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Therapeutically activating RB: reestablishing cell cycle control in endocrine therapy-resistant breast cancer

Chellappagounder Thangavel[1,2], **Jeffry L Dean**[1,2], **Adam Ertel**[1,2], **Karen E Knudsen**[1,2], **C Marcelo Aldaz**[4], **Agnieszka K Witkiewicz**[3], **Robert Clarke**[5,6], and **Erik S Knudsen**[1,2]

[1]Department of Kimmel Cancer Center, Philadelphia, Pennsylvania 19107, USA

[2]Department of Cancer Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, USA

[3]Department of Pathology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, USA

[4]Department of Carcinogenesis, M.D. Anderson Cancer Center, Houston, Texas 78957, USA

[5]Department of Lombardi Cancer Center, Washington, District of Columbia 20007, USA

[6]Department of Oncology and Physiology and Biophysics, Georgetown University, Washington, District of Columbia 20057, USA

Abstract

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.

(Correspondence should be addressed to Kimmel Cancer Center, Thomas Jefferson University, BLSB Room 1002, 233 South 10th Street, Philadelphia, Pennsylvania 19107, USA; eknudsen@kimmelcancercenter.org) .

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Introduction

Current breast cancer treatment is based on the status of a limited number of molecular markers (Bosco & Knudsen 2007, Musgrove & Sutherland 2009,Hammond *et al.* 2010, Harris & McCormick 2010). 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 (Jordan *et al.* 1987, Musgrove & Sutherland 2009, Hammond *et al.* 2010, Harris & McCormick 2010). In this context, only those tumors which are ER-positive will respond to such hormonal interventions (Jordan *et al.* 1987, Ariazi *et al.* 2006), and a combination of aromatase inhibitors which attenuate estrogen synthesis (e.g. Letrazole), selective ER modulator (e.g. Tamoxifen), or specific ER antagonists (e.g. ICI 182 780) are deployed in distinct clinical settings (Musgrove & Sutherland 2009). Importantly, ER-positive breast cancer constitutes ~70% of cases, and millions of such tumors have been treated with endocrine therapy (Wakeling *et al.* 1991, Musgrove & Sutherland 2009).

Estrogen antagonists are effective in ER-positive breast cancer; as such, tumors are dependent on estrogen signaling for proliferation and survival (Varma *et al.* 2007, Musgrove & Sutherland 2009). Thus, antagonizing ER signaling leads to cell cycle arrest and reduced tumor cell viability (Sutherland *et al.* 1983, Coser *et al.* 2009). Substantial preclinical study has demonstrated that cell cycle regulatory control is a key mechanism through which such agents act to prevent tumor growth (Watts *et al.* 1995, Carroll *et al.* 2000, Foster *et al.* 2001). Specifically, the withdrawal of estrogen (mimicking aromatase inhibitors) or use of estrogen antagonists (e.g. ICI 182 780 or Tamoxifen) results in an arrest in the G0/G1 phase of the cell cycle (Watts *et al.* 1995, Carroll *et al.* 2000, Foster *et al.* 2001,Markey *et al.* 2007). In this context, reduced ER signaling leads to the attenuation of CDK/cyclin complexes at multiple levels (Watts *et al.* 1995, Carroll *et al.* 2000, Foster *et al.* 2001). Most dramatically, cyclin D1 is a known and direct transcriptional target of the ER signaling network (Watts *et al.* 1994, Eeckhoute *et al.* 2006). Furthermore, culmination of the many ER-mediated downstream mechanisms coalescence in the control of net CDK activity (Foster *et al.* 2001,Planas-Silva & Weinberg 1997, Watts *et al.* 1995). As such, inhibition of CDK activity results in the maintenance of the retinoblastoma tumor suppressor protein (RB) in a hypophosphorylated and active state (Watts *et al.* 1995). In its hypophosphorylated state, RB serves to repress E2F-regulated genes (e.g. Cyclin A) and inhibits progression through S-phase and G2/M (Markey *et al.* 2002, Knudsen & Knudsen 2006). Despite the potent anti-proliferative activity of current hormone-based therapeutic strategies, acquired resistance is a critical clinical problem even with highly effective ER antagonists (Musgrove & Sutherland 2009).

To understand the basis of progression to therapeutic resistance, multiple preclinical and correlative clinical studies have been performed (Musgrove & Sutherland 2009). Functional analyses have suggested that deregulation of a multitude of signal transduction cascades can contribute to acquired resistance to endocrine therapy (Shou *et al.* 2004, Lee & Sicinski 2006, Perez-Tenorio *et al.* 2006). Specifically, deregulated mitogenic signaling through aberrant ErbB2, Grb10 and/or AKT signaling have all been shown to contribute to such therapeutic failure in preclinical models (Hu & Mokbel 2001, Jordan *et al.* 2004, Iorns *et al.* 2008, Miller *et al.* 2009). Additionally, deregulation of specific cell cycle components (e.g. $p27^{Kip1}$ or RB) can directly compromise the efficacy of ER inhibition (Cariou *et al.* 2000, Bosco *et al.* 2007). Such findings are supported by wide-ranging analyses of breast tumors by single markers or gene expression profiling (Musgrove & Sutherland 2009). Due to the heterogeneous pathways deregulated in the progression of ER-positive breast cancer, 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, which could be targeted for the treatment of ER-positive breast cancer that progresses in the presence of endocrine therapy.

Materials and methods

In silico analysis of the RB-loss signature in breast cancer microarray samples

Breast cancer samples with relapse-free survival information from patients who received adjuvant hormone (Tamoxifen) therapy were selected from a large dataset compiled from public microarray databases (Ertel *et al.* 2010). This dataset includes multiple cohorts identified under ArrayExpress accession number E-TABM-158 and GEO accession numbers GSE12093, GSE1456, GSE6532, and GSE9195. Samples were classified as either Luminal A or Luminal B based on their correlation against the intrinsic gene list profiles that define these two subtypes (Hu *et al.* 2006). Survival analysis was performed using Kaplan–Meier curves for relapse-free survival in the Luminal A and Luminal B breast cancer samples. The Log-rank test was used to evaluate differences in the Luminal A and Luminal B survival distributions. The magnitude of a previously defined RB-loss signature (Ertel *et al.* 2010) was used to order the breast cancer samples from low to high signature expression. The Kolmogorov–Smirnov test was used to evaluate the null hypothesis that Luminal A and Luminal B samples are evenly distributed across the spectrum of RB-loss signature expression. One-way ANOVA was used to test for differential expression between the Luminal A and Luminal B populations. An optimal threshold for low expression of RB-loss signature associated with good outcome was previously identified for the combined breast cancer microarray dataset (Ertel *et al.* 2010). Separating the Luminal B samples around this threshold identified a minority of samples (11%) with low RB-loss signature expression. Kaplan–Meier curves were used to perform survival analysis for low versus high RB-loss signature groups within the Luminal B samples and demonstrated that low RB-loss is associated with better outcome within the Luminal B subtype (P=0.0545). This association was strengthened slightly by adjusting the low RB-loss expression threshold to include one additional sample (Fig. 1, P=0.0401).

RB-loss signature

The RB-loss signature consists of 159 genes that were previously identified as RB-dependent targets in other model systems, as described by Ertel *et al.* (2010). The magnitude of the RB-loss signature, assessed over transcript measures available for 138 of the 159 genes, was used to order the breast cancer samples from low to high signature expression. The Kolmogorov–Smirnov test was used to evaluate the null hypothesis that Luminal A and Luminal B samples are evenly distributed across the spectrum of RB-loss signature expression. One-way ANOVA was used to test for differential expression between the Luminal A and Luminal B populations.

RB pathway expression in Tamoxifen-sensitive versus Tamoxifen-resistant samples

Samples among the same five microarray datasets were also identified as Tamoxifen-sensitive or Tamoxifen-resistant, in order to identify differential expression of RB pathway genes among these populations. Tamoxifen-resistant patients were defined as those who had disease recurrence within 5 years, while Tamoxifen-sensitive patients were defined as those who were disease free at 5 years. RB pathway genes were tested for differential expression between Tamoxifen-resistant, Tamoxifen-sensitive, and healthy reference samples using one-way ANOVA.

Cell culture

F100-16 and F40-7 cells were a generous gift from Dr Toshi Shioda, Harvard Stem Cell Institute, Cambridge, MA, USA. Tamoxifen-resistant MCF7/FBS/Tam^R and MCF7/CSS/Tam^R were a generous gift from Richard J Santen, Department of Internal Medicine, University of Virginia Health Sciences, Charlottesville, VA, USA. All the above-mentioned cell lines, in addition to MCF7, were maintained in DMEM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin/streptomycin and 2 mM L-glutamine. Cells were maintained at 37 °C in a humidified incubator at an atmosphere containing 95% air: 5% CO_2.

RB knockdown

RB knockdown was carried out using pMSCV-LMP-miRB vector expressing shRNA targeting the human RB (699-719), a generous gift from Dr Scott Lowe (Cold Spring Harbor Laboratory, New York City, NY, USA). Non-specific controls (miNS) were used. Retroviral infections were carried out in the presence of polybrene for a minimum of 6 h. Infected populations were selected with puromycin for a period of 2 weeks. RB knockdown was confirmed by immunoblot analysis.

Treatments and growth assessment

miNS- and miRB-expressing MCF7 cells were maintained in DMEM supplemented with 10% FBS or steroid hormone-free DMEM medium supplemented with 10% charcoal/dextran-treated (CDT) FBS for populations exposed to hormone-based therapies. Cells were treated with 500 nM PD-0332991 (provided by Pfizer, Inc., San Diego, CA, USA) or equal volume of DMSO control for a period of 24 h. ICI 182 780 (Tocris Bioscience, Ellisville, MO, USA) treatments were performed at a final concentration of 1.0 and 0.1 μM. For Tamoxifen (Sigma–Aldrich) based studies, a final concentration of 1.0 μM or equal volumes of vehicle control were utilized. For acute studies, ICI and Tamoxifen were employed for 48 or 72 h. Cells were harvested at the indicated times and processed for flow cytometry, chromatin immunoprecipitation assay (ChIP) or immunoblot. For long-term growth assessment, media supplemented with PD-0332991 or ICI replaced every 48 h for up to 10 days. For cell growth assays, cells were harvested and counted every 48 h using trypan blue exclusion. At the conclusion of growth assays, cells were fixed and stained with 1% crystal violet for visualization of cellular morphology and abundance.

Flow cytometric analysis

Flow cytometry was performed as previously described (Zagorski *et al.* 2007). Prior to harvesting, cells were pulse-labeled with BrdU for 1 h (GE Healthcare, Piscataway, NJ, USA).

Cyclin D1 mRNA analysis by real-time PCR

Using TRIzol reagent (Invitrogen), total RNA was isolated from MCF7, LCC9 under FBS or ICI. Total RNA (5 μg) was reverse transcribed using random hexamers and SuperScript III reverse transcriptase (Invitrogen). cDNA (200 ng) was used in quantitative PCR (qPCR) to amplify *Cyclin D1a* transcripts with forward and reverse primers (forward 5′-CTC TCC AGA GTG ATC AAG TGT GAC CC-3′ and reverse 5′-TGT GCA AGC CAG GTC CAC C-3′) or *GAPDH* mRNA primers (forward 5′-TGG AAA TCC CAT CAC CAT CT-3′; 5′-TTC ACA CCC ATG ACG AAC AT-3′) as described by Comstock *et al.* (2009).

Immunoblot analysis

Total cell lysate was prepared using standard methods, subjected to SDS-PAGE analysis and transferred to Immobilon-P membranes. Membranes were probed with antibodies against

RB (G3-245; BD Pharmingen, Rockville, MD, USA), ppRB Ser 780 (C84F6; Cell Signaling Technology, Boston, MA, USA), Cyclin A (C-19), $p27^{KIP1}$ (M-197), PCNA (PC10), RNRII (I-15), ERα (C-311) and Lamin B (M-20) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and RB (1F8) and Cyclin D1 (Ab-3) antibodies (NeoMarkers, Fremont, CA, USA). Lamin B served as a loading control.

β-Galactosidase assay

Cells were grown in the presence of PD-0332991, DMSO, FBS, CDT, or CDT/ICI 182 870 and were processed for β-galactosidase activity. Staining was carried out according to manufacturers provided protocol (Cell Signaling Technology). β-Galactosidase-positive cells were scored and calculated as a percentage of total population.

Chromatin immunoprecipitation assays

ChIP was carried out as previously described by Stengel *et al.* (2009). Briefly, cells were grown in the presence or absence of treatment, and cross-linked with formaldehyde. Next, cells were harvested and nuclei were isolated, lyzed, and sheared. Sheared chromatin was measured and equal amounts of chromatin from all conditions were pre-cleared with protein A/G beads (GE Healthcare). Ten percent of total chromatin was used as input control. The remaining chromatin was used for immunoprecipitation with antibodies against Sin3B (AK-2), Dbf4 (H-300), GFP (B-2) (Santa Cruz Biotechnology, Inc.), and RB (1F8, NeoMarkers). Immunoprecipitation was carried out in 500 μl of total volume of RIPA buffer containing sheared chromatin and protease inhibitors. Incubation of beads with extraction buffer (0.1 M $NaHCO_3$, 1% SDS, 0.3 M NaCl and 2 μl of 10 mg/ml RNase A) at 65 °C overnight was performed in order to de-crosslink. Immunoprecipitated DNA was purified using a PCR purification kit (Qiagen) and subjected to semi-qPCR.

PCR

Purified total chromatin or from ChIP was amplified by PCR using the following E2F target gene promoter primers: *Plk-1* – forward 5′-GGT TTG GTT TCC CAG GCT AT-3′, reverse 5′-GCT GGG AAC GTT ACA AAA GC-3′; *RNRII* – forward 5′-GAG GCA TGC ACA GCC ATT-3′, reverse 5′-GAC ACG GAG GGA GAG CAT AG-3′; *Cyclin A* – forward 5′-CCC CAG CCA GTT TGT TTC T-3′, reverse 5′-AGT TCA AGT ATC CCG CGA CT-3′; and negative control *Albumin* – forward 5′-CAG GGA TGG AAA GAA TCC TAT GCC-3′, reverse 5′-CCA TGT TCC CAT TCC TGC TGT-3′. Anti-Dbf4 IP and albumin promoter amplification served as a negative control. PCR-amplified products were subjected to 2% agarose gel electrophoresis and were visualized with ethidium bromide.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 4.0c) software (GraphPad Prism Software, Inc., La Jolla, CA, USA). All data were analyzed for statistical significance using Student's *t*-test and s.d. For all experiments, $P<0.05$ was considered significant. ***$P<0.0001$.

Results

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 (Bosco & Knudsen 2007, Sharma *et al.* 2010). Using an RB-loss expression signature that was developed previously from preclinical models (Ertel *et al.* 2010), the relationship of RB function to defined subtypes of ER-positive breast cancer was determined (Fig. 1). In a collection of

201 ER-positive breast cancer cases, classified as either luminal A or luminal B molecular subtypes, RB-signature expression were significantly associated with the luminal B subtype (Fig. 1A, $P<10^{-7}$). Specifically, luminal B breast cancer exhibited elevated expression of the signature indicative of RB loss of function (Fig. 1B). Importantly, luminal B breast cancers exhibit a poor durable response to endocrine therapy (Fig. 1C). Furthermore, the RB-signature further defined cases within the luminal B subtype that are sensitive (RB function) or resistant (RB deficient) to endocrine therapy (Fig. 1D). In spite of these provocative findings related to RB function, there was little evidence for loss of the *Rb1* transcript in ER-positive breast cancer (not shown). Together, these findings suggest that despite maintenance of the *Rb1* gene in ER-positive breast cancers that fail endocrine therapy, the function of RB is perturbed.

RB activation is important for the full response to estrogen antagonists

To decipher the functional involvement of RB-mediated transcriptional control in response to endocrine therapies, estrogen-dependent MCF-7 human breast cancer cell line 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 ER antagonist ICI 182 780 (ICI) in 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 (Fig. 2A). This hormone-directed therapy results in the degradation of the ERα and downregulation of cyclin D1 (Fig. 2B). In concert, an accumulation of $p27^{Kip1}$ and dephosphorylation of the RB tumor suppressor protein were observed, which were accompanied by downregulation of the RB/E2F-regulated gene cyclin A (Fig. 2B). These effects on transcription are associated with the specific recruitment of RB to target gene promoters, as detected by ChIP, only in the context of full cell cycle inhibition (Fig. 2C). Further experimentation demonstrated that this event is directly associated with the assembly of a Sin3b-containing repressor complex (Fig. 2D). Using shRNA approaches, RB protein levels were efficiently knocked down in MCF7 cells (Fig. 2E), and RB deficiency precluded the efficient assembly of Sin 3b repressor complexes (Fig. 2F), and limited the cell cycle arrest induced by estrogen withdrawal and estrogen antagonists (Fig. 2G). 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/acquired resistance. Thus, MCF7-derived cell models that were demonstrated to proliferate in the presence of continued exposure to endocrine therapies were utilized (Brunner *et al.* 1997, Wu *et al.* 2007, Coser *et al.* 2009). Acquisition of therapeutic resistance has been attributed to the selection of resistant sublines (i.e. LCC9, MCF7/FBS/Tam^R, and MCF7/CSS/Tam^R) or the presence of a subpopulation of cells that are intrinsically resistant to endocrine therapy (F100-16 and F40-7), as depicted in Fig. 3A (Brunner *et al.* 1997, Wu *et al.* 2007, Coser *et al.* 2009). Using these diverse models, their response to endocrine therapies was characterized. In LCC9 cells, treatment with ICI resulted in significant attenuation of ERα; however, cyclin D1 expression and RB phosphorylation were effectively maintained (Fig. 3B). Accordingly, RB was not engaged 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 (Supplementary Figure 1, see section on supplementary data given at the end of this article). Moreover, models resistant to tamoxifen demonstrated a failure to activate RB-mediated transcriptional repression in the presence of tamoxifen (Fig. 3E and Supplementary Figure 1, see section on supplementary data given at the end of this article). Cyclin D1 is regulated by complex coordination of protein and RNA levels; therefore, to determine how cyclin D1 levels are maintained in the context of endocrine-resistant models, qRT-PCR was performed. As shown, while basal levels of cyclin D1 RNA remained similar between the models, only the sensitive lines showed the attenuation of RNA levels and resultant suppression of cyclin D1 protein (Fig. 3F and Supplementary Figure 1, see section on supplementary data given at the end of this article). These findings suggest that compromised RB-mediated transcriptional control, presumably as a consequence of cyclin D1 deregulation, 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, it was postulated that activation of RB may represent a viable means to re-establish cell cycle inhibition downstream from endocrine therapy. To interrogate this possibility, the CDK4/6-specific inhibitor PD-0332991 was used to treat ICI-resistant LCC9 populations (Fry *et al.* 2004, Toogood *et al.* 2005). As shown, PD-0332991 resulted in the effective dephosphorylation of RB protein and the attenuation of the downstream target genes cyclin A and RNRII (Fig. 4A). Consonantly, effective recruitment of the RB protein to regulatory promoter elements (Cyclin A) was observed (Fig. 4B). These molecular events were associated with a profound inhibition of cell cycle progression as measured by BrdU incorporation (Fig. 4C). Importantly, similar response was observed across all of the models employed (Fig. 4D–F and Supplementary Figure 2, see section on supplementary data given at the end of this article). Thus, CDK4/6 inhibition represents a means to intercede in tumors that have failed endocrine therapy through deregulated RB phosphorylation and maintenance of E2F activity.

Therapeutic CDK4/6 inhibition promotes cellular senescence of breast cancer cells

Since there is a 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 and PD-0332991, and their ultimate influence on the proliferation of MCF7 cells. These data demonstrated that both PD-0332991 and ICI treatment are highly effective at mediating the suppression of cell growth in naïve MCF7 cells (Fig. 5A and B). However, while ICI treatment resulted in arrested cells, prolonged PD-0332991 exposure was specifically associated with the appearance of large, flat cellular morphology and β-galactosidase expression that is associated with induced senescence (Fig. 5C). Interestingly, while PD-0332991 treatment resulted in a significant fraction of cells staining positively for senescence-associated β-galactosidase (in agreement with other studies) (Michaud *et al.* 2010), endocrine therapy did not mediate this response (Fig. 5C and D). Thus, the induction of a senescence phenotype is specific to CDK4/6 inhibition, and not a general hallmark of cytostatic agents. In general, senescence is associated with cell cycle arrest in the presence of mitogenic signaling (Demidenko & Blagosklonny 2008, Demidenko *et al.* 2009). Since estrogen is a critical mitogen in ER-positive breast cancer cells, the coordinate impact of ICI and PD-0332991 was evaluated. Pre-treatment with ICI, while preventing proliferation, resulted in a significant reduction in the percent of senescent cells arising with PD-0332991 exposure (Supplementary Figure 3, see section on supplementary data given at the end of this article). Thus, these findings suggest that although both ICI and PD-0332991 result in inhibition of CDK4/6 activity, biological endpoints were distinct (i.e. quiescence versus senescence). 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), PD-0332991 remained effective at inducing durable cell cycle arrest and mediating the induction of senescence (Fig. 5E–G). Together, these studies underscore that distinct endocrine therapy and CDK4/6-inhibitors differ in their capacity to induce sustained growth arrest and senescence and ultimately mediate therapeutic response. Additionally, while events upstream of the RB/E2F axis are capable of mediating bypass of traditional endocrine-based therapies, restoration of RB function significantly impacts tumor cell growth.

Discussion

Approximately 70% of breast cancer patients are ER-positive, and have been treated with endocrine-based therapies as a first-line modality (Bosco & Knudsen 2007, Musgrove & Sutherland 2009,Hammond *et al.* 2010, Harris & McCormick 2010). While such therapies are initially effective, the acquisition of resistance represents a critical clinical problem that will influence ~50% of such patients during their lifetime (Riggins *et al.* 2007, Fan *et al.* 2009). Fortunately, there is an ever-increasing understanding of pathways which contribute to these therapeutic failures, and new diagnostic tools (e.g. OncotypeDx) to predict the course of disease (Paik 2007). Currently, due to the diverse mechanisms capable of driving and contributing to endocrine therapy bypass, most refractory lesions are treated with cytotoxic chemotherapies. The studies herein suggest that the use of agents which can re-establish cell cycle control – downstream from multiple signaling pathways – could be particularly useful in the treatment of both primary and refractory breast cancers.

Multiple pathways have been suggested to contribute to the bypass of endocrine therapy (Shou *et al.* 2004, Lee & Sicinski 2006, Perez-Tenorio *et al.* 2006,Musgrove & Sutherland 2009). Using models for acquired resistance, it is apparent that, in this context, the most prevalent mechanism of bypass is through signaling pathways that maintain mitogenic signaling to cyclin D1 in the absence of ER activity. In fact, in LCC9 cells, the activation of AKT and ERK are maintained in the presence of ICI (Supplementary Figure 4, see section on supplementary data given at the end of this article). This conclusion is similar to that observed in multiple xenograft models and in the analyses of primary tumors (Creighton *et al.* 2008, Massarweh *et al.* 2008, Musgrove & Sutherland 2009). In this context, a critical factor is the ability of such tumors to maintain sufficient cyclin D1/CDK activity to phosphorylate and inactivate RB. This signaling is likely quite complex, as overexpression of cyclin D1 is not sufficient to phenocopy the acquired resistance phenotype (Barnes & Gillett 1998, Pacilio *et al.* 1998,Li *et al.* 2003).

Importantly, the findings described above suggest that many tumors that progress in the context of ER antagonists may harbor a largely intact cell cycle control program. This finding differs substantially from cytotoxic therapies, wherein massive evolution/heterogeneity of the refractory tumors may compromise any targeted second-line therapy (McClendon *et al.* 2010, Oliver *et al.* 2010). To directly test the concept of therapeutically targeting the cell cycle machinery in this specific setting, the PD-0332991 compound was used to treat endocrine therapy-resistant breast cancer cells. Unlike first-generation CDK-inhibitors (e.g. flavopiridol), this compound is highly specific for CDK4/6 and will specifically inhibit the proliferation of cells with an intact RB pathway (Fry *et al.* 2004, Toogood *et al.* 2005, Finn *et al.* 2009, Dean *et al.* 2010, Rivadeneira *et al.* 2010). Among all of the models that represent intrinsic or acquired resistance used herein, PD-0332991 was exceedingly effective in limiting cell cycle progression. These findings directly support the concept (currently being tested in a phase II clinical trial) that PD-0332991 may forestall progression of ER-positive disease. Whether concordant treatment is the most appropriate means to use this agent in the clinic is unclear. However, the results herein would suggest

that CDK4/6 inhibitors could be effectively employed as a second-line therapy. In spite of that promise, there are clearly mechanisms for intrinsic resistance to such compounds (i.e. RB loss) or mechanisms for the evolution of resistance. However, in specific tumor types (e.g. malignant teratoma), PD-0332991 was shown to forestall disease progression for >1 year, indicating that such agents can clearly yield clinical benefit. One of the more interesting aspects of endocrine-based therapies pertains to the induction of a cell cycle arrest that is indicative of cellular quiescence. In contrast, PD-0332991 induces a cell cycle arrest that is associated with the molecular and physiological characteristics of cellular senescence. Thus, there are intrinsic differences through which these cytostatic therapies ultimately impinge on tumor cell biology that could be exploited in the context of therapeutic scheduling.

In sum, the aforementioned studies indicate that upstream deregulation of the RB pathway is an important determinant of the therapeutic response to endocrine-based therapies. However, restoration of RB-mediated tumor suppressive activity represents a key means to consider in the treatment of those tumors that fail first-line hormonal therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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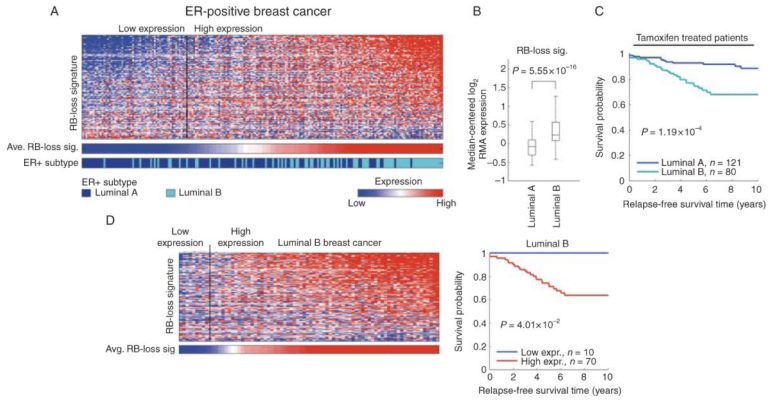

Figure 1.

Differential RB-loss signature expression and survival among Luminal A and Luminal B breast cancer subtypes. (A) Gene expression heatmap depicting Luminal A and Luminal B breast cancer samples arranged by RB-loss signature expression level. (B) Boxplots depicting differential expression of the RB-loss signature in Luminal A and Luminal B subtypes. (C) Kaplan–Meier curves for relapse-free survival in Luminal A and Luminal B breast cancer subpopulations. (D) Stratification of Luminal B breast cancers based on an optimized RB-signature threshold and associated Kaplan–Meier curves for relapse-free survival.

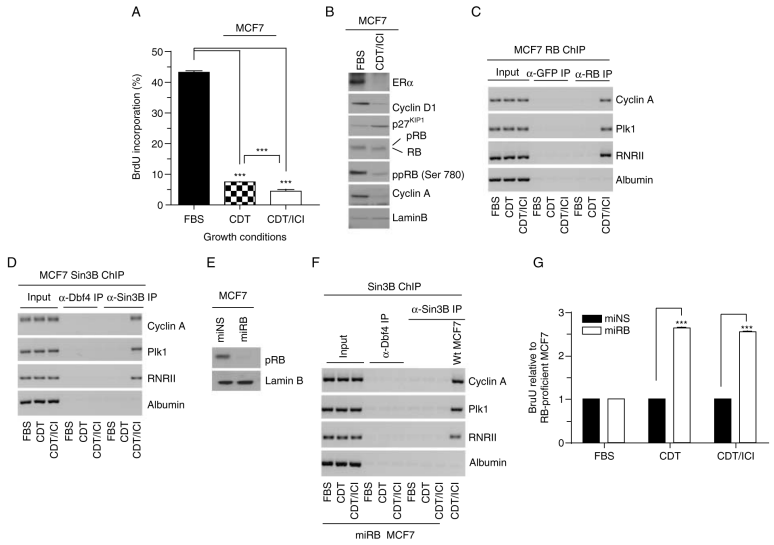

Figure 2.

Response to anti-estrogen therapy requires RB-mediated transcriptional repression. (A) Quantification of percent BrdU incorporation in MCF7 cells exposed to FBS, CDT, or CDT/ICI 182 780 (1.0 μM). (B) Immunoblot analysis of ERα, cyclin D1, $p27^{KIP1}$, pRB, ppRB (ser 780), cyclin A, and Lamin B in MCF7 cells exposed to FBS or CDT/ICI. (C) Chromatin immunoprecipitation experiments to examine the presence of RB at E2F target gene promoters in MCF7 cells treated with FBS or CDT/ICI. α-GFP IP and albumin promoters served as negative control and input lanes served as positive control. (D) Chromatin immunoprecipitation analysis to examine localization of Sin3B co-repressor at E2F target gene promoters in response to anti-estrogen exposure. α-Dbf4 IP and albumin promoters served as negative control and input lanes served as positive control. (E). Immunoblot analysis of pRB in miNS- and miRB-expressing MCF7 cells. (F) Sin3B ChIP assay on miRB-expressing MCF7 cells in response to FBS, CDT or CDT/ICI (1.0 μM) with CDT/ICI-treated wild-type MCF7 cells as a positive control. (G) Quantification of relative percent BrdU incorporation in miNS- and miRB-expressing MCF7 cells exposed to FBS, CDT, or CDT/ICI. ***, $P<0.0005$.

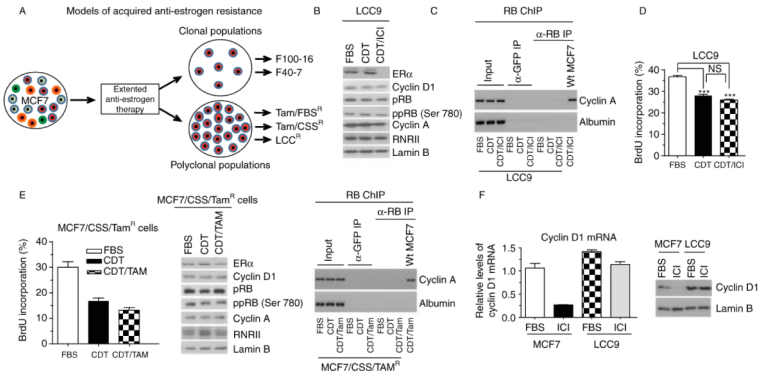

Figure 3.

Inability of RB to mediate repression of gene transcription in models of anti-estrogen resistance. (A) Schematic diagram demonstrating the generation of multiple models of anti-estrogen resistance. (B) Immunoblot analysis of ERα, cyclin D1, pRB, ppRB (ser 780), cyclin A, RNR II, and Lamin B in ICI-resistant LCC9 cells postexposure to FBS, CDT, or CDT/ICI for 72 h. (C) Detection of RB protein on E2F target gene promoters cyclin A by chromatin immunoprecipitation assay in ICI-resistant LCC9 cells with CDT/ICI-treated wild-type MCF7 cells as a positive control. (D) Percent BrdU incorporation of ICI 182 780-resistant LCC9 cells postexposure to FBS, CDT, or CDT/ICI for 48 h. (E) BrdU incorporation, protein expression, and RB chromatin immunoprecipitation to examine occupancy on cyclin A promoter in MCF7/CSS/Tam^R cells exposed to FBS, CDT, or CDT/Tamoxifen for 72 h. (F) Analyses of cyclin D1 mRNA levels by qRT-PCR and concurrent analyses of protein levels from MCF7 and LCC9 models. ***, P<0.0005.

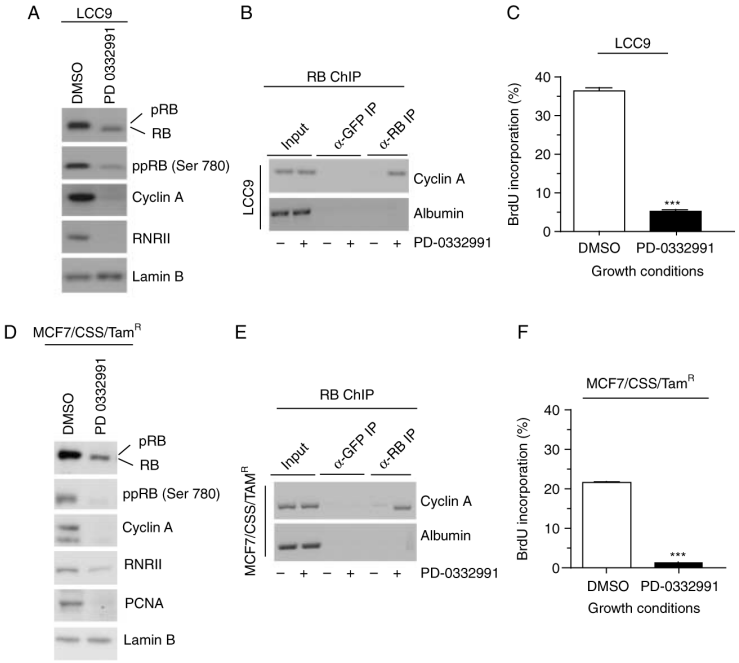

Figure 4.

CDK4/6 inhibition promotes RB-mediated transcriptional repression and decreases cellular proliferation in models of antiestrogen resistance. (A) Immunoblot analysis of pRB, ppRB (Ser 780), cyclin A, RNRII, and Lamin B (loading control) in LCC9 cells in response to DMSO or PD-0332991 (500 nM) for 24 h. (B) Identification of pRB on E2F target gene promoter cyclin A by ChIP in LCC9 cells treated with 500 nM PD-0332991 for 24 h (E2F target gene cyclin A as experimental gene, α-GFP IP and albumin promoter as negative controls and input served as positive control). (C) Percent BrdU incorporation in ICI 182 780-resistant LCC9 cells in response to 500 nM PD-0332991 for 24 h. (D) Immunoblot analysis of pRB, ppRB (ser 780), E2F target genes cyclin A, RNRII, PCNA, and Lamin B (loading control) in Tamoxifen-resistant MCF7/CSS/Tam^R cells postexposure to DMSO or PD-0332991 (500 nM) for 24 h. (E) Identification of pRB on E2F target gene promoter cyclin A by ChIP in Tamoxifen-resistant MCF7/CSS/Tam^R cells in response to DMSO or PD-0332991 (500 nM) exposed for 24 h (E2F target gene cyclin A as experimental gene, α-GFP IP and albumin promoter as negative controls, and input served as positive control). (F) Percentage BrdU incorporation in Tamoxifen-resistant MCF7/CSS/Tam^R cells in response to DMSO and PD-0332991 (500 nM) exposed for 24 h. ***, $P<0.0005$.

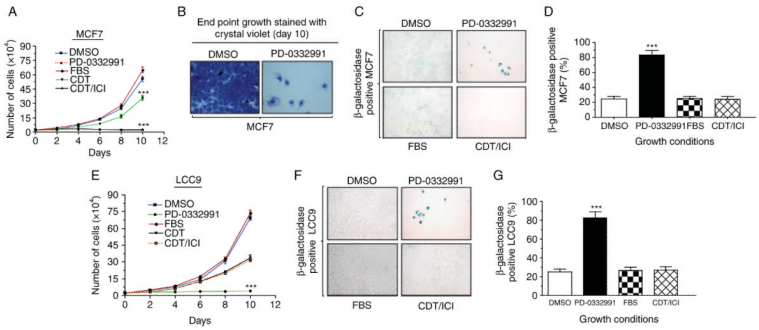

Figure 5.

Long-term proliferation control and pRB-mediated cellular senescence in MCF7 and LCC9 cells in response to ICI 182 780 and PD-0332991. (A) Growth of MCF7 cells in response to DMSO, PD-0332991 (500 nM), FBS, CDT, or CDT/ICI (1.0 μM) treated for 10 days. (B) Representative bright field microscopy images of crystal violet-stained MCF7 cells treated with PD-0332991 and vehicle (DMSO) for 10 days. (C) Representative images of β-galactosidase-positive MCF7 cells treated with DMSO, PD-0332991, FBS, and CDT/ICI. (D) Graphic representation of percentage (%) of β-galactosidase-positive MCF7 cells treated with DMSO, PD-0332991, FBS, or CDT/ICI. (E) Growth curve of ICI-resistant LCC9 cells in response to DMSO, PD-0332991 (500 nM), FBS, CDT, or CDT/ICI (1.0 μM) exposed for 10 days. (F) Representative microscopy images of β-galactosidase-positive LCC9 cells treated with DMSO, PD-0332991 (500 nM), FBS, or CDT/ICI (1.0 μM). (G) Graphic representation of percentage (%) of β-galactosidase-positive LCC9 cells treated with DMSO, PD-0332991 (500 nM), FBS, or CDT/ICI (1.0 μM). ***, $P<0.0005$.