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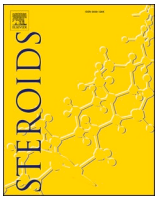

Effects of estradiol, progesterone or cAMP on expression of PGRMC1 and progesterone receptor in a xenograft model of human endometrium and in endometrial cell culture

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ABSTRACT

Estradiol and progesterone are key regulators of the menstrual cycle. In the human endometrium, progesterone induces morphological changes required for blastocyst implantation. Dysregulated response to progesterone can lead to endometrial pathologies including uterine bleeding and endometriosis. Besides the canonical nuclear progesterone receptor (encoded by the *PGR* gene), alternative response pathways include Progesterone Receptor Membrane Component 1 (PGRMC1), suspected to be involved in pathogenesis of endometrial diseases. We previously reported the spatiotemporal profile of *PGRMC1* expression in the human endometrium along the menstrual cycle, highlighting progressive increase and decrease during the proliferative and secretory phases, respectively. Here we directly addressed its regulation by estradiol and progesterone, with systematic comparison with regulation of *PGR* expression. We found a direct correlation between expression of both genes during the proliferative and secretory phases in the cycling endometrium, but not during the menstrual phase. In a xenograft model mimicking the cycle phases, estradiol significantly increased and progesterone significantly decreased *PGR* expression but changes were not significant for *PGRMC1*. Finally, we did not find any significant effect of the ovarian steroids on expression of *PGR* or *PGRMC1* in primary culture of endometrial stromal cells, except for a small increase in *PGR* expression by estradiol. Altogether, our experiments do not allow a major advance in our understanding of the mechanisms of cyclic variation of *PGRMC1* expression, in particular regarding potential regulation by the ovarian steroids.

1. Introduction

The endometrium is the mucous membrane lining the uterine cavity and in which an embryo can implant and develop. In the human endometrium, the *basalis* is the deepest layer in contact with the myometrium while the *functionalis* is the superficial layer. These two layers contain glands, surrounded by a stroma rich in fibroblasts, blood vessels and inflammatory cells. The human endometrium is a dynamic tissue that undergoes cyclic morphological remodeling in response to the variations in the blood levels of estradiol and progesterone throughout the three phases of the menstrual cycle [1]. During the proliferative phase, estradiol (E) stimulates tissue growth, while during the secretory phase, the increase of progesterone (P) concentration stabilizes the tissue and promotes the differentiation of stromal cells into decidual cells. These events are essential to prepare for a potential blastocyst implantation. Decidualization includes morphological changes by which stromal cells become rounder with increased cytoplasmic content, acquire myofibroblast characteristics and start secreting various proteins such as prolactin. In the absence of fertilization, blood levels of the two ovarian steroids drop, thereby triggering shedding of the endometrial functional layer and menstrual bleeding [2]. This tissue degradation is restricted to the upper *functionalis*, despite widespread expression of the estradiol and progesterone receptors in the *basalis*. Menstrual breakdown is caused by matrix metalloproteinases (MMPs), whose expression and activity are

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repressed by progesterone [1,3].

Perturbations of this tissue remodeling are associated with gynecological pathologies such as abnormal uterine bleeding and endometriosis [4]. Abnormal uterine bleeding is characterized by menstrual features, including induction of MMP activity, local tissue lysis and bleeding, occurring outside the expected menstrual phase of the cycle [5]. Abnormal uterine bleeding can also often result from use of progesterone derivatives (progestins) for contraception [6]. Endometriosis is characterized by the implantation and growth of endometrial tissue outside the uterus and is responsible for reduced fertility, inflammation and severe pain. Endometriosis is a hormone-dependent disease since growth of ectopic lesions requires an estrogenic stimulus [7]. In addition, it also involves local degradation of the host tissue by MMPs to ensure implantation and development of the lesions [8,9]. Treatment based on progesterone or progestins are prescribed to patients to reduce progression of endometriosis lesions. Unfortunately, not all patients respond to this type of treatment and the mechanisms underlying progesterone resistance are not fully understood [10]. In some cases, a lack of expression or activity of the canonical nuclear progesterone receptor (nPR) can be highlighted, but not systematically [11]. There are other nongenomic pathways able to modulate the response to progesterone via other proteins/receptors, such as the membrane Progesterone Receptors (mPRs) encoded by Progesterone and AdipoQ Receptor (*PAQR*) genes and the Progesterone Receptor Membrane Component (PGRMC) proteins encoded by Membrane-Associated Progesterone Receptor (*MAPR*) genes. These other pathways have received little attention so far compared to the classical nPR pathway.

Progesterone Receptor Membrane Component (PGRMC) 1 is an intracellular protein belonging to the MAPR family. It is expressed in different organs and was originally cloned in the liver under the name 25-Dx [12] or Hpr6.6 [13,14]. Like the other members of its family, PGRMC1 is a b5-like heme/steroid-binding protein. It can dimerize via heme to interact with other proteins and the presence of other motifs for molecular interaction opens a wide range of cellular functions [15–20].

In models of reproductive tissues, particularly in the human and murine breast and ovary, it has been shown that PGRMC1 can mediate non-genomic effects of progesterone [15,21,22] and interacts with other cellular pathways [23–26]. PGRMC1 was also reported to be implicated in steroidogenesis [27], to promote cell migration and invasion [28,29] and to regulate cell proliferation and apoptosis [30–32]. In addition, several studies showed an overexpression of PGRMC1 in breast, ovarian and endometrial cancer [15,33–36]. PGRMC1 was shown to modulate the effects of progesterone or progestins on cell proliferation in breast cancer cell lines [37,38].

In contrast, very little is known about the role of PGRMC1 in mediating progesterone response in the uterus. A study has reported that conditional *Pgrmc1* KO in the mouse female reproductive tract induced subfertility linked to a severe endometrial phenotype involving the spontaneous development of endometrial cysts [39]. PGRMC1 is also implicated in the decidualization of primary endometrial stromal cells [40]. In addition, endometrium of patients suffering from recurrent miscarriage was characterized by a decreased expression of PGRMC1 compared to healthy patients [41]. In another study, expression of PGRMC1 and PGRMC2 (both at the gene and protein levels) was decreased during the secretory phase in eutopic endometrium from women with endometriosis [42].

In a previous publication, our laboratory studied the spatiotemporal profile of PGRMC1 expression in the human endometrium along the menstrual cycle [43]. In particular, we showed that PGRMC1 expression progressively increases during the proliferation phase and decreases during the secretory phase. These changes in PGRMC1 expression were in line with previous studies based on microarray or proteomic analyses. [44–46]. Stimulation of *PGRMC1* expression by estradiol and inhibition by progesterone was reproduced *in vivo* in simian experimental models [47,48]. The former study also highlighted a similarity of the cyclic variations between *PGRMC1* and *PGR* expression, which corroborated earlier studies on cyclical *PGR* regulation in the human endometrium [49,50]. More recently, a 6-month exogenous administration of levonorgestrel to women via intrauterine device was shown to downregulate endometrial expression of both *PGR* (~10% from baseline) and *PGRMC1* (~15% from baseline) [51].

Although these studies suggest PGRMC1 regulation by the ovarian steroids, we still lack direct demonstration of the underlying molecular mechanisms, especially in the human endometrium. To address this question, we here used human endometrial tissue, either collected during normal menstrual cycle, or used in a mouse xenograft model (archived samples) and in primary culture of stromal cells to investigate the potential influence of estradiol and progesterone on *PGRMC1* expression in comparison with that of *PGR* (encoding nPR). We also questioned the effect of cAMP, involved in the decidualization stimulus, on *PGRMC1* and *PGR* expression.

2. Materials and methods

2.1. Collection of clinical specimens

This study was approved by the Ethics Committee of the Université Catholique de Louvain (2017/10JUL/362). Endometrial samples were provided by the Biolibrary of Saint-Luc University Clinics and collected from hysterectomy leftovers from patients operated for clinical reasons independent from the study. The included patients were under 50 years old, had spontaneous menstrual cycle, had not received sex hormone-related drugs for at least 3 months prior to surgery and had no malignant tumor or endometritis (Table 1). All the samples used in this study were analyzed by an expert pathologist (E.M.) to determine the cycle phase and the pathology following histological criteria. Based on a classical menstrual cycle of 28 days, the different phases were considered as follows: menstrual phase, days 1–5; the early, mid and late proliferative phases, days 6–8, 9–11 and 12–14 respectively; and the early, mid and late secretory phases, days 15–18, 19–22 and 23–28 respectively. The *functionalis* layer of human endometrium was scraped from the underlying basal layer and myometrium. For RNA analysis, a

Table 1
Endometrial samples used in this study.

# Sample	Age	Menstrual Phase	Pathology	Use
1	42	LS	Menorrhagia, polyp	Xenograft
2	45	LP	No identified gynecologic pathology	Xenograft
3	45	LS	Ovarian and tubal endometriosis, adenomyosis	Xenograft
4	43	EP	Adenomyosis	Xenograft
5	41	/	Fibroma, non-malignant adenomatoid tumor	pESC
6	40	EP	Leiomyoma, metrorrhagia	pESC
7	44	LP	Cornual polyp, cervix leiomyoma, adenomyosis	pESC
8	44	MS	Dysmenorrhea, menorrhagia, adenomyosis	pESC
9	45	MP	Leiomyoma	pESC
10	47	/	No identified gynecologic pathology	pESC
11	42	LP	Leiomyoma, Adenomyosis	pESC
12	39	MS	Adenomyosis	pESC
13	46	/	Rectovaginal endometriosis nodule	pESC
14	44	MS	Leiomyoma, chronic cervicitis, atheromatosis, menometrorrhagia	pESC
15	44	Mens	Leiomyoma, endometrial polyp	pESC
16	43	P	Adenomyosis	pESC
17	37	ES	Leiomyoma	pESC

Abbreviations: EP/MP/LP, Early/mid/late proliferative phase; ES/MS/LS, Early/mid/late secretory phase; /, unknown menstrual phase; pESC, primary endometrial stromal cells.

part of the tissue was kept into RNA lysis buffer (SV total RNA Isolation System; Promega) at −80 °C until total RNA isolation. The remaining tissue was used for cell purification in primary culture.

2.2. Cell isolation and culture

Primary endometrial stromal cells were obtained after digestion of endometrial tissue using bacterial collagenase (Type I; Sigma-Aldrich, C0130, 1 mg/mL). Digested endometrial fragments were then successively filtered through a 300-μm filter and a 30-μm filter to collect primary stromal cells. Primary endometrial stromal cells were grown in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12; Gibco 11039-021, ThermoFisher Scientific), supplemented with 10% Fetal Bovine Serum, 100U/mL penicillin and 100 μg/mL streptomycin (ThermoFisher Scientific) and at 37 °C in a humidified atmosphere containing 5% CO_2. Primary endometrial stromal cells were used until passage 6.

2.3. Archived samples of human endometrial xenografts

Experiments analyzing xenografted human endometrium were performed using archived RNA samples from a previous study [52]. Steps including housing of ovariectomized female CB17 mice with severe combined immunodeficiency (SCID), collection of human endometrium fragments, preparation of endometrial functionalis samples, xenografting of human endometrial fragments into mice and hormone supply were previously described in [52]. This previous study had been carried out in compliance with the rules of animal ethics in application at that time. No additional animal was used for the present study.

2.4. Cell incubation with hormones and cAMP

17β-estradiol-2-hydroxypropyl-β-cyclodextrin (E; Sigma) and progesterone-2-hydroxypropyl-β-cyclodextrin (P; Sigma) were dissolved in RNase free water and the progestin medroxyprogesterone 17-acetate (MPA; Cayman) in 100% dimethyl sulfoxide (DMSO) to achieve the stock concentration of 1 mM. These hormones were then further diluted in DMEM/F12 (Gibco 11039-021, ThermoFisher Scientific) to reach the working solution. Cells were seeded at a concentration of 2.10^4 cells/mL in DMEM/F12 supplemented with 10% FBS, 100U/mL penicillin, 100 μg/mL streptomycin. After 24 h, medium was changed and supplemented or not with hormones: 1 nM E or the combination 1 nM E + 100 nM P (EP) with or without 0.5 mM 8-bromoadenosine-3′,5′-cyclic monophosphate (8-Br-cAMP or cAMP; Sigma). Addition of 0.5 μg/ml 2-hydroxypropyl-β-cyclodextrin (HP-β-CD; Sigma) was used as control for E and P, and 0.01% DMSO was used as control for 100 nM MPA. Culture media were changed every 24 h and lysates were recovered after 72 h in the presence of hormones.

2.5. RNA extraction

Adherent cells were lysed with TRIzol Reagent (Ambion, Life Technologies), vortexed for 10 *sec* and incubated for 10 min at room temperature. Samples were then homogenized for 30 *sec* before adding 20 ng of tRNA. After vortexing for 1 min, 200 μL chloroform was added to each sample and mixtures were vortexed for another 30 *sec*. Mixtures were incubated for 15 min at room temperature and then centrifuged for 15 min at 12,000g and 4 °C. After centrifugation, upper aqueous phases were collected and 200 μL isopropanol 100% was added to each sample. Mixtures were vortexed for 30 *sec* and incubated at least 1 h at −80 °C before a centrifugation for 30 min at 12,000g and 4 °C to precipitate RNAs. RNA pellets were washed with 450 μL ethanol 70% and centrifuged for 10 more min at 12,000g and 4 °C. Finally, RNA pellets were dried and resuspended in autoclaved H_2O_d.

2.6. Quantitative Real-Time PCR

Total RNA concentration was quantified using Nanodrop ND-8000 spectrophotometer. Reverse transcription was performed with 500 ng RNA using SuperScript III Reverse Transcriptase kit (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. Real-Time PCR was conducted using KAPA SYBR FAST qPCR Master Mix (2X), 0.25 μM primers (forward and reverse) and cDNA corresponding to 15 ng RNA. Gene-specific oligonucleotides for *PGRMC1* amplification were previously published [42,53]. Other primers used were designed in our laboratory and are listed in Table 2. Except in experiments involving cAMP addition, values were normalized to *RPL13a* and *β-actin* housekeeping genes (HKGs) mRNA levels, which did not vary in response to E and/or P (see Fig. S5a for cell culture). On the other hand, HKGs varied in response to cAMP and were not used in corresponding figures (see text and Figs. S5b and Fig. 5 for details).

2.7. Immunolabeling

Immunohistofluorescence (IHF) was performed on paraffin-embedded tissue samples cut in serial sections of 6 μm. First, tissue samples were dewaxed in Histosafe before performing an antigen retrieval in citrate buffer for 75 min at 100 °C. Then tissue sections were permeabilized for 15 min in a solution of phosphate-buffered saline (PBS) containing 0.3% Triton X-100. After blocking the nonspecific binding sites for 1 h at RT in a solution of PBS, 10% bovine serum albumin (BSA), 3% non-fat milk and 0.3% Triton X-100, the slides were incubated overnight at 4 °C with primary antibody (Table 3) diluted in the blocking solution. Tissue sections were then washed 3 times during 5 min in PBS and 0.1% Triton X-100 before incubation for 60 min at RT with the adequate secondary antibodies (Table 3) diluted in a solution of PBS, 10% BSA and 0.3% Triton X-100. Nuclei were counterstained with Hoechst (BisBenzimide H33342, 1 ng/mL final concentration, Sigma) during this incubation.

Immunocytofluorescence (ICF) was realized with cells cultured on glass coverslips. After culture for the appropriate time, cells were washed in PBS and fixed during 15 min in 4% paraformaldehyde. Cells were then washed 5 times for 5 min in PBS before a permeabilization step for 15 min in a solution of PBS, 0.1% Triton X-100. Nonspecific binding sites were blocked for 1 h at RT in a blocking solution of PBS, 5% normal goat serum and 0.3% Triton X-100 and samples were then incubated overnight at 4 °C with primary antibodies (Table 3) diluted in blocking solution. The next day, cells on coverslips were washed 3 times for 5 min in PBS before incubation with appropriate secondary antibodies (Table 3) diluted in a solution of PBS, 1% BSA and 0.3% Triton X-100 for 1 h30 at RT. During the incubation with the secondary antibodies, nuclei were counterstained with Hoechst.

Table 2
Sequences of primers for specific amplification by quantitative real-time PCR.

Target	Primers	Sequence
PGRMC1	Forward	5′-TGACCTTTCTGACCTCACTGC-3′
	Reverse	5′-GCCCACGTGATGATACTTGA-3′
PGRMC2	Forward	5′-GTGTTCGAGAATGGGAAATGC-3′
	Reverse	5′-CTGATGGTTCTTCTCCTGGT-3′
PGR	Forward	5′-GCGCATATTCCAGAGCTGA-3′
	Reverse	5′-AGCAGTCCGCTGTCCTTTT-3′
Prolactin	Forward	5′-TGCTGCTGCTGGTGTCAAAC-3′
	Reverse	5′-TTGGGCTTGCTCCTTGTCTT-3′
MMP-3	Forward	5′- TCATTTTGGCCATCTCTTCC-3′
	Reverse	5′- GGGAAACCTAGGGTGTGGAT-3′
MMP-1	Forward	5′-GCAAACACATCTGACCTACAGG-3′
	Reverse	5′-TCTCAATGGCATGGTCCAC-3′
RPL13a	Forward	5′-GGCTAAACAGGTACTGCTGG-3′
	Reverse	5′-GGTTGGTGTTCATCCGCTTG-3′
β-actin	Forward	5′-AGCCTCGCCTTTGCCGA-3′
	Reverse	5′-TCACGCCCTGGTGCCTG-3′

Table 3
List of antibodies used for immunolabeling.

Primary antibodies	Species/ Isotype	Dilution	Company	Reference
Anti-Type III Collagen	Rabbit	1/50 (IHF)	Monosan	PS043
Anti-E-cadherin (Clone 36)	Mouse IgG2a mAb	1/1000 (IHF) 1/500 (ICF)	BD Transduction Laboratories	610,182
Anti-Vimentin (Clone V9)	Mouse IgG1 mAb	1/100 (ICF)	Santa Cruz	Sc-6260
Anti-ERα (Clone F-10)	Mouse IgG2a mAb	1/100 (ICF)	Santa Cruz	Sc-8002
Anti-nPR (Clone F-4)	Rabbit IgG mAb	1/200 (ICF)	Thermo Fisher	RM-9102-S0
Secondary antibodies	**Species**	**Dilution**	**Company**	**Reference**
Anti-Rabbit IgG, Alexa 488	Goat	1/1000	ThermoFisher	A11034
Anti-Mouse IgG2a, Alexa 568	Goat	1/1000	ThermoFisher	A21134
Anti-Mouse IgG1, Alexa 647	Goat	1/1000	ThermoFisher	A21240

Abbreviations: IHF, Immunohistofluorescence; ICF, Immunocytofluorescence.

2.8. Microscopy and signal quantification

Fluorescence was detected with a Cell Observer Spinning Disk (COSD) confocal microscope (Zeiss, Jena, Germany) and with a Pannoramic P250 Flash III slide scanner (3DHistech, Budapest, Hungary). The signal was analyzed and quantified with the image analysis software HALO (Indica Labs, Albuquerque, NM, USA). This software allowed the counting of cells positive for each signal after nucleus detection by Hoechst signal. Cells expressing E-cadherin were considered as epithelial cells and cells expressing vimentin as stromal cells. In each experiment, a negative control without primary antibody was used to correct the background noise.

2.9. Protein extraction and western blot analysis

After appropriate incubation, cells were washed with PBS and lysed with RIPA buffer (150 nM NaCl, 0.5% sodium deoxycholate, 50 mM Tris, 0.1% SDS, 1% Triton X-100, supplemented with 1 tablet per 50 mL of Complete protease inhibitor cocktail; Sigma). All lysates were sonicated and protein concentration was determined by the bicinchoninic acid (BCA; Sigma-Aldrich; B-9643) colorimetric method. Sample buffer 5x was added to each sample (0.25 M Tris-HCl, 10% SDS, 20% glycerol, 0.005% bromophenol blue, 5 mM dithiothreitol (DTT), pH 6.8) and the mixtures were heated for 5 min at 100 °C before centrifugation for 5 min at 14,000 g. Proteins were separated by SDS-PAGE in a running buffer (Tris 0.025 M, glycine 0.192 M, SDS 0.1%) and a 12% polyacrylamide gel. After separation, proteins were transferred to polyvinyl difluoride (PVDF) membranes (Perkin-Elmer, Zaventem, Belgium). Nonspecific binding sites on the membranes were blocked for 2 h at RT with a solution of Tris Buffer Saline (TBS: 20 mM Tris-HCl, 0.5 M NaCl, pH 7.5), supplemented with 0.05% Tween-20 (TBST) and 5% non-fat milk. Membranes were then incubated overnight at 4 °C with a solution of primary antibodies (Table 4). The next day, membranes were washed 3 times for 10 min with TBST before being incubated for 1 h at RT in horseradish peroxidase-conjugated appropriate secondary antibodies (Table 4) diluted in TBST, 5% BSA. Membranes were washed 3 times for 10 min with TBST and then 10 more min in TBS. Immunoreactive bands were finally visualized by chemiluminescence (SuperSignalTM West Femto Maximum Sensitivity Substrate; ThermoFisher Scientific) using the Fusion Solo S (Vilber Lourmat, Collegien, France). To ensure equal loading of samples, membranes were then incubated for 20 min in a stripping buffer (ReBlot Plus Strong Antibody Stripping Solution (10x); Merck Millipore), blocked as previously described, and re-probed with a primary anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody solution (Table 4) for 30 min at RT. The membrane was incubated with secondary antibody (Table 4) and immunoreactive bands were detected by chemiluminescence as described above.

Table 4
List of antibodies used for western blotting.

Primary antibodies	Species/ Isotype	Dilution	Company	Reference
Anti-PGRMC1	Mouse IgG2a mAb	2,5μg/mL (IF)	Abnova	H00010857-M03
Anti-GAPDH	Mouse IgG1 mAb	1/8000	Ambion	AM4300
Secondary antibodies	**Species**	**Dilution**	**Company**	**Reference**
Anti-Mouse IgG Horseradish Peroxidase conjugate	Goat	1/10000	Life Technologies	G21040

2.10. Statistical analysis

All statistical analyses were performed using GraphPad Prism 8.4.3. software (GraphPad, San Diego, CA). For RT-qPCR data in samples of cycling endometrium and in xenografts, statistical significance was tested using Kruskal-Wallis test with Dunn's post-hoc. For RT-PCR data in cell culture, statistical analyses were performed on ΔCt values from paired samples, i.e. cells from a same patient at the same passage and prepared at the same time. Outlier data was excluded using Grubb's test. Statistical significance was tested using Wilcoxon paired test. Differences were considered significant for $P < 0.05$.

3. Results

3.1. Expression of human endometrial PGRMC1 and PGR varies similarly during the menstrual cycle

Our laboratory has shown that *PGRMC1* mRNA concentration varies during the menstrual cycle in the human endometrium. Using samples of human endometrium collected at different times of the menstrual cycle (menstrual, proliferative or secretory phase), we reported that endometrial *PGRMC1* mRNA levels increases from the menstrual phase until the end of the proliferative phase before decreasing until the end of the secretory phase, to levels lower than those measured during the menstrual phase [43].

In the present study, we attempted to identify potential mechanisms of co-regulation between expression of *PGRMC1* and expression of two related molecules within the same human endometrial samples: *PGRMC2*, another member of the same *MAPR* gene family as *PGRMC1*; and *PGR*, encoding the canonical nuclear progesterone receptor nPR. Our results by quantitative RT-PCR show that unlike *PGRMC1*, the expression of *PGRMC2* mRNA was lower and did not vary during the menstrual cycle (Fig. 1a). In contrast, *PGR* expression varied during the menstrual cycle. Like *PGRMC1*, it increased from the menstrual phase to the proliferative phase and then decreased during the secretory phase (Fig. 1a). These results are in line with previous studies [49,50]. The expression of *PGRMC1* and that of *PGR* were correlated during the proliferative and secretory phases (Fig. 1b). However, in contrast with *PGRMC1* which had a lower expression level during the secretory phase than during the menstrual phase, the expression level of *PGR* during the secretory phase was equivalent to or even slightly higher than during the menstrual phase. Altogether, these data suggested hormonal regulation

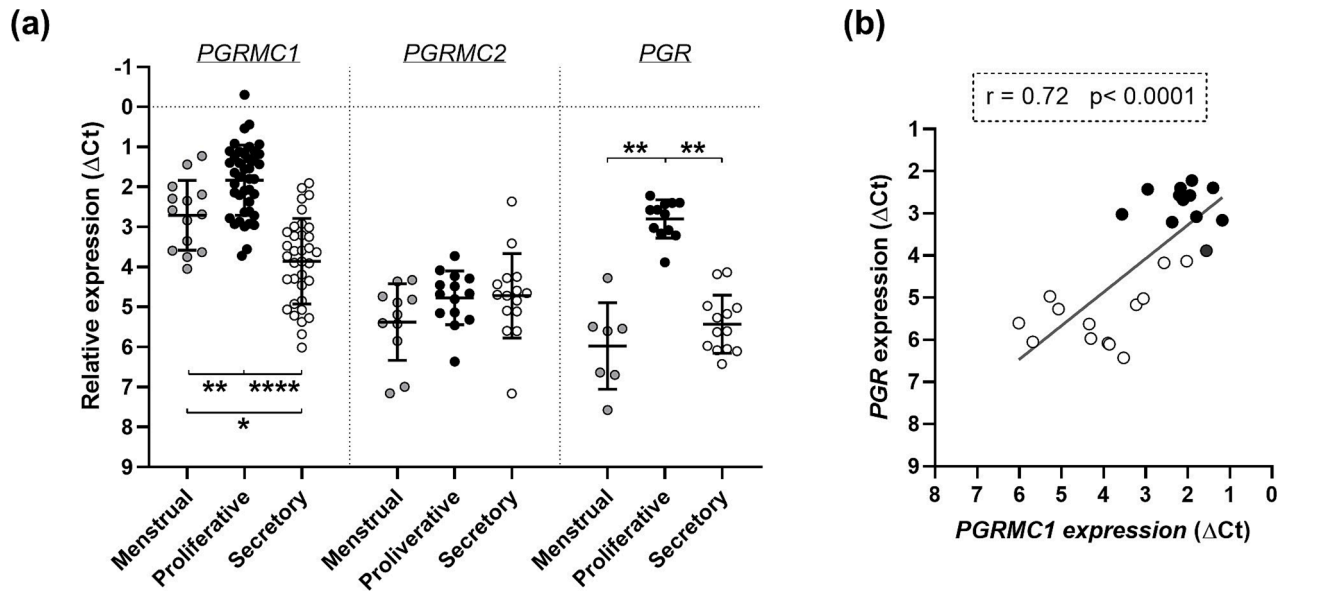

Fig. 1. Relative expression of *PGRMC1*, *PGRMC2 and PGR* during the menstrual cycle in the human endometrium. (a) Relative concentrations of *PGRMC1, PGRMC2* and *PGR* mRNA were measured by RT-qPCR in samples of the functional layer of the endometrium collected during the menstrual, proliferative or secretory phase of the menstrual cycle(n = 7–41). Results were normalized according to HKGs and are presented as dots for individual ΔCt values, and as means ± SD for each phase. Statistical test: Kruskal-Wallis with Dunn's post-hoc. Only statistically significant differences are shown. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$. (b) Pearson correlation test between *PGRMC1* and *PGR* expression in selected samples from (a) representing patients in proliferative (black) or secretory (white) phase. Results are presented as dots for individual ΔCt values with the simple linear regression.

of *PGRMC1* and *PGR* expression in the human endometrium during the menstrual cycle, although with divergent mechanisms, at least during menstruation.

3.2. Estradiol induces and progesterone represses PGR but not PGRMC1 mRNA expression in a mouse model of human endometrial xenograft

In order to better understand the correlation between the ovarian steroids and the expression of *PGRMC1* and *PGR* in the human endometrium, we used archived RNA samples from a study previously performed in our laboratory with a mouse model of human endometrial xenografts [52]. Briefly, fragments of the upper layer of human endometrium had been grafted subcutaneously into the flank of ovariectomized SCID mice (Fig. S1). Pellets delivering estradiol (E) and pellets delivering progesterone (P) had also been implanted into the back of these mice simultaneously with the graft. After 21 days of graft establishment, both hormonal pellets had been removed from some mice to mimic the menstrual phase (-H). Four days later, on day 25, new E or E + P pellets were implanted into some mice to mimic the proliferative and secretory phases, respectively. Mice had been finally sacrificed on

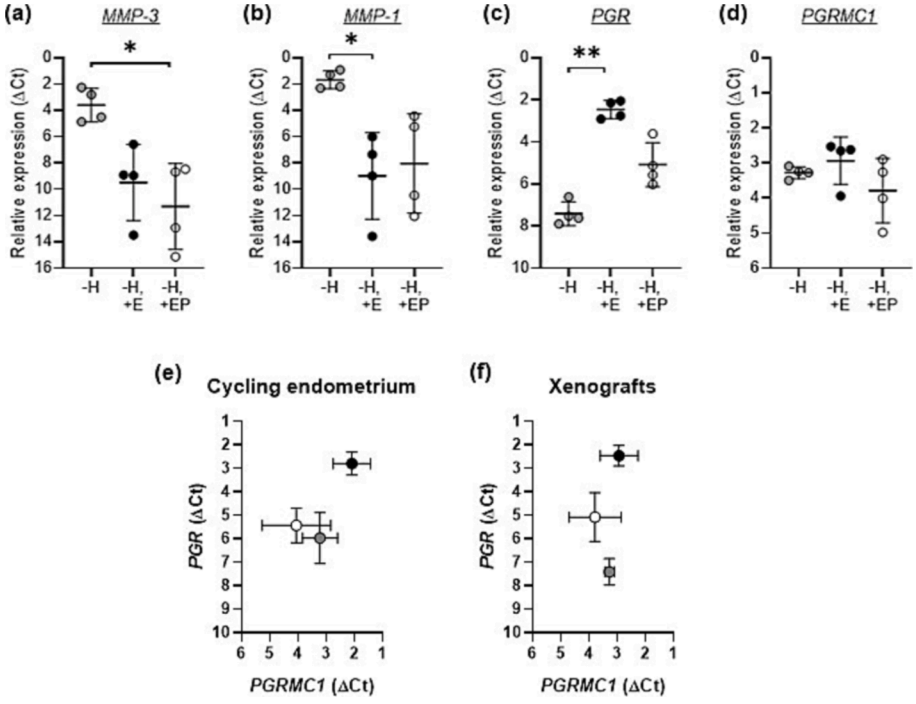

Fig. 2. Relative expression of *MMP-3, MMP-1, PGR* and *PGRMC1* in a murine model of human endometrial xenografts mimicking menstrual cycle phases. Human endometrial xenografts prepared as explained in Fig. S1 were collected from mice after 6 days of pellet removal (-H), after 4 days of pellet removal followed by 4–8 days of estradiol pellet re-implantation (-H, +E) or after 4 days of pellet removal followed by 4 days of estradiol and progesterone pellets re-implantation (-H, +EP). The relative concentration of *MMP-3* (a), *MMP-1* (b), *PGR* (c) and *PGRMC1* (d) mRNA was measured by RT-qPCR and normalized according to HKGs. Results are presented as dots for individual ΔCt values, and as means ± SD (n = 4). Statistical test: Kruskal-Wallis with Dunn's post-hoc. Only statistically significant differences are shown. * $p < 0.05$; ** $p < 0.01$. (e,f) Comparison of *PGRMC1* and *PGR* expression in the cycling endometrium and in xenografts using selected values from Fig. 1a in (e) or Fig. 2c, d in (f). Values are presented as mean relative expression (ΔCt) of menstrual or pseudo-menstrual (–H) conditions (in grey), proliferative or pseudo-proliferative (-H, +E) conditions (in black) and secretory or pseudo-secretory (-H, +EP) conditions (in white).

days 27, 29 or 33 and explants recovered for downstream analyses such as RNA extraction. Tissue and RNA leftovers were stored at −80 °C.

We quantified by RT-qPCR the expression of *MMP-3*, *MMP-1*, *PGR* and *PGRMC1*. *MMP-3* and *MMP-1* were first used to validate sample conservation, by comparison with data from the original study. As expected, both MMPs showed very high expression levels when the hormonal pellets were removed to mimic a menstrual phase (Fig. 2a,b; -H condition). Their expression was reduced in all samples collected in mice after new addition of E or E + P pellet, although this reduction was not always significant, most likely due to the small number of samples.

In the same xenograft samples, *PGR* mRNA expression (Fig. 2c) was significantly increased following re-addition of E (pseudo-proliferative phase) compared to -H condition (pseudo-menstrual phase) and was at an intermediate level between the other two conditions following re-addition of E + P (pseudo-secretory phase). This nicely reproduced the variations of *PGR* mRNA expression previously observed in cycling human endometrial samples, with < 1ΔCt difference between the two models for the average values for the three phases (compare *PGR* mRNA ΔCt values in Fig. 1a and Fig. 2c).

In contrast, we did not measure significant differences of *PGRMC1* expression between the different hormonal treatments. However, this was not totally surprising when integrating two important observations from cycling endometrial samples (Fig. 1). On the one hand, individual values were widely dispersed (interpatient variation) within each cycle phase, especially for *PGRMC1*. And on the other hand, differences between mean values by cycle phases were larger for *PGR* expression than for *PGRMC1* expression.

Finally, the direct comparison between natural cycles and xenograft values (Fig. 2e,f) emphasized another important difference between the two genes: the minimal values were measured during the (pseudo) menstrual phase for *PGR* but during the (pseudo)secretory phase for *PGRMC1*.

3.3. Estradiol stimulates mRNA expression of PGR but not of PGRMC1 in stromal cells in primary culture

Although the xenograft model is a more physiological and integrated model, it relies on comparison of different groups of animals, with inherent risk of large variability between individuals. We therefore switched to primary culture of endometrial cells since this model offers the advantage to allow statistical pairwise comparisons between different hormonal conditions processed at the same time from a same batch of cells. Considering recent publications suggesting a potential contribution of PGRMC1 during decidualization of stromal cells (see below), we focused on primary culture of endometrial stromal cells (pESC) to further address hormonal regulation of PGRMC1 expression. Only cultures with > 95% vimentin-positive stromal cells were retained (Fig. S2a). The presence of the nuclear receptors ERα and nPR was also confirmed by immunostaining (Fig. S2b).

The effects of estradiol addition were evaluated to mimic the switch between the menstrual and the proliferative phase of the menstrual cycle. We first ensured that the presence of 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), used as complexing agent for hydrophobic hormones (E and P), did not impact expression of *PGRMC1* and *PGR* (Fig. S3a). Then, cells precultured in the absence of hormones were incubated for 72 h in the presence of HP-β-CD alone (as control) or 1 nM E complexed with HP-β-CD. *PGR* mRNA expression was significantly increased when E was added by comparison with the control condition (Fig. 3a). This observation was in line with our results in the cycling endometrial samples and in the xenografts, although the magnitude of the increase was smaller. *PGRMC1* expression was not significantly modified by E (Fig. 3b).

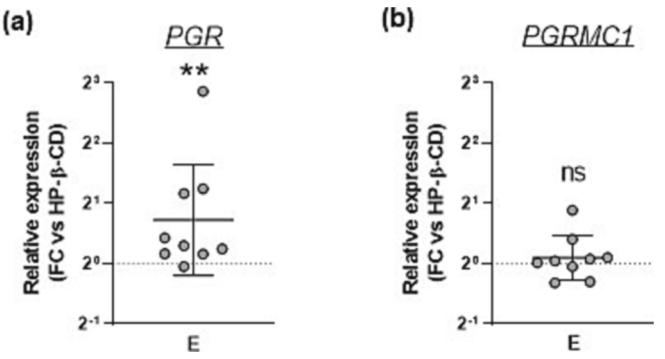

Fig. 3. Effects of estradiol addition on the relative concentration of *PGR* and *PGRMC1* mRNA in primary culture of endometrial stromal cells. Endometrial stromal cells in primary culture were incubated for 72 h with estradiol (1 nM; E) or with HP-β-CD as control. The relative concentration of *PGR* (a) and *PGRMC1* (b) mRNA was measured by RT-qPCR and normalized according to HKGs (n = 9). Individual values are presented as fold change (FC) in log2 scale by comparison with the corresponding HP-β-CD control in the same culture. Graphs also present geometric means with geometric SD. Statistical test: Wilcoxon paired test; not significant (ns); ** $p < 0.01$.

3.4. Neither progesterone nor progestin MPA modifies mRNA expression of PGRMC1 in primary culture of endometrial stromal cells

In a second step, we assessed the influence of progesterone or the progestin medroxyprogesterone 17-acetate (MPA) on *PGR* and *PGRMC1* expression. For this purpose, pESC cultured in the absence of hormones were incubated during 72 h in the presence of 1 nM E or the combination of 1 nM E with 100 nM P (EP) or with 100 nM MPA (EMPA). These conditions were used to mimic the secretory phase of the menstrual cycle. We also ensured that DMSO (1/10 000 final concentration) used to solubilize MPA, did not impact *PGRMC1* expression (Fig. S3b).

As in xenografts, we first used *MMP-3* and *MMP-1* to evaluate progesterone repression in the cell culture model. Their expression was decreased upon EMPA addition by comparison with E alone, as expected, but not upon EP addition (Fig. 4a,b). Moreover, the magnitude of *MMP-3* and *MMP-1* repression by EMPA was much lower (<4-fold between means) than that observed with EP in the xenografts (>6ΔCt between means, corresponding to > 64-fold; Fig. 2a,b). Surprisingly, *PGR* expression did not show significant variation following addition of EP or EMPA by comparison with E (Fig. 4c). Coherently, *PGRMC1* mRNA expression also remained stable upon EP or EMPA addition by comparison with E (Fig. 4d).

The same experiments were replicated using higher hormone concentrations (10 nM E and 1 μM P or 1 μM MPA), longer incubation times (up to 6 days), variations in serum supply (no serum or charcoal-treated serum). Similar results were obtained with all tested conditions (not shown) and no significant effect in *PGRMC1* expression could be found either upon E addition or upon EP or EMPA addition by comparison with E.

3.5. 8-Br-cAMP does not modify PGRMC1 and PGR expression in primary culture of endometrial stromal cells

The secretory phase of the menstrual cycle is notably characterized by decidualization of stromal cells. This step is essential to prepare the endometrium for the possible implantation of an embryo. We therefore hypothesized that decidualization could be necessary to mimic physiological regulation of *PGRMC1* expression during the secretory phase, at least in stromal cells. Decidualization of stromal cells can be mimicked in cell culture by incubating cells (i) with 8-bromoadenosine-3′,5′-cyclic monophosphate (8-Br-cAMP or cAMP) alone or (ii) with progesterone or a progestin such as MPA, or, (iii) for optimal differentiation, by a combination of hormones and cAMP [54,55]. Therefore, pESC

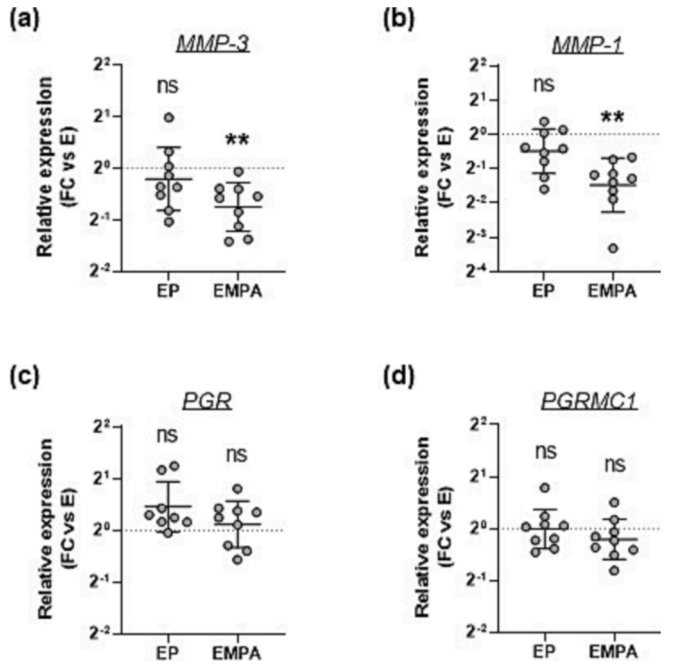

Fig. 4. Effects of progesterone or MPA addition on the relative concentration of *PGR* and *PGRMC1* mRNA in primary culture of endometrial stromal cells. Endometrial stromal cells in primary culture were incubated for 72 h with 1 nM estradiol (E), with combined 1 nM estradiol and 100 nM progesterone (EP) or with combined 1 nM estradiol and 100 nM MPA (EMPA). The relative concentration of *MMP-3* (a), *MMP-1* (b), *PGR* (c) and *PGRMC1* (d) mRNA was measured by RT-qPCR and normalized according to HKGs (n = 9). Individual values are presented as fold change (FC) in log2 scale by comparison with the corresponding E condition in the same culture. Graphs also present geometric means with geometric SD. Statistical test: Wilcoxon paired test; not significant (ns); ** $p < 0.01$.

precultured in the absence of hormones were incubated for 72 h in the presence of 0.5 mM cAMP combined with 1 nM estradiol and 100 nM progesterone. Decidualization of pESC was first validated by monitoring their morphological changes. As expected, in the presence of the combination EP + cAMP, pESC adopted an epithelial cell-like appearance consisting in more rounded and swollen cells, due to accumulation of glycogen droplets, by comparison with the corresponding EP condition without cAMP (Fig. S4a). Unexpectedly, expression of both HKGs, which was unaffected by hormones (Fig. S5a), significantly decreased in response to cAMP addition (Fig. S5b), thereby preventing their use to normalize qPCR data. PCR results in Fig. S4b,d and Fig. 5 are therefore presented as differences of the Ct values for the gene of interest, between paired samples from a same experiment (therefore with cells from a same patient at the same passage number and processed at the same time). Normalization between samples is ensured by adjustment of the same initial amount of total RNA for RT-qPCR. As expected, a strong increase in *prolactin* expression, a marker of decidualization, was measured (Fig. S4b). However, the mRNA concentration of both *PGR* and *PGRMC1* was not modified by the combination EP + cAMP, by comparison with EP without cAMP (Fig. 5a). At the protein level, western blots supported the absence of cAMP effect on PGRMC1, while suggesting a reduction in GAPDH that was used as a potential loading control (Fig. 5b).

In the same primary stromal cell cultures, addition of cAMP alone (i.e. without concomitant EP) was sufficient to induce the morphological changes in pESC corresponding to decidualization (Fig. S4c), as well as to stimulate their *prolactin* expression (Fig. S4d). However, the addition of cAMP alone did not modify *PGR* and *PGRMC1* expression (Fig. 5c). In western blotting, similar results were obtained (Fig. 5d) as in the presence of EP. Altogether, these experiments did not indicate any effect of cAMP on expression of *PGR* and *PGRMC1* in pESC and did not support the hypothesis that cAMP was necessary to induce reduction of PGRMC1 expression during the secretory phase.

4. Discussion and conclusion

Progesterone is a crucial regulator of the menstrual cycle and inappropriate response is involved in various gynecological pathologies. Two isoforms (PR-A and PR-B) of the nuclear progesterone receptor (nPR), both encoded by the *PGR* gene, have been largely documented since their discovery, decades ago. Alternative receptors and/or regulatory pathways, including PGRMC1, were identified more recently and the understanding of their precise contribution to hormonal response is still only partial.

In a previous study, we identified a temporal variation of PGRMC1 expression profile (mRNA and protein) along the menstrual cycle in the human endometrium, with a progressive increase during the proliferative phase and progressive decrease during the secretory phase [43]. In

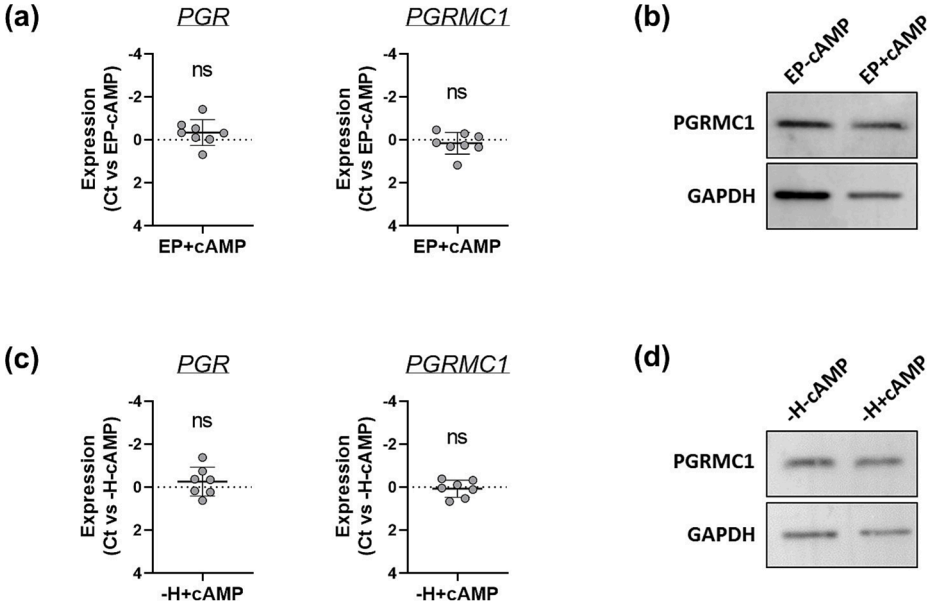

Fig. 5. Effects of 8-Br-cAMP combined or not with estradiol and progesterone on expression of *PGR* and *PGRMC1* in endometrial stromal cells in primary culture. Stromal cells in primary culture were incubated for 72 h with (EP, a,b) or without (-H, c, d) combined 1 nM estradiol and 100 nM progesterone and with (+cAMP) or without (-cAMP) 0.5 mM 8-Br-cAMP. HP-β-CD was used as control when appropriate. (a,c) The concentration of *PGR* and *PGRMC1* mRNA was measured by RT-qPCR (n = 6–8) but was not normalized according to HKGs. Individual results are presented as differences in Ct values vs the corresponding condition without cAMP and as means with SD. Statistical test: Wilcoxon paired test, not significant (ns). (b,d) Immunoblots of PGRMC1 (24 kDa) and GAPDH (36 kDa; used as loading control) in representative samples.

the present study, we further investigated the potential hormonal co-regulation of *PGRMC1* and *PGR* expression in the human endometrium, by combining and comparing analyses on cycling samples, xenografts and primary endometrial stromal cell culture. We first confirmed that expression of *PGR* and *PGRMC1* clearly varies along the menstrual cycle, unlike that of *PGRMC2* which remains constant. Moreover, although our data in cycling samples and xenografts converged to confirm that expression of *PGR* and *PGRMC1* is co-related during the proliferative and secretory phases, they also highlighted that their expression diverges during the menstrual phase. Indeed, whereas this phase is characterized by minimal values for *PGR* expression, *PGRMC1* mRNA concentration is minimal at the end of the secretory phase and increases thereafter. The mechanisms underlying this menstrual increase in *PGRMC1* expression remain unknown.

The cyclical variations were better reproduced in xenografts for *PGR* than for *PGRMC1*. It is important to note that expression of both genes was measured in the same xenograft samples, which excludes a major bias due to the low number of samples (4 by condition). Moreover, the range between the different phases or pseudo-phases (in xenografts) was more pronounced for *PGR* than for *PGRMC1*. Indeed, the minimal expression level measured for *PGR* during the proliferative phase in cycling samples (Fig. 1a) was higher than the maximal levels in both the menstrual and the secretory phase. Accordingly, *PGR* expression in the 4 xenografts mimicking the pseudo-proliferative phase was higher than in the 8 other xenografts (Fig. 2c). In striking contrast, individual *PGRMC1* expression levels were more heterogeneous and overlapped between phases, both in the cycling endometrium and in xenografts. This clearly suggests that regulation is tuned differentially for the two genes.

Since RT-PCR results reflect the mean expression level in the sample lysates used for RNA purification, spatial variation of expression could contribute to explain this individual tuning. Indeed, in our previous study, we showed by immunolocalization that, besides cyclical regulation, PGRMC1 is also locally controlled, especially during the proliferative phase, with a gradient of decreasing PGRMC1 concentration extending from the surface epithelium toward the *basalis* [43]. This major spatial orientation axis is not reproduced in the xenograft model, which only contained tissue originating from the superficial layer, but additional immunolabeling experiments should be performed with xenografts tissue sections to determine whether PGRMC1 expression profile is also spatially heterogeneous and whether nPR is colocalized or not. In addition to the lack of global orientation, xenografts in immunodeprived animals also lack part of immune and inflammatory cell populations. Besides human cells that were present at the time of grafting, xenograft infiltration by immune and inflammatory host cells is limited and could be biased by species specificities.

Although our two *in vivo* approaches converged to support cyclical hormonal regulation by the ovarian steroids, primary culture of isolated endometrial stromal cells at low passage numbers failed to directly confirm this hypothesis. This is in part explained by the substantial reduction in the amplitude of the hormonal effects in cell culture, by comparison with corresponding *in vivo* situations. For instance, we previously measured that the median concentration of *MMP1* and *MMP3* mRNA increases >1000-fold between the secretory and the menstrual phase, in cycling human endometrial samples [56]. Accordingly, expression of both genes was higher by a factor close to 100-fold (for *MMP3*) or more (for *MMP1*) in xenografts (Fig. 2a,b), when comparing samples collected from animals mimicking a pseudo-menstrual phase (after pellet removal) with animals mimicking a pseudo-secretory phase (new EP pellets). However, in striking contrast, expression of the two genes was not significantly repressed by addition of combined EP in primary culture, even at higher (10-fold) hormonal concentration and for up to 6 days. A significant but very limited repression (around 2-fold) was only achieved when replacing progesterone by MPA. This clearly underlines the complexity of the molecular mechanisms of hormonal response in the human endometrium, especially for a repressive role by progesterone, such as for *MMP3*, *MMP1*, but also *PGR* and *PGRMC1*. Accordingly, *PGR* mRNA expression in our stromal cell cultures was not significantly modified by combined EP or EMPA in cell culture by comparison with E alone (Fig. 4c), although it was repressed by ~6-fold both in cycling endometrial samples when comparing the secretory and proliferative samples (Fig. 1a) and in xenografts when comparing the pseudo-secretory (-H/+EP) and the pseudo-proliferative (-H/+E) samples (Fig. 2c). Moreover, *PGR* expression was only weakly induced by E addition (<2-fold) in primary stromal cell culture (Fig. 3a) by comparison with the stimulation observed in proliferative endometrial tissues (~10-fold or 3.2 cycles, Fig. 1a) and in xenografts from mice implanted with E pellet (~30-fold or 5 cycles, Fig. 2c). Altogether, our results do not exclude the involvement of estradiol and progesterone in the physiological control of endometrial *PGRMC1* expression, but suggest (partially) different mechanisms of response than for *PGR*.

Estradiol is known to induce endometrial expression of both nPR isoforms, PR-A and PR-B, during the proliferative phase [57,58]. We confirmed the presence of the receptors ERα and nPR in the stromal cells in primary culture. However, expression of ERα was weak and could have been insufficient to generate an estradiol-induced increase in *PGRMC1* expression, which would in turn prevent any subsequent repression by progesterone or MPA. This hypothesis can be tested by overexpressing ERα.

Loss of hormone response could also result from the procedure used to purify stromal cells, including limited tissue proteolysis. We reproduced these experiments with a cell line, the telomerase-transformed endometrial stromal cells *T*-HESC [59] and obtained similar results. However, a previous study highlighted the lack of progesterone-responsiveness of *T*-HESC cells and suggested that it was due to lack of nPR expression [60]. Moreover, it has previously been shown that very few genes acutely respond to progesterone treatment, either in combination with estradiol or not, in undifferentiated endometrial stromal cells in primary culture [61]. Once again, overexpression of the receptor could allow to test the hypothesis, but this would further increase the lack of physiological relevance of the cell culture model.

Addition of cAMP in stromal cell culture induced decidualization and prolactin expression, but failed to reduce mRNA or protein amounts of PGRMC1, both in the presence and the absence of EP. The interplay between progesterone and cAMP in the human endometrium is complex. Incubation of stromal cells with combined EP for 8 days is a common procedure to induce decidualization, but addition of cAMP to progesterone or progestins is a frequent alternative as it allows more rapid cell differentiation (in 2 or 3 days). It has been known for over two decades that cAMP and nPR signaling pathways converge for the induction of decidualization-specific transcription factors [62]. PR-A was suspected to be the major isoform involved in mediating the action of progesterone in stromal cells during the secretory phase, notably for decidualization [58]. Appropriate receptor levels are required. Indeed, the maintenance of elevated nPR levels inhibits the onset of decidualization. This was demonstrated by experiments in which transient transfection to overexpress PR-A or PR-B in primary human endometrial stromal cells dramatically reduced the activity of a *prolactin* promoter-reporter construction in response to cAMP. It was concluded that intracellular cAMP levels contribute to induction of decidualization in part by sensitizing stromal cells to the effect of progesterone by downregulating the expression level of PR [62]. However, in our experiments, *PGR* mRNA amounts in pESC were not modified by cAMP addition for 72 h, further questioning the regulation of PGR expression in this model. Another team has reported that treatment of endometrial stromal cells for 72 h with 1 μM MPA and 0.5 mM 8-Br-cAMP for 3 days resulted in expected decidualization and concomitant relocalization of PGRMC1 protein in the nuclear fraction at the expense of the membrane/organelle fraction [40]. However, this study did not highlight any quantitative change in PGRMC1 expression in response to decidualization, in agreement with our results.

Cell culture is a remarkable model for directly testing the effect of agents added to the culture medium or of genetic modifications.

However, it suffers from limitations, especially the lack of interaction with other cell types and with the surrounding extracellular matrix. Cell-cell and cell-matrix communications play key roles in cell adaptation to its environment. Therefore, 3D models such as organoids and assembloids combining epithelial and stromal cells are emerging and represent a very attractive alternative approach to further dissect the regulation of endometrial PGRMC1 in the human endometrium, be it by hormones or through additional mechanisms [63,64].

Mechanisms involved in transcriptional control of human *PGR* expression (reviewed in [65]) involve transcription factor-binding elements in the promoter, such as Sp1 and AP-1 binding sites, and a half-palindromic ER binding site in the PR-A promoter. Moreover, *PGR* expression is also regulated by microRNAs such as miR-194-3p and miR-196a which repress *PGR* expression in the eutopic endometrium of women with endometriosis. Furthermore, DNA methylation of CpG islands present in *PGR* promoter represses its expression. By comparison with *PGR*, the transcriptional regulation of *PGRMC1* remains largely unknown and requires further investigation.

Besides PR and PGRMC1, mPRs are important regulators of the progesterone response also in the endometrium [66]. Although mPRα is the predominant mPR isoform in reproductive tissues, other isoforms, including mPRβ and mPRγ have been detected in endometrial tissue. A study reported increased *PAQR7* (mPRα) expression and decreased *PAQR5* (mPRγ) expression during the secretory phase [67]. Expression of *PAQR7*, *PAQR8* (mPRβ) and *PAQR5* is decreased in the endometrium of women with endometriosis compared to healthy women [68]. Moreover, expression of *PAQR7* and *PAQR8* is downregulated in endometrial cancer [69]. As a reminder, *PGRMC1* is overexpressed in cancers including endometrial and breast tumors. Surprisingly, overexpression of PGRMC1 increased mPRα expression on the cell membrane of PR-negative breast cancer cells [23]. This apparent contradiction clearly underlines that additional investigation is required to better understand regulation of expression of the various progesterone receptors and their mutual interactions. Indeed, in the latter study [23], the authors found a close association of mPRα and PGRMC1 in several breast cancer cell lines, suggesting that the two proteins act as a receptor complex.

In conclusion, our direct comparison between *PGRMC1* and *PGR* expression in different experimental models derived from the human endometrium does not provide direct evidence of regulation of *PGRMC1* expression by estradiol and/or progesterone. However, our data do not refute the hypothesis of hormonal control of both genes in the human endometrium but further underline that specific mechanisms finely tune *PGRMC1* expression in space and time.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.steroids.2023.109284.

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