The Institute for Cancer Research

Annual Progress Report: 2010 Formula Grant

Reporting Period

July 1, 2013 – June 30, 2014

Formula Grant Overview

The Institute for Cancer Research (formerly Fox Chase Cancer Center) received $2,851,328 in formula funds for the grant award period January 1, 2011 through June 30, 2014. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

The Role of Histone-Complexes in Residue Specificity of Post-Translational Modifications - The purpose of this project is to develop the technology to determine the impact of histone presentation on the location(s) of post-translational modification of histones. *In vivo*, histones are predominately either bound to DNA (nucleosome) or bound to a histone chaperone. While there are clear correlations between changes in histone modifications and histone chaperones, their relationship is still unclear. Histone chaperones could alter post-translational modifications by interacting with the modification enzyme, protecting specific residues, or by increasing their apparent concentration by disrupting specific complexes. We aim to determine the effects of histone complexes on the location and identity of histone modifications by developing mass spectroscopy based assays to monitor multiple modifications at once.

Duration of Project

1/1/2011 - 12/31/2012

Summary of Research Completed

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

Research Project 2: Project Title and Purpose

DUSP6 Regulates the Response to EGFR Inhibitors in Cancer - Much effort has been devoted to the development of drugs targeted at signaling proteins thought to be essential for tumor cell viability. A central disappointment has been the fact that many of these inhibitors have limited efficacy in cancer patients in spite of potently inhibiting their drug targets. DUSP6 is a cytosolic member of the family of dual specificity phosphatases, a group of proteins that regulate ERK1/2 and p38. We have found that depletion of DUSP6 significantly increases the apoptosis caused by

Epidermal Growth Factor Receptor (EGFR) blockade. Although DUSP6 expression is associated with poor prognosis in cancers, the mechanism for DUSP6 action is not well understood. The purpose of this project is to discover the mechanism by which DUSP6 regulates tumors responsiveness to clinically important EGFR antagonists.

Duration of Project

1/1/2011 - 12/31/2013

Project Overview

Our immediate objective is to characterize the mechanism by which DUSP6 regulates tumor responsiveness to EGFR-targeting drugs. DUSP6 is known to be a cytosolic phosphatase for mitogen-activated protein kinases ERK1/2 and p38. Although changes in expression of DUSP6 have been reported as associated with specific prognoses in cancers, no clear mechanism for DUSP6 action has been established. Using stably integrated shRNA, we recently found that silencing DUSP6 markedly increased the cytotoxicity of the EGFR antagonists cetuximab, erlotinib and dasatinib, but not of inhibitors of RAF, MEK, PI3K, and PKC. Subsequent phosphoproteomic analysis of 46 candidate DUSP6 dephosphorylation targets in A431 cells treated with erlotinib revealed markedly increased phospho-p38 and p-CHK2 (activated) proteins in DUSP6-depleted cells. We thus hypothesized that DUSP6 regulates apoptosis commitment in EGFR-dependent cancers via regulation of the G2/M checkpoint. We will continue to investigate this hypothesis in the following Specific Aims:

Aim 1. Investigate the role of DUSP6 in regulation of cell growth and the G2/M checkpoint. We will probe whether DUSP6-regulated activation p38 regulates the cell cycle via the ATM-Chk2 pathway, and if DUSP6-mediated anti-EGFR sensitivity is dependent on p53 status in the tumor cell. We have created all necessary tools, including cell lines stably expressing inducible DUSP6-targeting shRNA, and overexpression plasmids for wild type, truncated and catalytically inactive mutants of DUSP6, to directly study this mechanism.

Aim 2. Model the DUSP6-p38-CHK2 interaction network. We will perform systematic analyses of the interactions between DUSP6 and the MAPK signaling network. We hypothesize that genetic and physical interactions will be conserved between the yeast and human orthologs at the level of functional modules. We will define and evaluate DUSP6-associated functional modules for the effects on cell cycle checkpoints, growth and viability in DUSP6-silenced or DUSP6-overexpressing cells.

Aim 3. Investigate the impact of DUSP6 expression on outcomes in head and neck squamous cell carcinoma (HNSCC). We wish to determine the relevance of our results to the clinic. Elevated DUSP6 levels have been associated with poor outcomes in some tumor types. We will establish whether EGFR-targeted therapies, and use of radiotherapy, which induce cell cycle checkpoints correlate with DUSP6 expression in HNSCC, with the goal of generating a biomarker for therapy selection.

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Expected Research Outcomes and Benefits

This research will establish DUSP6 as a novel regulator of cancer cell transition through the G2/M phase of the cell cycle, and will link this mechanism to the sensitivity of tumors to inhibitors of EGFR. We predict this mechanism is of particular relevance to head and neck squamous cell carcinomas harboring mutant p53 (associated with treatment refractoriness and metastatic behavior), in the context of cell treatment with EGFR-targeting inhibitors. Such a possibility is supported by our preliminary results showing significantly increased p38 and ATM/CHK22 activity, impaired growth kinetics and delayed G2/M transition following DUSP6 depletion. Conversely, we expect that increased expression of DUSP6 will confer resistance to EGFR targeting or chemotherapy agents with specific activity in G2/M phase of the cell cycle (inhibitors of topoisomerase I and II, cisplatin, doxorubicin, or ionizing radiation). These agents are commonly used to treat squamous cell carcinomas of the head and neck, and other cancers. By delineating the mechanism of treatment-sensitizing activity of DUSP6 inhibition, we ultimately hope to establish DUSP6 as a prognostic or predictive biomarker that will allow the more effective stratification of patients for treatment with different therapeutic regimens. Success in this goal would both improve cancer survival and reduce treatment costs based on more appropriate alignment of patients with the most appropriate therapeutic choices.

Summary of Research Completed

We have established DUSP6 as a novel regulator of cancer cell transition through the G2/M phase of the cell cycle, and linked this mechanism to the sensitivity of tumors to inhibitors of EGFR and chemotherapy. We demonstrated that this mechanism is of particular relevance to head and neck squamous cell carcinomas harboring mutant p53 (associated with treatment refractoriness and metastatic behavior), in the context of cell treatment with EGFR-targeting inhibitors. Such a possibility was supported by our published results showing significantly increased p38 and ATM/CHK22 activity, impaired growth kinetics and delayed G2/M transition following DUSP6 depletion.

We report that some of the results of this research were published in the recent 2013 issue of the British Journal of Cancer (2013): Bagnyukova TV, Restifo D, Beeharry N, Gabitova L, Li T, Serebriiskii IG, Golemis EA, Astsaturov I. DUSP6 regulates drug sensitivity by modulating

DNA damage response. Br J Cancer. 2013 Jul 9. doi: 10.1038/bjc.2013.353. PMID: 23839489

In Aim #3, we attempted to correlate expression of DUSP6 with head and neck cancers responsiveness to EGFR targeting or chemotherapy agents with specific activity in G2/M phase of the cell cycle (inhibitors of topoisomerase I and II, cisplatin, doxorubicin, or ionizing radiation). However, our extensive attempts to validate available anti-DUSP6 antibody reagents did not produce satisfactory results.

Contrastingly, DUSP6 is commonly deleted or epigenetically silenced in pancreatic adenocarcinoma (PDAC). This indicates the centrality of KRAS-MAPK-DUSP6 signaling axis in PDAC. We, therefore, chose to focus on studies of pancreatic ductal adenocarcinoma (PDAC) to probe into the mechanism that could lead to dismantling of this signaling axis. In further studies, we focused on: 1) development of a panel of patient-derived xenograft models (PDX); and 2) discovery of new drug targets to deregulate KRAS-MAPK signaling in PDAC.

Following our successful publication on DUSP6, we made the next logical step into the investigation of therapeutic targets in pancreatic cancer. Our focus on this type of tumor is two-fold: 1) the centrality of KRAS-MAPK-MYC signaling axis is further supported by the loss of DUSP6 in these tumors; 2) the grave nature of pancreatic cancer needs urgently novel therapy strategies, one of which we believe should be based on the disruption of the critical MAPK pathway.

Our preliminary results for this new direction of our studies are as follows:

1. Small molecule screen for inhibitors of primary PDAC cells. Our goal was to identify the most effective inhibitors of PDAC tumor growth. To avoid artifacts often associated with use of cell lines cultured for many passages on plastic, we adapted an innovative, recently described approach to screen primary pancreatic tumor cells. Briefly, PDAC surgical samples are dispersed to individual cells, treated with Rho inhibitors, and cultured for 5-6 passages on a fibroblast feeder system; this results in many features of in vivo tumors. In collaboration with Dr. Louis M. Weiner (Lombardi Cancer Center, Washington, DC), we conducted 6 parallel chemosensitivity screens of PDAC cells from 6 independent patients, using a focused library of 867 clinically relevant drugs (the NCI Clinical Collection and the FDA-approved drug set). This screen assessed tumor cell viability at 6 drug concentrations ranging from 16 nM-10 μM. Ten of the top 16 hits inhibit the transcriptional machinery. Of these, only triptolide (consistently one of the most potent hits) has been previously considered for PDAC therapy. Other transcriptional inhibitors included actinomycin D, epirubicin (and other anthracycline antibiotics) and plicamycin.

2. Selective activity of triptolide in MYC-amplified tumors. We have developed and genotyped a set of patient-derived xenografts (PDXs) from pancreatic patients. Most standard therapies used for treatment of pancreatic cancer patients produce little or no effect in this system (*Fig. 1A*), mimicking their poor performance in the clinic. In subsequent validation of hits from the primary screen for PDAC inhibitors, we noted that whereas two MYC-amplified cell lines grew as robustly as two non-MYC-amplified cell lines in the absence of drug treatment (*Fig. 1B*), there was a dramatic difference in their sensitivity to triptolide (*Fig. 1C*). The two MYC-amplified

models (PNX001 and YT037) showed complete regression by end of 21-day treatment period, and mice remained tumor-free for at least 20 more days and exhibited no signs of toxicity, while the two lines lacking MYC amplification showed only transient response.

We predicted that MYC stability is one of the key determinants of activity of triptolide against PDAC. This would also predict that stabilized MYC would confer resistance to anti-MYC drugs. Activity of KRAS-ERK has been shown to regulate MYC half-life via phosphorylation. This may offer the opportunity to further deregulate KRAS-ERK-MYC axis via a combined targeting of MYC transcription and KRAS-ERK pathway signaling. We are going to test these possibilities in vitro and in vivo on our established platform of PDAC PDX models.

In sum, our results suggest that: 1) targeting the transcription process can cause rapid regression of some PDAC xenografts; 2) MYC is an indirect secondary target of triptolide inhibition of ERCC3, and MYC inhibition can reciprocally cause loss of ERCC3; 3) stabilized phosphorylated MYC protein may be associated with resistance to transcriptional inhibitors; and hence, combined targeting of transcription and ERK pathway signaling may be synergistic in PDAC, particularly in the context of MYC-amplified, MYC-dependent tumors. Given that MYC is amplified in as many as 30% of PDACs, our study could be of value to a significant subset of patients with PDAC, who would be highly responsive to triptolide used as a lower dose monotherapy or in conjunction with signaling inhibitors.

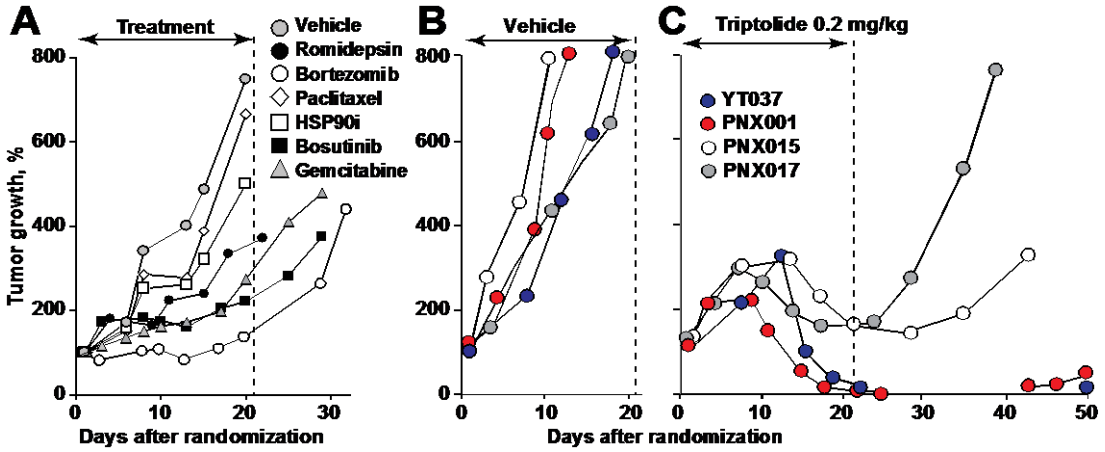

Figure 1. **(A)** 21-day therapy of PNX001 "xenopatient" shows limited efficacy of FDA-approved drugs. **(B)** Robust growth of control PDX tumors in vivo (vehicle) vs. **(C)** complete regression of MYC-amplified PDX models PNX001 and YT037 after 21-day treatment with triptolide. YT037 (blue circles) and PNX001 (red circles), n=5 mice, 10 tumors in each group] MYC-normal tumors showed only transient shrinkage. Lines are averaged volumes per group.

Research Project 3: Project Title and Purpose

A Gene Methylation Progression Model of Bladder Cancer - The purpose of this exploratory project is to identify the difference in aberrant gene methylation between non-invasive, low grade and high grade stromal invasive, and muscle-invasive bladder tumors. The central question in the management of superficial bladder tumors, which comprise the majority of bladder cancer, is how to identify the superficial tumors that will progress from those that will not, and thereby avoid overtreatment or undertreatment. Knowledge of the molecular steps in progression of bladder cancer will likely provide insight into the biology of the disease as well as identify novel targets for therapy. It is likely that genes identified will also be involved in other types of cancer with similar management issues such as prostate cancer.

Duration of Project

1/1/2011 - 06/30/2014

Project Overview

Bladder cancer is the sixth most common cancer in the western world. Currently, the clinician is unable to differentiate between more aggressive or indolent superficial bladder cancer. Despite the finding of pathologically confined disease at the time of surgery, 10-20% of patients will have progression to life-threatening muscle-invasive cancer. There is no molecular marker for this risk. Furthermore, the biological basis for invasion is poorly understood.

Our group has helped to pioneer the study of methylation in cancer. We have evidence that aberrant gene methylation in bladder cancer is frequent, early, neoplastic cell specific, and occurs in all pathways to tumorigenesis.

The goal of this project is to have our team use our expertise and state of the art genome-wide technology to rapidly screen for genes differentially methylated between pathological stages of bladder cancer. This will address the key clinical issue that the subset of individuals with superficial bladder cancer that will progress cannot be identified in advance.

The successful completion of this project could have future impact through improved care and outcome of bladder cancer. Our work could lead to an aggressiveness index to stratify aggressive superficial tumors for potentially curative cystectomy while still allowing patients with more indolent lesions to have bladder-sparing treatment. The genes identified may also have clinical utility in other tumor types with similar management issues, e.g., indolent versus aggressive prostate tumors. Lastly, we have expertise in translational application to bladder cancer and gene methylation tests have reached the clinic.

Our Specific Aims are
1. To profile genome-wide aberrant methylation of genes in pathological stages of bladder cancer
2. To validate aberrant methylation by direct bisulfite sequencing of the genes identified
3. To identify functional themes, pathways and networks in bladder tumor invasion and

progression.

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Expected Research Outcomes and Benefits

We expect to identify genes with a statistically significant different frequency of aberrant methylation between pathological stages based on depth of invasion of bladder cancer.

We expect to verify 25 genes aberrantly methylated in bladder cancer but unmethylated in normal transitional cells.

We expect to identify functional themes, pathways or networks within the 25 genes.

The project will improve health status through:

- Further elucidation of the methylome of the human bladder cancer cell
- Candidate markers for risk stratification of superficial bladder tumors to be tested further
- Candidate markers for diagnosis, and novel targets for chemoprevention or therapy
- Insight into the biology of bladder cancer and, in particular, tumor invasion
- Insight into the biology of, and markers for, other cancers e.g. prostate cancer.

Summary of Research Completed

Aim 1. To profile genome-wide aberrant methylation of genes in pathological stages of bladder cancer.
This aim was completed in a prior reporting period.
This data as well as data from Aims 2 and 3 was published as "A global profile of gene promoter methylation in treatment-naïve urothelial cancer" in Epigenetics 9:760-773, 2014. This research paper is publically accessible at http://www.landesbioscience.com/journals/epigenetics/

Aim 2. To validate aberrant methylation by direct bisulfite sequencing of the genes identified.
Our publication included data on verification of 4 of 7 genes by direct bisulfite pyrosequencing. Subsequently, a further 18 genes were verified as unmethylated in normal urothelium and hypermethylated in urothelial cancer by mining the Cancer Genome Atlas (TCGA) database available at https://tcga-data.nci.nih.gov/tcga/. We chose this "dry" validation as preferable to the original plan of direct sequencing. In total, 25 genes were verified, 18 as above and 7 by direct

sequencing. The correlation between the Infinium methylation score and the independent verification was deemed to be excellent. Representative genes are shown in Ibragimova et al 2014. Aim 2 is now completed.

Aim 3. To identify functional themes, pathways and networks in bladder tumor invasion and progression.

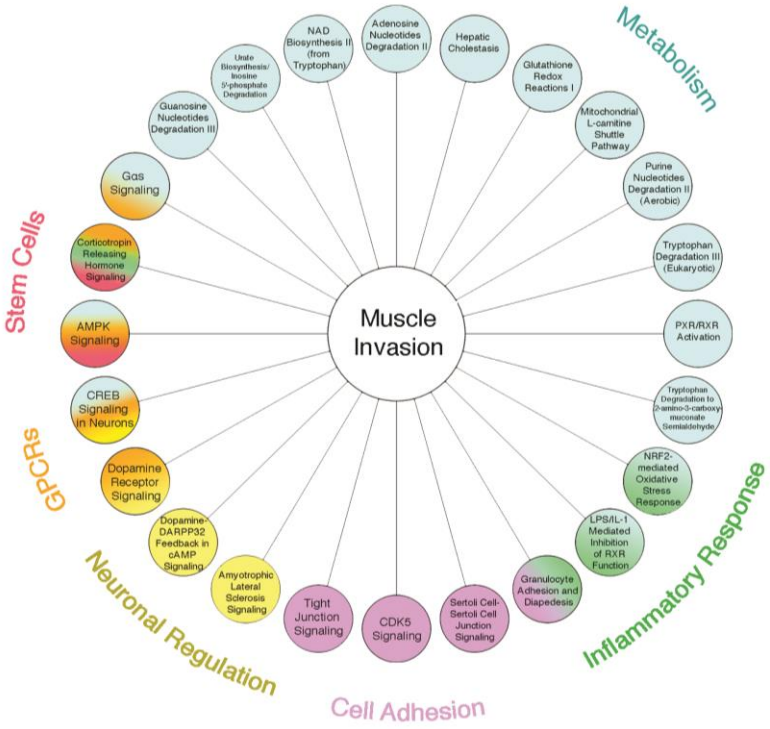
Ingenuity Pathways Analysis (IPA) of the 40 genes significantly more frequently hypermethylated in muscle-invasive urothelial cancer (UC) compared to superficial UC (S UC) found 24 canonical pathways to be significantly over-represented (IPA pathways with enrichment scores ≥1.3 equivalent to a non-log scale p value <0.05). The pathways are shown in *Figure 1* and may be broadly categorized as functional themes of cell adhesion, metabolism, inflammatory response, neuronal regulation, stem cells, G-protein coupled receptors and signal transduction including of the PI3K/AKT/mTOR pathway.

We performed separate analyses of high grade versus low grade UC, non-invasive pTa UC versus invasive pT1-4 UC, as well as high grade S UC vs. low grade S UC. IPA analysis of the genes significantly more frequently hypermethylated in HG UC found 22, and in invasive UC 27 canonical pathways to be significantly over-represented. There was considerable overlap between the significant genes in these analyses that suggests we have identified a signature of aggressiveness or progression.

Analysis by Database for Annotation, Visualization and Integrated Discovery (DAVID) highlighted G-protein signaling coupled to cyclic nucleotide second messenger, cyclic-nucleotide-mediate signaling, intracellular signaling cascade and response to endogenous stimulus and also cellular homeostasis, response to wounding, and transcription.

Aim 3 is now completed.

Figure 1. Pathways significantly overrepresented by aberrant gene methylation in muscle-invasive (MI) compared to S UC.
IPA analysis of genes significantly more frequently hypermethylated in MI UC compared to S UC identified 24 canonical pathways that may be broadly grouped as shown.



Research Project 4: Project Title and Purpose

Targeting STAT3 in Ovarian Cancer - The purpose of this project is to determine whether therapeutic targeting of signal transducer and activator of transcription 3 (STAT3) is effective for the treatment of ovarian cancer. Using an immunocompetent genetically engineered mouse model of epithelial ovarian cancer, we will determine the effect of STAT3 inhibition on tumor incidence latency and progression. Since activation of STAT3 in both tumor cells and immune/inflammatory cells in the tumor microenvironment is thought to contribute to cancer, this project is designed specifically to determine the effects of STAT3 inhibition on both cell populations.

Duration of Project

1/1/2011 - 12/31/2013

Project Overview

Epithelial ovarian cancer (EOC) is the most fatal of all of the gynecological malignancies.

Approximately 22,000 new cases are diagnosed and nearly 15,000 women die of ovarian cancer each year in the United Sates. The disproportionately high mortality rate of EOC is largely related to the fact that most cases are diagnosed at advanced stage, when disease has spread beyond the ovary. Patients generally respond well to aggressive surgical debulking and a standard combination chemotherapy regimen consisting of a platinum agent and taxane. However, the majority of patients eventually experience disease recurrence and, ultimately, drug resistant disease. There is a critical unmet need in identifying therapeutic agents that can improve patient outcomes. In order to discover alternative therapeutic agents, considerable effort has been devoted to defining the underlying molecular and genetic changes that contribute to the development and progression of EOC.

One target that has been identified is signal transducer and activator of transcription 3 (STAT3). STAT3 has been shown to be persistently activated in EOC and is associated with highly aggressive and invasive cases of EOC. Although STAT3 activation has been recognized in EOC for several years, until now, agents targeting STAT3 activation that are suitable for clinical use have been lacking. Recently, a small molecule Janus kinase 2 (JAK2) inhibitor, AZD1480, was identified that has direct effects on inhibition of STAT3 activity. The objective of our project is to test the therapeutic efficacy of this agent in an immunocompetent genetically engineered mouse model of EOC. In addition to evaluating the efficacy of AZD1480 in inhibiting ovarian cancer progression in mice, a second major objective is to assess the effects of AZD1480 treatment on the immune tumor microenvironment and ovarian cancer associated inflammation.

Our specific aims are to:

1) Evaluate the effects of a novel small molecule JAK2 inhibitor on epithelial ovarian cancer cells *in vitro*.
2) Determine the therapeutic efficacy of STAT3 inhibition on ovarian carcinoma development and dissemination *in vivo*.
3) Test the hypothesis that targeting the STAT3 pathway will inhibit *in vivo* tumor development by direct effects on both tumor cells and on inflammatory cells present in the tumor microenvironment.

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Expected Research Outcomes and Benefits

Based on extensive *in vitro* studies of STAT3 activation and signaling in our laboratory, we are convinced that it is a highly relevant target for therapeutic inhibition for the treatment of ovarian cancer. Activation of STAT3 is known to have pleiotropic effects supporting the growth and progression of many solid tumor types including epithelial ovarian cancer (EOC). Moreover, recent studies show that STAT3 activation in the tumor microenvironment also supports tumor initiation, growth and progression.

The development of a highly promising new Janus kinase 2 (JAK2) inhibitor, AZD1480, shows significant activity in preclinical cancer models. The goal of our project is to evaluate the therapeutic efficacy of AZD1480 for treatment of EOC using a genetically engineered mouse model of EOC developed in our lab. Importantly, EOC occurs spontaneously and these mice have a completely intact immune system. Therefore, using this model, we are uniquely capable of evaluating the effects of AZD1480 on not only ovarian tumor growth and progression, but on the immune tumor microenvironment as well. We predict that AZD1480 will show both tumor inhibitory activity as well as shifting the tumor microenvironment from tumor supporting inflammation to anti-tumor immunity. As AZD1480 is currently in Phase I development, demonstration of therapeutic efficacy in our mouse model of EOC would be expected to be rapidly translatable to a preclinical trial for ovarian cancer patients.

Summary of Research Completed

Effects of the novel small molecule JAK2 inhibitor AZD1480 on STAT3 DNA binding in cultured ovarian carcinoma cells (Aim 1).

In our prior analysis of the effects of the JAK2 inhibitor AZD1480 on cultured cells, we observed reduction of activated (phosphorylated) STAT3 at low (≤1 μM) concentrations of AZD1480. However, inhibition of cell viability and induction of apoptosis were observed only at higher concentrations (≥5 μM) of AZD1480, suggesting the possibility that these effects could be related to off-target effects. As an additional method to evaluate whether AZD1480 treatment affects JAK2-mediated activation of STAT3 directly, we performed electrophoretic molecular shift assay (EMSA) to determine if AZD1480 treatment reduces or abrogates pSTAT3 DNA binding. OVCAR-5 and OVCAR-8 cells were plated in 10 cm dishes and treated for 24 h with 0.1% dimethyl sulfoxide (DMSO, vehicle) or 1μM AZD1480 dissolved in 0.1% DMSO. Cells were pre-treated with 25ng/ml interleukin 6 (IL-6) for 3h to stimulate STAT3 activation and DNA binding. Cells were harvested and nuclear extracts isolated by incubation for 20 min with lysing buffer A (10mM HEPES, 10mM KCl, 1.5mM $MgCl_2$, 1mM dithiotreitol, 10% IGEPAL, protease and phosphatase inhibitors) and followed by incubation with lysing buffer B (20mM HEPES, 400mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 1mM DTT, 5% glycerol, protease and phosphatase inhibitors) for an additional 30 min. Nuclear extracts were incubated with ^{32}P-labeled STAT3 consensus oligonucleotide (5'-AGCTTCATTTCCCGTAAATCCC-3') or a mutant oligonucleotide (5'-GATCCTTCTGGGCCGTCCTAGATC-3') and resolved in non-denaturing 5%PAGE. The pSTAT3 DNA binding specificity was determined by super shifting of the specific band caused by additional pre-incubation with anti-STAT3 antibodies or by absence of specific band caused by incubation of the sample with mutant STAT3 consensus oligonucleotide.

Resolved gel was dried and exposed to radiography film at -80C. Results of these experiments confirmed that AZD1480 treatment reduced pSTAT3 DNA binding (data not shown).

Effects of AZD1480 on migration and adhesion of epithelial ovarian cancer cells (Aim 1).

The effects of AZD1480 on ovarian carcinoma cell migration and adhesion was evaluated previously in OVCAR-8 cells, showing AZD1480-mediated inhibition of both. To ensure that these results were not specific or unique to this single cell line, we extended these analyses to evaluate migration and adhesion in two additional cell lines, A1847 and OVCAR-5, using OVCAR-8 cells as a benchmark. To evaluate migration, equal numbers (4×10^4) of A1847, OVCAR-5, and OVCAR-8 cells were resuspended in serum free medium containing 0.1% DMSO (vehicle) or AZD1480 (1μM in 0.1% DMSO) and plated in duplicate in the upper chamber of trans-well chambers with membranes containing 8μm pores. To stimulate chemotactic migration, complete 10% FBS medium was added to the lower chamber. Cells were allowed to migrate through membrane toward the serum containing medium for 24 hours. Non-migrating cells in the upper chamber and top of the membranes were removed with a cotton swab and membranes were washed in PBS and then fixed in 4% paraformaldehyde. Fixed membranes were sealed on glass slides and evaluated by microscopy at 10X magnification. Five random fields for each membrane were photographed and the number of migrated cells was counted. Migration experiments were performed three times and the mean ± SEM migrating cells/field calculated. Data were analyzed by two sided t-test and *p*-values lower than 0.05 were considered as significant. Results of these experiments confirmed the effect of AZD1480 on ovarian carcinoma cell migration is uniform and not cell line-specific (Figure 1).

The effects of AZD1480 on adhesion of A1847 and OVCAR-5 were evaluated, again using OVCAR-8 cells as a benchmark. Equal numbers of A1847, OVCAR-5 and OVCAR-8 cells (3×10^3) were plated in quadruplicate wells of a 96-well plates pre-coated with 20μg/ml type I collagen, 2μg/ml fibronectin or 3% bovine serum albumin (control). Cells were allowed to adhere for 1 hour, then fixed in 4% paraformaldehyde and stained with 1% crystal violet in 25% methanol. Assays were performed three times. Adherent cells present in each well were counted and the mean ± SEM adherent cells/well calculated. Data were analyzed by two sided t-test and *p*-values lower than 0.05 were considered as significant. AZD1480 treatment significantly inhibited adhesion of A1847 cells to fibronectin and OVCAR-5 and OVCAR-8 cells to type I collagen. Thus, the effect of AZD1480 on ovarian carcinoma cell adhesion is not specific to OVCAR-8 cells (Figure 1).

AZD1480 alters STAT3-mediated gene expression in ovarian tumors in mice (Aim 2).

To discover the underlying molecular mechanisms contributing to tumor growth inhibition, we compared gene expression changes in a separate *in vivo* experiment in which ovarian tumor bearing MISIIR-TAg mice were treated acutely (6 hours) with vehicle or AZD1480 (n=4 mice/group). Gene expression differences were compared in RNA isolated from tumors from vehicle- and drug-treated mice by microarray analysis. Unsupervised clustering analysis allowed separation into two groups; drug-treated tumors clustered separately from vehicle treated controls (data not shown). Using 2-fold change and $p<0.01$ cutoff we identified a list of 10 upregulated and 87 downregulated genes in AZD1480 treated tumors (Figure 2). Notably, the list

of differentially expressed genes included a large number of genes previously shown to be regulated by STAT3; including genes involved in cancer and wound healing (e.g., *Ccnd1*, *Ccl2*, *Cepd*, *Ifiti1*, *Mx1*, *Mx2*, *Oas1*, *Oas2*, *Usp18*). Gene ontology (GO) analysis identified genes enriched for ribonucleoside metabolic process and interferon and cytokine-mediated signaling pathways. A subset of genes was selected for individual validation by qRT-PCR. Differential expression was shown for *Ccdn1*, *Ccl12*, *Ifi204* and *Stat3* (Figure 2). These results confirm the direct inhibitory effects of AZD1480 on STAT3 transcriptional activity *in vivo*, and strongly suggest that the observed tumor growth inhibition was at least partly mediated by inhibition of STAT3.

AZD1480 treatment affects the number and fractions of inflammatory cells present in the tumor microenvironment (Aims 3).

By the use of flow cytometry analysis of immune cells subpopulations present in mouse spleens, tumors and peritoneal washes, we previously showed that AZD1480 treatment had significant effects on immune cell populations in the peritoneal tumor microenvironment. To confirm these initial observations and to better understand the effects of AZD1480 on immune cells, an additional experiment was performed to carefully assess leukocyte populations present in spleens, ovaries or ovarian tumors, mesenteric lymph nodes and peritoneal washes collected from age-matched, transgenic mice and wild type littermates that were treated with vehicle or AZD1480 for 30 days. An additional four wild-type mice that were naïve to treatment were evaluated as controls. Immune cell sub-populations, isolated from peritoneal cavities, spleens, lymph nodes or tumors, were evaluated with enlarged number of antibodies recognizing mouse CD45, CD4, FoxP3, CD8, CD44, CD19, NK1.1, CD3e, F4/80, Gr-1 by flow cytometry. The data from these and the prior extensive experiment evaluating two large groups of mice treated with vehicle (n=11) or AZD1480 (n=17) were submitted to Dr. Samuel Litwin in the Biostatistics Facility. Dr. Litwin performed detailed statistical analyses of these data sets. Using X methods, the analysis confirms the finding that the numbers of CD45+ leukocytes, CD45+CD4+ helper T cells, and CD4+FoxP3+ regulatory T cells in the peritoneal microenvironment are significantly decreased in drug-treated mice compared to vehicle-treated controls. These subpopulations remain unchanged in the spleens of AZD1480-treated mice compared to controls. (Figure 3).

We have successfully completed the following milestones as stated in the original application during the final 6 months of the project:

Evaluated and confirmed the potential of the JAK2 inhibitor AZD1480 to disrupt binding of activated STAT3 to DNA oligonucleotides harboring consensus STAT3 binding sites.

Expanded analysis of the effects of AZD1480 on migration and adhesion by evaluation of additional cell lines (OVCAR-5 and A1847) and confirmation of the inhibitory effects of AZD1480 on ovarian carcinoma cell migration and adhesion.

Performed bioinformatics analysis of gene expression microarray data from tumor tissue isolated from AZD1480- and vehicle-treated mice and validated differential gene expression in tumors from drug-treated mice compared to vehicle-treated controls.

Finalized the extensive statistical analysis of data sets resulting from flow cytometry analysis of peritoneal washes, spleens, lymph nodes, and tumors collected from mice treated or untreated (vehicle) with AZD1480, confirming that AZD1480 results in significant reduction of CD45+ leukocytes, CD45+CD4+ T helper, and CD45+CD4+FoxP3+ T regulatory cells in the peritoneal tumor microenvironment.

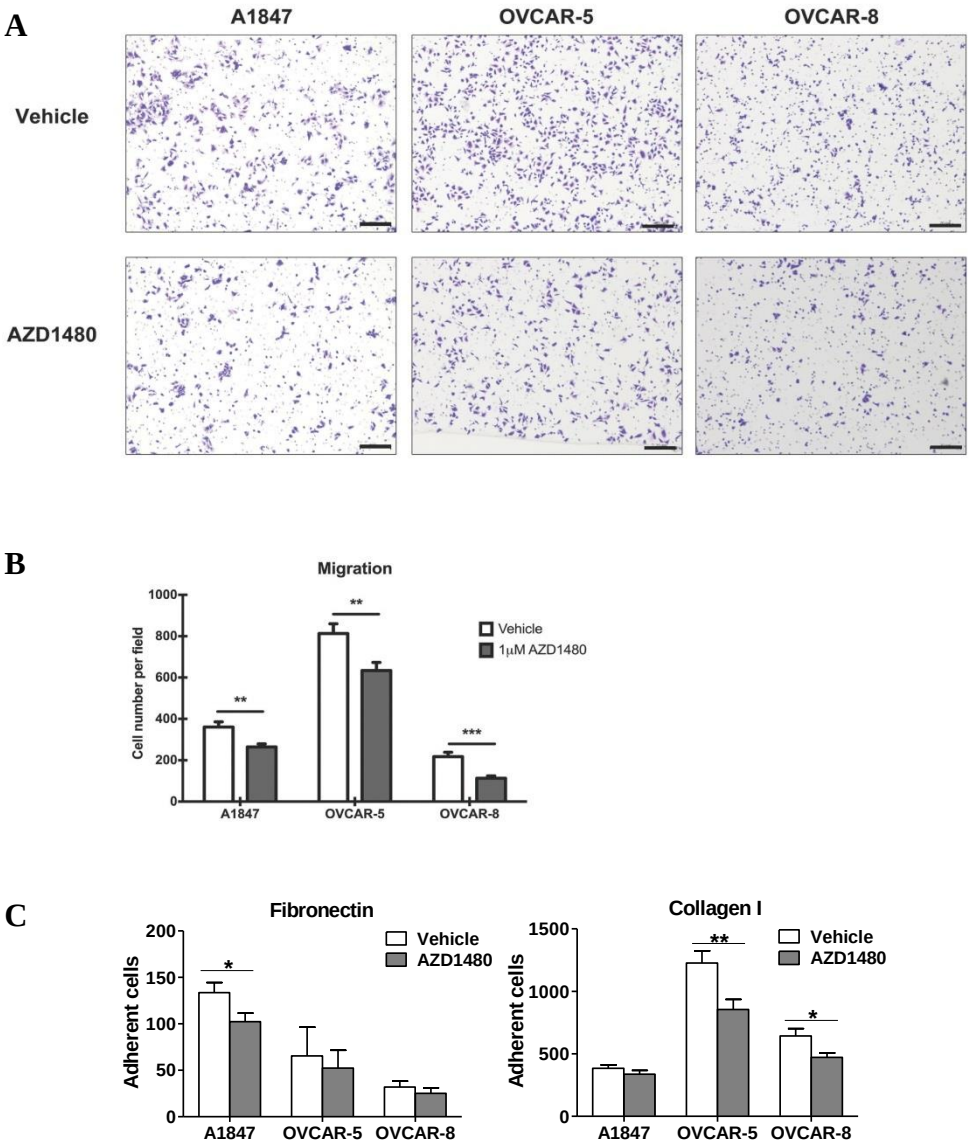

Figure 1. *AZD1480 treatment inhibits ovarian carcinoma cell migration***.** A) Representative light microscopy images of vehicle- and 1μM AZD1480-treated A1847, OVCAR-5 and OVCAR-8 cells migrated through 8μm pore membranes of transwell chambers. Scale bars = 200μm. B) Quantification was performed by counting the number of migrated cells present in 5 random fields of each transwell chamber. The mean ± SEM (n= 3 experiments) shows significant (**p<0.01, ***p<0.001) reduction in migration in ovarian carcinoma cells treated with AZD1480. C) A1847, OVCAR-5 and OVCAR-8 cells were treated with vehicle or AZD1480 and allowed to adhere to plates coated with fibronectin or type I collagen for one hour. AZD1480 treatment significantly reduced A1847 cells adhesion to fibronectin and OVCAR-5 and OVCAR-8 cells to collagen I. The mean ± SEM from three independent experiments are shown (*p <0.05, **p <0.01).

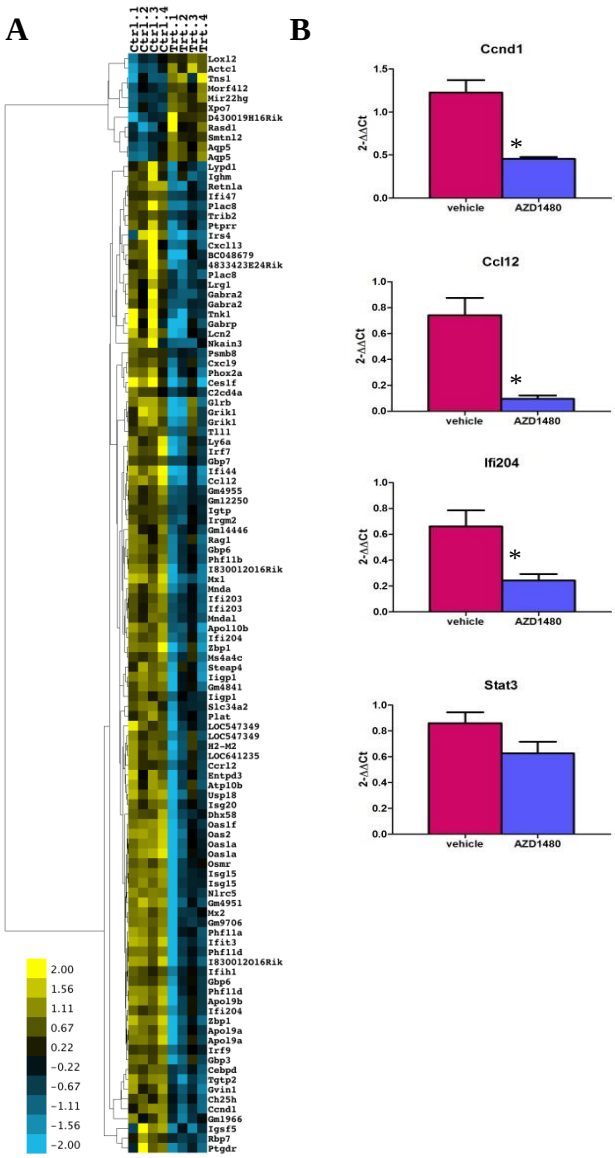

Figure 2. *Gene expression is altered in AZD1480-treated tumors.* MISIIR-TAg mice were treated with vehicle or AZD1480 for 6 hours (n=4 mice/group) and RNA was extracted from tumor tissue and subjected to microarray analysis. A) Heatmap showing 97 differentially expressed genes (10 upregulated, 87 downregulated) identified in AZD1480-treated tumors using a cutoff of >2-fold change and $p<0.01$ (FC>2; $p<0.01$). B) Validation of differential expression of selected genes (*Ccnd1*, *Ccl2*, *Ifi204*, *Stat3*) by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). *$p=0.0286$.

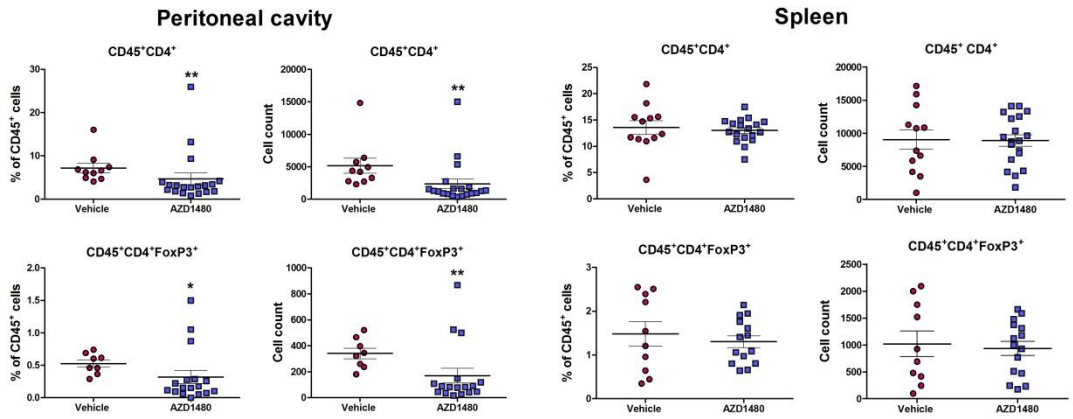

Figure 3. *AZD1480 treatment of ovarian tumor-bearing mice decreases the presence of helper T cells and regulatory T cells in the peritoneal tumor microenvironment.* Immune cells subpopulations present in samples collected from peritoneal washes and spleens of mice treated with vehicle or AZD1480 were subjected to FACS analysis. Graphs show the distribution of CD45+CD4+ helper T cells and CD45+CD4+FoxP3+ regulatory T cells in peritoneal washes (A) and spleens (B). Data are presented as the percent of CD45+ leukocytes and as total cell counts for each population. The fraction of CD45+CD4+ and CD45+CD4+FoxP3+ cells in peritoneal washes significantly decreased in AZD1480 treated mice. *$p<0.05$, **$p<0.01$.

Research Project 5: Project Title and Purpose

Classification and Prediction of Protein-Protein Interactions in Biology and Medicine - Protein interactions are central to all biological processes, including those leading to disease. Many proteins exist in complexes with other copies of the same protein and/or with other kinds of proteins critical to their function. Structural information on these interactions is essential for understanding basic biological processes and the development of therapeutics and diagnostics. However, the structures of these interactions are often unknown or ambiguous. We hypothesize that we can obtain far more information than currently available by exploiting evolutionary relationships among proteins and analysis of data from multiple biophysical experiments. To do this, we will classify domains in multi-domain proteins, cluster similar forms of interaction, and predict the structures of protein interactions that occur in signaling pathways associated with cancer.

Duration of Project

1/1/2011 – 6/30/2013

Summary of Research Completed

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

Research Project 6: Project Title and Purpose

Use of Somatic Cell Nuclear Transfer to Generate TCR Monoclonal Mice - A key approach in experimental immunology is to generate mice that express single T or B cell receptors (TCR, or BCR) in order to examine their specificity, function, and effect on development. Somatic cell nuclear transfer (SCNT) is a promising technique for the rapid generation of such TCR monoclonal mice, which involves replacing the oocyte's nucleus with a donor nucleus from a somatic cell. Here we propose experiments to improve the efficiency of SCNT for T lymphocytes and apply the approach to dissecting the mechanism of CD4/CD8 lineage commitment.

Duration of Project

7/1/2011 - 6/30/2013

Summary of Research Completed

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