INTRODUCTION

The role of progesterone in regulating reproductive physiology in animals is complex and difficult to evaluate because the direction and extent of the effects of progesterone depend on the endogenous hormonal milieu. The actions of progesterone typically require previous exposure to 17β-oestradiol (E₂) because E₂ regulates nuclear progesterone receptor expression. In the pituitary...
gland, the role of progesterone with respect to controlling lactotroph proliferation and prolactin (PRL) secretion is controversial. Progesterone can facilitate the effect of E₂, although it can also prevent oestradiol-induced PRL surges and PRL gene expression during prolonged progesterone treatment. This can also be observed in the experimental model of prolactinoma generated in female rats by chronic treatment with diethylstilbestrol (DES), a synthetic oestrogen, where cotreatment with progesterone antagonises the proliferative effects of DES on lactotrophs, thereby decreasing tumour size and serum PRL levels. Therefore, it appears that progesterone serves an antiproliferative and protective role in the pituitary gland. On the other hand, in E₂-primed animals, acute progesterone treatment increases PRL release and may stimulate proliferation depending on the hormonal environment and type of receptor involved.

However, it is important to recognise that progesterone also exerts an indirect impact on PRL secretion through regulating the activity of tyrosine hydroxylase (TH) in the hypothalamus, the main enzyme involved in the biosynthesis of dopamine, which is the major inhibitory regulator of PRL secretion.

Aside from other well-documented genomic effects, progesterone exerts rapid "nongenomic" actions through activation of membrane progesterone receptors (mPRs). These actions are relatively unaffected by inhibitors of transcription, as mimicked by steroids coupled to nonpermeant cell membrane molecules and persist in cells that do not express the classic genomic progesterone receptor. The mPRs belong to the progestin and adipoQ receptor (PAQR) family and were first identified in teleost fish, although it was later discovered that 5 subtypes (mPRα, mPRβ, mPRγ, mPRδ and mPRε) are found in tissues of several mammalian species, including humans. These receptors contain 7 transmembrane domains, display high-affinity progesterone binding and rapidly activate G proteins during their downstream signalling pathways following progesterone binding. Several studies have demonstrated that mPRα, mPRβ and mPRγ activation induces inhibitory G protein (Gi) signalling pathways, thereby decreasing adenylyl cyclase activity and cellular cAMP accumulation. mPRα and mPRε, on the other hand, are coupled to stimulatory G proteins (Gs) and increase cAMP accumulation after activation. Moreover, mPRα and mPRβ are expressed more strongly in different tissues of mammals such as the rat and mouse brain, as well as in reproductive tissues. mPRγ is largely expressed in the lung, liver, kidney and fallopian tubes; meanwhile, mPRδ and mPRε have been described in the human brain and pituitary.

Among the mPRs subtypes, mPRα is the most extensively characterised in vertebrates. Studies have revealed that mPRα is involved in the regulation by progesterone of numerous functions, including uterine function in humans and the inhibition of gonadotrophin-releasing hormone (GnRH) release in rodents. mPRα is also thought of as an intermediary of progesterone antitumourigenic effects in ovarian cancer cells, which may provide a new treatment option for patients undergoing advanced stage ovarian cancer therapy. Although there is substantial evidence that progesterone initiates rapid, cell-surface actions in several cell types through mPRs, there are no published studies reporting the exact role of these receptors in the pituitary gland.

In the present study, we hypothesise that mPRs mediate rapid actions of progesterone in the pituitary gland. Accordingly, to test this, we studied the expression of mPRs in rat pituitaries and in rat pituitary tumour-derived GH3 cells and investigated cell-type localisation, as well as the role of the mPR most likely involved in mediating the actions of progesterone in the rat pituitary.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated, including DES or (E)-3,4-bis(4-hydroxyphenyl)-3-hexene, a synthetic oestrogen. Progesterone was purchased from Steraloids (Newport, RI, USA). Org OD 02-0 was obtained from Organon (Oss, the Netherlands). R5020 was purchased from GE Healthcare (Piscataway, NJ, USA). Although Org OD 02-0 was initially described as a human mPRL agonist, its selectivity on mPRs in rat cells has also been demonstrated in the rat brain and hypothalamus. [2,4,6,7]pilotopine-8-deutero-4-14 C progesterone (H[P4]) was purchased from American Radiolabeled Chemicals (St Louis, MO, USA). Antibodies against extracellular signal regulated kinase (ERK) (p42/44) and phosphorylated ERK (p-p42/44) were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.2 | Animals

Adult female Sprague-Dawley (SD) rats (3 months old, 250 ± 30 g) were maintained at under a 12:12 hour light/dark cycle (lights on 07:00 hours) at 22 ± 2°C. Cycling rats were monitored daily, by vaginal smears, during 4-5-day oestrous cycles, and killed during dioestrus. Anterior pituitary glands were removed within minutes after decapitation.

The animals were provided with food and water ad libitum. The Institutional Animal Care and Use Committee of the Institute of Biology and Experimental Medicine (IByME) approved all animal procedures, and the studies were conducted in accordance with National Institutes of Health guidelines for animal research (8th edition 2010, NRC, USA) and also in accordance with the European Communities Council Directive of November 2010 (2010/63/UE).

2.3 | Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated from anterior pituitaries of female adult SD rats in dioestrus with Trizol reagent (Ambion, Life Technologies, Grand Island, NY, USA) in accordance with the manufacturer’s instructions. RNA purity and quantity were determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Purity was determined by 260/280 nm absorbance ratios, and absorbance ratios above 1.8 were considered as acceptable. Random
primers (Tecnolab, Buenos Aires, Argentina) and Moloney murine leukaemia virus reverse transcriptase (MMLV-RT; Promega, Madison, WI, USA) were used for cDNA synthesis. Reverse transcription was performed with 1 μg of RNA at 70°C for 5 minutes, 37°C for 60 minutes and 95°C for 5 minutes because the minimum specification of first-strand cDNA made from 1 μg of RNA is 120 ng. Then, a working solution of cDNA (6 ng μL⁻¹) was prepared by diluting samples 1:20 with diethyl pyrocarbonate-treated water. cDNA working solution (5 μL) was added to a 5 μL master mix containing 2 μL of EVA green quantitative PCR mix (Solis BioDyne, Tartu, Estonia) and 0.5 μL of each forward primer (0.25 μmol L⁻¹) and reverse primer (0.25 μmol L⁻¹) in accordance with the manufacturer’s instructions. Specific primers (Table 1), located on separate exons, were designed using primer3 (http://primer3.sourceforge.net) and synthesised by Tecnolab SA (Buenos Aires Argentina). Quantitative real-time PCR analysis was carried out using a Bio-Rad real-time PCR detection system and cfx manager (Bio-Rad Laboratories, Hercules, CA, USA). Standard thermocycler conditions were: 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 20 seconds and 72°C for 1 minute. All cDNA samples were assayed in duplicate for each gene, and melt curve analyses were performed to ensure specificity of amplification. The quantitative PCR efficiency of each pair of primers was previously tested using serially diluted samples. All primers showed similar efficiencies of ~95–100%. A housekeeping gene, Cyclophilin B (Cyp B), was used as an internal control to normalise differences in the amount of starting template between samples, as described previously. Target gene expression was quantified using the comparative CT method (ΔCT). To analyse the relative mRNA abundance among all the receptor types, we compared the 2^(-ΔΔCT) (2^(-[Ct mPR- Ct Cyp B]) values of each receptor. The expression of nPRs was analysed using a primer set to amplify total PRs and a second primer set to amplify only PR-B.

2.4 | Ex vivo assay

Female SD rats in dioestrus were killed by decapitation and anterior pituitaries were collected. Anterior pituitaries were cut in pieces and incubated in 500 μL of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% horse serum, 2.5% fetal bovine serum, penicillin/streptomycin (20 μg mL⁻¹; Gibco, Gaithersburg, MD, USA) and amphotericin B (0.25 μg mL⁻¹; PAA, Linz, Austria) for 2 hours at 37°C. Then, tissues were washed and stimulated for 1 hour at 37°C with 100 nmol L⁻¹ progesterone (P4) or with the selective mPR agonist, 10-ethynyl-19-norpregesterone Org OD 02-0, in serum-free medium (vehicle).

At the end of the treatment period, secreted medium and pituitaries were collected and PRL levels were measured by radioimmunoassay (RIA). Serum PRL levels were determined by RIA using a primary antibody of defined specificity raised in rabbit against rat PRL, with synthetic PRL as a reference preparation and [125I]PRL as tracer (all reagents provided by National Hormone and Pituitary Program). The inter- and intra-assay coefficients of variation were 6.7% and 12.6%, respectively.

2.5 | Immunostaining analysis of mPrα receptor and pituitary hormones (PRL, lutenising hormone [LH] and growth hormone [GH]) by confocal laser scanning microscopy

Pituitaries from 3-month-old female SD rats in dioestrus were removed immediately after death, and the pituitary cells were dispersed and seeded on glass coverslips (13 mm) at a density of 105 cells per well. Then, the cells were maintained in DMEM supplemented with 4% foetal calf serum and 8% horse serum (Gibco) in an incubator with a humidified atmosphere of 5% CO₂ and 95% air at 37°C for 3 days and then finally fixed in 4% formaldehyde.

For mPRα receptor detection, dispersed pituitary cells in coverslips were blocked for 1 hour in 5% phosphate-buffered saline-bovine serum albumin (PBS-BSA), incubated overnight in primary antibody (anti-rat mPRα 306t; Dr P. Thomas, University of Texas at Austin, Port Aransas, TX, USA; dilution 1:50) and exposed to Alexa 594 anti-rabbit secondary antibody (Invitrogen, Grand Island, NY, USA; dilution 1:1000) for 1 hour. Then, the cells were permeabilised with 0.50% Triton X-100/PBS, blocked for 1 hour in 5% PBS-BSA, and incubated with guinea pig antiserum directed against rat PRL; or rat LH; or rat GH (Dr A. Parlow, National Hormone and Pituitary Program, Torrance, CA, USA; dilution 1:1000) and further incubated with Alexa 488 anti-guinea pig secondary antibody (Invitrogen; dilution

**TABLE 1** Quantitative polymerase chain reaction primers

| Gene   | Accession number | Forward primer sequence 5′- to 3′ | Reverse primer sequence 5′- to 3′ |
|--------|------------------|-----------------------------------|-----------------------------------|
| CypB   | NM_022536        | GACCCTCCGTTGCAAGCAGAT             | GTCACTGCTCTCAGGTGTGGTCCTC         |
| mPrα   | NM_001034081     | CGGATCATTAGTGTCTTCCCC             | TGCCAGTTGCAAGGTGAATG             |
| mPrβ   | NM_001014099     | TACATGAAGTGGCTTCTAATGGGGCCAGAGCAG | ATTCGAGCCCAAGGTGAATG             |
| mPrγ   | NM_001014092     | GTCCAGGGTGCTTCAATGAAGGTGGAGG     | CTGCAGAGCCCAAGGTGAATG             |
| mPrδ   | NM_001191077     | CACTTGGTACTTTGGTTCCTGGAAGG      | CGAGGCGCATGAGGTGAATG             |
| mPrτ   | NM_001271152     | CACGTGAGCTTGGTCTGGAAGA           | AAGATTGCGGCGTGAAGATG             |
| PGRMC1 | NM_021766        | GTTCACATCTAACGATTCACCAACAGC      | CTTCCAGGCAACGGTGCAGCTC           |
| PR     | NM_0022847       | GACAACACAAGACCCGACGAC             | CGGAAATCCGAGCGAGATTG             |
| PR-B   | NM_0022847       | GCATCGCTCTGAATTCTGGCCTACCATAC    | GCTCTGGATTCTGGCTTCTCG             |
Control cells were incubated with the corresponding normal serum or immunoglobulin (IgG) subtype instead of primary antibody. Images were obtained using the inverted confocal laser scanning microscope FluoView FV 1000 (Olympus, Tokyo, Japan). The analysis of confocal microscopy images was performed using the FV10-ASW 1.6 VIEWER (Olympus).

2.6 | Flow cytometry
Control rats were killed, and anterior pituitary glands were removed after decapitation and collected in 1000 μL of DMEM. First, anterior pituitary glands were washed with DMEM-BSA containing 3 mg mL⁻¹ BSA. Then, anterior pituitaries were cut into small fragments and dispersed enzymatically by successive incubations in DMEM-BSA containing 0.75% trypsin, 10% charcoal-dextran-adsorbed foetal calf serum and 45 U mL⁻¹ deoxyribonuclease type I (DNase). Finally, the cells were dispersed by extrusion through a Pasteur pipette in Krebs buffer without Ca²⁺ and Mg²⁺. Dispersed cells were washed and resuspended in PBS. Cell viability, as assessed by trypan blue exclusion, was more than 95%. Cells were fixed using 0.2% paraformaldehyde for 15 minutes at room temperature, then permeabilised with saponine 0.05%, washed and centrifuged. Immunostaining of mPRα-positive cells and of PRL (lactotrophs) was performed using mPRα antibody (anti-rat mPRα 306; Dr P. Thomas; dilution 1:50) and a guinea pig antiserum directed against rat PRL (Dr A. Parlow, National Hormone and Pituitary Program; 1:1000) for 1 hour at 37°C. Then, cells were washed in PBS and incubated with a PE-conjugated anti-rabbit (Chemicon International, Temecula, CA, USA) (dilution 1:67; 40 minutes at 37°C) and fluorescein isothiocyanate-conjugated anti-guinea pig antibody (Chemicon International) (dilution 1:75; 40 minutes at 37°C). For isotype controls, cells were incubated with guinea pig serum instead of PRL antiserum and rabbit IgG instead of specific anti-rabbit antibody. Cells were washed, resuspended in PBS and analysed by fluorescence-activated cell sorting. Fluorescence intensity of ≥10 000 gated cells per tube was analysed using a FACScalibur (Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA). Data were analysed using WINMDI (http://www.cyto.purdue.edu/flowcyt/software/Winmdi.htm) and FLOWJO (https://www.flowjo.com) software.

2.7 | Culture of GH3 cells
GH3 cells (ATCC® CCL-82.1; American Type Culture Collection, Manassas, VA, USA) were grown as monolayer culture in DMEM supplemented with 5% foetal bovine serum and 5% horse serum, 2 mmol L⁻¹ glutamine, 1% minimum essential medium amino acids and 1% penicillin/streptomycin (pH 7.4). The cells were cultured in 15-cm culture dishes and replicated every 3-5 days in the exponential phase. Cells were harvested once a week by treatment with PBS containing trypsin (2.5 mg mL⁻¹; Gibco) and reseeded at 20% of the original density, and 4 subcultures were made before each experiment. In some experiments, the culture media was replaced with serum-free DMEM before experimentation.

2.8 | Preparation of plasma membranes
GH3 cells were subcultured in 15-cm culture dishes until they were 90% confluent and then harvested with a cell scraper and collected in ice-cold HAED buffer (25 mmol L⁻¹ Hepes, 10 mmol L⁻¹ NaCl, 1 mmol L⁻¹ ethylenediaminetetraacetic acid, 1 mmol L⁻¹ dithioerythritol, pH 7.6) with a protease inhibitor cocktail (Thermo Fisher Scientific). Cells were rinsed twice followed by a 5-minute centrifugation at 1000 g for 30°C and cell pellets resuspended in freshly prepared ice-cold HAED buffer. Cells were lysed by sonication for 10 seconds and then centrifuged at 1000 g at 30°C for 7 minutes to remove any nuclear and heavy mitochondrial material (nuclear fraction). The supernatant was centrifuged at 20 000 g at 30°C for 20 minutes to obtain the plasma membrane fraction, which was resuspended with HAED buffer.

2.9 | Single-point competitive binding assay
A single-point competitive assay was conducted as described previously using GH3 cells. Briefly, plasma membranes were incubated with 4 nmol L⁻¹ [2,4,6,7-³H]-progesterone ([³H]-P4) alone (total binding) or in the presence of 1 μmol L⁻¹ P4, Org OD 02-0 or R5020 for 30 minutes at 30°C. Bound [³H]-P4 was separated from free by rapid filtration over Whatman GF/B filters (GE Healthcare, Little Chalfont, UK) with a 36-well cell harvester (Brandel, Gaithersburg, MD, USA) and radioactivity bound to the filters was measured by scintillation counting (model LS6000; Beckman Coulter, Fullerton, CA, USA). The displacement of radiolabelled steroid binding by the progestin competitors was expressed as a percentage of the maximum specific [³H]-P4 binding.

2.10 | Western blot analysis of membrane progesterone receptors
GH3 plasma membranes were solubilised and mixed with 4× Lane Marker Reducing Sample Buffer (Thermo Fisher Scientific) boiled for 10 minutes and run on a 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel (15 μg protein per lane). Proteins were then transferred onto nitrocellulose membranes at 30°C for 1 hour and blocked in PBS-Tween-20 with 5% nonfat dried milk. Membranes were incubated overnight at 4°C in PBS + Odyssey® Blocking Buffer (LI-COR Biosciences, Lincoln, NE, USA) containing previously validated polyclonal antibodies²³: mPRα (dilution 1:500); mPRβ, mPRγ and mPRε (dilution 1:1000), as well as antibodies directed against progesterone receptor membrane component 1 (PGRMC1) (dilution 1:1000) or nuclear progesterone receptor (nPR) (dilution 1:1000) (Table 2). The nitrocellulose membranes were then incubated with fluorescence-conjugated secondary antibodies...
TABLE 2 Antibodies

| Peptide/protein target | Antigen sequence | Name of antibody | Manufacturer and/or name of individual providing the antibody | Species raised in: monoclonal or polyclonal | Dilution used |
|------------------------|------------------|------------------|------------------------------------------------------------|---------------------------------------------|---------------|
| Membrane progesterone receptor alpha (mPRα) | N-terminal TVDRAEVPLFLWKPC | 306t | P. Thomas | Rabbit, polyclonal | 1:500 for WB; 1:50 for IHC and ICC |
| Membrane progesterone receptor beta (mPRβ) | N-terminal KILEDGLPKMPTVC | A9830-4 | P. Thomas | Rabbit, polyclonal | 1:1000 for WB; 1:100 for ICC |
| Membrane progesterone receptor gamma (mPRγ) | N-terminal TDIKNDSYSWMLC | H833-4 | P. Thomas | Rabbit, polyclonal | 1:1000 for WB; 1:100 for ICC |
| Membrane progesterone receptor delta (mPRδ) | N-terminal CQGPLEGTAKQQ | PAQR6 | P. Thomas | Rabbit, polyclonal | 1:1000 for WB; 1:100 for ICC |
| Membrane progesterone receptor epsilon (mPRε) | N-terminal RNSHSAASPPASC | PAQR9 | P. Thomas | Rabbit, polyclonal | 1:1000 for WB; 1:100 for ICC |
| Progesterone receptor membrane component 1 (PGRMC1) | Internal region of PGRMC1 of human origin | PGRMC1 (D-16) | Santa Cruz Biotechnology, Inc., 82684 | Goat, polyclonal | 1:1000 for WB; 1:100 for ICC |
| Nuclear progesterone receptor | Amino acids 731-909 of PR of human origin | PR (2C11F11) | Santa Cruz Biotechnology, Inc., 130071 | Mouse, monoclonal | 1:1000 for WB |

ICC, immunocytochemistry; IHC, immunohistochemistry; WB, western blotting.

(dilution 1:10 000) (LI-COR Biosciences) for 1 hour at room temperature washed 3 times with PBS and scanned with the Odyssey® Infrared Imaging System (LI-COR Biosciences).

2.11 Immunocytochemical analysis of progesterone receptors

The presence of mPRs in GH3 cells was evaluated by immunofluorescence staining. GH3 cells (~2 \times 10^5 cells/well) were grown on glass coverslips and fixed with 4% paraformaldehyde in PBS for 20 minutes. Then, the cells were washed with PBS, blocked with 2% bovine serum albumin for 1.5 hours and incubated with mPRs (dilution 1:50; mPRγ, mPRδ, mPRβ, or PGRMC1 antibodies (dilution 1:100) (Table 2) overnight at 4°C. Subsequently, cells were washed and incubated with an Alexa Fluor 488 donkey anti-rabbit secondary antibody or an Alexa Fluor 488 donkey anti-goat secondary antibody (dilution 1:1000) (Thermo Fisher Scientific) for 1 hour at room temperature. The cells were washed, and the coverslips were mounted on glass slides with ProLong Gold antifade reagent with DAPI (Invitrogen). Localisation of fluorescent-labelled mPRs was assessed using an inverted microscope (Nikon, Tokyo, Japan) with a MetaVue Research Imaging System (Molecular Devices, Sunnyvale, CA, USA).

2.12 Measurement of prolactin secretion in GH3 collected medium after progesterin treatment

GH3 cells were cultured in 12-well plates (~1 \times 10^6 cells/well) and then incubated with 20 nm P4, Org OD 02-0, R5020 or vehicle in supplemented medium at 37°C for 30 minutes. At the end of the treatment period, the supernatants were collected and PRL levels were measured using a specific Rat Prolactin enzyme immunoassay kit (EIA) kit (Cayman Chemicals, Ann Arbor, MI, USA) in accordance with the manufacturer’s instructions.

2.13 Pertussis toxin assay

Pertussis toxin (List Biological Laboratories, Inc., Campbell, CA, USA) was activated by incubation with 15 mmol L^{-1} dithiothreitol at 35°C for 30 minutes (PTXa). For control groups, an aliquot of PTX was heat-inactivated by further incubation for 10 minutes at 99°C (PTXhi). Cells were pretreated with PTXa and PTXhi (2.5 μg mL^{-1}; List Biological Laboratories Inc., Campbell, CA, USA) for 30 minutes and then incubated with Org OD 02-0 (20 nmol L^{-1}) for 4 hours at 37°C. The PRL concentration in cell supernatants was measured using a specific Rat Prolactin EIA kit in accordance with the manufacturer’s instructions (Cayman Chemicals).

2.14 cAMP assay

GH3 cells were cultured in 12-well plates (~1 \times 10^6 cells/well) and serum-starved for 72 hours prior to a 10-minute treatment with progestin (20 nmol L^{-1}) or vehicle. After treatment, cells were washed twice with ice-cold PBS and lysed by a 20-minute incubation with 100 μL of 0.1 mol L^{-1} HCl at room temperature, followed by scraping and pipetting to homogenise samples. The cell suspension was clarified by a 10-minute centrifugation at 12 000 g and the supernatant was diluted 3×. Cellular cAMP concentration was determined with a Cyclic AMP EIA Kit (Cayman Chemical) in accordance with the manufacturer’s instructions.
2.15 | ERK activation assay

GH3 cells grown in 12-well plates (~1 × 10^6 cells/well) and serum-starved for 48 hours were incubated with P4, Org OD 02-0, R5020 (20 nmol L^-1) or vehicle for 15 minutes. Epidermal growth factor (100 nmol L^-1) was used as positive control (data not shown). After treatment, 100 μL of RIPA buffer (EMD Millipore, Billerica, MA, USA) containing phosphatase and a protease inhibitor cocktail (Thermo Fisher Scientific) was added and cells were collected by lysis with vortexing for 30 minutes. Then, lysates were centrifuged at 15,000 g for 5 minutes at 4°C to remove the cell debris. An aliquot of 5 μL of the supernatant was taken from each sample to quantify proteins by the Bradford assay. Samples containing ~15 μg were prepared for western blot analysis as described above. Total ERK and phosphorylated ERK were detected using monoclonal antibodies directed against total p42/44 and phospho-p42/44, respectively (p42/44 #9102; p-p42/44 #9106; Cell Signaling Technology) (dilution 1:2000). Membranes were then incubated with fluorescence-conjugated secondary antibodies (LI-COR Biosciences) and scanned with the Odyssey® Infrared Imaging System (LI-COR Biosciences). Phosphorylated ERK was normalised to total ERK using IMAGEJ software (NIH, Bethesda, MD, USA).

2.16 | Active transforming growth factor (TGF) β1 detection

GH3 cells were cultured in 12-well plates (~1 × 10^6 per well) and incubated with DMEM-BSA 2% without serum 24 hours before treatments. Cells were then treated, as previously described, with P4, Org OD 02-0 and R5020 (20 nmol L^-1) or vehicle for 15 minutes. After treatment, cell supernatants were collected and the content of active TGFβ1 (μg mL^-1 medium) of each sample was measured using a TGFβ1 DuoSet enzyme-linked immunosorbent assay (ELISA) kit (DY1679-05; R&D Systems, Minneapolis, MN, USA) in accordance with the manufacturer’s instructions.

2.17 | Knockdown of mPRα with small interfering RNA (siRNA)

GH3 cells were transfected twice with antisense siRNA oligos for mPRα (ON-TARGET plus Rat Psa7 siRNA; Dharmacon, Lafayette, CO, USA) or nontargeting oligos (CTL) (100 nmol L^-1) at 0 and 16 hours, using Lipofectamine 2000 (Invitrogen) as transfection reagent. After 48 hours of incubation, the siRNA mix was replaced with normal medium and then cells were cultured for additional 24 hours with DMEM-BSA 2% before use in the experiments.

2.18 | Inhibitor treatments

Org OD 02-0 for 15 minutes. Similarly, a specific inhibitor of ERK (10 μmol L^-1, PD98059; Biomol International, Plymouth Meeting, PA, USA) was used to pretreat cells for 1 hour prior to treatment with Org OD 02-0. At the end of the treatment periods, active TGFβ1 levels were measured by ELISA as described previously.

2.19 | Statistical analysis

Results are expressed as the mean ± SEM. Experiments were repeated between 3 and 5 times, with at least 3 replicates. P4, Org OD 02-0 and R5020 treatments results were evaluated by One-way ANOVA followed by Tukey’s post-hoc test. Two-way ANOVA was performed when the effects of 2 factors (progestin treatments and PTX, inhibitors treatments or siRNA treatment) were evaluated, followed by Bonferroni’s post-hoc multiple comparison test. P < .05 was considered statistically significant. The data set was transformed when required and analysed using PRISM, version 5 (GraphPad Software, San Diego, CA).

3 | RESULTS

3.1 | Expression of nPRs and mPRs by qRT-PCR in female rat pituitary

The mRNA expression of each progesterone receptor was assayed in pituitaries from adult female rats in dioestrus. The mPRα, mPRβ, mPRγ, mPRδ and mPRε genes were found to be expressed in the rat pituitary gland (Figure 1). According to the data, a qualitative analysis of the putative abundance of each mRNA shows that mPRs would represent approximately the 15% of the total progesterone receptors. Although the values obtained are related to the ΔCT results from quantitative PCR assays, they show that mPRα and mPRβ are greatly expressed in the pituitary and are probably more abundant than the other mPRs.

3.2 | mPRα is expressed in lactotrophs, gonadotrophs and somatotrophs

With mPRα being probably the most abundant mPR found in the pituitary, we next performed double immunocytochemistry (ICC) to assay mPRα protein coexpression with PRL, LH and GH cells. Immunostaining of mPRα was detected in lactotrophs, gonadotrophs and somatotrophs (Figure 2).

To explore the percentage of mPRα-positive cells among the total anterior pituitary cells and among the lactotroph population, we ran double indirect immunofluorescence assays for both mPRα and PRL. Using an approach that preserves the integrity of the cell membrane,22 we detected the percentage of anterior pituitary cells and lactotrophs expressing mPRα by flow cytometry (Figure 3). The results revealed that 42.1 ± 11.6% of total anterior pituitary cells were mPRα-positive (Figure 3A) and 63% of the mPRα-positive cells were also PRL-positive (37% of the mPRα-positive cells were non-lactotrophs) (Figure 3B).
Involvement of second messengers in mPRα-mediated progestin effects on PRL secretion

The specific binding of mPRα agonist Org OD 02-0 to GH3 cells membranes was demonstrated by a single-point ligand competition assay. A significant displacement of [3H]-P4 binding to GH3 plasma membranes was observed by both progesterone and Org OD 02-0.
whereas the nuclear PR agonist R5020 was ineffective (Figure 6A). This result shows the specificity of progestin binding sites in GH3 cell membrane extracts, as is characteristic of mPRs.

Because it was observed that progesterone and Org OD 02-0 inhibit PRL secretion in ex vivo pituitary incubations, this effect was studied in the GH3 cell line (Figure 6B). The results showed that PRL was significantly decreased by 20 nmol L\(^{-1}\) progesterone and Org OD 02-0 treatments, but not with R5020 treatment, suggesting the involvement of mPR\(\alpha\) in this action of progesterone. Hence, the involvement of second messengers in mPR\(\alpha\)-mediated effects was examined. mPR\(\alpha\) has been shown to alter second messenger pathways in a variety of cells (i.e., MCF-7 and SK-BR-3 human breast cancer cells and human umbilical vein endothelial cells) through activation of a pertussis toxin-sensitive inhibitory G protein. To test...
this in our model, GH3 cells were pretreated with activated pertussis toxin (PTXa, 2.5 μg mL⁻¹) or heat-inactivated pertussis toxin (PTXhi) 30 minutes prior to Org OD 02-0 treatment (20 nmol L⁻¹, 4 hours) and then PRL levels were measured. PTXa treatment completely abolished the inhibition of PRL secretion induced by Org OD 02-0 (Figure 6C), whereas PTXhi was ineffective, suggesting the involvement of an inhibitory G protein in this effect. The role of cAMP signalling was also investigated. Treatment with either progesterone or Org OD 02-0 resulted in a significant decrease in cAMP accumulation after 10 minutes (Figure 6D), probably as a result of progesterone activation of an inhibitory G protein (Gai), with a consequent decrease in adenylyl cyclase activity and cAMP cellular levels. Finally, the involvement of the mitogen-activated protein kinase (MAPK) cascade in mPRα-mediated effects of progesterin on PRL secretion was evaluated by western blot analysis of ERK1/2 phosphorylation after treatment with P4, Org OD 02-0 and R5020. Both progesterone and Org OD 02-0 stimulation induced a significant increase in ERK phosphorylation, whereas R5020 did not (Figure 6E), indicating the involvement of mPR, but not nPR, in this effect.

3.6 | TGFβ1 involvement in mPRα-mediated effects

TGFβ1 is a multifunctional and ubiquitous cytokine, well known for its ability to inhibit cell proliferation in epithelial cells. In the pituitary, TGFβ1 and its receptor TβRII are expressed in lactotrophs, and TGFβ1 is a known inhibitor of lactotroph function.25,26 Stimulatory effects of progesterone on TGFβ1 activation have been reported previously in different tissues.27-30; however, the receptors and mechanisms involved were not investigated in those studies. To explore the possible TGFβ1-mediated action in PRL inhibition induced by mPRα stimulation, active TGFβ1 levels after progesterin stimulation in GH3 cells were measured by a specific ELISA (Figure 7). Treatment with either P4 or Org OD 02-0 significantly increased active TGFβ1 levels after 15 minutes, whereas the nPR agonist R5020 had no effect (Figure 7A), suggesting that the rapid progesterone effect on TGFβ1 activation was mediated by mPRα.

In addition, signalling pathways involved in this effect were investigated using pertussis toxin (2.5 μg mL⁻¹), a cAMP analogue (10 μmol L⁻¹, 8-Br-cAMP) and a specific ERK inhibitor (10 μmol L⁻¹, PD98059). Pretreatment with PTX completely blocked the activation of TGFβ1 by Org OD 02-0 (Figure 7B), suggesting the involvement of the inhibitory G protein Gαi in this effect. Similarly, cotreatment with 8-Br-cAMP, which increases cAMP levels, also abolished Org OD 02-0-induced TGFβ1 activation, indicating the involvement of decreased cAMP levels in this effect of Org OD 02-0 (Figure 7C). However, Org OD 02-0 treatment was able to increase active TGFβ1 levels even in the presence of ERK inhibitor, PD98059 (Figure 7D), suggesting that the MAPK pathway may not be involved in Org OD 02-0-activation of TGFβ1 through mPRα.

Finally, to determine the specific role of mPRα in mPRα-mediated TGFβ1 activation, siRNA directed against mPRα was used to knockdown its expression in GH3 cells (Figure 8). Transfection with mPRα siRNA knocked down mPRα protein expression in GH3 cell membranes (Figure 8B) and completely abolished the progesterone and Org OD 02-0-induced increase in TGFβ1 activation (Figure 8A).

4 | DISCUSSION

mPRs mediate a wide variety of the nongenomic actions of proges- tins and are localised in the major reproductive tissues of mammals. The results of the present study clearly demonstrate that mPRs, protein and transcripts are expressed in the rat pituitary gland, as well as in the GH3 cell line. The observation that mPRα would appear to be the most abundant in both models is consistent with previous studies showing mPRα as a major mPR subtype expressed in the pituitary gland of other species.20,40,41 mPRα expression was found in gonadotrophs, somatotrophs and mainly in lactotrophs. The localisation
in gonadotrophs emphasises the involvement of these receptors in the control of GnRH-gonadotroph axis and deserves future investigation. In agreement, previous results showed the potential role of mPRα in the progesterone-mediated inhibitory effect on GnRH release and LH secretion in rodents, even in PR-knockout mice. mPRα expression was also found in rat somatotrophs, although the significance of this finding is currently unknown. On the other hand, the fact that mPRα was found highly expressed in the lactotroph population suggests a physiological role for this receptor in the lactotroph function.

The role of progesterone in the regulation of PRL secretion is controversial. Some studies have reported inhibition, whereas others have reported enhancement of PRL secretion. The findings of the present study support the role of progesterone as a negative modulator. This is in agreement with previous observations of Cramer et al., showing that in vivo progesterone treatment stimulates dopamine release into the hypophysial portal blood in female rats. Similarly, Arbogast et al. reported that progesterone enhances TH activity, which consequently leads to the inhibition of PRL secretion. On the other hand, it is largely assumed that most of the progesterone direct or indirect effects on PRL secretion depend on the activation of the nuclear PRA/B receptors. However, the results of the present study, obtained using an ex vivo assay to avoid hypothalamic influence, demonstrate that either progesterone or the mPRα selective agonist significantly decreased PRL secretion, suggesting the involvement of mPRα in this inhibitory effect. However, because mPRα expression was also found in both somatotrophs and gonadotrophs, we do not discard the paracrine interactions.

To deepen our understanding of the mechanisms involved, studies in the GH3 cell line were performed. First, the expression of the 5 mPRs isoforms was demonstrated in GH3 cells by real-time quantitative PCR, western blotting and ICC, with mPRα being the most abundant. Second, progesterone, as well as Org OD 02-0, but not R5020, was able to displace [3H]-P4 binding to GH3 cell membrane extracts, demonstrating the specificity of progestin binding sites in GH3 membranes, as is characteristic of mPRs. Third, prolactin secretion was inhibited by progesterone and Org OD 02-0, but not R5020, in GH3 cells, thereby supporting our previous results obtained from the ex vivo experiment with pituitary tissue. In addition, the fact that the effect of Org OD 02-0 on PRL secretion was blocked by pretreatment with pertussis toxin shows that this is dependent on the activation of an inhibitory G protein (Gi). Accordingly, treatment with either progesterone or Org OD 02-0 decreased cAMP levels probably as a result of a decrease in the activity of adenylyl cyclase. Interestingly, this mechanism of action, reducing intracellular cAMP levels through the inhibition of adenylyl cyclase activity, has also been described for dopamine as the primary mechanism by which dopamine suppresses PRL gene expression and PRL release.

Finally, a novel progesterone inhibitory mechanism mediated by mPRα in lactotrophs was described. It has been shown previously that TGFβ1 mediates, at least in part, the dopamine inhibitory action on lactotrophs. Dopamine, acting through the Drd2, up-regulates TGFβ1 expression and secretion, contributing to the inhibitory effect of dopamine. Recent evidence obtained in our laboratory indicates that pituitary TGFβ1 activity is regulated by dopamine and oestriadiol, which are the main factors involved in the control of lactotroph function, although how progesterone regulates this cytokine in the
pituitary remains unknown. Other studies have reported stimulatory progesterone effects on TGFβ1 activation in breast epithelial cells and lung cells, although the receptors and mechanisms involved were not investigated. One of the major findings of the present study is that mPRα is the main receptor mediating the rapid, nongenomic actions of progesterone, resulting in TGFβ1 activation in GH3 cells.
The fact that the Org OD 02-0 mimicked the stimulatory effects of progesterone on TGFβ1 activation, whereas the nPR agonist R5020 was ineffective, supports the involvement of mPRα in this effect. In addition, the knockdown studies with mPRα siRNA provided definitive evidence showing that mPRα is the principal receptor regulating this rapid effect of progesterone on TGFβ1 activation.

Moreover, the stimulatory effect of Org OD 02-0 on TGFβ1 activation was found to be a result of the activation of an inhibitory G protein with a consequent reduction in cAMP levels because these actions were blocked by pretreatment with pertussis toxin and the cotreatment with a cAMP analogue (8-Br-cAMP), respectively. This signalling pathway has been previously proposed for TGFβ1 activation in response to dopamine through D2 receptors coupled to an inhibitory G protein in rat lactotrophs. In addition, Pastoric and Sarkar demonstrated that treatment of anterior pituitary cells with forskolin, which activates adenylyl cyclase, significantly inhibited TGFβ1 levels in culture medium and TGFβ1 mRNA levels in cellular extracts. Thus, it is possible that inhibition of cAMP-dependent mechanisms may be involved in progestin regulation of TGFβ1. Besides the inhibition of the cAMP system, the mPRs have been shown to alter other second messenger pathways in a variety of target cells through the activation of the MAPK system. However, in our experimental model, a pharmacological inhibition of this pathway did not block Org OD 02-0-induced TGFβ1 activation, suggesting that the MAPK cascade is not involved in this effect.

In summary, the results of the present study demonstrate that mPRs are expressed in the female rat pituitary, with mPRα being the most abundant. We found an inhibitory effect of progesterone on PRL secretion acting through mPRα in ex vivo incubated
**Figure 7** Effect of progestin treatments on transforming growth factor (TGF)β1 activation in GH3 cells. (A) Effect of progesterone (P4), Org OD 02-0 (02) and R5020 on active TGFβ1 levels measured by an enzyme-linked immunosorbent assay. One-way ANOVA followed by Tukey’s post-hoc test (n = 4), with 3 replicates. **P = .01; ***P < .0001. (B) PTXa (PTX activated, 2.5 μg mL−1) effect on Org OD 02-0 (02) stimulatory effect on TGFβ1 activation. PTXhi (heat-inactivated, 2.5 μg mL−1) was used as a control. Two-way ANOVA followed by Bonferroni’s post-hoc test (n = 3), with 3 replicates. **P = .0035 02 vs V; significant interaction (P = .0453). (C) Effect of 8-Br-cAMP treatment on 8-Br-cAMP (10 µM) induced TGFβ1 activation. Two-way ANOVA followed by Bonferroni’s post-hoc test (n = 3), with 3 replicates. **P = .002 02 vs V; significant interaction (P = .0046). (D) Effect of ERK pathway inhibitor (PD98059) on Org OD 02-0 (02)-induced TGFβ1 activation. Two-way ANOVA followed by Bonferroni’s post-hoc test (n = 3), with 3 replicates. *P = .01 02 vs V; no significant interaction (P = .4696)

**Figure 8** Effect of membrane progesterone receptor (mPR)α knockdown expression in GH3 cells with small interfering RNA (siRNA) treatment on progestin-induced transforming growth factor (TGF)β1 activation. (A) Effects of progesterone (P4) and Org OD 02-0 (02) on active TGFβ1 levels in GH3 cells transfected with nontargeting oligos as control group of siRNA transfection (CTL) and GH3 cells transfected with siRNA mPRα (si-mPRα). Two-way ANOVA followed by Bonferroni’s post-hoc test (n = 2), with 3 replicates. **P = .01 P4 vs V; ***P = .0007 02 vs V; significant interaction (P = .0006). (B) Representative western blot of mPRα in GH3 cell membrane extracts of control GH3 cells transfected with nontargeting oligos (CTL) and GH3 cells transfected with mPRα siRNA (si-mPRα). mPRα expression was measured by western blotting in GH3-plasma membrane extracts. si-mPRα showed lower mPRα expression (n = 2)
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AUTHOR CONTRIBUTIONS

MAC, DP, JF, PT, SG and GDT conceived and designed the research. MAC, JF, AAM, AC, SG and EYF performed the experiments. MAC, JF and GDT analysed the data. MAC, JF, PT and GDT interpreted results of the experiments. MAC prepared the figures. MAC and GDT drafted the manuscript. MAC; GDT, JF, SG and PT edited and revised the manuscript. MAC, JF, PT, SG and GDT approved the final version of manuscript submitted for publication.

CONFLICT OF INTERESTS

No conflicts of interest, financial or otherwise, are declared by the authors.

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