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) is 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 co-localization of activated ERα with PR. Treatment with the pure anti-estrogen 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 demonstrated 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 co-localization 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.
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 etiological factors in breast cancer (2), experimental and epidemiological 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 (PRB) is necessary to activate c-Src/p21ras/Erk cascade by progestins (7), which in turn phosphorylates PR. Moreover, the regions through which both receptors interact have been identified (8). Conversely, Boonyatatanakornkit et al. 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 demonstrate 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 co-localization 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 (sc-2027) are rabbit polyclonals (Santa Cruz); 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 GAPDH (#2118) are rabbit polyclonals (Cell Signaling Technology). Mouse monoclonal pSer162 PRB, pSer190 PR and pSer294 PR were a gift from Dr. D. Edwards; 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.** DAPI, medroxyprogesterone acetate (MPA, 10nM) and RU-38486 (RU, 10nM) 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 (20mg) was used as a progestin. C4-HD tumors were subcutaneously (s.c.) transplanted into MPA-treated BALB/c mice as previously
described (10). When tumors reached a size of approximately 50 mm², 6 mice were treated s.c. 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 ATCC 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 performed as described previously (16). Cell proliferation was evaluated by either [³H]-thymidine uptake (16) or cell counting. C4-HD and T47D cells were plated with DMEM/F12 (Sigma) plus 10% fetal calf serum (FCS; BioSer) for 48 h. After starving for 24 h 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 siRNAs 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 non-specific siRNA (SI03650318, QIAGEN) using HiPerFect transfection reagent (QIAGEN). Cells were used 48 h post-transfection.
**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 (HPFs, 1000X) 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 co-localization.** *Tumors.* Frozen tumor sections were fixed in formalin, post-fixed in 70% ethanol, blocked, and incubated with the primary antibodies and FITC/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 co-localization of PR and ERα, we used the Pearson's correlation coefficient ($R_f$). Nuclei (200) of selected samples were analyzed by using PSC Co-localization plug-in (ImageJ-NIH; 19). $R_f$ 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 (IP) assays.** Nuclear extracts containing 0.5-1 mg of proteins were subjected to 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 IP 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 (22) and assays were performed as described previously (18).

**RNA preparation and real-time quantitative PCR (qPCR).** 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; Table S1). GAPDH (NM_002046.3) expression was used as a normalization control. Data from three experiments were combined to determine gene expression changes using $2^{(-\Delta \Delta C_{T})}$ 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% PFA for 30 min; ChIP assays performed as recommended by Diagenode using the HighCell# ChIP kit. Specific oligos for human CCND1 and MYC promoters were designed using Primer-Blast (NCBI; Table S1). The data from each IP (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 performed using the Re-ChIP-IT kit (Active Motif). Data from each sequential IP (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’s \( t \) test was used to compare means of two different groups. In all graphs, the mean ± SEM is shown, and experiments were repeated at least three times. Significant differences between control and treated cells were indicated with asterisk (*, \( p<0.05 \); **, \( p<0.01 \); ***, \( p<0.001 \)).

**RESULTS**

1) **ERα play 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, while MPA downregulates PR expression, it may be required to maintain high levels of ERα expression \textit{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 two 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 indicate that activated ERα contribute to progestin-dependent tumor growth.

2) ERα and PR interact in the nuclei of MPA-stimulated C4-HD cells \textit{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 \textit{in vitro}. In C4-HD cultures, ICI inhibited MPA-induced proliferation as demonstrated by $[^{3}H]$-thymidine uptake (16), cell counting (Fig. 2A) or BrdU 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 h), while high levels of PR were still detected after 24 h (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 co-localization was observed in MPA-treated cells, (Fig. 2B). A time course analysis of the interaction revealed that they start co-localizing as early as 5 min after MPA incubation with a decrease after 1 h (Fig. 2B). In cells treated for 30 min with MPA+ICI, there was a decrease in nuclear and an increase in cytosolic ERα staining (Fig. 2C left, arrows). These results suggest that ICI disrupts the molecular interaction induced by MPA. Similar incubations were performed with the corresponding phospho-receptor antibodies. Phospho-Ser118 ERα staining increased after 30 min of MPA treatment and co-localized with pSer162 PRB (Fig. 2C, middle) or pSer294 PR (Fig. 2C, right). These observations suggest that ERα and PR may be forming part of the same complexes in their active state (24). No cytosolic or membrane co-localization 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 co-localization 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 two different PR or ERα antibodies and blotted accordingly (Fig. 2D, right). These results suggest that both PR isoforms can participate in a nuclear complex with ERα.
3) **Nuclear interaction between ERα and PR in human breast cancer.**

To investigate whether the co-localization 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 (two ductal and two lobular carcinomas) we found a high degree of nuclear co-localization (Fig. 3A). We found a mild co-localization in three samples and a sporadic co-localization in other two samples. No staining was observed in receptor negative tumors (Fig. 3B). Co-IP assays performed using purified nuclear extracts from 2 positive samples and a negative control confirmed the nuclear interaction between ERα and PR (Fig. 3C). These results suggest that the interaction between ERα and PR has an important and yet unexplored role in human breast cancer.

4) **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 co-localization between ERα and PR during the first 5-10 min, and then a decrease was observed after 30 min of treatment (Fig. 4A). No cytosolic or membrane co-localization of PR and ERα was observed. Using phospho-specific antibodies, we demonstrated that pSer162 PRB and pSer294 PR co-localized with ERα after 10 min of MPA incubation (Supplementary Figs. S3A and S3B). Purified nuclear extracts from untreated or MPA-treated cells were IP 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 IPs from untreated cells (Fig. 4B). Cellular fractionation was controlled by Western blot using anti-tubulin or anti-Sp1.
antibodies (Supplementary Fig. S3C). These results demonstrate 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 μM) dramatically inhibited DNA-synthesis to levels similar to those of the antiprogestin RU (Fig. 4C). Additionally, we used two different siRNAs that decreased ERα expression (Fig. 4D, left), and also inhibited MPA-induced [3H]-thymidine uptake (Fig. 4D, right).

5) 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 min) after MPA incubation that lasted 24 h, except for a decrease observed 1 h (CCND1) or 3 h (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 (Figs. 5C and 5D). All this data suggest 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.
6) 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, while PR was still expressed even after 48 h of ICI incubation (Supplementary Fig. S3D). Moreover, MPA induced a higher PRE-luc activity in MDA-MB-231 cells stably transfected with PRB, when they were co-transfected with ERα (Supplementary Fig. S5). These results strongly suggest 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 Figure 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 min) and the chromatin subjected to IP with PR- or ERα-specific antibodies. DNA fragments were amplified by qPCR with three pairs of primers for each gene, previously used by others to report PR binding to those sequences (31, 33, 34; Fig. 6B). The recruitment of ERα and PR to the sites at +5-6 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 (Figs. 6C and 6D, 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 min of MPA stimulation (Figs. 6C and 6D, right panels). These findings suggest 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 both gene promoters was unaffected by the presence of ICI (Fig. 7A) or siRNA to ERα (Fig. 7B), while they did prevent ERα binding. These data indicate 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 demonstrated that a progestin can induce a direct and transient nuclear interaction between ERα and both PR isoforms at the promoters of two 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 co-treatment with antiprogestins plus selective estrogen receptor modulators (SERMs) 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 where 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 co-express 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 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 (RTKs), 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 EMSA assays (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 non-genomic mechanisms (7, 9, 34). The two models of cytoplasmic signaling pathways activated by Pg are shown in Figure 7C. Model -i- proposes that an early interaction between ERα and PRB 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 demonstrated (21), we were not able to find PR co-localizing with ERα at these sites. Activated growth factor (GF) receptors, usually RTKs, may 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 non-genomic effects of Pg-activated MAPKs utilize TF at the CCND1 promoter, inducing gene transcription and subsequently cell proliferation (43; Fig. 7C -v-).

However, it has been recently demonstrated that PR may have genomic effects at the CCND1 promoter (34, 44), even as a co-activator 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 (Fig. 7C -iii-). Interestingly, we found distinct consensus PRE half-sites (40) at the CCND1 promoter (Fig. 6B), which might bind activated PR, as demonstrated 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 co-activator) 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 (≤ 1μM) 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, non-genomic interaction between PR\(_B\) and ER\(\alpha\) 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/ER\(\alpha\) 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 like EGF (48). On the other hand, MYC can also be involved in the activation of cyclins (D1, D2, E1, 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/ER\(\alpha\) co-localization may have a better response to a combined antiprogestin-antiestrogen therapy.

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LEGENDS TO FIGURES

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/week, arrow), or operated for MPA removal. Animals were followed for 25 days and the tumor size (length x width) plotted (mean ± SEM). (B) Western blots of PRb (115 kDa), PRa (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 h after treatment initiation. Bar: 60 μm. **Right.** Quantification of protein expression.

Figure 2. MPA increases ERα and PR nuclear co-localization while 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 h. Cells were counted before (T0) and after 6 days (T6) of treatment. A representative experiment of the three is shown (mean ± SEM). (B) Confocal images of cells showing the increase in the nuclear co-localization 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 co-localization was performed as described in Materials and Methods using the Pearson's correlation coefficient (Rr). (C) **Left.** Confocal images of cells double stained for PR and ERα after 30 min of incubation with MPA and ICI (1 μM). Arrows: cytoplasmic or membrane ERα staining. Rr (mean ± SEM): #: p<0.001 MPA vs. Ctrl, and &: p<0.001 MPA vs. MPA+ICI. **Middle and right.**
Nuclear co-localization of pPR (Ser162 PR and Ser294 PR) and pSer118 ERα after 30 min of MPA incubation. Bar: 30μm. $R_e$: #: 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: 30μm. **Right.** Nuclear extracts of MPA-treated tumors were IP using protein A-agarose beads coupled with PR or ERα antibodies and immunoblotted with the corresponding antibodies (PR: Ab7 and ERα: MC-20). IP extracts with rabbit anti-IgG were used as controls. Input: C4-HD nuclear extracts. A representative experiment of three is shown.

**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 co-localization between total PR (M3568), pPR and ERα (SP1). Bar: 40μm. **(B)** No staining is observed in a PR-/ER- sample under the same conditions. Nuclei were counterstained with DAPI. Bar: 40μm. **(C)** Nuclear extracts from two PR+/ER+ tumors (A and B) and one PR- tumor (C) were IP using PR (M3568) or ERα (SP1) antibodies and immunoblotted with the corresponding antibodies. Input: purified nuclear extracts from each tumor used in IP assays.

**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 Figure 2. Bar: 15μm. The nuclear PR/ERα co-localization was estimated through the Pearson's correlation coefficient. **(B)** Nuclear extracts of
untreated or MPA-treated cells were IP 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 three is shown. (C) [3H]-thymidine uptake assays. After attachment, cells were starved and treated for 48 h with experimental solutions. A representative experiment of three is shown. (D) Left. Western blots showing ERα (SP1) expression in extracts from cells either treated or not with ICI for 48 h, transfected with two different siRNAs for human ERα or a non-specific siRNA. GAPDH was used as a loading control. Right. [3H]-thymidine uptake assays. Transfected cells with siRNAs were seeded, starved and either treated or not with MPA for 48 h. A representative experiment of three is shown.

**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 two different siRNA pools, prevents the MPA-induced increase in [3H]-thymidine uptake. Cells were transfected and treated as described in Figure 4D. A representative experiment (mean ± SEM) of three 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 h. (C and D) CCND1 (left) and MYC (right) mRNA expression relative to GAPDH was evaluated by qPCR using cells treated for 15 min with MPA and/or ICI, or transfected with two siRNAs for human ERα.
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 h and processed to measure luciferase. A representative experiment of three 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 et al (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 Figure 6B, with C serving as a negative control region of nuclear receptor binding. Data from ChIP-reChIP experiments using ChIP primers A (Fig. 6B) on CCND1 (C, right) and MYC (D, right) promoters are shown. Cells treated or not with MPA for 10 min were first IP with PR (H-190X) or ERα (HC-20X) antibodies, and then IP using either ERα or PR antibodies. qPCR and data analysis were performed as detailed in Materials and Methods.

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 (10nM) or MPA + ICI (1μM) for 10 min 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 Figure 6C. (B) Cells were treated with Ctrl siRNA or ERα siRNAs as shown in Figure 4D, starved, and either treated or not with MPA (10nM) for 10 min and processed for ChIP/qPCR studies (using ChIP primers A for both genes, Figure 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 non-genomic models for PR/ERα interaction after progestin treatment. See text for details.
Giulianelli et al., Figure 1

A

![Graph showing tumor size over days for MPA, MPA + FUL, and - MPA conditions.](image)

B

![Western blot diagram showing PR_b, PR_a, ER_a, and Erk1/2 expression over time (48 h and 96 h) for MPA and FUL conditions.](image)

C

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**Graphs showing quantitative analysis of PR, ER_a, CCND1, and MYC expression levels.**

**Legend:**
- **MPA**
- **FUL**
A

ChIP CCND1

IP: PR

Fold Induction

IP: ERα

Fold Induction

ChIP MYC

IP: PR

Fold Induction

IP: ERα

Fold Induction

ChIP Primers A B C

Ctrl MPA MPA + ICI

** B P < 0.05

A vs. B P < 0.05

B

ChIP CCND1

Fold Induction

MPA (10nM) + - + - + - -

siRNA Ctrl + - + - + - -

siRNA ERα (ESR1_8) + - + - + - -

siRNA ERα (ESR1_10) + - + - + - -

IP: PR IP: ERα

** P < 0.001

a vs. b P < 0.001

ChIP MYC

Fold Induction

MPA (10nM) + - + - + - -

siRNA Ctrl + - + - + - -

siRNA ERα (ESR1_8) + - + - + - -

siRNA ERα (ESR1_10) + - + - + - -

IP: PR IP: ERα

** P < 0.001

a vs. b P < 0.001

C

Graphical representation of the pathway involving PR, ERα, c-Src, Ras/Raf, MEK, PI3K, Akt, MAPKs, JAK STAT, and transcriptional regulation of CCND1 and MYC.
Estrogen receptor alpha mediates progestin-induced mammary tumor growth by interacting with progesterone receptors at the Cyclin D1/MYC promoters

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