Research Article

Cyclin D1 Is Necessary for Tamoxifen-Induced Cell Cycle Progression in Human Breast Cancer Cells

Robin L. Kilker and Maricarmen D. Planas-Silva

Department of Pharmacology, Penn State College of Medicine, Hershey, Pennsylvania

Abstract

Despite the success of tamoxifen in treating hormone-responsive breast cancer, its use is limited by the development of resistance to the drug. Understanding the pathways involved in the growth of tamoxifen-resistant cells may lead to new ways to treat tamoxifen-resistant breast cancer. Here, we investigate the role of cyclin D1, a mediator of estrogen-dependent proliferation, in growth of tamoxifen-resistant cells using a cell culture model of acquired resistance to tamoxifen. We show that tamoxifen and 4-hydroxytamoxifen (OHT) promoted cell cycle progression of tamoxifen-resistant cells after growth-arrest mediated by the estrogen receptor down-regulator ICI 182,780. Down-regulation of cyclin D1 with small interfering RNA blocked basal cell growth of tamoxifen-resistant cells and induction of cell proliferation by OHT. In addition, pharmacologic inhibition of phosphatidylinositol 3-kinase/Akt or mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 pathways decreased basal cyclin D1 expression and impaired OHT-mediated cyclin D1 induction and cell cycle progression. These findings indicate that cyclin D1 expression is necessary for proliferation of tamoxifen-resistant cells and for tamoxifen-induced cell cycle progression. These results suggest that therapeutic strategies to block cyclin D1 expression or function may inhibit development and growth of tamoxifen-resistant tumors. (Cancer Res 2006; 66(23): 11478-84)

Introduction

Tamoxifen, a selective estrogen receptor (ER) modulator, has been a mainstay in treating ER-positive breast cancer. Tamoxifen use is limited, however, by the presence (*de novo* resistance) or acquisition of tamoxifen-resistant growth (acquired resistance). Therefore, to inhibit growth of tamoxifen-resistant breast tumors, it is important to understand the pathways driving tamoxifen-resistant growth. Several mechanisms have been proposed to explain tamoxifen resistance (1–3). Commonly, tamoxifen-resistant tumors retain functional ER and, therefore, can respond to other hormone therapies, such as aromatase inhibitors or ER down-regulators (4, 5). In some tissues, such as the uterus, tamoxifen acts as a partial agonist of ER and stimulates cell proliferation (6). Thus, it is possible that a subset of tamoxifen-resistant tumors may acquire the ability to use tamoxifen as a partial agonist (7). In these cases, it is likely that tamoxifen mimics the effect of estrogen in cell cycle proliferation.

A key regulatory molecule mediating estrogen-dependent proliferation is cyclin D1 (8, 9). Blocking cyclin D1 function can prevent estrogen-dependent proliferation (10), whereas ectopic expression of cyclin D1 can abrogate antiestrogen-mediated arrest (11, 12) or rescue cells from an antiestrogen-induced arrest (13, 14). Moreover, growth factors that induce cyclin D1 may promote tamoxifen-resistant growth (15). In several, but not all, clinical studies, overexpression of cyclin D1 in breast tumors correlated with early relapse, poor prognosis, and refractoriness to tamoxifen treatment (16–21). Collectively, these studies suggest that cyclin D1 is a key mediator of estrogen-dependent proliferation.

The role of cyclin D1 in proliferation of tamoxifen-resistant cells is unknown. Although cyclin D1 is crucial for the growth of tamoxifen-sensitive breast cancer cells (10, 22), it is possible that mechanisms leading to tamoxifen-resistant growth may override the need for cyclin D1. The potential different roles of cyclin D1 in breast cancer development have been elegantly shown using animal models of breast cancer (23). Transgenic mice expressing the *ras* and *neu* oncogene in their mammary glands develop breast tumors. However, when these mice are crossed with cyclin D1 knockout mice, tumor formation is prevented in the absence cyclin D1. In contrast, transgenic *myc* or *Wnt-1* mice can still cause breast tumors, in spite of the lack of cyclin D1 expression. All these studies indicate that, depending on the oncogenic pathway driving tumor growth, cyclin D1 can play an essential role in breast cancer development and growth.

Multiple mechanisms and signaling pathways can regulate cyclin D1 expression. In breast cancer cells showing *de novo* resistance to tamoxifen due to overexpression of HER-2/*neu*, the ability of tamoxifen to promote growth was correlated with activation of phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) 1/2 pathways and cyclin D1 induction (24). In tamoxifen-sensitive cells, several studies have shown that inhibiting PI3K/Akt or MAPK/ERK1/2 activity impairs estrogen-dependent cyclin D1 expression and proliferation (25–28). The role of the PI3K/Akt or MAPK/ERK1/2 activity in cyclin D1 expression has not yet been investigated in cells with acquired resistance to tamoxifen.

We described previously development and characterization of a cell culture model of acquired resistance to tamoxifen derived from MCF-7 cells (29). In the present study, using a variant of MCF-7 cells whose proliferation is not inhibited by tamoxifen, we examined the requirement of cyclin D1 in growth of these human breast cancer cells that now can show tamoxifen-induced cell proliferation. In addition, we determine whether PI3K/Akt and MAPK/ERK1/2 signaling pathways regulate cyclin D1 expression in these cells. Here, we provide evidence that cyclin D1 is required for tamoxifen-induced cell proliferation and growth of tamoxifen-resistant cells and that both PI3K/Akt and MAPK/ERK1/2 signaling pathways regulate cyclin D1 expression in tamoxifen-resistant cells.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Maricarmen D. Planas-Silva, Department of Pharmacology (H078), Penn State College of Medicine, 500 University Drive, Hershey, PA 17033. Phone: 717-531-4569; Fax: 717-531-5091; E-mail: mcplanas@psu.edu.

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Materials and Methods

Cell culture. MCF-7 cells were grown in phenol red–containing DMEM [Irvine Scientific (Santa Ana, CA) or Life Technologies/Invitrogen (Carlsbad, CA)] supplemented with 5% fetal bovine serum (FBS) and antibiotics. Tamoxifen-resistant variants were developed as described previously (29). The tamoxifen-resistant cells (MTR-3) were maintained in phenol red–free DMEM (Irvine Scientific or Life Technologies) supplemented with 5% charcoal/dextran-stripped FBS (CSS; Hyclone, Logan, UT), antibiotics, and 1 μmol/L tamoxifen (Sigma, St. Louis, MO).

Cell proliferation assays. Before seeding, MTR-3 cells were cultured for 9 to 16 days in CSS medium without tamoxifen. Cells were then plated at approximately 4×10^4 to 6×10^4 per well on 12-well plates in CSS medium. MCF-7 cells were plated at approximately 1.2×10^5 on six-well plates in FBS medium. At day 0, both MCF-7 and MTR-3 cells were switched to CSS medium with or without 5 nmol/L estradiol (E_2) or 1 μmol/L tamoxifen. Medium was changed on days 2 and 4. On day 5, cells were fixed, sulforhodamine B assay (SRB) was done (30), and absorbance of wells was measured at 570 nm.

Cell cycle arrest and rescue. MCF-7 and MTR-3 cells were plated at approximately 1.2×10^5 per well on six-well plates in FBS or CSS medium, respectively. For cell cycle arrest, MCF-7 and MTR-3 cells were given CSS medium containing either ICI 164,384 or ICI 182,780 (ICI; Tocris, Ellisville, MO) for 48 hours (29). At this time (t = 0 hour), the indicated hormones were added directly to cultures or in fresh CSS medium. Thymidine incorporation assay was done at indicated times as described previously (31). For studies using signaling pathway inhibitors, DMSO vehicle, PD98059 (Calbiochem, San Diego, CA), or LY294002 (Tocris) was added, directly or in fresh medium, 30 minutes before hormone rescue.

Small interfering RNA studies. Cells were plated in CSS medium without antibiotics at 4×10^4 to 6×10^4 per well on a 12-well plate or 1×10^5 to 1.5×10^5 per well on a six-well plate. Cells were mock transfected with nothing added or transfected with reagent alone, 80 nmol/L nonspecific pooled small interfering RNA (siRNA), or 80 nmol/L anti-cyclin D1 pooled siRNA (Dharmacon, Lafayette, CO) using LipofectAMINE Plus or LipofectAMINE 2000 (Invitrogen) as per manufacturer's instructions. Medium was changed after 1 day and every other day thereafter until the end of the experiment. SRB or thymidine assays were done on indicated days. For rescue experiments, ICI was added to the fresh medium on the day following transfection and rescue was done after 48 hours.

Western immunoblot analyses. Preparation of cell extracts was done using conditions described previously (31). Equal amounts of total protein were electrophoresed on 4% to 12% polyacrylamide Bis-Tris gradient gels (Invitrogen) and transferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA) for blotting. Immunoblotting was done with antibodies specific for cyclin D1 [DCS-6 (Santa Cruz Biotechnology, Santa Cruz, CA), HD45 (31), or AB-3 (Neomarkers, Fremont, CA)], phosphorylated Akt (Ser^{473}, Cell Signaling, Beverly, MA), Akt1/2, phosphorylated ERK1/2, ERK2, and enolase (Santa Cruz Biotechnology) followed by enhanced chemiluminescence detection (Amersham Biosciences, Piscataway, NJ). For quantitation of immunoblots, immunoblot image was analyzed with LAS-1000 image analyzer.

Statistical analyses. Experimental values are means ± SD of representative experiments or an average of multiple experiments ± SE. To determine if the differences between two groups were significant, the Student's t test was used. For experiments with multiple groups, significance was determined using one-way ANOVA. Where significance was established in the ANOVA test, significance between groups was evaluated with the Student's t test.

Results

Tamoxifen stimulates growth of MTR-3 cells. We first assessed the effect of estrogen and tamoxifen on the growth of MTR-3 and MCF-7 cells. MCF-7 and MTR-3 grew to comparable levels in the presence of estrogen (Fig. 1*A*). Tamoxifen, as expected, reduced the growth of parental, tamoxifen-sensitive, MCF-7 cells compared with control (CSS) medium. However, in tamoxifen-resistant MTR-3 cells, tamoxifen increased growth relative to control medium. After growing in 1 μmol/L tamoxifen for 5 days, there was a significant difference ($P = 0.0002$) between growth of MTR-3 cells and that of MCF-7 cells (Fig. 1*B*). Furthermore, as we have reported previously (29), MTR-3 cells remained estrogen sensitive and, therefore, responded to the full agonistic effects of estrogen with growth stimulation over control medium. However, the effect of tamoxifen in the growth of tamoxifen-resistant cells was only a fraction of the effect of estrogen, indicating that, with regard to cell proliferation, tamoxifen acted as a partial agonist in tamoxifen-resistant cells.

Tamoxifen and 4-hydroxytamoxifen promote S-phase progression. To evaluate cell cycle progression in response to tamoxifen, we needed first to arrest cells in G_0/G_1. We have shown previously that MTR-3 cells remain sensitive to the growth-inhibitory effects of ICI 182,780 or its analogue ICI 164,384 (29), compounds that down-regulate the ER and cause cell cycle arrest in G_0/G_1. Therefore, after arresting the cells with these compounds, we evaluated whether tamoxifen can promote cell cycle reentry. Tamoxifen was able to promote entry into the S phase of the cell cycle in MTR-3 cells but not in the parental MCF-7 cells (Fig. 2*A*). These results suggested that, in tamoxifen-resistant cells, tamoxifen mimics estrogen effects in promoting cell cycle reentry.

To further evaluate this response, we compared the effect of tamoxifen, its metabolite, 4-hydroxytamoxifen (OHT), and E_2 on induction of cell cycle progression. E_2 would be expected to give the highest induction as per its full agonistic properties. OHT is a more potent form of tamoxifen; therefore, we suspected that it would compete against ICI more effectively than its parent compound. Indeed, even at 100-fold lower concentrations, OHT

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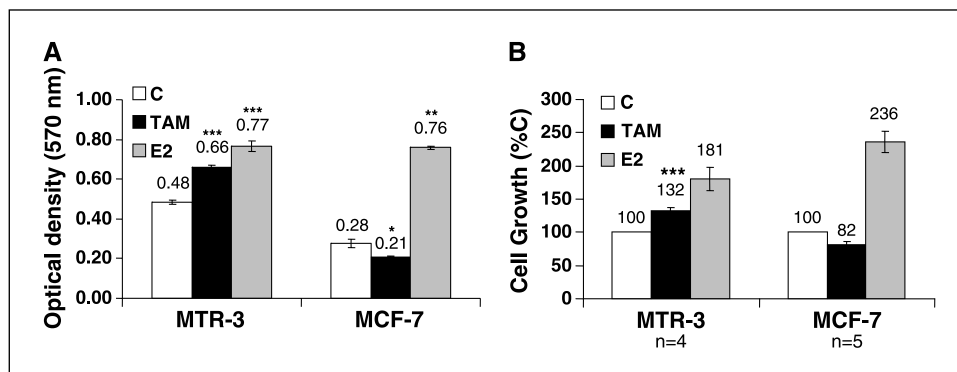

Figure 1. Tamoxifen induces growth in MTR-3 cells but not in MCF-7 cells. At the start of the experiment, medium was changed to CSS medium with 0.1% ethanol control (*C*), 1 μmol/L tamoxifen (*TAM*), or 5 nmol/L E_2. *A,* relative cell number was determined by SRB assay. Representative experiment of at least four independent experiments. *, $P = 0.046$, significantly different than control; **, $P = 0.001$; ***, $P = 0.0001$. *B,* values were normalized to percentage of control for each cell line. *Columns,* average of four independent experiments; *bars,* SE. ***, $P = 0.0002$, significantly different than MCF-7.

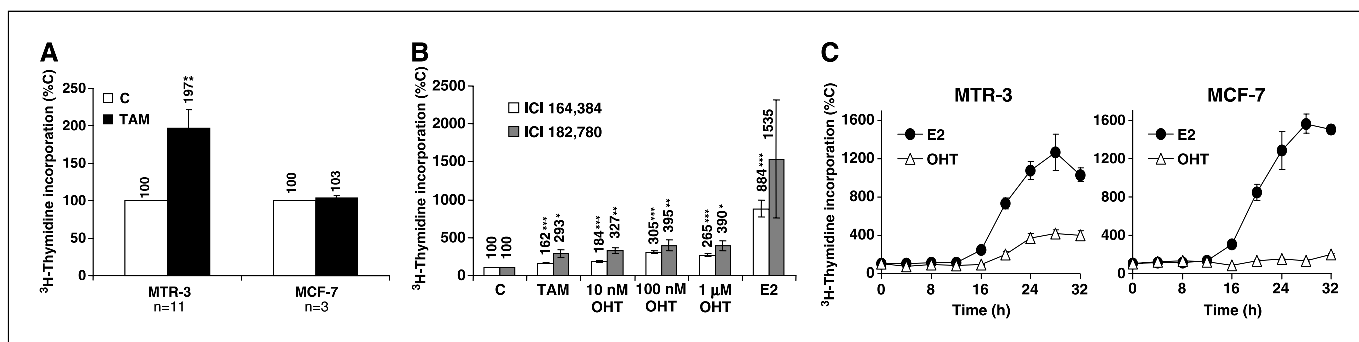

Figure 2. Tamoxifen and OHT can stimulate cell cycle progression in MTR-3 cells. *A,* cells were treated with CSS medium plus 50 nmol/L ICI 164,384. After 48 hours, 0.1% ethanol control or 1 μmol/L tamoxifen was added to the culture medium. [^{3}H]thymidine incorporation was done 24 hours following rescue hormone treatment. *Columns,* mean of at least three independent experiments; *bars,* SE. **, $P = 0.02$, significantly different from control. *B,* MTR-3 cells were cultured in CSS medium with 50 nmol/L ICI for 48 hours. Then, cells were treated with the following: 0.1% ethanol control; 1 μmol/L tamoxifen; 10 nmol/L, 100 nmol/L, or 1 μmol/L OHT; or 5 nmol/L E_2. [^{3}H]thymidine incorporation was done 24 hours later. *Columns,* average of at least four independent experiments; *bars,* SE. *, $P < 0.02$, significantly different from control; **, $P \leq 0.01$; ***, $P \leq 0.001$. *C,* following 48 hours of incubation with 50 nmol/L ICI, cells were treated with either 1 μmol/L OHT or 5 nmol/L E_2. [^{3}H]thymidine incorporation was done at the indicated time points. Data are representative of at least three independent similar experiments done with ICI 164,384 or ICI 182,780. *Points,* average of triplicate wells; *bars,* SD.

produced a more robust cell cycle induction than 1 μmol/L tamoxifen (Fig. 2*B*). Nevertheless, as the highest induction was achieved with at least 100 nmol/L OHT or with 1 μmol/L OHT, we used 1 μmol/L OHT in all subsequent experiments. Comparing the ICI analogues, 164,384 and 182,780, we found, as expected, similar activities (Fig. 2*B*). Because of its clinical relevance and its commercial availability, we continued to use the ICI 182,780 (fulvestrant) compound for further studies. It is of note that even the strongest response with OHT fell short of the induction achieved with 5 nmol/L E_2, indicative of the partial agonistic effects of OHT.

To study the kinetics of the cell cycle induction, we did a time course study. Estrogen promotes cell cycle progression in both cell lines. In contrast, S-phase progression was induced by OHT in MTR-3 cells but not in MCF-7 cells (Fig. 2*C*). Thymidine incorporation peaked at approximately 24 to 28 hours and we used the 24-hour time point for further studies. Therefore, all these data indicate that tamoxifen promotes proliferation of tamoxifen-resistant cells by acting as a partial agonist of ER.

Cyclin D1 is induced by OHT in tamoxifen-resistant cells. The estrogenic proliferative response in MCF-7 cells is mediated through up-regulation of cyclin D1. We have shown previously (29) that, in MTR-3 cells cyclin, D1 levels remain under ER regulation; therefore, cyclin D1 levels are reduced by treatment with ICI. Because OHT can overcome ICI arrest in the tamoxifen-resistant cells, similar to the ability of estrogen in parental cells, we hypothesized that OHT promotes MTR-3 cell cycle progression through cyclin D1 up-regulation as well. Addition of OHT to ICI-arrested MTR-3 cells increased levels of cyclin D1 expression (Fig. 3*A*). Again, this effect was not as potent as the response to estrogen. These data correlate with the effect observed in S-phase progression showing that OHT is acting as a partial agonist. Quantification reveals that, in 6 to 8 hours, E_2 produces a 2-fold induction (206 ± 14%; $P < 0.0005$; Fig. 3*B*). Cyclin D1 induction by OHT is also significant, falling slightly short of that by E_2 (179 ± 17%; $P < 0.0005$). In tamoxifen-sensitive MCF-7 cells, addition of E_2 to ICI-arrested cells also produced a 2-fold induction of cyclin D1 expression (Fig. 3*C* and *D*). However, in contrast to what was observed in MTR-3 cells, addition of OHT to arrested MCF-7 cells did not increase the levels of cyclin D1 expression (Fig. 3*C* and *D*). Therefore, these results support a role for cyclin D1 in tamoxifen-induced cell cycle progression in cells that have acquired resistance to tamoxifen.

Cyclin D1 is necessary for S-phase progression and optimal cell growth. To determine the necessity for cyclin D1 in growth and cell cycle progression of tamoxifen-resistant cells, we used siRNA technology. A siRNA pool against cyclin D1 was able to reduce protein levels by 78 ± 5% compared with a control siRNA consisting of a pool of nonspecific sequence (Fig. 4*A* and *B*). This reduction in cyclin D1 correlated with reduced S-phase progression and reduced proliferation. Cells treated with cyclin D1 siRNA incorporated tritiated thymidine at a significantly reduced level (50 ± 9%; $P = 0.007$) compared with a nonspecific pool siRNA control (Fig. 4*C*). In parallel, growth was significantly reduced in cyclin D1 siRNA-treated cells over control (58 ± 10%; $P = 0.003$; Fig. 4*D*). These data indicate that cyclin D1 expression is required for basal growth of tamoxifen-resistant cells.

Cyclin D1 is necessary for OHT-induced cell cycle progression. After establishing that cyclin D1 is necessary for basal growth of MTR-3 cells, we sought to determine whether abrogation of cyclin D1 would affect the ability of OHT to rescue the cells from an ICI-mediated cell cycle arrest. In control samples, OHT treatment mediated a significant (367 ± 7%) induction of cell cycle progression into the S phase. In contrast, OHT-mediated cell cycle progression was blocked significantly ($P = 0.03$) in tamoxifen-resistant cells treated with siRNA against cyclin D1 (Fig. 5). Estrogen induction was also reduced significantly (951 ± 14% to 287 ± 24%; $P = 0.009$). Therefore, reducing cyclin D1 levels is sufficient to inhibit the partial agonist response to OHT as well as the response to estrogen in tamoxifen-resistant cells.

Pharmacologic inhibition of PI3K/Akt or MAPK/ERK1/2 pathways in tamoxifen-resistant cells. To identify alternative ways to reduce cyclin D1 expression in tamoxifen-resistant cells, we evaluated whether cyclin D1 expression requires the PI3K/Akt and MAPK/ERK1/2 pathways, as has been established for tamoxifen-sensitive cells (27, 28). To pursue this part of the study, we first did dose-response studies with pharmacologic inhibitors of these pathways to determine the minimum dose that efficiently blocks the respective signaling pathways in cells with acquired resistance to tamoxifen. Specifically, we showed that LY294002, an inhibitor of PI3K, can block phosphorylation of Akt, a PI3K-dependent event, in a dose-dependent manner (Supplementary Fig. S1). In addition,

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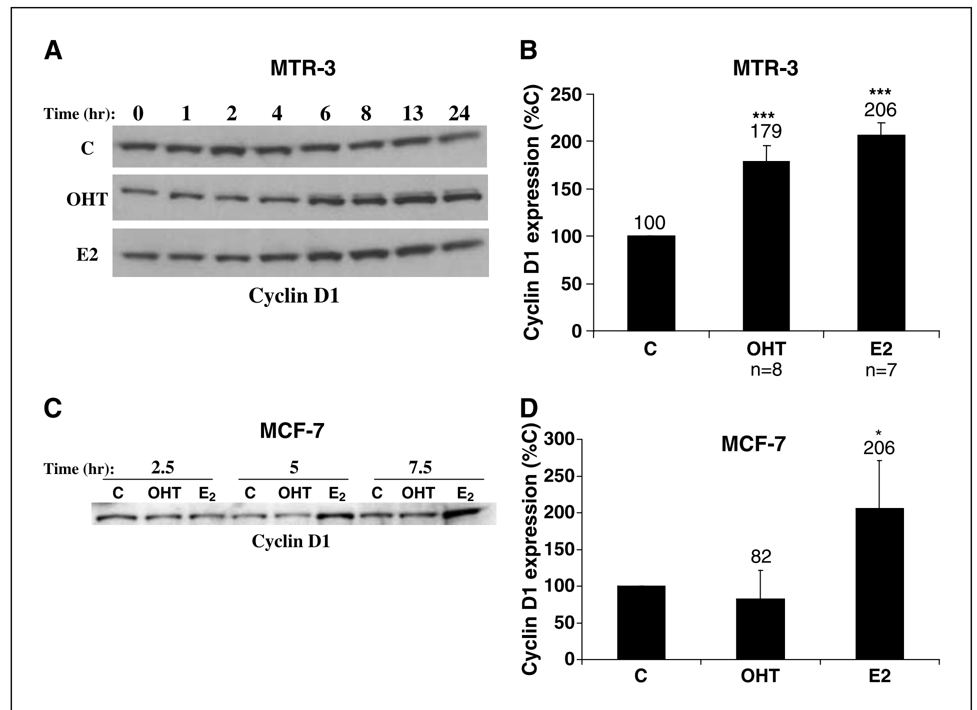

Figure 3. Cyclin D1 expression is induced by OHT in MTR-3 cells but not in MCF-7 cells. After 48 hours of treatment with ICI 182,780, either 0.1% ethanol control, 5 nmol/L E_2, or 1 μmol/L OHT was added to the medium. *A,* equal amounts of protein from MTR-3 cell lysates obtained from cells treated for the indicated times were used to evaluate cyclin D1 expression by Western blotting. Representative blot of at least four similar experiments done after cell cycle arrest with either 10 nmol/L or 50 nmol/L ICI. *B,* average cyclin D1 induction in MTR-3 cells after 6 to 8 hours of treatment, quantified from seven or eight independent experiments. ***, $P < 0.0005$, significantly different from control. *C,* cyclin D1 expression in MCF-7 cells treated as indicated after cell cycle arrest with 10 nmol/L ICI. *D,* quantification of the changes in cyclin D1 expression in parental MCF-7 cells measured between 6 to 8 hours of treatment after ICI-mediated cell cycle arrest. *Columns,* mean of four independent experiments; *bars,* SD. *, $P = 0.017$, significantly different from control.

PD98059, an inhibitor of the MAPK/ERK1/2 pathway, blocked signaling as measured by phosphorylation of ERK1/2 (Supplementary Fig. S1). Therefore, to examine the roles of these signal transduction pathways in the growth of tamoxifen-resistant cells, we used, in subsequent experiments, concentrations of these compounds that effectively inhibit the specific signaling pathway in these tamoxifen-resistant cells.

Requirement of PI3K/Akt and MAPK/ERK1/2 pathways on cyclin D1 expression in tamoxifen-resistant cells. To determine whether these signal transduction inhibitors affect cyclin D1 expression in tamoxifen-resistant cells, we first evaluated their effects on asynchronously growing MTR-3 cells. Treatment with the signal transduction pathway blockers quickly reduced levels of cyclin D1 (Fig. 6*A*). PD98059 decreased cyclin D1 levels but not as efficiently as LY294002. These results suggest that cyclin D1 expression in tamoxifen-resistant cells is more sensitive to inhibition of the PI3K pathway.

PI3K/Akt and MAPK/ERK1/2 pathways are involved in OHT-induced cell cycle progression and cyclin D1 expression. To examine the possible roles of the PI3K/Akt and MAPK/ERK1/2

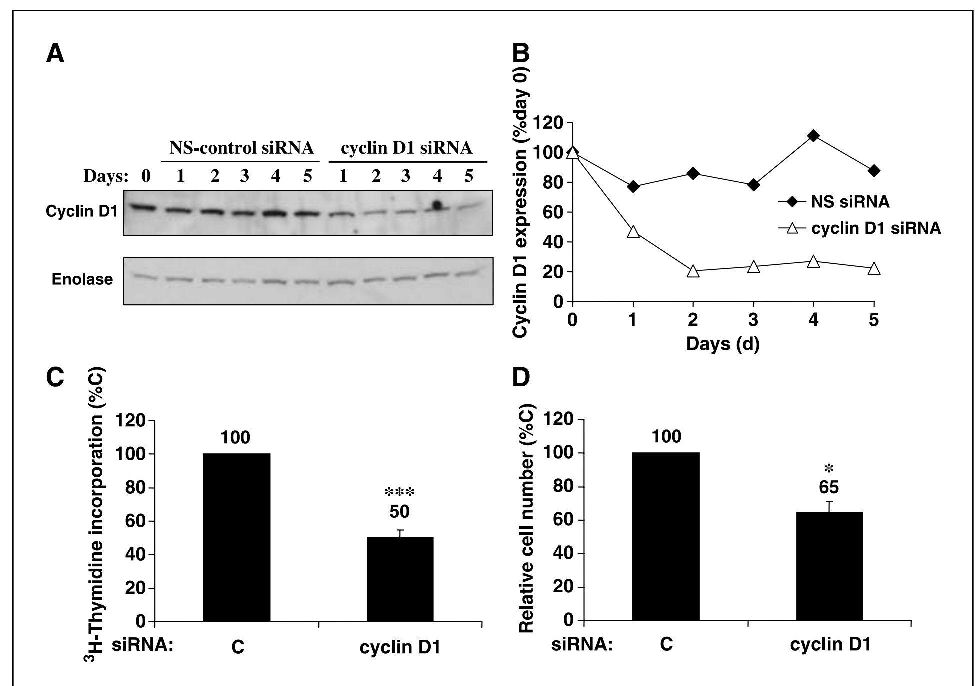

Figure 4. Cyclin D1 knockdown blocks growth of tamoxifen-resistant cells. MTR-3 cells were transfected with cyclin D1 siRNA or nonspecific control (*NS*) siRNA. *A,* cells were harvested each day for 5 days and proteins from cell extracts were immunoblotted for cyclin D1 and enolase as a loading control. Representative of at least three similar independent experiments. *B,* graphic representation of cyclin D1 blot quantification. *C,* [^{3}H]thymidine incorporation was done on day 5. *Columns,* average of three independent experiments; *bars,* SE. ***, $P = 0.0003$, significantly different from control. *D,* cell growth was assessed on day 4 or 5 by SRB assay. *Columns,* mean; *bars,* SE. *, $P = 0.03$, significantly different from control.

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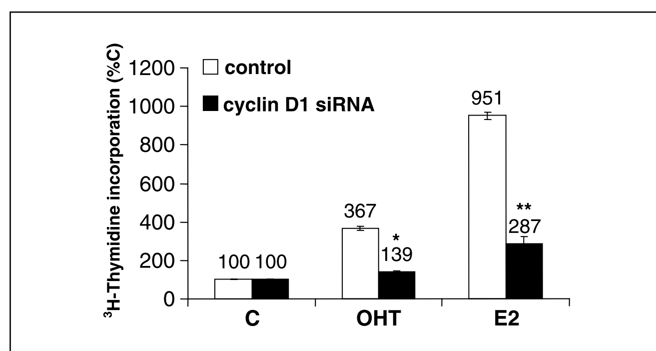

Figure 5. Cyclin D1 knockdown impairs the ability of OHT to induce cell cycle progression. At the start of the experiment, MTR-3 cells were transfected with cyclin D1 siRNA or reagent control. After 24 hours, medium was changed to CSS medium without antibiotics, with 10 nmol/L ICI 182,780. Following 48 hours of incubation, medium was changed to CSS medium with either 0.1% ethanol control, 1 μmol/L OHT, or 5 nmol/L E_2. [^{3}H]thymidine incorporation was done 24 hours later. *Columns,* mean; *bars,* SE. *, *P* = 0.03, cyclin D1 siRNA-transfected significantly different than control; **, *P* = 0.009, cyclin D1 siRNA-transfected significantly different than control.

pathways in the agonistic effects of OHT, we did rescue experiments from an ICI-mediated cell cycle arrest in the presence or absence of the inhibitors LY294002 and PD98059, respectively. The PI3K inhibitor, LY294002, effectively blocked the OHT agonistic response (*P* = 0.05; Fig. 6*B*). Although the MAPK pathway inhibitor, PD98059, was able to reduce the rescue response from OHT as measured by [^{3}H]thymidine incorporation, this effect did not achieve statistical significance due to a high variability between independent experiments (Fig. 6*B*). These data suggest that both PI3K/Akt and MAPK/ERK1/2 pathways are required for OHT-mediated cell cycle progression, although, again, the PI3K pathway may have a more crucial role.

In addition, we also determined whether blocking the PI3K/Akt and MAPK/ERK1/2 pathways prevented OHT induction of cyclin D1 expression. After pretreating the cells with inhibitors of the PI3K/Akt and MAPK/ERK1/2 pathways, we evaluated the ability of OHT to up-regulate cyclin D1. Both the PI3K and MAPK pathway inhibitors blocked basal levels of cyclin D1 expression and abrogated OHT-stimulated cyclin D1 induction (Fig. 6*C* and *D*). Thus, activation of the PI3K and MAPK is required for maintenance and induction of cyclin D1 expression in tamoxifen-resistant cells. All these data argue that cyclin D1 expression is necessary for the growth of tamoxifen-resistant cells and suggest that blocking cyclin D1 expression and function may impair growth of tamoxifen-resistant cells.

Discussion

This study provides evidence that cyclin D1 is a potential therapeutic target in breast cancer cells with acquired resistance to tamoxifen. The effect of inhibiting cyclin D1 expression on the growth of tamoxifen-resistant cells or on tamoxifen-induced cell cycle progression has not been evaluated previously. Here, we show that cyclin D1 is necessary for basal proliferation of tamoxifen-resistant cells. Moreover, we also establish a role of cyclin D1 in tamoxifen-induced cell cycle progression in cells with acquired resistance to tamoxifen. In these cells, tamoxifen can act as a partial

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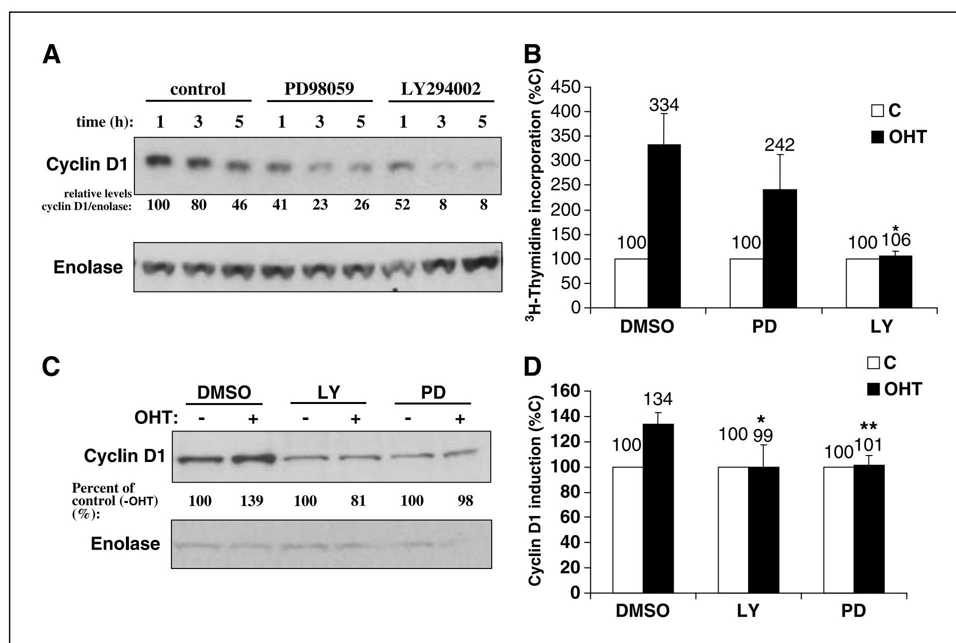

Figure 6. Cyclin D1 expression and OHT-dependent cell cycle progression require the PI3K/Akt and MAPK/ERK1/2 pathways. *A,* MTR-3 cells growing in CSS medium were treated with DMSO control, 50 μmol/L PD98059, or 10 μmol/L LY294002. At the indicated time points, cells were washed and directly lysed in their culture dish. Equal amounts of protein were prepared from the lysates and immunoblotted for cyclin D1. Enolase was used as loading control. Representative blot. *B,* MTR-3 cells were arrested in CSS medium containing ICI. After 48 hours of incubation, cells were treated with 0.1% DMSO control, 50 μmol/L PD98059 (*PD*), or 10 μmol/L LY294002 (*LY*) immediately followed by 0.1% ethanol control or 1 μmol/L OHT. [^{3}H]thymidine incorporation was done 24 hours later. *Columns,* average of four independent experiments; *bars,* SE. *C,* MTR-3 cells arrested with ICI were pretreated with inhibitor by adding 0.1% DMSO control, 10 μmol/L LY294002, or 50 μmol/L PD98059 to the culture medium. After 30 minutes of pretreatment, 1 μmol/L OHT was added. Seven hours later, cells were harvested. Equal amounts of protein lysate were prepared for Western blot and immunoblotted with a cyclin D1–specific antibody. Enolase was used as loading control. Data are representative of at least three independent experiments. *D,* level of cyclin D1 was quantified and is represented graphically. *Columns,* average of three independent experiments; *bars,* SE. *, $P \leq 0.05$, significantly different induction from DMSO control; **, $P \leq 0.01$.

agonist by inducing cyclin D1 expression and cell proliferation and this tamoxifen-induced proliferation also requires cyclin D1.

The agonistic response to tamoxifen may be clinically important as suggested by reports of both tumor flare with tamoxifen treatment (32–34) and clinical withdrawal response on cessation of treatment (7). Our results argue that tamoxifen can indeed act as a partial agonist and stimulate growth of breast cancer cells that acquire resistance to tamoxifen. The ability of tamoxifen to promote cell cycle progression correlates with induction of cyclin D1 expression following similar kinetics to those seen after estrogen-dependent cell cycle progression. These results are consistent with microarray studies in tamoxifen-sensitive MCF-7 cells showing that tamoxifen can up-regulate most of the same genes involved in estrogen-dependent proliferation, except cyclin D1 (35). This observation led the authors to suggest that the ability of tamoxifen to modulate cell cycle progression depends on its effect on cyclin D1 expression (35). Thus, in the tamoxifen-sensitive state, lack of cyclin D1 induction prevents cell cycle progression. Therefore, all these data together indicate that the ability of tamoxifen-resistant cells to up-regulate cyclin D1 expression in response to tamoxifen is a necessary step in the acquisition of tamoxifen-stimulated cell cycle progression.

A requirement for cyclin D1 in tamoxifen-stimulated cell cycle progression is indicated by our findings that blocking cyclin D1 expression with siRNA prevents OHT-mediated cell cycle progression. Moreover, inhibiting cyclin D1 expression also affects basal growth of tamoxifen-resistant cells, providing evidence of the important role of cyclin D1 for the growth of ER-positive breast cancer cells. Inhibition of cyclin D1 can also block proliferation and mimic antiestrogen-mediated arrest in tamoxifen-sensitive cells (10, 36, 37). Therefore, these data together suggest that cyclin D1 is a necessary component for the growth of ER-positive breast cancer cells whether in the tamoxifen-sensitive or tamoxifen-resistant state.

Our results show that inhibition of PI3K/Akt and to a lesser extent inhibition of MAPK/ERK1/2 reduced the ability of OHT to promote cyclin D1 expression and cell cycle progression in breast cancer cells with acquired resistance to tamoxifen. In tamoxifen-sensitive cells, blocking the PI3K/Akt or MAPK/ERK1/2 pathways impairs estrogen-dependent cyclin D1 expression and proliferation (25–28). Moreover, cyclin D1 overexpression can partially overcome the cell cycle arrest induced by blocking the PI3K/Akt or MAPK/ERK1/2 pathways in tamoxifen-sensitive cells (28). In support of a role of the PI3K/Akt or MAPK/ERK1/2 pathways in the agonist effect of tamoxifen, MCF-7 cells overexpressing HER-2/*neu* activate both PI3K/Akt and MAPK/ERK1/2 and induce cyclin D1 expression in response to tamoxifen (24). In addition, a recent study with cells that have acquired resistance to tamoxifen has shown that levels of activated PI3K/Akt are basally increased compared with tamoxifen-sensitive MCF-7 cells (38). Nevertheless, although our tamoxifen-resistant cells do not seem to exhibit increased levels of activated PI3K/Akt or MAPK/ERK1/2 pathways, expression of cyclin D1 in these tamoxifen-resistant cells is clearly sensitive to inhibitors that block activation of these pathways. Therefore, PI3K/Akt and MAPK/ERK1/2 activities are required for the partial agonist response of OHT in tamoxifen-resistant cells. Hence, blocking these signal transduction pathways represents alternative strategies to modulate cyclin D1 expression and cell proliferation in tamoxifen-resistant cells.

Our data indicate that cyclin D1 is a key cell cycle regulator for growth of tamoxifen-resistant cells. Recently, the role of cyclin D1 as a potential therapeutic target for tamoxifen-sensitive ER-positive breast cancer has been validated using MCF-7 breast cancer cells as a cell culture model (22). Therefore, a potential strategy in the treatment of ER-positive breast cancer would be to combine hormonal treatment and blockade of cyclin D1 function. Several strategies have already been successfully used to block cyclin D1 expression, such as antisense oligonucleotides, a CRE-decoy oligonucleotide, a novel DNA-binding ligand, or siRNA (37, 39, 40). An important lesson learned from knocking out cyclin D1 in the mouse genome was that cyclin D1 expression was required for full mammary gland development but not required for normal development and function of most tissues (41). Hence, these animal studies indicated that therapeutic strategies to block cyclin D1 expression and/or function in breast cancer patients may have low toxicity because they will not affect proliferation of normal cells. Moreover, recent studies have shown that cyclin D1 and its partner cyclin-dependent kinase-4 are necessary for development and maintenance of HER-2-driven breast tumors in transgenic animals, suggesting a crucial role of cyclin D1 in HER-2-driven breast cancers (42, 43). In addition to the value of cyclin D1 as a therapeutic target, as cyclin D1 is commonly expressed in breast tumors, cyclin D1 antisense peptide nucleic acid probes could be used to detect early recurrences in a noninvasive way (44). Therefore, cyclin D1 is not only a potential therapeutic target in tamoxifen-resistant breast tumors but could also help to detect recurrences of ER-positive breast cancer.

In summary, tamoxifen-resistant breast cancer cells require cyclin D1 for proliferation. In some cases, as shown here, tamoxifen can also act as a partial agonist in breast cancer cells with acquired resistance to tamoxifen promoting proliferation via up-regulation of cyclin D1 expression. Enhanced expression of cyclin D1 is required for tamoxifen-induced cell cycle progression of breast cancer cells and depends on both PI3K/Akt and MAPK/ERK1/2 signaling pathways. Therefore, blocking cyclin D1 expression may represent a more specific and less toxic way to treat ER-positive breast cancer and prevent both tamoxifen flare and acquisition of tamoxifen-resistant growth.

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