Thomas Jefferson University

Annual Progress Report: 2007 Formula Grant

Reporting Period

July 1, 2009 – June 30, 2010

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.

Principal Investigator

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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 are focused specifically on the following two objectives: 1) Determining additional approaches to treat tamoxifen-resistant cancers. 2) Exploring the action of the RB-pathway as a central determinant of molecular targeted therapeutics.

Current breast cancer treatment is based on the status of a limited number of molecular markers. Particularly, the status of the estrogen receptor (ER) is used to direct treatment of disease with endocrine therapies that target the critical dependence of such breast cancers on estrogenic signaling. In this context, only those tumors which are ER-positive will respond to such hormonal interventions and a combination of aromatase inhibitors which attenuate estrogen synthesis (e.g. Letrazole), selective estrogen receptor modulator (e.g. Tamoxifen), and specific estrogen receptor antagonists (e.g. Fulvestrant), which are deployed in distinct clinical settings. Importantly, ER-positive breast cancer constitutes approximately 70% of cases, and millions of such tumors have been treated with endocrine therapy.

By all measures, estrogen antagonists are effective in ER-positive breast cancer, as such tumors are dependent on estrogen signalling for proliferation and survival. Thus, antagonizing ER signalling leads to cell cycle arrest and reduced viability. Substantial preclinical study has demonstrated that cell cycle regulatory control is a key mechanism through which such agents act to prevent tumor growth. Specifically, the withdrawal of estrogen (mimicking aromatase inhibitors) or use of estrogen antagonists (e.g. Fulvestrant or Tamoxifen), results in an arrest in the G1 phase of the cell cycle. In this context, reduced ER signalling leads to the attenuation of CDK/cyclin complexes at multiple levels. Most dramatically, cyclin D1 is a known and direct transcriptional target of the ER signalling network. Furthermore, culmination of the many ER-mediated downstream mechanisms coalescence in the control of net CDK activity. Such inhibition of CDK activity results in the maintenance of the retinoblastoma tumor suppressor protein(RB) in a hypophosphorylated and active state. RB serves to repress E2F-regulated genes (e.g. Cyclin A) and inhibits progression through S-phase and G2/M. In spite of the potent anti-proliferative activity of the current therapeutic strategies which ultimately mediate cell cycle progression through attenuation of the cell cycle machinery, acquired resistance is a critical clinical problem even with highly effective estrogen receptor antagonists.

To understand the basis of progression to therapeutic resistance, multiple preclinical and correlative clinical studies have been performed. Functional analyses have suggested that deregulation of a multitude of signal transduction cascades can contribute to acquired resistance to endocrine therapy. Specifically, deregulated mitogenic signalling such as ErbB2, Grb10, AKT, CDK10 have all been shown to contribute to such therapeutic failure in preclinical models. Additionally, deregulation of specific cell cycle components (e.g. p27Kip1 or RB) can directly compromise the efficacy of estrogen receptor attenuation. Such findings are supported by wide-ranging analyses of breast tumors by single markers or gene expression profiling. Due to the wide-ranging and heterogeneous pathways deregulated in progression of ER-positive breast cancer, the second-line therapies deployed are generally conventional cytotoxic chemotherapies that exhibit varied success. These clinical realities underscore the importance of defining common nodes of therapeutic failure in ER-positive breast cancer, and thereby illustrate the importance of developing new and targeted approaches for the treatment of ER-positive breast cancer that progresses in the presence of endocrine therapy.

METHODS

The transcriptional signature of RB loss is associated with luminal B breast cancers: As RB can be inactivated via multiple mechanisms, gene expression profiling is particularly useful in

understanding the functionality of RB in clinical specimens. Using an RB-loss expression signature that was developed previously from preclinical models, the relationship of RB function to defined subtypes of ER-positive breast cancer was determined (Fig. 1). In a cohort of breast cancer cases, the RB-signature was a strong classifier for defining luminal B breast cancer cases (Fig. 1A). Specifically, luminal B breast cancer exhibited an elevated expression of the signature indicative of RB loss of function (Fig. 1B). Importantly, luminal B breast cancer exhibit a poor durable response to endocrine therapy, and this finding is paralleled by the impact of the RB-signature in this cohort (Fig. 1C). In spite of these provocative findings related to RB function, there was little evidence for loss of the *Rb1* transcript in ER-negative breast cancer (not shown). Together, these findings suggest that in ER-positive breast cancers that fail endocrine therapy the function of RB is being perturbed, although the gene remains present.

RB activation is important for the full response to estrogen antagonists: To decipher the functional involvement of RB-mediated transcriptional control the estrogen dependent MCF-7 model was employed. Endocrine therapy directed against such ER-positive breast cancer models is known to induce profound cell cycle inhibition. Consistent with this concept, treatment of ER-positive MCF-7 cells with the pure estrogen receptor antagonist ICI 182,780 in charcoal dextran treated (CDT) media (lacking steroid hormones) resulted in a profound cell cycle inhibition (Fig. 2A). As shown by flow cytometry, while >40% of cells were actively progressing through the cell cycle as measured by BrdU incorporation, culture in CDT resulted in a significant reduction in proliferation that was further augmented by the inclusion of the estrogen antagonist ICI 182,780 (Fig. 2B). This hormonal therapy results in the degradation of the estrogen receptor alpha (ER alpha) and down-regulation of cyclin D1 (Fig. 2C). In concert, there is an accumulation of $p27^{Kip1}$ and dephosphorylation of the RB tumor suppressor protein, which is accompanied by down-regulation of the RB/E2F regulated gene cyclin A. These effects on transcription are associated with the specific recruitment of RB to target gene promoters by chromatin immunoprecipitation only in the context of full cell cycle inhibition (Fig. 2D). This event is directly associated with the assembly of a Sin3b containing repressor complex at these promoters (Fig. 2E). Using shRNA approaches, RB protein levels were efficiently knocked down in MCF7 cells (not shown) In this context, RB-deficiency precluded the efficient assembly of repressor complexes, and limited the cell cycle arrest induced by estrogen withdrawal and estrogen antagonists (not shown). *Together these findings suggest that RB transcriptional control may be a key node in the control of therapeutic response to ER antagonists.*

Models of acquired anti-estrogen resistance exhibit a lack of Rb-mediated transcriptional repression. The evolution of resistance to endocrine therapies is a major clinical problem, and an unbiased means to interrogate the underlying basis of such resistance is to use models of spontaneous resistance. For these studies, we focused on MCF7 breast cancer cell models that were defined to proliferate in the presence of continued exposure to endocrine therapies. The ultimate acquisition of therapeutic resistance has been attributed to selection of resistant sub-lines (i.e., LCC9, MCF7/FBS/Tam^R and MCF7/CSS/Tam^R) or the presence of a sub-population of cells that are intrinsically resistant to endocrine therapy (F100-16 and F40-7) as depicted in Fig. 3A. Using these diverse cell lines, their response to endocrine therapies was initially characterized. In LCC9 cells, treatment with ICI 182,780 results in significant attenuation of ER-alpha; however, cyclin D1 expression and RB phosphorylation are effectively maintained

(Fig. 3B). As such, there is a failure to engage RB at target promoters (Fig. 3C). In keeping with these molecular events, LCC9 cells are resistant to the growth-inhibitory effects of CDT and CDT/ICI, as determined by BrdU incorporation (Fig. 3D). Similar results were observed with additional ICI-resistant cell lines (not shown). In keeping with the general nature of these findings, similar models resistant to tamoxifen demonstrated a failure to activate RB-mediated transcriptional repression (Fig 3E). Again, analogous findings were observed in an additional tamoxifen resistant cell line (not shown). *These findings suggest that loss of RB-mediated transcriptional control, even with the RB gene intact, could be a particularly important facet of ER-positive breast cancer biology.*

RB activation as a means to treat endocrine therapy resistant disease: Based on the findings of the preclinical and *in silico* analyses, we postulated that activation of RB may represent a viable means to re-instate cell cycle inhibition down-stream from endocrine therapy. To interrogate this possibility, we used the CDK4/6-specific inhibitor PD-0332991 to treat Fulvestrant-resistant LCC9 populations. At a 500nM concentration, PD-0332991 resulted in the effective dephosphorylation of RB protein and the attenuation of the downstream target genes cyclin A and RNRII (Fig. 4A). In keeping with the restoration of RB function, we observed effective recruitment of RB protein to promoter elements (Cyclin A) in the LCC9 model (Fig. 4B). These molecular events were associated with a profound inhibition of cell cycle progression as measured by BrdU incorporation (Fig. 4C). Importantly, this response was observed across all of the models employed (Fig. 4D-4F). *Thus, CDK4/6 inhibition could represent a means to intercede in tumors that have failed endocrine therapy through deregulated RB phosphorylation and maintenance of E2F activity.*

Therapeutic CDK4/6 inhibition can promote cellular senescence of breast cancer cells: As there is significant concern that acquired resistance to cytostatic therapies can develop (similar to that observed in endocrine therapies), we interrogated the impact of prolonged exposure to ICI 182,780 and PD-0332991, and their ultimate influence on the proliferation of MCF7 cells. These data demonstrated that both PD-0332991 and ICI 182,780 treatment are highly effective at mediating the suppression of cell growth in naïve MCF7 cells (Fig. 5A). However, prolonged PD-0332991 exposure was specifically associated with the appearance of flat large cells that are associated with induced senescence (Fig. 5B). Interestingly, while PD-0332991 treatment resulted in a significant fraction of senescent cells (in agreement with other studies), endocrine therapy did not mediate this response (Fig. 5C and 5D). Thus, the induction of a senescence phenotype is specific to CDK4/6 inhibition and not a general hallmark of cytostatic agents. Importantly, similar experiments carried out in the LCC9 model revealed that while these cells can proliferate in the presence of endocrine therapies (i.e., CDT or CDT/ICI 182,780), PD-0332991 remained effective at inducing durable cell cycle arrest and mediating the induction of senescence (Fig. 5E-5G). *Together, these studies underscore that distinct cytostatic therapies differ in their capacity to induce senescence and mediate therapeutic response. Additionally, while events upstream of the Rb/E2F axis are capable of mediating bypass traditional endocrine-based therapies, restoration of normal Rb/E2F function is capable of mitigating the associated cell cycle effects.*

CONCLUSION

These combined studies support the concept that inhibition of CDK4/6 activity by agents such as PD-0332991, could represent a viable treatment option for treating those patients that fail endocrine therapies. These preclinical models are predicted to enable direct the use of the drug more effectively in a clinical setting.

Figure 1

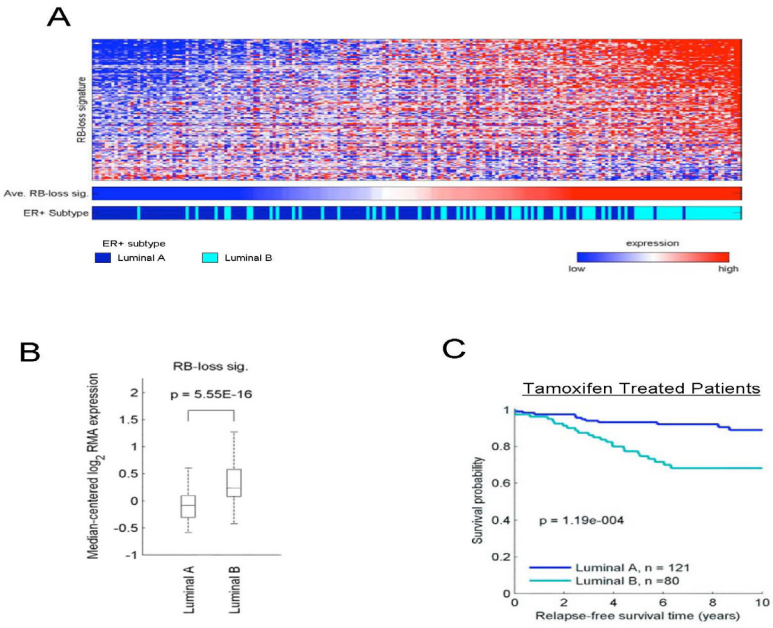
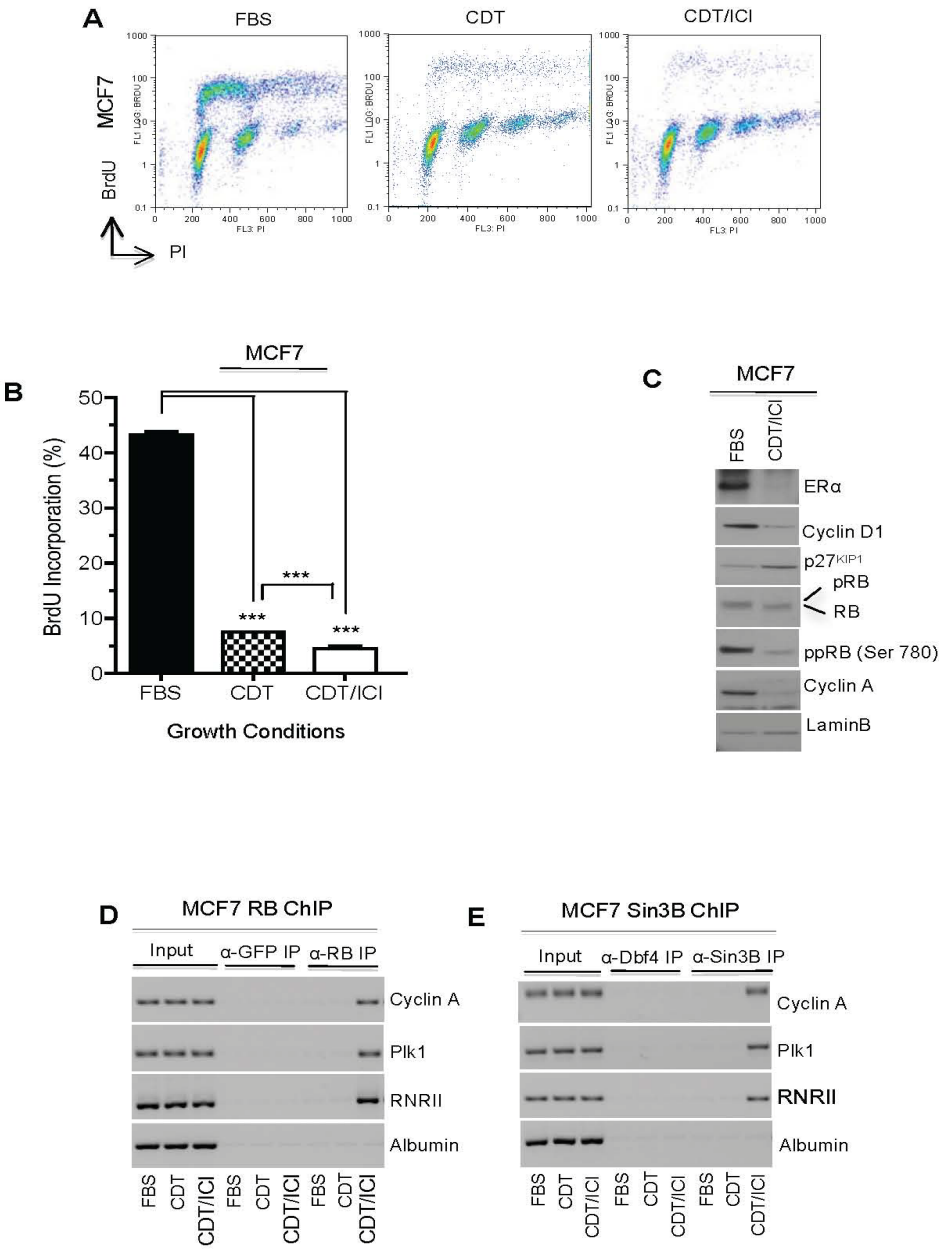


Figure 2


A
FBS
CDT
CDT/ICI
MCF7
BrdU
PI
B
MCF7
BrdU Incorporation (%)
FBS
CDT
CDT/ICI
Growth Conditions
C
MCF7
FBS
CDT/ICI
ERα
Cyclin D1
p27KIP1
pRB
RB
ppRB (Ser 780)
Cyclin A
LaminB
D
MCF7 RB ChIP
Input
α-GFP IP
α-RB IP
Cyclin A
Plk1
RNRII
Albumin
E
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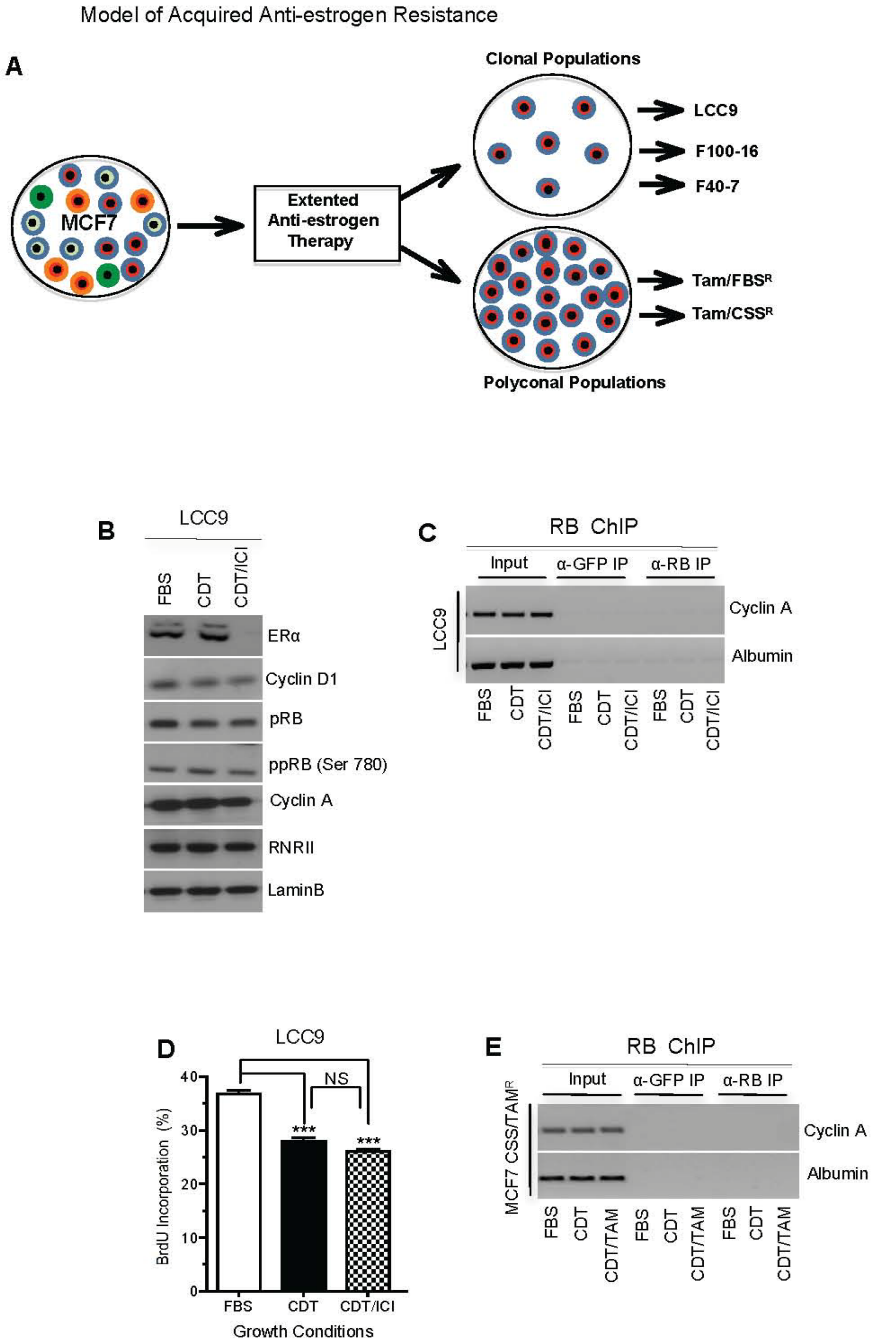
Input
α-Dbf4 IP
α-Sin3B IP
Cyclin A
Plk1
RNRII
Albumin

Figure 3


Model of Acquired Anti-estrogen Resistance
A
Clonal Populations
MCF7
Extented Anti-estrogen Therapy
LCC9
F100-16
F40-7
Tam/FBS^R
Tam/CSS^R
Polyconal Populations
B
LCC9
FBS
CDT
CDT/ICI
ERα
Cyclin D1
pRB
ppRB (Ser 780)
Cyclin A
RNRII
LaminB
C
RB ChIP
Input
α-GFP IP
α-RB IP
LCC9
Cyclin A
Albumin
FBS
CDT
CDT/ICI
D
LCC9
NS
***
BrdU Incorporation (%)
0
10
20
30
40
FBS
CDT
CDT/ICI
Growth Conditions
E
RB ChIP
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α-RB IP
MCF7 CSS/TAM^R
Cyclin A
Albumin
FBS
CDT
CDT/TAM

Figure 4

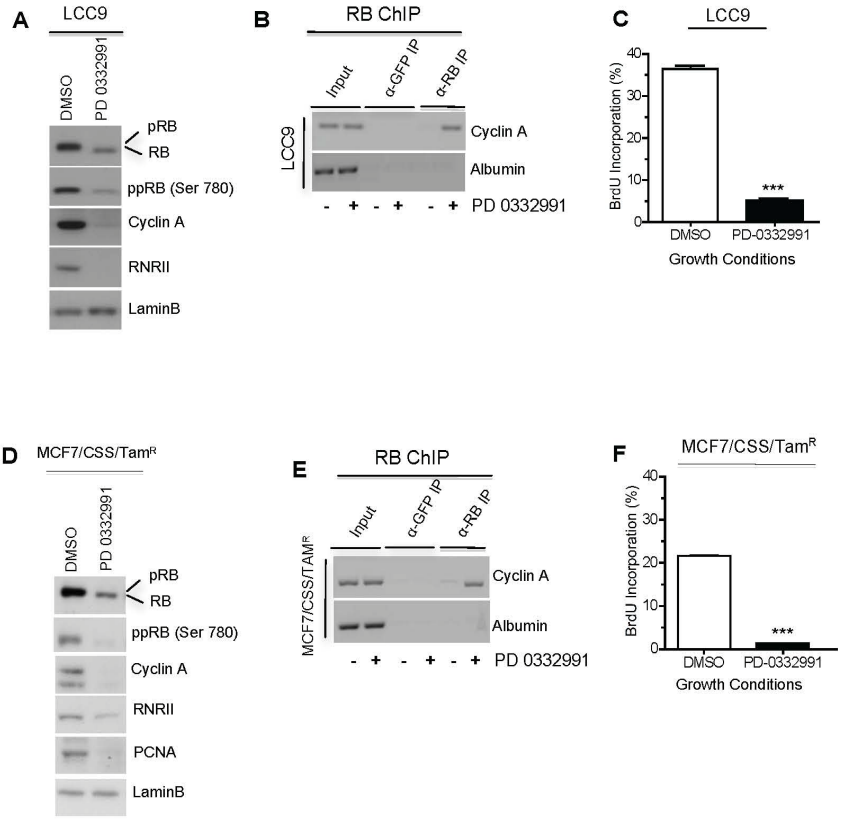
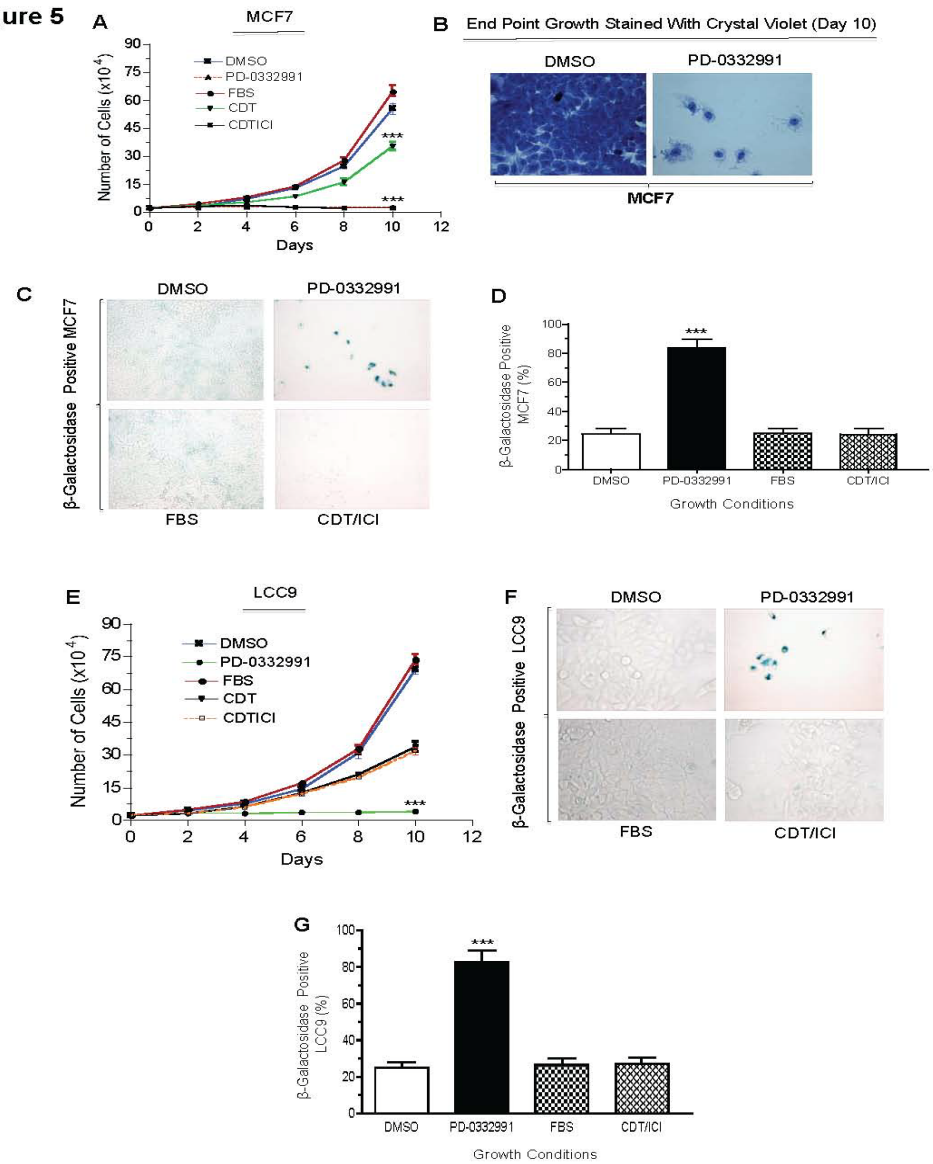

A
LCC9
DMSO
PD 0332991
pRB
RB
ppRB (Ser 780)
Cyclin A
RNRII
LaminB
B
RB ChIP
Input
α-GFP IP
α-RB IP
LCC9
Cyclin A
Albumin
- + - + - + PD 0332991
C
LCC9
BrdU Incorporation (%)
40
30
20
10
0
***
DMSO
PD-0332991
Growth Conditions
D
MCF7/CSS/Tam^R
DMSO
PD 0332991
pRB
RB
ppRB (Ser 780)
Cyclin A
RNRII
PCNA
LaminB
E
RB ChIP
Input
α-GFP IP
α-RB IP
MCF7/CSS/TAM^R
Cyclin A
Albumin
- + - + - + PD 0332991
F
MCF7/CSS/Tam^R
BrdU Incorporation (%)
40
30
20
10
0
***
DMSO
PD-0332991
Growth Conditions

Figure 5


A
MCF7
Number of Cells (x10^4)
DMSO
PD-0332991
FBS
CDT
CDTICI
***
Days
B
End Point Growth Stained With Crystal Violet (Day 10)
DMSO
PD-0332991
MCF7
C
DMSO
PD-0332991
β-Galactosidase Positive MCF7
FBS
CDT/ICI
D
β-Galactosidase Positive MCF7 (%)
***
DMSO
PD-0332991
FBS
CDT/ICI
Growth Conditions
E
LCC9
Number of Cells (x10^4)
DMSO
PD-0332991
FBS
CDT
CDTICI
***
Days
F
DMSO
PD-0332991
β-Galactosidase Positive LCC9
FBS
CDT/ICI
G
β-Galactosidase Positive LCC9 (%)
***
DMSO
PD-0332991
FBS
CDT/ICI
Growth Conditions

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 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

The goal of this grant is to assess cyclin D1 action in cancer cells. As planned, there are two aims for the proposal. The first aim is to determine factors that cause abnormal cyclin D1 action, and the second assess the importance of abnormal cyclin D1 in relevant cancer models. From these studies, long-term goals are to evaluate treatment strategies and outcomes related to abnormal cyclin D1 activity in this tumor type. In the last 12 months of funding, we have made significant progress toward these the goals of Aims 1A and 1B, and just published these data in multiple manuscripts. In addition, we have published data associated with Aim 2A. These accomplishments are described below:

<u>Aim 1: Define the ways that cyclin D1 becomes abnormal in cancer cell models and human tumors:</u>

The cyclin D1b oncogene arises from alternative splicing of the CCND1 transcript, and harbors markedly enhanced oncogenic functions not shared by full-length cyclin D1 (cyclin D1a). Recent studies showed that cyclin D1b is selectively induced in a subset of tissues as a function of tumorigenesis; however, the underlying mechanism(s) that control tumor-specific cyclin D1b induction remain unsolved. Here, we identify the RNA-binding protein ASF/SF2 as a critical, allele-specific, disease-relevant effector of cyclin D1b production. Initially, it was observed that SF2 associates with cyclin D1b mRNA (transcript-b) in minigene analyses and with endogenous

transcript in prostate cancer (PCa) cells. SF2 association was altered by the CCND1 G/A870 polymorphism, which resides in the splice donor site controlling transcript-b production.

This finding was significant, as the A870 allele promotes cyclin D1b in benign prostate tissue, but in primary PCa, cyclin D1b production is independent of A870 status. Data herein provide a basis for this disparity, as tumor-associated induction of SF2 predominantly results in binding to and accumulation of G870-derived transcript-b. Finally, the relevance of SF2 function was established, as SF2 strongly correlated with cyclin D1b (but not cyclin D1a) in human PCa. Together, these studies identify a novel mechanism by which cyclin D1b is induced in cancer, and reveal significant evidence of a factor that cooperates with a risk-associated polymorphism to alter cyclin D1 isoform production. Identification of SF2 as a disease-relevant effector of cyclin D1b provides a basis for future studies designed to suppress the oncogenic alternative splicing event. (c)2010 AACR.

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

Human cyclin D1 is expressed as two isoforms derived by alternate RNA splicing, termed D1a and D1b, which differ for the inclusion of intron 4 in the D1b mRNA. Both isoforms are frequently upregulated in human cancers, but cyclin D1b displays relatively higher oncogenic potential. The splicing factors that regulate alternative splicing of cyclin D1b remain unknown despite the likelihood that they contribute to cyclin D1 oncogenicity. In this study, we report that Sam68, an RNA-binding protein frequently overexpressed in prostate cancer cells, enhances splicing of cyclin D1b and supports its expression in prostate cancer cells. Chromatin immunoprecipitation and RNA coimmunoprecipitation experiments showed that Sam68 is recruited to the human CCND1 gene encoding cyclin D1 and that it binds to cyclin D1 mRNA.

Transient overexpression and RNAi knockdown experiments indicated that Sam68 acts to enhance endogenous expression of cyclin D1b. Minigene reporter assays showed that Sam68 directly affected alternative splicing of CCND1 message, with a preference for the A870 allele that is known to favor cyclin D1b splicing. Sam68 interacted with the proximal region of intron 4, and its binding correlated inversely with recruitment of the spliceosomal component U1-70K. Sam68-mediated splicing was modulated by signal transduction pathways that elicit phosphorylation of Sam68 and regulate its affinity for CCND1 intron 4. Notably, Sam68 expression positively correlates with levels of cyclin D1b, but not D1a, in human prostate carcinomas. Our results identify Sam68 as the first splicing factor to affect CCND1 alternative splicing in prostate cancer cells, and suggest that increased levels of Sam68 may stimulate cyclin D1b expression in human prostate cancers.

Alternative CCND1 splicing results in cyclin D1b, which has specialized, protumorigenic functions in prostate not shared by the cyclin D1a (full length) isoform. Here, the frequency, tumor relevance, and mechanisms controlling cyclin D1b were challenged. EXPERIMENTAL DESIGN: First, relative expression of both cyclin D1 isoforms was determined in prostate adenocarcinomas. Second, relevance of the androgen axis was determined. Third, minigenes were created to interrogate the role of the G/A870 polymorphism (within the splice site), and findings were validated in primary tissue. Fourth, the effect of G/A870 on cancer risk was assessed in two large case-control studies. RESULTS: Cyclin D1b is induced in tumors, and a

significant subset expressed this isoform in the absence of detectable cyclin D1a. Accordingly, the isoforms showed noncorrelated expression patterns, and hormone status did not alter splicing.

Whereas G/A870 was not independently predictive of cancer risk, A870 predisposed for transcript-b production in cells and in normal prostate. The influence of A870 on overall transcript-b levels was relieved in tumors, indicating that aberrations in tumorigenesis likely alter the influence of the polymorphism. CONCLUSIONS: These studies reveal that cyclin D1b is specifically elevated in prostate tumorigenesis. Cyclin D1b expression patterns are distinct from that observed with cyclin D1a. The A870 allele predisposes for transcript-b production in a context-specific manner. Although A870 does not independently predict cancer risk, tumor cells can bypass the influence of the polymorphism. These findings have major implications for the analyses of D-cyclin function in the prostate and provide the foundation for future studies directed at identifying potential modifiers of the G/A870 polymorphism.

Aim 2 : 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.
* As part of this subaim, studies to generate new mouse models are currently underway. We have generated animals with a knock-in of cyclin D1b, and are currently initiating the characterization process.

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

Regulation of the androgen receptor (AR) is critical to prostate cancer (PCa) development; therefore, AR is the first line therapeutic target for disseminated tumors. Cell cycle-dependent accumulation of cyclin D1 negatively modulates the transcriptional regulation of AR through discrete, CDK4-independent mechanisms. The transcriptional corepressor function of cyclin D1 resides within a defined motif termed repressor domain (RD), and it was hypothesized that this motif could be utilized as a platform to develop new strategies for blocking AR function. Here, we demonstrate that expression of the RD peptide is sufficient to disrupt AR transcriptional activation of multiple, prostate-specific AR target genes. Importantly, these actions are sufficient to specifically inhibit S-phase progression in AR-positive PCa cells, but not in AR-negative cells or tested AR-positive cells of other lineages.

As expected, impaired cell cycle progression resulted in a suppression of cell doubling. Additionally, cell death was observed in AR-positive cells that maintain androgen dependence and in a subset of castrate-resistant PCa cells, dependent on Akt activation status. Lastly, the ability of RD to cooperate with existing hormone therapies was examined, which revealed that RD enhanced the cellular response to an AR antagonist. Together, these data demonstrate that RD is sufficient to disrupt AR-dependent transcriptional and proliferative responses in PCa, and can enhance efficacy of AR antagonists, thus establishing the impetus for development of RD-based mimetics.

In addition, the following advances have been made, which pertain to the importance of cyclin D1 on clinical markers of prostate cancer growth and progression:

D-type cyclins regulate cellular outcomes in part through cyclin dependent kinase (CDK)-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 (PCa) was used as a paradigm, as 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 (Figure 1). Unexpectedly, cyclin D1 was found to both enhance and suppress androgen-dependent signaling events, demonstrating that cyclin D1 is a selective effector of hormone action (Figure 2). Clustering and gene validation analyses showed that androgen-induced target genes that are directly regulated by AR were suppressed by cyclin D1. Analyses of AR levels on target gene loci, of clinical relevance, demonstrated that cyclin D1 limits AR occupancy after hormone stimulation (Figures 3 and 4). Together, these findings reveal a new function for cyclin D1 in controlling hormone-dependent transcriptional outcome, and demonstrate a role for cyclin D1 in regulating transcription factor dynamics.

FIGURES

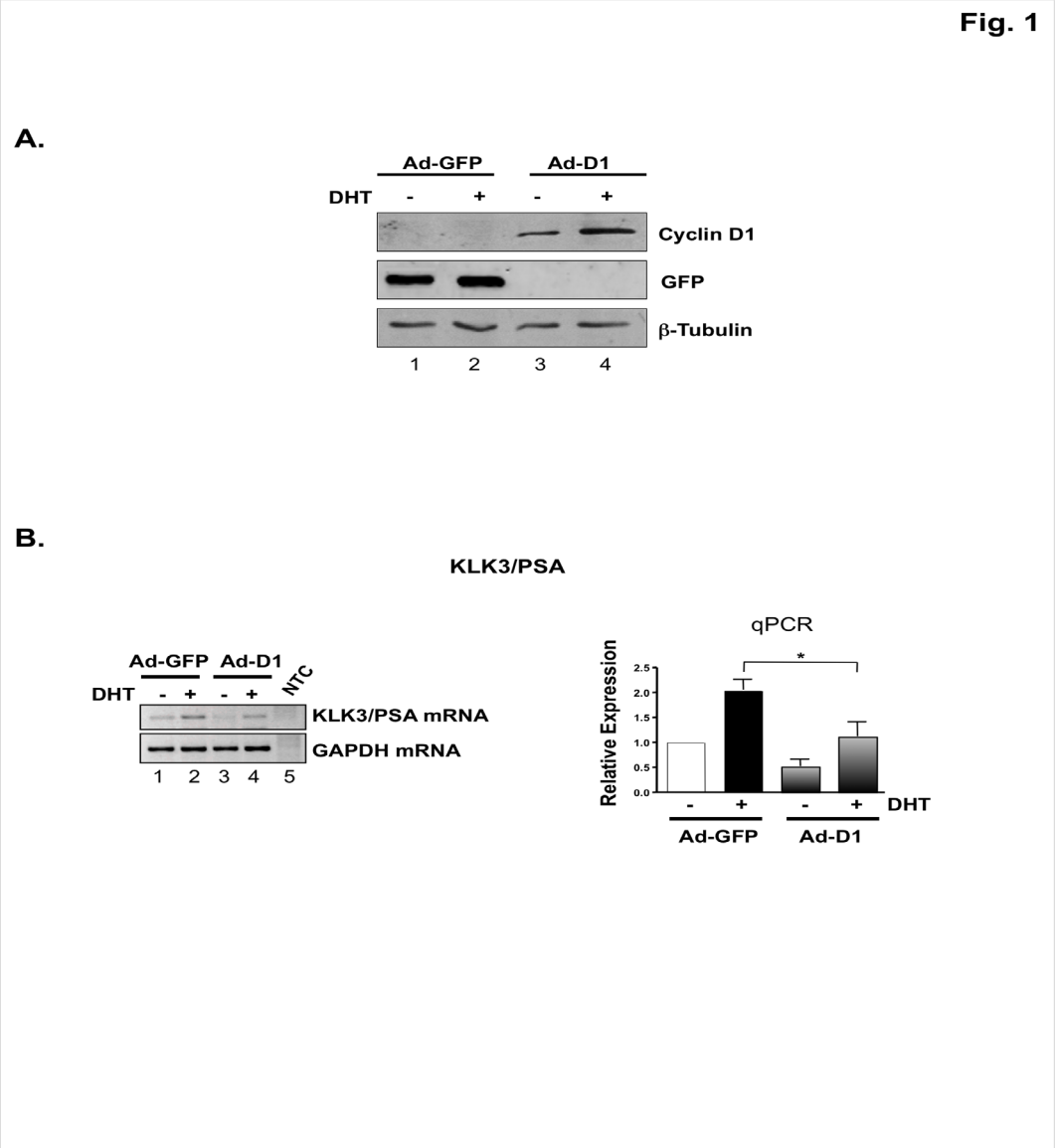

Fig. 1. Cyclin D1 regulates prostate-specific tumor marker expression. To examine the cyclin D1-regulated transcriptional outcome in response to androgen, LNCaP prostate cancer cells were incubated in charcoal-dextran treated (CDT, 5%) serum to naturally deplete D-type cyclins, then transduced with cyclin D1 (Ad-D1) or control (Ad-GFP), and subsequently treated with a physiological dose of androgen (DHT, 1nM) or ethanol (EtOH) control. *A.* To evaluate cyclin D1 protein expression, representative LNCaP cell lysates treated as described above, were immunoblotted for cyclin D1, GFP, and loading control β-Tubulin. *Note*: The post-transcriptional induction of cyclin D1 protein by androgen is maintained under cyclin D1 constituted conditions (lanes 3 and 4). *B. KLK3/PSA* expression was determined by conventional PCR (left) and Taqman-based qPCR (right) to assess the ability of cyclin D1 to regulate prostate-specific tumor marker expression. Non-template control is indicated by NTC. Histogram is represented as the mean fold change ± SEM of 3 independent experiments where each control sample (Ad-GFP + EtOH) is set to "1". Significant ($p<0.05$) down-regulation by cyclin D1 of androgen-induced expression is indicated by an asterisk.

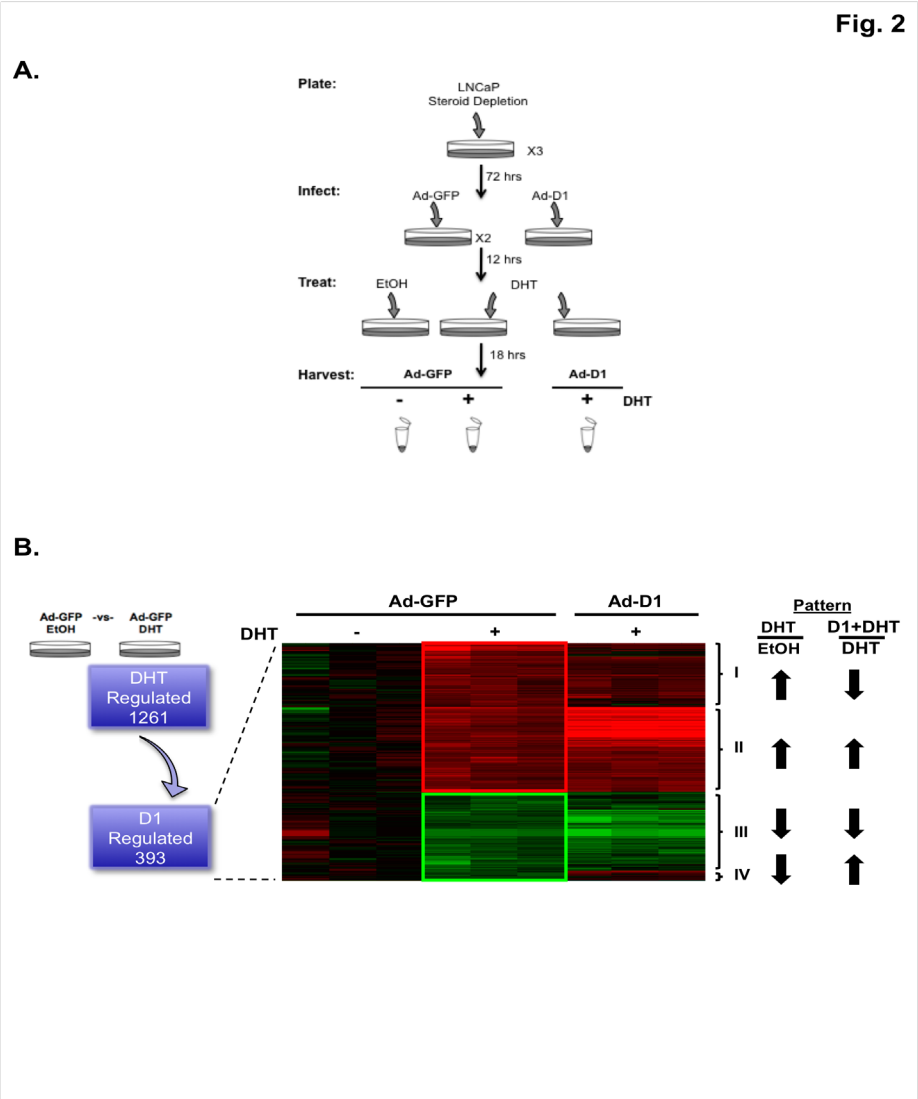

Fig. 2. Cyclin D1 modulates androgen-dependent gene expression. *A*. Schematic of the experimental design for unbiased gene expression array analyses to identify androgen-regulated transcripts that are sensitive to cyclin D1. Microarray analysis was performed in triplicate for each treatment condition on the HG-U133plus2 platform (Affymetrix). Individual samples were normalized and overlaid onto a custom GeneChip library file to provide a more accurate interpretation of the expression data. All statistical comparisons and visualizations were performed using GeneSpring GX v7.3.1 (Agilent). *B. Schematic*: to identify androgen-regulated transcripts responsive to cyclin D1, a statistical ($p<0.05$) comparison between GFP-transduced LNCaP cells treated with EtOH or DHT was performed. Transcripts were then selected using a 1.2-fold cutoff and the corresponding expression values in the presence of cyclin D1 and DHT are shown. *Heatmap*: to identify expression patterns (as indicated, Patterns: I-IV), transcripts were empirically assigned to clusters using a k-means algorithm. Red and green indicate up-regulated and down-regulated transcripts, respectively. The androgen response and influence of cyclin D1 on the androgen response are indicated by arrows.

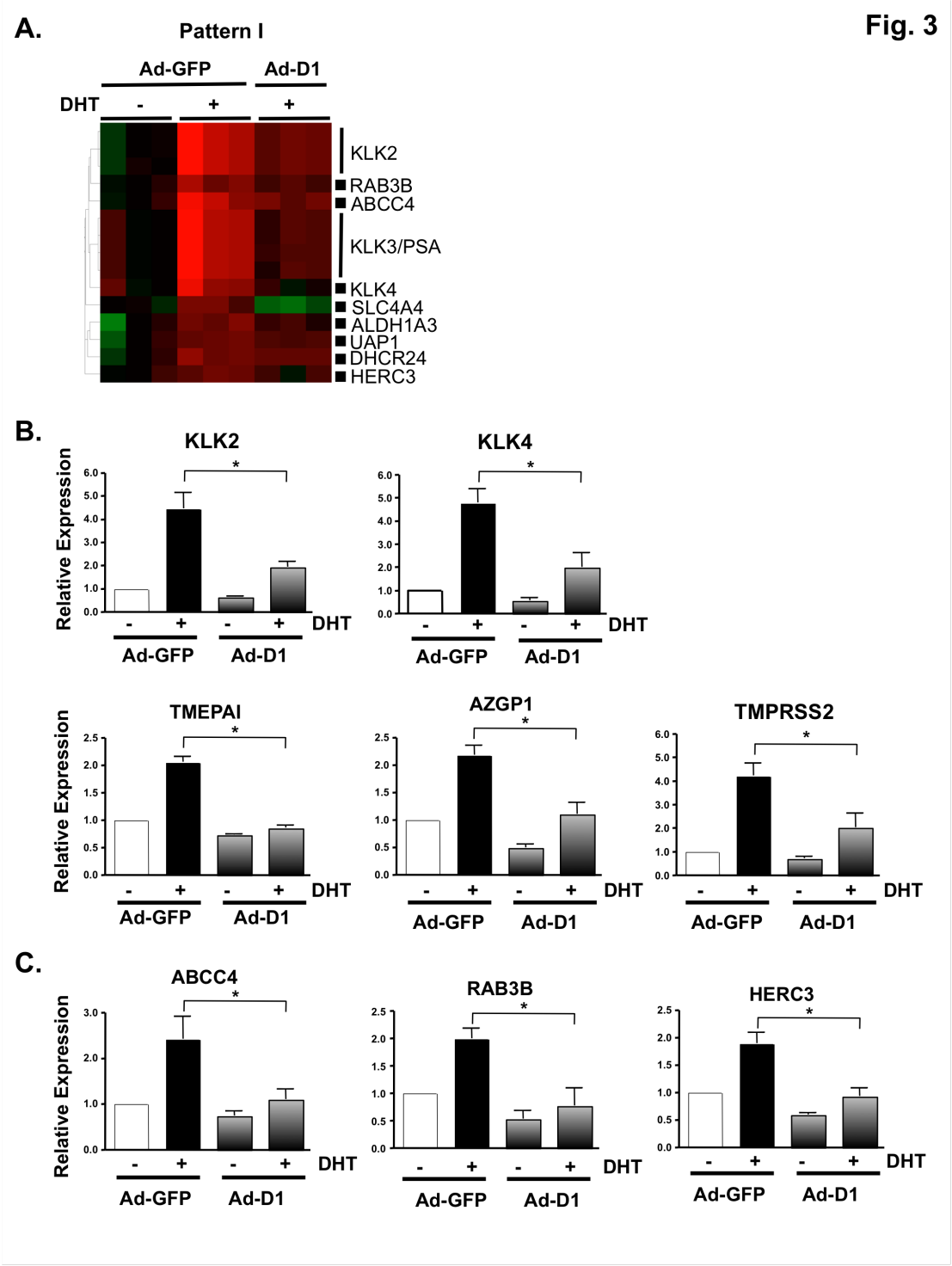
Fig. 3
A.
Pattern I
Ad-GFP
Ad-D1
DHT
-
+
+
KLK2
RAB3B
ABCC4
KLK3/PSA
KLK4
SLC4A4
ALDH1A3
UAP1
DHCR24
HERC3
B.
KLK2
KLK4
TMEPAI
AZGP1
TMPRSS2
Relative Expression
DHT
Ad-GFP
Ad-D1
C.
ABCC4
RAB3B
HERC3

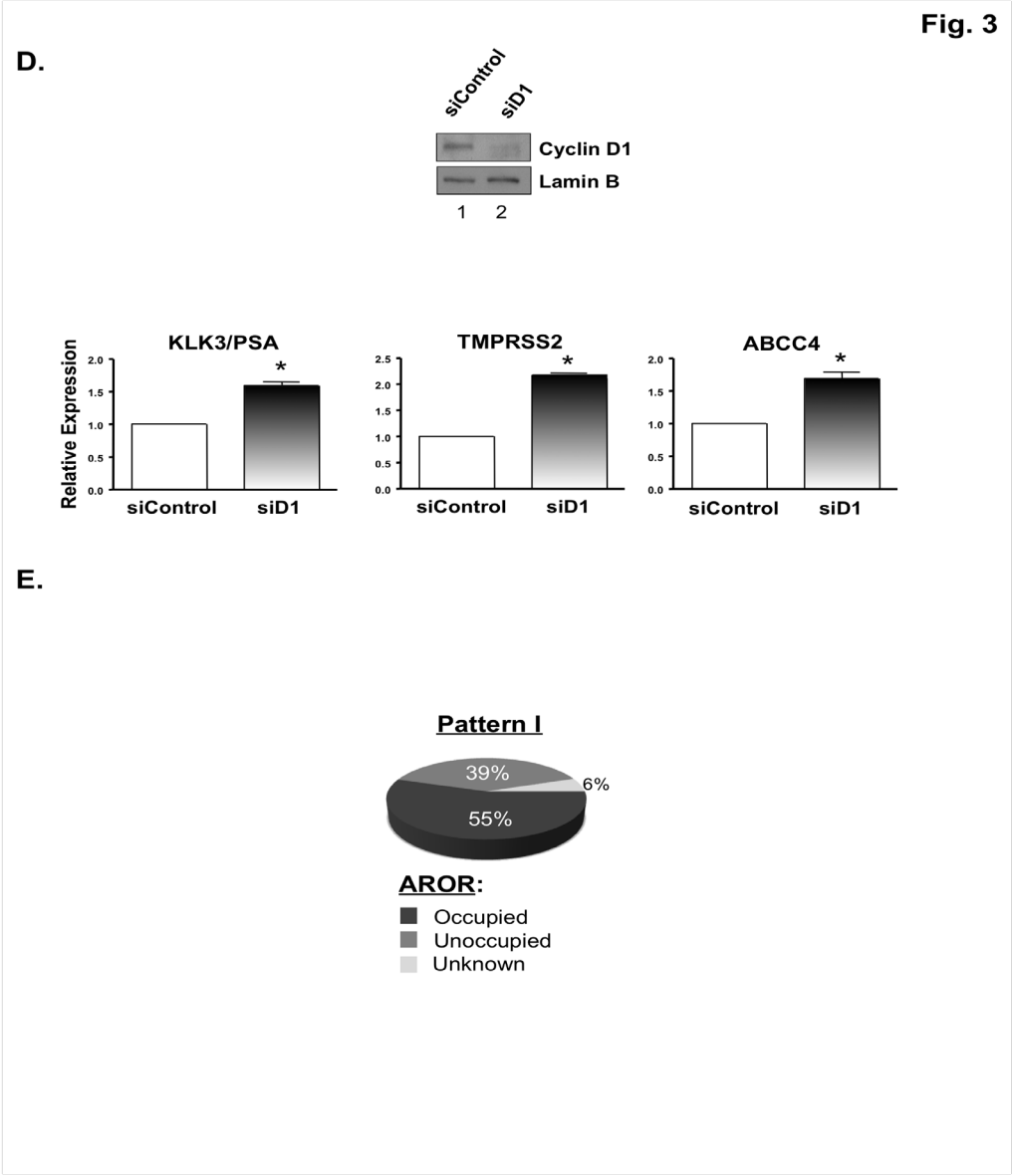

Fig. 3. Cyclin D1 attenuates AR-dependent gene expression. To determine the role of cyclin D1 on AR-dependent gene expression: *A.* Heatmap analysis was performed on selected Pattern I transcripts that are frequently observed as androgen/AR-regulated transcripts. The relative expression of *B.* known AR-target genes: *KLK2*, *KLK4*, *TMEPAI*, *AZGP1*, and *TMPRSS2*; and *C.* putative AR-target genes: *ABCC4*, *RAB3B*, and *HERC3* was performed by SYBR-based qPCR from 3-4 independent experiments and presented as described in Fig. 1. Cyclin D1-transduced cells treated with ethanol were included in the validation to assess the impact of cyclin D1 on basal transcription. All transcripts tested validated as androgen-dependent; however, only the transcripts that demonstrated a significant ($p<0.05$) difference in the presence of cyclin D1 are indicated by an asterisk. *D.* Knockdown of cyclin D1, in LNCaP cells cultured under standard androgen conditions (i.e., FBS 5%), was performed to further validate the influence of cyclin D1 on AR target gene expression. A representative immunoblot for cyclin D1 knockdown is provided. The relative expression of KLK3/PSA, TMPRSS2, and ABCC4 was determined by qPCR, from 3 individual experiments, and plotted as described above. *E.* The transcripts from Pattern I were assessed for AR occupied regions (AROR) within 50kb of the transcriptional start site (TSS) using a publically available dataset, as described in the Materials and Methods, to determine the overall potential of AR to regulate these gene loci.

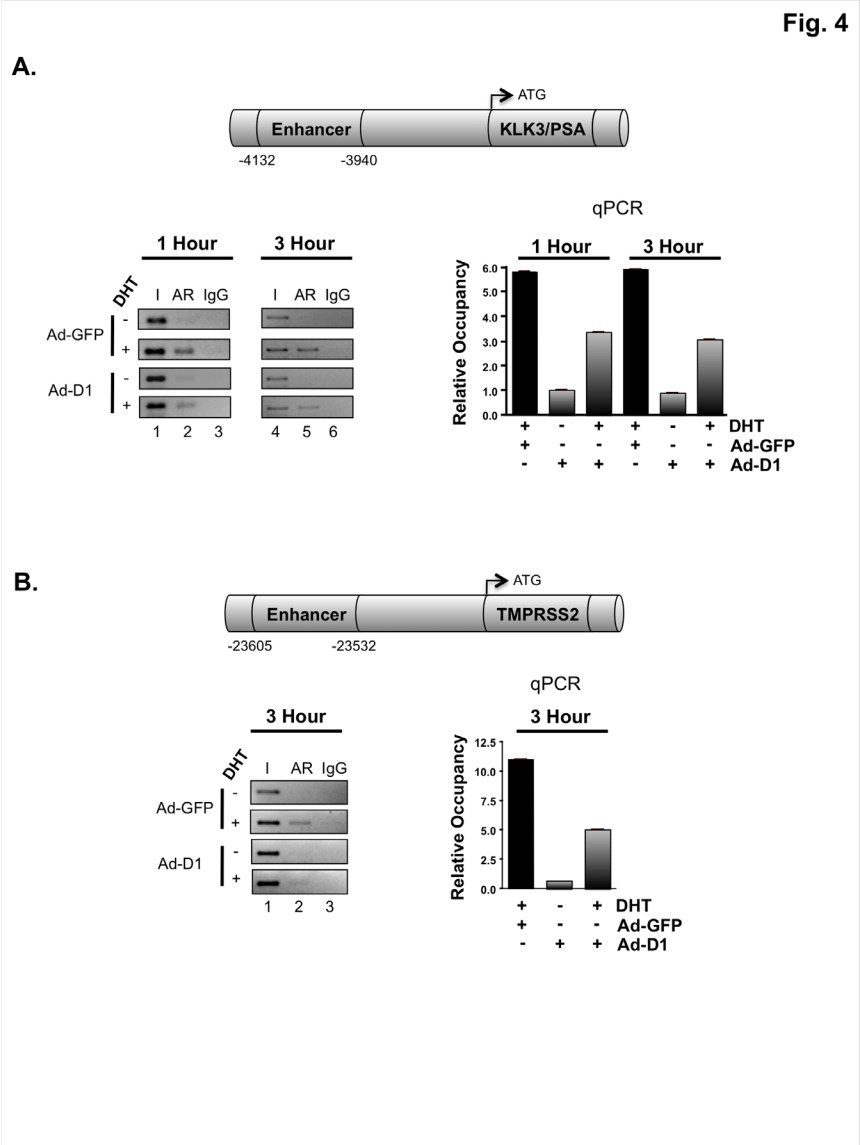

Fig. 4. Cyclin D1 displaces AR occupancy at target-genes loci. Chromatin immunoprecipitation (ChIP) analysis was performed to determine the influence of cyclin D1 on AR occupancy. LNCaP cells were treated as described in Fig. 2 except cells were stimulated with 10nM DHT for 1-3 hours. Histograms represent the relative occupancy ± SD from a representative AR ChIP where each condition is a biological triplicate. *A. Schematic*: *KLK3/PSA* locus showing the location of the well-characterized, AR-responsive enhancer region upstream of the transcriptional start site (TSS). *Histogram*: qPCR analysis for the enhancer region from AR ChIP assays at 1 and 3 hours. Representative conventional PCR is provided (left). *B. Schematic*: *TMPRSS2* locus showing the location of the ARE V region. *Histogram*: qPCR analysis for the ARE V region from an AR ChIP assay at 3 hours. Representative conventional PCR is provided (left).

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

Previously, we examined the expression of CAPER using a human breast cancer tissue microarray, consisting of 84 invasive ductal carcinomas (IDCs) and 20 normal breast tissue samples. Quantitative analysis of CAPER expression was achieved by using the PM2000 AQUA automated quantitative analysis system (HistoRx, Inc). Our results show that CAPER protein expression is significantly elevated in all three tumor grades (well, moderately and poorly differentiated) . We also assessed the expression and distribution of CAPER by immunohistochemical staining of paraffin-embedded tissue sections. Our results show 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 significantly higher levels in ductal carcinoma in situ (DCIS) and IDC samples, where it assumes a predominantly nuclear distribution Thus, our results directly establish that there is a cytoplasmic-to-nuclear shift in the distribution of CAPER during the transition from pre-malignancy to malignancy.

In the current reporting period, related to Aim 1, we stained all the paraffin-embedded tissue sections necessary to correlate the expression of CAPER with other biomarkers (ER, PR, HER2), patient progression, survival, and recurrence data. The CAPER staining has already been scored by a human breast cancer pathologist and we will now perform the statistical analyses using SPSS and SAS software under guidance of Dr. Terry Hyslop and the Biostatistics Core Facility at Kimmel Cancer Center.

Regarding Aim 2, we already generated the MCF-10A cells overexpressing CAPER and MCF-7 cells harboring a CAPER siRNA necessary to the completion of this aim. We also identified new downstream targets of CAPER through unbiased screening methods. In addition, MCF-10A, MCF-7 and control (pBABE alone) cells have already been injected in the flank of 8 week-old female nude mice. We will now start injecting MCF-10A cells overexpressing CAPER and MCF-7 cells harboring a CAPER siRNA in the flank of 8 week-old nude mice.

Other Related Findings from our Group Include these Novel Biomarkers Related Studies:

Loss of stromal caveolin-1 expression predicts poor clinical outcome in triple negative and basal-like breast cancers. We investigated the possible predictive value of stromal caveolin-1 (Cav-1) as a candidate biomarker for clinical outcome in triple negative (TN) breast cancer patients. A cohort of 85 TN breast cancer patients was available, with the necessary annotation and nearly 12 years of follow-up data. Our primary outcome of interest in this study was overall survival. Interestingly, TN patients with high-levels of stromal Cav-1, had a good clinical outcome, with >50% of the patients remaining alive during the follow-up period.

In contrast, the median survival for TN patients with moderate stromal Cav-1 staining was 33.5 months. Similarly, the median survival for TN patients with absent stromal Cav-1 staining was 25.7 months. A comparison of 5-year survival rates yields a similar pattern. TN patients with high stromal Cav-1 had a good 5-year survival rate, with 75.5% of the patients remaining alive. In contrast, TN patients with moderate or absent stromal Cav-1 levels had progressively worse 5-year survival rates, with 40% and 9.4% of the patients remaining alive.

In a parallel analysis, the levels of tumor epithelial Cav-1 had no prognostic significance. As such, the prognostic value of Cav-1 immunostaining in TN breast cancer patients is compartment-specific, and selective for an absence of Cav-1 staining in the stromal fibroblast compartment. A recursive-partitioning algorithm was used to assess which factors are most predictive of overall survival in TN breast cancer patients. In this analysis, we included tumor size, histologic grade, whether the patient received surgery, radiotherapy or chemotherapy, CK5/6, EGFR, p53 and Ki67 status, as well as the stromal Cav-1 score. This analysis indicated that stromal Cav-1 expression was the most important prognostic factor for overall survival in TN breast cancer. Virtually identical results were obtained with CK5/6 (+) and/or EGFR (+) TN breast cancer cases, demonstrating that a loss of stromal Cav-1 is also a strong prognostic factor for basal-like breast cancers. Our current findings may have important implications for the close monitoring and treatment stratification of TN and basal-like breast cancer patients.

Tumor cells induce the cancer associated fibroblast phenotype via caveolin-1 degradation: Implications for breast cancer and DCIS therapy with autophagy inhibitors. Loss of stromal

caveolin 1 (Cav-1) is a novel biomarker for cancer-associated fibroblasts that predicts poor clinical outcome in breast cancer and DCIS patients. We hypothesized that epithelial cancer cells may have the ability to drive Cav-1 downregulation in adjacent normal fibroblasts, thereby promoting the cancer associated fibroblast phenotype. To test this hypothesis directly, here we developed a novel co-culture model employing (i) human breast cancer cells (MCF7), and (ii) immortalized fibroblasts (hTERT-BJ1), which are grown under defined experimental conditions. Importantly, we show that co-culture of immortalized human fibroblasts with MCF7 breast cancer cells leads to Cav-1 downregulation in fibroblasts.

These results were also validated using primary cultures of normal human mammary fibroblasts co-cultured with MCF7 cells. In this system, we show that Cav-1 downregulation is mediated by autophagic/lysosomal degradation, as pre-treatment with lysosome-specific inhibitors rescues Cav-1 expression. Functionally, we demonstrated that fibroblasts co-cultured with MCF7 breast cancer cells acquire a cancer-associated fibroblast phenotype, characterized by Cav-1 downregulation, increased expression of myofibroblast markers and extracellular matrix proteins, and constitutive activation of TGFbeta/Smad2 signaling.

siRNA-mediated Cav-1 downregulation mimics several key changes that occur in co-cultured fibroblasts, clearly indicating that a loss of Cav-1 is a critical initiating factor, driving stromal fibroblast activation during tumorigenesis. As such, this co-culture system can now be used as an experimental model for generating "synthetic" cancer associated fibroblasts (CAFs). More specifically, these "synthetic" CAFs could be used for drug screening to identify novel therapeutics that selectively target the Cav-1-negative tumor micro-environment. Our findings also suggest that chloroquine, or other autophagy/lysosome inhibitors, may be useful as anti-cancer agents, to therapeutically restore the expression of stromal Cav-1 in cancer associated fibroblasts. We discussed this possibility, in light of the launch of a new clinical trial that uses chloroquine to treat DCIS patients: PINC (Preventing Invasive Breast Neoplasia with Cholorquine) [See http://clinicaltrials.gov/show/NCT01023477].

Loss of stromal Caveolin-1 leads to oxidative stress, mimics hypoxia and drives inflammation in the tumor microenvironment, conferring the "Reverse Warburg Effect": A transcriptional informatics analysis with validation. Cav-1 (-/-) deficient stromal cells are a new genetic model for myofibroblasts and cancer-associated fibroblasts. Using an unbiased informatics analysis of the transcriptional profile of Cav-1 (-/-) deficient mesenchymal stromal cells, we have now identified many of the major signaling pathways that are activated by a loss of Cav-1, under conditions of metabolic restriction (with low glucose media). Our informatics analysis suggests that a loss of Cav-1 induces oxidative stress, which mimics a constitutive pseudo-hypoxic state, leading to 1) aerobic glycolysis and 2) inflammation in the tumor stromal microenvironment. This occurs via the activation of 2 major transcription factors, namely HIF (aerobic glycolysis) and NF-kB (inflammation) in Cav-1 (-/-) stromal fibroblastic cells.

Experimentally, we showed that Cav-1 deficient stromal cells may possess defective mitochondria, due to the over-production of nitric oxide (NO), resulting in the tyrosine nitration of the mitochondrial respiratory chain components (such as complex I). Elevated levels of nitro-tyrosine were observed both in Cav-1 (-/-) stromal cells, and via acute knock-down with siRNA targeting Cav-1. Finally, metabolic restriction with mitochondrial (complex I) and glycolysis

inhibitors was synthetically lethal with a Cav-1 (-/-) deficiency in mice. As such, Cav-1 deficient mice show a dramatically reduced mitochondrial reserve capacity. Thus, a mitochondrial defect in Cav-1 deficient stromal cells could drive oxidative stress, leading to aerobic glycolysis, and inflammation, in the tumor microenvironment. These stromal alterations may underlie the molecular basis of the "Reverse Warburg Effect", and could provide the key to targeted anti-cancer therapies using metabolic inhibitors.

In direct support of these findings, the transcriptional profile of Cav-1 (-/-) stromal cells overlaps significantly with Alzheimer's disease, which is characterized by oxidative stress, NO over-production (peroxynitrite formation), inflammation, hypoxia, and mitochondrial dysfunction. We concluded that Cav-1 (-/-) deficient mice are a new whole-body animal model for an activated lethal tumor micro-environment, i.e., "tumor stroma" without the tumor. Since Cav-1 (-/-) mice are also an established animal model for pro-fibrotic disease, our current results may have implications for understanding the pathogenesis of scleroderma (systemic sclerosis) and pulmonary fibrosis, which are also related to abnormal mesenchymal stem cell function.

Publications Resulting From This Work

Martinez-Outschoorn, U.E., Pavlides, S., Whitaker-Menezes, D., Daumer, K.M., Milliman, J.N., Chiavarina, B., Migneco, G.M.,Witkiewicz, A.K., Martinez-Cantarin, M.P., Flomenberg, N., Howell, A., Pestell, R.G., Lisanti, M.P., and F. Sotgia. 2010. Tumor Cells Induce the Cancer Associated Fibroblast Phenotype Via Caveolin-1 Degradation: Implications for Breast Cancer and DCIS Therapy with Chloroquine. Cell Cycle, 9: 2423-2433. http://www.landesbioscience.com/journals/6/article/12048/

Witkiewicz, A.K., Dasgupta, A., Sammons, S., Er, O., Potoczek , M.B., Guiles, F., Sotgia, F., Brody, J.R., Mitchell, E.P., and M.P. Lisanti. 2010. Loss of Stromal Caveolin-1 Expression Predicts Poor Clinical Outcome in Triple Negative and Basal-Like Breast Cancers. Cancer Biology and Therapy, volume 10 (July 15th issue), In Press. http://www.landesbioscience.com/journals/5/article/11983/

Pavlides, S., Tsirigos, A., Vera, I., Flomenberg, N., Frank, P.G., Casimiro, M.C., Wang, C., Fortina, P., Addya, S., Pestell, R.G., Martinez-Outschoorn, U.E., Sotgia, F., and M.P. Lisanti. 2010. Loss of Stromal Caveolin-1 Leads to Oxidative Stress, Mimics Hypoxia, and Drives Inflammation in the Tumor Microenvironment, Conferring the "Reverse Warburg Effect": A Transcriptional Informatics Analysis with Validation, Cell Cycle, 9: 2201–2219. http://www.landesbioscience.com/journals/6/article/11848/

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

By the end of the project period, our imaging capabilities will be greatly enhanced, thereby providing the resources to support our ongoing research mission and maximize the states investment.

Since the last reporting period, TJU has engaged in the implementation of a master space strategic plan which impacts the location of intended facility upgrades as previously identified. Infrastructure deficiencies such as HVAC and emergency power capabilities, in addition to upgrades needed to co-locate investigators utilizing the equipment purchased in the current and

previous grant cycle, have prompted adjustments to the 5th floor and 2nd floor where the imaging equipment resides.

Through the use of awarded Federal stimulus dollars, pending state research support and a significant institutional commitment, we will be able to provide an environment that will better leverage the equipment investments made to date. It is our expectation that the investment of critical capital equipment via the grant funding of Project 4 will be of greater benefit than the original vision at the time of the initial grant submission.

In support of the anticipated facilities upgrades on several floors of the Jefferson Alumni Hall building and since the last reporting period the following purchases have been made to further the aims of the project:

- Additional enhancements have been made to the FEI Technai 12. Specifically a serious of purchases has resulted in an upgrade to the package purchased in the last reporting period. Conventional transmission electron (TEM) micrographs are acquired at zero tilt and provide ultra-structural characterization in three dimensions (3D) or cellular organelles, which is essential to understand cellular processes. Thick sections prepared for conventional TEM are examined in a tilt series where digital images are captured from plus 70 to minus 70 degrees in 1 degree increments. After data alignment the digital images can be reconstructed into a 3D volume. This research method can be profitable for the investigation of virtually any cellular organelle and its physical relationship with surrounding structures. The purchases included software upgrades-Xplore 3D retrofit package (FEI), tomography holder (Fischione Instruments) system maintenance package (FEI), and PC hardware (Dell). $115,517.67 was spent to enable these upgrades.

- An EM CCD camera system (Leica Microsystems) The ProEm 1024 B will further enhance our electron microscopy program and will complement the microscopy equipment actively in place. The ProEM is the most advanced EMCCD camera available today delivering a single photon sensitivity, a large field of view and a high speed EM mode and utilizes a Gigabit Ethernet interface. The cost for the purchase was $60,235.50.

- To enhance our microscopy capabilities a DG4/Fs30 Rapid Wavelength Switcher 330W and support control equipment was purchased from B&B Miscroscopes Limited. DG4/Fs30 Rapid Wavelength Switcher.The Lambda DG-4/DG-5 is a complete illumination system offering speed and versatility for experiments requiring rapid wavelength switching. The instrument retains all the advantages of interference filter based systems, yet eliminates the temporal constraints imposed by traditional filter changing devices like filter wheels. Switching between any two wavelengths is achieved in less than the 1.2msec vertical retrace period of a video signal, allowing the user to perform real-time video imaging. The high switching speed of the Lambda DG-4/DG-5 facilitates the ability to follow fast changes in ion concentrations in dual wavelength ratio imaging applications and to monitor other concomitant changes in the studied system at additional wavelengths. Total cost was $28,041.65.

- A Mosiac Digital Illumination System for an Olypus IX81 Microscope. The Mosaic Digital Illumination Systems uses a computer controlled spatial light modulator to map a diffraction limited mask onto the specimen plane. In combination with its continuous wave (CW) laser or arc lamp light fluorescence excitation sources, it accommodates laser scanning confocal, spinning disk confocal and widefield microscopes. The package purchase price was $62,865.00