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PRECLINICAL STUDY

Classical membrane progesterone receptors in murine mammary carcinomas: agonistic effects of progestins and RU-486 mediating rapid non-genomic effects

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Abstract In this article, we demonstrate the expression of functional progesterone binding sites at the cell membrane in murine mammary carcinomas that are stimulated by progestins and inhibited by antiprogestins. Using confocal immunofluorescence, ligand binding and cell compartment-specific western blots, we were able to identify the presence of the classical progesterone receptors. Medroxyprogesterone acetate (MPA) and RU-486 (1×10^{-11} and 1×10^{-8} M) behaved as agonists activating extracellular signal-regulated kinases (ERKs) and progestin-regulated proteins, except for Cyclin D1 and Tissue factor which failed to increase with 1×10^{-8} M RU-486, an experimental condition that allows PR to bind DNA. These results predicted a full agonist effect at low concentrations of RU-486. Accordingly, at concentrations lower than 1×10^{-11} M, RU-486 increased cell proliferation in vitro. This effect was abolished by incubation with the ERK kinase inhibitor PD 98059 or by OH-tamoxifen. In vivo, at a daily dose of 1.2 μg/kg body weight RU-486 increased tumor growth, whereas at 12 mg/kg induces tumor regression. Our results indicate that low concentrations of MPA and RU-486 induce similar agonistic non-genomic effects, whereas RU-486 at higher concentrations may inhibit cell proliferation by genomic-induced effects. This suggests that RU-486 should be therapeutically administered at doses high enough to guarantee its genomic inhibitory effect.

Keywords Membrane progesterone receptors · Mammary carcinomas · Progesterone receptor isoforms: membrane-initiated steroid signaling · Antiprogestins · Breast cancer treatment · Non-genomic effects · Progestins

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Abbreviations

chFCS	Steroid-stripped fetal calf serum
ERα	ER alpha
MISS	Membrane initiated steroid signaling
MPA	Medroxyprogesterone acetate
mPR	Membrane progesterone receptors
OH-Tam	OH-Tamoxifen
PD	PD 98059
Pg	Progesterone
PI	Propidium iodide
PR	Progesterone receptor
PR-A	PR isoform A

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PR-B	PR isoform B
s.c	Subcutaneous

Introduction

Progesterone (Pg) interacts with the intracellular isoforms of progesterone receptors (PR) A and B, which function as ligand-dependent transcription factors to control the expression of specific genes. This cellular response to Pg is known as the "classical" or genomic action of Pg. Upon ligand binding, receptors dimerize and bind to regulatory DNA sequences on target genes and activate transcription. Several co-regulatory proteins have been identified that bind to and regulate the activity of the receptors [1, 2]. These actions require nuclear localization of the PR, and measurable gene transcription takes about 30–60 min [3].

Rapid, non-classical steroid hormone actions were first described in 1941 by Selye when he observed the anesthetic effects of high Pg doses [cited in 4] and in 1977 by Pietras and Szego [5], who showed the presence of estrogen binding sites in the membrane of endometrial cells. Because these effects were not abrogated by transcriptional inhibitors and did not seem to require hormone binding to intracellular receptors, they were called non-genomic actions [6]. The non-genomic actions of steroids are generically known as membrane-initiated steroid signaling (MISS) mechanisms [7, 8]. Some of these effects are now considered to be non-receptor-mediated actions at the plasma membrane and are thought to arise from alterations in membrane fluidity. High hormone concentrations are necessary to elicit these responses, which are not blocked by classical antagonists [9].

Other Pg effects are mediated by different types of known membrane receptors such as the oxytocin receptor [10], the nicotinic acetylcholine receptor [11], or the GABA A receptor [12]. More recently, other specific Pg binding sites have been characterized. A membrane-associated Pg binding protein, progesterone membrane component 1 (PGMC-1, also known as 25-Dx), is regulated by Pg in brain regions involved in reproductive behavior [13]. Pg treatment increased mRNA levels of this receptor in rats with spinal cord injuries [14]. Zhu et al. [15, 16] described a gene with the characteristics of a membrane progestin receptor in spotted seatrout, and identified and cloned other members of this family of membrane PRs (mPRs) from several vertebrate species, including human, mouse, pig, *Xenopus*, zebra fish, and *Fugu*. Members of this family have highly conserved nucleotide and predicted amino acid sequences and structures similar to those of the spotted seatrout mPR [15, 16]. Phylogenetic analysis indicates that these cDNAs comprise three distinct groups (α, β, and γ) within this gene family. Structural analyses of the translated cDNAs suggest that they encode membrane proteins with seven transmembrane domains. The transcripts showed distinct distributions in reproductive, neural, kidney, and intestinal tissues. The three receptors have been detected in MCF-7 and SK-BR-3 human breast cancer cells, and higher levels of mPRα have been observed in breast cancer as compared to normal breast tissue [17]. Although the role of these proteins as membrane receptors is still controversial [18], a decrease in cAMP has been reported after Pg exposure in mPR-transfected cells. Several synthetic progestins and antiprogestins, including RU-86486 (RU-486), which have relatively high binding affinities for nuclear PR, displayed no binding affinity for recombinant membrane human proteins [19].

In mouse mammary ductal carcinomas induced by medroxyprogesterone acetate (MPA) [20], we have characterized two PR binding sites: a high affinity, low capacity site (K_d: 43 pM; $Q = 9$ fmol/mg protein) and the classical lower affinity, high capacity site (K_d: 9.2 nM; $Q = 376$ fmol/mg protein) [21]. Antiprogestins bind to both sites, suggesting structural similarities. In the same study, we described significant proliferative effects at concentrations lower than those required to activate the classical PR [21]. In vitro, MPA was stimulatory along a curve with two slopes, one at very low concentrations (EC_{50}: 1.5 fM), and the other with an EC_{50} of 0.33 nM. These values are compatible with the described K_d for the classical PR. As membrane receptors may have either very low or very high K_d, these results were considered compatible with the hypothesis that a small number of very high affinity classical PRs could be located at the cell membrane.

Although there are many studies regarding classical membrane estrogen receptor alpha (ERα) [22–24], relatively few reports have evaluated the classical PR. Welter et al. [25] identified classical PRs in human aortic cells and Younglai et al. [26] identified them in granulose cells. In sperm, proteins with a lower molecular weight than the classical PR have been identified that are recognized by antibodies that bind to the ligand-binding domain of the classical PR. Activation is associated with increased calcium influx, tyrosine phosphorylation of sperm proteins including extracellular signal-regulated kinases (ERKs), chloride efflux and an increase in cAMP [27]. In breast cancer cells, there is only some preliminary evidence suggesting the expression of the classical PR on the membrane of MCF-7 cells [28].

The aim of this study is to investigate the expression of classical PRs at the cell membrane in murine mammary carcinomas. We also sought to evaluate the non-genomic effects of MPA, a synthetic progestin, and RU-486, a classical antiprogestin, using the activation of ERK as an indicator of receptor tyrosine kinase (RTK) activation.

Materials and methods

Animals

Two-month-old virgin female BALB/c mice (Instituto de Biología y Medicina Experimental Animal Facility) were used. The animals were housed in groups of four per cage in an air-conditioned room at 20 ± 2°C under a 12-h light/dark cycle, and had access to food and water ad libitum. Animal care and manipulation were in agreement with institutional guidelines and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources 1996).

Tumors

C4-HD is a transplantable ductal mammary tumor induced by MPA in a BALB/c female mouse [29] and is maintained by serial subcutaneous (s.c.) transplantations into syngeneic MPA-treated female mice. C4-HD expresses high levels of ER and PR [30], has a mutated p53 [31], and shows a near-diploid karyotype [31]. The C4-HI variant, derived from a C4-HD tumor that started to grow in a mouse that had not been treated with MPA, also shows high ER and PR levels, but it has acquired a polyploid karyotype [31].

Cell lines

LM3 is an ER and PR negative cell line [32] and was used as a negative control for immunocytochemical studies. Cells were maintained as described previously [29, 32]. Human T47D cells were purchased from the ATCC (Manassas, VA).

Primary cultures

Reagents

DMEM/F12 (1:1 Dulbecco's modified Eagle's medium: Ham's F12, without phenol red), trypsin, EDTA, MPA, OH-Tamoxifen (OH-Tam) and RU-486 were all purchased from Sigma–Aldrich (St. Louis, MO). PD98059 was obtained from Calbiochem, La Jolla, CA. Fetal calf serum (FCS) was purchased from Bioser (Buenos Aires, Argentina).

The epithelial cells were separated from the fibroblasts by differential sedimentation, as previously described [30], and 5×10^5 cells/ml were plated in culture dishes with 10% FCS. Epithelial cells were allowed to attach for 48 h. The medium was replaced with fresh medium with 10% FCS for C4-HI epithelial cells or with medium with 5% steroid-stripped FCS (chFCS) plus 10-nM MPA for C4-HD and was thereafter changed every 2–3 days. At confluence or when cell clusters looked overcrowded, the cells were detached with 0.25% trypsin, washed, resuspended in fresh standard medium, and plated again.

Immunofluorescence

Cells growing on chamber slides were arrested at 80% for 24 h, as previously described. Membrane PR expression was evaluated in cells incubated for 20 min–2 h with FGF-2 (Sigma-Aldrich; 50 ng/ml) or vehicle. For phospho-ERK (pERK), pSer 190 PR and pSer 294 PR studies, cells were incubated for different times with 1×10^{-11} M or 1×10^{-8} M MPA, Pg or RU-486. After washing with PBS, the cells were fixed in formalin or ice cold ethanol 70% for 30 min, blocked in 10% FCS and incubated ON with either primary ERK and pERK antibodies (both from Santa Cruz Biotech., Santa Cruz, CA), PR (Ab-7), pSer190 PR (Ab-11) or pSer 294 (Ab-12) (all three from Neomarkers, Union City, CA), at a 1/100 dilution in blocking buffer at 4°C. The pSer190 and pSer 294 PR antibodies were originally developed by Clemm et al. [33]. Cells were then incubated with FITC-conjugated secondary antibodies 1/100 dilution (sheep anti rabbit or horse anti mouse, Zymed San Francisco, CA) for 1 h at room temperature. The nuclei were stained with propidium iodine (PI), and the slides were mounted with Vectashield. For co-localization studies, cells treated with FGF-2 were fixed as described above, washed with PBS, blocked in 10% FCS and incubated with the E-cadherin antibody (Rabbit mAb 3195, Cell Signaling Tech, Danvers MA) at a 1/100 dilution for 1 h at RT. They were then incubated with the pSer 190 PR or the pSer 294 PR antibodies at a 1/100 dilution in blocking buffer ON at 4°C. After washing with PBS three times, the cells were incubated with either FITC- or Texas red- conjugated secondary antibodies (1/100 dilution) for 1 h at room temperature. The slides were then mounted with Vectashield. Cells were analyzed using a Nikon Laser Confocal Microscope (Plan Apo 60×/1.40 oil).

Extract preparation

Membrane extract preparation

Reagents Tris (Tris[hydroxymethyl]aminomethane) was purchased from USB Corporation (Cleveland, OH); sodium molybdate and NaOH were from Merck Química (Buenos Aires, Argentina); NaCl and Triton X-100 were purchased from Anedra (Buenos Aires; Argentina); protease inhibitors and $MgCl_2 \cdot 6H_2O$ were purchased from Sigma-Aldrich (St Louis, USA); and sucrose was purchased from ICN Biomedicals, Inc (Aurora, OH).

C4-HI tumor transplants were excised when they reached 100 mm^2, snap-frozen and kept in liquid nitrogen. Fifteen grams of tumor were pooled and processed for membrane isolation as previously described [22] with modifications. The tissues were homogenized in two volumes of isotonic buffer pH 8.2 (20-mM Tris, 10-mM sodium molybdate, 140-mM NaCl, 5-mM $MgCl_2 \cdot 6H_2O$) with protease inhibitors (0.5-mM PMSF, 0.025-mM ZPCK, 0.0025-mM TLCK, 0.025-mM TPCK, 0.025-mM TAME). All procedures were performed at 4°C. The nuclear components and unbroken cells were removed by centrifuging twice at 2300 rpm for 15 min in isotonic buffer plus inhibitors. The supernatants were combined and centrifuged at 33000 rpm for 1 h. The pellet was resuspended in 0.5 ml of buffer, and the suspension was layered on top of a 4 ml, 41% (wt/v, in isotonic buffer) sucrose layer and centrifuged at 29000 rpm for 65 min. Located on the top of the layer, membranes were removed and washed with 4-ml isotonic buffer by centrifugation at 33000 rpm for 50 min. The pellet was resuspended in a solution of 0.2% (v/v) Triton X-100/0.1 N NaOH and extracted by mixing it with a magnetic stir bar on ice for 2 h. The solution was centrifuged at 33000 rpm for 30 min, the supernatant was recovered and tested for protein concentration by the Lowry assay [34] and the purity of the membrane fractions was assessed by lactate dehydrogenase analysis. Membrane extracts from C4-HI or C4-HD primary cultures were obtained as reported previously [35]. To study the regulation of classic mPR by MPA or RU-486 using Western blots, 10 P100 Petri dishes/group of primary cultures of purified epithelial C4-HD cells were used. Cells were starved for 24 h and then incubated with vehicle, 1×10^{-8} M MPA or RU-486 for 20 min and they were processed as described above and resuspended in a final volume of 30 µl.

Preparation of cell extracts for ERK studies

The cells were serum-starved for 48 h before hormonal treatments. Then, they were incubated for 1–20 min with MPA, Pg, RU-486 or control media. The incubation medium was removed, the cells were washed three times with ice-cold PBS and lysed using M-Per (Mammalian protein extraction reagent; Pierce, Rockford, IL, USA) plus protease inhibitors, following the instructions of the manufacturer. Protein concentration was determined by the Lowry assay [34]. Tumor extracts were obtained as previously described [36].

Lactate dehydrogenase assay (LDH)

The activity of LDH, a cytoplasmic enzyme, was analyzed by the conversion of pyruvate to L-lactate. A decrease in absorbance at 340 nm with time is indicative of NADH oxidation. The spectrophotometer was set at 340 nm and 25°C, and the following solutions were added into the cuvette: 2.8 ml 0.2 M Tris-HCl pH 7.3, 0.1 ml 6.6 mM NADH (Sigma-Aldrich), and 0.1 ml 30 mM sodium pyruvate (Sigma–Aldrich). The cuvette was left in the spectrophotometer 4–5 min to achieve temperature equilibration and then established a blank rate. For each determination, 0.05 ml of the extract was added and recorded ΔA_{340}/min.

Binding assays

C4-HI or LM3 cells were cultured as described above in 30 p100 Petri dishes and incubated for 20 min with FGF-2 (50 ng/ml). The rationale for this design was our previous observation that FGF-2 increased PR membrane immunoreactivity. Cell extracts were processed to obtain standard membrane extracts. Briefly, after a first centrifugation of 10 min at 1000 rpm, the pellet was resuspended in 1 ml of binding buffer (10 mM Tris–HCl, pH 7.4, 1.5 mM EDTA, 10% glycerol, 10 mM sodium molybdate) and centrifuged at 2300 rpm for 15 min to discard nuclei. The supernatant was then centrifuged at 13000 rpm for 20 min, and the pellet was now resuspended in binding buffer. Extracts from three experiments were pooled and 100 µl of extracts (quintuplicates) were incubated with 2.2 nM ^{3}H-R5020 (progesterone receptor agonist; specific activity: 84.8 Ci/mmol; NEN, Boston, MA) and an excess of R5020 (1 µM final concentration; NEN, Boston, MA) or buffer. After 24 h of incubation at 0°C, 1 ml of cold buffer was added and the solutions were immediately filtered using glass fiber filters (GF/B 12.5 cm, Whatman, Maidstone, UK). Tubes were rinsed with another 1 ml of cold buffer and the procedure was repeated. Filters were left at room temperature until they were dry, and then they were immersed in scintillation solution (OptiPhase, HiSafe**,** Perkin Elmer, Boston, MA) and counted in a liquid scintillation counter. The experiment was repeated twice.

Western blots

The reagents were purchased from Invitrogen Life Tech. (Carlsbad, CA). Methanol was purchased from Merck Química. Rainbow prestained molecular weight markers and HyBond-ECL nitrocellulose membranes were from Amersham Life Science (Buckinghamshire, UK). KCl was purchased from Anedra.

Tumor or cell extracts (30–50 µg total protein/lane) were separated on 8% (PR, ERα, FGFR-2, Integrin-β1 or Sp1), 10% (EGF-R, tissue factor, c-myc and STAT 5), or 12% (ERK 1 and 2, p21 and cyclin D1) discontinuous polyacrylamide gels (SDS-PAGE). The proteins were dissolved

in sample buffer (6 mM Tris pH 6.8, 2% SDS, 0.002% bromophenolblue, 20% glycerol, 5% mercaptoethanol) and boiled for 2 min. After electrophoresis, they were blotted onto a 0.4-μm nitrocellulose membrane and blocked overnight in 5% skimmed milk in 0.1% PBST (0.8% w/v NaCl, 0.02% w/v KCl, 0.144% w/v Na_2PO_4, 0.024% w/v KH_2PO_4, pH 7.4, 0.1% v/v Tween 20). The membranes were washed several times with PBST and then incubated with different PR antibodies: PR Ab-7 (Ab7; Neomarkers) or C-19 (sc-538; Santa Cruz Biotech.) or with ERα (MC-20; Santa Cruz Biotech.) or integrin β1 (MAB 1997; Chemicon, Billerica, MA); or Sp1 (RB-10475; Thermo Fisher Scientific, Fremont, CA) or cyclin D1 (Ab-1, Clone DCS-6; Thermo Scientific, Fremont, CA) or FGFR-2 (Bek, C-17; Santa Cruz Biotech.) or tissue factor (FL-294; Santa Cruz Biotech.) or p21 (sc-397; Santa Cruz Biotech) or STAT5 (sc-835; Santa Cruz Biotech) or EGFR (sc-03; Santa Cruz Biotech) or c-myc (9E10; Covance Research products; Denver, Co) antibodies. For pERK evaluation, ERK (ERK 1; sc-94, Santa Cruz Biotech.) or pERK (sc-7383; Santa Cruz Biotech.) antibodies were used and for pAKT evaluation in T47D cells, phosphorylated Ser 473 AKT (9271; Cell Signaling Tech, Danvers MA), and total AKT (610837; BD Transduction Laboratories) antibodies were used. All antibodies were incubated overnight at 4°C, at a 1:500 dilution in PBST except for PR (Ab-7) that was used at a 1:100 dilution and ERK, pAKT, and AKT at a 1:1000 dilution. Blots were probed with sheep anti-mouse IgG or donkey anti-rabbit IgG horseradish peroxidase-conjugated whole antibody (Amersham Life Science; 1:1000 dilutions). The luminescent signal was generated with an ECL Western blotting detection reagent kit (Amersham Pharmacia Biotech, Buckinghamshire, UK), and the blots were exposed to an autoradiographic film (Curix RP1, Agfa, Argentina) for 10 s–20 min. Band intensity was quantified only in unsaturated films.

RT-PCR

The cells were grown until 80% confluence, arrested with medium plus 1% chFCS for 24 h., washed in sterile PBS, scraped with Tri Reagent (Molecular Research Center Inc., Cincinnati, OH) and incubated for 5 min at 30°C. Chloroform was added, and the samples were incubated for other 3 min and centrifuged at 12000 rpm for 15 min at 4°C. The aqueous phase was recovered; cold isopropanol was then added, and the mixture was incubated 10 min at 30°C and centrifuged at 12000 rpm for 10 min. The pellet was recovered, washed with 75% ethanol, centrifuged at 6800 rpm for 5 min, dried, resuspended in DEPC water, and kept at 55°C for 10 min before measuring the purified RNA concentration in a spectrophotometer. After DNAse treatment (Deoxyribonuclease I, Amplification grade, Invitrogen), reverse transcription was performed using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Life Tech.). PCR were performed with the cDNA obtained as a template. Two pairs of primers were designed for mPRα, mPRβ, and mPRγ. mPRα: (a) forward 5′-GTGATGCTGCTAGCCAATCC-3′, reverse 5′-TCAGGAGTCCTTCATCTCTGC-3′; (b) forward 5′-CTTGGCCTCCTTCACCTACC-3′, reverse 5′-GAAGCTGTAATGCCAGAACTCC-3′. mPRβ: (a) forward 5′-CTTGCAGGTCAGGAGATTGG-3′, reverse 5′-GATCCGCTTGACATAGTCTGC-3′; (b) forward 5′-AGGCACTGTCCGTGATATGG-3′, reverse 5′-GGTTCATGCAGAAGACAGTCC-3′. mPRγ: (a) forward 5′-TAAGAAGCTTGCTGCCAAGG-3′, reverse 5′-GAGGCCATCACACCTTATCGGAGGCCATCACACCTTATCG-3′; (b) forward 5′-CTACATTGGCCACAGTCACC-3′, reverse 5′-GGTCTTGTCCAGAAGGATGG-3′. For PR, the primers were forward 5′-AAAGGATCCGCAGGTTCTC-3′ and reverse 5′-CCAGGGAGATCGGTATAGGC-3′. The accession number of the sequences used to design the RT-PCR primers were: mPRα, NM: 027995.2; mPRβ, NM: 028829.3; mPRγ, NM: 028748; and PR, NM: 008829.2. Finally, the samples were loaded on a 2% agarose gel.

^{3}H-Thymidine uptake assay

Cell proliferation assays were performed as previously described [37]. Briefly, cells were seeded in 96-well microplates. After attachment (24 h), the cells were incubated for 48 h with the experimental solutions to be tested in 1% chFCS. Fifty percent of the medium was replaced with fresh medium every 24 h. The cells were incubated with 0.4 μCi of ^{3}H-thymidine (specific activity: 20 Ci/mmol) for 24 h, trypsinized and harvested in a cell harvester. Filters were counted in a liquid scintillation counter. The assays were performed in octuplets; means and standard deviations were calculated for each solution tested.

Preparation of nuclear extracts

C4-HD epithelial cells were collected and lysed on ice for 10 min in TEDGS buffer (50 mM Tris pH 7.4, 7.5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 0.25 M sucrose). Protease inhibitors (0.5 mM PMSF, 0.025 mM ZPCK, 0.0025 mM TLCK, 0.025 mM TPCK, 0.025 mM TAME) were added before preparing the extracts. The homogenate was centrifuged for 10 min at 3300 rpm at 4°C, and the nuclei on the pellet were washed in TEDGS buffer—0.01% NP-40. The nuclei were resuspended in TEDGS containing 0.4-M KCl and incubated at 4°C for 30 min, and the nuclear homogenate was centrifuged at 12000 rpm for 20 min at 4°C. The nuclear extract was

diluted 1:2 in TEDGS buffer with 30% glycerol, to reduce the salt concentration. Proteins were quantified by Lowry.

Electrophoretic mobility gel shift assay (EMSA)

To detect DNA-binding activity of PR in nuclear extracts of tumor cells, we used a synthetic 27-bp oligonucleotide containing a progesterone response element (PRE) 5′-GATCC**TGTACA**GGA**TGTTCT**AGCTACA-3′ (DNAgency). This assay was performed as previously described [38]. Briefly, the PRE oligonucleotide was end-labeled with [γ^{32}P] ATP (New England Nuclear, where) by T4 Polynucleotide Kinase (New England Biolabs) to a specific activity of 30000–50000 cpm/0.1 ng. The nuclear extracts (20 μg) were incubated with ^{32}P-PRE for 40 min at room temperature in a total reaction volume of 30 μl. The DNA-binding reaction also consisted of 40 ng/μg nuclear extract of poly dI–dC in a binding buffer containing 10-mM Tris pH 7.4, 50-mM NaCl, 1-mM dithiothreitol, 5-mM $MgCl_2$, 0.5 μg/μl gelatin, 10% glycerol, 0.5-mM PMSF, 5-μg/ml aprotinin, 5-μg/ml leupeptin, 5-μg/ml pepstatin, 0.15-mM spermin, 0.5-mM spermidine. The samples were subjected to electrophoresis on nondenaturing 5% acrylamide gels in low ionic strength TBE buffer (22.5-mM tris, 20-mM boric acid, 0.5-mM EDTA), at 20 mA/gel at room temperature for 3 h. Gels were dried under vacuum and were autoradiographed by exposure to medical X-ray film (CP-BU New, Agfa Argentina) at −70°C. The specificity of PR–PRE complexes was studied by competition with 50-fold mass excesses of unlabeled PRE or an unrelated oligonucleotide (DNAgency). In the supershift assays, the rabbit polyclonal anti-PR antibody C-19 (Santa Cruz Biotech) was used at a concentration of 4 μg/assay.

In vivo RU-486 treatment

BALB/c mice bearing s.c. transplanted HI tumors of a size of approximately 50 mm^2 were treated s.c. with RU-486 (12 mg/kg body weight/day). Several mice were euthanized one or 6 h after treatment for pMAPK evaluation and control mice were euthanized 1 h after vehicle administration. The remaining animals were used for the evaluation of tumor size. Tumors were processed for Western blot or immunohistochemical studies.

To evaluate the effect of low RU-486 concentrations, tumors were transplanted s.c. and after 4 days, animals were ovariectomized and RU-486 (1.2 μg/day/kg body weight) treatment was started once the tumors became palpable. Animals were observed for 10 days, and tumor size was measured with a Vernier Caliper. Three different experiments using four animals per group were conducted.

Statistical analysis

Differences in tumor size between two groups were evaluated using the Student's *t* test. The intensity of the bands corrected to protein loading and ^{3}H-thymidine uptake data were compared using one-way ANOVA analysis followed by Tukey *t* test. Data of one representative experiment is shown. All experiments, unless indicated, were performed using triplicates at least three different times.

Results

C4-HD tumors grow only in progestin-treated mice. C4-HI, on the other hand, is one of the several variants generated that can grow in untreated mice [40]. Both C4-HD and C4-HI regress with RU-486 treatment. This is also observed with other antiprogestins (ZK 98299 and ZK 230211) and with antisense oligonucleotides to PR, consistent with a role for PRs in mediating the growth of both tumor types [36, 41, 42]. In vitro, both tumors showed similar hormone responsiveness. MPA stimulated and RU-486 inhibited cell proliferation [38].

Classical PRs are also located at the cell membrane in mammary carcinomas

Due to the large amounts of PR expressed in tumors from the MPA-breast cancer model, we decided to explore the possible presence of membrane-associated progesterone binding sites. We assayed different growth conditions and fixation protocols. As we have shown in previous studies, FGF-2 mimics the effects of progestins inducing PR phosphorylation, PR activation, and PR-mediated cell proliferation [38]. Thus, we decided to evaluate the possible PR membrane localization using quiescent, progestin, RU-486, or FGF-2-treated cells using first the Ab-7 monoclonal antibody. An optimal positive membrane signal was evident by confocal microscopy mainly in C4-HI cells incubated with FGF-2 (50 ng/ml; Fig. 1a, left), and fixed with ethanol but not with formaldehyde. Under these experimental conditions both nuclear and cytosolic PR signals were also observed. However, mainly nuclear staining was observed using this antibody in MPA (1×10^{-8} M)- or RU-486 (1×10^{-8} M)-treated cells (Fig. 1a, left). No immunoreactivity was observed in PR-negative murine LM3 cells (Fig. 1b).

This prompted us to investigate membrane pPRs using the Ab-12 and the Ab-11 antibodies originally developed by Clemm et al. [33], which react with PR phosphorylated at serine 294 or 190 residue, respectively. Phosphorylation of these serines increases with ligand binding [33]. An increase in membrane staining was observed after 20 min of incubation

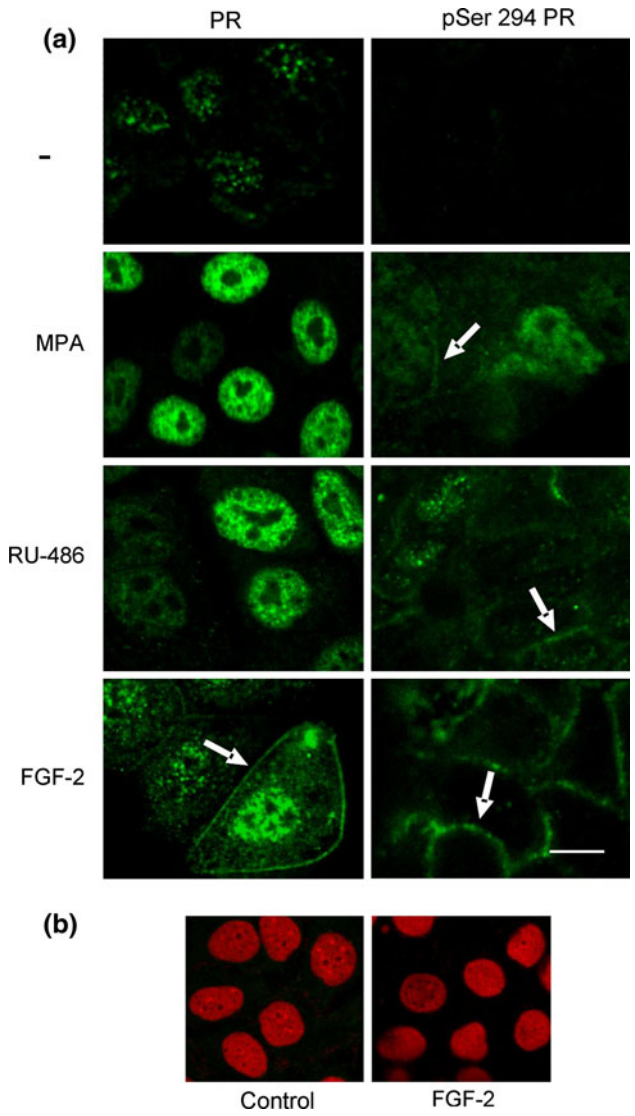

Fig. 1 Classic PR showing membrane localization. **a** Immunofluorescence of primary cultures of C4-HI cells incubated for 20 min–2 h, with or without MPA (1×10^{-8} M), RU-486 (1×10^{-8} M), or FGF-2 (50 ng/ml). Cells were then fixed with ice cold ethanol 70% for 30 min and incubated with PR Ab 7 (*left*) or pSer 294 PR (Ab-12; *right*) monoclonal antibodies and a secondary anti-mouse FITC as described in "Materials and methods". MPA and RU-486 induced an increase in nuclear PR staining, whereas membrane (*arrow*) and nuclear staining were observed in FGF-2-treated cells. All treatments induced membrane pSer 294 PR staining (*arrows*). **b**. As a negative control, the PR negative LM3 cells treated or not with FGF-2 were used and immunostained with Ab-7. In this case, PI was used for nuclear counterstaining; *bar*: 9 μm

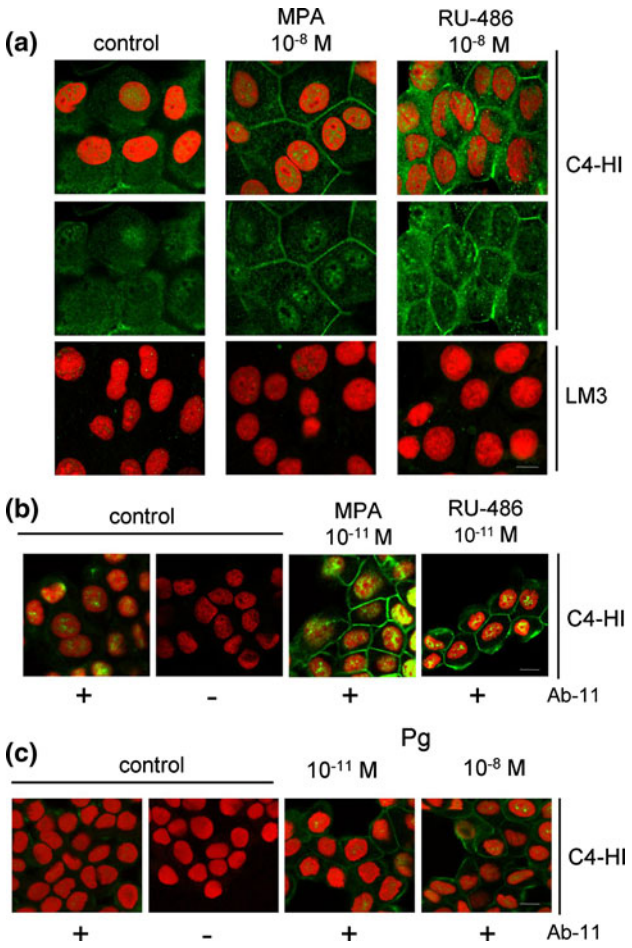

Fig. 2 Membrane pSer 190 PR localization increases with low or high concentrations of progestins or RU-486. **a** C4-HI primary cultures were treated for 20 min with either MPA (1×10^{-8} M), RU-486 (1×10^{-8} M), or vehicle and fixed with ice cold ethanol 70% for 30 min. Then they were immunostained with pSer190 PR Ab (Ab 11, Neomarkers), and with a secondary FITC-coupled mouse antibody. PI was used for nuclear counterstaining. As a negative control the PR negative LM3 cells were used; *bar*: 10 μm. **b** C4-HI cells were treated with 1×10^{-11} M MPA, RU-486 or vehicle and they were processed as described in (**a**); *bar*: 15 μm. **c** C4-HI cells were treated with 1×10^{-11} or 1×10^{-8} M Pg and they were processed as described above; *bar*: 15 μm. An increase in membrane staining was observed in all treated cells

with MPA (1×10^{-8} M), RU-486 (1×10^{-8} M) or FGF-2 (50 ng/ml) using the pSer 294 antibody (Fig 1a, right).

We next studied the pSer 190 PR expression in progestin- or RU-486-treated cells. As observed in Fig 2, both compounds increased membrane staining. Thus, we chose this antibody to evaluate if lower concentrations of MPA and RU-486 had the same effect and to evaluate if Pg also increased membrane staining. As shown in Fig. 2a, membrane staining increased when cells were treated for 20 min with 1×10^{-8} M (Fig. 2a) or 1×10^{-11} M (Fig. 2b) MPA, RU-486, or Pg (Fig. 2c). PR-negative LM3 cells did not react with this antibody.

To corroborate membrane PR staining, we also performed co-localization studies in FGF-2-treated cells, the experimental condition that showed the highest PR membrane staining. We used the pSer 190 PR and the pSer 294 PR antibodies, together with an E-cadherin antibody, and found that pPR and E-cadherin line together at the cell membrane (Fig. 3).

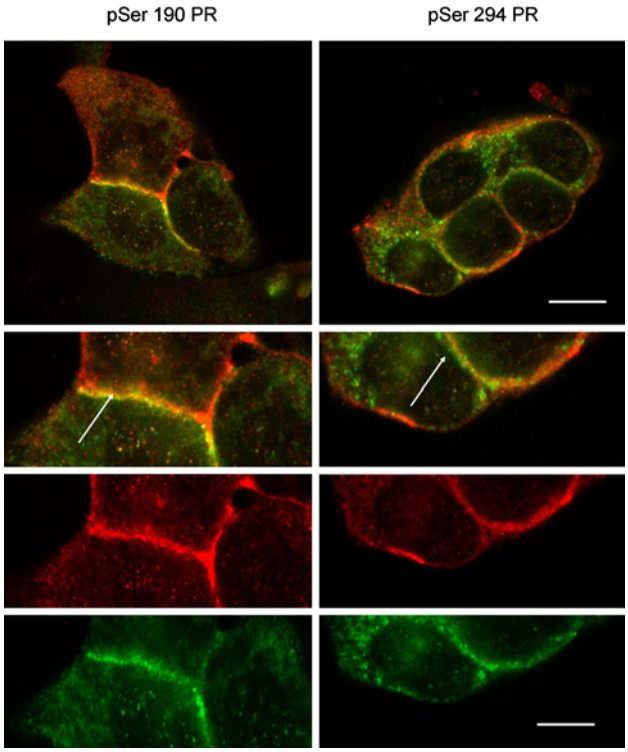

Fig. 3 pPR colocalizes at the cell membrane with E-cadherin. Immunofluorescence of primary cultures treated for 20 min with FGF-2 (50 ng/ml). Cells were fixed as described above and incubated with the monoclonal mouse pSer190 PR (Ab 11) or pSer 294 (Ab12) antibodies and the rabbit monoclonal E-cadherin antibody as described in "Materials and methods". A secondary FITC-coupled mouse antibody and a Texas red-coupled rabbit antibody were used. *Top* clusters of cells showing membrane staining for both antibodies; *bar*: 10 μm. *Bottom* A high power view of a membrane area in which the co-localization can be appreciated (*arrows*). *Middle panel*: E cadherin; *lower panel*: pSer PR stainings

To further confirm the results of our immunocytochemical analysis, we performed Western blots using purified membrane fractions prepared as described by Powell et al. [22] to detect membrane ERα in MCF-7 cells. Starting with 15 g of tumors, we purified 1–6 mg of membrane proteins. Since each gram of tumor contains approximately 100 μg of soluble protein out of 1500 μg of total protein, we believe that our procedure purifies the extracts by 250–1500-fold. Immunoreactive bands, corresponding to PR-A (83 kDa) and PR-B (115 kDa), were detected in the same extracts using both a monoclonal (Ab-7) and a polyclonal (C-19) antibodies. Similar results were obtained using the monoclonal Ab-11 antibody that recognizes pSer190 PR-A and B (Fig. 4a). In almost all Western blots, regardless of the antibody used, a band of 105 kDa was observed in both total and membrane extracts. ERα (66 kDa) was also present in these membrane fractions (Fig. 4b). To rule out contamination with cytosolic or nuclear proteins during separation procedures, several different cell compartment markers were included. In all the cases, they were assayed using the diluted 30 μg/ml protein extracts used for membrane extracts. β1-integrin (144 kDa) and FGFR-2 (120 kDa) were used as membrane control proteins and at this protein dilution they were highly expressed only in the membrane fraction. Similarly, cyclin D1 and Sp1 nuclear proteins were only observed in the total fractions (Fig. 3b). In addition to the Western blot controls, the absence of significant NADH oxidation further validated the membrane fraction (Fig. 4c).

Membrane extracts were also used to detect PR via binding techniques. Membrane extracts from FGF-2-treated cells were obtained and processed for ^{3}H-R5020 binding as described in "Materials and methods". For this particular experiment, we selected FGF-2-treated cells, because their increased expression of PR at the cell membrane. In single-point assays at 2.2-nM ^{3}H-R5020 concentration, we were able to detect specific binding (20983 ± 2333 vs. 16300 ± 1832 cpm) corresponding to 70 fmoles of R5020 binding sites per mg membrane proteins. No specific binding was observed in LM3 which lack PR expression.

As a whole these data suggested that classic PR are also located at the cell membrane and that progestins and RU-486 exert similar effects regarding mPR regulation in a tumor model in which MPA stimulates cell proliferation and RU-486 exerts inhibitory effects.

Non-classic mPR are also expressed in C4-HD and C4-HI cells

The expression of novel mPR receptors was evaluated by PCR using two pairs of primers for each receptor as explained in "Material and methods", and the three types were detected in both C4-HD and C4-HI (not shown). However, since during the course of this study, it was demonstrated that they do not bind MPA or RU-486, they were not considered in further studies.

MPA and RU-486 activate ERK

We evaluated the ability of MPA and RU-486 to activate ERK. MAPK mediate a paradigmatic pathway that can be modified by non-genomic effects of estrogens [43], androgens [44], and progestins [45, 46]. Epithelial tumor cells from C4-HD or C4-HI cultures were incubated with different concentrations of MPA, Pg, and/or RU-486 for varying short periods. ERK activation was analyzed by Western blotting and immunofluorescence using pERK antibodies. Only the experiments showing low basal levels of pERK were used for further analysis. In the experiments in which basal levels were high, no modulation was

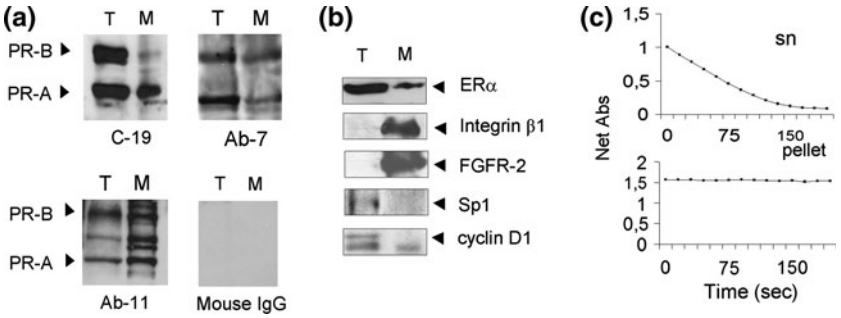

Fig. 4 Detection of classical PR and ERα in purified membrane extracts (M) obtained from C4-HI tumors (T). **a** *Top* Western blots of the same membrane extract purified from 15 g of tumor tissue blotted with two different antibodies against PR: C-19, a polyclonal antibody, and the Ab-7 monoclonal antibody. PR-A (83 kDa) and PR-B (115 kDa) were detected with both antibodies. A whole cell extract from the C4-HD tumor was used as a positive control (T). *Bottom* similar extracts were blotted with the pSerPR antibody, Ab 11 (Neomarkers), and the bands corresponding to the PR isoforms were also observed. A negative control using only the secondary anti mouse antibody is shown. **b** Western blot showing the immunoreactive band corresponding to ERα (66 kDa, MC-20 Santa Cruz Biotech.) in the same T and M fractions used in a. Integrin $\beta 1$ (144 kDa, Chemicon) and FGFR-2 (120 kDa; Santa Cruz Biotech.) were detected with high intensity only in the membrane fractions, while Sp1 (106 kDa) and cyclin D1 (36 kDa) were only observed in the total fractions. These controls were performed seeding 30 µg/lane to use the same protein dilutions as those used in membrane extracts. **c** A lactate dehydrogenase assay was used to confirm the purity of the membrane extracts. In the last centrifugation performed to obtain the purified membrane fractions, no enzyme activity, suggestive of cytosolic contaminant, was detected in the pellet as compared with the supernatant (sn)

observed. Figure 5a shows representative Western blots of extracts from C4-HI cells incubated with 1×10^{-11} M MPA or RU-486 (left panel), 1×10^{-8} M MPA or RU-486 (middle panel), or 1×10^{-8} M Pg with or without 1×10^{-8} M RU-486 (right panel). Even low concentrations of MPA or RU-486 increased levels of pERK 1 or 2, or both. Pg also increased pERK, and RU-486 did not reverse Pg-induced activation. Immunofluorescence studies support the results of the Western blot analysis (Fig. 5b). An increase in cytoplasmic and nuclear pERK was observed in MPA-treated cells. Pg and RU-486 induced a similar effect and did not abolish the MPA-induced increase (not shown).

We have previously observed a complete decrease in pERK 24–48 h after RU-486 administration in vivo, concomitant with PR-down regulation and tumor regression [36]. To investigate whether the rapid increase in pERK observed in vitro was also observed in vivo, we evaluated pERK/ERK in tumors growing in mice treated for 1–6 h with RU-486. An increase in pERK was also observed as early as 1 h after RU-486 administration ($P < 0.001$; Fig. 5c). These results indicate that ERK activation precedes tumor involution, and that activation of these kinases is a common pathway leading to increases or decreases of cell proliferation.

RU-486 mimics the effect of MPA regarding the expression of progestin-regulated proteins except for the expression of cyclin D1 and tissue factor at 1×10^{-8} M

To further corroborate our data suggesting the presence of agonistic effects of RU-486 and MPA, we evaluated the expression of several Pg-regulated proteins [47, 48] after 24 h of treatment. RU-486 showed at low concentrations a similar pattern of protein expression regulation as MPA. At higher concentrations, however, it behaved as an agonist for some of the analyzed proteins (myc, STAT 5 and p21) or as an antagonistic for others such as Cyclin D1 and Tissue factor (Fig. 6a, b). No regulation was observed for EGF-R. pERK was also evaluated and was still activated, even with 1×10^{-8} M RU-486. These results suggest a different mechanism of action between low ($<1\times10^{-11}$ M) or high ($>1\times10^{-8}$ M) concentrations of RU-486 depending on the protein involved.

PRs activated by 1×10^{-8} M RU-486 bind DNA

To corroborate that PRs activated by RU-486 are able to bind naked DNA, and potentially induce genomic effects, we performed EMSA studies using two concentrations of RU-486 (1×10^{-11} M and 1×10^{-8} M). Only 1×10^{-8} M RU 486 was able to induce a mobility gel shift using a PRE probe (Fig. 7), confirming that although lower and higher doses of RU-486 were able to activate membrane PR or pERKs, only the high concentrations, compatible with the standard receptor's Kd [21], are able to bind DNA to induce genomic effects.

Low concentrations of RU-486 stimulate cell proliferation and in vivo tumor growth

RU-486 proved inhibitory throughout our experiments, even at 1×10^{-9} M [30]. However, the similar agonistic non-genomic effects of MPA and RU-486 suggested that at

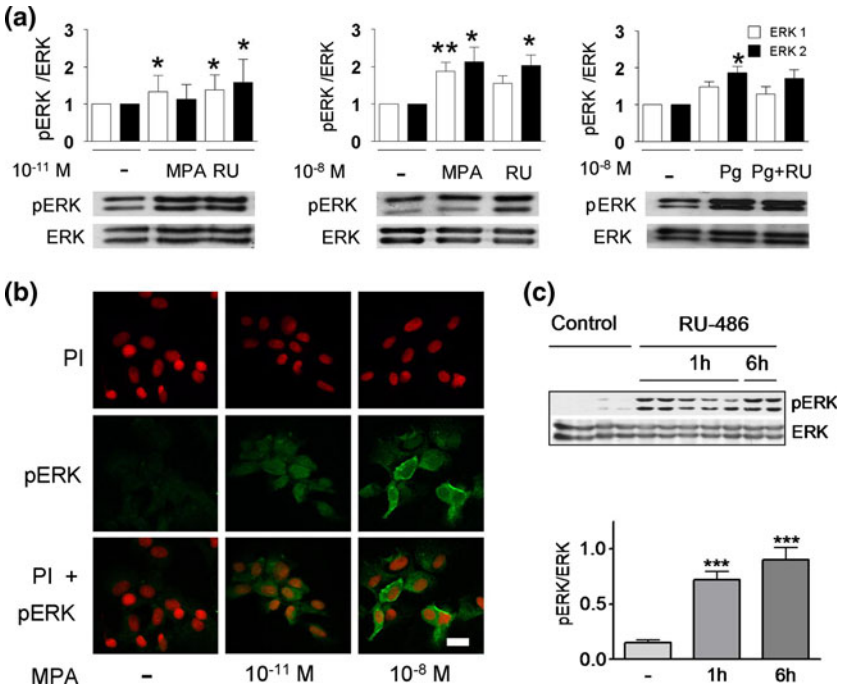

Fig. 5 ERK activation induced by MPA and RU-486. **a** Representative Western blots of pERK 1 and 2 and total ERK in primary cultures of C4-HI treated or not for 10 min with 1×10^{-11} M MPA or RU-486 (RU; *left*) or 1×10^{-8} M MPA or RU (*middle*) or 1×10^{-8} M Pg with or without 1×10^{-8} M RU (*right*). The intensities of pERK 1 (44 kDa) and pERK 2 (42 kDa) in relation to the amount of total ERK 1 or 2 in eight different extracts/group for MPA and RU and in three for Pg were quantified. Experimental values were referred to the mean value observed in the control group. ** $P < 0.01$ and * $P < 0.05$, experimental versus control group. **b** Immunocytochemical studies showing primary cultures of C4-HI treated for 10 min with 1×10^{-11} M and 1×10^{-8} M MPA. The primary monoclonal antibody against pERK (Santa Cruz Biotech.) was used followed by a mouse secondary FITC-labeled antibody. PI was used for nuclear counterstaining (*bar*: 20 μm). An increase in both cytosolic and nuclear staining is observed. **c** Immunoblots of pERK and ERK (Santa Cruz Biotech), using tumors obtained from mice treated with saline or RU-486 (12 mg/kg body weight), for 1 or 6 h. Protein bands were quantified by densitometry and levels of pERK (1 + 2) were calculated after normalization to the ERK content of each sample**.** An increase in pERK was observed after 1 and 6 h of RU-486 treatment; *** $P < 0.001$ RU-486-treated versus control

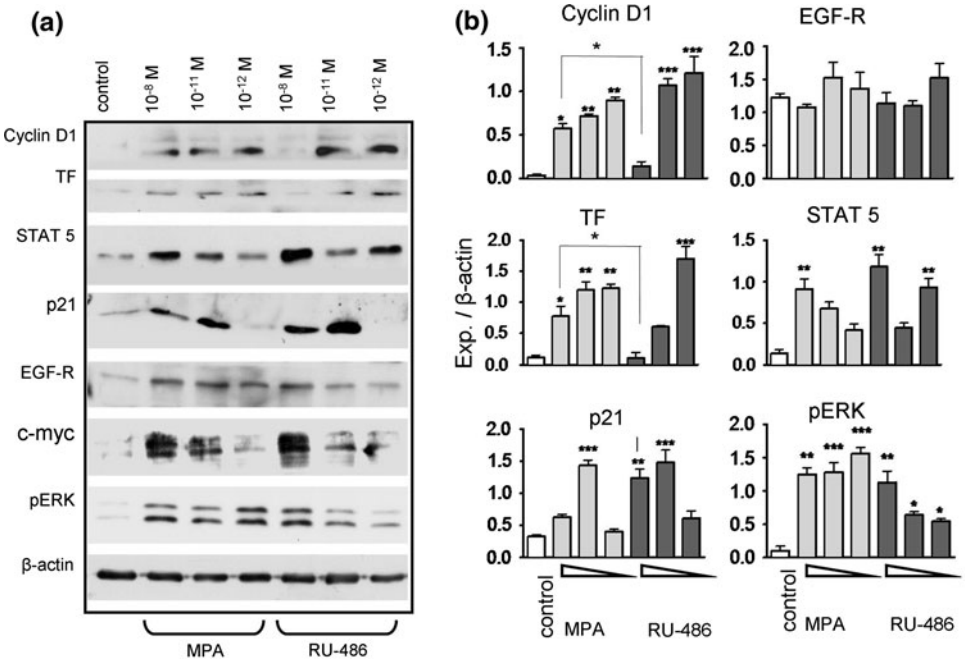

Fig. 6 Effect of different concentrations of MPA and RU-486 on the expression of progestin-regulated proteins. **a** Regulation of Pg-regulated proteins by MPA and RU-486. Representative Western blots of pERK 1 and 2, STAT 5, c-myc, cyclin D1, p21, tissue factor (TF), and EGF-R in primary cultures of cells treated or not for 24 h with MPA or RU-486. Cells were incubated in the absence of serum 24 h previous to treatment. **b** The intensities of the bands in relation to the loading control (β-actin) of two different experiments are shown (right). *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$, experimental versus control group

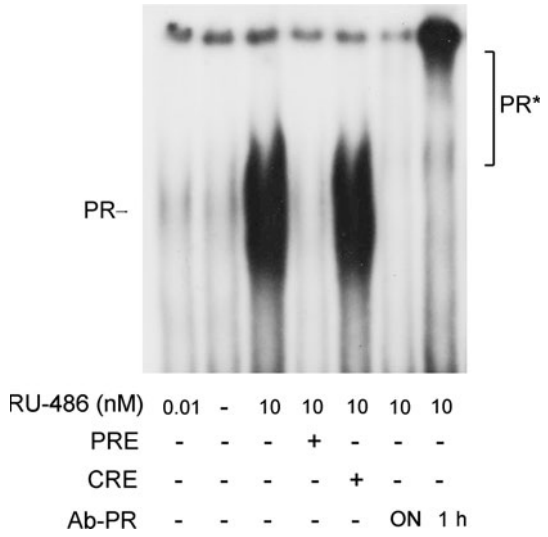

Fig. 7 Effect of different concentrations of RU-486 on EMSA. Primary cultures of epithelial C4-HD cells were stimulated with 1×10^{-11} M and 1×10^{-8} M RU-486 for 1 h and nuclear extracts were normalized by protein content and used in EMSA. Different controls were used to assess the specificity of the bands: the displacement with excess of unlabeled PRE, or the unrelated sequence CRE, and the supershift assay (PR*) induced by the incubation with the polyclonal antibody anti-PR (C-19, Santa Cruz Biotech). The ON incubation blocked PR–DNA binding and the incubation for 1 h induced upshifted bands

concentrations lower than 1×10^{-11} M, a stimulatory effect on cell proliferation should be observed. To further analyze this possibility, we studied the effect of RU-486 on ^{3}H-thymidine uptake over a concentration response curve ranging from 1×10^{-15} to 1×10^{-7} M in C4-HI cells; and as observed in Fig. 8a, low concentrations of RU-486 stimulated cell proliferation. Moreover, while 1×10^{-8} M RU-486 was able to completely abolish MPA-induced cell proliferation ($P < 0.001$), at low concentrations, RU-486 increased MPA-induced cell proliferation ($P < 0.01$; Fig. 8b). The simultaneous incubation of low concentrations of MPA or RU-486 together with the MEK inhibitor PD 98059 (PD) or with OH-Tam prevented the stimulatory proliferative effects (Fig. 8c). These results indicate, on one hand, that ERKs mediate cell proliferation induced by RU-486 and, on the other hand, they support the use of combined antiprogestins/antiestrogens treatments for breast cancer to prevent any potential agonistic effect of antiprogestins.

To investigate if low concentrations of RU-486 were able to stimulate cell proliferation in a human breast cancer we used T47D cells. As shown in Supplemental Fig. 1a, low concentrations of RU-486 are also stimulatory for these human cells. Moreover, the increase in cell proliferation correlated with increases in ERK and AKT phosphorylation (Supplemental Fig. 1b).

To move to an in vivo setting, female mice inoculated s.c. with C4-HI tumors were ovariectomized (ovx) 4 days after tumor inoculation. Once the tumors were palpable, the mice were treated with daily doses of RU-486 at 1.2 μg/kg. These RU-486 doses, which are 10^{4} times lower than those used to inhibit tumor growth, induced a reproducible increase in tumor size (Fig. 9, left). When the same experiment was carried out in non-ovx animals the differences were not significant, probably because endogenous Pg levels masked the slight proliferative effect induced by RU-486. In contrast, as previously reported, daily doses of 12 mg/kg induced almost complete tumor regression (Fig. 9, right). These data are consistent with the agonistic effect of RU-486 observed at very low concentrations and support the in vitro data regarding cell proliferation.

Discussion

In this study, we report the identification of active progestin binding sites at the cell membrane of epithelial malignant cells in a mouse model of breast cancer. Our results suggest that these sites are functionally identical to the classical PR isoforms A and B. Classical ERα was also detected in the same membrane extracts. The series of tumors used in this study has been extensively characterized. They express high levels of PR and ERα. This, together with our use of a highly efficient method of cell membrane purification, has allowed us to specifically identify classical membrane PRs in tumors for the first time. Most studies regarding membrane steroid receptors in mammary tissue have been performed using cell lines [28], in many cases cell lines transfected with steroid hormone receptors [43].

The membrane localization of classic steroid receptors remains a controversial issue and anchoring mechanisms for steroid receptors in the cell membrane are being studied by several groups. Recently Pedram et al. [28] demonstrated PR palmitoylation, as previously reported for other membrane steroid receptors [49]; this post-translational modification is important for membrane anchoring. Razandi et al. [24] have also suggested that ERα may act as G-coupled receptor by binding to caveolin 1 and PR co-immunoprecipitates with caveolin 1 in our tumor model [50]. In this report, we have focused on identifying PRs using a set of highly specific antibodies that recognize sequences in the C-terminal region (C-19) or in the N-terminal half of the PR (Ab-7). The incubation of our target cells with FGF-2, MPA, Pg, or RU-486 for short times induced an increase in membrane pPR immunoreactivity.

In order to obtain the best possible immunofluorescence signal, we tested different procedures and fixation

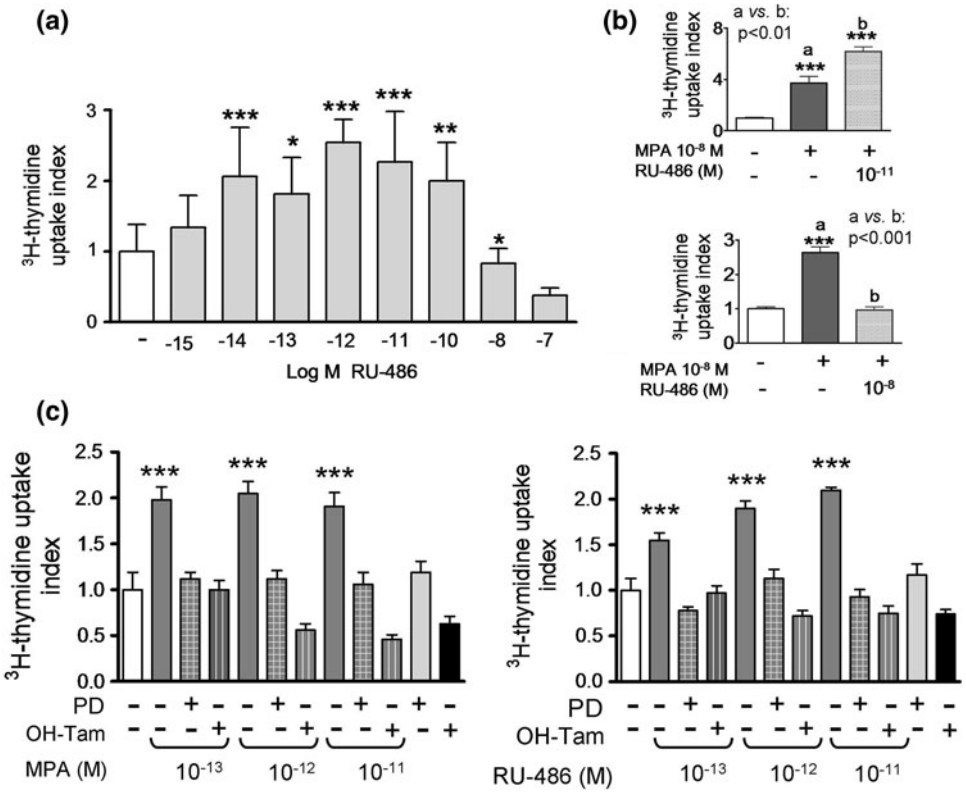

Fig. 8 Effects of low concentrations of RU-486 on cell proliferation. C4-HI primary cultures, plated in 96-well plates, were treated with RU-486 ranging from 1×10^{-15} M to 1×10^{-7} M (**a**) or with 1×10^{-8} M MPA with or without RU-486 (**b**) or with low concentrations of MPA or RU-486 with or without 1×10^{-5} M PD 98059 (PD) or 1×10^{-6} M OH-Tam (**c**) for 48 h. ^{3}H-thymidine was added for the last 24 h. ^{3}H-Thymidine labeling index was calculated as experimental cpm/control cpm. A representative experiment is shown using octuplets for each experimental group. *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$ between experimental and control group. PD and OH-Tam inhibited the stimulatory effects induced by MPA or RU-486 ($P < 0.001$)

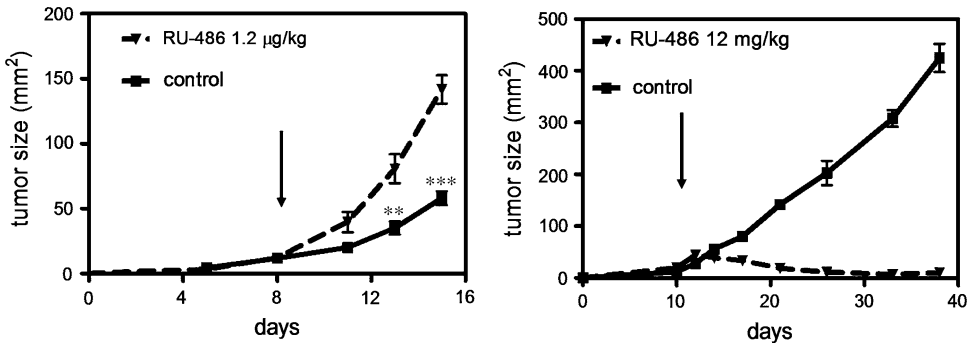

Fig. 9 Effects of low concentrations of RU-486 on tumor growth. *Left* ovariectomized BALB/c mice were transplanted with C4-HI s.c. and when tumors reached a size of 25 mm^2 they were treated with 1.2 µg/kg/day of RU-468 s.c. or vehicle for 10 days. Tumor size was measured with a Vernier caliper every 2 days. Three independent experiments using 4 mice/group were performed. ** $P < 0.01$, *** $P < 0.001$ experimental versus control in the last two time points, respectively. *Right* BALB/c mice were transplanted s.c. with C4-HI tumors. When the tumors had a size of approximately 25–50 mm^2 (*arrow*), animals were treated with RU-486 (12 mg/kg body weight/day, s.c.) or remained untreated; $n = 4$/group; three different experiments

protocols and found that fixation in ethanol for 30 min at 4°C provided the best results. Only discrete staining was observed when cells were fixed using formaldehyde. This may explain why we have not detected the membrane PR in previous studies. It is possible that alcohol fixation preserves membrane epitopes. The best membrane PR staining was observed in FGF-2-treated cells using three different antibodies (Figs. 1, 3). Along the same line, we also demonstrated specific ^{3}H-R5020 binding to membrane extracts of FGF-2-treated cells.

We also found that the tumors we tested express the recently described non-classical mPRs, α, β, and γ [15, 16]. The localization of these mPRs remains controversial [18, 51]. Possible crosstalk between these mPRs and the classical PRs has been proposed [17]. Since these mPRs do not bind MPA, RU-486, or R5020, we focused on the classical

membrane PR; we used MPA and Pg as PR agonists [30] and RU-486 as an antagonist [37]. Even though MPA is a synthetic progestin that may also have androgenic [52] or glucocorticoid actions [53], we had demonstrated in previous studies that its proliferative effects are mediated by the PR [30, 54]. Similarly, RU-486 has antiglucocorticoid and antiandrogenic properties in addition to its antiprogestin effect [55, 56] and we have also addressed the participation of PR in the inhibitory effects [36, 54].

We also showed that MPA, Pg, and RU-486 induced a rapid increase in ERK phosphorylation. Since it has been reported that RU-486 may inhibit the MPA-induced increase of ERK activation [57, 58], we repeated these experiments exhaustively to overcome variability across primary cultures. As already demonstrated [38], the presence of a few fibroblasts may results in secretion of FGF-2 and other factors that stimulate ERKs. The agonistic effect of RU-486 on ERK activation has also been reported in human T47D cells that overexpress PR-B [59]. Interestingly, we were able to demonstrate that low concentrations of RU-486 were also able to increase ^{3}H-thymidine uptake, ERK and Akt activation in T47D cells. In these cells, although the expression of membrane PR has been suggested, by confocal microscopy PR has been mainly localized in the nuclei [60]. This is in our opinion the reason we believe that the description of the membrane localization of PR, as compared with other steroid receptors, is delayed.

In our tumor model, all agonists, as well as RU-486, induced ERK activation. However, this increase did not necessarily correlate with cell proliferation. Increases in ERK activation associated with inhibition of cell proliferation have already been observed in other tumor models [61]. Moreover, Marshall [62] proposed that the duration of ERK activation was important for determining cell fate. Overexpression of tyrosine kinase receptors and changes in ligand concentrations have been shown to regulate the extent of ERK activation. Interestingly, in PC12 cells, increases in nerve growth factor concentrations were shown to induce neurite differentiation and lower concentrations induced a slight proliferative response [63]. In the cells studied here, ERKs were still activated 24 h after MPA or RU-486 treatment in vitro. In vivo, however, a decrease in ERK activation was observed in tumors treated for 24–48 h with RU-486 [36].

We have previously shown that MPA can induce cell proliferation even at concentrations as low as 1×10^{-11} M [21]. Here, we demonstrate that low concentrations of RU-486 exert similar effects, and that the stimulatory effects induced by low concentrations of MPA or RU-486 were abolished by a MEK inhibitor and by OH-Tam. RU-486 is a type-II antiprogestin [56] that binds PR with a higher affinity than progesterone itself. It induces PR phosphorylation and PR–DNA binding, but recruits repressors instead of coactivators to certain gene promoters to modulate gene transcription [64, 65]. Accordingly, we demonstrated that only 1×10^{-8} M RU-486 promoted the upshift of PR mobility after electrophoresis on SDS-gels. This upshift is associated with phosphorylation, suggesting the existence of different mechanisms of action for RU-486 administered at concentrations of 1×10^{-8} M or lower. When we characterized the expression of six Pg-regulated proteins [48, 66–69], in all cases low concentrations of RU-486 induced effects similar to those induced by low concentrations of MPA. At 1×10^{-8} M MPA or RU-486, a concentration that is proliferative for MPA and inhibitory for RU-486, similar regulation of STAT5 and c-myc was observed, and RU-486 induced greater expression of p21 as compared to MPA. However, MPA increased tissue factor and cyclin D1 expression, whereas both the proteins were down regulated by RU-486, illustrating the complexity of the mechanisms involved in cell growth regulation and highlighting the hierarchical role of cyclin D1. The pivotal role of cyclin D1 in mouse mammary tissue is not surprising since PR knock out mice and cyclin D1 knock out mice share the same mammary gland phenotype [70, 71]. Moreover, in a gene array study of different PR-B mutants, Quiles et al. [48] noted the role of cyclin D1 in a central pathway in which genomic and non-genomic pathways converge. Since the promoter of cyclin D1 does not have a regular PRE, different genomic and non-genomic mechanisms have been proposed to explain cyclin D1 regulation [48, 72, 73]. Activated PRs have been detected at the promoter of cyclin D1 [48, 69] and STAT binding sites (GAS), Sp 1, AP1, or even NF-kB sites might participate in PR-mediated cyclin D1 expression. Increases in c-myc, on the other hand, have been related to apoptosis or mitosis and it is possible that c-myc in the absence of cyclin D1 may signal growth arrest or apoptosis when high levels of p21 are present [74].

The possibility that low concentrations of MPA or RU-486 induce a PR tethering to different promoters such as Sp1, AP1, or GAS containing promoters [48, 75] cannot be ruled out. Ongoing experiments and gene array studies will help to determine the differences in gene activation between low concentrations of MPA and RU-486 in this model.

The interpretation of the effects of MPA is more complex since we know from previous studies that incubation with 10-nM MPA increases expression of growth factors or growth factor receptors and inhibits TGFβ1, which is a cell proliferation inhibitory factor in this model. All this machinery can contribute to the maintenance of a highly proliferative state [40]. This is aligned with data from other

laboratories showing that MPA also sensitizes cells to growth factor signaling [76]. This loop may be responsible for the increased proliferation at higher MPA concentrations.

The data reported herein suggest that MPA and/or RU-486 elicit rapid non-genomic effects such as ERK activation. These stimuli may prime the cells for further signaling. In the absence of additional stimuli, MPA and RU-486, induce a small increase in cell proliferation. Concentrations of MPA high enough to elicit genomic effects also induce a further increase in cell growth. In contrast, concentrations of RU-486 higher than 10^{-9} M block cyclin D1 expression and induce a complete inhibition of cell proliferation despite of the previous increase in pERK or the agonistic effect that RU-486 might have in some proliferative genes. The mechanisms by which membrane PR may participate in these phenomena remain to be elucidated.

Another possible explanation for these results is that RU-486 and MPA at low concentrations bind only to PR-B exerting agonistic effects and that at higher concentrations RU-486 could induce the PR-A activation responsible for the inhibitory effects. In this case, we would have expected that lower RU-486 levels induce a shift in the EMSA.

There is increasing evidence that suggest that PRs are potentially interesting breast cancer therapeutical targets [40, 77–79]. The results reported herein indicate that if antiprogestins will be clinically used, a high concentration enough to guarantee their genomic effects should be used to prevent possible agonistic non-genomic effects of the antiprogestins that may lead to cell proliferation. The fact that antiestrogens blocked the stimulatory effects of RU-486 also suggest that combined treatments may be preferable to a single antiprogestin treatment.

In summary, in this study we have described for the first time the presence of classic membrane PR that may be responsible for the progestin-mediated non-genomic effects. We have also demonstrated that the inhibitory effect of RU-486 correlated with the ability of activated PR to bind DNA, and to inhibit cyclin D1 expression suggesting that the genomic effects may be responsible for this inhibition of tumor growth.

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Conflict of interest statement The authors declare that they have no competing interests.

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