Thomas Jefferson University

Annual Progress Report: 2007 Formula Grant

Reporting Period

July 1, 2010 – June 30, 2011

Formula Grant Overview

The Thomas Jefferson University received $3,591,514 in formula funds for the grant award period January 1, 2008 through December 31, 2011. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Role of the RB Tumor Suppressor in Breast Cancer - Breast cancer is a major health concern in the United States, wherein approximately 1 woman in 8 will be diagnosed with the disease. While significant strides have been made in improving therapy for breast cancer, an estimated 41,000 women in the United States will die from this disease in 2008. Therefore, there is clear need for providing better therapeutic options for women with breast cancer. The finding that a protein called RB plays a critical role in the response to therapeutic agents is of high clinical importance, as RB is known to be inactivated in a large percentage of breast cancers. Thus, understanding more about RB function may lead to more appropriate and effective therapy for women with breast cancer.

Anticipated Duration of Project

1/1/2008 - 12/31/2011

Project Overview

Approaches currently used to treat breast cancer include hormonal, cytotoxic, and targeted therapeutic agents. At present, breast cancer represents one of the few cancers wherein treatment options are based on molecular targets. Most notably, estrogen receptor status is a critical determinant for therapy, wherein estrogen receptor positive tumors are treated with estrogen antagonists such as tamoxifen. This level of testing is critical as ER-deficient tumors invariably fail to respond to tamoxifen. However, even a large percentage of ER-positive tumors still fail tamoxifen therapy. Means to discover the determinants of therapeutic failure and provide enhanced diagnostic power is therefore of great importance. Many targets of therapeutic agents function through common down-stream pathways that effect cellular proliferation--particularly, they all impinge upon the cell cycle machinery. One component of cell cycle control which is compromised in breast cancer at high-frequency is the retinoblastoma tumor suppressor-pathway. Retinoblastoma tumor suppressor, RB, is a critical determinant of hormonal therapies, such that RB-deficient tumors fail to effectively respond to tamoxifen. Additionally,

deregulation of the RB-pathway is associated with early relapse in breast cancer patients treated with tamoxifen. In contrast, loss of RB enhances sensitivity to specific cytoxic agents.

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

The overall hypothesis of this study is that the RB-pathway is a critical determinant for therapeutic response in breast cancer.

The objectives are to:

1. Define those mechanisms through which the RB-pathway is disrupted in therapy-resistant cancer. These analyses will define mechanisms of therapeutic bypass.

2. Elucidate the function of E2F activity and RB-regulated genes in the response to tamoxifen. These studies will enable the development of diagnostics to allow for improved application of therapeutics in the treatment of breast cancer

3. Delineate those targeted therapeutics that are modulated by loss of RB function and to define additional mechanisms to treat tamoxifen-resistant breast cancer. These studies will provide insight into new therapeutic combinations to be utilized in the treatment of breast cancer.

4. Produce new animal models for breast cancer. Such animal models are critical for rigorously testing new compounds and diagnostics developed through this research programs.

Combined, successful attainment of these objectives will provide new diagnostic and therapeutic avenues that will have a significant impact on the treatment of breast cancer.

Summary of Research Completed

These studies for the last year were focused on the following objectives: 1) Determine the impact of pharmacologically activating RB on standard chemotherapeutic regimens. 2) Explore the ability to activate RB to protect normal tissue from the side effects of chemotherapy.

Triple negative breast cancer (TNBC) is an aggressive disease that lacks established markers to direct therapeutic intervention. Thus, these tumors are generally treated with cytotoxic chemotherapies that cause severe side effects. Loss of the retinoblastoma tumor suppressor (RB) protein occurs in a substantial fraction of TNBC cases, providing heterogeneity that can be rationally targeted. Here we utilize RB-status not only to direct treatment of TNBC with a combination of chemotherapy and CDK-inhibition, but also to protect normal tissue from chemotherapy-associated toxicity. We found that while CDK4/6-inhibition provides a cooperative cytostatic effect with anthracycline-based chemotherapy in RB-proficient tumor models, it ultimately protects cells from chemotherapy-mediated toxicity. In contrast, RB-deficient cells are refractory to CDK4/6-inhibition but remain highly sensitive to chemotherapy, suggesting that CDK inhibitors could be rationally deployed to modulate the impact of chemotherapy in tumor vs. normal tissue. Consistent with this hypothesis, CDK4/6-inhibition was able to protect cardiac tissue from chemotherapy-associated damage. These data provide preclinical rationale for using RB-status to direct cancer treatment with the combination of chemotherapy and CDK inhibitors while also ameliorating side effects that negatively influence patient health.

TNBC accounts for 15-20% of all breast cancers, yet approximately 50% of breast cancer deaths. Such tumors are ER/PR/Her2-negative and unresponsive to both endocrine therapy and Her2-targeted agents. Consequently, TNBC is often treated with cytotoxic chemotherapy regimens, most of which include anthracyclines (e.g. doxorubicin), and yield significant side effects (e.g. cardiotoxicity) that can preclude treatment of patients with existing health conditions and further compromise quality of life. While significant research has been devoted to development of cardioprotective interventions, there are currently few agents that can protect patients from cardiotoxicity without compromising the efficacy of chemotherapy.

RB-pathway inactivation is a frequent occurrence in TNBC that is associated with both the aggressive behavior of these tumors and improved response to cytotoxic chemotherapy. This sensitivity is thought to be the result of a predilection toward cell death associated with bypass of RB-mediated cell cycle checkpoints. Conversely, RB-proficient tumors generally experience a cytostatic response following therapeutic challenge, which could ostensibly prove to be the basis of recurrent disease. This role for RB in modulating cytotoxicity could be of particular importance in the context of chemotherapy-associated tissue damage, as targeted RB-pathway activation would potentially protect RB-proficient tissues without altering chemotherapeutic response in RB-deficient tumors.

In this context, highly specific CDK4/6 inhibitors have been developed and represent a viable mechanism for systemic activation of the RB-pathway. Preclinical studies from our lab and others have demonstrated that CDK4/6-inhibition blocks DNA synthesis by prohibiting G_1 to S-phase progression, resulting in a potent cytostatic effect that is strictly dependent on a functional RB-pathway. The current study tests the hypothesis that RB-status is a critical determinant of cellular response to chemotherapy in both tumor and non-tumor tissue by determining the effect of pharmacological CDK4/6-inhibition on the response of TNBC to anthracycline-based chemotherapy, as well as the ability of CDK4/6-inhibition to serve as a protective mechanism against a common chemotherapeutic side effect in normal tissue.

RESULTS:

RB-Pathway activation cooperates with cytostatic action of chemotherapy, but antagonizes cytoxicity: To first determine the potential additive or antagonistic impact of pharmacological CDK4/6-inhibition (i.e. PD-0332991) and cytotoxic chemotherapy (i.e. doxorubicin), RB-proficient TNBC cell lines (MDA-MB-231, Hs578T) were ultilized. PD-0332991 treatment alone resulted in a dramatic decrease in BrdU incorporation associated with cell cycle arrest in G_1-phase (Fig. 1a), and a corresponding decrease in proliferation-associated proteins regulated by RB (cyclin A, topoisomerase IIa) (Fig. 1b). In contrast, doxorubicin treatment did not inhibit BrdU incorporation, but lead to accumulation of cells in S-phase and G_2-M, and enhanced levels of proliferation-associated proteins. PD-0332991 and doxorubicin co-treatment resulted in an intermediate cell cycle distribution with significantly decreased BrdU incorporation and proliferation-associated proteins (Fig. 1a,b).

These data indicate that RB-pathway activation is dominant to effects of doxorubicin in the context of proliferation. As has been previously reported, cyclin D1 protein levels accumulated with PD-0332991 treatment (Fig. 1b). Interestingly, doxorubicin treatment led to degradation of cyclin D1 irrespective of CDK4/6-inhibition, suggesting a fully operable DNA damage response in cells treated with doxorubicin despite inhibition of CDK4/6-activity. This was confirmed by phospho-gH2AX (p-gH2AX) staining, as doxorubicin-treated cells harbored increased p-gH2AX foci irrespective of CDK4/6-inhibition (Fig. 1c). In contrast, while doxorubicin treatment resulted in upregulation of pro-apoptotic factor E2F1 and induction of cleaved PARP, these signaling events were attenuated by PD-0332991 (Fig. 1c). Combined, these results indicate that RB-pathway activation enhances cytostatic response, yet inhibits doxorubicin-mediated cell death signaling.

To examine the impact of CDK4/6-inhibition on *in vivo* tumor response to doxorubicin, we utilized mice harboring MDA-MB-231 xenografts. While doxorubicin alone significantly inhibited tumor growth beginning 4 days post-treatment, PD-0332991 alone or in combination with doxorubicin was highly effective at suppressing tumor growth from Day 1 (Fig. 1d). Consistent with cell culture studies, CDK4/6-inhibition resulted in significantly decreased proliferation as determined by Ki67 staining in tumor tissue (not shown), as well as decreased BrdU incorporation (not shown). Interestingly, while doxorubicin alone did not inhibit proliferation, it did exhibit a cooperative cytostatic effect with PD-0332991 (Fig. 1d). In contrast, phospho-histone H3 (pSer10) staining of mitotic chromosomes suggested a possible antagonism in the context of doxorubicin-mediated cytotoxicity.

While doxorubicin treatment resulted in a dramatic increase in cells displaying aberrant mitotic figures and chromosome fragmentation commonly associated with apoptosis, this phenotype was completely inhibited by co-treatment with PD-0332991 (not shown). This was not associated with DNA damage burden, as the percent of p-gH2AX-postive tumor cells was not influenced by PD-0332991 (not shown); thus, it is most likely due to the cytostatic response specifically modulated by CDK4/6-inhibtion (not shown). These studies confirm that doxorubicin and CDK4/6-inhibition yield a cooperative cytostatic response; however, there is antagonism related to apoptotic processes and catastrophic mitotic events associated with the cytoxocity of chemotherapy.

RB-dependent pharmacological modulation of chemotherapy response: To assess the impact of CDK4/6-inhibition on the therapeutic response of RB-deficient TNBC to chemotherapy, we utilized two RB-deficient TNBC cell lines (MDA-MB-468, MDA-MB-436). As expected, PD-0332991 was completely ineffective at suppressing proliferation in RB-deficient cells (Fig. 2a). Importantly, PD-0332991 and doxorubicin co-treatment resulted in cell cycle profiles and proliferation rates virtually identical to those observed with doxorubicin alone. Furthermore, there was no effect of PD-0332991 on the expression of proliferation-associated proteins, or doxorubicin-mediated degradation of cyclin D1 (Fig. 2b), induction of p-gH2AX or apoptotic signaling (Fig. 2c). These data confirm that CDK4/6-inhibition in combination with chemotherapy does not alter therapeutic response of RB-deficient TNBC to the cytotoxic agent.

To determine the long-term impact of CDK4/6-inhibition on cell viability following doxorubicin treatment, clonal cell outgrowth assays were performed. Cells were treated with PD-0332991 and/or doxorubicin for 24h, allowed to recover in fresh media for the indicated times, and stained with crystal violet to visualize post-treatment outgrowth (Fig. 2d). CDK4/6-inhibition was able to maintain viability of RB-proficient cells (MDA-MB-231) in the presence of doxorubicin, which then have the capacity to repopulate the culture (Fig. 2d). This phenomenon was dependent on a functional RB-pathway, as RB-deficient cells (MDA-MB468) were not protected by PD-0332991 (Fig. 2d). The percentage of individual cell populations displaying outgrowth is shown. These findings suggest that CDK4/6-inhibition could be used to specifically influence RB-proficient tissues, allowing for more specific elimination of RB-deficient tumors.

RB activation can protect against chemotherapy induced cardiac damage: To test the hypothesis that CDK4/6-inhibition could protect normal tissue from chemotherapy-associated damage, we examined the impact of PD-0332991 on doxorubicin-mediated cardiotoxicity in mice. Histological analyses of cardiac tissue in doxorubicin-treated mice revealed marked cardiomyocyte central clearing consistent with significant loss of sarcomeres (Fig. 3a), a classic finding with doxorubicin cardiotoxicity, which was not observed in mice treated with vehicle or PD-0332991 alone (not shown). Strikingly, cardiac tissue from mice co-treated with PD-0332991 and doxorubicin were virtually indistinguishable from control mice (Fig. 3a). Additionally, cardiac tissue from doxorubicin-treated mice displayed focal p-gH2AX staining and BrdU incorporation in areas of tissue damage, which were completely inhibited by PD-0332991 (Fig. 3a). Interestingly, these events were not associated with increased Ki67 expression (not shown). This general phenomenon was also observed to a lesser extent in other typically non-proliferative tissues, including liver and kidney (not shown).

To determine whether the observed tissue damage influenced cardiac function, echocardiography was performed. While mice receiving vehicle or PD-0332991 alone displayed normal cardiac contractile function as determined by Ejection Fraction (EF) and Fractional Shortening (FS) measurements, doxorubicin-treated mice demonstrated a significant decrease in contractile function (Fig. 3b). Critically, mice receiving both PD-0332991 and doxorubicin displayed contractile function comparable to control mice, and significantly better than doxorubicin-treated mice. Thus, pharmacological CDK4/6-inhibition resulted in protection of cardiac tissue from the functional damage associated with doxorubicin-mediated toxicity.

CONCLUSION

Combined, these findings provide significant preclinical rationale for using RB-status to direct the treatment of TNBC while ameliorating common side effects associated with chemotherapy.

Publication Resulting from this Work:

Dean, JL, Thangavel, C, McClendon, AK, Reed, CA, Knudsen, ES. (2010) Therapeutic CDK4/6 inhibition in breast cancer: key mechanisms of response and failure. Oncogene 29, 4018-4032 doi:10.1038/onc.2010.154.

Figure 1

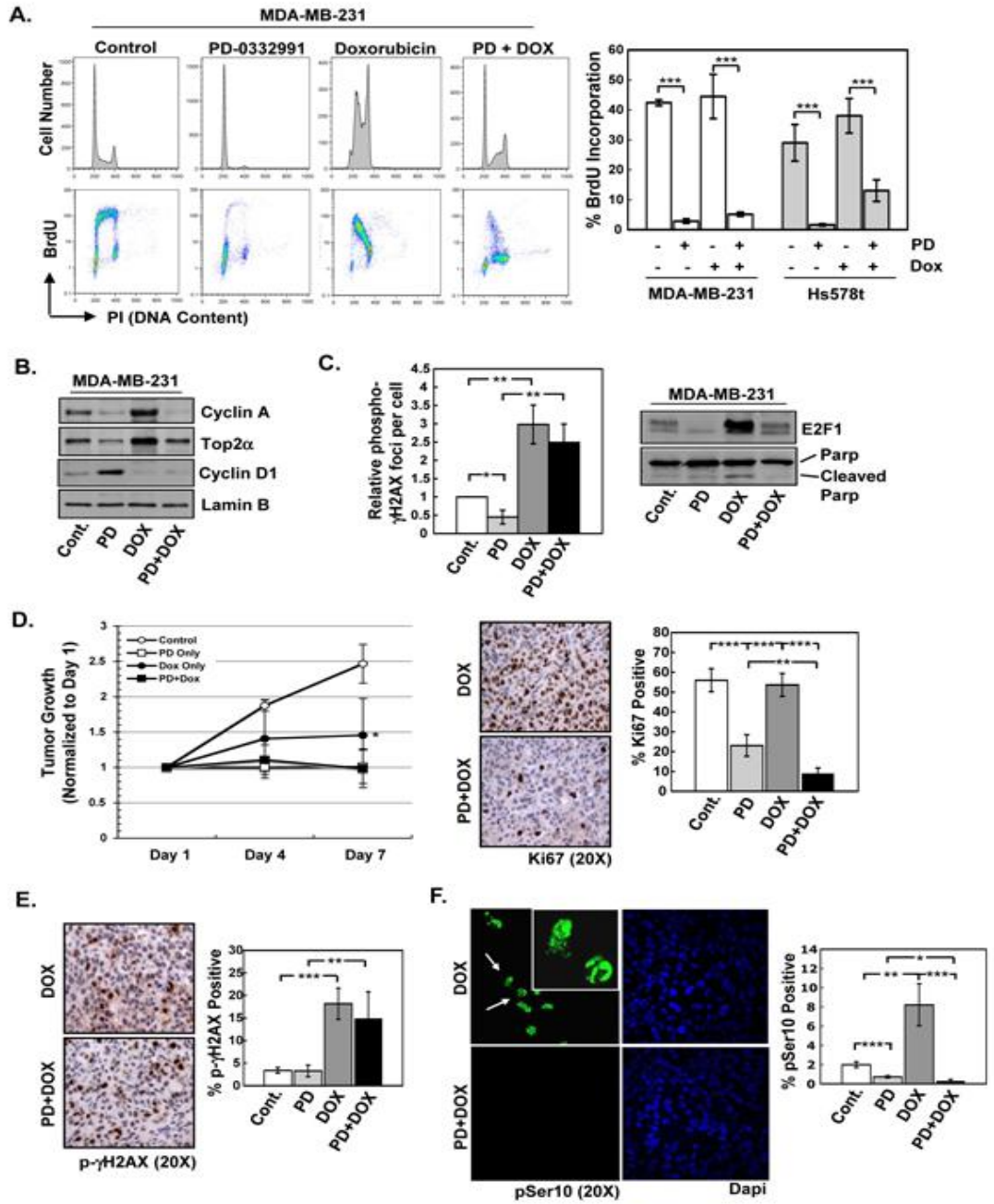


Figure 2

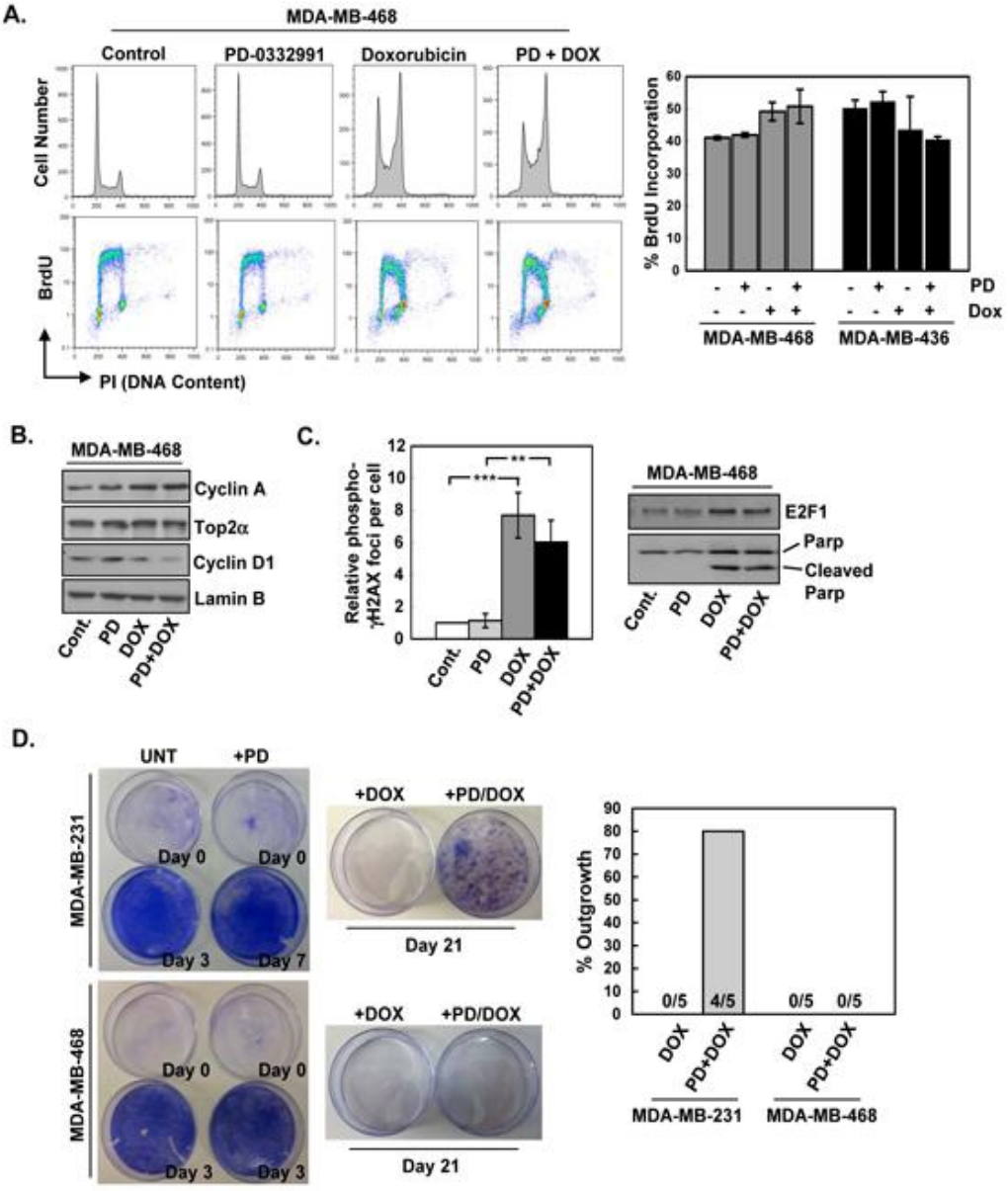
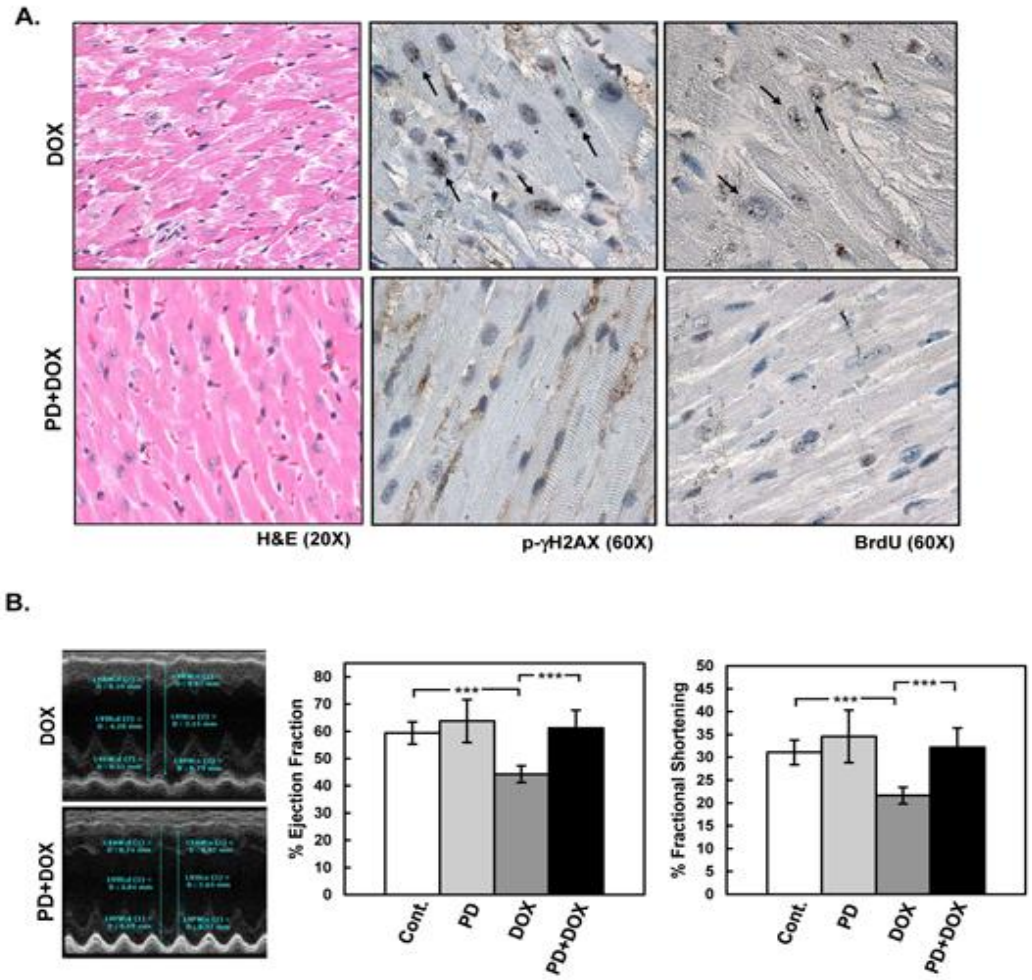

A.
MDA-MB-468
Control
PD-0332991
Doxorubicin
PD + DOX
Cell Number
BrdU
PI (DNA Content)
% BrdU Incorporation
PD
Dox
MDA-MB-468
MDA-MB-436
B.
MDA-MB-468
Cyclin A
Top2α
Cyclin D1
Lamin B
Cont.
PD
DOX
PD+DOX
C.
Relative phospho-γH2AX foci per cell
MDA-MB-468
E2F1
Parp
Cleaved Parp
D.
UNT
+PD
MDA-MB-231
Day 0
Day 3
Day 7
+DOX
+PD/DOX
Day 21
MDA-MB-468
% Outgrowth
0/5
4/5
0/5
0/5
DOX
PD+DOX
MDA-MB-231
MDA-MB-468

Figure 3



Research Project 2: Project Title and Purpose

How the Loss or Aberrant Cyclin D1 Regulation Impacts AR Activity and Prostate Cancer Progression - It is possible that cyclin D1 action can be altered in Prostate Cancer (PCa) by somatic mutation. Cyclin D1 mutations are generally thought to occur at low frequency in human disease, mostly based on observations in colorectal and breast cancer. However, recent studies showed that somatic alterations occur in some types of cancer. Dramatically, these mutations can significantly alter subcellular localization of cyclin D1. To date, no study has examined cyclin D1 mutation in Prostate Cancer (PCa). Of 30 primary tumors screened to date, one tumor-derived mutation occurring in a region that controls splicing of the exon that encodes the nuclear export signal has been detected. These data indicate that cyclin D1 gene alterations may be a mechanism to promote aberrant cyclin D1 expression and /or localization in PCa.

Anticipated Duration of Project

1/1/2008 - 12/31/2011

Project Overview

One of the most important functions of androgen receptor (AR) is the stimulation of cancer growth. Therefore, the typical course of action has been to block cancer growth through the attenuation of androgen action. This approach is initially effective through the elimination of a significant number of cancer cells but those cells remaining eventually find a way to turn AR activity back on, and at this stage the cancer is difficult to treat. We are dedicated to understanding the factors that regulate AR-dependent cancer growth and have characterized a unique function for cyclin D1, a well known factor for regulating cancer growth, which can block AR activity. Human prostate tumors containing cyclin D1 had lower PSA levels, suggesting that cyclin D1 is actively engaged in reducing AR activity. By contrast, a significant number of tumors had lost cyclin D1, and these tumors correlated with higher PSA levels. These findings suggest that cyclin D1 loss allows AR to regain its activity. Since inhibiting AR is the goal of PCa therapy, these observations many reveal one way by which tumors evade therapy, and that idea will be tested here. In addition, a second class of tumors were identified wherein cyclin D1 was "misplaced" in the cell, and likely prevented from doing its job to keep AR activity low. This may represent a second way by which tumors boost AR activity and tumor growth. Together, these observations have culminated into the idea that loss or abnormal cyclin D1 may provide cancer cells a means to turn AR back on and circumvent therapy.

Aim 1 will define the ways that cyclin D1 becomes abnormal in cancer cell models and human tumors:
1. Determine additional factors that cause abnormal cyclin D1 action.
2. Identify genetic components in cyclin D1 that may cause its abnormal action.

Aim 2 will determine the importance of abnormal cyclin D1 in mouse tumor models:
1. Develop new mouse models to mimic abnormal cyclin D1 as seen in human tumors.
2. Evaluate treatment strategies and outcomes related to abnormal cyclin D1 in mouse models.

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

It is expected that these studies will provide a monumental leap in the understanding of cyclin D1 regulation in prostate cancer. If successful, the outlined strategies will provide the

justification for developing future pre-clinical strategies to effectively manage tumors with de-regulated cyclin D1. In summary, the studies outlined in this project will have translatable outcomes and completion of this work will:

1. Lead to the development of new tumor markers.
2. Reveal insight for novel therapeutic design.
3. Uncover new areas of prostate cancer research.
4. Define the role of cell cycle components in a therapeutic model

Summary of Research Completed

Aim 1 Goal: Define the ways that cyclin D1 becomes abnormal in cancer cell models and human tumors:

Aim 1A . Determine additional factors that cause abnormal cyclin D1 action

In vitro work has shown that Cav-1 can act as an AR coactivator by enhancing its transciptional activity. However, it is unknown how Cav-1 affects androgen-dependent growth and signaling *in vivo*. To explore this role, a novel mouse model of Cav-1 overexpression was developed with a hormone-insensitive promoter. Cav-1 transgenic (Tg) mice subjected to castration and androgen stimulation display enlarged prostate weights and increased DNA synthesis. Through gene transcript and proteomic profiling, we demonstrate that Cav-1 overexpression favors androgen-regulated responses and enhances processes involved in transcription, cell cycle and protein synthesis. Interestingly, Cav-1 overexpression was associated with an increase in the phosphorylation of AR on serine 210, a post-translational modification linked to its activity in androgen-stimulated conditions.

In addition, these mice exhibited an increase in the phosphorylation of ribosomal S6 protein on serine 235/236 (pS6), a marker of protein synthesis and downstream component of the mTOR pathway. Thus, Cav-1 Tg mice could serve as a novel model for studying AR-regulated pathways involved in prostate growth and proliferation.

Aim 1B. Identify genetic components in cyclin D1 that may cause its abnormal action

D-type cyclins regulate cellular outcomes in part through cyclin-dependent, kinase-independent mechanisms that modify transcription factor action, and recent *in vivo* studies showed that cyclin D1 associates with a large number of transcriptional regulators in cells of the retina and breast. Given the frequency of cyclin D1 alterations in cancer, it is imperative to delineate the molecular mechanisms by which cyclin D1 controls key transcription factor networks in human disease. Prostate cancer was used as a paradigm because this tumor type is reliant at all stages of the disease on androgen receptor (AR) signaling, and cyclin D1 has been shown to negatively modulate AR-dependent expression of prostate-specific antigen (KLK3/PSA).

Strategies were employed to control cyclin D1 expression under conditions of hormone depletion, and the effect of cyclin D1 on subsequent androgen-dependent gene expression was determined using unbiased gene expression profiling. Modulating cyclin D1 conferred widespread effects on androgen signaling and revealed cyclin D1 to be a selective effector of

hormone action. A subset of androgen-induced target genes, known to be directly regulated by AR, was strongly suppressed by cyclin D1. Analyses of AR occupancy at target gene regulatory loci of clinical relevance demonstrated that cyclin D1 limits AR residence after hormone stimulation. Together, these findings reveal a new function for cyclin D1 in controlling hormone-dependent transcriptional outcomes and demonstrate a pervasive role for cyclin D1 in regulating transcription factor dynamics.

Aim 1B

In breast cancer, inactivation of the RB tumor suppressor gene is believed to occur via multiple mechanisms to facilitate tumorigenesis. However, the prognostic and predictive value of RB status in disease-specific clinical outcomes has remained uncertain. We investigated RB pathway deregulation in the context of both ER-positive and ER-negative disease using combined microarray datasets encompassing over 900 breast cancer patient samples. Disease-specific characteristics of RB pathway deregulation were investigated in this dataset by evaluating correlation among pathway genes as well as differential expression across patient tumor populations defined by ER status. Survival analysis among these breast cancer samples demonstrates that the RB-loss signature is associated with poor disease outcome within several independent cohorts.

Within the ER-negative subpopulation, the RB-loss signature is associated with improved response to chemotherapy and longer relapse-free survival. Additionally, while individual genes in the RB target signature closely reproduce its prognostic value, they also serve to predict and monitor response to therapeutic compounds, such as the cytostatic agent PD-0332991. These results indicate that the RB-loss signature expression is associated with poor outcome in breast cancer, but predicts improved response to chemotherapy based on data in ER-negative populations. While the RB-loss signature, as a whole, demonstrates prognostic and predictive utility, a small subset of markers could be sufficient to stratify patients based on RB function and inform the selection of appropriate therapeutic regimens.

Aim 2 Goal: Determine the importance of abnormal cyclin D1 in mouse tumor models:

Aim 2A. Develop new mouse models to mimic abnormal cyclin D1 as seen in human tumors.

During the progress reporting period, two new mouse models were generated. Both models are knock-ins of the cyclin D1 locus. The first resulted in development of a cyclin D1b knock-in animal. Recent evidence from our lab has demonstrated cyclin D1 is replaced in prostate cancer by a splice variant of D1 dubbed cyclin D1b. D1b is markedly impaired in its ability to limit AR signaling, allowing for sustained and durable AR activity in response to ligand. Globally, cyclin D1b has been shown to elicit oncogenic potential in a host of different systems (breast, bladder, esophageal, skin) and is the sole isoform of cyclin D1 expressed in nearly 1/3 of primary prostate tumors. It is thus vital to understand the *in vivo* consequence of cyclin D1b expression, in order to understand how it elicits its oncogenic potential.

As such, development of the cyclin D1b knockin animal will be of broad relevance for a number of disciplines. A schematic of the animal generation screen is provided below as Figure 1.
In the progress reporting period, the construct was injected into ES cells, ES cells were selected and screened, blastocyst injections were performed, chimeric animals were obtained and shown to allow for germline transmission, and heterozygous mice were generated. These are

remarkable accomplishments for a single progress reporting period. While the characterization process has only just begun, expression of cyclin D1b has been verified in both the heterozygous and wild-type animals (Figure 2). We expect in the next reporting period to describe the phenotypes of these animals.

The second animal line generated is a knock-in of a cyclin D1 mutant deficient in transcriptional control (cyclin D1-delta RD). At present, the construct has been generated, ES cells have been screened, and blastocyst injections have been performed. Thus, we anticipate characterization of these animals as part of the next progress report.

Aim 2B. Evaluate treatment strategies and outcomes related to abnormal cyclin D1 in mouse models

The majority of estrogen receptor (ER)-positive breast cancers are treated with endocrine therapy. While this is effective, acquired resistance to therapies targeted against ER is a major clinical challenge. Here, model systems of ER-positive breast cancers with differential susceptibility to endocrine therapy were employed to define common nodes for new therapeutic interventions. These analyses revealed that cell cycle progression is effectively uncoupled from the activity and functional state of ER in these models. In this context, cyclin D1 expression and retinoblastoma tumor suppressor protein (RB) phosphorylation are maintained even with efficient ablation of ER with pure antagonists. These therapy-resistant models recapitulate a key feature of deregulated RB/E2F transcriptional control. Correspondingly, a gene expression signature of RB-dysfunction is associated with luminal B breast cancer, which exhibits a relatively poor response to endocrine therapy.

These collective findings suggest that suppression of cyclin D-supported kinase activity and restoration of RB-mediated transcriptional repression could represent a viable therapeutic option in tumors that fail to respond to hormone-based therapies. Consistent with this hypothesis, a highly selective CDK4/6 inhibitor, PD-0332991, was effective at suppressing the proliferation of all hormone refractory models analyzed. Importantly, PD-0332991 led to a stable cell cycle arrest that was fundamentally distinct from those elicited by ER antagonists, and was capable of inducing aspects of cellular senescence in hormone therapy refractory cell populations. These findings underscore the clinical utility of downstream cytostatic therapies in treating tumors that have experienced failure of endocrine therapy.

Publications Resulting from this Progress:

1) Bryant, KG, Camacho, J, Jasmin, JF, Casimiro, MC, Addya, S, Fortina, P, Wang, C, Balasubramaniam, S, Pestell, RG, Knudsen, KE, Schwarting, R, Lisanti, M, and Mercier, I (2010) Caveolin-1 overexpression enhances androgen dependent growth and proliferation in the mouse prostate. *Int J Biochem Cell Biol*., 2011 May 12. [Epub ahead of print].

2) Thangavel, C, Dean JL, Ertel, A, Knudsen KE, Aldaz, MC, Witkiewicz, AK, Clarke, R, and Knudsen ES (2011) Therapeutically activating RB: reestablishing cell cycle control in endocrine therapy resistant breast cancer. *Endocr Relat Cancer*, 2011 Apr 28;18(3):333-45. Print 2011.

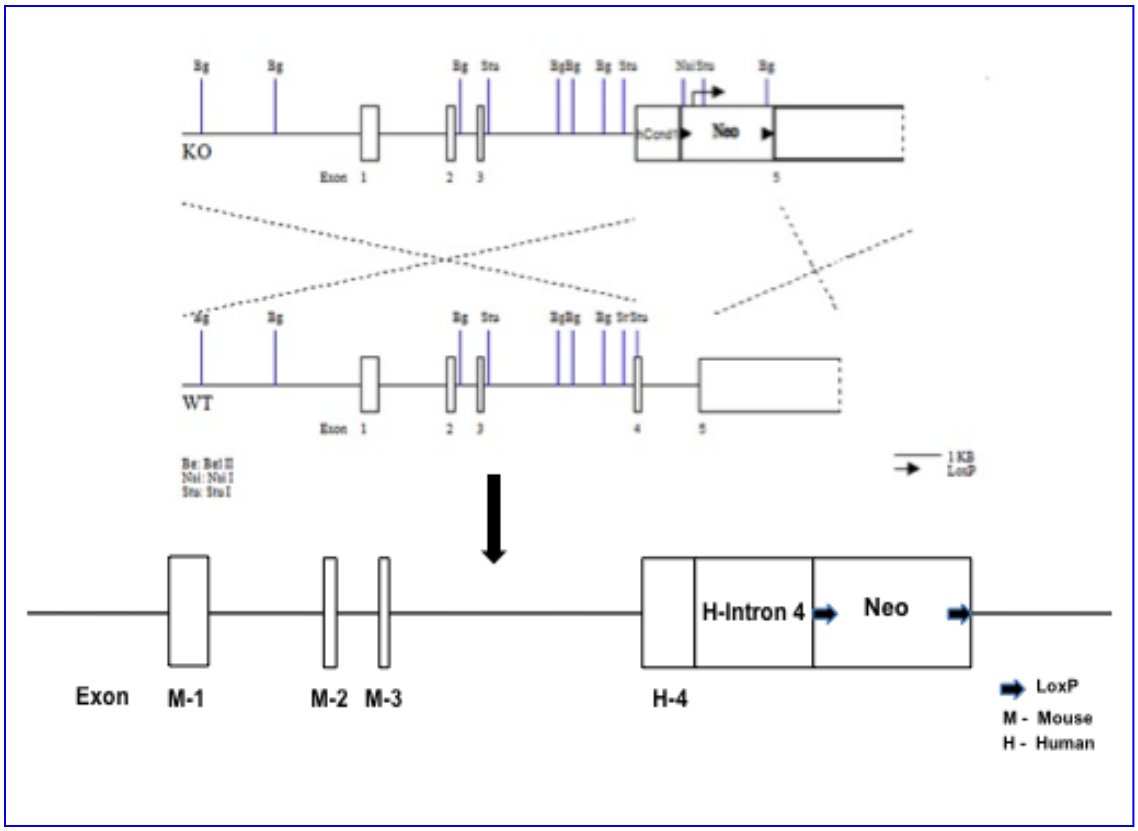

Figure 1: Schematic of the Cyclin D1b knock-in construct. As shown, human exon 4 and ntron 4 was knocked into the murine CCND1 using standard recombination technology. Exon 5 was also removed. The resulting contstruct will allow formation of cyclin D1b but not cyclin D1a.

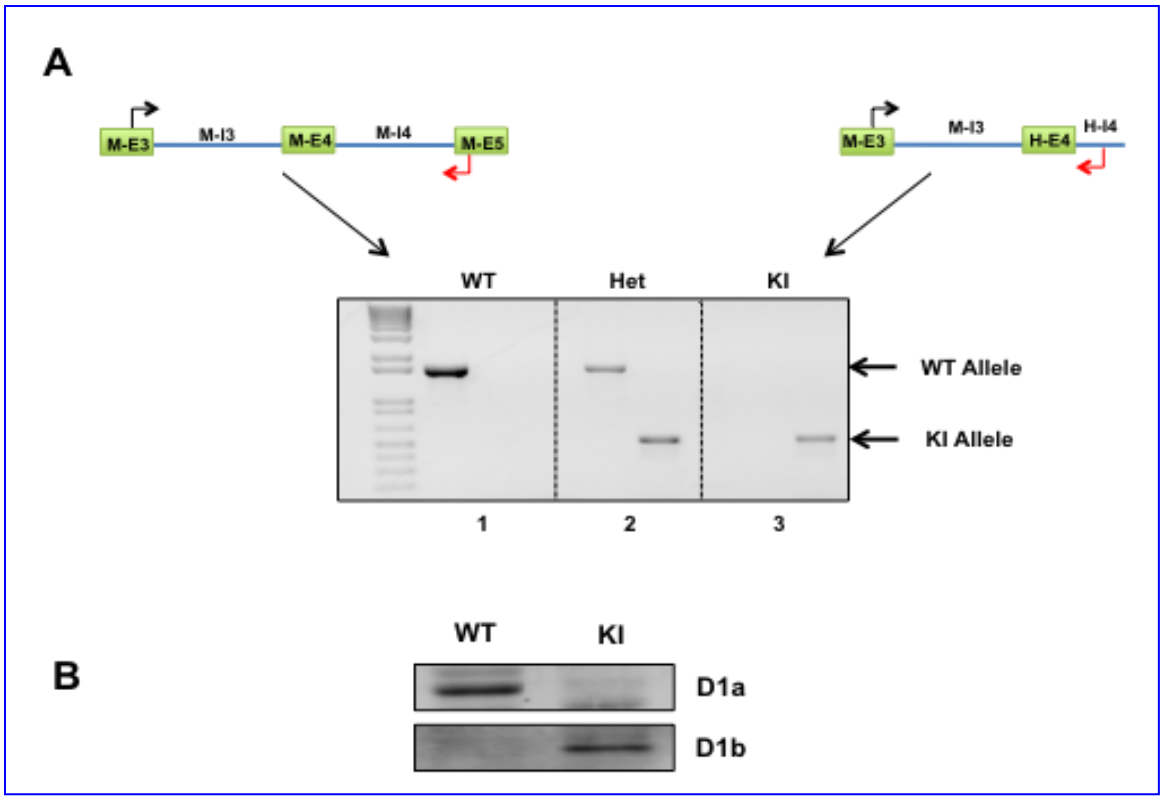

Figure 2. Expression of the cyclin D1b construct. A. As shown, animals of the heterozygous phenotype (Het) or homozygous for the knockin allele (KI) were assessed for cyclin D1a (wt allele) and cyclin D1b (KI allele) expression by qPCR. B. Formation of a stable protein was verified by immunoblot.

Research Project 3: Project Title and Purpose

Novel Therapeutic and Prognostic Markers in Breast Cancer - Breast cancer is a major cause of death in the United States and the western world. Advanced medical technologies and therapeutic strategies are necessary for the successful detection, diagnosis, and treatment of breast cancer. Novel technologies (tissue microarrays (TMA) and automated quantivative bioimaging (AQUA)) will be used to identify new therapeutic and prognostic markers for human breast cancer. This new molecular marker will allow researchers to improve diagnostic accuracy for individual patients, enhancing both the prognostic predictions as well as the prediction of drug responsiveness for a given patient.

Anticipated Duration of Project

1/1/2008 – 12/31/2011

Project Overview

Breast cancer is a serious health threat and a very common cause of death and disability. Certain

abnormalities of the breast, including ductal carcinoma *in situ* (DCIS) commonly lead to invasive cancer. However, not all of these early lesions lead to invasive breast cancer and this is a major source of worry for women with such lesions. Currently, women with these lesions typically receive toxic therapies with potentially serious side-effects although many of the patients may not need or benefit from such added therapy. In this study several promising molecular markers of these early lesions will be examined to help predict which women will go on to develop breast cancer and require such therapy in this early stage.

CAPER is a novel estrogen-receptor (ER) co-activator gene that has not yet been implicated in the pathogenesis of human breast cancer. Unpublished preliminary results directly show that CAPER is upregulated during the transition from pre-malignancy (hyperplasia and DCIS) to invasive ductal carcinoma (IDC).

The two specific aims of the project will be:

1) Determine the Role of CAPER in the Human Breast Cancer Onset and Progression, from Pre-Malignancy and DCIS to Invasive Ductal Carcinoma. The expression of CAPER in human pathological specimens will be examined and its expression will be correlated with other biomarkers (ER, PR, HER2), and patient progression, survival, and recurrence data. The possible use of CAPER as a diagnostic predictive biomarker for breast cancer progression from pre-malignancy will be assessed.

2) Determine the Functional Role of CAPER in Human Breast Cancer, using Xenograft Models. Unpublished preliminary results show that CAPER is highly expressed in malignant breast cancer cell lines, such as MCF7 cells, but fails to be expressed in "normal" immortalized cell lines, such as MCF10A cells. Thus, this research will determine if over-expression of CAPER is sufficient to allow MCF10A cells to form tumors in nude mice. The expression of CAPER siRNA will be used to prevent MCF7 tumor formation in nude mice. Finally, the therapeutic potential of a cell-permeable dominant-negative peptide inhibitor that contains the 44 amino acid ER-interacting domain of CAPER will be assessed.

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

Identifying the majority of DCIS patients whose tumors are likely to remain local is critical to avoid overtreatment and to focus therapy on the patients with true invasive potential. The current classification for DCIS based on nuclear grade, architectural differentiation and presence of necrosis, does not adequately predict the likelihood of recurrence after breast conserving therapy. Previous studies have attempted to identify prognostic molecular markers. Many of the molecules thought to play critical roles in progression of invasive breast cancer, such as estrogen receptors, progesterone receptors, Her-2/neu, p21, Ki-67, p53, bcl-2 have not been shown to be independent prognostic markers for local recurrence or progression of DCIS. Therefore, there is a critical need to identify novel predictors of DCIS progression and potential targets for therapy.

Summary of Research Completed

CAPER is a novel ER co-activator gene that couples transcription with pre-mRNA processing. Interestingly, we recently reported increased expression of CAPER in human breast cancer specimens. We demonstrated that CAPER is undetectable or expressed at relatively low levels in normal breast tissue and assumes a cytoplasmic distribution. In contrast, CAPER is expressed at higher levels in ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC) specimens, where it assumes a predominantly nuclear distribution. However, the functional role of CAPER in breast cancer progression remains to be determined. Here, we used gene silencing techniques to reduce the expression of CAPER in the human breast cancer cell line MCF-7. The growth of MCF-7 cells was then assessed by measuring cell proliferation (cell counts and BrdU incorporation).

Interestingly, knockdown of CAPER expression significantly reduced the growth of MCF-7 cells in vitro. MCF-7 cells (5 x 106) infected with either control or CAPER shRNAs were also injected in mammary fat pads of eight-week old female nude mice. Six week after the injection, mice were euthanized and tumors were harvested, weighed and measured. Importantly, nude mice injected with MCF-7 cells harboring CAPER shRNAs developed smaller tumors than mice injected with MCF-7 cells harboring control shRNAs (See Figures 1-3). Mechanistically, tumors derived from the mice injected with MCF-7 cells harboring CAPER shRNAs display reduced expression of MCM7, cyclin D1 and PCNA. In summary, knockdown of CAPER expression markedly reduces the growth of human breast cancer cells in both in vitro and in vivo settings. Mechanistically, knockdown of CAPER is also associated with reduced expression of proliferation markers.

Related to these studies, we have also recently published the following papers:

Molecular profiling of a lethal tumor microenvironment, as defined by stromal caveolin-1 status in breast cancers. Witkiewicz AK, Kline J, Queenan M, Brody JR, Tsirigos A, Bilal E, Pavlides S, Ertel A, Sotgia F, Lisanti MP. Cell Cycle. 2011 Jun 1;10(11):1794-809. Epub 2011 Jun 1.

Evidence for a stromal-epithelial "lactate shuttle" in human tumors: MCT4 is a marker of oxidative stress in cancer-associated fibroblasts. Whitaker-Menezes D, Martinez-Outschoorn UE, Lin Z, Ertel A, Flomenberg N, Witkiewicz AK, Birbe RC, Howell A, Pavlides S, Gandara

R, Pestell RG, Sotgia F, Philp NJ, Lisanti MP. Cell Cycle. 2011 Jun 1;10(11):1772-83. Epub 2011 Jun 1.

Cytokine production and inflammation drive autophagy in the tumor microenvironment: Role of stromal caveolin-1 as a key regulator. Martinez-Outschoorn UE, Whitaker-Menezes D, Lin Z, Flomenberg N, Howell A, Pestell RG, Lisanti MP, Sotgia F. Cell Cycle. 2011 Jun 1;10(11):1784-93. Epub 2011 Jun 1.

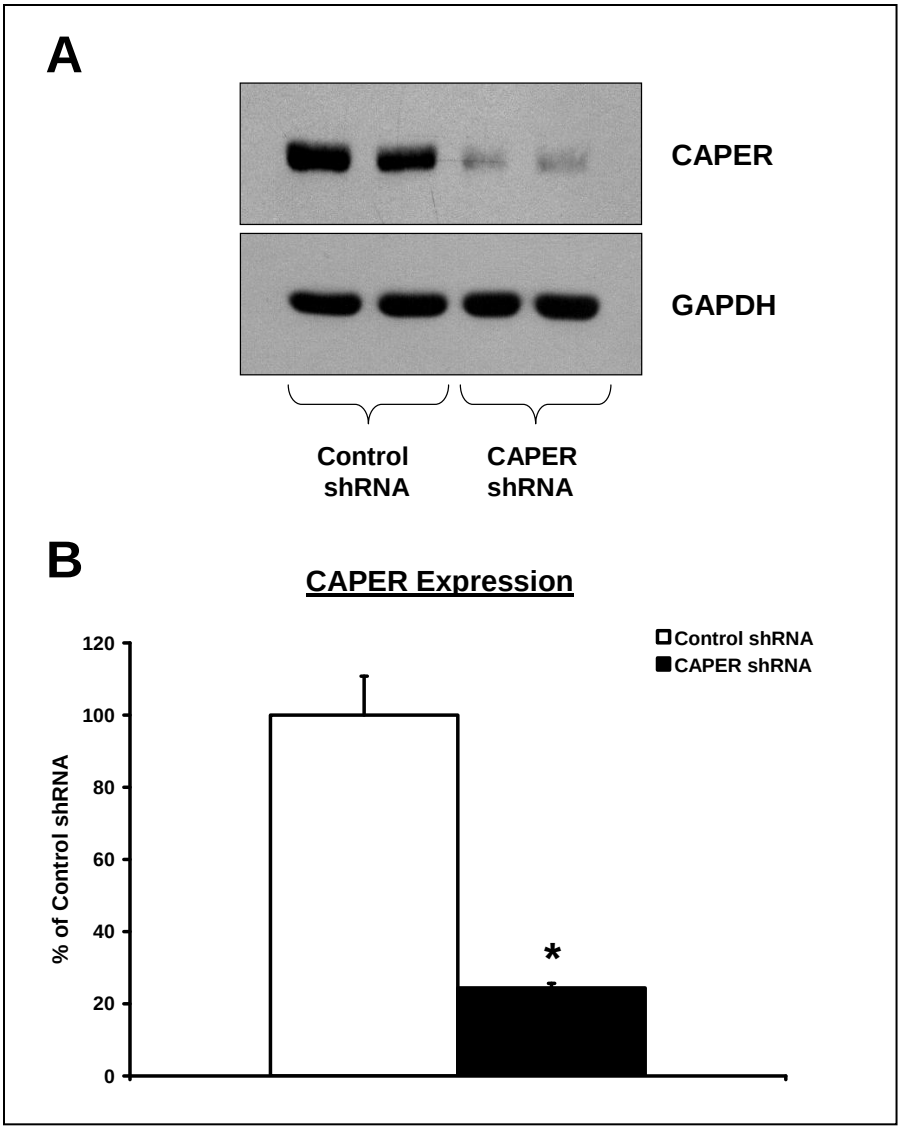

FIGURE 1. (**A**) Western blot analyses show that MCF-7 cells infected with CAPER shRNA lentiviral particles display reduced CAPER protein levels as compared to MCF-7 cells infected with control shRNA lentiviral particles. Immunoblot analysis with GAPDH is shown as a control for equal loading. (**B**) CAPER expression was quantified with the NIH ImageJ software and expressed as a percentage of the control group (*$p<0.05$ vs Control shRNA, n=4 for each group).

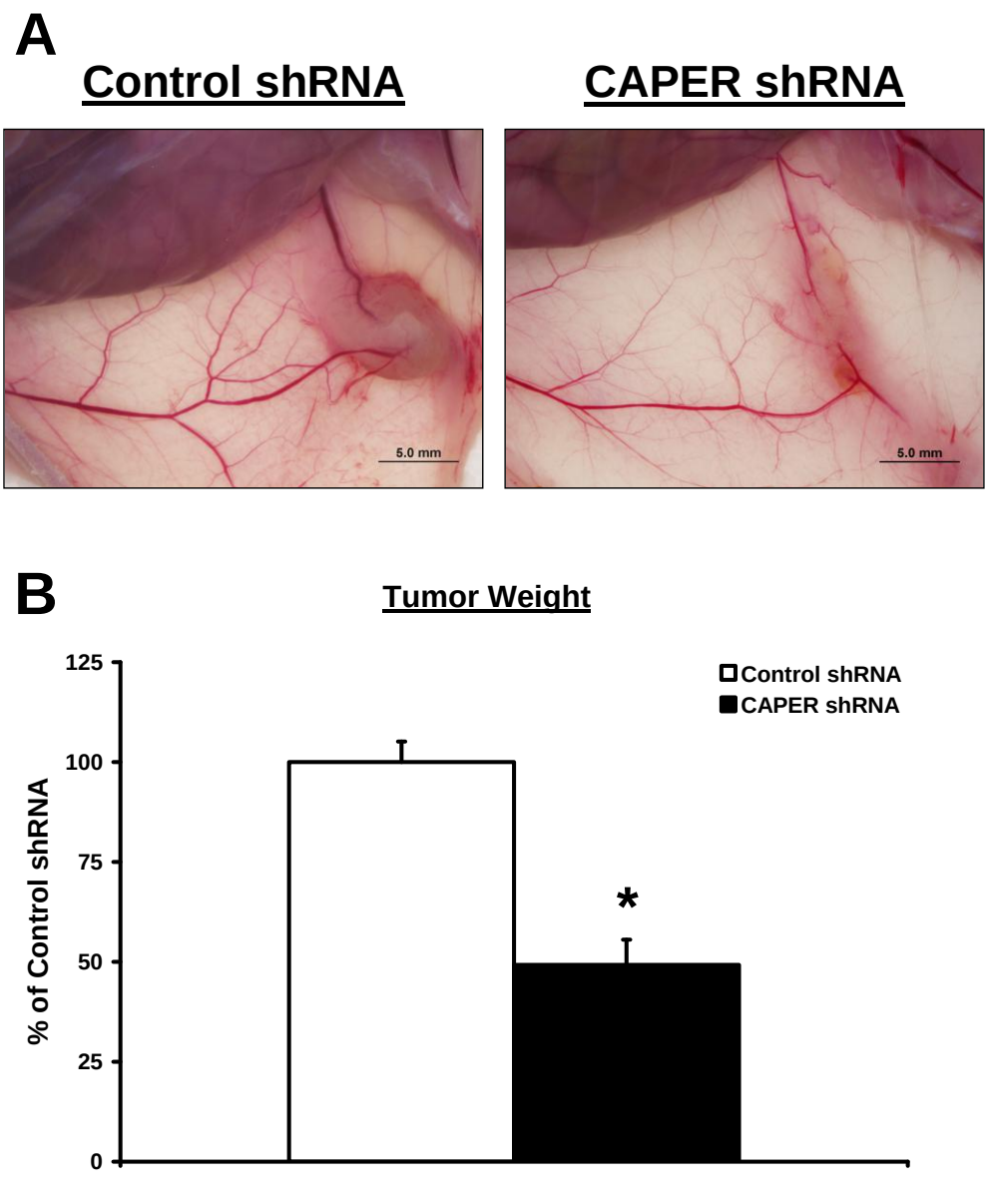

FIGURE 2. (**A**) Female nude mice injected with MCF-7 cells harboring CAPER shRNAs show smaller tumors than those injected with MCF-7 cells harboring control shRNAs. Accordingly, tumors derived from mice injected with MCF-7 cells harboring CAPER shRNAs display reduced weight (**B**) (*$p<0.001$ vs control shRNA, n=10-22 for each group).

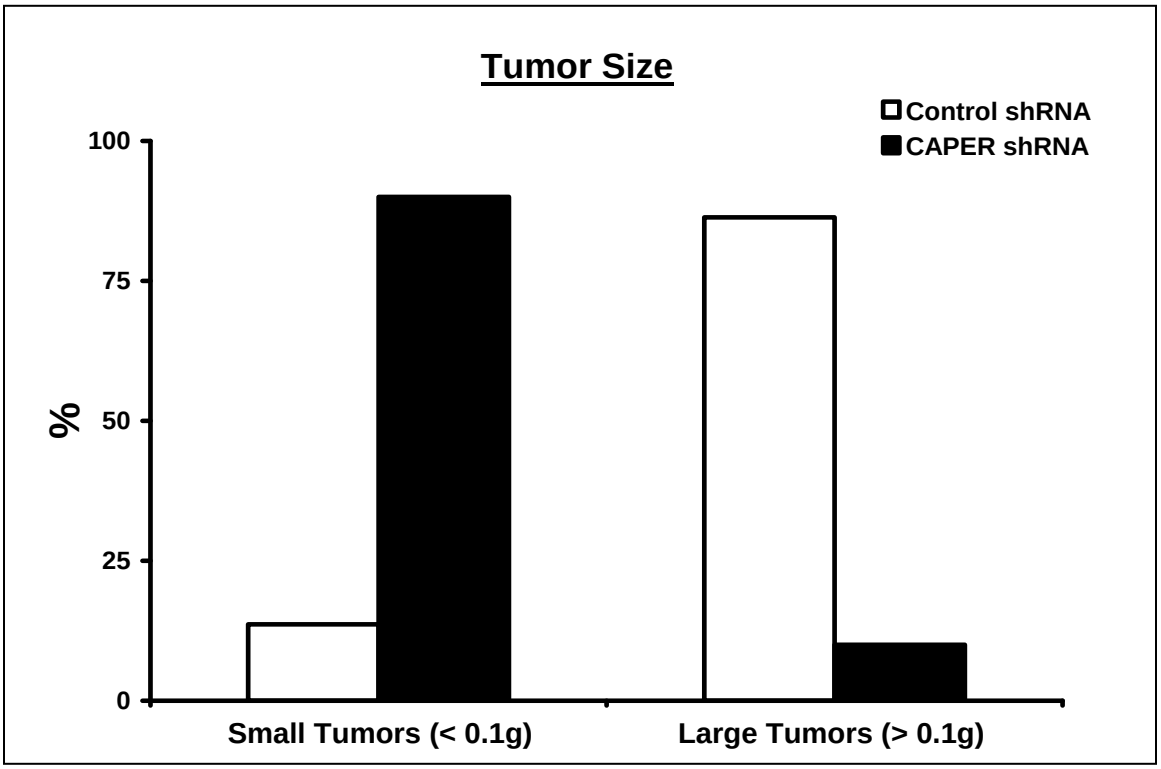

FIGURE 3. When tumors were stratified as either small (<0.1 g) or large (>0.1 g) tumors, only 10% of mice injected with breast cancer cells harboring CAPER shRNAs showed large tumors as compared to 86.4% of mice injected with cells harboring control shRNAs (n=10 to 22 for each group).

Research Infrastructure Project 4: Project Title and Purpose

Core Equipment and Renovations for Pathology Research–A Research Infrastructure Facilities Renovation Project - The purpose of this project is to establish a shared equipment facility containing state-of-the-art analytical equipment to facilitate the research objectives of the Department of Pathology, Anatomy and Cell Biology of Jefferson Medical College.

Anticipated Duration of Project

1/1/2008 - 12/31/2011

Project Overview

The major uses of the facility are expected to enable enhanced analysis of biological samples by light-based fluorescence microscopy and electron microscopy. The facility will be located in

Rooms 552/552A and 528/529 in Jefferson Alumni Hall. The space is net ~1,818 square feet.

The specific aim is to implement an infrastructure project for the support of shared electron microscopy resources. The specific project is to have digital camera and processing equipment in an appropriately outfitted environment for temperature, humidity, electrical interference and dust control.

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Expected Research Outcomes and Benefits

The indication of success of this project will come from publications of original research that have been facilitated by use of the equipment requested. The long-term success will come from gaining funding of new grant proposals that emanate from the work done.

Summary of Research Completed

The original scope of work identified specific rooms (552/552A and 528/529) that were, at the time of the award, the focus of the Pathology Imaging program. Pathology has traditionally performed its scientific activity on floors 2 and 5. The Pathology imaging program does not just involve the previously identified rooms on the 5th floor but also was housed on the 2nd floor.

However, in the past 3 years, and after the CURE grant award from the Commonwealth in 2008, TJU has performed a strategic planning process to reevaluate the entire building and its associated programming. It was determined that although the building was 42 years old, with improvements in infrastructure and the renovation of general lab and core support space, the building would provide 40 more years of service. TJU submitted and was awarded an ARRA infrastructure grant (NIH/NCRR notice of award January 2010) and a grant from the state (RACP awarded fiscal year 2011) which will allow our institution the ability to begin building state of the art facilities for several programs including Pathology. Most of the space Pathology occupied on the 2nd floor was slated to be demolished and renovated. The 2nd floor imaging program- specifically imaging equipment not capable of being housed on the 5th floor was housed between lab suites 259-261 which is in the renovation zone.

It was determined, based on the master space utilization strategic plan for the building, that further investment on the 5th floor would be contrary to the final program location once all renovations are completed and would be an inefficient use of the funds available (CURE, RACP and NIH/NCRR ARRA and substantial institutional capital funds). An investment on the 2nd floor would dovetail with the renovation project and leverage all funding sources towards a unified imaging program. The current use of funds enhances, not contradicts, the aims of the intended result. Below are the expenses associated with that activity for the past reporting period.

- Imaging equipment purchased in prior project periods on this grant has been permanently housed in a newly renovated space (Room 238 Jefferson Alumni Hall). This additional 470+ square foot space (under the control and management of the Department of Pathology) will leverage the Commonwealth's investment in equipment to more efficiently and effectively complete the aims of the project. The entire project cost totaled over $165,000, of which the institution committed over $130,000 and the balance of the project was supported by the grant in the amount of $34,672.

- To complete the imaging center, it was necessary to support the purchase of one final piece of equipment. The department purchased from Carl Zeiss Microimaging a Confocal Laser Scanning Microscope. This state-of-the-art laser microscopy system with accessories provides technology beyond the limits of traditional confocal systems. The total cost was $847,380 of which the grant support totaled only ~18% or $154,105. The significant balance was provided by institutional funding.