86	1353.4±60.2	1272.9±102.8	2691.9±290.6	2258.7±245.9	<0.0001	0.343	0.3906
CD83	211.9±75.9	224.1±16.3	820.0±221.0	819.1±56.8	0.0007	0.931	0.4084
CD40	332.6±18.2	328.1±27.9	1423.4±237.1	1260.1±197.8	<0.0001	0.6084	0.5054
HLA-DR	4671.8±625.2	2032.1±218.5	23642.1±9005.3	11153.4±3651.1	0.0128	0.3809	0.3822



Research Project 4: Project Title and Purpose

Repurposing Cholinesterase Inhibitors for Smoking Cessation – The purpose of this project is to evaluate cognitive enhancing interventions for smoking cessation. The focus will be on the cholinesterase inhibitor, galantamine.

Anticipated Duration of Project

1/1/2013 – 12/31/2016

Project Overview

The broad objective is to develop better treatments for nicotine dependence. To achieve this objective, we will test the effects of a cholinesterase inhibitor medication, Galantamine, on withdrawal-related cognitive deficits (i.e., working memory, sustained attention, and behavioral inhibition), and short-term quitting success.

Aim 1: To evaluate the effects of galantamine when administered as 8 and 16 mg oral once daily dose for 46 days, compared to placebo, on short-term abstinence
H1: Galantamine will increase the number of days of abstinence during a short-term quit attempt, compared to placebo.

Aim 2: To examine the effects of galantamine on cognitive performance following 72 hours of mandatory abstinence from smoking
H2a: Galantamine will attenuate withdrawal-related cognitive deficits in attention, working memory, response inhibition, and cognitive flexibility, compared to placebo.
H2b: Reduction in withdrawal-related cognitive deficits, particularly working memory, will predict ability to remain abstinent during the 7-day observation period.

Principal Investigator

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Other Participating Researchers

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Expected Research Outcomes and Benefits

This research will test whether a medication currently approved for the treatment of Alzheimer's disease, galantamine, may be an effective medication for smoking cessation. Findings from this research may have important implications for helping more smokers quit by increasing the number of available treatment options.

Summary of Research Completed

Since accrual began, 110 (36%) of the 301 completed phone screens resulted in a subject eligible for Intake. Of these subjects, 107 (89%) scheduled an in-person screening visit. Of the 63 subjects (70%) who attended this visit, 24 were ineligible, 32 subjects were deemed eligible, and 7 decided not to participate.

Of the 32 subjects who initiated the study, 3 were deemed ineligible for non-compliance (failure to remain abstinent during the mandatory abstinence period (n=1) and positive urine drug screen (n=2)), 3 were withdrawn after missing a mandatory study visit, and 2 withdrew. As a result, 20 subjects have completed the study and 4 are currently active. Of the 2 subjects who withdrew for their own reasons, 1 withdrew because he decided to try to quit smoking using a different medication and the other participant withdrew due to time constraints. To enhance recruitment, we have increased our advertising efforts (i.e., television ads). In the next year, we anticipate

recruiting 4-5 smokers per month.

To date, 20 subjects have completed the study and adherence to the study regimen is excellent (98% by pill count). “Loss of appetite”, “tiredness”, “headache”, and “difficulty sleeping” were the most common side effects reported, but all were rated as mild or moderate. All subjects to date have proceeded with the Standard dosing schedule and no subjects have withdrawn due to adverse events.

Research Project 5: Project Title and Purpose

Clinical-pathologic Correlates in Neuroendocrine Tumors – Gastrointestinal neuroendocrine tumors (GI NETs) are rare tumors of the alimentary tract or pancreas that commonly metastasize to the liver and beyond. They arise from neuroendocrine cells distributed throughout the body and have a low incidence, but high prevalence because their clinical behavior is generally indolent. It has been estimated that there are about 100,000 people in the United States with GI NETs including in the State of Pennsylvania. How these tumors develop is not well understood. We are interested in understanding more about the patients who develop these tumors and the biology of the tumors themselves in order to ultimately improve the diagnosis, treatment, and clinical outcome of individuals who develop GI NETs.

Anticipated Duration of Project

1/1/2013 – 12/31/2016

Project Overview

The objectives of this project are: (1) to recruit patients with gastrointestinal neuroendocrine tumors (GI NETs) and develop an annotated, longitudinal, clinical database including biosamples (tumor tissue and buffy coat) for use by our research group to facilitate better understanding of the genetics and biochemical signaling pathways leading to tumor formation and malignancy and (2) to determine the mechanistic basis for Hedgehog signaling in the pathogenesis of NETs as a basis for future clinical therapeutic intervention**.** This will build upon our University of Pennsylvania Interdisciplinary Neuroendocrine Tumor Program, the only one of its type in the state of Pennsylvania.

Aim 1: Consenting eligible subjects will undergo complete review of their entire medical records. Clinical and demographic data will be extracted using a standard form before being inserted into a Redcap coded database that has been developed by us. Information about tumor pathology, metastases, family history, diagnosis, timing of symptoms and interventions, clinical genetic screening, biochemical profile, and treatment information will be extracted from the subject's medical chart (our electronic medical record is EPIC) and pathological records. Fresh frozen tumor samples (not needed for diagnostic purposes) will be obtained from the collaborating pathologists and surgeons when enrolled subjects undergo surgery. Previously stored, paraffin embedded tumor blocks will be examined as required. All subjects will also be asked to provide a blood sample (40 mL) for DNA, RNA, and/or protein extraction.

Aim 2: Our second objective is to test the hypothesis that Hedgehog (HH) signaling is active in both Multiple Endocrine Neoplasia type 1 (MEN 1) and sporadic NETs. We have recently shown in pancreatic NETs from mice as well as in a limited number of human tumor samples, that the HH signaling pathway is activated. A key effector of the HH signaling pathway, Gli1, is highly expressed in the tumor samples as detected by immunohistochemistry (IHC) or in situ hybridization (ISH). Moreover, inhibitors of the HH signaling pathway suppress NET growth in mice. Furthermore, DAXX/ATRX, another pair of proteins involving telomere and histone function, has been reported recently to be mutated or reduced in NETs. We will also examine potential changes of these proteins in NETs. Results from these studies will provide a strong foundation for us to design future clinical trials to target the HH signaling pathway in NET patients.

Principal Investigator

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Other Participating Researchers

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Expected Research Outcomes and Benefits

The University of Pennsylvania has an increasingly prominent reputation and currently provides state-of-the-art clinical care to a growing number of Pennsylvanians as well as national (and international) GI NET patients. In its first year (October 2010-October 2011), the GI NET programs saw 217 patients. We anticipate significant growth in clinical activity as the program matures and are expecting a 10% increase per year for the next three years. Consequently, we expect that our tumor and buffy coat biosample banking and database development efforts will expand concurrently. Together with enhanced infrastructure development we will be poised to dramatically accelerate our research program in human genetics, tumor biology, novel therapies and imaging modalities that will directly affect the clinical care provided to our growing number of patients and move the field forward on the discovery and translational research fronts.

We anticipate establishing a robust, relational database for NETs, the first of its type in the state of Pennsylvania, during this funding period. This database will link clinical, pathological, radiological and molecular information. Importantly, it will foster hypothesis-driven research to determine the functional roles of HH signaling in NETs, which we anticipate will be completed during this funding period.

Summary of Research Completed

Aim1: *Establish an accurate, real time, searchable patient database as well as a centralized tumor and buffy coat bank for germline and tumor tissues, including fresh and paraffin embedded specimens to be used for innovative translational research in genetics, epigenetics, cell and molecular biology, and clinical outcomes..*

Our Redcap database is now operational. To date 96 study subjects have been consented for our protocol and 96 have been entered into the database (49 have 100% of the data fields completed). Figure 1 represents a sample of the Redcap database dashboard. Figure 2 represents a sample of the Redcap data collection form (page 1 of 17).

In addition, fresh neuroendocrine tumor samples were obtained from 34 patients undergoing surgery through our collaboration with the Cooperative Human Tissue Network (CHTN) and the University of Pennsylvania Department of Pathology and Laboratory Medicine. We also obtained archival paraffin-imbedded tissue on 5 patients and we have stored buffy coat samples from 31 subjects and serum samples from 7 subjects (a recent protocol amendment). We elected to target the initial subject recruitments towards patients with pancreatic neuroendocrine tumors (PNETs) who were undergoing surgery in order to enhance the ability to satisfy aim #2 (see below).

Aim 2: To determine the functional roles of the HH signaling pathway in NETs.**.**

We have performed Patch 1 immunohistochemistry (IHC) staining on fresh and archival tissue specimens from 23 patients with PNETs. These included both genetically confirmed MEN-1 patients as well as others with sporadic disease. Samples were mainly obtained from primary tumors and local lymph node metastases but some were from metastases further afield (e.g., the liver or ovary). We also obtained normal pancreas tissue in the tissue procurement process and this has allowed us to compare hedgehog (HH) staining in normal and malignant tissues. An example of Patch 1 staining is shown in Figure 3.

In addition to continuing to recruit new NET patients visiting our Center for the database and tissue bank programs, we are currently engaged in analyzing the data obtained thus far to begin to describe the clinicopathologic correlation between HH staining and clinical and demographic features of our patients with PNETs. Preliminary analysis appears to indicate that Patch 1 staining is specific for NETs (in contrast to normal pancreatic tissue) and that about two-thirds of patients exhibit Patch-1 staining (both MEN-1 and sporadic disease). A manuscript describing these findings is in process and we are hopeful that we may identify predictors of Patch 1 positivity that may permit future clinical trials of HH inhibitors in patients with metastatic PNETs.

Drs. Metz and Hua both attended the Caring For Carcinoid Foundation Annual Scientific NET and Carcinoid Tumor Workshop in Boston, MA on May 22, 2014 as invitees. Moreover, we have also begun to collaborate with Michele Kim, MD at Mt. Sinai School of Medicine whereby our Redcap database (deidentified data only) will be included in her analysis of the natural history and clinical outcomes of NET patients at our two institutions.

Figure 1: Sample dashboard depicting the Redcap database status report for patients enrolled into our protocol (red dots indicated incomplete data collection, yellow is complete but unverified, green indicates complete collection). Each subject's data are categorized according to baseline demographics, genetic information, baseline clinical information and yearly updates.

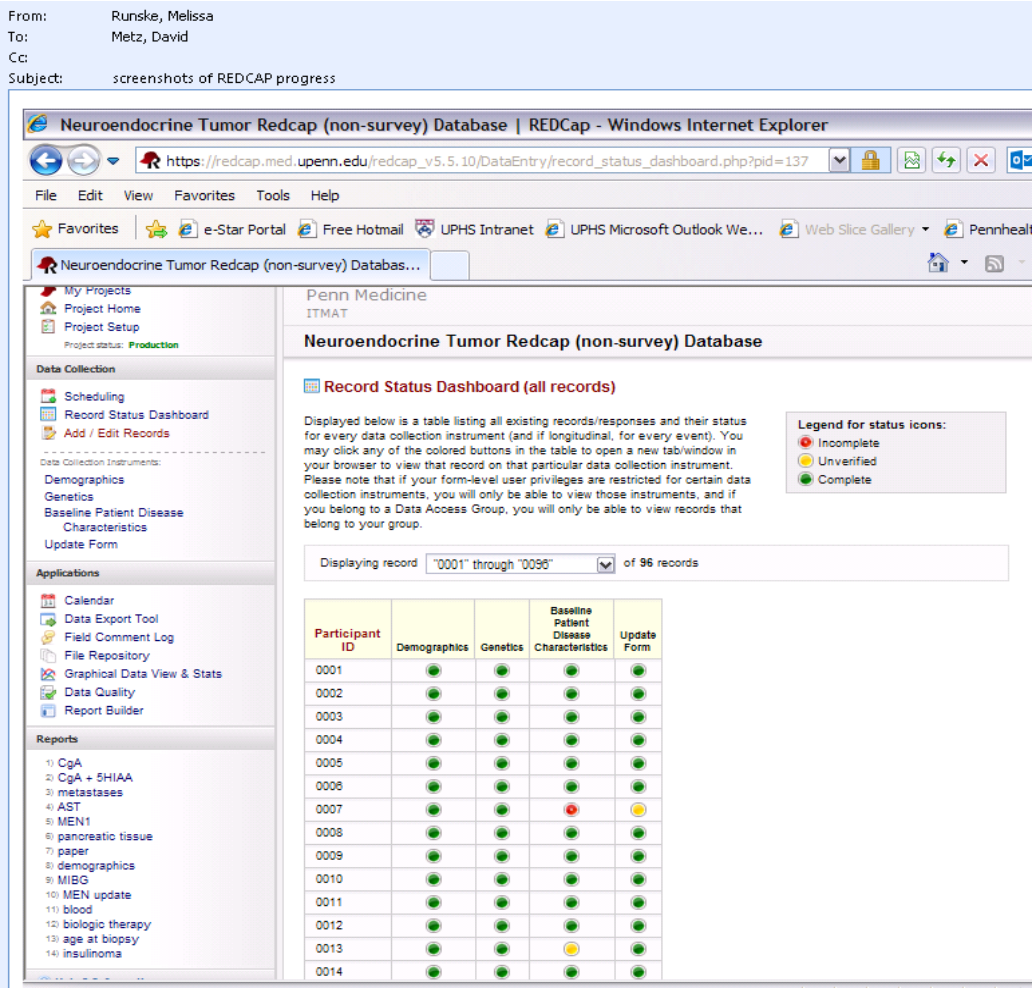


Figure 2: Sample of the Redcap data collection form (Page 1 of 17). Each subject's data are categorized according to baseline demographics, genetic information, baseline clinical information and yearly updates.

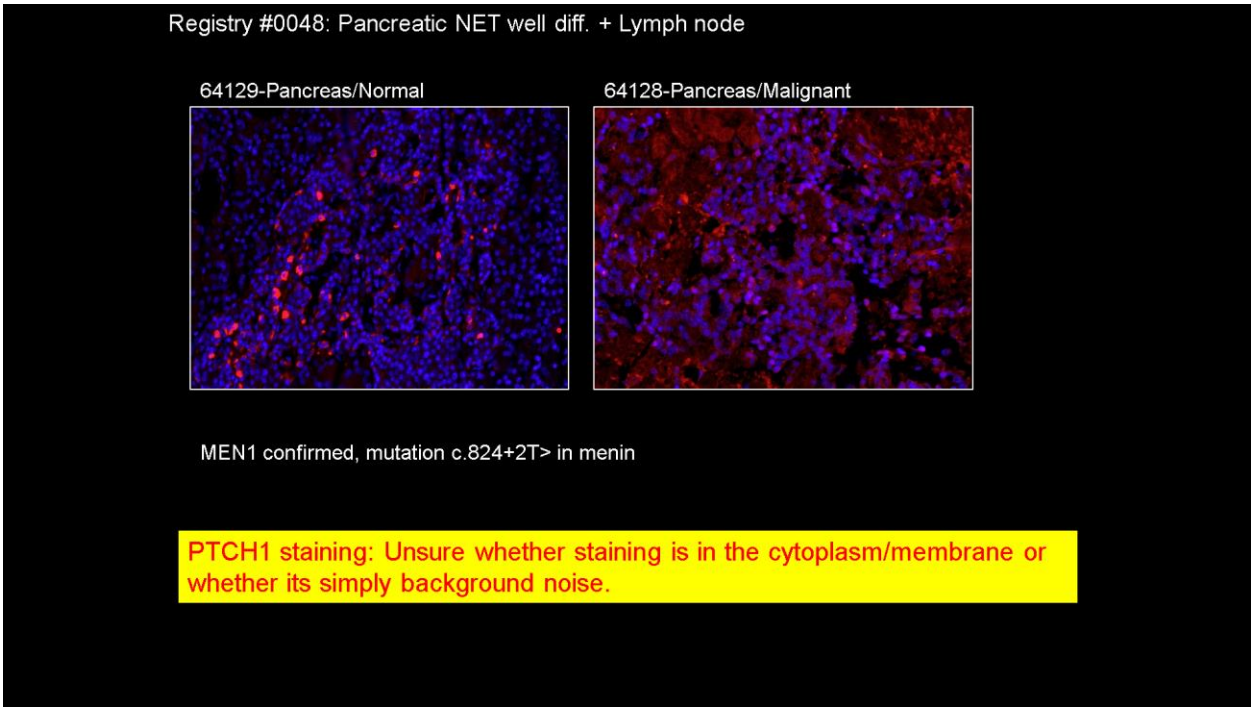
Demographics

Participant ID	_____
Xian Patient	☐ Yes ☐ No
This patient is a confirmed NET patient	☐ Yes ☐ No
Date of Informed Consent	_____
Type of consent form signed	☐ Database ☐ Genetics ☐ Combined
Future use of samples	☐ Samples may be used for any scientific puposes involving this or any other project ☐ Samples may be used for this project only and for other projects with permission ☐ Samples may be used for this project only
Upload signed informed consent	
Person Obtaining Informed Consent	☐ David Metz ☐ Bonita Bennett ☐ Julie Starr ☐ Other
If "other", please indicate:	_____
Type of consent process	☐ Telephone ☐ In Person

Demographics Information

First Name	_____
Last Name	_____
Date of Birth	_____
age	_____ (age)
MRN	_____
Gender	☐ Female ☐ Male
Marital Status	☐ Married ☐ Not married
Education Level	☐ Did not finish High School ☐ High School Diploma or GED ☐ Some College ☐ B.S or B.A Degree ☐ Post-Graduate Degree ☐ Unknown
Insurance	☐ yes ☐ no

Figure 3: Patch 1 staining of NET tissue obtained from subject #0048 (an MEN-1 positive individual with a metastatic PNET). The normal pancreas tissue adjacent to the NET does not stain for Patch 1 (left) whereas the tumor tissue stains robustly (right).



Research Project 6: Project Title and Purpose

Notch Signaling and Esophageal Carcinogenesis – Little is known about the role of Notch signaling in esophageal squamous cell carcinoma (ESCC), one of the most deadly cancers. In particular, there is a fundamental gap in understanding how Notch signaling regulates a subset of ESCC cells that are more capable of forming tumors, prone to metastasize and foster resistance to conventional chemotherapy. This research aims at elucidating how Notch signaling contributes to the pathogenesis of esophageal squamous cell carcinoma (ESCC).

Anticipated Duration of Project

1/1/2013 – 12/31/2016

Project Overview

The objective of this research is to elucidate how Notch signaling contributes to the pathogenesis of esophageal squamous cell carcinoma (ESCC). The *central hypothesis* is that Notch signaling activates mitophagy and epithelial-mesenchymal transition (EMT) to induce highly tumorigenic ESCC cells defined by high expression of CD44 (CD44H cells), the receptor for hyaluronic acid. This hypothesis will be tested by pursuing two interrelated *Specific Aims*: (1) To determine how mitophagy is activated to facilitate EMT and tumorigenicity of ESCC cells; (2) To delineate how

Notch signaling regulates mitophagy in ESCC cells undergoing EMT. Genetic gain-of-function and loss-of-function experiments will be done in an inducible fashion in both aims. In addition, genetically engineered ESCC cells will be characterized in xenograft tumors. Under the second aim, a genetically-engineered mouse model of ESCC will be used to determine the role of Notch signaling in mitophagy and EMT.

Principal Investigator

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

This research will delineate how Notch signaling contributes to disease progression and poor prognosis in esophageal squamous cell carcinoma (ESCC). Specifically, this research will reveal a novel relationship between Notch signaling and mitophagy (i.e., mitochondria-targeted autophagy) in the maintenance of highly tumorigenic ESCC cells defined by high expression of CD44 (CD44H cells), the receptor for hyaluronic acid. CD44H cells are also metastatic and resist chemotherapy and radiation therapy. Activation of Notch signaling leads to the induction of CD44H cells via mitophagy. Thus, pharmacological selective modulations of Notch signaling or mitophagy may efficiently reduce CD44H cells. Therefore, the proposed research is expected to vertically advance and expand understanding of mechanisms by which mitochondrial homeostasis is regulated and in turn, deregulated in esophageal cancer. Such knowledge will lead to development of innovative pharmacological strategies that will manipulate Notch signaling and mitophagy in esophageal cancer cells and have the potential to advance the therapy of ESCC and prevention of disease progression. This research may also trigger similar approaches in the therapy of other squamous cell carcinomas.

Summary of Research Completed

Aim 1: *To determine how mitophagy is activated to facilitate EMT and tumorigenicity of ESCC cells.*

Aim 1A: When and how is mitophagy activated during EMT?
We used flow cytometry to visualize epithelial-mesenchymal transition (EMT) in esophageal cancer cell lines EPC2T and TE11. We carried out flow cytometric analyses to reveal two discrete cell populations CD44L and CD44H in culture in a reproducible manner. We isolated CD44L and CD44H cells by fluorescence-activated cell sorting (FACS). In response to

transforming growth factor (TGF)-β1 stimulation, FACS-purified CD44L cells gave rise to CD44H cells along with an intermediate cell population transitioning from CD44L to CD44H as a function of time. This transition was confirmed as EMT by morphological and biochemical assessment of CD44L and CD44H cells. Thus, we reproducibly validated EMT in the conversion of CD44L cells to CD44H cells. We also visualized mitophagy (i.e. autophagy targeting mitochondria) in cells undergoing EMT. We used cyto-ID staining to detect autophagosomes. Cyto-ID staining results were corroborated by biochemical markers of autophagy including LC3B. We used a lentivirus-based reporter to detect Parkin2 (a regulator of mitophagy) without a success. Alternatively, we documented mitophagy by performing immunofluorescence to co-localize mitochondrial markers (MTCO1 and TOM20) and either cyto-ID or LC3B. In addition, we carried out transmission electron microscopy (TEM) analyses of cells to document structural changes in mitochondria and autophagosomes during EMT induced by TGF-β1 stimulation. In aggregate, we assessed successfully mitophagy in CD44L and CD44H cells during EMT. We started determining mitochondrial activities by flow cytometric detection of MitoTracker® dye stained cells.

Aim 1A-(i) and (ii): How do mitochondrial ROS influence mitophagy and EMT?
We determined mitochondrial reactive oxygen species (ROS) by flow cytometry using 2',7'-dichlorofluorescein (DCF) and MitoSox dyes in CD44L and CD44H cells following TGF-β1 stimulation. We found that ROS were induced in CD44L cells at the onset of EMT. We determined the expression level of SOD2, an essential mitochondrial antioxidant. SOD2 appeared to be downregulated in CD44L cells at the onset of EMT, suggesting that SOD2 downregulation may promote ROS induction in CD44L cells. Such a premise was further corroborated in RNA interference (RNAi) experiments directed against SOD2, where we evaluated the functional consequences of altered SOD2 suppression.

Aim 1A-(iii): How inhibition of mitophagy or Parkin2 may affect mitophagy and EMT?
We started generating lentivirus expressing tetracycline-inducible Parkin2 for RNAi experiments in EPC2T CD44L and CD44H cells. In addition, we started experiments using chloroquine to inhibit mitophagy pharmacologically. We have confirmed chloroquine blocks autophagy.

Aim 1B: How altered mitochondrial ROS and mitophagy may influence tumorigenicity?
We optimized conditions in xenograft transplantation models for flow cytometry and FACS, immunohistochemistry (IHC) and TEM to determine tumorigenicity, EMT and mitophagy. We started documenting mitophagy by TEM in xenograft tumors.

Aim 2: *To delineate how Notch signaling regulates mitophagy in ESCC cells undergoing EMT*.

Aim 2A: How does notch signaling influence mitophagy and tumorigenesis?
In Aim 2A, we generated and started validating essential reagents used in this Aim. They include Notch reporter 8xCSL-GFP, tetracycline-inducible activated forms of Notch1 (ICN1), tetracycline-inducible short hairpin RNA (shRNA) directed against Notch1. We performed Western blotting to document ICN1 expression and theNotch1 shRNA effect upon endogenous Notch1 expression in EPC2T and TE11 cells.

Aim 2B: Is mitophagy activated during invasive growth in L2Cre;p120ctn$^{fl/fl}$ mouse model of ESCC?

In Aim 2B, we started optimizing conditions for flow cytometry, immunohistochemistry and immunofluorescence to detect cyto-ID positive cells, CD44H cells, mitophagy, EMT and Notch target genes in primary mouse ESCC tissues. We also started breeding necessary mice strains proposed to in this aim. They include L2Cre;p120ctn$^{fl/fl}$ and mice carrying the Rosa26 locus containing tdTomato (tdT) fluorescent protein cDNA under the loxP-stop-loxP (LSL) sequence (*R26tdT*)(Jackson Lab) to generate *p120ctn$^{fl/+}$;R26tdT$^{+/-}$*.

Research Project 7: Project Title and Purpose

Calcineurin-NFAT Signaling in Megakaryocytic Leukemia of Down Syndrome – Down syndrome is the most common genetic disorder and causes many clinical effects including an increased incidence in the blood cancer, acute megakaryoblastic leukemia (AMKL). This project is based on preliminary data showing that the *DSCR1* and *DYRK1A* genes located on chromosome 21 play a role in leukemia in Down syndrome patients. These genes block the calcineurin-NAT signaling pathway. Thus, the goal of this project is to determine whether this pathway is important in megakaryocyte development and whether increased expression of these 2 genes are necessary for AMKL in Down syndrome patients. This project may lead to new treatments for Down syndrome AMKL and other diseases with defects in megakaryocytes.

Anticipated Duration of Project

1/1/2013 – 12/31/2016

Project Overview

Down syndrome (DS) occurs extremely frequently (1/700 live births) and produces significant morbidities, including altered cancer susceptibility. An unsolved problem of biological and clinical importance is how 50% increased dosages of one or more chromosome 21 genes impacts tissue development and malignant transformation. This problem is illustrated by a 500-fold elevated incidence of acute megakaryoblastic leukemia (AMKL) in young children with DS.

The Specific Aims are

Aim 1: To investigate the role of calcineurin-NFAT signaling and increased Dscr1 and Dyrk1A expression in megakaryopoiesis.

Aim 2: To investigate the role of calcineurin-NFAT signaling and increased DSCR1 and DYRK1A expression in the initiation and/or progression of mouse megakaryoblastic leukemia.

Our preliminary data indicate that DS alone enhances the expansion of megakaryocytes and their precursors. This effect is mediated in part via suppression of the calcineurin-NFAT signaling pathway in megakaryocytes by increased expression of two chromosome 21-encoded calcineurin inhibitor genes, DSCR1 and DYRK1A. Calcineurin, a ser/thr phosphatase, activates NFAT family transcription factors to regulate the development of many tissues. We hypothesize that 50% increased DSCR1 and DYRK1A gene dosages in DS suppress calcineurin-NFAT to stimulate megakaryopoiesis and are thus drivers of DS-associated AMKL. We propose to

explore novel, clinically relevant roles for this signaling pathway in DS-associated AMKL. Specifically, we aim to investigate the role of calcineurin-NFAT signaling and increased Dscr1 and Dyrk1A expression in the initiation and/or progression of mouse megakaryoblastic leukemia. We will use genetic and pharmacologic approaches to manipulate DSCR1, DYRK1a and NFAT activity and determine the effects on malignant megakaryopoiesis identifying the relevant NFAT target genes. We will analyze two different transgenic mouse strains that overexpress Dscr1 and/or Dyrk1A to levels approximating those that occur in DS. We will use these genetic models, along with pharmacologic manipulation, to better define how calcineurin-NFAT regulates megakaryoblast expansion, differentiation and apoptosis. Through functional studies, gene profiling and chromatin immunoprecipitation, we will identify key NFAT target genes in megakaryocytes.

Principal Investigator

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Other Participating Researchers

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Expected Research Outcomes and Benefits

How subtle alterations in gene dosage exert profound influences on tissue development and malignant transformation is poorly understood. Down syndrome, caused by an inherited extra copy of chromosome 21, provides an important illustrative example. This common disorder affects virtually all organ systems, producing morbidities and mortality with considerable medical, economic and social implications. Chromosome 21 contains approximately 230 protein encoding genes and 16 microRNA loci. While many of these genes are extensively studied, how 50% increased dosages, either individually or collectively, causes DS phenotypes is largely unknown. Unraveling such mechanisms will increase our basic knowledge of tissue development and identify medically relevant genetic pathways and interactions.

The study of DS is relevant to cancer since this population is highly susceptible to leukemia. In particular, DS children are prone to blood cancers including acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML) and acute megakaryoblastic leukemia (AMKL). Here, we focus on AMKL, a relatively rare AML that occurs at 500-fold increased rates in young children with DS. Our proposed studies will specifically investigate how an increase in gene dosage of the chromosome 21 genes, DSCR1 and DYRK1A, suppress calcineurin-NFAT signaling to stimulate megakaryopoiesis and AMKL. Insights gained through our studies of DS-AMKL are relevant to normal hematopoiesis and to other leukemias of early childhood that are thought to arise in the fetus. This work may also lead to new, less toxic therapies for DS-AMKL and perhaps other

forms of leukemia that are associated with chromosome 21 duplication, as calcineurin-NFAT signaling is amenable to pharmacologic manipulation.

Summary of Research Completed

Aim 1: *To investigate the role of calcineurin-NFAT signaling and increased Dscr1 and Dyrk1A expression in megakaryopoiesis.*

Work for this Aim includes characterization of circulating platelet levels and megakaryopoiesis in our *Dyrk1A* transgenic mice. These mice were generated by targeting a single extra copy of HA-tagged *Dyrk1A* cDNA with its native promoter into the ROSA26 locus. Unfortunately these mice have had difficulty breeding with very small litter sizes (1-2 pups) limiting our experimental analysis. We have begun to breed the transgenic mice to wild-type mice to determine whether we could increase the number of pups with the transgene. Initial studies on platelet counts of n=2 mice suggest an elevation in the number of circulating platelets however, the number of mice analyzed are still too few.

Other progress on this Aim included studies to assess how thrombopoietin (TPO) regulates calcineurin-NFAT activation. As described in our original proposal, since TPO is the major megakaryocyte cytokine and critical for megakaryopoiesis, we have been investigating activation of the calcineurin-NFAT pathway by TPO *in vitro.* Our initial studies have utilized murine fetal liver-derived megakaryocytes to examine development and/or differentiation *in vitro*. Fetal liver-derived megakaryocytes were cultured for 3 days either in the presence or absence of 0.2 μM cyclosporin A, the immunosuppressant drug that very specifically inhibits calcineurin activation. Fetal liver-derived megakaryocytes were also cultured in TPO doses ranging from 0, 1, 5 and 10 ng/ml with the media replaced daily and fresh TPO and cyclosporin A added. At days 2, 4, 6 and 8, cells were stained with CD41 and CD42 to identify megakaryocyte progenitors and 7AAD for live/dead staining. Studies using fetal liver-derived megakaryocytes showed minimal expansion during the 8-day culture period with only the presence of TPO, thus we altered our experimental design and added IL6 and stem cell factor (SCF) to the culture medium. These factors are all known to play a role in megakaryopoiesis although the relative contribution of each of these factors is not well understood. Since our studies indicate that fetal liver-derived megakaryocytes may not require calcineurin-NFAT activation, we began to use bone marrow-derived megakaryocytes for our studies to determine the contribution of calcineurin-NFAT activation towards TPO mediated megakaryocyte development and expansion. Bone marrow megakaryocytes were isolated and cultured in the presence of IL6, SCF and TPO at doses ranging from 0, 0.5, 0.75, 1, 10 and 50 ng/ml in the presence and absence of cyclosporin A at 0.2 μM. Double positive CD41/CD42 megakaryocytes are the progenitor population so we monitored the double positive population at days 2, 4 and 6 after culture. Our data shows a significant increase in megakaryocyte progenitors beginning 4 days after culture in the presence of CsA at TPO doses of 0.5, 0.75 and 1 ng/ml but not at higher TPO doses.

We have also made progress investigating NFATc1/c2 specific targets in megakaryocytes by chromatin immunoprecipitation. We have been optimizing NFATc1 ChIP experiments. For ChIP experiments, ideally 1-5 $x10^6$ cells as starting material would be preferable for high resolution and genome wide analysis of DNA-protein interactions. While the NFATc1 and

NFATc2 mAb (both available from Santa Cruz) have been validated in other cell types for ChIP-Seq experiments, they have not yet been used in megakaryocytes. This past year, our studies have been focused on optimizing NFATc1 ChIP conditions using primary endothelial cells with known targets transactivated by NFATc1 and with primary megakaryocyte precursors by validating NFATc1 binding to CDK4, which contains a known NFAT binding site. One complication of ChIP-seq experiments with primary megakaryocytes is the ability to isolate sufficient numbers of primary megakaryocytes. For our initial studies, we isolated bone marrow megakaryocytes using a BSA gradient, however, expansion of these primary bone marrow megakaryocytes in culture using the megakaryocyte cytokine, thrombopoietin (TPO) led to alterations in calcineurin-NFAT signaling. Thus, we attempted to utilize fetal liver megakaryocytes for NFATc1 ChIP experiments. During our initial studies, we generated sufficient numbers of fetal liver-derived megakaryocytes for ChIP-Seq experiments, however, we were unable to detect NFATc1 binding to CDK4 by PCR after NFATc1 ChIP was performed on these cells. There are inherent differences in signaling pathways between fetal liver-derived megakaryocytes (fetal cells) and bone marrow-derived megakaryocytes (adult cells). Thus, it is likely that calcineurin-NFAT regulation of megakaryopoiesis is most important in adult and not during fetal development.

Aim 2: *To investigate the role of calcineurin-NFAT signaling and increased DSCR1 and DYRK1A expression in the initiation and/or progression of mouse megakaryoblastic leukemia.*

Work for this Aim includes the expansion of our colony of *Dscr1* x *Gata1s* transgenic mice to examine whether a single gene in trisomy is sufficient for progression to AMKL in the context of a *Gata1s* allele and overexpression of a dominant negative TPO receptor (DA-MPL). In our initial experiment, we isolated and purified double mutant *Dscr1* x *Gata1s* hematopoietic stem cells and infected these cells with the DA-MPL retrovirus for transplantation into lethally irradiated hosts. We co-infected hematopoietic stem cells isolated from our *Dscr1* x *Gata1s* transgenic mice and wild-type mice with the DA-MPL retrovirus and a GFP-retrovirus in a 1:10 ratio and found no GFP+ cells when analyzed by flow cytometry. We are currently optimizing our retroviral infection protocol of hematopoietic stem cells.

Research Project 8: Project Title and Purpose

p53 and Tumor Cell Metabolism – The tumor suppressor p53 is most frequently mutated gene in human tumors, and recent evidence indicated a critical role for p53-mediated metabolic regulation and senescence in tumor suppression. However, it is still poorly understood how p53 regulates metabolism and senescence, and how p53 may connect these two processes. Our preliminary studies suggest reciprocal regulation between p53 and two tricarboxylic acid (TCA) cycle-associated enzymes that modulates both metabolism and senescence. We plan to elucidate this reciprocal regulation and its deregulation in tumor cells. This project will lead to better understanding of p53 biology and the molecular mechanisms of tumor suppression.

Anticipated Duration of Project

1/1/2013 – 12/31/2016

Project Overview

The tumor suppressor p53 plays a preeminent role in protection against malignancies. Among a range of cellular processes elicited by p53, senescence and metabolic regulation have recently emerged as central mechanisms for tumor suppression. However, it is unclear how p53 induces senescence and which p53-regulated metabolic enzymes are crucial for tumor growth. Nor is it clear how p53-mediated senescence and metabolic regulation are linked. Metabolism in tumor cells is markedly altered to meet the demands of these cells for energy, biosynthesis, and defenses against reactive oxygen species (ROS). Our preliminary studies suggest a previously unexpected function of p53 in suppressing two metabolic enzymes that are important for meeting these demands of tumor cells. Interestingly, down-regulation of these enzymes reciprocally activates p53 in a feed-forward manner, bolstering this pathway and enhancing p53 activation. Furthermore, down-regulation of these enzymes modulates the outcome of p53 activation leading to strong induction of senescence, but not apoptosis. Our findings suggest a positive feedback mechanism that sustains p53 activation, as well as a connection between metabolism and senescence mediated by p53. We plan to test the hypothesis that *mutual regulation between metabolic enzymes and p53 modulates metabolism and senescence, and that deregulation of these enzymes due to p53 inactivation contribute to the survival and growth of tumor cells*. We propose the following two interrelated specific aims: 1) Examine how p53 modulates metabolism through malic enzymes (MEs); and 2) Define role of MEs in p53 activation. By focusing on the previously unknown role of p53 in controlling ATP production, synthesis of macromolecules, and anti-oxidant defense, this project will generate novel insights into p53-mediated metabolic regulation, its connection with senescence, and its role in tumor suppression.

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Expected Research Outcomes and Benefits

Given the remarkably high frequency of p53 mutations in various cancers, and given that metabolic enzymes are among the best targets for small compounds, an attractive therapeutic approach for cancer is to target p53-regulated metabolic enzymes that are important for cancer progression. However, such p53-regulated metabolic enzymes are still not well understood. Our preliminary studies suggest that *p53 may regulate bioenergetics, biosynthesis, and ROS detoxification via inhibiting two enzymes associated with the TCA cycle*. We plan to further investigate this novel function of p53 and the role of these enzymes in oncogenic growth. The proposed work will likely provide important insights into the metabolic reprogramming in tumor

cells, its effect on tumor cell growth, and the mechanisms and regulation of p53 in tumor suppression.

Summary of Research Completed

Aim 1: *Examine how p53 modulates metabolism through MEs.*

The broad objective of this aim is to investigate the role of the p53 family proteins in regulating metabolism. TAp73 is a member of the p53 family, and it shares extensive similarity in sequence and structure organization to p53. However, while the role of p53 in tumor suppression is well established, the role of p73 in tumorigenesis is still a matter of debate. The complex role of p73 is in part due to the expression of two major p73 isoform classes: the transactivation-proficient (TAp73) and the transactivation-deficient (ΔN) isoform, through the use of alternative promoters. In transfection assays TAp73 often behaves like p53 and regulates genes involved in apoptosis and cell cycle arrest, while ΔNp73 exerts a dominant negative effect on TAp73 and p53 likely through the formation of non-functional hetero-complexes with them and/or the competition with them for DNA binding. Nevertheless, while p53 is mutated in 50-70% of all human tumors, TAp73 is almost never mutated in human tumors. These observations raise important questions as to whether TAp73 confers an advantage to tumor cells, and if so, what may be the molecular mechanism.

We recently reported that TAp73 enhances the expression of G6PD. This function is unique to TAp73, and is not shared by p53 or the other p53 family protein p63. It is required for maintaining G6PD expression in unstressed cells, promoting glucose flux to the pentose phosphate pathway (PPP) for biosynthesis and anti-oxidant defense. Previously, Rufini *et al.* revealed another proliferative targets of the PPP, the mitochondrial complex IV (cytochrome C oxidase) subunit 4 (Cox4i1). In this study, we examined the role of TAp73-mediated G6PD expression in additional cell lines. TAp73 deficiency primarily leads to the formation of lung tumors; and hence we used a human lung cancer cell line, H1299.

p73 regulates G6PD expression in H1299 cells – As reported in our recently published paper, p73 silencing reduces G6PD expression and cell proliferation in a number of cell lines including human osteosarcoma U2OS cells, mouse embryonic fibroblast cells (MEFs), and isogenic human colon cancer HCT116 cells that are wild-type for both p53 and its target gene p21 or null for either one of them. Similarly, knock down of p73 reduced G6PD expression in H1299 cells (Figure 1A). PPP is a major source of cellular NADPH and ribose. In H1299 cells, the lack of p73 reduced NADPH levels (Figure 1C) and DNA synthesis (Figure 1C and D), impeding cell proliferation (Figure 1E). As expected, G6PD knockdown also resulted in a decline in NADPH levels, DNA synthesis, and cell proliferation, to extents similar to or greater than p734 knockdown (Figure 1B-1E). Of note, in G6PD knockdown H1299 cells, silencing TAp73 no longer affected these parameters (Figure 1B-E). These results suggest that, in H1299 cells, p73 controls the expression of G6PD and promotes cell proliferation.

G6PD overexpression completely restores the growth of p73-depleted H1299 cells – To further assess the importance of TAp73-mediated G6PD expression in H1299 cells, we forced the expression of G6PD. As expected, this restored the activity of G6PD (Figure 2A and B).

Interestingly, unlike in the other cell lines where G6PD overexpression only partially restored the growth of p73-depleted cells, p73-depleted H1299 cells expressing exogenous G6PD grew nearly as well as control cells (Figure 2C and D). This data suggests that induction of G6PD expression is both necessary and sufficient for TAp73-mediated cell proliferation in H1299 cells.

p73 fails to affect ROS homeostasis in H1299 cells – Our recent data in other cancer cell lines indicates that ROS detoxification and biosynthesis of nucleosides are essential for TAp73-mediated cell growth. Since depletion of either p73 or G6PD in H1299 cells resulted in a strong reduction in DNA synthesis (Figure 2C and D), both p73 and G6PD may play an important role in nucleotide synthesis in these cells. We measured the effect of p73 and G6PD on ROS content using the cell-permeably radical dye 2',7'-dichlorofluorescein diacetate (DCFDA). While G6PD-depleted cells displayed an increasing content of ROS as expected, p73-depleted cells failed to accumulate ROS (Figure 3A). This result suggests that in p73-depleted cells the expression of G6PD might not have been reduced to a level that affected cellular ROS. Forced expression of G6PD in either control cells or p73-depleted cells had minimal effect on ROS levels (Figure 3B), consistent with the notion that the levels of endogenous G6PD in these cells might be sufficient for ROS detoxification.

To extend these analyses, we examined the effect of p73 on cellular capacity to withstand oxidative stresses. H1299 cells were highly resistant to oxidative stress, even when they were treated with 250 μM H_2O_2 for 24 hours (Figure 3C). Silencing p73 or enforcing the expression of G6PD had nearly no impact on cell survival in the presence of H_2O_2 (Figure 3C). Taken together, these data suggest that H1299 cells possess a robust anti-oxidant capacity even when G6PD levels are reduced, and that p73 has a minimal role in maintaining the redox state of these cells although it is important for DNA synthesis.

METHODS

Cell culture and gene knockdown with siRNA – U2OS cells were maintained in McCoy's 5A Medium, IMR90 cells in MEMα medium, and H1299 cells in RPMI1640 medium (Life Technologies). All mediums were supplemented with 10% fetal bovine serum (FBS). The following siRNAs were purchased from Invitrogen (Carlsbad) with sequences indicated: human G6PD, 5'-AAACCCACUCUCUUCAUCAGCUCGU-3'; human p73, 5'-GAGCUCGGGAGGGACUUCAACGAAG-3'.

Semi-quantitative PCR with reverse transcription PCR – Total RNA was isolated from cells by TRIzol reagent (Invitrogen) and 2 μg RNA of each sample was reversed to cDNA by the First-strand cDNA Synthesis System (Marligen Biosciences). cDNA (0.2 μg) of each sample was used as a template to perform PCR. The primer pairs for human genes were: G6PD, 5'-ATGGCAGAGCAGGTGGCCCT-3' and 5'-TCATGCAGGACTCGTGAATG-3'; β-actin, 5'-GACCTGACTGACTACCTCATGAAGAT-3' and 5'-GTCACACTTCATGATGGAGTTGAAGG-3'; TAp73, 5'-ATGGCCCAGTCCACCGCCACC-3' and 5'-TCGAAGGTGGAGCTGGG-3'.

G6PD enzyme activity, NADPH and ROS levels – G6PD enzyme activity was determined as described previously. NADPH levels were determined using the NADPH Quantification kit (BioVision). ROS levels were analyzed as described previously. Briefly, cells were incubated at

37 °C for 30 min in phosphate buffered saline (PBS) containing 10 µM 2',7'-dichlorodihydrofluorescein diacetate (H2-DCFDA). Cells were then washed twice with PBS, treated with trypsin, and re-suspended in PBS. Fluorescence signal was immediately measured using a FACScan Flow Cytometer (Becton Dickinson).

BrdU incorporation assay – H1299 cells were treated with p73 siRNA, G6PD siRNA or control siRNA. Then, cells were pulse-labelled with BrdU for 1 h and analyzed by anti-BrdU immunostaining. In brief, BrdU-labeled cells were washed with PBS and fixed with 2% paraformaldehyde and 70% ethanol at 4 degree. Afterwards, cells were permeabilized with 0.2% Triton X-100 and followed with Tris-buffered saline (TBS) wash. After treatment of 4N HCl, 0.2% Triton X-100, staining with anti-BrdU antibody and FITC-labeled BrdU-specific antibody, the images were acquired with an Olympus DP71X microscope (Olympus). The FITC signal was converted to a red color for better visualization.

Aim 2: *Define role of MEs in p53 activation.*
No work was done on this aim during the reporting period.

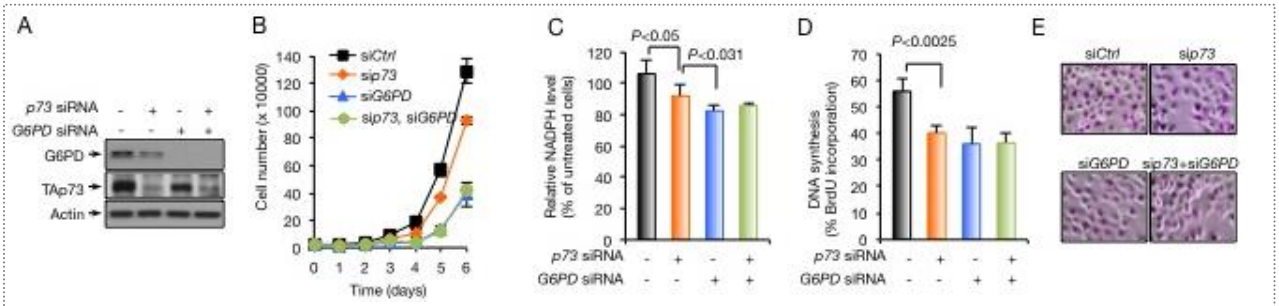

Figure 1. Regulation of G6PD expression, NADPH levels, and DNA synthesis by p73 in H1299 cells. (**A** and **B**) H1299 cells were transfected with control (-), *p73*, and *G6PD* siRNA as indicated. Protein expression was analyzed (A) and cell proliferation is shown (B). (**C**) NADPH levels in H1299 cells treated with control, *p73*, and *G6PD* siRNA as indicated. (**D** and **E**) H1299 cells were transfected with control, *p73*, and *G6PD* siRNA as indicated. Cells were assayed for BrdU incorporation (**D**). Representative images of cells stained with BrdU (**E**).

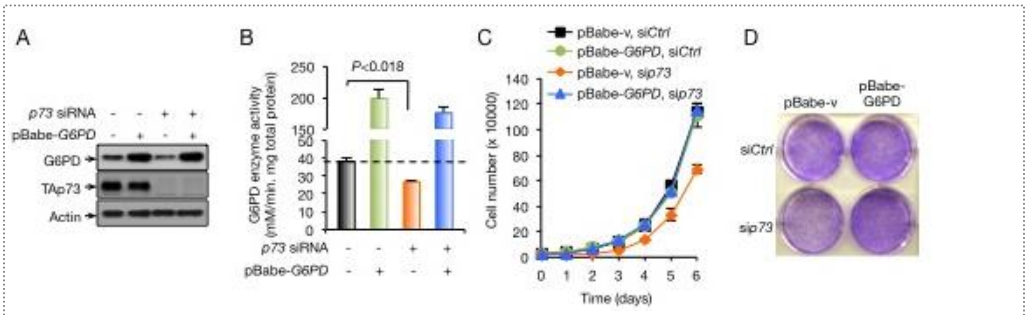

Figure 2. Over-expression of G6PD rescues growth defects of p73-depleted cells. (**A** and **B**) Protein expression (A) and G6PD activity (B) in H1299 cells stably overexpressing G6PD or vector control in the presence or absence of *p73* siRNA are shown. (**C** and **D**) H1299 cells stably overexpressing G6PD or vector control were treated with *p73* or control siRNA as indicated. Cell proliferation (C) and representative images of cells stained with crystal violet at day 6 (D) are shown.

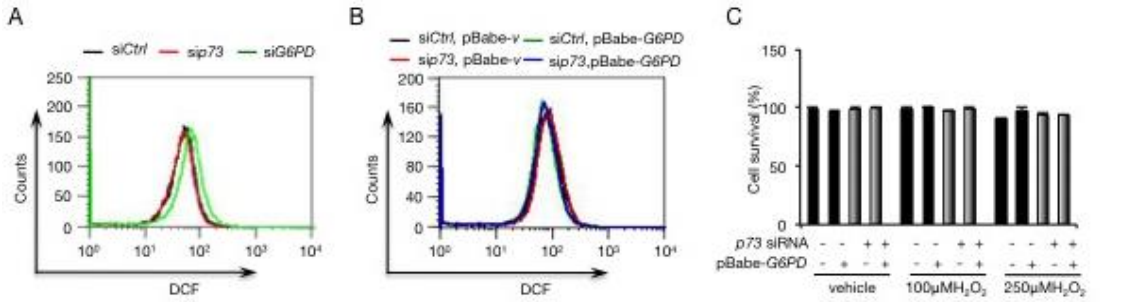

Figure 3. p73 depletion fails to affect ROS in H1299 cells. (**A**) ROS levels in H1299 cells transfected with indicated siRNA. (**B**) H1299 stably overexpressing G6PD or vector control were transfected with *p73* siRNA or control siRNA as indicated. ROS levels were analyzed. (**C**) H1299 stably overexpressing G6PD or vector control were transfected with *p73* siRNA or control siRNA as indicated. Cells were treated with or without 100 μM or 250 μM H_2O_2 for 24 hours, and cell viability was analyzed by trypan blue staining.

Research Project 9: Project Title and Purpose

Single Cell Transcriptomics for High Resolution Cell Characterization – The purpose of this project is to develop new technologies for assessing the transcriptome at the high resolution level of individual cells and use the characterization to understand the role of hidden molecular subtypes in modulating cell phenotype, including those involved in development and response to therapeutics.

Anticipated Duration of Project

1/1/2013 – 12/31/2016

Project Overview

Specific aims:
Aim 1: Develop an efficient and robust method for single cell transcriptome profiling in dispersed cell cultures and in intact tissues.
Aim 2: Using the new technology developed in Aim 1, assess single cell transcriptomes from two model organisms, mouse and *C. elegans*.

In this project, we will test the hypothesis that hidden molecular subtypes of cells exist in multi-cellular organisms and that the molecular subtypes will be predictive of cell variability both during organismal development and under environmental changes, such as with animal aging or drug treatment. To address this hypothesis, in Aim 1, we will develop novel biotechnologies to robustly assess single cell RNA quantities. The novel technologies will utilize next generation RNA sequencing and laser-capture of RNA pools from individual cells. Briefly, we will develop a new technique to add unique sequence tags to pools of RNA and use next generation sequencing to identify the association between unique digital tags and transcripts. This method will help increase the efficiency of single cell RNA profiling by at least 5-fold and make the technique sufficiently robust for broad use. We will also refine the application of a laser-

mediated reagent to capture RNA in intact tissue slices. In Aim 2, the technology will be applied to understanding individual cell variation during *C. elegans* development and in mouse cell response to non-steroidal anti-inflammatory drugs. These experiments in model organisms will provide information for understanding the interaction of hidden molecular variation and cell responses. The results of this study will generate novel biotechnologies and fundamental information that can be used in understanding cell phenotype and degeneration.

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Expected Research Outcomes and Benefits

The results of this project are expected to have two key outcomes and benefits. First, while technologies currently exist for measuring RNA from single cells, the methods are difficult, time-consuming, and non-standardized such that only a few laboratories in the world can carry out the assays with robustness. We will develop new, efficient and robust technologies for quantitatively characterizing the RNA contents of individual cells both in cell culture and in tissue contexts. These technologies will help disseminate single cell measurement techniques to a much broader research community allowing applications in such areas as the study of cell degenerative diseases and cancer. To maximize benefit, we will directly train researchers in state-of-the-art single cell RNA profiling techniques. Second, the project will help delineate the genomic factors that govern cell phenotype variability in both mature and developing organisms, which will generate fundamental knowledge for understanding how to apply cell-based therapeutics and for understanding how the variation in a cell's molecular subtype modulates variation in response to drug treatment.

Summary of Research Completed

During this reporting period, we have made progress on Specific Aim 1: Develop an efficient and robust method for single cell transcriptome profiling in dispersed cell cultures and in intact tissues. We have made two advances in this area, one for in vivo profiling of RNA from individual cells and second for digitally tagging RNA sequences for better quantification.

Transcriptome In Viva Analysis (TIVA):
We developed a novel reagent called TIVA-tag (Lovatt, D., et al., *Transcriptome in vivo analysis*

(TIVA) of spatially defined single cells in live tissue. Nat Methods, 2014. 11(2): p. 190-6). We synthesized a photo-activatable poly-A RNA capture reagent called TIVA-tag shown in Fig 1. Briefly, the TIVA-tag consists of a polyT (probe)-polyA (blocking strand) oligonucleotide hairpin, with photocleavable linkers. The TIVA-tag is conjugated to a cell-penetrating peptide, which allows entry into any cell. Once applied to culture or tissue, it can be photo-activated within a single cell or subcellular compartment to reveal the polyT probe, which anneals to endogenous cellular RNA. The biotin group that is also part of the TIVA-tag allows efficient RNA isolation using streptavidin-labeled beads. The activation of the TIVA-tag can be monitored by a change in FRET signal from two spectrum-coupled dyes (see Fig 2). This figure shows an extension of the method where we have used a two-photon laser to activate the tag in only a single dendrite. Comparison of TIVA-isolated single dendrite transcriptome and mechanically dissected transcriptome from 300 dendrites show a correlation of 0.6, which is average range for single cell-to-cell variation.

In our second development, we have developed a transposon-based method to add digital sequence signatures into cDNA from transcriptomes. Currently, next-generation sequencing for RNA quantification is carried out by amplifying RNA and then sequencing millions of reads of a library made from the amplified RNA pool. The sequenced reads are mapped back to transcript models of the studied genome and the multiplicities of the mapped reads are used to quantify the degree of expression of that gene (transcript). A problem here is that RNA amplification may give distorted representation of the original transcriptome. One way to overcome this problem is to tag each of the original RNA with a unique sequence bar-code such that after amplification, RNA is quantified by counting the number of unique bar-codes rather than the number mapped reads. There are several approaches in the literature for such “digital” tagging of DNA or RNA. A problem with all of the approaches is that the method of incorporating the digital signature tends to yield molecular artifacts making the techniques difficult with low reproducibility. We have developed a strategy based on Tn5 transposon. We designed a custom transposon with the Tn5 mosaic ends, which allows incorporation into double-stranded DNA by the Tn5 transposases. The custom transposon was designed with a unique pattern framework to setup a 15bp random sequence tag. Using 15 base positions gives us 2^{30} ~10^{9} different tags, which should be sufficient to uniquely associate with most small pools of RNA. The transposon also has a restriction and cloning site designed into it such that the incorporation can be also used to generate a fragmentation library and cloned if needed. We have successfully demonstrated incorporation and sequencing the results yielded expected digital tagging. We are working on increasing the sensitivity such that the method can be used to tag single cell transcriptomes.

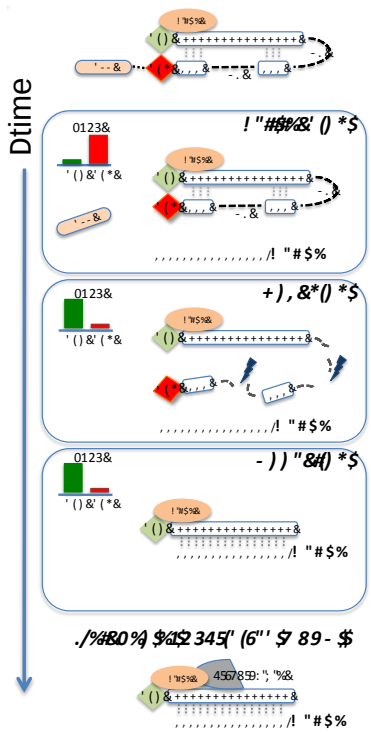

Fig 1:.Schematic of the TIVA-Tag protocol and how it is used to isolate endogenous cellular mRNA populations.

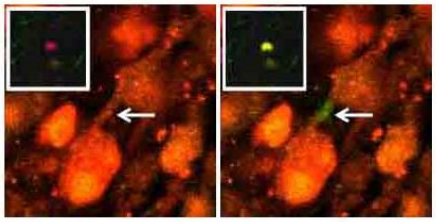

Fig 2: Activation of the TIVA-tag in a single dendrite in the live slice preparation. The arrow points to the dendrite that is activated. The inset shows a single cell soma in the slice that fluoresces red until the TIVA-tag is activated by UV-uncaging.

Research Project 10: Project Title and Purpose

Research Infrastructure: Construction of the Neuro-Behavioral Sciences – The purpose of this research infrastructure project is to contribute to the creation of a 45,000 gross square foot research facility that facilitates basic science research at the boundaries of Psychology and Biology. This research facility will occupy the contiguous upper 3 floors of a larger 75,000 GSF building dedicated to (1) research at the boundaries of Psychology and Biology to (2) undergraduate instruction in both Biology and Psychology and to (3) creation of a shared home for four of the undergraduate life science programs in the School of Arts and Sciences. Health research grant funds shall be used for construction of a facility that shall be used solely for the purpose of biomedical, clinical or health research as defined by Act 2001-77.

Anticipated Duration of Project

1/1/2013 – 12/31/2016

Project Overview

The purpose of this research infrastructure project is to contribute to the creation of a 45,000 gross square foot research facility that facilitates basic science research at the boundaries of

Psychology and Biology. This research facility will occupy the contiguous upper 3 floors of a larger 75,000 GSF Neuro Behavioral Sciences building dedicated to (1) research at the boundaries of Psychology and Biology to (2) undergraduate instruction in both Biology and Psychology and to (3) creation of a shared home for four of the undergraduate life science programs in the School of Arts and Sciences

A total of 45,000 gsf in the new Neuro Behavioral Sciences Building will be dedicated to research at the boundaries of modern psychology with a focus on the areas of Animal Learning and Behavior, Behavioral and Cognitive Neuroscience, Decision Processes, Developmental Psychology, Language and Communication, Memory and Learning, Sensation and Perception, Developmental Psychology, Evolutionary Psychology, Positive Psychology, Psychopathology and Psychotherapy, Social and Cultural Psychology. These research areas will be located with and physically connected on every floor to the Department of Biology - creating a nexus of study in the broad strategic area of Genes to Brains to Behavior. The research floors will include faculty offices for the Department of Psychology, graduate student and postdoctoral researcher offices, research rooms for the various needs of the Department of Psychology, and various seminar and meeting spaces for the occupants of the research facility. The research facility will be fully segregated via electronic security from the remainder of the building.

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

The principal outcomes of this infrastructure project will be to improve the workspace quality and ease of collaboration of the faculty performing research under the aegis of the School's strategic focus on Genes to Brains to Behavior

The project is designed with sufficient space such that the complex (Lynch, NBS, Leidy, Goddard) houses both the offices and research laboratories of all faculty in Biology and Psychology. A key architectural achievement for the complex is that it will enhance existing interactions and facilitate new ones, particularly interactions that promote integrated studies of genes, brains, and behavior. The building will also be sufficiently versatile to meet current needs and to adapt rapidly to accommodate new research technologies as the fields evolve.

Summary of Research Completed

During the reporting period substantial progress was made on the construction of this research facility. Construction documents were completed, bids were awarded, site clearing was completed, excavation was completed, and construction of the foundation and core support structures in the facility was begun.

Project 11: Project Title and Purpose

VEGF Receptor Inhibition in Lung and Liver Metastases – Therapies that target vascular endothelial growth factor (VEGF) pathways are being increasingly utilized in the treatment of metastatic cancers. This project focuses on tissue-specific differences in the tumor microenvironment that may affect the efficacy of anti-angiogenic therapies. This project specifically explores the effectiveness of VEGF receptor 1 and 2 inhibition in preventing the establishment or growth of lung and liver metastases and may lead to the identification of the optimal combination for site specific anti-angiogenic strategies. Thus, this work could have substantial impact in treatment approaches for metastatic disease.

Anticipated Duration of Project

1/1/2013 – 12/31/2016

Project Overview

Aim 1: To examine VEGFR-1 and VEGFR-2 inhibition on liver and lung metastases.
Subaims:

I. Determine the effects of VEGFR-1 and VEGFR-2 inhibition on oxaliplatin-based chemotherapy for experimental colorectal cancer lung and liver metastases.
II. Assess the toxicity of long-term VEGFR-1 and/or VEGFR-2 inhibition in mice.

In this project, we aim to examine VEGFR-1 and VEGFR-2 inhibition on liver and lung metastases. We will investigate both the effects and the role of VEGF receptor inhibition on metastatic disease using transplantable tumor models and experimental metastases models with antibody inhibition of either VEGFR-1 or VEGFR-2 as well as a combination of R1 and R2 inhibition together with oxaliplatin-based chemotherapy and assess the toxicity of long-term VEGFR-1 and/or VEGFR-2 inhibition in mice.

Patients with solid tumors usually do not die from their primary tumors but rather from uncontrolled metastatic disease, and the liver and lung are two of the most common sites of metastases for many cancers. The primary treatment of patients with known metastatic disease to the liver and lung is usually cytotoxic chemotherapy, which is uncommonly curative but can often delay disease progression. Anti-angiogenic therapies, usually in combination with chemotherapy, are now standard in the treatment of metastatic colorectal cancer and other cancers and are being increasingly evaluated in the adjuvant setting. Many patients do not have identifiable metastatic disease at the time of diagnosis but rather harbor radiologically occult micrometastases that ultimately grow to lethal sizes. These micrometastases require angiogenesis

to grow beyond a couple millimeters in size. Vascular endothelial growth factor (VEGF), a key angiogenic factor in both primary tumors and metastases, exerts most of its effects though VEGF receptors 1 and 2 (VEGFR-1, VEGFR-2). Given the significant differences in vasculature of different organs, it has been postulated that key differences exist in the angiogenic pathways used by metastases in organs.

Published studies and preliminary data from my lab and our collaborators, have shown that VEGF mediates its pro-angiogenic effects on *lung* endothelial cells primarily via VEGFR-2 and its effects on *liver* endothelial cells primarily via VEGFR-1. These studies also found that the use of VEGFR-1 neutralizing antibody suppressed experimental *liver* metastases but not *lung* metastases, while VEGFR-2 neutralizing antibody suppressed experimental *lung* metastases but not *liver* metastases. Here we propose to explore the differences in angiogenic pathways utilized by metastases in different host organ environments as a prerequisite to the development of site-specific anti-angiogenic strategies. *Specifically, we aim to examine VEGFR-1 and VEGFR-2 inhibition on liver and lung metastases*. We will investigate both the effects and the role of VEGF receptor inhibition on metastatic disease using transplantable tumor models and experimental metastases models in mice by antibody inhibition of VEGFR-1 or VEGFR-2 as well as a combination of both receptors together with oxaliplatin-based chemotherapy. We will also assess the toxicity of long-term VEGFR-1 and/or VEGFR-2 inhibition in mice.

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Expected Research Outcomes and Benefits

The cause of death from cancer is more often due to metastatic disease, which commonly occurs in the liver or lung, rather than due to the primary tumor. Once microscopic metastases are established at distant sites, angiogenesis, or the formation of new blood vessels, is a critical requirement for metastases to grow beyond a microscopic size. The long-term goal of this project is to elucidate differences in angiogenic pathways utilized by metastases in different organs as a prerequisite to the development of site-specific anti-angiogenic strategies.

The best-characterized angiogenic factor is vascular endothelial growth factor (VEGF), which exerts its effects primarily through two tyrosine kinase receptors VEGFR-1 (VEGFR-1, Flt-1) and VEGFR-2 (VEGFR-2, Flk-1, KDR). The central hypothesis of this proposal is that the effects of VEGFR-1 and VEGFR-2 inhibition on the establishment and growth of metastases

differs significantly in the lung and liver host organ environments. We will focus on elucidating the effects of VEGFR-1, and VEGFR-2 inhibition on the establishment and growth of metastases in the liver and lung in experimental metastases models in mice. Insights gained through our studies towards understanding of the VEGF pathways utilized in lung and liver metastases may lead to improved therapies targeting these pathways. This work may also lead to the identification of optimal combinations for site-specific anti-angiogenic strategies in the treatment of metastatic disease and lay a foundation for future clinical trials of VEGFR-1 and VEGFR-2 inhibitors.

Summary of Research Completed

Aim I: Determine the effects of VEGFR-1 and VEGFR-2 inhibition on oxaliplatin-based chemotherapy for experimental colorectal cancer lung and liver metastases.

Studies for this Aim have been investigating the response of experimental lung and liver metastases of CT26 mouse colon carcinoma cells to VEGFR1 and VEGFR2 inhibition by the monoclonal antibodies MF-1 and DC101, respectively. We established the timeline for lung and liver metastases in Balb/C wild-type mice with 5 X 10^5 cells injected via tail vein demonstrating abundant lung metastases 18 days after tail vein injection. Similarly, intraportal injection of 5 X 10^5 CT26 mouse colon carcinoma cells into Balb/c wild-type mice leads to liver metastases after 14 days. Our initial attempts to generate large quantities of rat monoclonal antibodies against VEGFR-1 (referred to as MF-1) and VEGFR-2 (referred to as DC101) using commercially available hybridomas led to very low concentration of antibodies. Thus, we have begun to utilize a bioreactor for large-scale production of monoclonal antibodies from these 2 hybridomas and will concentrate the secreted antibody obtained from the bioreactor. Antibody production for mouse treatment studies is currently on-going.

Aim II: Assess the toxicity of long-term VEGFR-1 and/or VEGFR-2 inhibition in mice.

Progress on this Aim includes optimizing lung and liver metastases with CT26 mouse colon carcinoma cell lines into $Rag1^{-/-}$ mice. Using both male and female $Rag1^{-/-}$ mice, our studies demonstrate that injection of 5 X 10^5 CT26 mouse colon carcinoma cells via tail vein leads to lung metastases 10-12 days after injection. As anticipated, metastatic tumor lesions formed earlier in $Rag1^{-/-}$ mice due to the lack of immunosurveillance. Similarly, intraportal injection of 5 X 10^5 CT26 mouse colon carcinoma cells into $Rag1^{-/-}$ mice demonstrated liver metastases after 8 days. We are currently reducing the number of cells we inject so that the timeline for the formation of lung and liver metastases will be extended to ensure adequate time for early treatment. As described above in progress for Aim 1, the difficulty in obtaining large concentrated quantities of MF-1 and DC101 monoclonal antibodies for VEGFR1 and VEGR2 inhibition have prevented us from beginning our studies on long-term toxicity. We are currently troubleshooting monoclonal antibody production using a bioreactor.

Research Project 12: Project Title and Purpose

Understanding Wnt5a Regulation of Protein Depalmitoylation During Cell Migration – Most cancer deaths are caused by dissemination of cancer cells away from the primary tumor to distant

sites in the body. However, little is known about what causes cancer cells to metastasize. Highly motile cells require cell polarity to establish the front and rear of the cell for efficient directional migration and chemotaxis, but the mechanisms that regulate cell polarity are poorly understood. Since starting my own lab I have found that Wnt5a induces protein depalmitoylation that causes polarized protein localization and increased invasion in melanoma cells. We will continue these studies to understand how Wnt5a regulates this process to promote metastasis.

Anticipated Duration of Project

1/1/2013 – 12/31/2016

Project Overview

Aim 1: Define the downstream Wnt5a signaling events that regulate protein depalmitoylation and polarized protein localization. *Hypothesis: MCAM depalmitoylation is induced by Wnt5a mediated downstream signaling events and identification of other palmitoylated targets will be identified by mass spectrometry.* a) Identify downstream Wnt5a components that regulate MCAM depalmitoylation. b) Identify and characterize Wnt5a regulated palmitoylated proteins by proteomics.

Aim 2: Determine the regulation and function of protein palmitoylation cycle during directional cell migration. *Hypothesis: Decreased palmitoylated MCAM caused by reduced protein acyl-transferase activity or Wnt5a induced APT1 activity promote invasive behavior.* a) Determine the effect of mutations in DHHC proteins identified in patient tumor samples on polarized MCAM localization and cell invasion. b) Identify novel regulators of Wnt5a signaling and protein depalmitoylation through immunopurification of APT1 interacting proteins. c) Determine the levels of palmitoylated MCAM in tumors.

My lab has focused on regulation of polarized MCAM localization in response to non-canonical Wnt signaling. We metabolically labeled cells with labeled palmitate and we found that cysteine 590 is palmitoylated in melanoma and the levels of palmitoylated MCAM are reduced within 30 minutes of Wnt5a treatment. Inhibition of the depalmitoylating enzyme APT1 (acyl protein thioesterase 1) with the drug palmitostatin B blocks MCAM depalmitoylation, polarized localization of MCAM, and invasion in 3D collagen.

Palmitoylation is the reversible lipid modification of cysteine that regulates protein trafficking and localization. Palmitoylation regulates protein localization at the immune synapse in T-cells and the localization of glutamate receptors at neuronal synapses. The membrane localization of Ras is also regulated by palmitoylation, but little is known about the function of protein palmitoylation during metastasis.

My lab recently mutated cysteine 590 to glycine (C590G) in MCAM and found that when expressed causes polarized localization of MCAM and increased invasive behavior of melanoma cells in 3D collagen. This gain of function mutation bypasses the need for added Wnt5a and has allowed us to address the *in vivo* function of dynamic polarized MCAM localization in cancer.

We have implicated regulation of MCAM depalmitoylation in tumor cell invasion. Human melanoma cells transiently expressing the palmitoylation resistant $C^{590}G$ MCAM-GFP mutant were injected subcutaneously into SCID mice and the resulting tumors displayed more invasive front with MCAM-GFP cells detached from the tumor compared to the compact tumors formed from wild type MCAM-GFP expressing cells

The proposed studies will begin to shed light on the role of protein palmitoylation during melanoma metastasis that has been mostly unexplored.

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Expected Research Outcomes and Benefits

The non-canonical Wnt5a signaling pathway plays a paramount role in important biological processes as it regulates cell polarity and polarized cell movement during embryonic development. Wnt5a is also upregulated in melanoma cell lines that exhibit invasive behavior and immunohistochemistry staining of melanoma biopsies that revealed high levels of Wnt5a in late stage metastatic tumors compared to early non-metastatic tumors.

We have demonstrated Wnt5a regulates the reversible modification of cell adhesion molecules with palmitate in melanoma, breast and ovarian cancer cells. We show that the Wnt5a induced depalmitoylation of the cell adhesion molecule MCAM regulates polarized localization of the proteins, increases cell invasion, and contributes to polarized cell movements during vertebrate development.

We have identified the 2 enzymes, DHHC20 and APT1 that regulate the palmitoylation state of MCAM in our system and have demonstrated their function during cell invasion. We are uniquely positioned to elucidate the molecular mechanism of Wnt5a regulation of protein palmitoylation during cell polarization and directional cell migration using biochemical, cell biological and genetic approaches.

As the biochemical regulation of cell polarity becomes understood strategies that specifically block cell polarized cell movement during cancer metastasis could lead to novel therapeutic approaches. Of equal importance is understanding the signaling mechanism of Wnt5a regulated

protein palmitoylation and cell polarization could provide much needed insight into cell movement and migration during embryogenesis that when disrupted can result in birth defects.

Summary of Research Completed

Aim 1: Define the downstream Wnt5a signaling events that regulate protein depalmitoylation and polarized protein localization. *Hypothesis: MCAM depalmitoylation is induced by Wnt5a mediated downstream signaling events and identification of other palmitoylated targets will be identified by mass spectrometry.*

a) Identify downstream Wnt5a components that regulate MCAM depalmitoylation.

Research Completed:
We identified the Wnt cytosolic protein Dvl2 as an interacting protein of APT1. We have determined that APT1 interacts with the PDZ and DEP domains of Dvl2 (Figure 1A). The PDZ domain interacts with MCAM and we hypothesize Dvl2 is a scaffold mediating interaction between APT1 and MCAM and is sufficient to attenuate the interaction between APT1 and MCAM in the presence of Wnt5a (Figure 1B, C). When Dvl2 is inhibited by siRNA we do not see labelling of MCAM with palmitate. However, treatment with the APT1 inhibitor palmostatin B does not increase the level of palmitoylation indicating the decrease in palmitoylated MCAM isn't caused by increased APT1 activity and is more likely caused by loss of labeling due to decreased turnover of palmitate on proteins. We are optimizing an alternative approach called an acyl-biotin exchange assay to measure protein palmitoylation. This assay directly labels palmitoylated cysteine residues eliminating the need to metabolically label cells with palmitic acid azide. This assay is independent of turnover of palmitate on proteins. We expect Dvl2 inhibition with either siRNA or by expressing different Dvl2 domains to block the MCAM-APT1 interaction and inhibit MCAM depalmitoylation.

b) Identify and characterize Wnt5a regulated palmitoylated proteins by proteomics.

Research Completed:
We have found that DHHC20, a palmitoyl transferase that we have characterized in melanoma, also palmitoylates the epidermal growth factor receptor (EGFR) in breast cancer cells (Figure 2A). The palmitoylation of EGFR is blocked when the C-terminal tail is deleted suggesting the C-terminal tail is the site of palmitoylation (Fig 1A, C). The region of the C-terminal tail that contains cysteine residues (exons 25 and 26) has been shown by other groups to be deleted in lung cancer patient samples due to complex genomic rearrangements (Figure 2D). Mutating the putative palmitoylation site in exon 25 (cysteine 1049) increases EGFR receptor signaling measured by autophosphorylation similar to levels observed in the deletion mutant (Figure 2B, D). Inhibition of DHHC20 expression increases the response of EGFR to EGF treatment by as much as 10 fold (Figure 2E). Inhibition of the palmitoyl transferase DHHC20 by shRNA causes increased cell migration and is blocked by treatment with the EGFR inhibitor gifitinib (Figure 2F). Furthermore, patient data in The Cancer Genome Atlas shows DHHC20 is mutated in 2% of lung adenocarcinoma tumors.

To profile Wnt5a regulated palmitoylated proteins we need a method for purifying the

palmitoylated proteins for mass spectrometry analysis. The acyl-biotin exchange assay allows us to directly label palmitoylated proteins with biotin to isolate on streptavidin beads. We have this assay working in the lab for small scale analysis by western blotting. We will now scale the process up for the proteomics study.

Aim 2: Determine the regulation and function of protein palmitoylation cycle during directional cell migration. *Hypothesis: Decreased palmitoylated MCAM caused by reduced protein acyl-transferase activity or Wnt5a induced APT1 activity promote invasive behavior.*

a) Determine the effect of mutations in DHHC proteins identified in patient tumor samples on polarized MCAM localization and cell invasion.

Research Completed: This sub aim was completed as of the last reporting period.

b) Identify novel regulators of Wnt5a signaling and protein depalmitoylation through immunopurification of APT1 interacting proteins.

Research Completed:
We have identified the known Wnt signaling protein Dvl2 as an APT1 interacting protein. This provides a useful positive control when searching for other APT1 interacting proteins. We continue to optimize our immunopurification of APT1 from control and Wnt5a treated cells. When we have a high enough yield of APT1 and interacting proteins we will excise bands and send them out for mass spectrometry.

c) Determine the levels of palmitoylated MCAM in tumors.

Research Completed:
We are optimizing the acyl-biotin exchange assay on tissue culture cells described in 2b to directly measure protein palmitoylation. When we have this assay working efficiently we will apply this to melanoma tumor samples. This will allow us to measure protein palmitoylation in human tissue samples by immunoblotting.

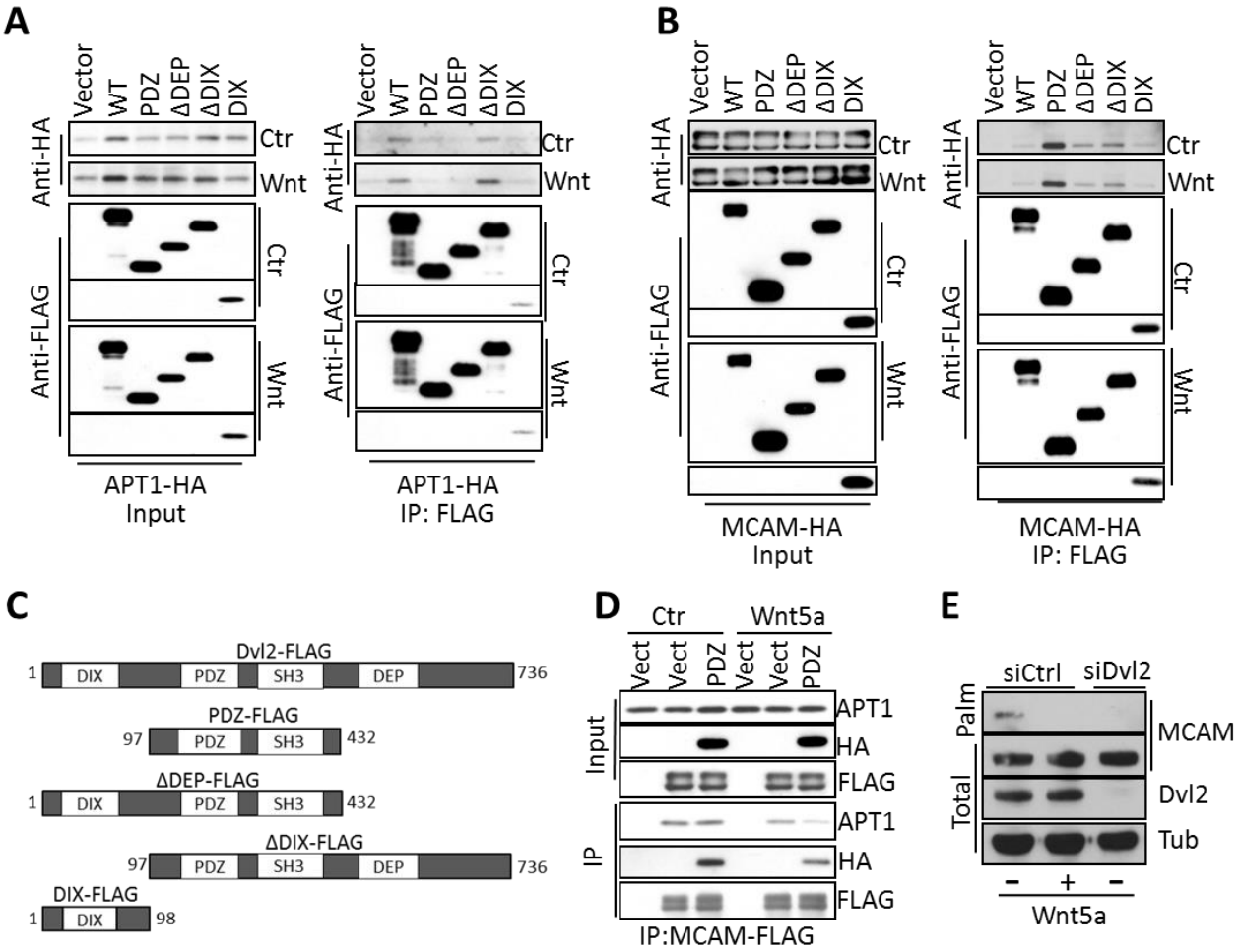

Figure 1. A) Expression of MCAM-HA, FLAG tagged Dvl2 and Dvl2 domains. Immunoprecipitation of FLAG reveals an interaction between Dvl2 and APT1 that requires both the PDZ and DEP domains (ΔDIX) of Dvl2. B) Expression of Dvl2 domains with HA tagged MCAM. The PDZ domain interaction with MCAM is greater than the full length WT Dvl2. C) Dvl2 deletion constructs used in A&B. D) Expression of the FLAG tagged Dvl2 domains and MCAM-HA. D) Overexpression of the HA tagged PDZ domain blocks the interaction between MCAM-FLAG and endogenous APT1 in the presence of Wnt5a. E) Inhibition of Dvl2 by siRNA blocks MCAM palmitoylation.

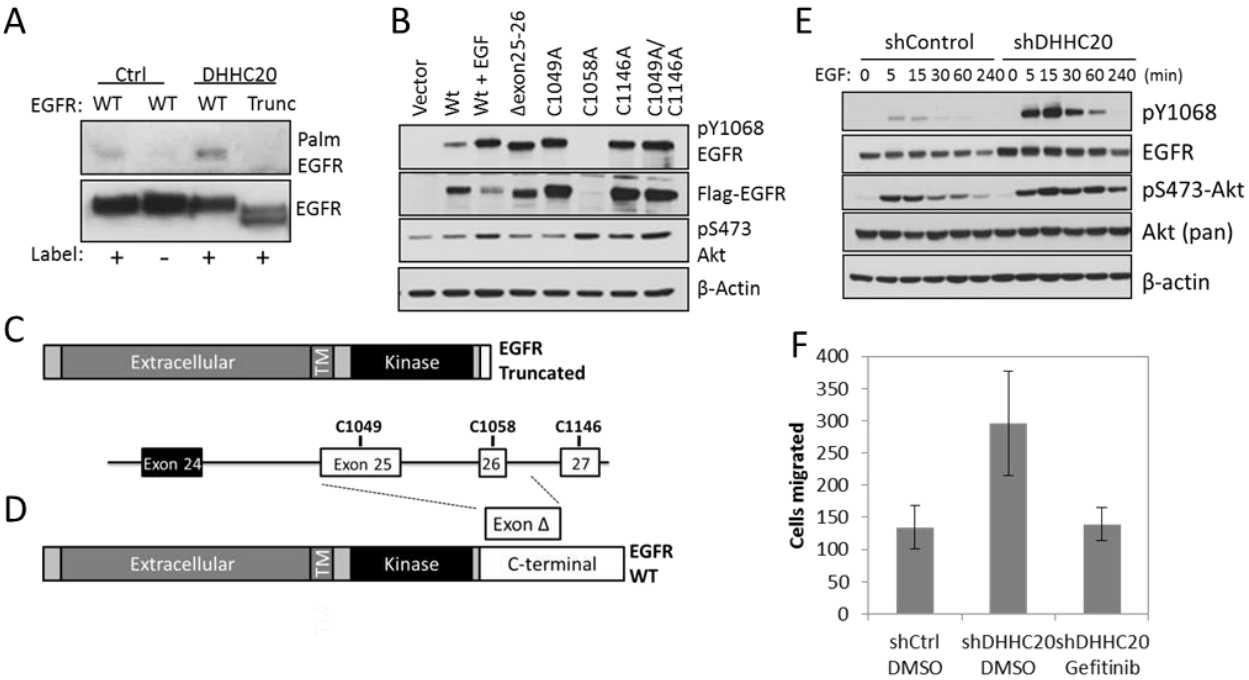

Figure 2. A) EGFR palmitoylation increases with ectopic DHHC20 expression and truncation of the C-terminal tail abolishes palmitoylation. B) Deletion of exons 25, 26 increases EGFR phosphorylation. Mutation of the cysteines in the C-terminal tail increases EGFR autophosphorylation to a level similar to the exon deletion in NIH3T3 cells and increases Akt activation. C) Position of the EGFR truncation used in the palmitoylation assay in (A). D) Positions of putative palmitoylation sites and the site of the EGFR exon deletion previously identified in lung cancer patient samples. E) Inhibition of the palmitoyl transferase DHHC20 increases the activation of EGFR in response to the ligand EGF. F) Inhibition of DHHC20 increases migration of breast cancer cells. The migration is inhibited by treatment with the EGFR inhibitor gifitinib.

Research Project 13: Project Title and Purpose

Long Noncoding RNAs Required for Human Female Preimplantation Development – This project will focus on recently discovered epigenetic regulators of expression called long noncoding RNAs (lncRNAs). We will utilize human cell culture models to identify lncRNAs important for early human female preimplantation development, in particular X-linked transcripts involved in X-chromosome silencing, a critical event required for normal female development. Our goal is to develop markers that can identify abnormal placental development, predict poor implantation, and signal problems with embryonic differentiation, a powerful tool for preimplantation genetic diagnosis screens.

Anticipated Duration of Project

12/18/2013 – 12/31/2016

Project Overview

Epigenetic regulation is defined by stable and heritable changes in gene expression and cellular phenotype that are independent of DNA sequence. Epigenetic mechanisms play important roles in a variety of biological processes, including development and cellular differentiation. As such, alterations in these regulatory pathways are responsible for birth defects and adult disease. Noncoding RNAs are critical epigenetic regulators of gene expression. The known repertoire of noncoding RNAs, especially long noncoding transcripts (defined as >200 nucleotides in length), has increased dramatically due to advances in high-throughput sequencing. Long noncoding RNAs (lncRNAs) outnumber proteins, yet few have been well characterized, especially during early human development.

We hypothesize that lncRNAs are required for normal developmental programs in human preimplantation embryos, and that perturbations in their expression will compromise growth and development of the embryo and placenta. This project will use Next-Generation Sequencing and lineage-specific reporter cell lines to identify lncRNAs important for early human female preimplantation development, in particular X-linked transcripts involved in X-chromosome silencing, a critical event required for normal female development.

Our Aims are:
(1) determine the complete expression profile of lncRNAs in human cell culture models of female hiPSCs and trophoblasts,
(2) identify lncRNAs required for human female epiblast and trophectoderm lineages using reporter cell lines, and
(3) determine lineage specificity for the lncRNAs found to function in epiblast differentiation and trophectoderm formation.

Our long-term goals are: (1) to understand the molecular details of lncRNA-mediated gene regulation required for human female blastocyst development; and (2) to develop robust lncRNA markers for assessing human female embryo quality for use in preimplantation genetic diagnostic screens.

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Expected Research Outcomes and Benefits

This project will be the first to identify female-specific human long noncoding RNAs from high-quality cell lines representing the first two developmental lineages: the epiblast (embryo) and the trophectoderm (placenta). We will distinguish long noncoding RNAs associated with normal development from those expressed abnormally.

We will develop human female reporter human pluripotent stem cell lines (OCT4-GFP for epiblast; CDX2-mCherry for trophectoderm lineages) and use them to screen for functionally relevant human long noncoding RNAs required for pluripotency, epiblast differentiation (OCT4 line), and trophectoderm formation (CDX2 line).

We will use genome engineering technology to generate stable female hiPSCs lines with transcriptional disruptions of important long noncoding RNAs. These lines will be differentiated *in vitro* to determine the lineage-specific molecular consequences when these long noncoding RNAs are deleted. We anticipate that the long noncoding RNAs identified by these experiments will be used as screening markers for assessing human embryo quality in preimplantation genetic screens.

Summary of Research Completed

Since the initiation of funding on the HRFF project entitled "Long noncoding RNAs required for human female preimplantation development" on December 1, 2013, we have made significant progress on the specific aims proposed in the strategic plan.

Aim 1: We proposed to examine the transcriptional profiles for 2 developmental stages: pluripotent stem cells and cells differentiated with BMP4, which form early trophoblast cells. We have completed the massively parallel sequencing of a group of the human female pluripotent stem cells, comparing XIST-expressing lines with those that have irreversibly silenced the XIST gene. For this project, we generated strand-specific libraries from 5 isogenic hiPS lines (XIST+ or XIST-) and the HUES-9 XIST-expressing human embryonic stem cell line. We sequenced these libraries at the BGI CHOP facility using their Hi-Seq instrument, and obtained roughly 50 million reads for each sample., Working in collaboration with Dr. Kyoung Jae Won (UPenn, Genetics), an expert in bioinformatics analyses, the reads were aligned to the Human Ref Seq database, and we used ran several scripts to identify transcriptional units across the genome. First, we compared expression between XIST-expressing and XIST-lacking cell lines, and observed that the greatest difference with expression occurred at the X-chromosome. XIST-lacking female hiPSCs had greater numbers of reads mapping to the X, suggesting that the loss of XIST RNA expression resulted in reactivation of the silent X-chromosome (Figure 1A). We also observed a reduction in the number of reads mapping to chromosome 6 in cells lacking XIST RNA expression, and future experiments are investigating how XIST gene silencing could correlate with altered expression from this autosome. Next, we developed a bioinformatics pipeline to identify novel long noncoding RNAs expressed in human female pluripotent stem cells. Unlike many of the recent publications profiling long noncoding RNA expression in human pluripotent stem cells, we did not exclude transcripts overlapping coding genes. We identified over 350 novel transcripts (not annotated in ENSMBL nor Ref Seq databases)

expressed throughout the genome, with the largest chromosomes containing more novel transcripts (Figure 1B). Next, we examined the average length for the group of novel long noncoding RNAs, and found no correlation with chromosome size. Interestingly, we observed that the X and chromosomes 6 and 13 had the largest gene sizes for these novel long noncoding RNAs (Figure 1C). Finally, we sought to determine the most biologically relevant novel long noncoding RNAs in female pluripotent stem cells. Previously, we found that XIST RNA expression is a robust marker of stem cell quality, where cells lacking XIST expression are low quality and have cancer-related characteristics. We assembled a list of the top 10 candidate long noncoding RNAs (for future investigation) that were exclusively (or abundantly) expressed in high quality, XIST-expressing lines (Figure 1D). We are now validating the expression of these transcripts using qRT-PCR, and will characterize their function in human stem cells in the future.

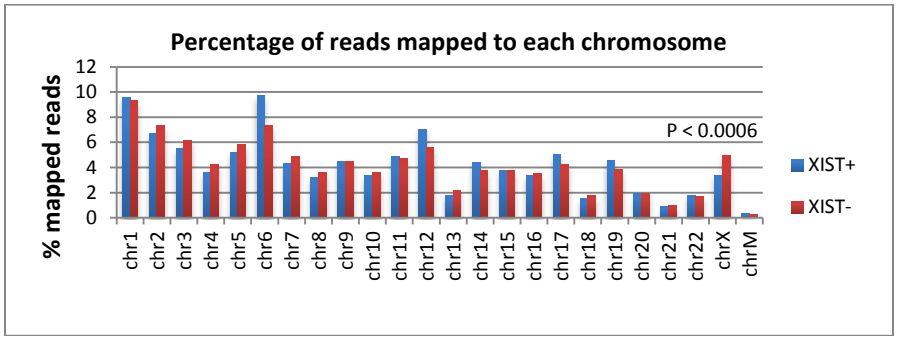
The second part of Aim 1 is to profile the transcriptional signature of BMP-4 differentiated human pluripotent stem cells in order to identify long noncoding RNAs that regulate the formation of progenitor cells for the placenta. First, we optimized the in vitro differentiation conditions that would yield the greatest expression of CDX2, a transcription factor that specifies the extra-embryonic lineage in mice. We reasoned that humans would also express CDX2 during the early stages of trophectoderm formation, which is observed in extra-embryonic cells of human preimplantation embryos. We profiled CDX2 expression and another lineage marker for placenta and mesoderm, cytokeratin-7 (CK7), in human embryonic stem cells and during BMP4-induced differentiation (Figure 2A). We found that day 2 of BMP4 treatment yielded the greatest CDX2 expression via immunofluorescence, and that the population of differentiated cells was nearly homogenous for CDX2 expression via FACS sorting (Figure 2B). We also verified BMP4 differentiation efficiency using qRT-PCR (Figure 3), and confirmed that day 2 exhibited the greatest expression of CDX2 transcripts, and that NANOG expression (a marker of pluripotency) rapidly decreases by differentiation day 2. We also observed expression of CK-7 appearing at day 2 of differentiation, and expression continued past day 4 (data not shown). These results indicate that CDX-2 specific purification of differentiated cells is not necessary prior to RNA-Sequencing, and confirm the utility of BMP4-mediated differentiation for generating trophoblast cells (expression of placental hormones and markers were also confirmed for d10-15; data not shown). We have differentiated human male and female pluripotent stem cell lines using BMP4 for 2 days, isolated RNA, and are ready to submit libraries for RNA-Sequencing analysis. We plan to continue collaborating with Dr. Won's group for the bioinformatics portion of this project.

Aim 2: We sought to generate two reporter cell lines to use in functional screening of long noncoding RNAs that affect either pluripotency or formation of the trophoblast lineage. For investigating the pluripotency/self-renewal function, we used an OCT4 reporter system, which was designed by Rudolf Jaenische's lab. We obtained two different targeting vectors that introduce eGFP immediately after the stop codon (Figure 2D), and TALENs designed to introduce double-strand breaks in this region. We targeted a male and female human embryonic stem cell lines with these constructs, and successfully obtained at least 4 clones for each (positive clones targeting OCT4 in HUES-9 cells are shown in 2D). To investigate long noncoding RNAs important for the trophectoderm lineage, we designed a donor vector that would introduce eGFP immediately following the stop codon of CDX2. This construct is designed to express eGFP when CDX2 is expressed from the endogenous locus. We designed 2 sets of TALENs to cut

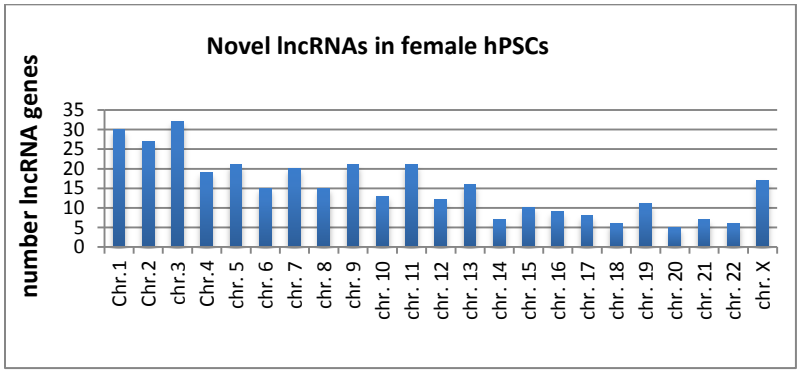
within the 3' region of the CDX2 gene, and have successfully generated 5 correctly targeted clones. We found that BMP4-induced differentiation of one positive CDX2 reporter line generates a very weak GFP signal (determined by live cell imaging), and we are working out conditions to increase signal strength (by removing the selection marker, which will reduce the space between the UTR and eGFP) and whether the signal is sufficient for FACS detection. In summary, we have successfully generated the two reporter cell lines that we proposed. Moving forward, we will test whether disruption of known protein coding genes important for each lineage (NANOG for the OCT4 reporter cells; CK-7 for the CDX2 reporter cells) disrupts GFP expression.

Figure 1

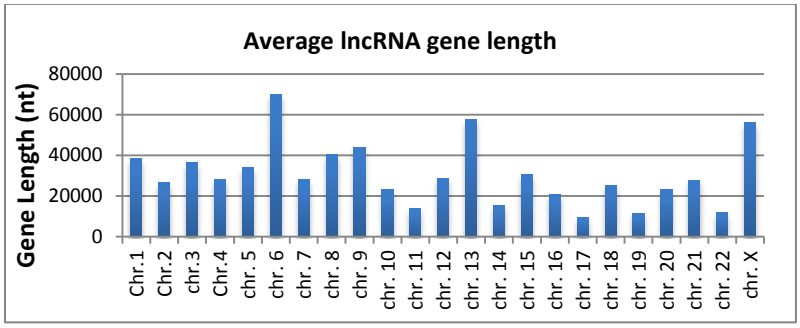
A.



B.



C.



D. Novel long noncoding RNAs (top 10 candidates) that are missing or absent in XIST-lacking pluripotent stem cells.

Symbol	Chr.	hiPS-2 XIST+(FKPM)	hiPS-2 XIST- (FKPM)	HUES-9 XIST+ (FKPM)
SCARNA22	4	423.316	0	730.433
AP000459.4	21	54.689	0	166.787
AC004696.2	19	30.5662	0.0115084	199.591
CTD-2554C21.2	19	31.9814	0	15.5157
CTC-228N24.1	5	9.03407	0.716773	12.257
RP11-219G10.3	4	9.71603	1.23972	29.8745
CTD-2554C21.3	19	16.4063	0	7.90883
RP5-1119A7.14	22	6.47842	0.142591	8.98101
RP4-755D9.1	X	6.24138	0.970557	10.5533
CTC-512J12.4	19	5.69285	0.392744	21.1337

Figure 2

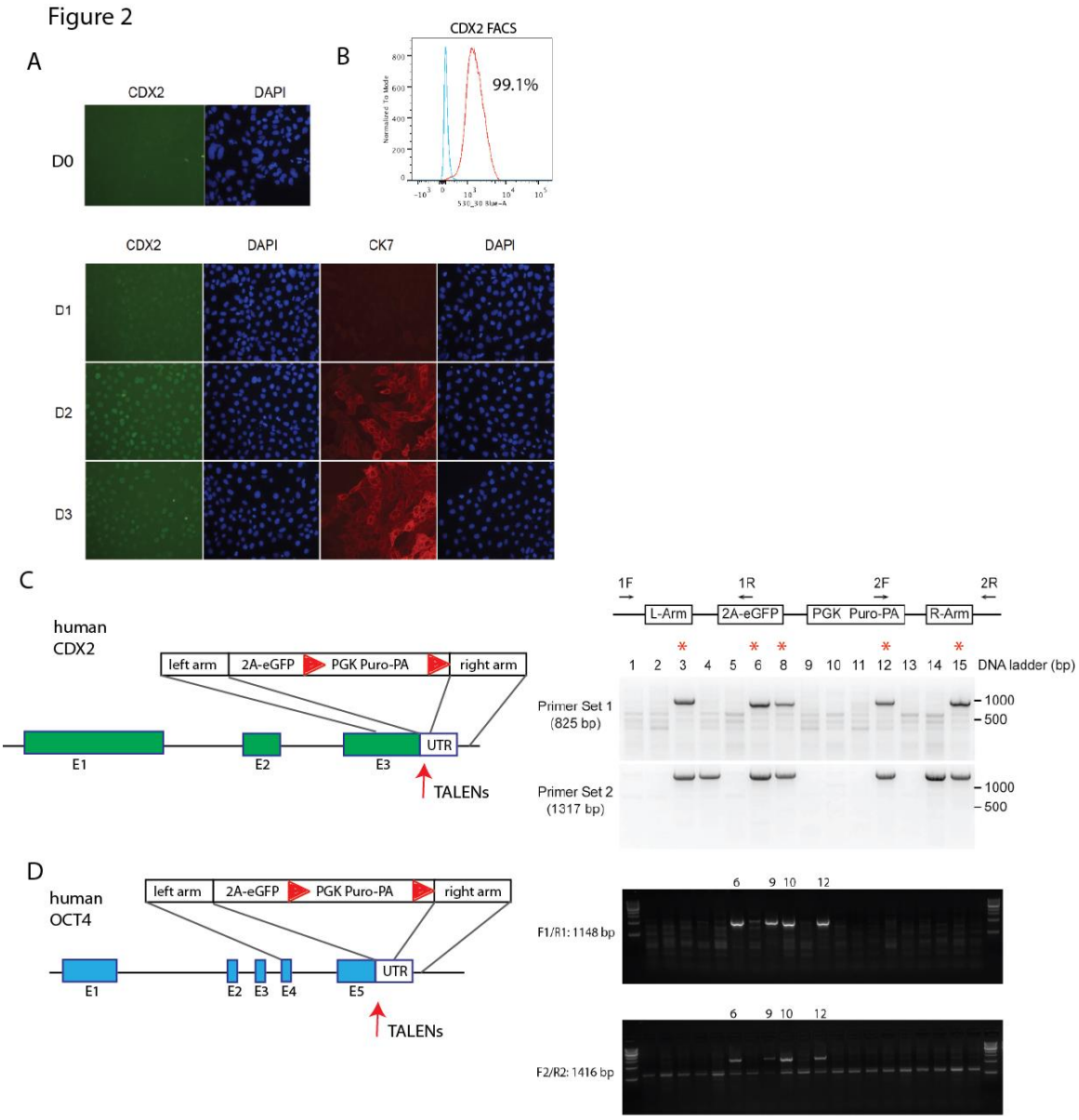
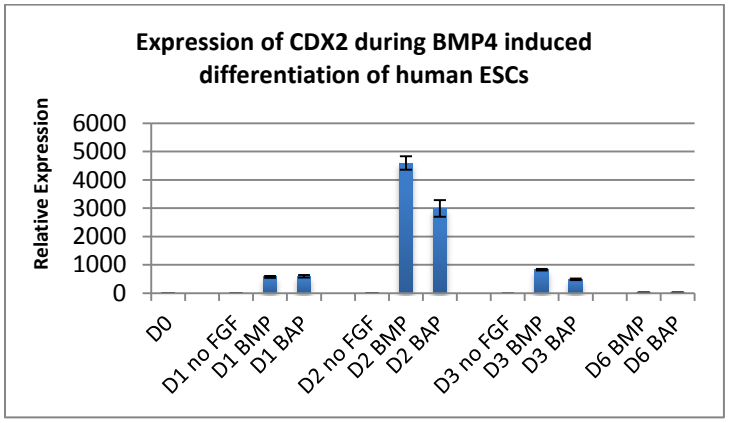
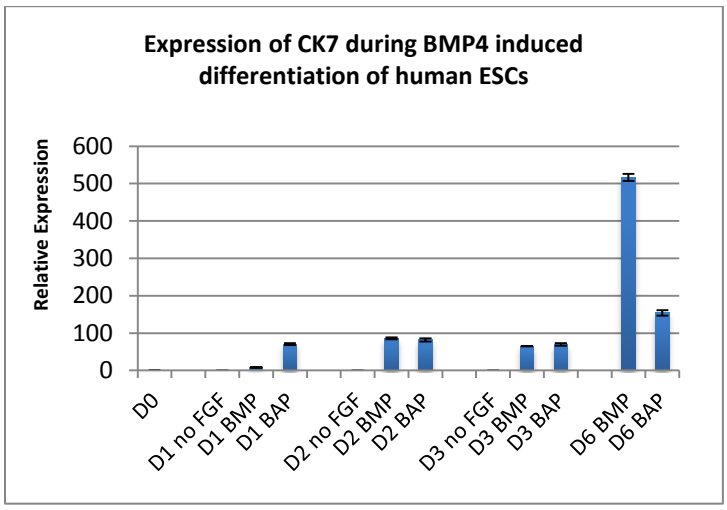

A
CDX2
DAPI
D0
B
CDX2 FACS
99.1%
CDX2
DAPI
CK7
DAPI
D1
D2
D3
C
human
CDX2
left arm
2A-eGFP
PGK Puro-PA
right arm
E1
E2
E3
UTR
TALENs
1F
1R
2F
2R
L-Arm
2A-eGFP
PGK Puro-PA
R-Arm
1 2 3 4 5 6 8 9 10 11 12 13 14 15 DNA ladder (bp)
Primer Set 1
(825 bp)
Primer Set 2
(1317 bp)
1000
500
D
human
OCT4
left arm
2A-eGFP
PGK Puro-PA
right arm
E1
E2 E3 E4
E5
UTR
TALENs
6 9 10 12
F1/R1: 1148 bp
6 9 10 12
F2/R2: 1416 bp

Figure 3

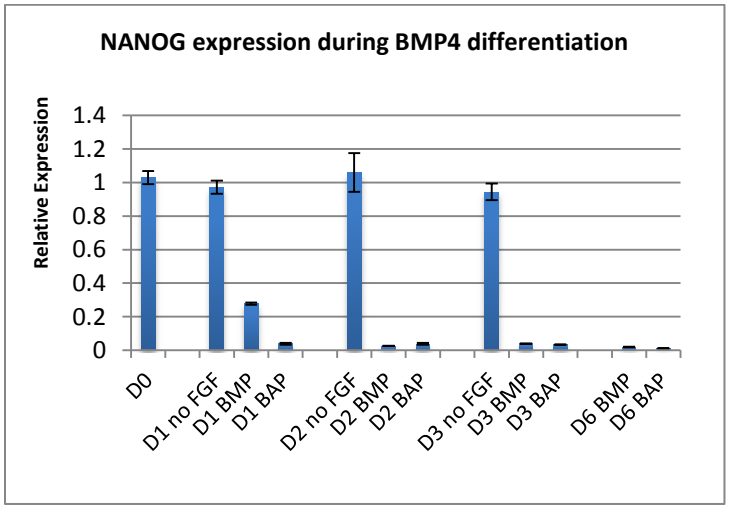
A.


Expression of CDX2 during BMP4 induced differentiation of human ESCs
Relative Expression
6000
5000
4000
3000
2000
1000
0
D0
D1 no FGF
D1 BMP
D1 BAP
D2 no FGF
D2 BMP
D2 BAP
D3 no FGF
D3 BMP
D3 BAP
D6 BMP
D6 BAP

B.


Expression of CK7 during BMP4 induced differentiation of human ESCs
Relative Expression
600
500
400
300
200
100
0
D0
D1 no FGF
D1 BMP
D1 BAP
D2 no FGF
D2 BMP
D2 BAP
D3 no FGF
D3 BMP
D3 BAP
D6 BMP
D6 BAP

C.


NANOG expression during BMP4 differentiation
Relative Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
D0
D1 no FGF
D1 BMP
D1 BAP
D2 no FGF
D2 BMP
D2 BAP
D3 no FGF
D3 BMP
D3 BAP
D6 BMP
D6 BAP

Research Project 14: Project Title and Purpose

John Morgan Building – Research Infrastructure Project for the Department of Radiology – The purpose of this research infrastructure project is to renovate space in the John Morgan Building in support of the University of Pennsylvania Perelman School of Medicine's Department of Radiology. The project will provide approximately 8,400 square feet of renovated space and infrastructural improvements in order to consolidate Radiology labs currently located in disparate locations across campus. This will greatly enhance scientific synergies within the department and increase economies of scale for equipment and other resources, as well as create research bridges with other programs which address critical needs common to Penn, the NIH road map, and the Commonwealth of Pennsylvania.

Anticipated Duration of Project

12/18/2013 – 12/31/2016

Project Overview

The renovations on the first floor, west wing of the John Morgan Building will provide necessary upgrades and enhancements to one of the most historic buildings of the medical school campus. As part of the overall campus master planning project, the John Morgan Building has been identified as a high priority for reinvestment. Renovations to the first floor will allow for the consolidation of the research of several investigators, thereby increasing research efficiencies and creating opportunities for collaborative research efforts on a broad range of disciplines in biomedical research.

Furthermore, the plan to move the Radiology department in the John Morgan Building will bring researchers closer to the adjacent Richards Building, which will house computational neurosciences (including several Radiologists). This plan unites the department in two main buildings rather than in several disparate locations across campus. Lastly, consolidating Radiology faculty from Blockley Hall, Stemmler Hall and Market Street will enable space to be cleared for additional programmatic advantages, which will ultimately support both school-wide and University-wide initiatives.

The total area of modifications is approximately 8,400 square feet, and the scope of this project includes the following:

1) Renovation of wet and dry laboratory space
2) Separation of one air handler into two systems which will allow for support of BSL2 lab practice
3) Replacement of laboratory utilities (such as DI water and the instrument air system)
4) Replacement of decaying utility infrastructure (such as piping, fittings, electrical and mechanical duct work).

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Expected Research Outcomes and Benefits

Evaluation of this project will be based on three sets of criteria: (1) the successful construction of research space in support of the mission and goals of the Department of Radiology; (2) the number of extramural sponsored projects awarded to the investigators directly supported in this space; and (3) the number of articles submitted to peer-reviewed publications by the investigators directly supported in this space.

The renovations to the John Morgan Building first floor, west wing are being completed to allow for increased programmatic efficiencies in support of the Department of Radiology. The research that will be conducted in the renovated space spans a wide disciplinary spectrum, with considerable strength in cancer research (particularly breast and animal models, as well as studies at the cellular level), cardiovascular, musculoskeletal, neurofunctional (positron emission tomography/magnetic resonance), and method development (magnetic resonance imaging techniques, imaging agents, image processing, and optical imaging). The space is also ideally positioned in the midst of the broader computational neurosciences research communities planned for the adjoining Richards Building.

Summary of Research Completed

Over the past year, research programming has been identified and documented through interviews with the eight groups proposed for co-location in the west wing. Extensive utility surveys of the west wing and budget constraints have defined a critical path for replacement and renovation of existing infrastructure which will support Biosafety Level 2 work in the labs, correcting a long term deficiency in this wing. Approximately sixty percent of the west wing will be constructed for this use while the remainder will be renovated for office, tech desk and non-lab support rooms. The south wing renovations will include offices and tech support spaces to support dry research functions.

Considering the age of the John Morgan building, logistical planning has been critical to keep the existing radiology labs in these wings operating with minimal shutdowns and disturbances. The planning requires that an initial phase occur to relocate access points to the Maidment offices and labs for continued operation. The majority of the construction will be in the second phase, which will include installation of the new air handling unit on the courtyard roof. The third phase will involve removing the old air handler and connecting the existing labs of Drs. Maidment and Zhou to the new air handler. Substantial surveys of areas well beyond the project areas have been necessary to evaluate deficiencies in the building and plan for corrections. We anticipate construction to start in early September.

Research Project 15: Project Title and Purpose

Cellular Transdifferentiation for Regenerative Therapies – The purpose of this work is to make substantial advancements toward the direct conversion of endogenous cell types into cells that provide therapeutic benefit for cardiovascular or neurodegenerative disease.

Anticipated Duration of Project

12/18/2013 – 12/31/2016

Project Overview

Advances in reprogramming have revealed that differentiated mammalian cells possess remarkable plasticity – from the induction of pluripotency *in vitro* to the conversion of pancreatic exocrine cells to endocrine cells *in vivo*. This pancreatic "lineage reprogramming" raises the intriguing possibility that other cell types might be capable of *in vivo* reprogramming. If this could be applied to the mammalian brain, it is conceivable that dopaminergic neurons (DNs), whose degeneration is the hallmark of Parkinson's Disease (PD), could be generated by direct reprogramming. Likewise, the *in vivo* conversion of cardiac fibroblasts in damaged hearts into functional cardiomyocytes provides a powerful new approach to the treatment of cardiovascular disease. In this research project, we aim to make significant strides toward the direct *in vivo* conversion of endogenous cell types into cells that provide therapeutic benefit for cardiovascular or neurodegenerative disease. In the case of cardiomyocytes, we will also study the mechanisms by which distinct myocyte subtypes (atrial, ventricular, or nodal) are produced.

Specific Aim 1: To Transdifferentiate porcine cardiac fibroblasts to induced cardiomyocytes in vitro and in vivo. We will identify the combination of transcription factors necessary for direct conversion of porcine cardiac fibroblasts to cardiomyocytes *in vitro*. We will evaluate the ability of several viral vectors to transduce scar tissue fibroblasts in infarcted pig hearts. We will then test the hypothesis that conversion of these fibroblasts to functional cardiomyocytes can be achieved via the delivery of a select group of cardiac transcription factors.

Specific Aim 2: To Direct the Generation of Distinct Cardiomyocyte Subtypes. We will test the hypothesis that cardiomyocyte subtype identity is specified by a "Tbx code" in which different Tbx genes combine to specify different subtypes.

Specific Aim 3: To Transdifferentiate non-human primate astrocytes to dopaminergic neurons in vitro and in vivo. We will use lentiviral vectors to deliver reprogramming transcription factors to monkey astrocytes both in vitro and in a monkey model of Parkinson's Disease. We will test the hypothesis that *in vivo* conversion of astrocytes to dopaminergic neurons can mediate functional improvement of MPTP-treated monkeys.

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Expected Research Outcomes and Benefits

We expect that the direct *in vivo* conversion of astrocytes to dopaminergic neurons will provide a powerful new therapeutic option for Parkinson's Disease. Similarly, we anticipate that direct conversion of scar tissue fibroblasts into functional cardiomyocytes of the appropriate subtype will constitute a major new advance in cardiovascular disease therapy.

Summary of Research Completed

Specific Aim1: To transdifferentiate porcine cardiac fibroblasts to induced cardiomyocytes in vitro and in vivo.

Our work in this aim involves the use of a clinically relevant porcine model of myocardial infarction for the study of *in vivo* cardiac reprogramming.

As a first step, we proposed to identify the combination of transcription factors necessary for direct conversion of porcine cardiac fibroblasts to cardiomyocytes *in vitro*. For this purpose, we used different combinations of factors, including HNGMT (Hand2, Nkx2.5, Gata4, Mef2c and Tbx5), which has been shown to convert mouse cardiac fibroblasts to cardiomyocytes, and new combinations that include new factors (transcription factors: myocardin, Mesp1; microRNAs and small molecules). Cardiac fibroblasts from a healthy pig were used in these experiments. The different combinations of factors were added a day after plating and 14 days later the cells were fixed and analyzed by immunohistochemistry. Although there has been some difficulty in the culture of these cells *in vitro*, mostly due to their high proliferation and subsequent peeling off from the culture dish before the end point of our experiment (conditions that are being improved), we were able to detect reprogramming of pig cardiac fibroblasts into cardimyocytes

in vitro. We observed expression of cardiac Troponin (cTnT) and myosin heavy chain (Myh11) in several of these combinations as shown in Figure 1.

Although the porcine model has been well characterized as a preclinical model of myocardium infarction, and multiple studies have used viral vectors to target cardiomyocytes in the pig heart, little is known about the ability of different viral vectors to specifically target non-myocytes. In collaboration with Dr. Robert Gorman and the clinicians in his laboratory, we have performed an *in vivo* experiment to determine the viral-mediated targeted transgene delivery to the heart following myocardial infarct in the pig model. Four male Yorkshire pigs had an anteroapical infarction performed through a left thoracotomy. Viral vectors were injected into the heart one week following infarction, since at this time point it is expected the infarct region to be depleted of cardiomyocytes and the infiltrating fibroblasts to be highly proliferative. Different types of viral vectors were used, all driving expression of GFP: Lentivirus: Vimentin Promoter driving expression of GFP (injected into 1 pig); Adeno-Associated Virus Pseudotype 1: AAV1.CMV.bi.nls.AcGFP, nuclear GFP (injected into 2 pigs); and Adeno-Associated Virus Pseudotype 9: AAV9.CMV.bi.nls.AcGFP, nuclear GFP (injected into 1 pig). The experimental procedure was performed accordingly to the diagram shown in Figure 2. Two weeks later, animals were sacrificed and tissues were collected for immunohistochemical analysis. GFP expression was detected in the infarcted heart injected with the AAV9.CMV.bi.nls.AcGFP (Figure 3) vector. This result demonstrates the ability of AAV9-mediated transgene delivery to target cardiac fibroblasts of an infarcted heart in the pig.

Specific Aim 2: To direct the generation of distinct cardiomyocyte subtypes

The goal of this aim was to determine if we could identify the transcription factors that could generate the various sub-types of cardiomyocytes via cell conversions. These experiments utilized human cells and as has been the case with other studies, the human cell conversions are proving to be problematic. After initial attempts towards this aim, we concluded that our efforts in SA 1, with the pig work and SA 3 were moving very quickly and successfully and we turned our attention to those studies.

Specific Aim 3: To transdifferentiate non-human primate astrocytes to dopaminergic neurons in vitro and in vivo.

In the past year, we have made progress toward the improvement of transdifferentiation of astrocytes to induced dopaminergic neurons (iDNs). We analyzed the population of astrocytes by immunohistochemistry in adult African green monkeys (cholorocebus sabaeus), the species studied at St. Kitts Biomedical Research Foundation (SKBRF). We used sections of monkey striatum and stained for astrocyte markers to better understand the biology of these cells. The markers investigated in the experiment were Glutamine synthetase, S100 calcium binding protein B (S100B*), Glutamate Aspartate Transporter* (*GLAST*), Glia fibrillary acidic protein (GFAP) and Vimentin (Vim). As the result demonstrates (Figure 4), GFAP and Vimentin (Vim) are robustly expressed in astrocytes and both are putative candidates to target astrocytes. This result suggests that using the Vimentin promoter to target astrocytes for i*n vivo* direct reprogramming could efficiently target the cell of interest. Moreover, to validate novel dopaminergic neurons in the striatum, we stained for dopaminergic markers in the substantia nigra by immunostaining. In

these experiments, we optimized our protocol and determined the ideal concentration of antibodies to stain for dopaminergic markers such as tyrosine hydroxylase (TH), forkhead box protein A2 (FOXA2), and the dopamine transporter (DAT). The staining revealed the endogenous expression of dopaminergic markers in the substantia nigra, as expected (Figure 5). This protocol will be used to validate the transdifferentiation of astrocytes to dopamine producing cells in the striatum in future experiments.

Furthermore, in effort to accomplish the generation and validation of viral vectors for transdifferentiation, we used monkey astrocytes *in vitro* to test if the inclusion of VP16 would improve the efficiency of reprogramming. To this end, we used the transactivated fusion of VP16-ASCL1 along with the native forms of LMX1B and NURR1 (vALN) for *in vitro* reprogramming. This combination did yield induced dopaminergic neurons and is now being evaluated in an *in vivo* system: MPTP-lesioned African green monkeys.

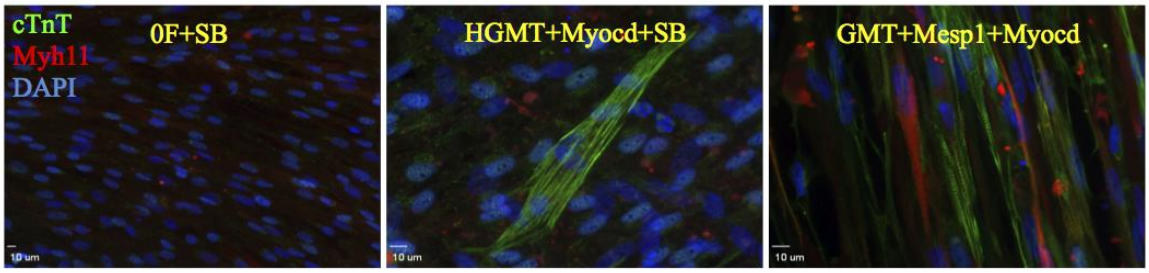

Figure 1. Representative immunostaining of porcine induced cardiomyocytes (iCMs) *in vitro* at Day 14 stained for cTnT (green) and Myh11 (red) and counterstained with DAPI (blue). Scale bar 10μm.

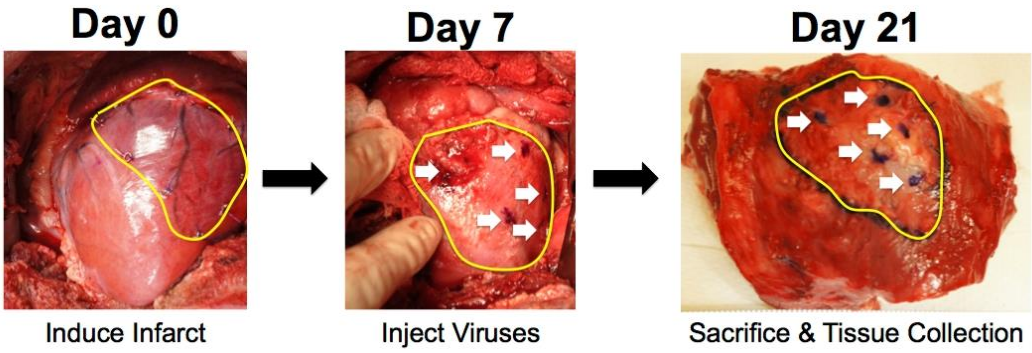

Figure 2: Experimental outline of the *in vivo* studies. For injections, 50 μL of virus was loaded into a syringe and injected into 5 areas of the infarct region (therefore a total of 250 μL). Injection sites were selected based on the architecture of the epicardium such that identification of site locations after sacrifice would be possible.

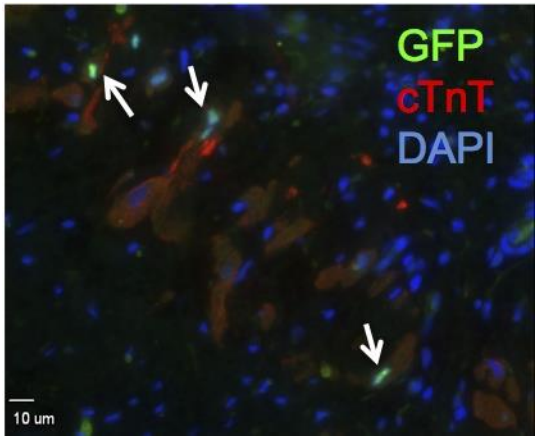

Figure 3. Representative immunostaining for GFP and cTnT at 2 weeks following viral delivery of the AAV9.CMV.bi.nls.AcGFP virus into the infarcted pig heart. Notice the GFP positive nuclei (arrows). Scale bar 10μm.

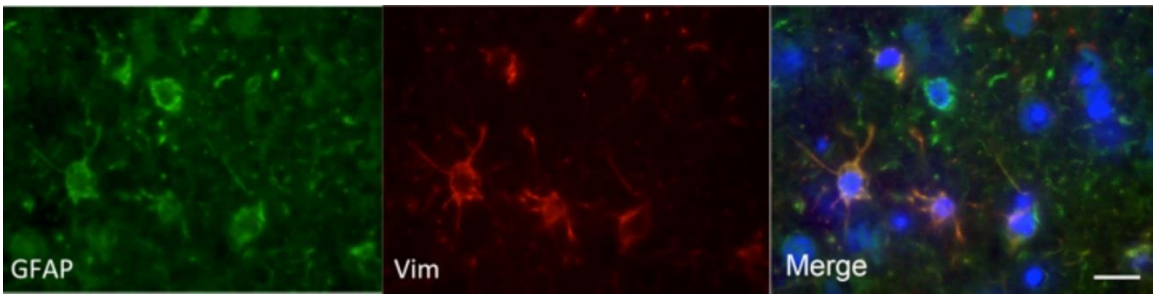

Figure 4: Immunohistochemistry analysis of endogenous astrocytes in the striatum. Astrocyte-specific markers GFAP (green) and Vimentin (red) are expressed in the striatum. DAPI stain (blue) marks nuclei. Scale bar 60 μm.

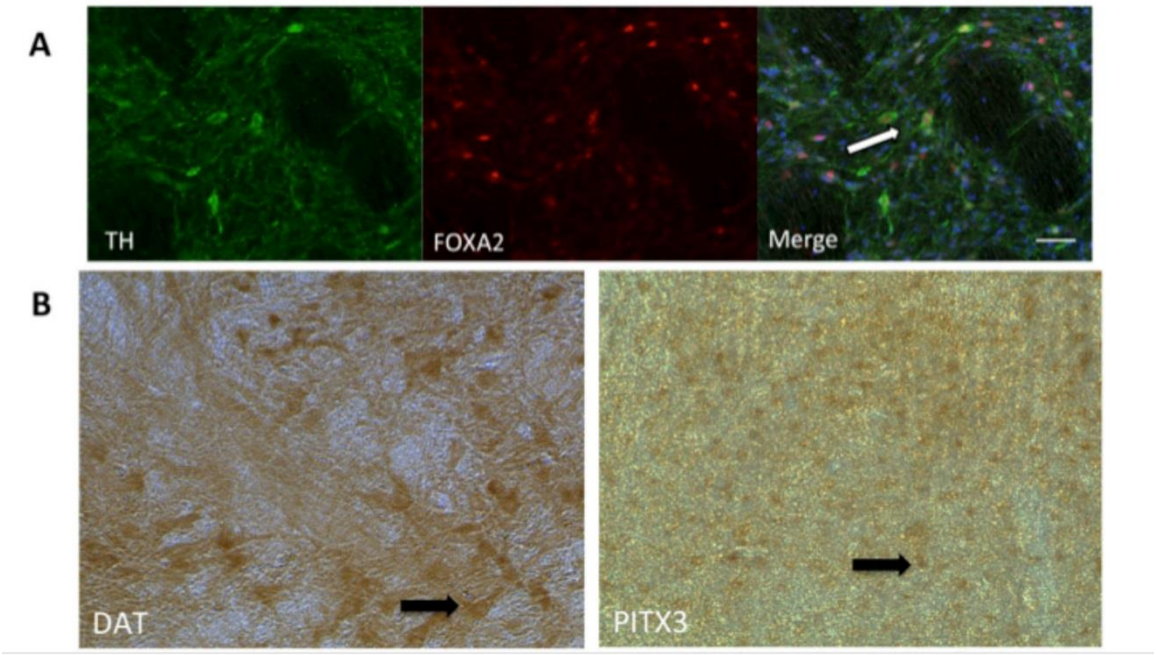

Figure 5: Immunohistochemistry analysis of endogenous dopaminergic markers in the substantia nigra. (A) Staining of TH-positive cells and transcription factor FOXA2 (arrow, co-staining of TH and FOXA2), scale bar 20µm. (B) Staining of cells positive for DAT and PITX3 (arrow), 40 X objective.

Research Project 16: Project Title and Purpose

Isolation, Biogenesis and Cargo of Microvesicles from Brain and Pancreatic Tumor Cells – The purpose of this project is to develop new techniques and knowledge about brain and pancreatic tumor microvesicles (MV) that will allow their use for detection, prognosis and identification of treatment targets in patients. We will develop a handheld device that will reduce the collection and measurement time and increase the sensitivity of detection compared to currently available methods. We will interrogate the biogenesis of MV under tumor-like microenvironmental conditions and determine the nature of the material (cargo) within them using RNA isolation techniques and deep sequencing.

Anticipated Duration of Project

2/11/2014– 12/31/2016

Project Overview

The analyses of the sparse molecular markers that are shed from tumors into the circulation have potential to be used for both disease diagnostics and monitoring. Microvesicles (MVs) are one type of "liquid biopsy." Under conditions of stress, blood elements and other cells (including

tumor cells) secrete a heterogeneous mixture of 250-2000 µm, membrane-derived, organelle-like structures into the interstitial spaces that eventually reach the circulation. MVs are mediators of intercellular communication due to their capacity to merge with and transfer bioactive molecular cargo to recipient cells. Cargo may include growth factors and receptors, proteases, adhesion and signaling molecules, DNA, messenger and microRNA. The increase of MV is almost always associated with a poor prognosis. The overall goals of this project are to understand the biogenesis of MV and assess their cargo to determine function. We will use the current standard and a novel, new method for MV collection. We propose 2 aims.

Specific Aim 1: The goal of this aim is to understand how changes in the tumor microenvironment (ME) and use of radiation therapy effect the formation and function of MV.

- Aim 1a: Assess the production of MV from tumor cells and spheroids under varying ME conditions.
- Aim 1b: Determine the effect of radiation MV collected under conditions described in SA1a.
- Aim 1c: Cryo-EM examination of MV formation under defined conditions of our developed cell models.
- Aim 1d: Assess the miRNA contained in a subset of MV produced in SA1a&b.

Specific Aim 2: The goal of this aim is to develop an assay to isolate and magnetically label MVs produced from a cellular model for whole blood on to microbeads.

- Aim 2a: Develop polystyrene bead capture assay.
- Aim 2b. Develop MNP labeling assay.
- Aim 2c. Detect culture harvested MVs on beads.

Aim 1 hypothesizes that (1) the biogenesis of MV is modulated by the tumor microenvironment, (2) radiation affects the production and content of MV, (3) MV contain biologically relevant cargo especially various types of RNA and cytokines. The specific molecules vary depending on the ME in which they were produced. For this aim we will use cells and spheroids originating from brain and pancreatic tumor cell lines. In our second aim, we will develop a micro-magnetic sensing handheld device that reduces the time for MV isolation and increases the high sensitivity of detection (100 MV/mL) compared to conventional techniques. This innovative approach to MV detection and analysis has great potential for minimally invasive, routine monitoring of disease progression and treatment efficacy, offering potential benefits for patients.

Principal Investigator

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Expected Research Outcomes and Benefits

The primary outcome of Specific Aim 1 is the understanding of the biogenesis and biology of circulating MV produced from pancreatic and brain tumors cells *in vitro* before and following treatment with radiation. High-grade tumors of both sites are known to have abnormal biochemical (hypoxic, hypoglycemic, low pH) and unique structural microenvironments. Therefore, we will study the morphological processes involved in the formation of MV and their cargo (primarily miRNA) under these biologically relevant conditions, recreated *in vitro.* We expect that the data resulting from these studies will closely reflect the actual biology of MV formation *in vivo* as compared to studies performed under less biologically relevant conditions. Understanding the effect of these factors, especially on the MV cargo will allow better use of MV as diagnostic and/or prognostic factors and as therapy targets.

Conventional molecular analyses of MVs require great quantities of MV and consist of time-consuming procedures performed on large pieces of equipment, such as ultra-centrifuges and flow cytometers. The device that we propose to develop in SA2 is handheld and aims to reduce measurement times from several hours to less than thirty minutes. Moreover, due to the high sensitivity of our micro-magnetic sensing method, the biomarker specific limit of detection (100 MV/mL) will exceed that of conventional techniques. This innovative, clinically practical, approach to CμV detection has potential for non-invasive, routine monitoring of disease progression and drug efficacy, offering benefits for patients.

Summary of Research Completed

Aim 1: to understand how changes in the tumor microenvironment (ME) and use of radiation therapy effect the formation and function of MV

In the 5 months since we received funding for this grant (February 2014), we have concentrated on optimizing our techniques for obtaining a large number of concentrated microvesicles (MV) from monolayer cultures and spheroids. We have made two important observations in our preliminary experiments. Most investigators use various types of differential centrifugation to concentrate MV and exosomes (EX). We differentiate these based on the allowable size for identification: MV detected by flow cytometry (>300-2000 nm diameter) and EX detected by electron microscopy or Nanosight (50-250 nm in diameter). All previous methods have utilized a low- and medium-speed spin to remove debris, followed by high-speed spin of the medium-speed supernatant. The high-speed pellet presumably contains MV, and the high-speed supernatant can then be placed under ultracentrifugation to collect EX. In attempting to quantify this step-wise purification process, we were surprised to find a preponderance of MV in the medium -speed pellet, and transmission EM demonstrated particles smaller than 300 nm in both the medium and high-speed pellets. This is major step forward in optimizing the number of particles (from the medium speed and high speed pellets by Flow Cytometry and/or EM),

characterizing their contents (cargo) including miRNA and identifying their biological function. We are presently trying to optimize the low- and medium-speed g-force and time to optimally collect all MV. The 2nd observation involves an analysis of the threshold requirements for the Flow Cytometer presently used (BD FacsCANTO). We have discovered that much smaller particles can be observed if we use forward scatter and the fluorescent channel for phosphatidyl serine (presently Pacific Blue labeled Annexin V), rather than the conventional forward scatter and side scatter (**Fig 1a).** In blood analysis, this also allows the differentiation of platelets vs MV, previously a difficult challenge.

One of sub-goals of Aim 1 involved the relative efficiency of MV formation from cell-monlayers versus the 3-dimensional state of multi-cell tumor spheroids. We have been successful at generating spheroids from both the U87 glioma and Panc1 pancreatic cell lines using a relatively new method that allows spheroid initiation on a concave surface (non-adherent) tissue-culture-grade agarose of on 24-well plates (In Vitrogen) {Catherine Kelley, personal communication}. The Panc1 cells grow with a substantially longer doubling time than do the U87s (~24 hr *versus* 16 hrs) *in vitro*, and this is reflected in a substantially longer time needed for initiation and growth of the Panc1 spheroids. Both spheroid types grow to greater than a mm in size, and the Panc1 but not U87 spheroids have a significant hypoxic component at this size (**Fig. 1b**). Since the spheroids are grown in individual wells of multi-well plates we are currently working towards minimizing the amount of medium required (to maximize the number of (MV) formed per cell, without toxicity). The eventual purpose of this is so we can lyse a large number of MV for RNA analysis.

Since the spheroids grow at tissue densities, we feel that their best model is using confluent cell monolayers is for with daily feeding (replacement of nutrient depleted media). This coincidentally allows the nearly continuous (daily) collection of large numbers of microvesicles to be used for the joint experiments with Dr. Issadore (Aim 2) as well as our own RNA isolation. In characterizing the growth of the two cell-lines under these conditions, we discovered an unexpected effect of serum concentration: at high cell densities, the U87 cells grew at almost the same rate for serum concentrations of 1.5% to 12%, whereas at low densities there was the expected decrease in colony forming ability as the serum concentration decreased. This has two important implications for our proposed studies: serum starvation was one of the environmental stresses we plan to test for its effect of MV formation, so this can now be accomplished without affect cell growth. Additionally, use of low serum removes the contamination of serum-derived MV in the total production. Most importantly, it is clear that the cells, at high density, are producing survival factors. Preliminary data suggest that these factors are pro-survival cytokines, but anti-survival MV. Although we are unaware of literature data documenting such serum effects, we have found them to be consistent for not only the U87 cell, but also the Panc1 cells and an additional human squamous cell carcinoma (SQ20B). Concurrent work on the effects of serum and glucose, cryoEM imaging and RNA isolation are being performed, but data is not complete enough to report.

Aim 2: to develop an assay to isolate and magnetically label MVs produced from a cellular model for whole blood on to microbeads.

We performed initial testing of our MV capture assay, as described in our grant's Aim 2a. The

major goal of Aim2a was to develop a microbead-based assay that can isolate MV that originate from tumors in whole blood (**Fig. 2a**). We have demonstrated this capability by capturing MV onto 3 µm polystyrene microbeads functionalized with specific antibodies to capture the target MV (**Fig. 2b**). We performed optimization and characterization of the technique (detailed below), and also demonstrated its generality by measuring both pancreatic tumor and neuron cell line-derived MV.

To quantify and optimize the MV capture assay, we used fluorescent labeling of the captured vesicles using a lipophilic membrane stain (DiI) that non-specifically stains any vesicle that had been captured onto the bead. The beads were analyzed using flow cytometry, where each bead's mean fluorescence intensity (MFI) corresponds to the quantity of captured microvesicles. Preliminary data shows that microvesicles from the pancreatic cancer cell line PD7591 could be captured onto microbeads using anti-EpCAM as an affinity ligand (**Fig. 2b**). The number of vesicles captured onto the beads increased with antibody concentration and was invariant to bead concentration (**Fig. 2c**), supporting the hypothesis that we can capture vesicles based on specific antibody interaction and that there is an excess of microvesicles.

In addition to demonstrating qualitatively that we can capture MV onto our beads, we also show that the quantity of MV captured correlates with the concentrations of MV in the serum. To demonstrate this claim, we used cultured neuron cells. The neuron cells that we used were embryonic rat cortical/hippocampal neurons (E17). The capture assay used anti-GluR2 antibody. Using serial dilutions of the cell serum, we perform the bead assay for several concentrations of MV (**Fig. 2d**). As expected, the MFI of the beads shifts linearly with the concentration of the MV. The dynamic range of the test was determined, with the upper bound given by the point at which the beads are entirely covered with vesicles and the lower bound given by the limit-of-detection of the detection modality to detect few vesicles on a given bead. To expand dynamic range, our chip will now include an automated platform to perform serial dilutions and do several measurements in parallel.

FIGURES

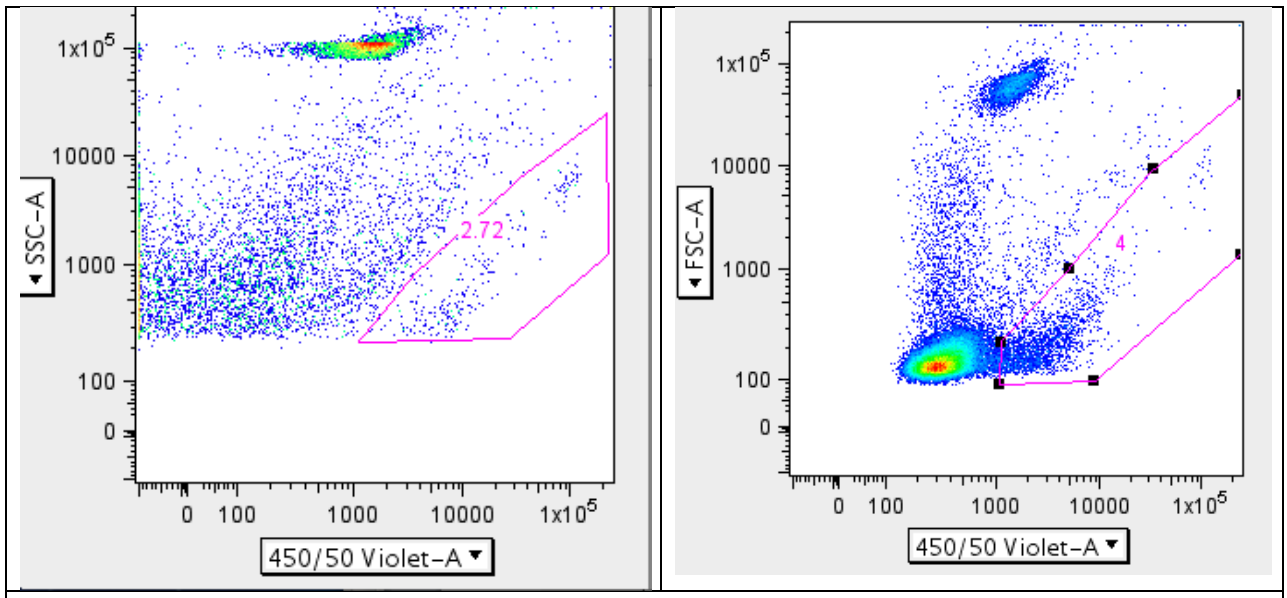

Figure 1a (FG): Flow cytometry data from a patient on treatment for GB. In the left image, the Annexin V positive population is identified in the region of interest, based on the conventional threshold (FSC vs. SSC). It accounts for 4% of the total population, 251 events The sample was re-run using a threshold based on Pacific Blue labeled Annexin V and FSC (right image). Using this threshold, more Annexin 5 events are seen, accounting for 4% of all of the events or 1880 events. The additional events are smaller (lower FSC) suggesting that we can identify and count MV < 300µm in diameter.

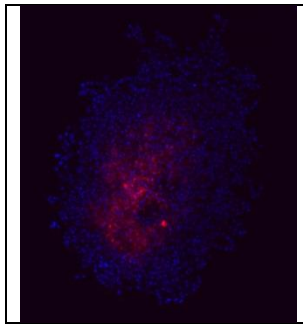

Figure 1b: Fluorescent immunohistochemical image of a panc1 spheroid that had been exposed to EF5, a 2-nitroimidazole hypoxia labelling agent for 3 hours before freezing. The spheroid is about 8 mm in diameter and the red is EF5-Cy3 staining of hypoxic cells. The blue is propidium iodide to counterstain all viable cells.

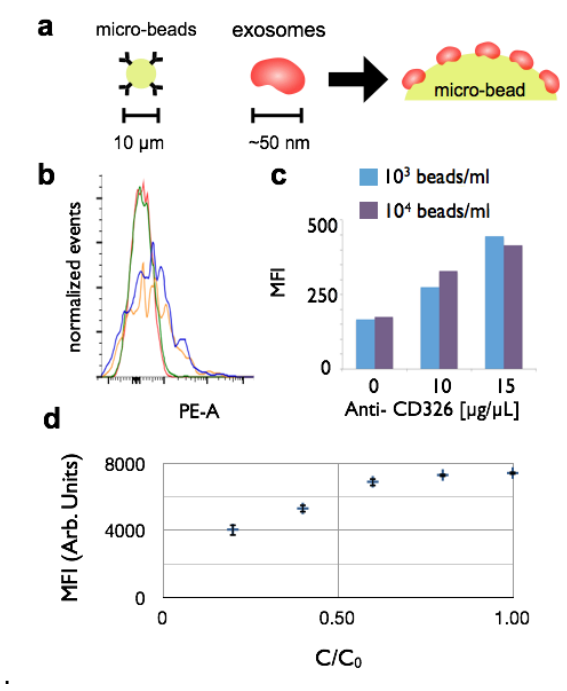

Fig. 2 a. MV originating from tumors are selectively isolated from 7.5 mL of whole blood using antibody functionalized microbeads. **b.** Preliminary flow cytometer data showed successful capture of microvesicles from a pancreatic cancer cell line onto beads using anti-EpCAM. Red and green are negative controls, beads only and beads and sample but no antibody. Blue and Orange are duplicates of 10^3 beads /mL with 15 µg/µL anti EpCAM. **c.** Mean fluorescence intensity of the MV covered beads increased with concentrations of anti-EpCAM (CD326), in agreement with selective capture. **d.** By performing serial dilutions of serum from cell culture, the dynamic range and sensitivity of the bead capture assay was characterized, using cultured neuron cells