Estrogen Receptor Alpha Mediates Progestin-Induced Mammary Tumor Growth by Interacting with Progesterone Receptors at the *Cyclin D1/MYC* Promoters

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Abstract

Synthetic progesterone used in contraception drugs (progestins) can promote breast cancer growth, but the mechanisms involved are unknown. Moreover, it remains unclear whether cytoplasmic interactions between the progesterone receptor (PR) and estrogen receptor alpha (ERα) are required for PR activation. In this study, we used a murine progestin-dependent tumor to investigate the role of ERα in progestin-induced tumor cell proliferation. We found that treatment with the progestin medroxyprogesterone acetate (MPA) induced the expression and activation of ERα, as well as rapid nuclear colocalization of activated ERα with PR. Treatment with the pure antiestrogen fulvestrant to block ERα disrupted the interaction of ERα and PR *in vitro* and induced the regression of MPA-dependent tumor growth *in vivo*. ERα blockade also prevented an MPA-induced increase in *CYCLIN D1* (*CCND1*) and *MYC* expression. Chromatin immunoprecipitation studies showed that MPA triggered binding of ERα and PR to the *CCND1* and *MYC* promoters. Interestingly, blockade or RNAi-mediated silencing of ERα inhibited ERα, but not PR binding to both regulatory sequences, indicating that an interaction between ERα and PR at these sites is necessary for MPA-induced gene expression and cell proliferation. We confirmed that nuclear colocalization of both receptors also occurred in human breast cancer samples. Together, our findings argued that ERα–PR association on target gene promoters is essential for progestin-induced cell proliferation. *Cancer Res; 72(9); 2416–27. ©2012 AACR.*

Introduction

Breast cancer is the most frequently diagnosed cancer and a leading cause of cancer death in women worldwide (1). Although most of the evidence suggests estrogens as the major etiologic factor in breast cancer (2), experimental and epidemiologic evidence, reviewed recently (3–5), also points to the involvement of progesterone receptors (PR) in breast cancer development and progression. However, the mechanisms by which PR participate in tumor growth are not yet well understood. Considering that PR is usually used as a marker of estrogen receptor alpha (ERα) functionality (6), it may be intuitive to think that there is a sequential effect on ERα inducing PR expression. It has been reported that an early cytoplasmic interaction between ERα and PR isoform B (PR_B) is necessary to activate c-Src/$p21^{ras}$/Erk cascade by progestins (7), which in turn phosphorylates PR. Moreover, the regions through which both receptors interact have been identified (8). Conversely, Boonyaratanakornkit and colleagues have proposed that a polyproline motif in the amino-terminal domain of PR is sufficient to mediate c-Src tyrosine kinase activation by progestins (9).

Using a progestin-dependent murine mammary carcinoma, C4-HD (10) and the human T47D breast cancer cells, which are also stimulated by progestins (11, 12), we show that a genomic interaction between ERα and PR is essential for progestin-induced gene expression and tumor cell proliferation. Chromatin immunoprecipitation (ChIP) using T47D cells, confirms that PR is activated in the absence of ERα. However, the presence of both activated receptors at the *MYC* or *CYCLIN D1* (*CCND1*) promoters is required to trigger gene expression and cell proliferation. Moreover, the nuclear colocalization of both receptors in human breast cancer samples suggests that a genomic interaction between activated ERα and PR may be a common event in breast cancer growth.

Materials and Methods

Antibodies

PR (C-19 and H-190X), Erk1/2 (sc-94), ERα (MC-20 and HC-20X), AIF (sc-5586), BAX (sc-493), BCL/XL (sc-634), and IgG

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(sc-2027) are rabbit polyclonals (Santa Cruz Biotechnology); PR (Ab7) and ERα (Ab10) are mouse monoclonals and ERα (SP1, #RM-9101) a rabbit polyclonal (Thermo Scientific); CCND1 (#2978), pSer118 ERα (#2515), pSer167 ERα (#2514), MYC (#5605) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; #2118) are rabbit polyclonals (Cell Signaling Technology). Mouse monoclonal pSer162 PR_B, pSer190 PR, and pSer294 PR were a gift from Dr. D. Edwards (BCM, Houston, TX); ERα (M7047) and PR (M3568) are mouse monoclonals (Dako); pSer294 PR (Ab61785) and Ki67 (Ab15580), are rabbit polyclonals (Abcam). Secondary antibodies were obtained from Vector Labs.

Reagents

4′, 6-Diamidino-2-phenylindole (DAPI), medroxyprogesterone acetate (MPA, 10 nmol/L), and RU-38486 (RU, 10 nmol/L) were purchased from Sigma. ICI 182.780 (ICI) was a gift from AstraZeneca.

Animals

Two-month-old virgin female BALB/c mice (IByME-Animal Facility) were used. Animal care and manipulation were in agreement with institutional and reference guidelines (13).

In vivo experiments

Depot MPA (20 mg) was used as a progestin. C4-HD tumors were subcutaneously transplanted into MPA-treated BALB/c mice as previously described (10). When tumors reached a size of approximately 50 mm^2, 6 mice were treated subcutaneously, as described (14), with Fulvestrant (FUL; AstraZeneca), 6 received no other treatment, and the MPA depot was removed in another 6 mice.

Human breast cancer tissue samples

Breast cancer resection specimens from 15 patients immediately frozen at −70°C were provided by Bancario Hospital, Buenos Aires. The study was approved by the Institutional Review Board.

Cell lines

Human T47D cells obtained from American Type Culture Collection were validated by Genetica DNA Laboratories Inc. by short tandem repeat profiling and maintained as described (15). Passages lower than 15 were used.

Cell proliferation

Primary cultures of C4-HD tumors were carried out as described previously (16). Cell proliferation was evaluated by either [^{3}H]-thymidine uptake (16) or cell counting. C4-HD and T47D cells were plated with Dulbecco's Modified Eagle's Medium/F12 (Sigma) plus 10% fetal calf serum (FCS; BioSer) for 48 hours. After starving for 24 hours with 1% steroid-stripped FCS (chFCS), the cultures were incubated with the experimental solutions.

Gene silencing

T47D cells were seeded in 12- or 96-well plates and transfected with short interfering RNAs (siRNA) to human ERα (ESR1_8 and ESR1_10, QIAGEN), human CCND1 (ON-TARGETplus SMARTpool CCND1 from Thermo, or a pool of CCND1_5 and CCND1_6 from QIAGEN), or a nonspecific siRNA (SI03650318, QIAGEN) using HiPerFect transfection reagent (QIAGEN). Cells were used 48 hours posttransfection.

Immunohistochemistry

Sections of formalin-fixed, paraffin-embedded tissues were reacted with different antibodies using the avidin–biotin peroxidase complex technique (Vector Lab) and counterstained with hematoxylin (17). Positive cells were counted in 10 high-power fields (HPF, 1,000×) of each section and expressed as the mean ± SEM of the percentage of the ratios between the number of events and the cell number/HPF.

Immunofluorescence and colocalization

Tumors. Frozen tumor sections were fixed in formalin, postfixed in 70% ethanol, blocked, and incubated with the primary antibodies and fluorescein isothiocyanate/TX-conjugated secondary antibodies, and counterstained with DAPI as described previously (18). Images were obtained using a Nikon Eclipse E800 Confocal Microscope and Nikon DS-U1 with ACT-2U software.

Cells. Cultures growing on chamber slides were fixed in 70% ethanol and processed as described previously (18). To quantify nuclear colocalization of PR and ERα, we used the Pearson's correlation coefficient (R_r). Nuclei (200) of selected samples were analyzed by using PSC Colocalization plug-in (ImageJ-NIH; ref. 19). R_r ranges between −1 (perfect negative correlation) to +1 (perfect positive correlation) with 0 meaning no correlation.

Tumor and cell extracts

Tumors were homogenized and processed to obtain nuclear purified fractions (20) and total cell extracts prepared using M-PER mammalian protein extraction reagent (Pierce). Nuclear cell culture extracts were obtained and proteins quantified as described previously (21).

Immunoprecipitation assays

Nuclear extracts containing 0.5 to 1 mg of proteins were subjected to immunoprecipitation (IP) using 2 μg of PR or ERα antibodies and rocked overnight at 4°C. The immunocomplexes were then captured by adding protein A-agarose (Santa Cruz) processed as described (18) and subjected to Western blots.

Western blots

Tumor, cell extracts (100 μg proteins/lane), or immunoprecipitated proteins were separated on discontinuous polyacrylamide gels and detected as previously described (20).

Activation of reporter genes

The PRE-Luc vector used was a gift from Dr. C. Gardmo (Karolinska Institutet, Stockholm, Sweden; ref. 22) and assays were conducted as described previously (18).

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RNA preparation and real-time quantitative PCR

Total RNA was isolated from cultures with TRIzol Reagent (Invitrogen) and converted to cDNA as described previously (18). Specific oligos for human *MYC* (NM_002467.4) and *CCND1* (NM_053056.2) were designed using Primer-Blast (NCBI; Supplementary Table S1). *GAPDH* (NM_002046.3) expression was used as a normalization control. Data from 3 experiments were combined to determine gene expression changes using $2^{(-\Delta C_t)}$ formula. A melting curve was generated for every run to confirm assay specificity.

ChIP and sequential ChIP assays

After treatment, cells were fixed with 1% paraformaldehyde for 30 minutes; ChIP assays carried out as recommended by Diagenode using the HighCell# ChIP kit. Specific oligos for human *CCND1* and *MYC* promoters were designed using Primer-Blast (NCBI; Supplementary Table S1). The data from each immunoprecipitate (IgG, PR, and ERα) was normalized to the corresponding inputs of chromatin before IP, normalized to IgG/input data, and expressed as relative to the control. Five experiments were combined to determine receptor binding to gene promoters. Sequential ChIP (ChIP-reChIP) was carried out using the Re-ChIP-IT kit (Active Motif). Data from each sequential immunoprecipitates (PR/ERα and ERα/PR) were normalized to the corresponding inputs before IP, normalized to IP IgG/IgG data, and expressed as relative to the control.

Statistical analysis

ANOVA and Tukey multiple post *t* test were used to evaluate differences of means of multiple samples, and Student *t* test was used to compare means of 2 different groups. In all graphs, the mean ± SEM is shown, and experiments were repeated at least 3 times. Significant differences between control and treated cells were indicated with asterisk (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Results

ERαs plays a key role in C4-HD tumor growth *in vivo*

We have previously shown that C4-HD tumors that express ERα and PR grow in MPA- or progesterone (Pg)-treated female mice (10) and that the blockade of PR induces complete tumor regression (23). This experimental system provided an opportunity to explore the role of ERα in progestin-induced tumor growth by using the pure antiestrogen FUL. Surprisingly, FUL induced a complete regression of tumors growing in the presence of MPA (Fig. 1A), and this was associated with a decrease in both PR isoforms and ERα expression, as evaluated by Western blot (Fig. 1B) and immunohistochemistry (Fig. 1C). Expression of ERα after MPA withdrawal was negligible, however, a significant increase in PR was observed after MPA removal, suggesting that, in the progestin-dependent C4-HD tumor, although MPA downregulates PR expression, it may be required to maintain high levels of ERα expression *in vivo* (Fig. 1C). Moreover, activated ERα (pSer167 and pSer118 ERα) was also high in MPA-treated tumors (Supplementary Fig. S1A). FUL-induced tumor regression was associated with a cytostatic effect, as shown by a decrease in the mitotic index (Ki67 quantification, Supplementary Fig. S1B), and in the expression of 2 progestin-regulated proteins, CCND1 and MYC (Fig. 1C). In addition, in FUL-treated tumors, an increase in apoptosis (Supplementary Fig. S1B), associated with a decrease in BCL/XL, and an increase in BAX and AIF (Supplementary Fig. S1C) expression were observed. These results indicated that activated ERα contributes to progestin-dependent tumor growth.

ERα and PR interact in the nuclei of MPA-stimulated C4-HD cells *in vitro* and this interaction is necessary to induce cell proliferation

The fact that high levels of PR, but not of ERα, were observed in the nuclei of C4-HD tumors after MPA removal, led to hypothesize that both receptors participated in growth stimulation. Therefore, we investigated the effect of the blockade of ERα on MPA-induced cell proliferation and the role of MPA on ERα and PR expression *in vitro*. In C4-HD cultures, ICI inhibited MPA-induced proliferation as shown by [^{3}H]-thymidine uptake (16), cell counting (Fig. 2A), or bromodeoxyuridine staining (Supplementary Fig. S2A). Similarly, blocking ERα expression using siRNAs also inhibited the MPA-induced increase in [^{3}H]-thymidine uptake (Supplementary Fig. S2B). A time course analysis of ERα and PR expression after ICI treatment showed an early downregulation of ERα (6 hours), although high levels of PR were still detected after 24 hours (Supplementary Fig. S2C), indicating that the blockade of MPA-induced cell proliferation by ICI was not associated with PR downregulation.

An increase in both nuclear ERα and PR immunoreactivity and nuclear colocalization was observed in MPA-treated cells (Fig. 2B). A time course analysis of the interaction revealed that they start colocalizing as early as 5 minutes after MPA incubation with a decrease after 1 hour (Fig. 2B). In cells treated for 30 minutes with MPA+ICI, there was a decrease in nuclear and an increase in cytosolic ERα staining (Fig. 2C left, arrows). These results suggested that ICI disrupts the molecular interaction induced by MPA. Similar incubations were done with the corresponding phospho-receptor antibodies. Phospho-Ser118 ERα staining increased after 30 minutes of MPA treatment and colocalized with pSer162 PR_B (Fig. 2C, middle) or pSer294 PR (Fig. 2C, right). These observations suggested that ERα and PR may be forming part of the same complexes in their active state (24). No cytosolic or membrane colocalization of PR and ERα was observed in MPA-treated cells and no staining was observed in hormone receptor–negative murine LM3 (25) breast cancer cells (data not shown). Moreover, using frozen samples from C4-HD tumors growing in MPA-treated mice, we confirmed the nuclear colocalization between PR/ERα *in vivo* (Fig. 2D, left). Finally, we corroborated the interaction between both receptors by co-IP assays using nuclear extracts from MPA-treated C4-HD tumors. Proteins were immunoprecipitated with 2 different PR or ERα antibodies and blotted accordingly (Fig. 2D, right). These results

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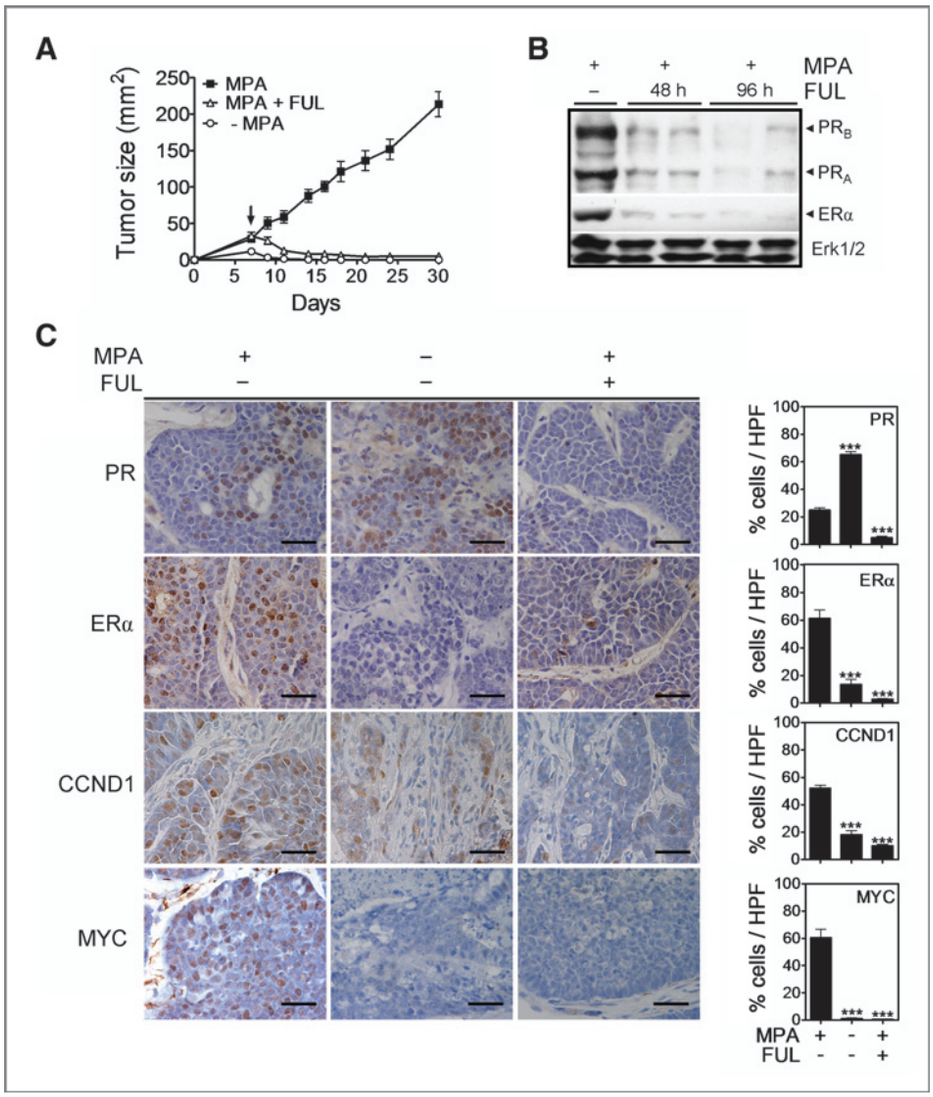

Figure 1. Antiestrogen treatment induces the regression of C4-HD tumors growing with MPA. A, MPA-treated mice carrying C4-HD tumors were FUL treated or not (5 mg/wk, arrow) or operated for MPA removal. Animals were followed for 25 days and the tumor size (length × width) plotted (mean ± SEM). B, Western blots of PR_B (115 kDa), PR_A (83 kDa, C-19), and ERα (66 kDa, MC-20) in tumors from the experiment shown in A. Total Erk1/2 was used as a loading control. C, immunohistochemical studies of PR (C-19), ERα (MC-20), CCND1, and MYC expression in tumor samples from A, 48 hours after treatment initiation. Bar, 60 μm. Right, quantification of protein expression.

suggested that both PR isoforms can participate in a nuclear complex with ERα.

Nuclear interaction between ERα and PR in human breast cancer

To investigate whether the colocalization between ERα and PR was unique for our murine model, we evaluated the expression of ERα, PR, and pPR in 15 frozen breast cancer samples. In 4 of them (2 ductal and 2 lobular carcinomas), we found a high degree of nuclear colocalization (Fig. 3A). We found a mild colocalization in 3 samples and a sporadic colocalization in other 2 samples. No staining was observed in receptor negative tumors (Fig. 3B). Co-IP assays carried out using purified nuclear extracts from 2 positive samples and a negative control confirmed the nuclear interaction between ERα and PR (Fig. 3C). These results suggested that the interaction between ERα and PR has an important and yet unexplored role in human breast cancer.

ERα and PR interaction in the nuclei of progestin-stimulated T47D cells is necessary to induce cell proliferation

To further investigate the role of ERα in MPA-induced cell proliferation we used T47D cells. MPA increased the nuclear colocalization between ERα and PR during the first 5 to 10 minutes and then a decrease was observed after 30 minutes of treatment (Fig. 4A). No cytosolic or membrane colocalization of PR and ERα was observed. Using phospho-specific antibodies, we showed that pSer162 PR_B and pSer294 PR colocalized with ERα after 10 minutes of MPA incubation (Supplementary Fig. S3A and S3B). Purified nuclear extracts from untreated or MPA-treated cells were immunoprecipitated with PR or ERα antibodies. We observed a significant increase in pSer294 PR ($P < 0.01$) and in ERα ($P < 0.05$), or in total PR ($P < 0.01$), respectively, as compared with immunoprecipitates from untreated cells (Fig. 4B). Cellular fractionation was controlled by Western blot using anti-tubulin or anti-Sp1 antibodies

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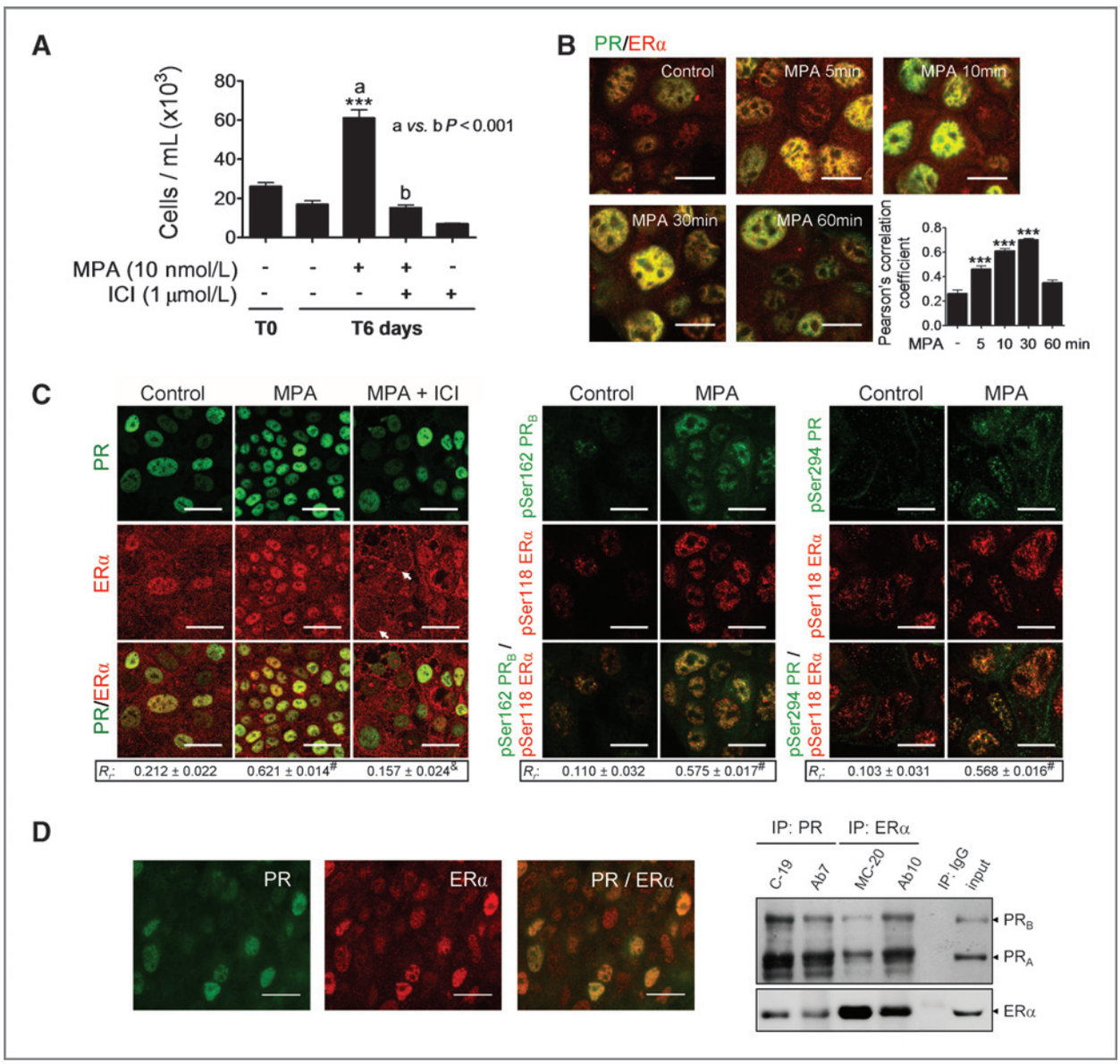

Figure 2. MPA increases ERα and PR nuclear colocalization whereas ICI disrupts this interaction, thereby inhibiting cell proliferation in C4-HD cells. A, C4-HD cells were grown and then starved in 1% chFCS for 24 hours. Cells were counted before (T0) and after 6 days (T6) of treatment. A representative experiment of the 3 is shown (mean ± SEM). B, confocal images of cells showing the increase in the nuclear colocalization of PR (Ab7) and ERα (MC-20) after a time-dependent incubation with MPA. All pictures were obtained the same day using the same microscope settings. Bar, 15 μm. The quantification of nuclear PR/ERα colocalization was carried out as described in Materials and Methods using the Pearson's correlation coefficient (R_r). C, left, confocal images of cells double stained for PR and ERα after 30 minutes of incubation with MPA and ICI (1 μmol/L). Arrows, cytoplasmic or membrane ERα staining. R_r (mean ± SEM): #, $P < 0.001$ MPA versus Ctrl, and &, $P < 0.001$ MPA versus MPA+ICI. Middle and right, nuclear colocalization of pPR (Ser162 PR_B and Ser294 PR) and pSer118 ERα after 30 minutes of MPA incubation. Bar, 30 μm. R_r: #, $P < 0.001$. D, left, confocal images of C4-HD tumors growing in MPA-treated mice double stained for PR (Ab7) and ERα (MC-20). Bar, 30 μm. Right, nuclear extracts of MPA-treated tumors were immunoprecipitated using protein A–agarose beads coupled with PR or ERα antibodies and immunoblotted with the corresponding antibodies (PR: Ab7 and ERα: MC-20). Immunoprecipitated extracts with rabbit anti-IgG were used as controls. Input, C4-HD nuclear extracts. A representative experiment of 3 is shown.

(Supplementary Fig. S3C). These results showed that both PR isoforms interact with ERα in the cell nuclei of human progestin-treated cells. We then explored the role of ERα in MPA-driven proliferative responses. ICI (0.1 and 1 μmol/L) dramatically inhibited DNA synthesis to levels similar to those of the antiprogestin RU (Fig. 4C). In addition, we used 2 different siRNAs that decreased ERα expression (Fig. 4D, left) and also inhibited MPA-induced [^{3}H]-thymidine uptake (Fig. 4D, right).

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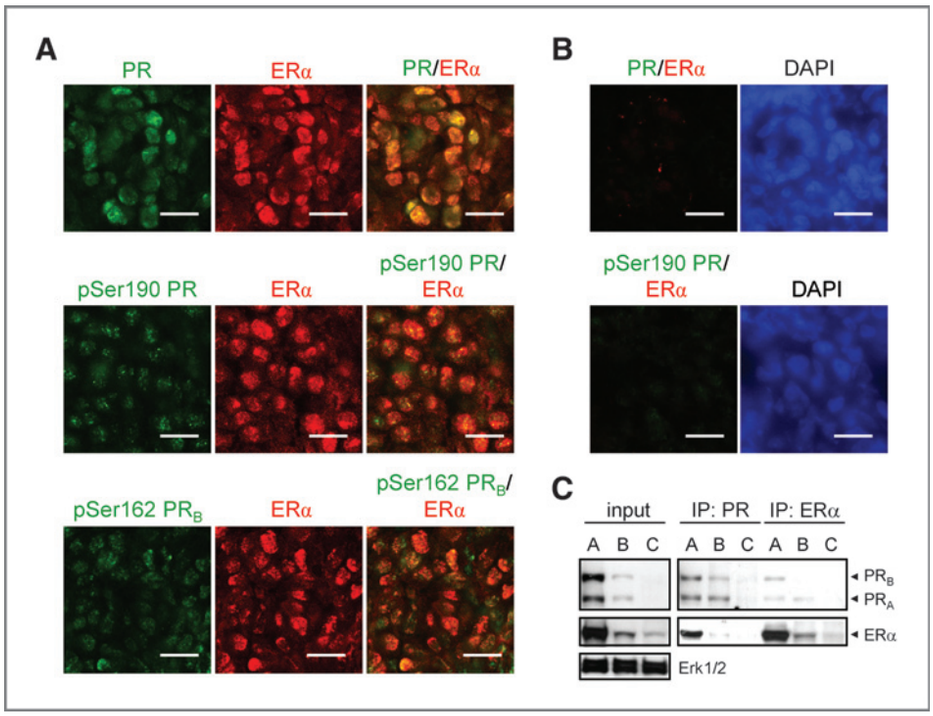

Figure 3. Interaction between PR and ERα in human breast cancer tissue samples. A, confocal immunofluorescence images of frozen sections from a PR^+/ER^+ invasive ductal carcinoma showing nuclear colocalization between total PR (M3568), pPR, and ERα (SP1). Bar, 40 μm. B, no staining is observed in a PR^-/ER^- sample under the same conditions. Nuclei were counterstained with DAPI. Bar, 40 μm. C, nuclear extracts from 2 PR^+/ER^+ tumors (A and B) and one PR^- tumor (C) were immunoprecipitated using PR (M3568) or ERα (SP1) antibodies and immunoblotted with the corresponding antibodies. Input, purified nuclear extracts from each tumor used in IP assays.

The inhibition of ERα expression prevents MPA-induced *CCND1* and *MYC* expression in T47D cells

As part of their proliferative activity, progestins induce the expression of *CCND1* (18, 26–30) and *MYC* (18, 31, 32) mRNA in T47D cells. We analyzed their time-dependent expression in response to MPA. We observed an early increase (15 minutes) after MPA incubation that lasted 24 hours, except for a decrease observed 1 hour (*CCND1*) or 3 hours (*MYC)* after treatment (Fig. 5A). The increase in mRNA correlated with an early and gradual increase in protein expression (Supplementary Fig. S4). The knockdown of CCND1 using siRNAs prevented DNA synthesis triggered by MPA (Fig. 5B). We therefore used ICI or siRNAs to analyze the contribution of ERα to gene transcription activated by MPA. The inhibition of ERα blocked the MPA-dependent transcription of both *CCND1* and *MYC* genes (Fig. 5C and D). All this data suggested that ERα activity, presumably through its ability to interact with PR by forming nuclear complexes, can control the expression of key proliferative genes in response to progestins.

ERα inhibition blocks the MPA-induced activation of reporter genes and prevents ERα, but not PR binding to *CCND1* and *MYC* promoters in T47D cells

To further understand the role of ERα mediating MPA transcriptional activities, we evaluated the effect of ICI on the activation of a reporter luciferase assay controlled by the progesterone response element (PRE) sequence in T47D cells. ICI inhibited MPA-induced PRE-luc expression (Fig. 6A) and induced the downregulation of ERα, whereas PR was still expressed even after 48 hours of ICI incubation (Supplementary Fig. S3D). Moreover, MPA induced a higher PRE-luc activity in MDA-MB-231 cells stably transfected with PR_B, when they were cotransfected with ERα (Supplementary Fig. S5). These results strongly suggested a role for the PR/ERα complexes in the regulatory elements of MPA-regulated genes. To confirm the binding of both receptors to the same promoter regions, we used ChIP analysis on *CCND1* and *MYC* regulatory sequences. In Fig. 6B we show a schematic representation of both gene promoters, highlighting the PRE and estrogen response element (ERE) sites in each case, as well as the primers used in ChIP/qPCR analysis. Cells were incubated with MPA (10 minutes) and the chromatin subjected to IP with PR- or ERα-specific antibodies. DNA fragments were amplified by qPCR with 3 pairs of primers for each gene, previously used by others to report PR binding to those sequences (refs. 31, 33, 34; Fig. 6B). The recruitment of ERα and PR to the sites at +5 to 6 Kb (ChIP primers C) was used as a negative control of receptor binding (Fig. 6B). Specific binding of both receptors was detected at the same promoter regions in each gene (ChIP primers A and B) after MPA treatment (Fig. 6C and D, left and middle panels). We then evaluated whether PR and ERα were simultaneously bound to the *CCND1* and *MYC* gene promoters by using a sequential ChIP assay. PR or ERα antibodies were used in the first IP, and ERα or PR antibodies in the sequential ChIP (reChIP). qPCR analysis clearly showed that PR and ERα co-occupy the *CCND1* and *MYC* promoters after 10 minutes of MPA stimulation (Figs. 6C and D, right panels). These findings suggested that progestins induce the assembly of PR/ERα protein complexes at both promoters to control its transcriptional activation in breast cancer cells.

To further understand the molecular mechanism driving these effects, we evaluated ERα and PR binding to these regulatory sequences when we inhibited ERα. PR binding to

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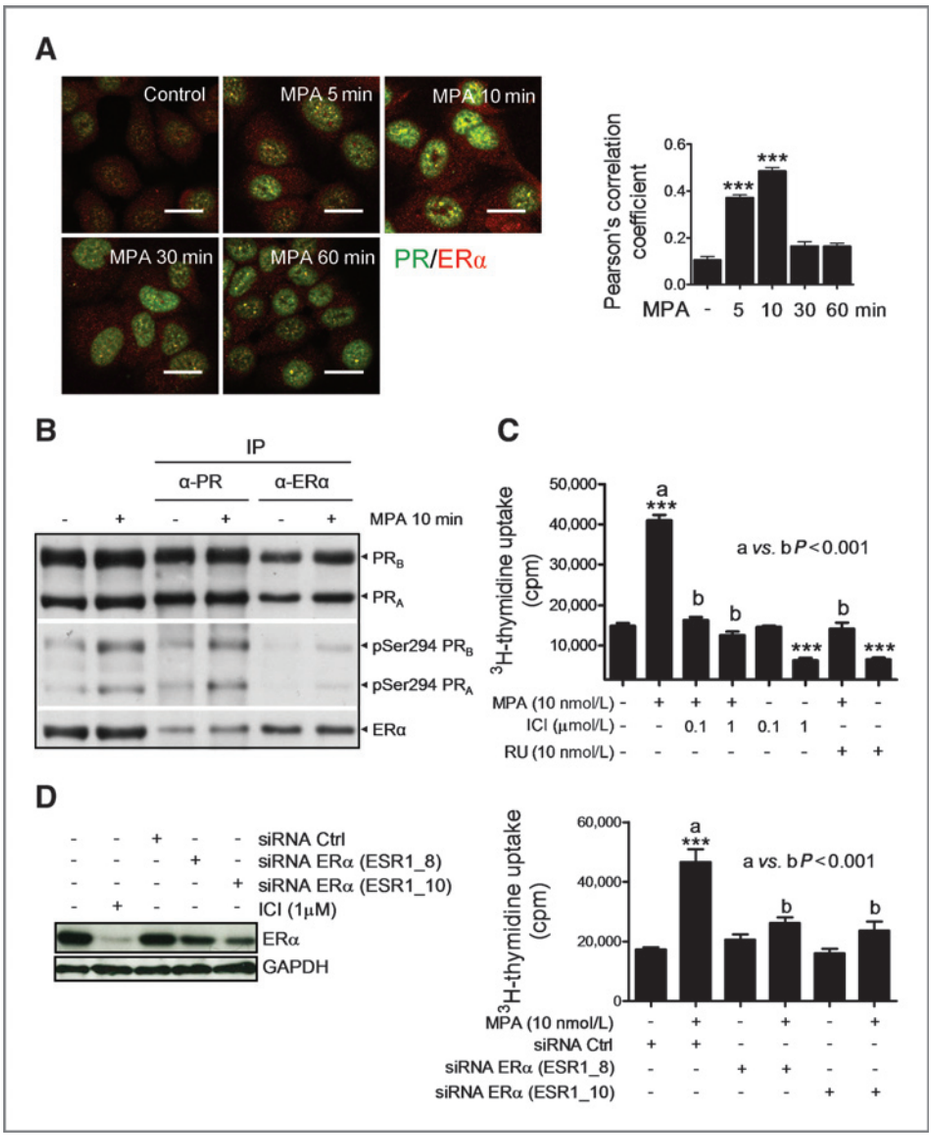

Figure 4. MPA increases cell proliferation and the ERα/PR nuclear interaction in T47D cells; the blockade of ERα prevents the MPA-induced proliferative effect. A, confocal images of immunofluorescence using PR (Ab7) and ERα (SP1) antibodies in cells treated as described in Fig. 2. Bar, 15 μm. The nuclear PR/ERα colocalization was estimated through the Pearson's correlation coefficient. B, nuclear extracts of untreated or MPA-treated cells were immunoprecipitated using protein A–agarose beads coupled with PR (C-19) or ERα (SP1) antibodies and immunoblotted with the corresponding antibodies (PR: C-19 and pSer294, ERα: SP1). A representative experiment of 3 is shown. C, [^{3}H]-thymidine uptake assays. After attachment, cells were starved and treated for 48 hours with experimental solutions. A representative experiment of 3 is shown. D, left, Western blots showing ERα (SP1) expression in extracts from cells either treated or not with ICI for 48 hours, transfected with 2 different siRNAs for human ERα or a nonspecific siRNA. GAPDH was used as a loading control. Right, [^{3}H]-thymidine uptake assays. Transfected cells with siRNAs were seeded, starved, and either treated or not with MPA for 48 hours. A representative experiment of 3 is shown.

both gene promoters was unaffected by the presence of ICI (Fig. 7A) or siRNA to ERα (Fig. 7B), although they did prevent ERα binding. These data indicated that both proteins need to interact at the *CCND1* and *MYC* promoters to induce gene transcription and cell proliferation, supporting our hypothesis that the presence of ERα at those promoters is required to induce PR-mediated gene expression.

Discussion

In this study we have shown that a progestin can induce a direct and transient nuclear interaction between ERα and both PR isoforms at the promoters of 2 progestin responsive proto-oncogenes, namely *CCND1* and *MYC*. Moreover, this activity can have dramatic effects on breast cancer cell proliferation and seems to be dependent on ERα actions, as its inhibition with ICI induced complete regression of C4-HD tumors growing in the presence of the progestin. Thus, our results suggest that a combined treatment with antiestrogens and antiprogestins can be beneficial to breast cancer patients. As it has previously been reported (35), the cotreatment with antiprogestins plus selective estrogen receptor modulators may have an additive effect. Moreover, MPA-independent murine mammary carcinomas, C4-HI, respond better to a combination of tamoxifen and mifepristone than to both single agents (36).

We confirmed our observations in the murine model, using T47D cells in which the inhibition of ERα activity resulted in a complete blockade of MPA-dependent *MYC* and *CCDN1* gene transcription and cell proliferation. The fact that progestins exerted growth inhibitory effects on MDA-MB-231 cells stably transfected with PR (37) but stimulated cell proliferation in models that coexpress ERα and PR (11, 12, 15, 16, 38), also suggests that both receptors cooperate to trigger cell proliferation. In this regard, it is known that the human *MYC* gene

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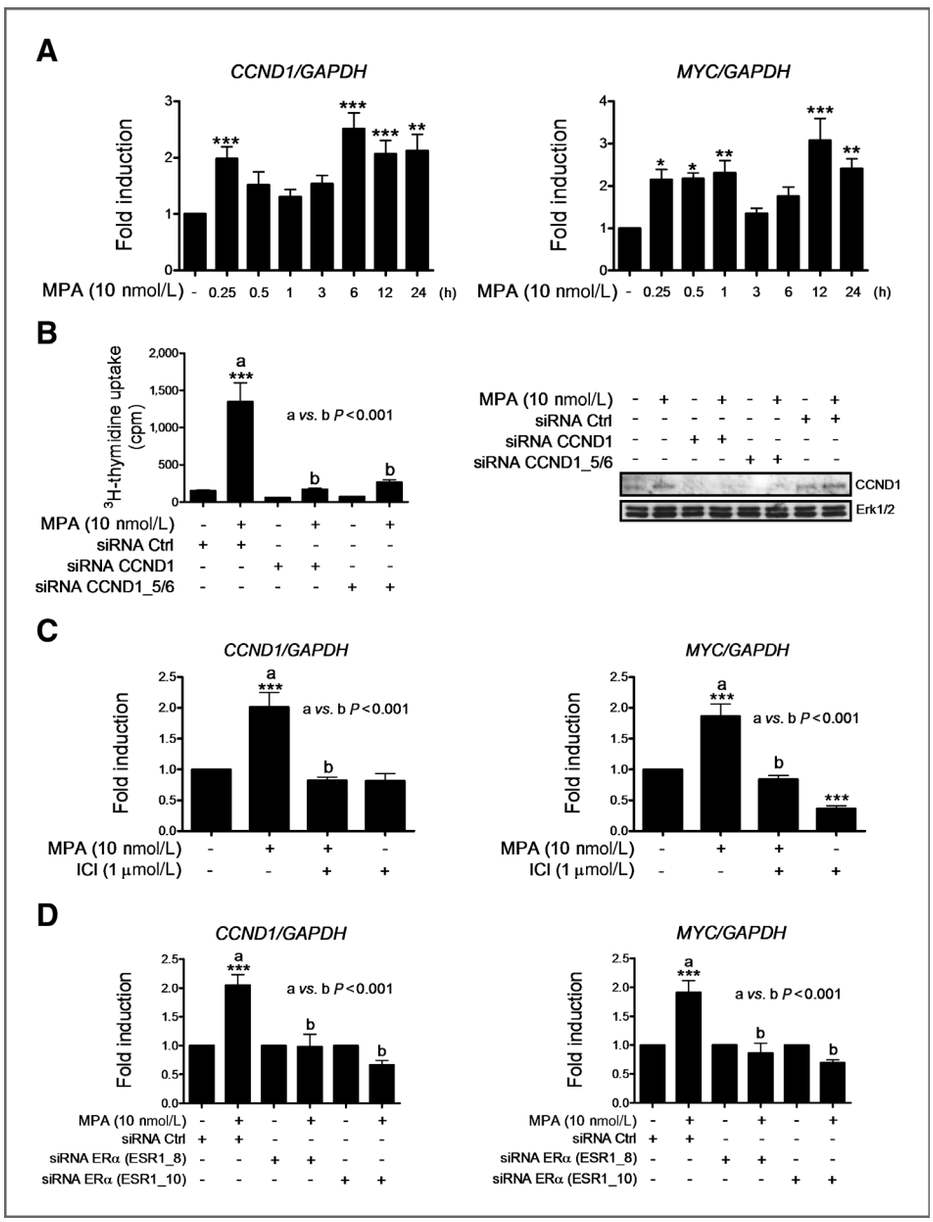

Figure 5. ERα mediates MPA-induction of *CCND1* and *MYC* in T47D cells. A, *CCND1* (left) and *MYC* (right) mRNA levels relative to *GAPDH* were measured by qPCR after MPA-treatment. B, left, the blockade of CCND1 expression by using 2 different siRNA pools prevents the MPA-induced increase in [^{3}H]-thymidine uptake. Cells were transfected and treated as described in Fig. 4D. A representative experiment (mean ± SEM) of 3 is shown. Right, the expression of CCND1 in untransfected or siRNA-transfected cells was evaluated by Western blot using Erk1/2 as a loading control. Cells were untreated or MPA-treated for 24 hours. C and D, *CCND1* (left) and *MYC* (right) mRNA expression relative to *GAPDH* was evaluated by qPCR using cells treated for 15 minutes with MPA and/or ICI, or transfected with 2 siRNAs for human ERα.

promoter contains a functional PRE that mediates the binding of activated PR (18, 31, 39) and we also identified other consensus PRE half sites (40) that might also bind PR (Fig. 6B). Moreover, it has recently been reported that ERE half sites at the *MYC* proximal promoter (Fig. 6B) are not responsive to estrogens (41). It may be possible that after progestin treatment, these sites might also bind ERα in complexes with PR. In addition, we have recently shown in T47D cells that MPA induces the binding of PR, transcription factors (TF), such as STAT5, and nuclear tyrosine kinase receptors (RTK), such as FGFR-2, to the same regions of the *MYC* promoter (18). The results reported herein indicate that activated ERα could be present in the same multimeric protein complexes as supported by NoShift electrophoretic mobility shift assays (ref. 18; Fig. 7C *-iii-*). The regulation of human *CCND1* by progestins may be more complicated, as no canonical PRE sites have been described in its promoter and accordingly, it has been suggested that PR regulates *CCND1* expression by nongenomic mechanisms (7, 9, 34). The 2 models of cytoplasmic signaling pathways activated by Pg are shown in Fig. 7C. Model *-i-* proposes that an early interaction between ERα and PR_B is necessary for c-Src/$p21^{Ras}$/Erk, PI3K/Akt, and JAK/STAT activation (7, 8, 42); conversely, model *-ii-* proposes that a polyproline motif in the amino-terminal domain of PR is sufficient to activate cell signaling pathways (9). Albeit a cytoplasmic as well as membrane localization of PR has been shown (21), we were not able to find PR colocalizing with ERα at these sites. Activated growth factor receptors, usually RTKs, may

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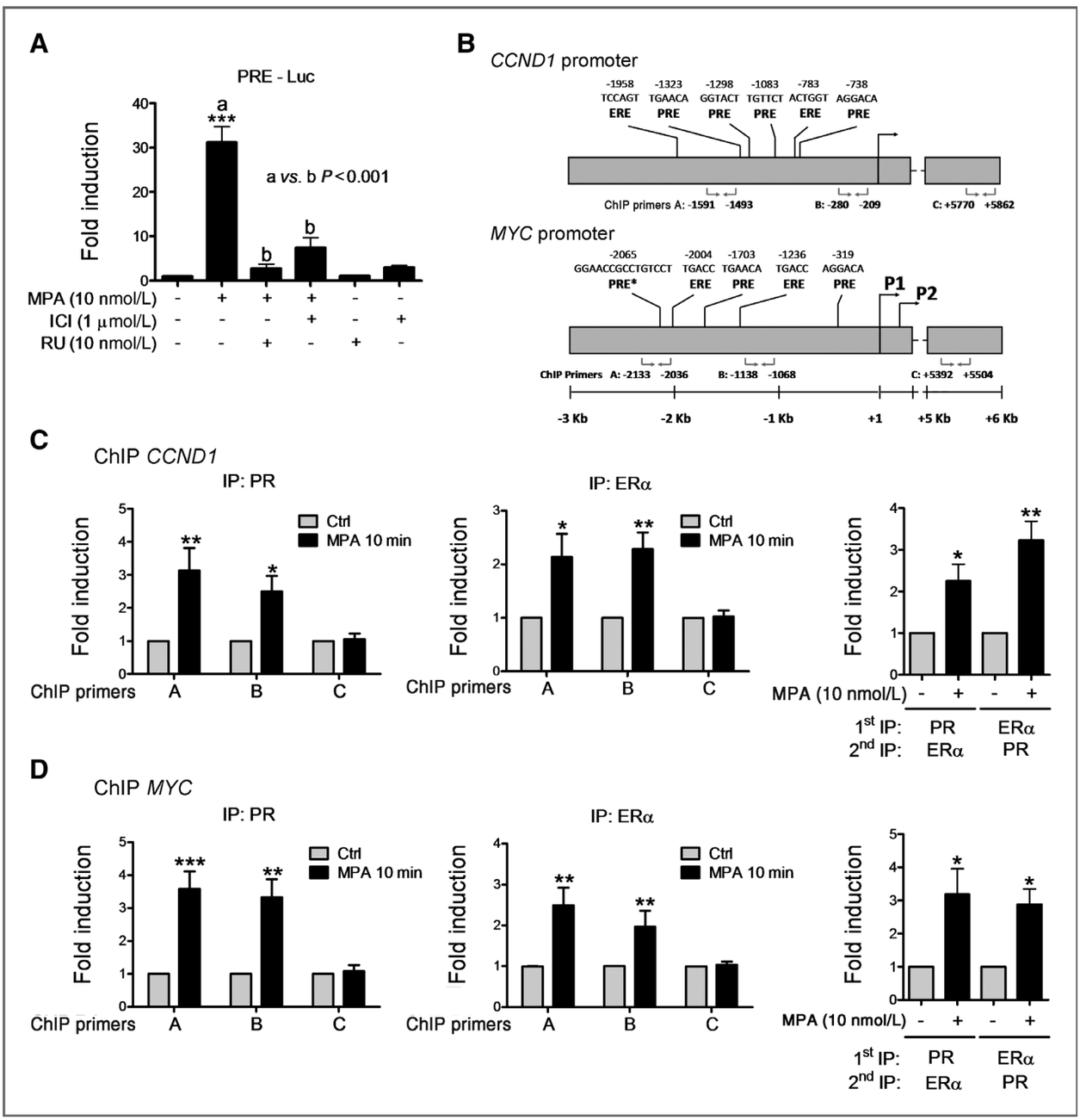

Figure 6. MPA induces the binding of PR and ERα to both *CCND1* and *MYC* promoters in T47D cells. A, cells transfected with a PRE-luc plasmid were treated or not for 24 hours and processed to measure luciferase. A representative experiment of 3 is shown (mean ± SEM). B, schematic representation of predicted PRE or ERE half sites in the upstream promoter regions of human *CCND1* (top) and *MYC* (bottom) genes, and qPCR primers used for ChIP assays. *, PRE-like sequence described by Moore and colleagues (31). Cells were either treated or not with MPA and processed for ChIP/qPCR studies to detect the presence of PR (H-190X, left) and ERα (HC-20X, middle) on *CCND1* (C) and *MYC* promoters (D). A, B, and C represent the ChIP primers shown in B, with C serving as a negative control region of nuclear receptor binding. Data from ChIP-reChIP experiments using ChIP primers A (B) on *CCND1* (C, right) and *MYC* (D, right) promoters are shown. Cells treated or not with MPA for 10 minutes were first immunoprecipitated with PR (H-190X) or ERα (HC-20X) antibodies, and then immunoprecipitated using either ERα or PR antibodies. qPCR and data analysis were carried out as detailed in Materials and Methods.

stimulate cytoplasmic signaling pathways, that in turn induce PR phosphorylation and activation in both the absence or presence of steroids (15, 43). Both models propose that these nongenomic effects of Pg-activated MAPKs use TF at the *CCND1* promoter, inducing gene transcription and subsequently cell proliferation (43; Fig. 7C -*v*-). However, it has been recently shown that PR may have genomic effects at the *CCND1* promoter (34, 44), even as a coactivator of STAT3 (26; Fig. 7C -*iv*-). In this study, we showed for the first time that PR and ERα share the same progestins-sensitive regions at *CCND1* and *MYC* promoters

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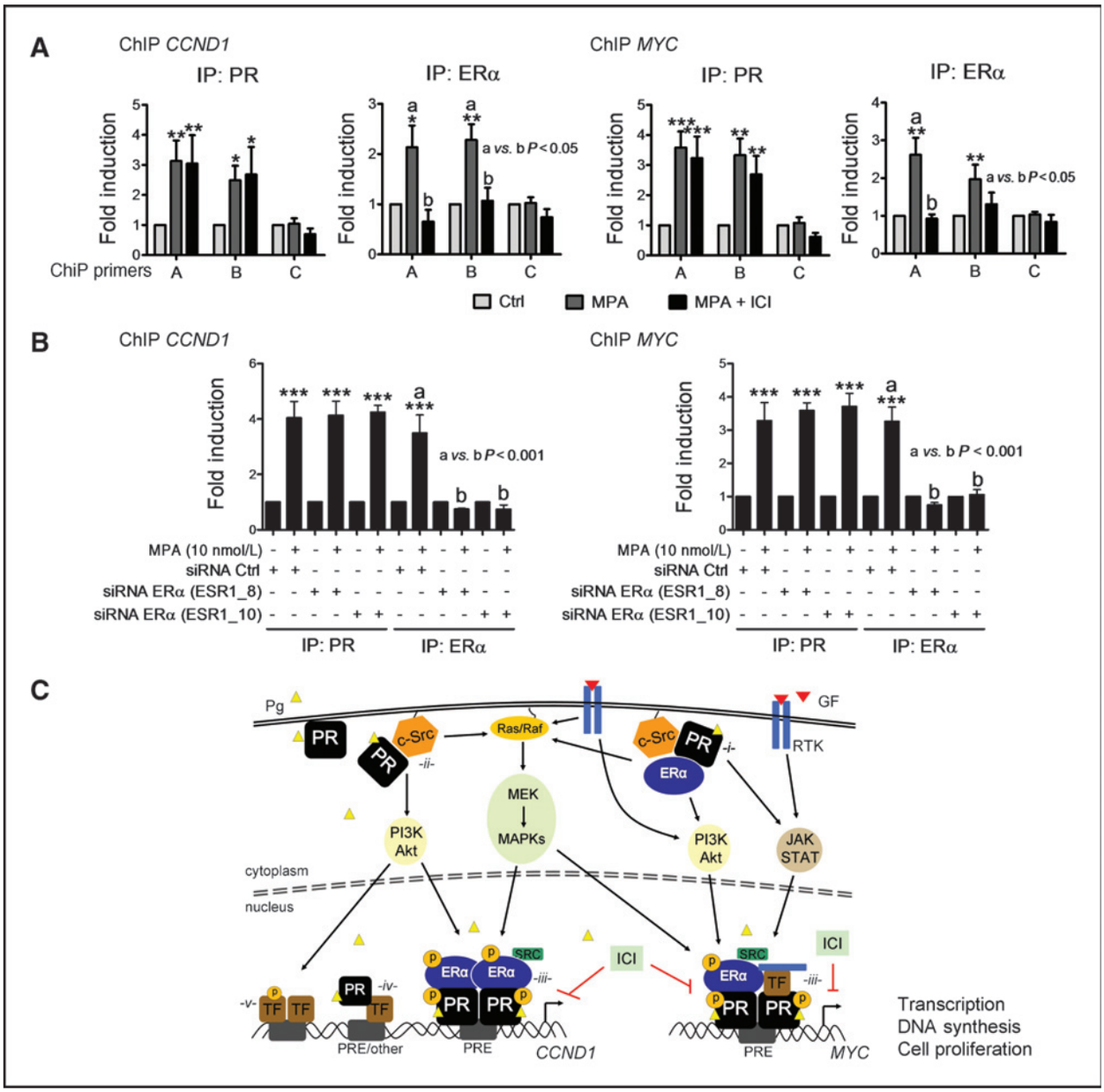

Figure 7. ICI or ERα siRNA prevent ERα binding in MPA-treated T47D cells, but not the binding of PR to *CCND1* and *MYC* promoters. A, cells were either treated or not with MPA (10 nmol/L) or MPA + ICI (1 μmol/L) for 10 minutes and processed for ChIP/qPCR studies to detect the presence of PR (H-190X) and ERα (HC-20X) on *CCND1* (left) and *MYC* (right) promoters. ChIP primers were used as in Fig. 6C. B, cells were treated with Ctrl siRNA or ERα siRNAs as shown in Fig. 4D, starved, and either treated or not with MPA (10 nmol/L) for 10 minutes and processed for ChIP/qPCR studies (using ChIP primers A for both genes, Fig. 6B) to detect the presence of PR (H-190X) and ERα (HC-20X) on *CCND1* (left) and *MYC* (right) promoters. C, integration of the proposed genomic and nongenomic models for PR/ERα interaction after progestin treatment. See text for details.

(Fig. 7C *-iii-*). Interestingly, we found distinct consensus PRE half sites (40) at the *CCND1* promoter (Fig. 6B), which might bind activated PR, as shown for others genes (45, 46). Aligned with our observations, the hypothesis that both ERα and PR can interact at the gene promoter level has been proposed by other authors in different contexts (44). However, this is the first report showing that PR and ERα are recruited to the same sites at the *CCND1* and *MYC* promoters after PR activation by MPA. It has been described that SRC (steroid receptor coactivator) proteins may also participate in this response (47), but we have not yet studied their involvement in this setting. Our results also show that antiestrogenic concentrations of ICI ($\leq$ 1 μmol/L) block the formation of MPA-induced PR/ERα nuclear complexes, inhibiting gene transcription and cell proliferation, without affecting the activation and binding of PR at the gene promoter. This implicates a change in the paradigm that a rapid, nongenomic

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interaction between PR_B and ERα is necessary to activate the c-Src/$p21^{ras}$/Erk cascade and PR by progestins. Whether the genomic interaction described here also involves ERID domains (8) at PR, remains to be investigated.

The expression of *MYC* and *CCND1* constitutes an early and transient event mediated by MPA, and it is quite conceivable that PR/ERα complexes driven effects are required to unwind the chromatin. This may be followed by the recruitment of other transcription factors, and full transcription of proliferative oncogenes. In addition, this activity could also be required for transcription events induced by other mitogens such as epidermal growth factor (48). On the other hand, MYC can also be involved in the activation of cyclins (D1, D2, E1, and A2), cyclin-dependent kinases (CDK4), and in the downregulation of cell-cycle inhibitors (49).

Finally, in this study we also showed that both receptors interact in the nuclei of selected human breast cancer samples, suggesting that ligand-independent hormone receptor activation may also be implicated in breast cancer tumor growth in patients. Thus, it is possible to speculate that patients showing higher levels of PR/ERα colocalization may have a better response to a combined antiprogestin–antiestrogen therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Giulianelli, R. Soldati, V. Wargon, S.I. Vanzulli, R. Martins, E. Zeitlin

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Giulianelli, J.P. Vaqué, A.A. Molinolo, C. Lanari

Writing, review, and/or revision of the manuscript: S. Giulianelli, J.P. Vaqué, A.A. Molinolo, L.A. Helguero, C.A. Lamb, J.S. Gutkind, C. Lanari

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Giulianelli

Study supervision: S. Giulianelli, C. Lanari

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