Université Catholique de Louvain
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Cell Unit

Progesterone Receptor Membrane Component (PGRMC) 1:

Study of its expression, regulation and potential functions in the human endometrium and in endometriosis

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LIST OF ABBREVIATIONS

25-Dx	25kDa-dioxin
8Br-cAMP	8Br-cyclic adenosine monophosphate
AF	Activating function
AP1	Activator protein 1
ARID1A	AT-rich interaction domain 1A
BMDSCs	Bone marrow-derived stem cells
CO	Carbon monoxide
COS-7	African green monkey kidney fibroblast cells
COX-2	Cyclooxygenase-2
CYP	Cytochrome P450
Cytb5	Cytochrome b5-like domain
Dap1p	Damage associated-response protein 1
DBD	DNA-binding domain
DIE	Deep infiltrating endometriosis
E2	Estradiol
EC	Endometriotic cyst
EGFR	Epidermal growth factor receptor
ERE	Estradiol response element
ERs	Estradiol receptors
FECH	Ferrochelatase
FSH	Follicle-stimulating hormone
G3BP2	GTPase-activating protein binding protein 2
GnRH	Gonadotropin-releasing hormone
GPER	G-protein coupled estrogen receptor
GPR30	G-protein coupled receptor 30
GRE	Glucocorticoid response element
HEK293	Human embryonic kidney-derived cell line
HKG	Housekeeping gene
HO	Heme oxygenase
HOX	Homeobox
Hpr6.6	Heme progesterone receptor 6.6
HP-βCD	2-Hydroxypropyl β-cyclodextrin
HRE	Hormone response elements
IGFBP1	Insulin-like growth factor-binding protein 1

IL-1	Interleukin-1
INSIG1	Insulin-induced gene 1
IZA	Inner zone antigen
IκB	NF-κB inhibitor
KO	Knock-out
KRAS	Kirsten rat sarcoma virus
LBD	Ligand-binding domain
LH	Luteinizing hormone
MAPRs	Membrane associated progesterone receptors
mERs	Membrane estradiol receptors
MET	Mesenchymal-to-epithelial cell transition
MIF	Macrophage migration inhibitory factor
MISS	Membrane-initiated steroids signaling
MMP	Matrix metalloproteinase
MPA	Medroxyprogesterone acetate
mPR	Membrane progesterone receptor
MRI	Magnetic resonance imaging
NF-κB	Nuclear factor of κ light polypeptide gene enhancer in B-cells
NK	Natural killer
NMR	Nuclear magnetic resonance
nPRs	Progesterone receptors
NR5A1	Nuclear receptor subfamily 5 group A member 1
NSAIDs	Non-steroidal anti-inflammatory drugs
P4	Progesterone
PDGFR-β	Platelet-derived growth factor receptor-β
PGE2	Prostaglandin E2
PGRMC1	Progesterone receptor membrane component 1
PGRMC2	Progesterone receptor membrane component 2
PHB-1/2	Prohibitin 1 and 2
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PPP2R1A	Protein phosphatase 2A regulatory subunit A alpha
PRE	Progesterone response element
PRL	Prolactin
RA	Retinoic acid
RERG	Ras-like estrogen-regulated growth inhibitor

ROS	Reactive oxygen species
SCAP	SREBP cleavage-activating protein
SCID	Severe combined immunodeficiency
SE	Superficial endometriosis
SERM	Selective estrogen receptor modulators
SF1	Steroidogenic factor 1
SGK1	Serum and glucocorticoid-regulated kinase 1
SH2	Src homology 2
SH3	Src homology 3
SIGCs	Spontaneously immortalized granulosa cells
SIMS	Secondary ion mass spectrometry
SP1	Specific protein 1
SPRMs	Selective progesterone receptor modulators
SREBP	Sterol-regulatory element-binding protein
SSEA-1	Stage specific embryonic antigen-1
Tcf/Lef	T cell factor/lymphoid enhancer factor
TGF-β	Transforming growth factor-β
TNFα	Tumor necrosis factor α
uNK	Uterine natural killer
VEGF	Vascular endothelial growth factor
ZEB	Zinc finger E-box-binding homeobox

I. INTRODUCTION

A. THE HUMAN ENDOMETRIUM

Main references:

- L'endomètre présent et avenir (Trévoux R., 2009)
- Human physiology : An Integrated Approach (Silverthorn, 2007)

i. General anatomy and histology

Along with the vulva, vagina, fallopian tubes and ovaries, the uterus is an organ of the female reproductive system. It is located in the pelvis, anterior to the rectum and posterior to the bladder and is covered by a reflection of the peritoneum. The uterus is composed of a serous layer - the perimeter, a smooth muscular layer - the myometrium, and a mucous lining - the endometrium (Figure 1).

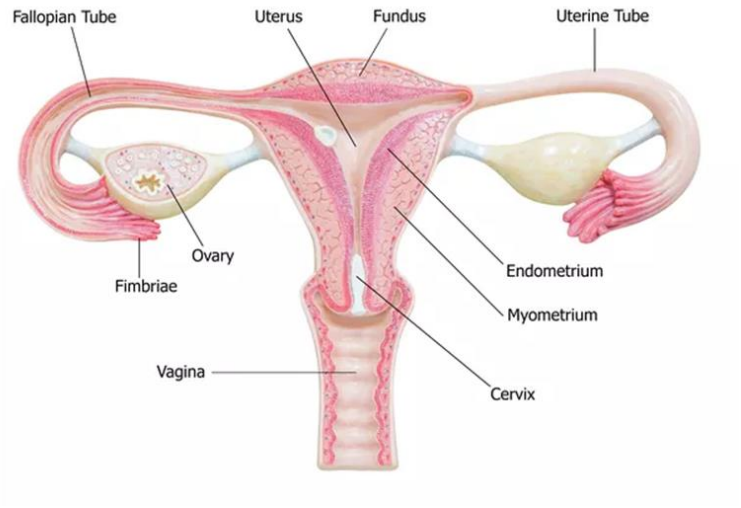

Figure 1: The female reproductive system. *Published in (Kwon, 2017).*

Based on histological and functional characteristics, the endometrium is divided into a basal layer, in contact with the myometrium, and a functional layer that extends from the basal layer to the uterine cavity (Figure 2a). The junction between the myometrium and the endometrial basal layer as well as between the basal and the functional layers are not sharp and regular but rather interdigitate.

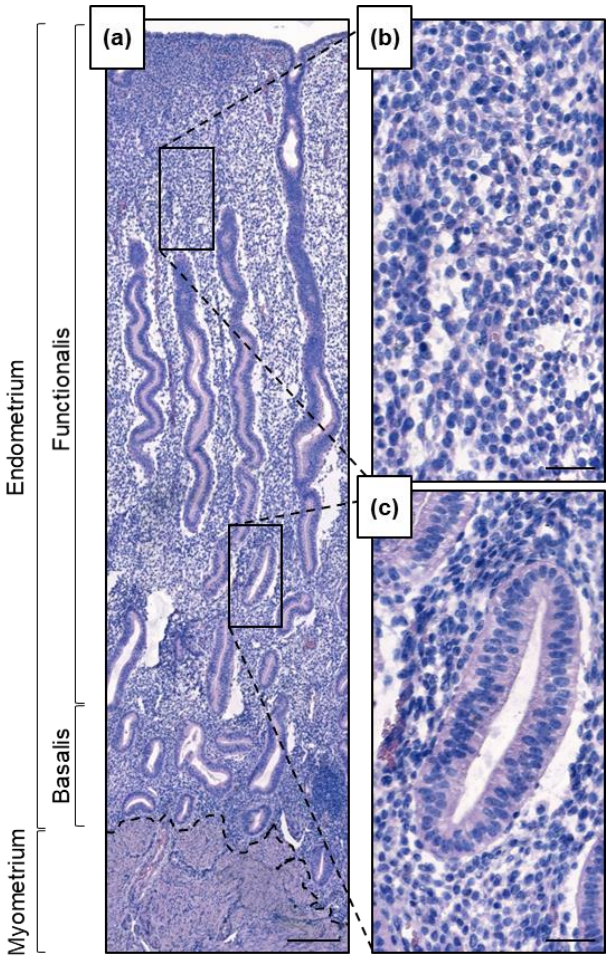

Figure 2: Histology of endometrium.
Endometrial section of a patient in the early secretory phase. **(a)** The human endometrium is divided into a functional superficial layer and a basal layer which lies on the myometrium. It is composed of a rich stroma **(b)** and glands **(c)**.

The endometrium is composed of both epithelial cells that form the surface epithelium or are organized into glandular structures, and a rich multicellular mesenchyme, or stroma (Figure 2b and c). The stroma is predominantly composed of fibroblasts. Nevertheless, immune cells such as lymphocytes, especially T-cells and natural killer cells (NK), and macrophages are periodically recruited and delivered by blood vessels.

The vascularization of the endometrium depends on the anastomosis of the uterine and ovarian arteries. In the myometrium, they divide into arcuate arteries which then enter the endometrium and branch off into straight or spiral arterioles. The straight arterioles supply the basal layer while the spiral arterioles supply the stroma of the functional layer. Below the surface epithelium, the capillary plexus drains into a venous sinus (Figure 3).

The cellular composition and the morphology of the endometrium, and in particular the functional layer, are extremely variable during the menstrual cycle.

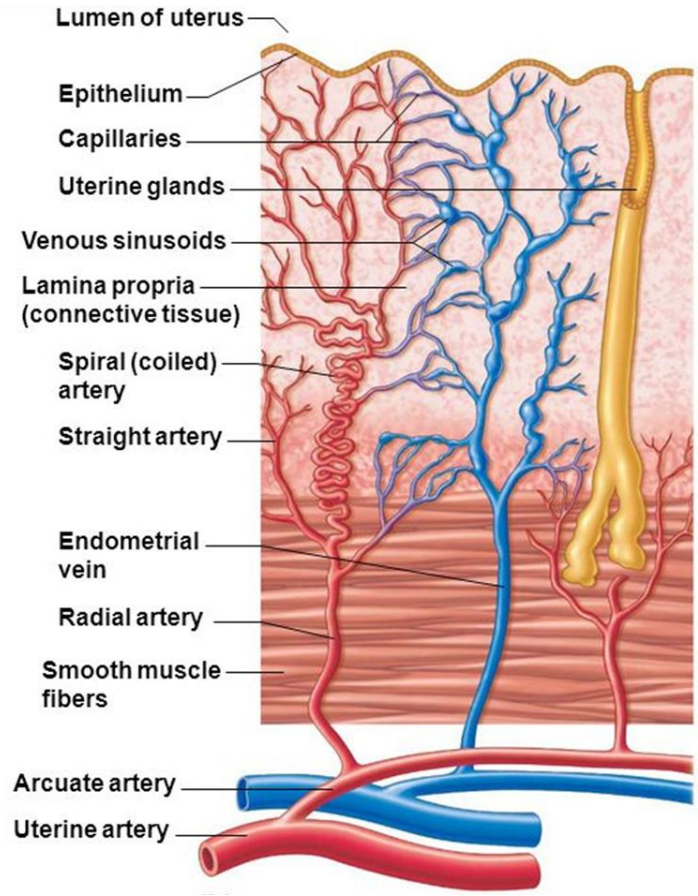

Figure 3: Vascularization of endometrium. *Reproduced from (Akerlund, 1994).*

ii. The menstrual cycle

The menstrual cycle arbitrarily begins with the first day of menstrual bleeding and classically lasts 28 days. However, the cycle length may vary between 25 and 35 days depending on the individual and may even be irregular between successive menstrual cycles.

The menstrual cycle is defined as the set of physiological changes that prepare the female reproductive system for fertility. It is indeed the cyclical variations in blood concentration of steroid hormones that regulate the tissue growth and differentiation to prepare the endometrium for the potential implantation of an embryo. The two major orchestrators of these structural changes are the hormones estradiol (17β-estradiol; E2) and progesterone (pregn-4-ene-3,20-dione; P4) produced by the ovaries. Therefore, the endometrial cycle is intrinsically linked to the ovarian cycle (Figure 4).

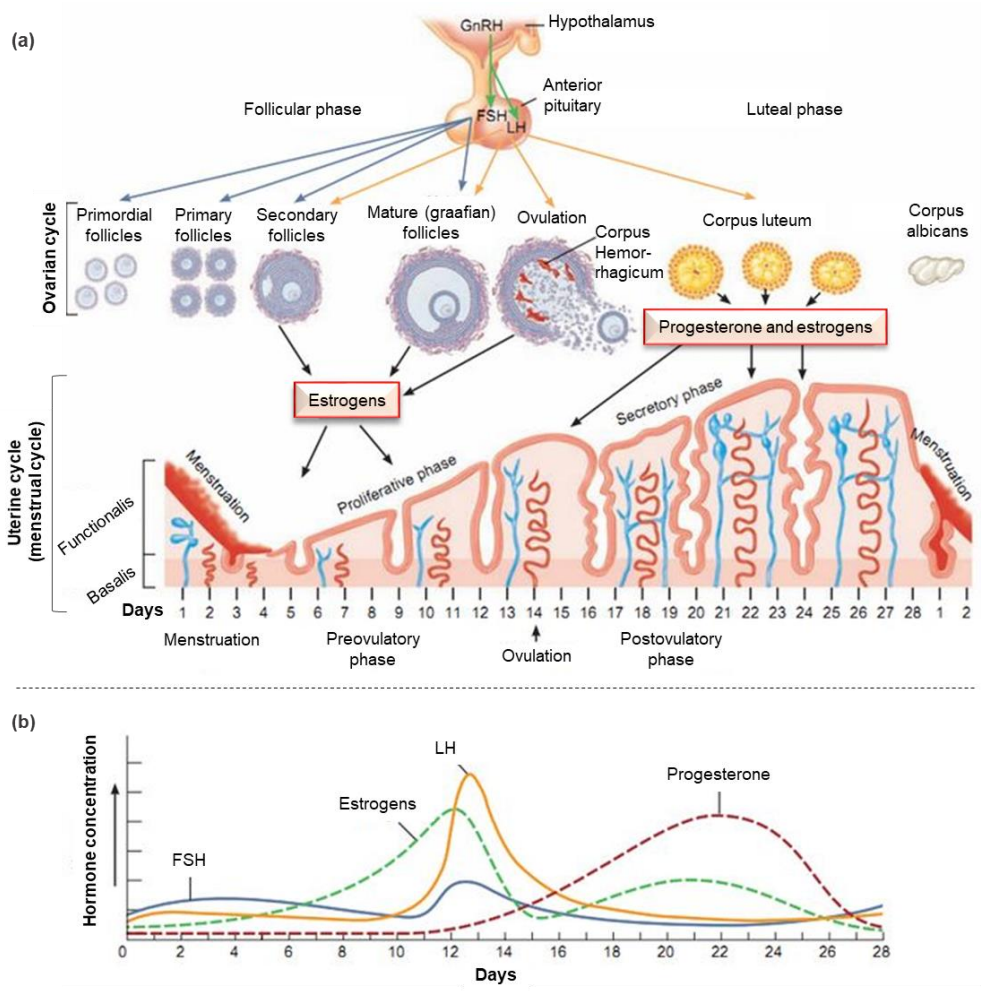

Figure 4: The menstrual cycle.
(a) Control by the hypothalamic-pituitary axis of the ovarian and endometrial menstrual cycle. **(b)** Variation of blood concentrations of the hormones LH, FSH, estrogens and progesterone during the menstrual cycle. *Adapted from (Schust, 2010).*

1. The ovarian cycle and the secretion of ovarian steroids

The ovarian cycle is controlled by the hypothalamus and the anterior pituitary gland which secrete successively the hormones gonadotropin-releasing hormone (GnRH) and follicle-stimulating hormone (FSH) and luteinizing hormone (LH), respectively.

The ovarian cycle consists of two phases; the follicular phase and the luteal phase (Figure 4).

The duration of the follicular phase is variable. It extends from the first day of menstruation until ovulation, which typically corresponds to day 14 in a conventional 28-day cycle. The luteal phase is more stable. It begins the day after ovulation and lasts 14 days.

During the follicular phase, under the effect of FSH, several antral follicles (Figure 5) enter the maturation process within the two ovaries. One of these will become the dominant follicle aka the Graafian follicle. The dominant follicle is composed of several layers surrounding the oocyte: a layer of glycoproteins, the zona pellucida, a layer of multi-layered epithelial cells, the granulosa, and beyond the basement membrane, a layer of connective tissue divided into inner and outer theca that forms the transition to the ovarian stroma. The inner theca is provided with abundant capillaries and contains connective cells whose cytoplasm contains lipid droplets. The external theca is less vascularized and consists mainly of fibroblasts, connective fibers and smooth muscle cells.

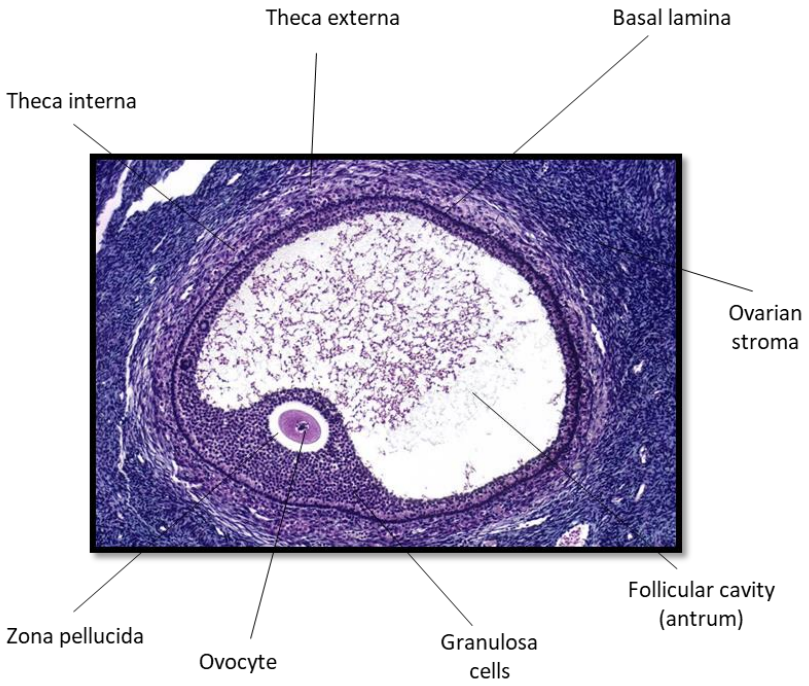

Figure 5: The antral follicle.
Structural composition of a antral ovarian follicle. *Adapted from (Gougeon, 2022)*.

After ovulation, which occurs 38 hours after the LH peak, the follicle transforms into a corpus luteum during the luteal phase. If no fertilization occurs, the corpus luteum only remains functional for about ten days. Otherwise, it is maintained during the first six months of pregnancy to ensure the synthesis of the necessary hormones.

Cholesterol is the precursor of all steroid hormones which include progestogens, glucocorticoids, mineralocorticoids, androgens and estrogens. The process of enzymatic transformation of cholesterol into various steroid hormones is called steroidogenesis and is dependent on the expression and activity of the enzymes involved in the various successive stages of their synthesis (Figure 6).

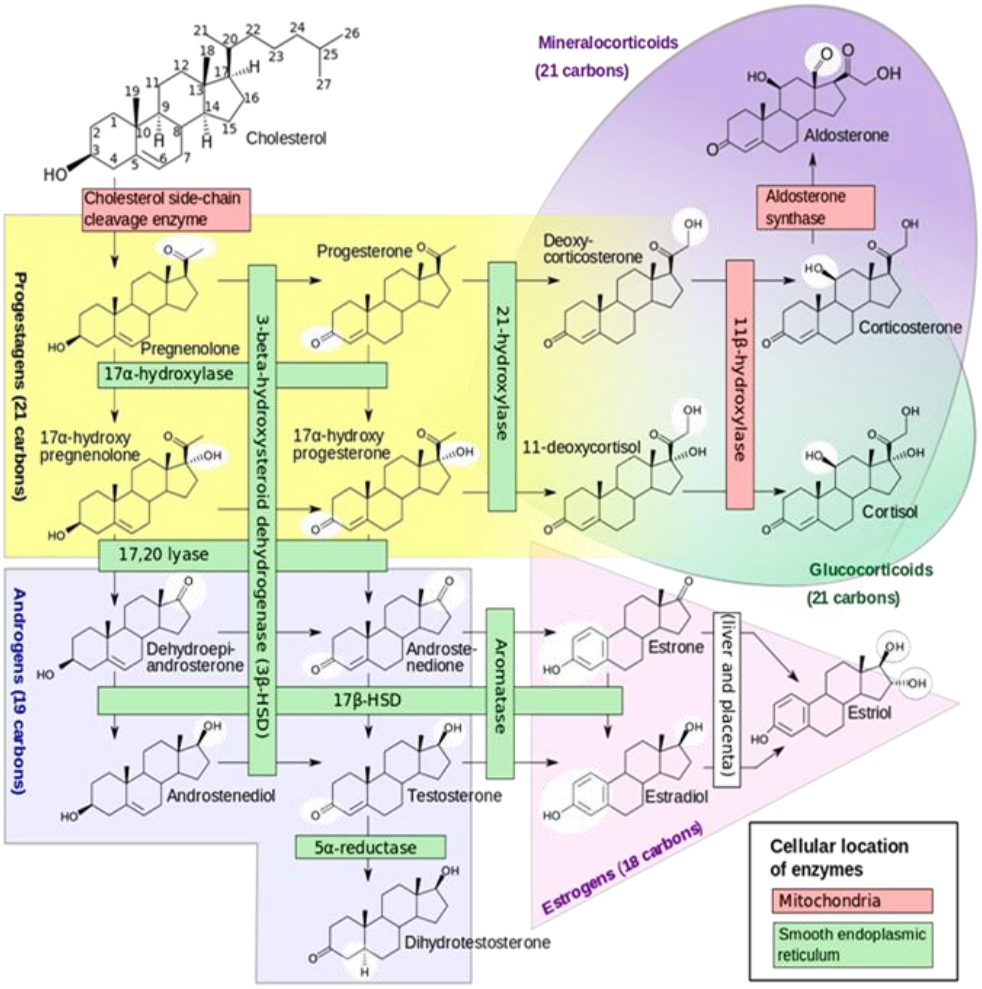

Figure 6: Steroidogenesis.
Enzymatic pathways involved in the transformation of cholesterol into progestogens, androgens, estrogens, glucocorticoids and mineralocorticoids. *Published in (Häggström, 2014)*

Although it is recognized that other tissues may be involved in the production of sex hormones, progestogens and estrogens are primarily synthesized by the ovaries. Estradiol is produced by the granulosa cells as early as the secondary follicle stage and is secreted into the follicular cavity, whereas progesterone is synthesized by the corpus luteum during the luteal phase. Ovarian steroidogenesis during the follicular phase is based on the cooperation of the granulosa and internal theca cells. In the granulosa cells, LH stimulates the expression of cholesterol side-chain cleavage enzyme which catalyzes the conversion of cholesterol to pregnenolone (Figure 6). Since granulosa cells do not express 17α-hydroxylase (Voutilainen, Tapanainen, Chung, Matteson, & Miller, 1986), the synthesized pregnenolone and progesterone then diffuse into the surrounding internal theca cells where they are converted to androstenedione. After returning into the granulosa cells, FSH promotes the conversion of androstenedione to estradiol by the enzymes aromatase and 17β-HSD successively (Miller & Auchus, 2011).

Once secreted into the bloodstream, the hormones progesterone and estradiol in turn exert negative feedback control on the hypothalamic-pituitary system and initiate numerous morphological and functional changes in the endometrium.

2. The endometrial cycle

The endometrial menstrual cycle is divided into three successive phases; the menstrual, proliferative and secretory phases. In a 28-day cycle, the menstrual phase lasts 5 days. The proliferative phase extends from day 6 to ovulation on day 14. This is followed by the secretory phase until day 28 (Figure 4).

The proliferative phase is concomitant with the follicular phase of the ovarian cycle and is synonymous with the growth of endometrial tissue. Under the influence of high blood concentrations of estradiol, the proliferation of epithelial, stromal and endothelial cells is stimulated. The thickness of the functional layer increases progressively until the end of the proliferative phase. The glands appear rectilinear, mostly pseudostratified and composed of columnar cells. The stroma, initially loose, becomes progressively denser.

During the secretory phase, the increase in blood progesterone concentration limits the proliferation and favors the differentiation of the endometrial cells. The glands become sinuous and the epithelial cells acquire a vacuolated cytoplasm, loaded with glycogen. The stroma, on the other hand, continues to grow denser at the surface of the endometrium. The number of uterine natural killers (uNKs), which are involved in immune tolerance towards the trophoblast, increases dramatically. Lymphocytes and macrophages progressively invade the stroma throughout the secretory phase.

At the end of the secretory phase, the glandular epithelium in the superficial layer presents a saw-toothed appearance to the point where it is characterized as the "uterine lace". Around the spiral arterioles, the fibroblasts present a pre-decidualized aspect characterized by an enlargement of their cytoplasm which becomes eosinophilic and finely granular.

In the absence of pregnancy, blood concentrations of estradiol and progesterone fall, which induces menstruation of the endometrium. Within a few days, the functional layer of the endometrium is proteolyzed and begins to regenerate while most debris are evacuated through the vagina.

The fall in progesterone blood levels initiates several morphological changes including increased blood flow and fragility and permeability of spiral arterioles which then become a source of local bleeding. These changes are accompanied by the secretion of prostaglandins, several inflammatory mediators and cytokines/chemokines that lead to tissue invasion by leukocytes, contractility of the uterus, vasoconstriction of the basal arteries and stimulation of matrix metalloproteinases (MMPs) responsible for degradation of the extracellular matrix (Figure 7).

At the same time, the menstrual endometrium shows signs of local repair (Gaide Chevronnay et al., 2009). To rapidly limit bleeding and protect the endometrium from infection, endometrial reconstruction begins with angiogenesis and restoration of the surface epithelium. Re-epithelialization begins with the migration of epithelial progenitor cells localized in the basal layer, particularly at the junction with the functional layer of the endometrium (Cousins, Pandoy, Jin, & Gargett, 2021). In addition, other factors are suspected to participate in endometrial tissue regeneration. They include a potential mesenchymal-to-epithelial cell transition (MET) and the influence of inflammatory cells, proangiogenic factors and growth hormones such as members of the transforming growth factor-β family (TGF-β). The expression of genes involved in extracellular matrix synthesis is also stimulated during the menstrual phase but, probably to facilitate epithelial cell migration, stromal regeneration is observed only in parallel with the increase in estradiol blood concentrations, at the

beginning of the proliferative phase (Critchley, Maybin, Armstrong, & Williams, 2020; Salamonsen, Hutchison, & Gargett, 2021).

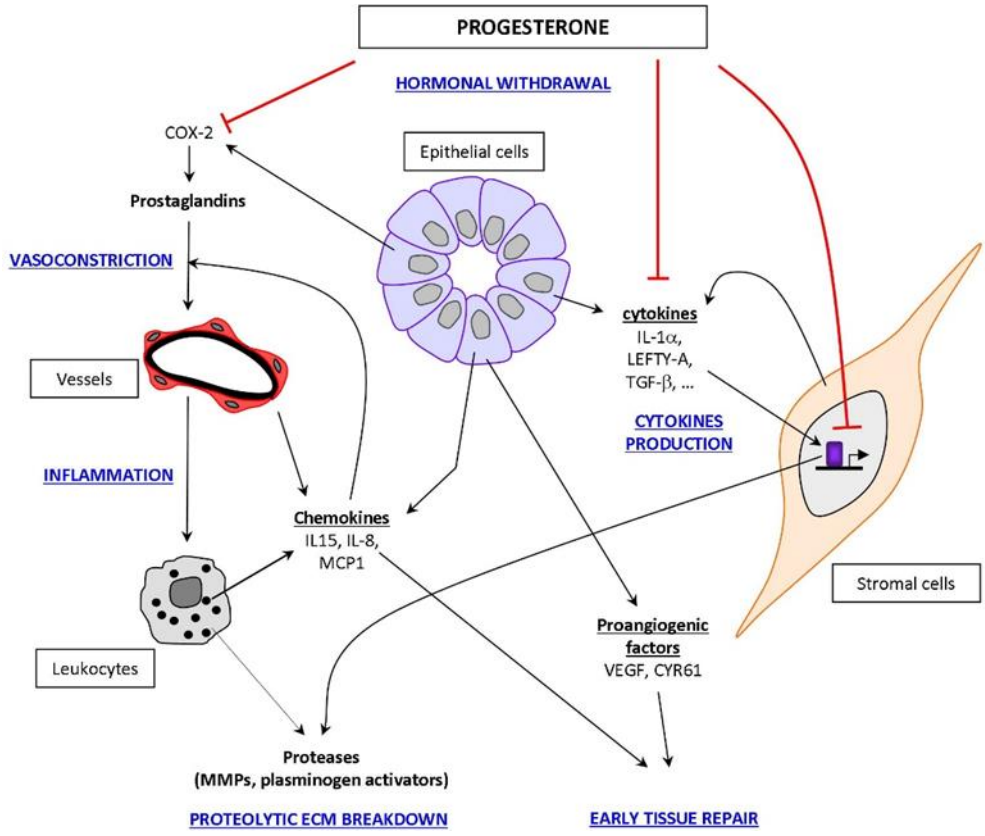

Figure 7: Hypothesis of spatiotemporal control of human endometrial tissue remodeling by progesterone.
In the secretory phase, progesterone prevents tissue degradation of the endometrium and prepares it for embryonic implantation by controlling cytokine secretion and inflammation. In the absence of pregnancy, the fall in progesterone levels leads to the lifting of its inhibitory effects and causes proteolytic degradation coupled with endometrial tissue repair. *Published in (Henriet, Gaide Chevronnay, & Marbaix, 2012).*

iii. Estradiol and progesterone receptors in the endometrium

To exert their activity on endometrial tissue, estradiol and progesterone bind to receptors capable of initiating a cellular response through genomic or non-genomic mechanisms.

These receptors are usually divided into two categories: nuclear and membrane receptors. Genomic signaling pathways reflect a slow response (hours) and combine all mechanisms by which steroid receptors regulate gene expression: by interaction (i) directly with DNA, (ii) with one or more transcription factors such as activator protein 1 (AP1), specific protein 1 (SP1), and the nuclear factor of the light κ polypeptide gene enhancer in B cells (NF-κB), or even (iii) independently of ligand binding (DeMayo & Lydon, 2020; Yu et al., 2022). In contrast, the non-genomic pathways are faster (seconds to minutes) and involve the activation of various signaling pathways resulting in the activation of kinases, the activation of kinases, ion channels and second messengers (Puglisi et al., 2019). Classically, the nuclear receptors induce the genomic pathways while the non-genomic pathways depend on the membrane receptors. But, non-genomic signaling can also regulate genomic pathways and vice versa. Hence, the final cellular effect of steroids is often the result of a convergence of events (Wilkenfeld, Lin, & Frigo, 2018).

1. The « classical » nuclear receptors

The nuclear estradiol receptors (ERs) and progesterone receptors (nPRs) are the best described and are therefore regularly referred to as the “classical” receptors.

The expression of nuclear estradiol and progesterone receptors is directly influenced by cyclic variations in blood concentrations of estradiol and progesterone. Although the mechanisms of regulation of their expression are not precisely identified, several mouse models

have demonstrated that estradiol stimulates the expression of ERs and nPRs while progesterone represses their expression (Graham & Clarke, 1997; Prange-Kiel, Rune, Zwirner, Wallwiener, & Kiesel, 2001).

ERs and nPRs have structural similarities (Figure 8). They are composed of an N-terminal domain that allows interaction with the transcriptional machinery, a "zinc finger" DNA-binding domain (DBD), a hinge (H) region and a ligand-binding domain (LBD).

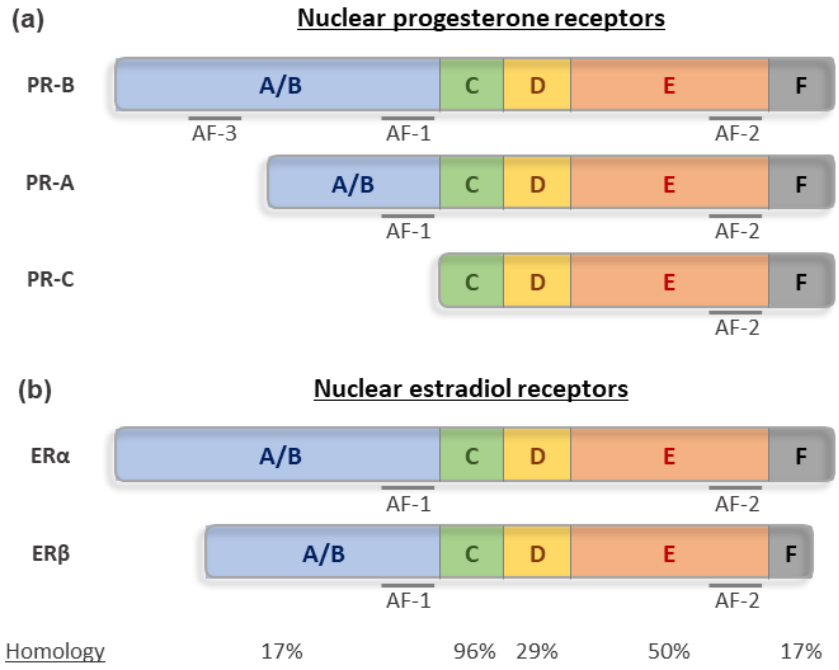

Figure 8: Illustration of ERs and nPRs structures.
The A/B domain is involved in the specific interaction of ERs and nPRs with the transcriptional machinery via the Activating function (AF) regions. The DBD is located in the C domain. The D domain represents the Hinge region. The LBD is located in the E region. The F domain is a variable region. **(a)** The 3 isoforms of nPR, PR-A, PR-B and PR-C, are derived from the same *PGR* gene. **(b)** Nuclear estradiol receptors, ER*α* and ERβ, are expressed by 2 distinct genes, respectively *ESR1* and *ESR2*. The homology levels between the two receptors are shown at the bottom of each domain. *Adapted from (Gaide Chevronnay, 2009).*

ERs include the ERα and ERβ proteins. They are encoded by two independent genes, respectively *ESR1* and *ESR2*. Apart from the DBD, the two isoforms show little homology which explains why their biological activities differ: ERα is a potent transcriptional activator whereas ERβ is known to inhibit ERα activity. Nevertheless, both ERα and ERβ independent deletion leads to early infertility of female mice related to dysregulation of serum E2 levels, of ovulation and of estrous cycle. In ERα KO mice, a hypoplastic uterus and hemorrhagic cysts in the ovaries are also observed (Antonson et al., 2020; Antonson, Omoto, Humire, & Gustafsson, 2012; M. Chen et al., 2009; Dupont et al., 2000; Lubahn et al., 1993).

The ERs expression is stimulated by estradiol until the beginning of the secretory phase but ERα is significantly more expressed than ERβ in the whole endometrial tissue. In the mid-secretory phase, ERα and ERβ concentrations gradually decrease until the end of the cycle. ERβ appears to decrease more in the glands than in the secretory stroma (Yu et al., 2022).

The three isoforms of the nPR (PR-A, PR-B, and PR-C) are all encoded by the same gene, *PGR*, but are the source of three different mRNAs. PR-B represents the total isoform while PR-A and PR-C are N-terminally truncated, causing the loss of one or two Activating Function (AF) sites respectively. As a result, PR-B is transcriptionally more active than PR-A which, like ERβ, acts more as a regulator of PR-B activity. However, PR KO mouse models have shown that PR-A and PR-B can have independent activities: PR-A is more required for uterine development and PR-B for mammary gland development (Conneely & Lydon, 2000). Although specific to the uterus, the expression and functions of the C isoform are unclear as it remains poorly investigated. However, a potential role in the repression of progestogen activity by sequestration of progesterone has been

suggested in late pregnancy (Condon, Hardy, Kovaric, & Mendelson, 2006; Wei, Gonzalez-Aller, Wood, Miller, & Horwitz, 1990).

Under the effect of estradiol, the expression of nPRs is stimulated during the proliferative phase, predominantly in the epithelium. Both the A and B isoforms are equivalently present in the epithelium, whereas the A isoform prevails in the stroma. In the secretory phase, PR-A and PR-B amounts decrease, especially in the glands, and the ratio PR-A/PR-B is unbalanced: first in favor of PR-A isoform at the beginning of the secretory phase and then in favor of PR-B.

The physiological changes in the endometrium during the menstrual cycle are more frequently attributed to the activity of the ERα and PR-B isoforms. The ERβ and PR-A isoforms, on the other hand, appear to function more as feedback controllers by interacting with the targets of their respective counterparts (Kowalik, Rekawiecki, & Kotwica, 2013).

In their inactive form, ERs and PRs interact with chaperone proteins, such as Heat Shock Protein -90 and -70, capable of regulating their subcellular localization, promoting their binding to ovarian steroids or even modulating their transcriptional activity. The binding of their respective ligand leads to the separation of chaperone proteins, the activation and homo-dimerization of the receptors, their nuclear translocation and their interaction with specific sites on chromatin called "hormone response elements" (HRE; estradiol response element (ERE) or progesterone response element (PRE)). Their activity can be modulated by post-translational modifications of the receptors, the type of ligand, the formation of homo- or hetero-dimers, or the composition and number of cofactors recruited for transcription.

2. The membrane receptors

In opposition to the nuclear receptors, the expression and contribution of membrane receptors to endometrial physiological mechanisms during the menstrual cycle are not yet clearly defined.

A subpopulation of ER was identified in the plasma membrane (mER), particularly at the caveolae/lipid raft where they constitute a pool of mER organized into functional signaling modules (Arnal et al., 2017; Yu et al., 2022). The mERs (α and β) are palmitoylated forms of nuclear ER capable to induce a membrane-initiated steroids signaling (MISS) pathway. Although most rapid actions of estrogen are mediated by full-length mER, many other truncated isoforms are also observed at the plasma membrane (Mauvais-Jarvis, Lange, & Levin, 2022). To date, there is still no information about the synthesis and location of the various mER isoforms in the physiological and pathological endometrium. The role of mERs in cardiovascular functions is currently investigated preferentially. However, in the mouse model carrying a mutation in the palmitoylation site of ERα, the loss of membrane-localized ERα induced an abnormal development of breast, ovaries and potentially uterus leading to female infertility (Adlanmerini et al., 2014; Pedram, Razandi, Lewis, Hammes, & Levin, 2014). These results indicated the importance of the cellular pool of mERα in reproduction and suggest that nuclear and membrane receptors have common functions and collaborate to transmit an estrogenic signal.

G-protein coupled receptor 30 (GPR30) as known as G-protein coupled estrogen receptor (GPER) is a seven transmembrane receptor localized in the endoplasmic reticulum or in the plasma membrane. Among all membrane estrogen receptors, GPER was recognized as the major mediator of the rapid cellular effects of estrogen (Yu et al., 2022). Its estradiol binding affinities are, however, lower (K_d = 3-6nM) than

those of classical ERs (K_d = 0.1-1.0nM) (Kuiper et al., 1997; Revankar, Cimino, Sklar, Arterburn, & Prossnitz, 2005; Thomas, Pang, Filardo, & Dong, 2005). Its direct interaction with estradiol is even questioned. Nevertheless, several studies demonstrate its ability to induce estrogenic activity *in vivo* in several organs. A collaboration with E2-activated membrane-localized, mERs, to transmit an estradiol signal is therefore suspected. The phenotype of GPER KO mice is clearly distinct from that of ER and mER KO mice. It does not show reproductive dysfunction but rather increased atherosclerosis, total and LDL cholesterol levels, and vascular inflammation with a tendency to obesity and diabetes (M. R. Meyer et al., 2014; Sharma et al., 2020). GPER is nevertheless expressed in the uterus. In the endometrium, its expression is predominantly epithelial and increases during the proliferative phase before decreasing drastically at the beginning of the secretory phase. In pregnancy, its expression appears to be increased, both in the epithelium and the stroma, and a role for GPER in oxytocin-induced myometrial contractility during labor is envisaged (Kolkova, Noskova, Ehinger, Hansson, & Casslén, 2010; Maiti et al., 2011). This expression profile of GPER suggests a hormonal input-dependent regulation of its expression that was demonstrated in an endometrial adenocarcinoma cell line (Ishikawa cells). In these cells, the presence of estradiol or an ERα agonist stimulated GPER expression while a decrease in its expression was observed in Ishikawa cells stably transfected with PR-A receptor (Plante et al., 2012).

The (potential) membrane progesterone receptors include the progesterone receptor membrane component 1 and 2 (PGRMC1-2) and the progestin and adipoQ receptors (PAQRs). PGRMC1, and PGRMC2 by analogy, is discussed in more detail in I.**C.**

The PAQRs family, also called the membrane progesterone receptors (mPRs), includes 5 members in humans, mPRα, mPRβ, mPRγ, mPRδ and mPRε, respectively encoded by the genes *PAQR7*,

PAQR8, *PAQR5*, *PAQR6* and *PAQR9*. The mPRs are seven transmembrane progesterone receptors localized at the plasma membrane capable of binding progesterone with high affinity (Kd ~4-8 nM). Although they are all expressed in a good number of organs, mPRα is predominantly expressed in reproductive tissues, mPRβ in neuronal tissues, mPRγ in kidney, colon and lung (Y. Zhu, Bond, & Thomas, 2003) and mPRδ and mPRε are expressed in different brain regions (Pang, Dong, & Thomas, 2013). In the endometrium specifically, mPRα, mPRγ and mPRε gene expression varies during the menstrual cycle, where mPRα is more expressed during the secretory phase whereas mPRγ and mPRε exhibit the highest expression during the proliferative phase. During preterm and term labor, the expression of mPRα expression is decreased, while mPRβ expression is only decreased during term labor (Fernandes et al., 2005; Valadez-Cosmes, Vázquez-Martínez, Cerbón, & Camacho-Arroyo, 2016). Although there is not yet an mPR KO mouse model, mechanistic studies in other models have identified some potential functions in reproduction. Thus, a defect in oocyte maturation and ovulation has been identified in zebrafish KO mPRs, mPRα promotes sperm hypermotility and mPRα and mPRβ are suspected to amplify PR-B activity in the myometrium to maintain it in a quiescent state during early pregnancy (Thomas, 2008).

iv. Estradiol and progesterone activities in the endometrium

Endometrial tissue remodeling occurs for only one purpose: to prepare for the potential implantation of a blastocyst. The endometrium is a complex and dynamic tissue whose composition and structure can be modulated spatially and temporally. This is finely orchestrated by estradiol and progesterone (Figure 9), and the slightest disturbance in expression or activity can lead to infertility or various endometrial pathologies such as endometriosis (Osteen, Yeaman, & Bruner-Tran, 2003).

1. Cell proliferation

In contrast with the early tissue repair mechanisms observed during the menstrual phase, endometrial regeneration during the proliferative phase is dependent on estradiol. Cell proliferation is, in fact, directly stimulated by estradiol and then limited by progesterone during the secretory phase. The anti-estrogenic activity of progesterone is caused by inhibiting the synthesis of ERs and by promoting the expression of the enzyme 17β-HSD which converts estradiol into estrone with less affinity for ERs.

2. Decidualization

Decidualization is the transformation of endometrial stromal cells into secretory decidual cells that participate in the microenvironment essential for blastocyst implantation. The decidualization process is initiated by progesterone in the secretory phase, before implantation, and continues in case of pregnancy. The decidual endometrial fibroblasts are large polygonal cells with a clear boundary and a central nucleus. They synthesize and secrete specific novel proteins that participate in implantation and/or maintenance of the embryo in the uterine lining (Brighton et al., 2017; Brosens, Hayashi, & White, 1999; Dunn, Kelly, & Critchley, 2003; Lockwood,

Krikun, Hausknecht, Papp, & Schatz, 1998; Lockwood et al., 2004; Schatz, Guzeloglu-Kayisli, Arlier, Kayisli, & Lockwood, 2016). Among them, prolactin (PRL) and tissue factor insulin-like growth factor-binding protein 1 (IGFBP1) represent the two most commonly used markers to assess endometrial stromal cell differentiation in culture. Several experiments performed in murine knock-out (KO), endometrial explants and primary endometrial stromal cultures have unequivocally demonstrated the role of progesterone in the decidualization process (Gellersen & Brosens, 2014). Nevertheless, differentiation of primary endometrial stromal cells in culture appears to be promoted by the convergence of the progesterone and cAMP signaling pathways. Activation of the cAMP pathway, independently of progesterone, would even be sufficient to increase prolactin and IGFBP1 expression to levels slightly lower than those observed in the presence of progesterone (Popovici, Kao, & Giudice, 2000; Samalecos et al., 2009; Telgmann, Maronde, Taskén, & Gellersen, 1997; Tierney, Tulac, Huang, & Giudice, 2003).

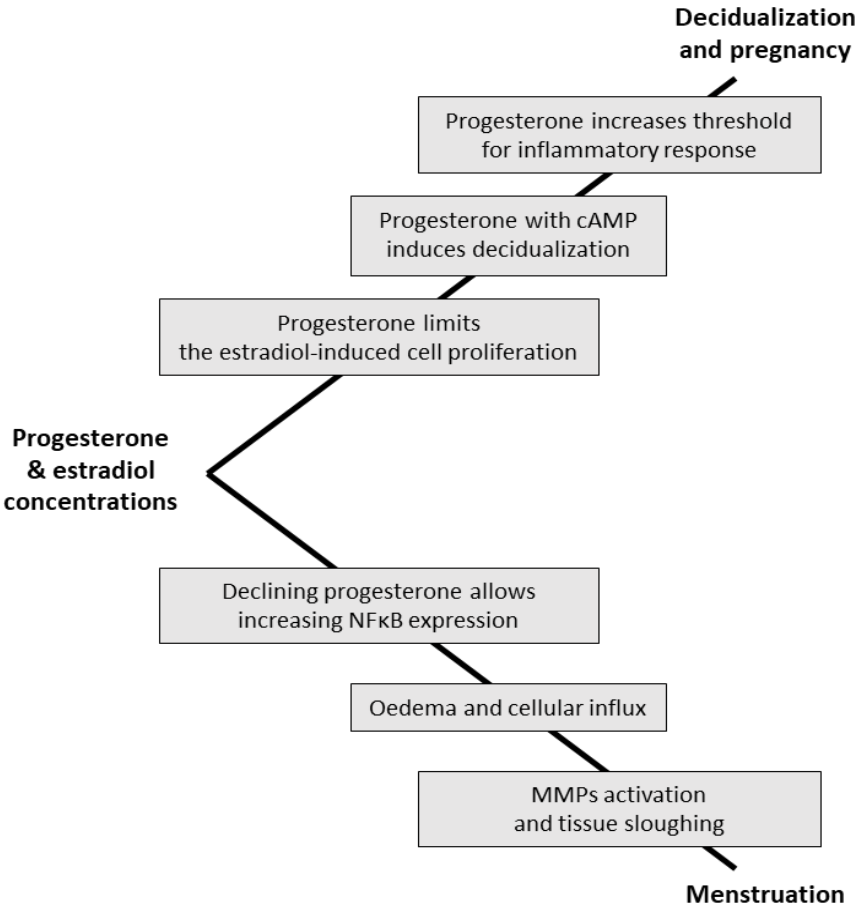

Figure 9: The physiology of the endometrium is defined by the blood levels of ovarian steroids.
Ovarian steroids, progesterone and estradiol, control cell proliferation, stromal cell differentiation and the inflammatory response of the endometrium in preparation for embryo implantation. Hormonal withdrawal induces increased expression and activity of the transcription factor NFκB, local secretion of cytokines and prostaglandins causing inflammation and tissue invasion by immune cells and destruction of the extracellular matrix by MMPs. *Adapted from (Critchley et al., 2020; Kelly, King, & Critchley, 2001).*

3. Inflammation

Progesterone also inhibits the NF-κB signaling pathway that regulates expression of genes involved in the inflammatory response (Kelly et al., 2001; King, Critchley, & Kelly, 2001). NF-κB is an inducible transcription factor that, in its inactive form, is present in the cytoplasm, coupled to inhibitory proteins such as NF-κB Inhibitor (IκB).

By increasing IκB or binding its recognition sites, progesterone prevents NF-κB activity. Withdrawal of progesterone therefore causes stimulation of the NF-κB pathway and consequently participates in menstruation through the production of endometrial cytokines and chemokines as well as the synthesis of the matrix metalloproteinases MMP-1, MMP-3 and MMP-9 (Chase, Bond, Crook, & Newby, 2002).

4. MMPs

MMPs represent a group of proteinases instrumental in local degradation of the extracellular matrix and, consequently, in endometrial remodeling (Dong, Dong, Campana, & Bischof, 2002; Gaide Chevronnay et al., 2012; Nagase, Visse, & Murphy, 2006). In the endometrium, the majority of MMPs are secreted by endothelial and stromal cells (fibroblasts or neutrophils) and their expression and activity are cyclically and locally regulated.

Most MMPs are expressed during the menstrual phase, when estradiol and progesterone concentrations are lowest. Furthermore, the increase in expression of successive MMPs upon withdrawal of estradiol and progesterone hormones has been demonstrated in experimental models ranging from simple to complex: human endometrial stromal cell and explant cultures, a mouse model of human endometrial xenograft, as well as ovariectomized macaques (Coudyzer et al., 2015; Marbaix et al., 1995; Slayden & Brenner, 2006;

Vassilev et al., 2005). Some of these studies show that the increase in MMPs expression and activity requires the lifting of the repressor effect of progesterone, either as a result of hormone concentration decrease or through addition of an antiprogestin such as mifepristone (Marbaix, Donnez, Courtoy, & Eeckhout, 1992; Marbaix et al., 1996; Spitz & Bardin, 1993).

Degradation of the extracellular matrix begins in the most superficial part of the endometrium and progressively extends to the entire functional layer without reaching the basal layer. By *in situ* hybridization (Figure 10), a study demonstrated that MMP-1 expression in the endometrium just before menstruation correlated with extracellular matrix degradation in the most superficial layer of the endometrial functional layer (Kokorine et al., 1996).

Several paracrine factors such as interleukin-1α (IL-1α), TGFβs, LEFTY2/EBAF, endothelin, and tumor necrosis factor α (TNFα), are suspected to be involved in the local regulation of MMPs expression (Henriet et al., 2012). Although the precise mechanisms have not yet been clearly identified for most of them, progesterone could indirectly and locally control MMPs by modulating the expression and/or secretion of paracrine factors by epithelial, stromal, and endothelial cells. As an example, IL-1α is an inducer of MMPs constitutively expressed by epithelial cells but progesterone prevents its secretion and consequently the paracrine stimulation of IL-1α synthesis by stromal cells (Pretto et al., 2008). IL-1α can also stimulate the production of endothelin by endometrial epithelial cells, another paracrine factor inducing MMP-1 and MMP-3 (Salamonsen, Marsh, & Findlay, 1999).

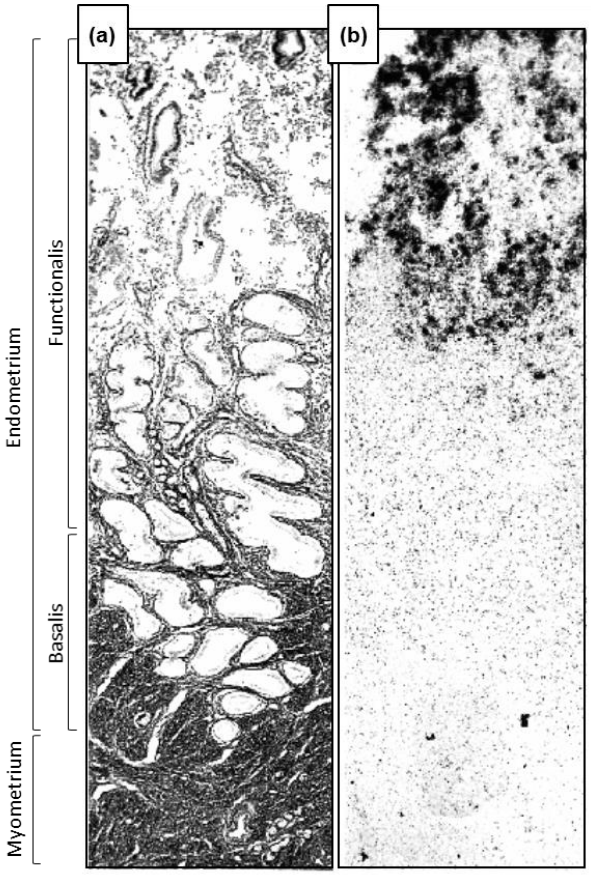

Figure 10: Correlation between the extracellular matrix degradation and focal induction of MMP-1 in the functionalis of the menstrual endometrium.
(a) The disappearance of the argyrophilic fibrillary network extends from the surface towards the basal layer that is preserved from degradation. **(b)** *In situ* hybridization of *MMP-1* mRNA reveals that *MMP-1* is expressed in the areas of the functional layer showing extracellular matrix breakdown. *Adapted from (Kokorine et al., 1996)*.

B. ENDOMETRIOSIS

i. General clinical and diagnostic features

1. Definition and classification

Endometriosis is a chronic inflammatory gynecological pathology which affects approximately 1 in 10 women of childbearing age worldwide. It is characterized by the localization of endometrial-like tissue outside the uterus. The eutopic endometrium is located in the uterus while the lesion constitutes the ectopic endometrium.

Endometriosis is categorized according to the location and histopathology of the endometriosis lesions (Figure 11). Thus, a distinction is made between superficial, deep and ovarian endometriosis. Deep infiltrating endometriosis lesions (DIE) are located in the muscular layer of the viscera, diaphragm and other organs whereas superficial, or peritoneal, endometriosis (SE) generally develops on the surface of the peritoneum. Ovarian endometriosis is characterized by the appearance of endometriotic cysts (EC) on the surface of the ovary(ies), called endometriomas (Nisolle & Donnez, 1997). Occasionally, endometriosis lesions have been identified in the thoracic cavity, in the brain and in men (Fukunaga, 2012; Ichida et al., 1993; Jabr & Mani, 2014; Kawaguchi et al., 2018; Pinkert, Catlow, & Straus, 1979; Sarma et al., 2004; Simsek et al., 2012; Thibodeau, Prioleau, Manuelidis, Merino, & Heafner, 1987).

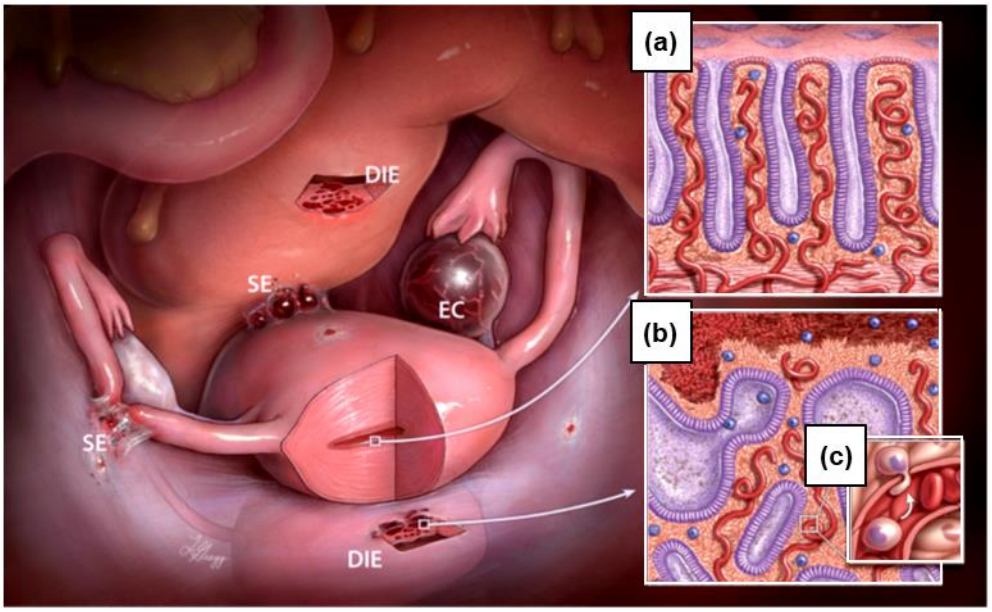

Figure 11: Different forms of endometriosis.
Deep Infiltrating Endometriosis (DIE), ovarian Endometriotic Cyst (EC), and Superficial Endometriosis (SE). Histology of eutopic endometrium **(a)** and endometriosis **(b)** is composed of both glandular epithelium and stroma. **(c)** Hypothesis for establishing deep endometriosis by transdifferentiation of a circulating stem cell. *Adapted from (Y. Wang, Nicholes, & Shih, 2020).*

2. Symptoms

The symptoms of endometriosis are multiple but the main ones are intense pelvic pain, notably caused by substantial inflammation, as well as reduced fertility in 30-50% of affected patients. They can also lead to significant psychological burden, which can result in a reduced quality of life.

In the most advanced stages of the disease, infertility can be explained by distortions and/or alterations of the uterine tubes and/or ovaries, thereby mechanically preventing the passage of gametes or embryos from/to the uterine cavity. In addition, the presence of ovarian lesions can slow down or disrupt ovulation. Indeed, innervation and vascularization may be compromised by a large

endometrioma; alternatively, inflammation induced by ectopic endometrial cells may prematurely stimulate folliculogenesis (Bulun et al., 2019).

Early stages of endometriosis are frequently detected in patients consulting for subfertility (Brichant, Audebert, & Nisolle, 2016). The impact of these lesions on fertility is still debated. The biological activity as well as the inflammation caused by endometriosis lesions could be harmful to sperm or egg. On the other hand, some authors suggest that the eutopic endometrium of patients suffering from endometriosis present biochemical and histological disparities with the non-endometriotic endometrium which could disturb implantation and be the cause of their infertility. These divergent factors include progesterone resistance, increased immune infiltrate, pro-inflammatory cytokines and chemokines but also decreased expression of genes such as $\alpha v\beta 3$ integrin, glycodelin, HOXA10 and HOXA11 (Cirkel et al., 1993; Garcia et al., 1992; Klein, Pérgola, Rao-Tekmal, Dey, & Schenken, 1993). Nevertheless, the actual impact of each of these factors on infertility in endometriosis is not yet clearly defined.

On the other hand, patients with endometriosis frequently have other gynecological pathologies such as adenomyosis and leiomyoma. In addition, ovarian endometriosis appears to be a risk factor for the development of ovarian cancer.

Adenomyosis is defined by the presence and development of endometrial tissue in the myometrium (Struble, Reid, & Bedaiwy, 2016). In many respects, adenomyosis shows similarities with endometriosis, particularly deep endometriosis. In addition to common histological features, adenomyosis and endometriosis seem to share symptoms, etiological hypotheses, diagnostic methods, therapeutic molecules, ... (Stratopoulou, Camboni, Donnez, &

Dolmans, 2021) so much so that some authors have suggested that they are simply different manifestations of a single disease (J. Donnez, Dolmans, & Fellah, 2019).

3. Diagnosis

Despite the high prevalence of the disease, the diagnosis of endometriosis is still long (4-11 years) and difficult. Indeed, in the absence of a specific indicator and biomarker, endometriosis is still very likely to be under-diagnosed. It is estimated that 65% of women are initially misdiagnosed (Greene, Stratton, Cleary, Ballweg, & Sinaii, 2009).

Diagnosis is based on a combination of parameters such as symptomatology, history and patient report, gynecological examination (palpation of uterosacral ligaments, nodules or adnexal mass), transvaginal ultrasound and pelvic magnetic resonance imaging (MRI) (Taylor, Kotlyar, & Flores, 2021). Despite this, not all endometriosis lesions can be detected, especially the smaller ones. Therefore, the identification of sufficiently sensitive and specific biomarker(s) of endometriosis remains a priority. As such, two combinations of circulating miRNAs (miR-let-7b/miR-let-7d/miR-let-7f/miR-3613/miR-451/miR-125b-5p and miR-125b-5p/miR-150-5p/miR-61/miR-342-3p/miR-451a/miR-3613-5p/miR-let-7b) have recently been considered as a potential candidate to specifically detect endometriosis in patients (Cosar et al., 2016; Moustafa et al., 2020).

ii. Origin of endometriosis

Mainly due to a lack of relevant experimental models, the origin of endometriosis is still unclear. Two major hypotheses of pathogenesis are nevertheless favored: retrograde menstruation and stem cells. In addition, other theories such as hematogenous or lymphatic dispersion, coelomic metaplasia and induction of Mullerian remnants have also been suggested. However, none of these theories alone can explain all diagnosed cases of endometriosis, suggesting that superficial, deep and ovarian endometriosis may develop by different mechanisms.

1. Retrograde menstruation

Retrograde menstruation occurs when endometrial fragments migrate through the fallopian tubes instead of being evacuated through the vagina during menstruation.

According to Sampson and his team, endometriosis lesions originate from retrograde menstruation that implants and persists ectopically (Sampson, 1927). This hypothesis is also supported by the fact that women with obstructed natural ducts have a higher risk of developing endometriosis ((Barbieri, 1998; Sanfilippo, Wakim, Schikler, & Yussman, 1986). Nevertheless, retrograde menstruation is frequent but only 10% of women develop endometriosis (Alderman, Yoder, & Taylor, 2017; D'Hooghe & Debrock, 2002). Moreover, it is only sufficient to explain the development of superficial and ovarian endometriosis.

2. Stem cells

From endometrial or extrauterine origin, stem cells represent an interesting perspective in the pathogenesis of endometriosis in order to explain the development of deep endometriosis lesions or those located outside the abdominal cavity.

In the endometrium, a population of stem cells is physiologically present to ensure tissue repair during the menstrual phase. This same population is suspected to passively enter the angio-lymphatic space of the endometrium during menstruation and subsequently be transported by the circulatory system towards an environment conducive to lesion development. Specific markers of endometrial stem cells have already been identified such as stage specific embryonic antigen-1 (SSEA-1) and N-cadherin for epithelial cells or platelet-derived growth factor receptor-β (PDGFR-β) for stromal cells (Cousins et al., 2021; Gargett, Schwab, & Deane, 2016; Nguyen et al., 2017; Valentijn et al., 2013). Yet, to date no study has been able to prove the role of endometrial stem cells in the pathogenesis of endometriosis.

On the other hand, and although their involvement remains modest, tissue regeneration of the endometrium may also involve bone marrow-derived stem cells (BMDSCs) including mesenchymal, endothelial and hematopoietic stem cells (Becker et al., 2011). During the menstrual and proliferative phase, the number of circulating BMDSCs increases. Therefore, some authors suggest that BMDSCs can stray and implant elsewhere than in the endometrium to initiate the development of endometriosis lesions. Subsequently, the ectopic endometrium would compete with the eutopic by secreting BMDSCs recruitment factors to promote its development (Moridi, Mamillapalli, Cosar, Ersoy, & Taylor, 2017).

To date, these recruitment factors have not been clearly identified but the ligand CXCL12 and its receptor CXCR4 seem to be involved. Their expression may be modulated by estradiol and progesterone and more stimulated in endometriotic epithelial cells than in the eutopic endometrium of patients without endometriosis (Leconte et al., 2014; Moridi et al., 2017).

iii. Lesion development

Several genetic and epigenetic, immunological, biochemical or endocrine factors are likely to influence lesion development after implantation (Figure 12). The next sections elaborate on the most relevant ones.

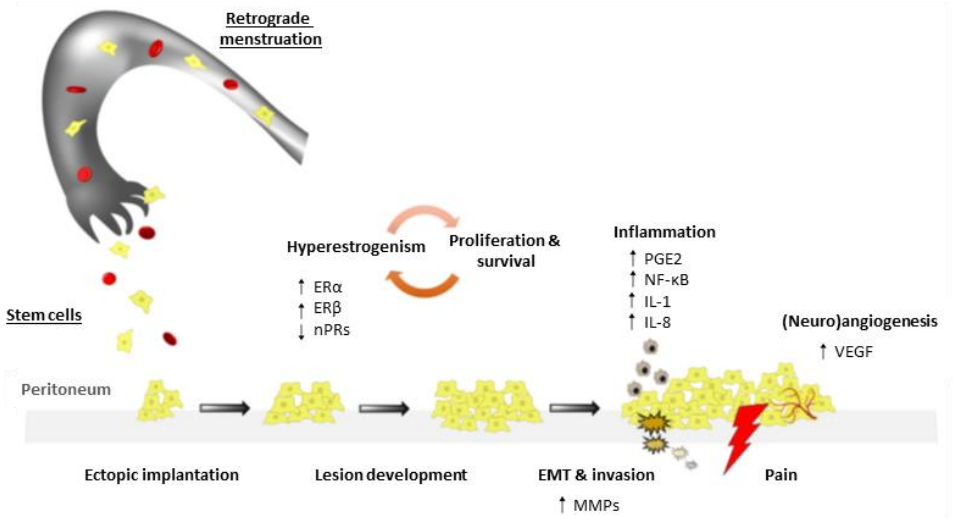

Figure 12: Summary of the mechanisms involved in endometriosis initiation and progression.
Implantation of ectopic endometrial cells (peritoneum) is initiated by retrograde menstruation or by circulating stem cells. The constant hyperestrogenism observed in endometriosis, followed by enhanced cell proliferation and survival, leads to lesion development and progression. At the same time, accumulating immune cells release a number of inflammatory mediators, that promote the invasion and angiogenesis/neuroangiogenesis leading to lesion growth and the generation of pain symptoms in endometriosis patients. *Adapted from (Kapoor, Stratopoulou, & Dolmans, 2021).*

1. Genetic and epigenetic factors

A recent study reports that 83% of deep endometriosis lesions contain somatic mutations and 26% of them are referenced as potentially leading to cancer development (Anglesio et al., 2015; Anglesio et al., 2017; Suda et al., 2018). For example, somatic mutations in *KRAS*, *PIK3CA*, *ARID1A*, and *PPP2R1A* genes have been identified and are also frequently observed in ovarian cancers or endometrial carcinomas. Although the simultaneous presence of several of these somatic mutations is required for cancer development, it has already been shown that some of them, such as *KRAS* and *ARID1A*, could independently contribute to the development of endometriosis lesions by limiting their progesterone response capacity, promoting their survival and/or invasion potential (C. W. Cheng et al., 2011; Wiegand et al., 2010; Yoo et al., 2017).

Epigenetic and microRNA expression profiles may also differ between eutopic and ectopic endometrium (Borghese et al., 2010; Dyson et al., 2014; L. Wang, Zhao, Li, Wang, & Kang, 2019).

Although the consequences for lesion development have not always been investigated, the differentially methylated genes identified are associated with immune surveillance, inflammatory response, cell adhesion, apoptosis control, ovarian steroid response, and/or MAPKs activation. For example, the promoters of genes suspected to be involved in the pathogenesis of endometriosis such as *ESR1-2*, *PGR*, *NR5A1*, *CYP19A1* and several *HOX* and *GATA* family genes, appear to be affected (Izawa, Taniguchi, Terakawa, & Harada, 2013; Y. Wu et al., 2005; Xue, Lin, Yin, et al., 2007).

MicroRNAs (miRNAs) are small (19-25 nucleotides) non-coding RNAs capable of repressing gene expression by degrading mRNA or interfering with their translation. It is envisaged that their transport, via microvesicles or exosomes, in blood or peritoneal fluid may

participate in the initiation and/or progression of endometriosis lesions (Y. Wang et al., 2020). Furthermore, decreased expression of miRNA-200b induces increased expression of Zinc finger E-box-binding homeobox (ZEB)-1 and -2 in endometriosis lesions, two transcription factors involved in invasion and proliferation of ectopic endometrial cells (Eggers et al., 2016).

2. Immunological factors

Local inflammation as well as immune imbalance are two primary factors for the development of endometriosis, which requires a perfect balance between pro-inflammatory and immune tolerance mechanisms.

It is suspected that the inflammation is caused by the cyclic tissue degradation induced by the fall in estradiol and progesterone hormone concentrations. Indeed, like the eutopic endometrium, the ectopic endometrium may respond to variations in blood concentrations of ovarian steroids. Without the possibility of exiting, blood and tissue fragments accumulate in the endometriosis lesions. The resulting increase in the production of reactive oxygen species (ROS) leads to cell death and inflammation.

It is therefore not surprising that endometriosis lesions represent a privileged inflammatory focus compared to the eutopic endometrium. Inflammatory mediators such as cyclooxygenase-2 (COX-2), IL-1β, IL-8, TNF-α and prostaglandin E2 (PGE2) appear increased. Different types of immune cells may also be recruited such as B cells, macrophages, dendritic cells, NK cells and regulatory T cells (Kapoor et al., 2021). Compared to the endometrium of patients without endometriosis, the eutopic endometrium has an elevated level of COX-2 expression and an infiltration of immune cells, particularly macrophages (Y. Wang et al., 2020).

NF-κB is constitutively activated in peritoneal lesions and, in mice and rats, activation of the NF-κB pathway stimulates the development and growth of endometriosis lesions (Azuma et al., 2017; Celik et al., 2016; González-Ramos et al., 2007; Lousse et al., 2008). As a modulator of immunity, the transcription factor NF-κB appears to play a crucial role in promoting the secretion of pro-inflammatory cytokines and growth factors (Malvezzi, Hernandes, Piccinato, & Podgaec, 2019). It can also stimulate the production of angiogenic factors (IL-8, macrophage migration inhibitory factor (MIF) and vascular endothelial growth factor (VEGF)), cell proliferation and tissue invasion, including through MMPs (González-Ramos et al., 2010; Kapoor et al., 2021).

3. Biochemical factors: Oxidative stress

Oxidative stress is a factor potentially involved in the pathophysiology of endometriosis and arises when there is an imbalance between the presence of ROS and antioxidants (J. Donnez, Binda, Donnez, & Dolmans, 2016; Van Langendonckt, Casanas-Roux, & Donnez, 2002). In excess, ROS alter several cellular functions by modulating protein activity and gene expression that can affect female fertility (Ruder, Hartman, Blumberg, & Goldman, 2008). For example, they play a critical role in the regulation of NF-κB (González-Ramos et al., 2010).

4. Endocrine factors: hormonal imbalance

Endometriosis is characterized by a profound imbalance between progesterone and estrogenic activity (Bulun, 2009). Indeed, particularly in lesions, progesterone activity appears to be reduced in favor of estrogenic activity.

The increase in estrogenic activity is related both to an increase in local estradiol concentration and to a disruption of its response pathways.

The sources of estradiol in endometriosis lesions are multiple. Through the bloodstream, estradiol levels may be of ovarian origin or from surrounding tissues that convert circulating androstenedione to estrone through the enzyme aromatase. In synergy with inflammation, endometriosis lesions themselves are able to convert cholesterol to estradiol (Attar et al., 2009; Bulun et al., 2005). Indeed, increased levels of PGE2 in endometriosis lesions stimulate the expression of enzymes involved in steroidogenesis via the transcription factor steroidogenic factor 1 (SF1).

In contrast to patients without endometriosis, the expression of the estradiol receptors is perturbed:

- ERα expression is decreased in stromal cells from ovarian endometriomas (Xue, Lin, Cheng, et al., 2007).
- ERβ is increased in endometriosis, in lesions as well as in the eutopic endometrium and particularly in stromal cells (Bulun et al., 2012; Bulun et al., 2019; Yilmaz & Bulun, 2019).
- GPER is more expressed in eutopic endometrium in proliferative phase and even more in the ectopic endometrium during the secretory phase (Plante et al., 2012; Yuguchi et al., 2013).

Epigenetic changes observed in *ESR1* and *ESR2* genes may explain the decreased expression of ERα and increased expression of ERβ, respectively (Monsivais et al., 2014; Xue, Lin, Cheng, et al., 2007). Furthermore, the repressive activity of ERβ toward ERα expression further accentuates this difference in expression. It is suggested that estradiol binding to ERβ stimulates the expression and/or activity of the Ras-like proteins estrogen-regulated growth inhibitor (RERG) and serum and glucocorticoid-regulated kinase 1 (SGK1), which are involved in endometriotic cell proliferation and survival, respectively (Monsivais et al., 2014; Monsivais et al., 2016). ERβ can also limit apoptosis through its interaction with the cytoplasmic apoptotic machinery as well as increase IL-1β production and consequently enhance cell adhesion and proliferation (Han et al., 2015). More recently, ERβ has also been shown to contribute to cellular evasion of immune surveillance by promoting CD47 expression in endometrial stromal cells (Hu, Zhang, Lu, Fu, & Hu, 2022). In addition, estradiol via ERβ rapidly induces COX-2 expression in uterine endothelial cells and, consequently, promotes macrophage infiltration and inflammation (Yilmaz & Bulun, 2019).

Although endometriotic tissues contain significant levels of progesterone, they show little evidence of response to this hormone, hence the concept of "progesterone resistance". A decrease in the production of retinoic acid (RA) is notably observed in progesterone-resistant endometriotic stromal cells. RA is a physiological paracrine factor capable of stimulating the expression of the 17β-HSD enzyme in epithelial cells and thus the conversion of estradiol to estrone (Y. H. Cheng, Imir, Fenkci, Yilmaz, & Bulun, 2007; Y. H. Cheng et al., 2008; Zeitoun et al., 1998). In other words, the loss of progesterone response may also participate in the estrogenic overstimulation of endometriotic cells.

Currently, the mechanisms responsible for progesterone resistance are only poorly deciphered. A decrease in nPRs is often observed (Attia et al., 2000; Jacques Donnez & Dolmans, 2021). Moreover, PR-B is sometimes even undetectable. Similar to the regulation of ERs expression, this decrease in nPRs expression originates from epigenetic modifications such as DNA methylation, histone modifications and miRNA expression (Patel, Rudnicki, Yu, Shu, & Taylor, 2017; Rocha-Junior et al., 2019; Y. Wu, Starzinski-Powitz, & Guo, 2008; Y. Wu, Strawn, Basir, Halverson, & Guo, 2006; Zubrzycka, Zubrzycki, Perdas, & Zubrzycka, 2020). Thus, the expression of nPRs isoforms is unbalanced. Nevertheless, the decrease in nPRs expression is not systematically observed in endometriosis lesions, and not significantly in the eutopic endometrium. Therefore, it cannot explain resistance to progesterone on its own (MacLean & Hayashi, 2022). On the other hand, the expression of the chaperone protein FKBP52, which is necessary for the functions of nPRs, also appears to be decreased in the endometrium of a baboon model of endometriosis and suggests the existence of alternative mechanisms (Jackson et al., 2007).

iv. Treatments: the problem of progesterone resistance

Treatments of endometriosis can be divided into two categories: surgical or drug-based. The choice of treatment will depend on the stage of development of the disease, the type of lesion, but also on the patient herself and in particular her possible desire for pregnancy. Nevertheless, none of them is able to cure the disease. On the one hand, some endometriosis lesions, which are too small to be detected during surgery, remain and create new endometriosis sites. On the other hand, current therapeutic molecules can only limit the development of lesions and the resulting pain, but can also, in many cases, be the cause of significant side effects (for example: risk of venous or arterial embolism, untimely menstrual bleeding, mood changes, weight gain, etc.). Therefore, the development of new therapeutic molecules is eagerly needed today.

The main objective of hormonal treatments is to prevent the menstrual cycle, in particular menstruation, and the inflammation that causes pelvic pain and the development of endometriosis lesions. This category of treatment includes GnRH agonists or antagonists, synthetic progestin compounds, selective progesterone receptor modulators (SPRMs) and selective estrogen receptor modulators (SERMs) (Vannuccini, Clemenza, Rossi, & Petraglia, 2021).

In addition, non-steroidal anti-inflammatory drugs (NSAIDs) are frequently prescribed. By inhibiting COX enzyme functions and reducing prostaglandin concentrations, they limit inflammation, endometriosis lesion growth, and associated pain (National Guideline, 2017).

1. GnRH agonists and antagonists

GnRH agonists initially stimulate gonadotropin secretion. Long-term exposure then induces repression of GnRH receptors and, as a

result, a decrease in FSH and LH levels. This results in a decrease in ovarian estradiol blood levels which influences the development of endometriosis lesions but also the eutopic endometrium.

GnRH antagonists (Elagolix, Relugolix and Linzagolix) represent a class of molecules that are promising. They act by competing with GnRH and directly prevent its binding to the GnRH receptor. Currently, studies show a rapid, dose-dependent and reversible decrease in estradiol.

2. Progestins and SPRMs/SERMs

Presently, there is a range of progestogen compounds among which it is necessary to select the molecule offering the best cost/benefit ratio for each patient. medroxyprogesterone acetate (MPA), levonorgestrel, desogestrel, nomegestrol, norethisterone acetate and dienogest were proven effective (Buggio, Somigliana, Barbara, Frattaruolo, & Vercellini, 2017; Troìa, 2022; Vannuccini et al., 2021). Alone or in combination with estrogenic compounds, low doses of these oral contraceptives are usually sufficient to impair lesion progression and treat endometriosis-related pain by limiting inflammation and promoting apoptosis. However, more than one-third of patients do not respond to progestin treatments (Bulun et al., 2019; Jensen, Schlaff, & Gordon, 2018), most often as a result of progesterone resistance.

SPRMs and SERMs represent a new class of therapeutic molecules capable of producing agonist or antagonist effects on the progesterone and estradiol receptors, respectively. These molecules are still under study, but some authors recommend caution about their efficiency against endometriosis lesions and observe adverse effects of SERMs on the eutopic endometrium (J. Donnez & Dolmans, 2016, 2020a, 2020b).

C. **PGRMC1**

i. Background and general characteristics

The Progesterone Receptor Membrane Component 1 (PGRMC1) protein was independently discovered in 1996 by two research groups. In rat liver, Selmin et al. identified a gene encoding a predicted 25kDa protein and overexpressed during dioxin treatment. Accordingly, PGRMC1 was named 25-Dx (Selmin et al., 1996). In the same year, Meyer et al. isolated, from pig liver microsomal membranes, a protein capable of binding ^{3}H-P4 (C. Meyer, Schmid, Scriba, & Wehling, 1996). This protein was later named membrane Progesterone Receptor or mPR by the same research team (Falkenstein et al., 2001).

Depending on the biological context, different names have been assigned to PGRMC1 orthologs in vertebrates (Cahill, 2007): mPR (membrane Progesterone Receptor), 25-Dx (25kDa-Dioxin), Hpr6.6 (Heme progesterone receptor 6.6), IZA (Inner zone antigen), ... It is only in 2006 that the name Progesterone Receptor Membrane Component 1, with acronym PGRMC1, were attributed (Peluso, Pappalardo, Losel, & Wehling, 2006) and became predominantly used.

Orthologs of PGRMC1 were also identified in less evolved eukaryotic organisms, thereby highlighting that its structure was highly conserved during evolution. In the yeast *Saccharomyces cerevisiae*, the PGRMC1 ortholog was first cloned in 2003 as Dap1p (damage associated-response protein 1) (Hand, Jia, Bard, & Craven, 2003). In the plant *Arabidopsis Thaliana*, the small synthetic molecule AG-205 was found to bind the protein AT2G24940 sharing structural features with PGRMC1 (Yoshitani et al., 2005). This compound was extensively used in a part of my experimental work and is further characterized in **III.C.***ii*.

1. Family

A related protein, PGRMC2, was identified in human liver in 1998 by sequence similarity with *PGRMC1* (Gerdes, Wehling, Leube, & Falkenstein, 1998).

PGRMC1 and PGRMC2 were used as founding members of the membrane associated progesterone receptors (MAPRs) family, a subfamily of the cytochrome-b5 family. MAPR proteins are characterized by the presence of a cytochrome-b5-like heme/steroid binding domain on which their bioactivity is claimed to depend (Kimura et al., 2012). MAPR proteins have an extremely well-conserved amino acid sequence in vertebrates, from zebrafish to humans. Four MAPR proteins are found in mammals: two transmembrane proteins, PGRMC1 and PGRMC2, and two secreted proteins, Neudesin and Neuferricin.

2. Expression

PGRMC1 protein is produced from the *PGRMC1* gene located on the X chromosome (Xq24).

PGRMC1 is expressed in most human organs but mainly in liver, kidney, cerebellum, testis, seminal vesicle, skin and endometrium (The human protein atlas, The human protein atlas, 2022). It also appears to be overexpressed in several cancers and/or cancer cell lines of gynecological (breast, ovarian and endometrial, cervical) or non-gynecological (colon, thyroid, lung, ...) origin (Crudden, Loesel, & Craven, 2005; Peluso & Pru, 2021).

The subcellular localization of PGRMC1 appears to be variable. The experimental model and conditions used seem to induce structural and/or post-translational modifications of PGRMC1 influencing its translocation (Sabbir, 2019). However, the protein is most frequently identified in mitochondria, the plasma membrane, and the

endoplasmic reticulum, particularly in the perinuclear region (Cahill, 2007; W. Wu, Shi, Huang, Balducci, & Garfield, 2010; L. Zhang et al., 2008). Although the PGRMC1 sequence does not contain a nuclear localization signal, PGRMC1 is also observed in the nucleus. Additional analyses have, in fact, demonstrated that some phosphorylated and/or sumoylated forms of PGRMC1 are preferentially localized in the nucleus (Beausoleil et al., 2004; Peluso, Lodde, & Liu, 2012; Sabbir, 2019; Sauer et al., 2005).

The physiological mechanisms of regulation of PGRMC1 expression are not yet clearly defined. Regulation of its expression by progesterone was suspected but studies aimed at further elucidating PGRMC1 regulation by progesterone generated contradictory results. However, most authors agree that progesterone represses *PGRMC1* expression (Peluso & Pru, 2021) and suggest that in the absence of progesterone receptor response element (PRE) in the *PGRMC1* promoter, nPR mediates the repressive activity of progesterone via the glucocorticoid response element (GRE) (Lösel, Besong, Peluso, & Wehling, 2008). Yet, this remains to be demonstrated.

3. Structure

The major PGRMC1 isoform is composed of 195 amino acids and its structure is different from those of the progesterone receptors nPR and mPR.

PGRMC1 comprises an N-terminal extracellular domain, a transmembrane domain, and a C-terminal cytoplasmic domain (Figure 13). As a member of the MAPR protein family, PGRMC1 has a cytochrome b5-like domain (cytb5) capable of binding heme. PGRMC1 also contains some predicted peptide sequences: a Src homology 3 (SH3)-binding sequence at P63, and two Src homology 2 (SH2)-binding sequences at positions Y139 and Y180. Upon phosphorylation of the tyrosine residues in the SH2-binding domains, the SH3 and SH2 target

sequences could allow PGRMC1 to interact with any other protein containing SH3 or SH2 domains respectively.

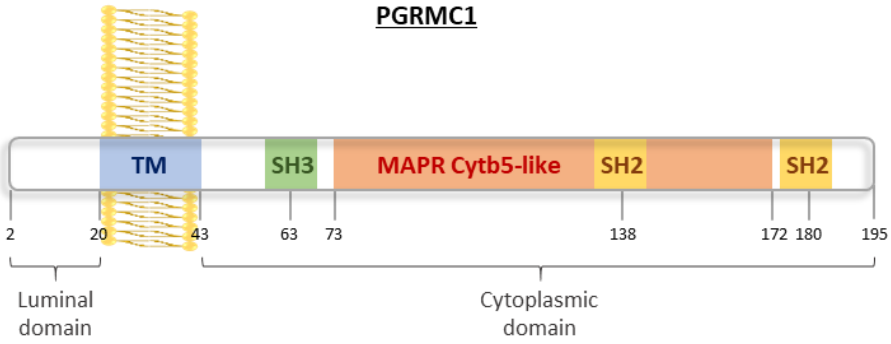

Figure 13: Representation of the amino acid sequence of PGRMC1.
The structure of PGRMC1 protein comprises an N-terminal extracellular domain, a transmembrane domain, and a C-terminal cytoplasmic domain. The cytoplasmic domain contains a cytochrome b5-like domain (MAPR Cytb5-like), two SH2-binding domains and one SH3-binding domain.

To date, the identification of the three-dimensional structure of PGRMC1 relies exclusively on crystallographic and nuclear magnetic resonance spectroscopy (NMR) analysis of its cytoplasmic domain (Kabe et al., 2016; Kaluka, Batabyal, Chiang, Poulos, & Yeh, 2015). Both studies demonstrated that PGRMC1 can form stable homo/hetero dimers/oligomers in the presence of heme (Figure 14). Indeed, the dimerization process was shown to be mediated by the binding of two heme molecules, with each PGRMC1 monomer binding a heme molecule via interaction of its tyrosinate ion at position 113 (Figure 14b; Y113) with the heme iron atom. The crystallographic structure of the heme-bound cytosolic domain of PGRMC1 (amino acids 72-195) also suggests interactions between heme and two tyrosine residues (Y107 and Y164) and one lysine (K163) in PGRMC1 (Kabe et al., 2016). The influence of the luminal and transmembrane domains of PGRMC1 on these interactions, however, has never been investigated so far.

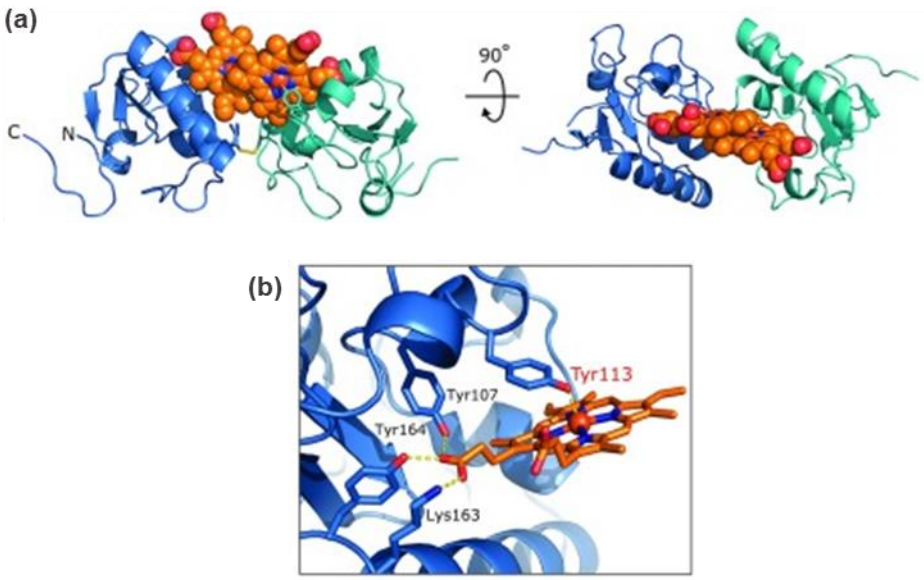

Figure 14: Three-dimensional conformation structure of dimerized PGRMC1 protein with heme molecules.
(a) Heme-stacking dimerization of PGRMC1. Two PGRMC1 subunits (blue and green ribbons) dimerize via stacking of the heme molecules (in orange and red). **(b)** Tyr-coordination of heme on PGRMC1. X-ray crystallographic analysis (PDB accession code: 4X8Y) revealed that heme forms a 5-coordinated bond with tyrosine residue 113 of PGRMC1. *Adapted from (Kabe, Handa, & Suematsu, 2018)*.

4. Post-translational modifications

The predicted molecular weight of PGRMC1 is 21.671kDa. However, the experimental value is slightly higher, around 25kDa, and some authors even identify by western blotting other bands with various higher molecular weights. Some forms can be explained by hetero/homo-dimerization/oligomerization of PGRMC1. Others are the result of post-translational modifications (Cahill, Jazayeri, Kovacevic, & Richardson, 2016; Peluso et al., 2012; Sabbir, 2019). In the human embryonic kidney-derived cell line (HEK293), forms of PGRMC1 with molecular weight shifts resulting from post-translational modifications are the most represented (Sabbir, 2019).

The amino acid sequence of PGRMC1 indeed presents several potential phosphorylation, sumoylation, acetylation and ubiquitination sites (Figure 15) that could modulate the structure, localization and/or molecular interactions of PGRMC1 and, consequently, its activity (Cahill & Medlock, 2017). However, their frequency as well as their influence on PGRMC1 functions are unknown or too poorly described, and mostly remain hypothetical.

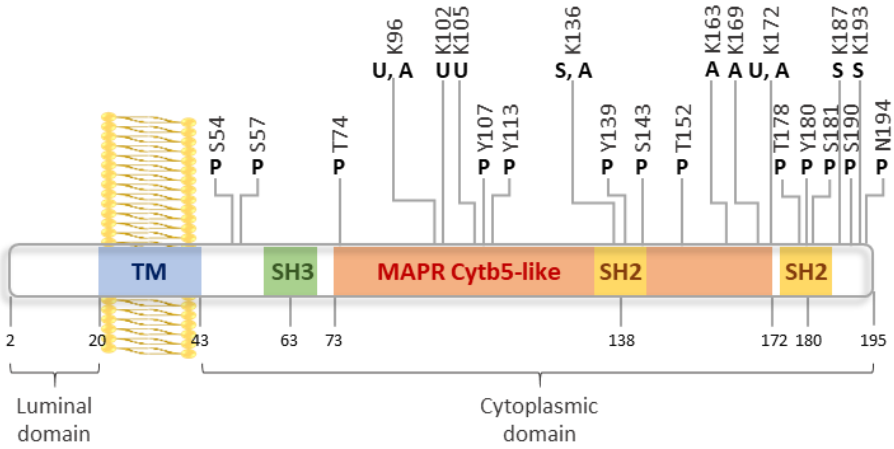

Figure 15: PGRMC1 post-translational modification sites.
PGRMC1 protein present several predictive post-translational modification sites that can modulate its expression and/or functions: 13 phosphorylation sites (P), 5 acetylation sites, 4 ubiquitinylation sites (U) and 3 sumoylation sites (S).

According to the PhosphoSite database, the phosphorylation sites most commonly detected *in vivo* are S57, Y113, Y180 and S181 (PhosphoSitePlus, 2022). Particularly well conserved among the four members of the MAPR family, the functional consequences of their phosphorylation can be relevant and multiple. Indeed, in agreement with Sabbir's observation in HEK293 cells, the phosphorylation of the Y113 site would notably prevent heme binding and, consequently, the functions that require the PGRMC1 dimerization. Moreover, two studies have recently demonstrated that the double mutation of the S57 and S181 phosphorylation sites and the triple mutation (with additional mutation of the Y180 phosphorylation site) affects several metabolic and/or genomic functions of the MIA PaCa-2 pancreatic cancer cell line, and of embryonic stem cells . Indeed, MIA PaCa-2 cells expressing the double mutant show morphological changes involving altered actin cytoskeletal organization, an increase in their migratory capacity and, conversely, a decrease in their invasion potential. In

addition, a broad-spectrum proteomic analysis of these cells identified a plethora of under-expressed proteins, compared to the control (non-mutated) condition, involved in glucose metabolism, morphology and mitochondrial functions (Thejer, Adhikary, Kaur, et al., 2020). In embryonic stem cells expressing the triple mutant, the same team also detected genomic instability, CpG hypermethylation, and disruption of the expression of proteins related to cell cycle control and DNA replication pathways (Thejer, Adhikary, Teakel, et al., 2020).

The S57 and S181 phosphorylation sites have been predicted to be constitutively phosphorylated by Casein Kinase 2 (CK2), a constitutively active Ser/Thr protein kinase (Neubauer et al., 2008). The resulting steric hindrance could inhibit protein interactions with the SH3-binding domain and Y180 site of PGRMC1, respectively (Cahill, Jazayeri, Kovacevic, et al., 2016).

To date, two sumoylated forms of PGRMC1, at 70kDa and 100kDa, have been identified (Peluso et al., 2012; Sabbir, 2019). Sumoylation prevents proteasomal degradation of a protein through competition with ubiquitination of a common lysine residue (Escobar-Ramirez et al., 2015). It can also correlate with decreased transcriptional activity, most commonly through modulation of chromatin structure (Wotton, Pemberton, & Merrill-Schools, 2017). In HEK293, the decrease in sumoylated forms of PGRMC1 correlated with an increase in ubiquitinylated forms as well as degradation of PGRMC1 by the proteasome (Sabbir, 2019). In SIGCs (spontaneously immortalized granulosa cells), transfection of a PGRMC1 protein in which all 3 sumoylation sites had been mutated modulated transcriptional activity of Tcf/Lef, transcription factors that ultimately mediate the transcriptional response to Wnt signaling (Peluso et al., 2012). Both of these studies also evidenced a progesterone-mediated decrease in PGRMC1 sumoylation and in depending functions.

ii. Interactions and functions of PGRMC1

PGRMC1 is referred to as a hub protein to highlight the large number of interaction partners to which this protein is assigned. As a result, the functions of PGRMC1 appear to be multiple and varied and most of them are of interest in cancer and/or in the female reproductive system. This section describes the interaction mechanisms of the main partners of PGRMC1 and their functional consequences.

1. PGRMC1 as a mediator of progesterone activity

PGRMC1 was originally considered a progesterone receptor, implying that it binds progesterone and that its functions are progesterone-related. Yet, its direct interaction with progesterone has never been indisputably confirmed (Cahill, Jazayeri, Catalano, et al., 2016). Furthermore, it is now clear that PGRMC1 functions can also be independent of progesterone.

The ability of PGRMC1 to mediate a progesterone response does not require direct binding to the hormone and has been demonstrated in several cellular models. Peluso et al. repeatedly identified in ovarian cells an anti-apoptotic activity of PGRMC1 requiring the presence of progesterone (Peluso, Gawkowska, Liu, Shioda, & Pru, 2009; Peluso et al., 2006; Peluso, Romak, & Liu, 2008; Peluso, Yuan, Liu, & Lodde, 2013). In the context of breast cancer, Hans Neubauer's group notably demonstrated that PGRMC1 overexpression promotes proliferation of two breast cancer cell lines induced by the progestins MPA, norethisterone and dydrogesterone (Bai et al., 2021; Zhou et al., 2013). In the endometrium, Salsano et al. proposed a role for PGRMC1 in the decidualization process of stromal cells (Salsano et al., 2020; Salsano et al., 2017).

However, further investigations are still needed to identify how PGRMC1 functions are influenced by progesterone. Nevertheless, three potential and nonexclusive scenarios have been suggested:

(i) Progesterone could indirectly modify some post-translational modifications of PGRMC1 and, consequently, influence its protein conformation, interactions, activity and functions (Bai et al., 2021; Peluso et al., 2012; Sabbir, 2019).

(ii) Conversely, some post-translational modifications of PGRMC1 could be necessary to interact directly with progesterone and other synthetic derivatives and transmit a progestogen signal. For example, prior phosphorylation of PGRMC1 by CK2 appeared to be required for induction of cell proliferation by the progestins MPA, norethisterone and dienogest in two breast cancer lines, MCF7 and T47D (Schneck et al., 2013; Willibald et al., 2017).

(iii) Finally, since it was demonstrated that PGRMC1 interacts with PGRMC2 and/or mPRα, some authors suggest that PGRMC1 could regulate the cellular response capacity to progesterone by modulating the bioavailability of its receptors at the plasma membrane (Cahill, Jazayeri, Catalano, et al., 2016; Sueldo, Liu, & Peluso, 2015; Thomas, Pang, & Dong, 2014)

2. Heme: its role in dimerization and the functions of PGRMC1

By homology with other related proteins, in particular cytochrome b5 proteins, some authors suggest and demonstrate that PGRMC1 can interact with heme (Min et al., 2005; Min et al., 2004; Oda, Nakajima, Toyoda, Fukami, & Yokoi, 2011).

Characterization of this binding was first performed in yeast and the proportion of heme-bound PGRMC1 was estimated to range between 17% and 21% without addition of exogenous heme (Ghosh et

al., 2005). However, this proportion could vary depending on the heme structure because PGRMC1 has a higher affinity for ferric (Fe^{3+}) heme than for ferrous (Fe^{2+}) heme (Thompson et al., 2007). In vertebrates, additional analyses are still needed to define the percentage of PGRMC1 bound to heme. To date, only one study measured heme-bound PGRMC1 levels by mass spectrometry in the HEK293 cell line: they were virtually undetectable (Sabbir, 2019).

Although the results obtained using the N-terminal-truncated forms of PGRMC1 still need to be confirmed with the full isoform, the binding of PGRMC1 to heme depends on an interaction between the tyrosinate ion in PGRMC1 (Y113) with the iron atom of heme as well as with three separate bonds of Y164, Y107, and K163, each with a propionate group in heme. The heme is a hydrophobic prosthetic group, so its binding on the surface of PGRMC1 appears unstable and favors its stabilization by the formation of dimers. The dimerization of two PGRMC1 monomers is based only on the hydrophobic apposition of the two heme molecules, so there is no interaction between the amino acids. This type of protein dimerization had not yet been observed in eukaryotes and was named "heme-stacking dimer" (Kabe et al., 2018).

The heme is required for the activity of multiple hemoproteins. It was therefore proposed that PGRMC1 could act as a heme chaperone and regulate hemoprotein activity. Several interactions between PGRMC1 and cytochrome P450 enzymes were suspected and partially confirmed (Cahill & Medlock, 2017; McGuire et al., 2021; Ryu, Klein, & Zanger, 2017). However, binding of heme-staking dimers in humans has only been proven for the cytochrome P450 proteins: CYP1A2, CYP3A4, and CYP51A1 (Kabe et al., 2016). Their functional consequences are mainly defined by analogy with the results of previous studies.

CYP1A2 and CYP3A4 are involved in the metabolism of chemotherapeutic agents. The role of PGRMC1 in chemoresistance has been repeatedly demonstrated. In breast, endometrial or uterine cancer models, PGRMC1 limited cell death by stimulating CYP3A4 activity and thus the transformation of doxorubicin into its inactive compound, doxorubicinol (Clark et al., 2016; Friel et al., 2015; Lin et al., 2012; Lin et al., 2013; Lin et al., 2015). The anti-apoptotic role of PGRMC1 was also observed in ovarian cancer cells under cytostatic treatment such as cisplatin and paclitaxel (Liu, Zhou, Zhao, & Chen, 2012; Peluso et al., 2009; Peluso et al., 2008; X. Zhu et al., 2013).

CYP51A1 catalyzes the demethylation of lanosterol in cholesterol biosynthesis. The interaction of PGRMC1 with this enzyme as well as its role in stimulating its activity has been demonstrated in both humans and yeast (R. J. Craven, Mallory, & Hand, 2007; Hughes et al., 2007; Szczesna-Skorupa & Kemper, 2011).

A binding was identified between heme-staking dimer and EGFR, a protein involved in signal transduction required for cell proliferation (Kabe et al., 2016). This interaction was disrupted in the presence of carbon monoxide (CO) (Kabe et al., 2018). CO can cause the dissociation of heme-stacking dimers but also bind to the iron atom of monomeric structures to stabilize this conformation and prevent their dimerization (Kabe et al., 2016). CO is produced during enzymatic degradation of heme in the smooth endoplasmic reticulum by a heme oxygenase (HO) (T. Wang et al., 2021). It was then proposed that the formation of heme-stacking dimers as well as their associated functions are dependent on both the levels of heme molecules as well as their degradation products (Cahill & Medlock, 2017; Kabe et al., 2018). Accordingly, a potential role for PGRMC1 as a regulator of heme homeostasis was suggested. This hypothesis is further supported by Piel et al. who identified an interaction between the N-terminal portion of PGRMC1 and ferrochelatase (FECH), an enzyme localized in the

mitochondrial membrane and involved in heme synthesis (Piel et al., 2016). In addition, PGRMC1 appeared to regulate the biosynthesis of the hormone hepcidin in hepatocytes (Li et al., 2016). Hepcidin controls the membrane concentration of the transporter ferroportin responsible for cellular iron export (Nemeth & Ganz, 2021).

The presence of heme has not been characterized in the endometrium, not even during menstruation. Therefore, it is difficult to estimate the importance of PGRMC1-heme interactions and heme-stacking dimer functions during the menstrual cycle. As a reminder, the only analysis that measured the proportion of PGRMC1 dimerized under physiological conditions found very low levels (Sabbir, 2019).

In contrast, their influence in tissues subject to hypoxia as well as in cancer was more intensively investigated. Indeed, PGRMC1 protein abundance was higher in hypoxic tumor environments (Neubauer et al., 2008) and hypoxia decreased HO activity and, consequently, intracellular CO level (Morikawa et al., 2012). Therefore, the formation of heme-staking dimers could be promoted and exert major effects.

3. Cholesterol biosynthesis and steroidogenesis

In the absence of sufficient cholesterol supply, cell types other than hepatocytes, including fibroblasts, are able to activate cholesterol biosynthetic pathways. Independently of heme binding, PGRMC1, or one of its homologues, can interact with several other cytochrome P450 enzymes involved in cholesterol biosynthesis and/or steroidogenesis (Hughes et al., 2007; McGuire et al., 2021; Min et al., 2005; Min et al., 2004; Oda et al., 2011; Peluso, 2013; Szczesna-Skorupa & Kemper, 2011; L. Zhang et al., 2008).

In addition to its ability to stimulate CYP activity, PGRMC1 was found to interact with a regulatory complex of cholesterol biosynthesis. Although its involvement in the complex has not yet

been determined, binding of PGRMC1 to insulin-induced gene 1 (INSIG1) was detected in the COS-7 (African green monkey kidney fibroblast cells) and Hela breast cancer cell lines (Suchanek, Radzikowska, & Thiele, 2005). When intracellular sterol concentration is insufficient, INSIG1 releases sterol-regulatory element-binding protein (SREBP) and SREBP cleavage-activating protein (SCAP) from the endoplasmic reticulum to ultimately stimulate the expression of genes involved in cholesterol biosynthesis (Figure 16). Furthermore, by increasing the concentrations of cholesterol, the substrate of steroidogenesis, PGRMC1 could indirectly promote steroid production.

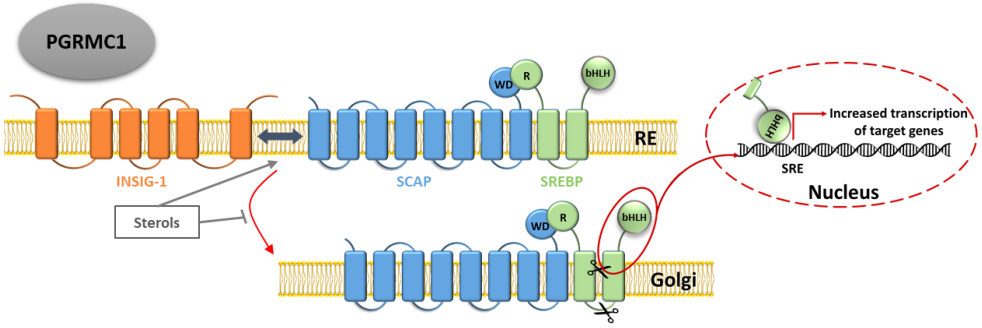

Figure 16: Potential interaction of PGRMC1 with the INSIG-1/SCAP/SREBP complex.
Through its potential interaction with INSIG-1, PGRMC1 could be part of the regulatory complex of intracellular cholesterol levels, INSIG-1/SCAP/SREBP. INSIG-1 sequesters SCAP and SREBP in the endoplasmic reticulum (ER) by interacting with the SCAP domain, which in turn is linked to the SREBP regulatory domain (R) through its WD 40 domain (WD). When intracellular sterol levels are insufficient, SCAP dissociates from INSIG and escorts SREBP into the Golgi apparatus where it is processed to release the basic-helix-loop-helix domain (bHLH). The bHLH domain translocates into the nucleus and initiates transcription of genes involved in cholesterol uptake or in cholesterol synthesis.

4. Cell proliferation

The functions of PGRMC1 on cell proliferation that will be discussed below appear, so far, to be shared with PGRMC2 as well. However, the nature of their respective activities has not yet been described. It could be complementary, competitive or compensatory.

PGRMC1 may act as a regulator of mitosis and apoptosis by regulating two aspects of cell division: mitotic spindle stability and cell cycle entry. Indeed, in culture of SKOV-3 cells, a rat ovarian epithelial cancer line, the addition of an antibody against PGRMC1 appears to both stimulate and slow down the cell proliferation (Lodde & Peluso, 2011): the percentage of mitotic figures increases while the cell number is reduced compared with the control group (with IgG antibody), suggesting a prolongation of cell cycle M phase.

The duration of mitosis may be influenced by the rate of disassembly and reassembly of spindle microtubules, especially composed of β-tubulin. In this same study, an interaction between PGRMC1 and β-tubulin was observed by *in situ* proximity ligation assay and the PGRMC1 depletion slowed the rate of the spindle microtubule disassembly (Lodde & Peluso, 2011). The same effects were observed in the presence of progesterone. The authors concluded that in the presence of progesterone, PGRMC1 could limit cell proliferation by promoting the stability of the mitotic spindle of ovarian cells.

Cycle entry following PGRMC1 depletion has been observed in other cell models and is sometimes accompanied by an increased rate of apoptotic cell death (Pedroza et al., 2020; Pedroza et al., 2021; Peluso, Griffin, Liu, & Horne, 2014; Sueldo et al., 2015; Terzaghi et al., 2016).

How PGRMC1 stimulates cycle entry is not yet precisely identified but some mechanisms have been proposed:

(i) Together with PGRMC2, GTPase-activating protein binding protein 2 (G3BP2) and NF-κB α (IκBα), PGRMC1 might form a complex capable of preventing NF-κB nuclear translocation on which cycle entry may depend (Peluso, Pru, Liu, Kelp, & Pru, 2019).

(ii) In breast cancer, Pedroza *et al.* demonstrated that PGRMC1 can promote cell survival and proliferation by promoting the phosphorylation and thus the activity of EGFR. As a consequence, the PI3K/AKT/mTor signaling pathway was stimulated as well as the expression of genes that depend on it (Pedroza et al., 2020). The role of PGRMC1 towards EGFR could potentially be modulated by progesterone or progestins but also indirectly by estradiol (Cai et al., 2021; Pedroza et al., 2021).

(iii) Another team suggested that by promoting phosphorylation of PGRMC1 at position S181, the progestins norethisterone and dydrogesterone, can stimulate breast cancer cell proliferation through activation of ERα (Figure 17). By competition, S181-phosphorylated PGRMC1 can interact with the ERα regulatory complex proteins, prohibitin 1 and 2 (PHB-1/2), and indirectly induce ERα translocation (Bai et al., 2021).

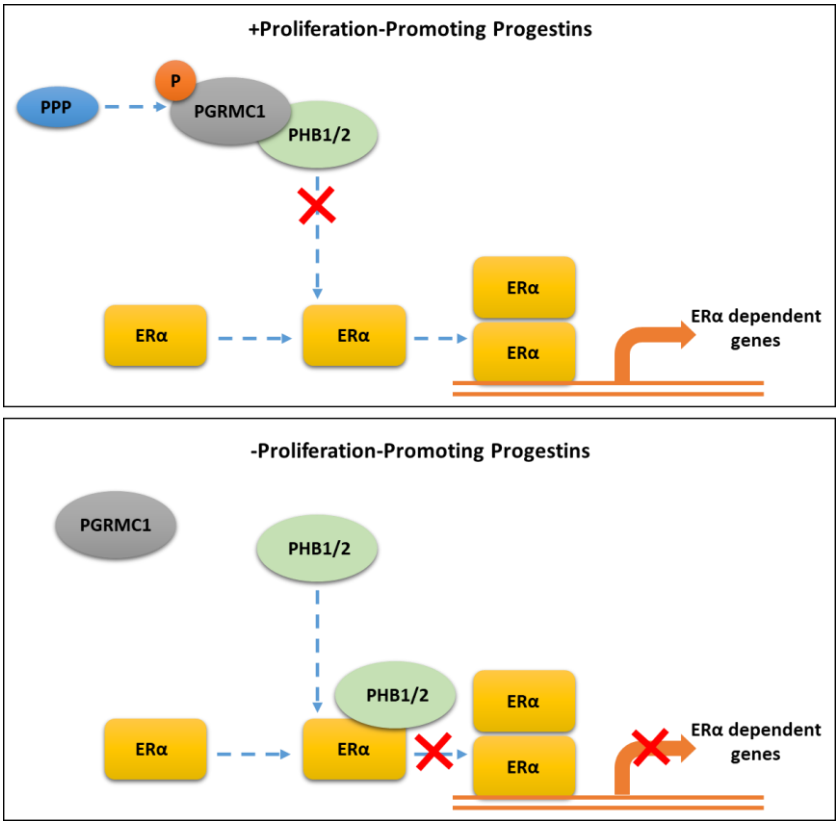

Figure 17: Potential crosstalk between PGRMC1 and PHB1/2 in ERα-signaling cascades.
S181-phosphorylation on PGRMC1 mediates interaction with prohibitin (PHB) 1/2 upon treatment with proliferation-promoting progestins (PPPs). In absence of PPPs: PHB1/2 act as ERα co-regulators to inhibit the transcription of ERα-dependent genes. In presence of PPPs: S181-phosphorylated PGRMC1 interacts with PHB1/2, possibly inhibiting their function as transcription factor regulators and enabling the transcription of ERα-dependent genes. *Reproduced from (Bai et al., 2021)*.

iii. PGRMC1, infertility and endometriosis

PGRMC1 seems correlated with fertility in humans and in several experimental models.

In zebrafish, invalidation of pgrmc1 limited oocyte maturation and a decrease of nPRs was observed in Pgrmc1/2$^{-/-}$ double knockout (KO) mutants (X. J. Wu, Thomas, & Zhu, 2018; X. J. Wu & Zhu, 2020).

In a mouse model, conditional KO *Pgrmc1* in the endometrium induced the development of endometrial cysts as well as a decrease in the number of pups per litter (McCallum et al., 2016).

In humans, PGRMC1 expression was reported to be lower in the endometrium of patients with recurrent miscarriage than in the endometrium of fertile patients (Feng et al., 2014; Lyzikova, Zinovkin, & Pranjol, 2020). *In vitro*, the role of PGRMC1 was considered in decidualization of human endometrial stromal cells required for the preparation of an environment conducive to embryonic implantation (Salsano et al., 2020; Salsano, González-Martín, Quiñonero, Pérez-Debén, & Domínguez, 2021; Salsano et al., 2017). Furthermore, Allen *et al.* identified a potential role for PGRMC1 in the progestational repression of MMP-9 expression and activity by MPA in cytotrophoblastic cells, suggesting its influence in embryonic implantation (Allen et al., 2015).

Although decreased fertility is observed in ~50% of patients with endometriosis, very few studies characterize and consider the role of PGRMC1 in this condition and none has been conducted in endometriosis lesions. To date, only two studies have measured and characterized the expression of PGRMC1 in the eutopic endometrium in endometriosis, one in the macaque and the other one in humans. In the secretory endometrium of ovariectomized macaques with endometriosis, PGRMC1 expression was measured by *in situ*

hybridization and immunohistochemistry but no difference was found in its expression or localization (C. S. Keator, Mah, & Slayden, 2012). However, another study identified a decrease in PGRMC1 expression in the secretory phase in the eutopic endometrium of patients with endometriosis, that was associated with a decrease in PGRMC1 protein signal in the eutopic stroma (Bunch et al., 2014).

However, all these studies remain preliminary, and none of them addresses precisely and thoroughly the expression, localization and/or role of PGRMC1 in endometriosis.

II. OBJECTIVES

Investigations and publications from other teams working on physiological and/or pathological functions of PGRMC1 in other gynecological organs suggest that this path of research should be extended to endometrium and endometriosis. Indeed, PGRMC1 expression or activity could be modified in endometriosis and thereby contribute to fertility issues observed in 50% of the patients and/or in progesterone resistance which jeopardizes progestin-based treatments.

My research project was therefore divided into three specific objectives aimed at defining PGRMC1 expression, localization, regulation and activity in the endometrium and in endometriosis.

A. Specific Aim #1: To characterize the expression of PGRMC1 in the human endometrium and endometriosis during the menstrual cycle.

When my PhD project was initiated, the expression and tissue localization of PGRMC1 in the endometrium and endometriosis were only minimally described. I therefore characterized the expression profile and localization of PGRMC1 (ARNm and protein) in the cycling human endometrium of control patients and compared with eutopic endometrium and lesions from patients with endometriosis.

The findings were gathered in a **manuscript** submitted and currently in minor editorial revision after the first peer reviewing (**III**.A.*i*.)

B. Specific Aim #2: To determine whether and how estradiol and progesterone are involved in the regulation of *PGRMC1* expression.

Since estradiol and progesterone are the major orchestrators of the cyclical changes in the endometrium during the menstrual cycle, we hypothesized that variations in PGRMC1 expression should result from direct or indirect regulatory mechanisms responding to changes in ovarian steroid concentrations. The influence of estradiol, progesterone and progestational compounds on PGRMC1 expression was briefly investigated *in vivo*, in a mouse model of human endometrial xenograft (**III**.B.*ii*), before being investigated in more details *in vitro* in various endometrial cell culture models (**III**.B.*iii*).

This objective resulted in acquisition of preliminary data that will require additional experiments to constitute the basis of a future publication.

C. Specific Aim #3: To identify potential roles for PGRMC1 in the endometrium and endometriosis.

Targeted investigation of potential endometrial roles of PGRMC1 in endometrial stromal cells was briefly performed before turning to a broad-spectrum transcriptomic analysis aiming to identify its functional targets. Based on the laboratory's field of expertise, a particular interest was given to endometrial tissue remodeling, and more precisely to the regulation by PGRMC1 of endometrial fibroblast cell proliferation and of the expression of two MMPs (*MMP-3* and *MMP-1*) crucial during menstruation (**III**.C.*i*.).

Some of the results obtained were disturbing for unexpected reasons but were valorized in a **publication** (**III.C.***ii*.). Additional preliminary data provided the proof-of-concept for another PhD project that was initiated for further identification of PGRMC1 targets.

III. RESULTS

A. CHARACTERIZATION OF PGRMC1 IN HUMAN ENDOMETRIUM AND ENDOMETRIOSIS/ADENOMYOSIS

i. "Spatiotemporal pattern of expression of Progesterone Receptor Component (PGRMC) 1 in endometrium from patients with or without endometriosis or adenomyosis"

This section describes the experimental work that was performed to investigate the spatiotemporal pattern of expression and localization of PGRMC1 in the uterine/eutopic endometrium of patients without or with endometriosis or adenomyosis.

The resulting data are presented as formatted in the manuscript that was accepted for publication in the Journal of Steroid Biochemistry and Molecular Biology.

Article

Spatiotemporal expression pattern of Progesterone Receptor Component (PGRMC) 1 in endometrium from patients with or without endometriosis or adenomyosis

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Abstract: The endometrium plays a crucial role in reproduction and, in humans, is cyclically remodeled under hormonal control. Estradiol favors tissue proliferation whereas progesterone inhibits tissue growth and induces morphological changes. Endometriosis is often associated with fertility issues and with exacerbated estrogen and reduced progesterone concentration or response in the eutopic endometrium. However, underlying mechanisms remain unclear. Progesterone Receptor Membrane Component (PGRMC) 1 is a protein able to modulate progesterone response and its murine knockout reduced fertility. However, the precise spatiotemporal pattern of PGRMC1 expression in the human endometrium is still poorly characterized. We investigated variations of eutopic endometrial PGRMC1 expression by combining RT-qPCR, immunofluorescence and *in situ* hybridization. We found that PGRMC1 expression progressively increases during the proliferative phase and decreases during the secretory phase. However, immunolabeling and identification of mRNA-containing cells were regularly heterogeneous in samples, according to tissue depth, with a gradient extending from the surface epithelium towards the basalis. There was no significant difference in PGRMC1 mRNA amounts between patients with or without endometriosis or adenomyosis, for any phase of the menstrual cycle, but cells with strong or moderate PGRMC1 immunolabeling were reduced during the proliferative phase in endometriotic patients. In conclusion, although the cyclical variation of PGRMC1 expression globally follows fluctuation of ovarian steroids, further work is required to precisely characterize hormonal control and identify the additional levels of regulation responsible for local adjustment of PGRMC1 concentration. This is particularly

important in the light of recent studies emphasizing the correlation between adequate PGRMC1 amounts and fertility.

Keywords: PGRMC1; endometrium; endometriosis

1. Introduction

The endometrium is the uterus mucosa composed of epithelial (glandular or luminal) cells, and a rich stroma. Histologically, the basal layer, in contact with the myometrium, can be distinguished from the functional layer, extending towards the uterine cavity. The endometrium is a dynamic tissue, continuously subjected to tissue breakdown, regeneration and maturation throughout the menstrual cycle. These events are globally orchestrated by circulating ovarian steroids in order to prepare for potential blastocyst implantation (Gaide Chevronnay et al., 2009). The molecular mechanisms responsible for response to estradiol and progesterone therefore need to be finely regulated, both spatially and temporally, to ensure the major function of the endometrium, fertility (Marquardt, Kim, Shin, & Jeong, 2019).

Endometriosis and adenomyosis are gynecological pathologies characterized by the growth of endometrial tissue either outside the uterus (ectopic lesion) or within the myometrium respectively. Endometriosis affects approximately 1 out of 10 women worldwide and is the cause of severe pelvic pain and reduced fertility. In many aspects, adenomyosis shows similarities with endometriosis, particularly deep endometriosis (Struble et al., 2016). In addition to common histological features, adenomyosis and endometriosis seem to share symptoms, etiological hypotheses, diagnostic methods, therapeutic molecules, ... (Stratopoulou et al., 2021) so much so that some authors have suggested that they are simply different manifestations of a single disease (J. Donnez et al., 2019).

Endometriotic lesions typically present an imbalance in their capacity for hormonal response which results in over-stimulation of estrogenic signaling pathways (estradiol dominance) and, conversely, in a decrease in their capacity for progestational response (progesterone resistance). Consequently, treatments which are mainly based on the

prescription of progestogen compounds (progestins) (Giudice, 2010; Vercellini et al., 2016) only succeed in limiting estradiol-primed cell proliferation. However, they do not eliminate the lesions of endometriosis (Liang et al., 2018) and are even ineffective in some patients (Barra et al., 2019; Jacques Donnez & Dolmans, 2021). The origin of progesterone resistance is multifactorial and not fully understood (Reis et al., 2020). A decrease in the synthesis and/or biological activity of the nuclear progesterone receptor (nPR) has already been observed, in particular in endometriosis lesions, but not systematically. Moreover, the results are often contradictory in the eutopic endometrium from a patient with endometriosis and therefore cannot explain the infertility encountered in 50% of patients (McKinnon, Mueller, & Montgomery, 2018). On the other hand, there are other receptors/proteins capable of transmitting or modulating a progesterone response that were poorly considered by comparison with nPR so far. A decrease in the expression and protein abundance of the membrane progesterone receptors α and β (also named PAQR7 and PAQR8 respectively) as well as of the protein PGRMC1 (Progesterone Receptor Membrane Component 1) has been observed in the eutopic endometrium (Bunch et al., 2014; Vázquez-Martínez et al., 2020).

A member of the MAPRs (Membrane Associated Progesterone Receptors) protein family, PGRMC1 has been the subject of several studies aimed at establishing its role in various gynecological pathologies and in particular in endometrial, ovarian and breast cancer where it appears to be overexpressed (Cahill, Jazayeri, Catalano, et al., 2016; Feng et al., 2014; Peluso & Pru, 2014; Y. Zhang, Ruan, Mi, & Mueck, 2017). Thus, PGRMC1 was reported to modulate cell proliferation and apoptosis (Pedroza et al., 2020; Pedroza et al., 2021; Peluso & Pru, 2014; Sletten, Smaglyukova, Ørbo, & Sager, 2020), promote cell migration and invasion (Lee et al., 2021), regulate the expression of enzymes involved in cholesterol metabolism, participate in the metabolism of therapeutic molecules (Cahill, Jazayeri, Catalano, et al., 2016; Cahill & Medlock, 2017) and interfere in some of their target interactions (Neubauer et al., 2011). Additionally, its implication in fertility has already been suggested. First, a conditional Pgrmc1

knockout in the murine endometrium induced the development of endometrial cysts and a decrease in the offspring (McCallum et al., 2016). In humans, PGRMC1 expression was decreased in endometrium of patients suffering from recurrent miscarriage (Feng et al., 2014; Lyzikova et al., 2020). Moreover, PGRMC1 was reported to participate in the decidualization of primary endometrial stromal cells, a differentiation that is essential for embryo implantation (Salsano et al., 2020; Salsano et al., 2017). Two studies, based on microarray (Talbi et al., 2006) or proteomic (J. I. Chen et al., 2009) analyses, determined that PGRMC1 is abundantly expressed during the proliferative phase and that it decreased in secretory phase, especially in the stroma. However, the precise spatiotemporal pattern of PGRMC1 expression in the human endometrium is still poorly characterized.

In this study, we investigated the spatiotemporal variations of PGRMC1 expression in the human endometrium by combining quantification and localization of both its mRNA and the protein. Endometria from control patients were compared to endometria from patients suffering from endometriosis or from adenomyosis.

2. Materials & methods

2.1 Tissue collection

The study was approved by the Ethical Committee of the Université Catholique de Louvain (2017/10JUL/362). Tissue samples were provided by the Biobank of Saint-Luc University Clinics as surgical leftovers from patients operated for clinical reasons independent from the study. Biomaterial was obtained by biopsy during laparoscopy or following hysterectomy from patients with spontaneous menstrual cycle (<50 years old), without progestin treatment for the last 3 months and without uterine cancer or endometritis. Other gynecological pathology(ies) for samples used for immunofluorescence and/or *in situ* hybridization are detailed in Table I. Samples of eutopic endometrium were separated in three groups: patients with endometriosis (E), patients with adenomyosis (A) and control patients without endometriosis or adenomyosis (CTL).
For each sample, the cycle phase was determined by an expert anatomo-pathologist on the basis of histological criteria. In a classical

menstrual cycle of 28 days, the menstrual phase was considered to extend from day 1 to 5, the early, mid and late proliferative phases respectively from day 6 to 8, from day 9 to 11 and from day 12 to 14, and the early, mid and late secretory phases respectively extend from day 15 to 18, from day 19 to 22 and from day 23 to 28.
Tissue for histological examination was fixed in 4% paraformaldehyde and embedded in paraffin whereas tissue for RNA analysis was placed into RNA lysis buffer (SV total RNA isolation system; Promega, Madison, WT, USA) at -80°C until RNA extraction.

2.2 RNA extraction and Quantitative Real-Time PCR

Total RNA was isolated from tissue samples using the SV total RNA isolation system according to the manufacturer's recommendations (Promega).
For all samples, the RNA concentrations were evaluated using a Nanodrop ND-8000 spectrophotometer. 500ng total RNA were used for reverse transcription using SuperScript III Reverse Transcriptase kit (Invitrogen, Waltham, MA, USA) according to manufacturer's recommendations.
Primers for quantitative amplification of PGRMC1 cDNA were previously published (Bunch et al., 2014) and amplification efficiency was checked before use. Real-time PCR amplification was performed with the KAPA SYBR FAST qPCR Master Mix (2X), 0.25µM primers (forward and reverse) and 15ng cDNA. Two housekeeping genes (HKGs), RPL13a and β-actin, were selected based on their stability of expression and the mean of their Ct values was used for normalization in each RT-qPCR experiment.

2.3 *In situ* hybridization

In situ hybridization analysis was performed on a selection of paraffin-embedded tissue samples cut in serial sections of 5µm (Table I). After dewaxing in xylene, antigen retrieval, detection of the *PGRMC1* mRNA and signal amplification were performed with the RNAscope kit (RNAscope 2.5 HD Reagent kit-RED; Bio Techne Ltd., Mineapolis, MN, USA) according to the manufacturer's recommendations (F. Wang et al., 2012). The PGRMC1 specific probe (RNAscope Probe-HS-PGRMC1-C1) was especially designed for our laboratory and produced by the manufacturer. A negative control probe (RNAscope Negative Control Probe - DapB-C2) was systematically used to evaluate the background noise of each endometrium sample. At the end of the RNAscope protocol, the slides were immediately incubated in the blocking solution to continue with protein detection by immunofluorescence.

Table I. Endometrial samples analyzed by immunofluorescence and/or *in situ* hybridization.

Sample #	Age	Menstrual Phase	Successful pregnancy(ies)	Group	Pathology(ies)	Experiment(s)
1	45	EP	Yes	CTL	Menometrorrhagia	IF-ISH
2	49	EP	No	CTL	Leiomyoma	IF
3	49	MP	Yes	CTL	Myoma, menorrhagia	IF-ISH
4	45	LP	Yes	CTL	/	IF-ISH
5	41	ES	Yes	CTL	Leiomyoma, fibroma, atheromatosis	IF-ISH
6	44	MS	Yes	CTL	Uterine tube cyst, hydrosalpinx	IF-ISH
7	44	MS	Yes	CTL	Leiomyoma, menometrorrhagia, atheromatosis, cervicitis	IF-ISH
8	37	MS	Yes	CTL	Prolapsus	IF-ISH
9	45	MS	Yes	CTL	/	IF
10	38	LS	No	CTL	/	IF
11	44	LP	Yes	A	Adenomyosis	IF
12	43	MS	Yes	A	Adenomyosis	IF
13	48	EP	Yes	E	Endometriosis	IF
14	44	MP	Yes	E	Peritoneal endometriosis, leiomyoma	IF
15	41	MP	Yes	E	Peritoneal endometriosis, dysmenorrhea, menorrhagia	IF
16	44	ES	Yes	E	Cervical endometriosis, salpingitis, hydrosalpinx, uterine tube cyst	IF
17	46	MS	No	E	Cervical endometriosis, fibroma, cervicitis	IF

Abbreviations: EP/MP/LP, Early/mid/late proliferative phase; ES/MS/LS, Early/mid/late secretory phase; /, with no identified gynecologic pathology; IF, immunofluorescence; ISH, *in situ* hybridization.

2.4 Immunolabeling

Immunofluorescence was performed on paraffin-embedded tissue samples (Table I) cut in serial sections of 6µm (or 5µM if after RNAscope *in situ* hybridization). After dewaxing in Histosafe, antigen retrieval was performed in citrate Buffer for 75 min at 100°C and tissue sections were permeabilized for 15 min in phosphate-buffered saline (PBS), 0.3% Triton X-100. Nonspecific binding sites were blocked for 1h at RT with PBS, 0.3% Triton X-100, 10% bovine serum albumin (BSA) and 3% milk before incubating the slides overnight at 4°C with the primary antibody (Table II). The next day, tissue samples were washed 3 times for 5 min in PBS, 0.1% Triton X-100, and incubated with 1/1000 secondary antibody (adequate combination of Goat anti-rabbit IgG (Alexa 488) for PGRMC1, Goat anti-mouse IgG2a (Alexa 568) or Goat anti-mouse IgG2a (Alexa 647) for E-cadherin; Life Technologies, Carlsbad, CA, USA) for 60 min at RT. Nuclei were counterstained with Hoechst (BisBenzimide H33342, 1µg/mL, Sigma, St Louis, MO, USA) during incubation with the secondary antibody.
Because the antigen retrieval solution of the RNAscope kit was stronger than the one used for single immunolabeling, the concentration of primary and secondary antibodies was multiplied by 2 for immunolabeling performed after the RNAscope *in situ* hybridization.

Table II. Primary antibodies used for immunolabeling.

	Isotype	**Company**	**Commercial reference**	**Dilution**
PGRMC1 (D6M5M)	Rabbit mAb	Cell Signaling	13856	1/250
E-cadherin (Clone 36)	Mouse IgG2a mAb	BD Transduction Laboratories	610182	1/1000

In accordance with the recommendations of Hewitt et al. (Hewitt, Baskin, Frevert, Stahl, & Rosa-Molinar, 2014), the specificity of the anti-PGRMC1 antibody was previously validated *in vitro* by our laboratory (Thieffry et al., 2021). To do this, the expression of PGRMC1 was specifically repressed by siRNA-PGRMC1 in two endometrial cell lines, one stromal (T-HESC) and one epithelial (HEC-1A), and

confirmed by RT-qPCR, immunocytofluorescence and Western blotting. Additionally, a negative control without primary antibody was used in each experiment to correct background noise.

2.5 Microscopy and signal quantification

Protein and mRNA were 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 signals were analyzed and quantified with the HALO software, an image analysis platform (Indica Labs, Albuquerque, NM, USA). This software allowed to (i) count the cells positive for each signal, (ii) define their epithelium-stroma tissue distribution, and (iii) distinguish 3 progressive thresholds of PGRMC1 protein signal intensity.

The analysis procedure is schematized in Figure S1. Each sample was virtually divided into 500µm thick sections from the surface epithelium down to the basal layer (Figure S1a). The cells were then identified by nucleus detection (Hoechst signal) followed by modelization by the artificial intelligence of the Halo software (Figure S1b). For each modelized cell, the average of the pixel intensities present in the cell was calculated and a colour was assigned according to the 3 intensity thresholds. The cells expressing E-cadherin marker were considered as epithelial cells. These modelized cells were then spatially plotted (Figure S1c, partial images presenting representative tissue areas) and quantified slice by slice (Figures S1d, the whole tissue area) to estimate their distribution according to the cell type and the depth of the endometrium.

The background noise was corrected with the appropriate negative control for each slide. Background variability from sample to sample was negligible for immunolabeling. Therefore, intensity thresholds were kept identical for all analysed samples. Conversely, background intensity between samples was extremely variable for *in situ* hybridization. Consequently, assignment of signal intensity based on fixed intensity thresholds was not recommended and was not used for

in situ hybridization. Cells are either positive or negative for the presence of *PGRMC1* mRNA.

2.6 Statistical analysis

All statistical analyses were performed with GraphPad Prism 8.4.3.
Analyses of RT-qPCR data were performed on ΔCt values and the outliers were identified and excluded using the Grubbs' test.
For the quantification of PGRMC1 immunolabeling, statistical comparisons were performed using values for virtual slices rather than for the whole endometria to account for differences in tissue thickness between endometrial samples (essentially due to cycle phases).

3. Results

3.1 *PGRMC1* mRNA concentrations vary during the menstrual cycle in the human endometrium

PGRMC1 expression was measured by RT-qPCR, in samples of the functional layer of human endometria covering the three phases of the menstrual cycle and collected from hysterectomy specimens from control patients (without endometriosis or adenomyosis).

The proliferative and secretory phases were subdivided into early, mid and late subphases to appreciate the progressive variation in the mRNA expression. Indeed, a gradual increase in endometrial *PGRMC1* mRNA concentration was observed from the menstrual phase until the end of the proliferative phase, reaching 2.3x between the means of these two phases. Values then decreased approximately 6.3x between averages of the late proliferative and the late secretory phases. Values increased again from the late secretory to the menstrual phase (Figure 1).

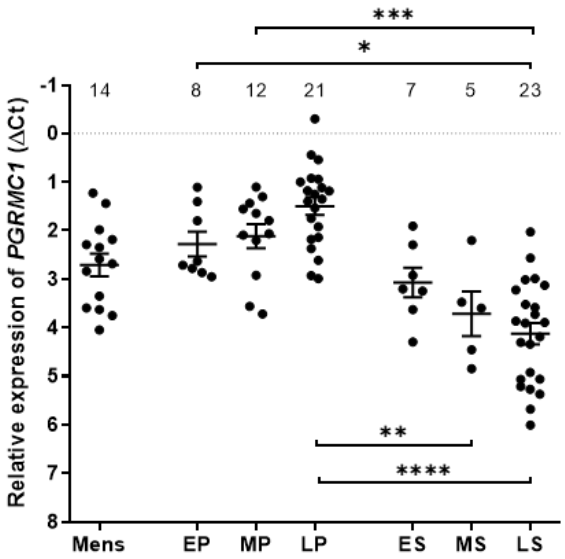

Figure 1: *PGRMC1* mRNA levels vary during the menstrual cycle in the human endometrium.

Relative concentrations of *PGRMC1* mRNA in functional layer of endometrium collected during the menstrual (Mens), early/mid/late proliferative (EP/MP/LP) or early/mid/late secretory phase (ES/MS/LS) were measured by RT-qPCR, normalized according to HKGs and are presented as dots for individual values (ΔCt), and as means ± SEM. The number of samples is indicated at the top of the graph. Statistical test: Kruskal-Wallis with Dunn's post-hoc, only statistically significant differences are shown.* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.2 PGRMC1 immunostaining varies with the menstrual cycle and the tissue depth in the human endometrium

The tissue localization of PGRMC1 was identified by immunofluorescence in 4 proliferative and 6 secretory human endometrial samples (Figure 2). The distinction between epithelium and stroma was achieved by co-labeling epithelial structures with E-cadherin.

Immunofluorescence images show that PGRMC1 is present in the stroma and epithelium and suggest a more intense immunostaining of PGRMC1 during the proliferative phase (Figure 2a-d) than during the secretory phase (Figure 2e-j), in agreement with the mRNA variations measured by RT-qPCR (Figure 1). Moreover, we suspected an intensity gradient of this signal as a function of tissue depth.

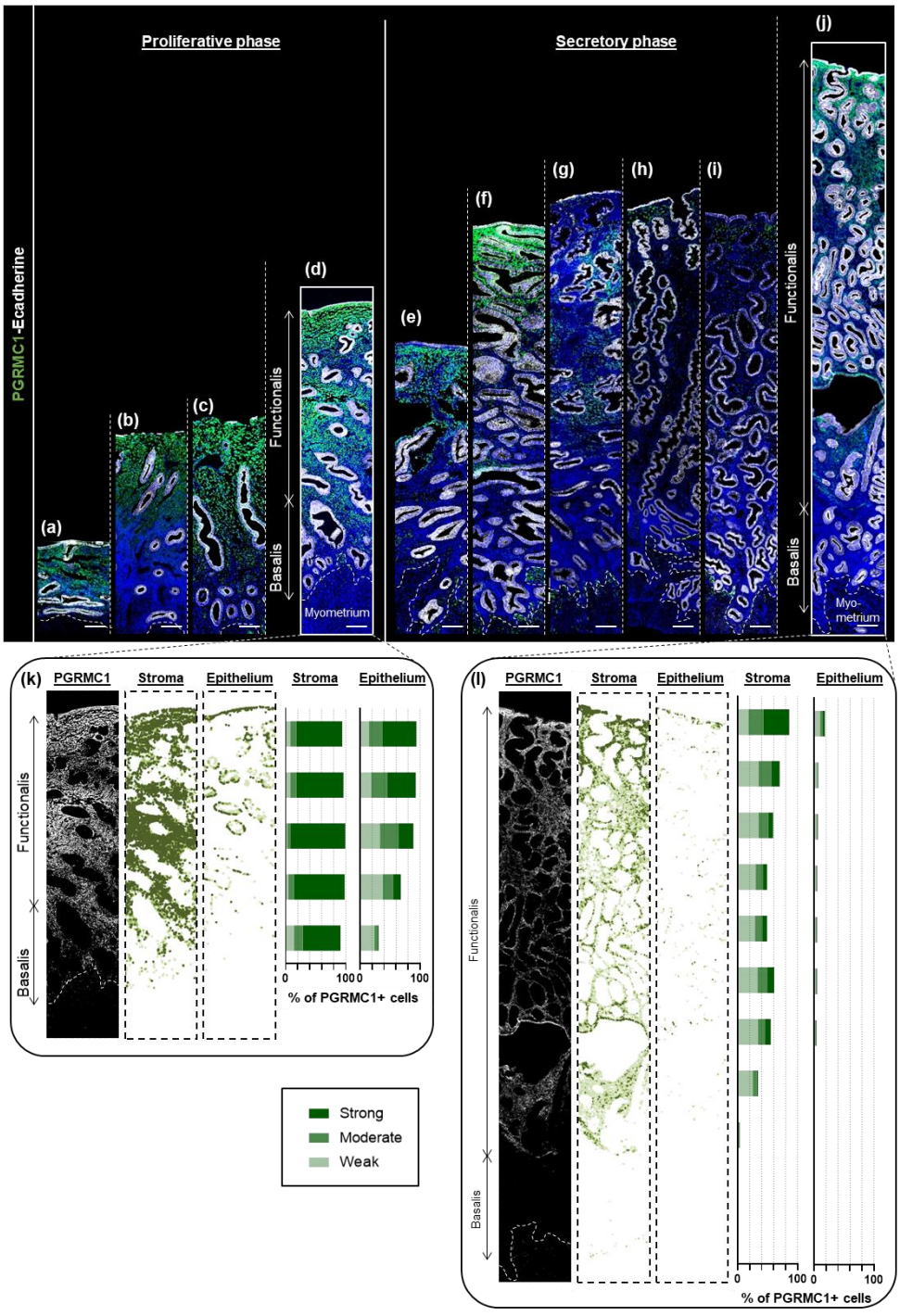
Proliferative phase
Secretory phase
PGRMC1-Ecadherine
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
Functionalis
Basalis
Myometrium
Myo-metrium
(k)
PGRMC1
Stroma
Epithelium
Stroma
Epithelium
0
100
% of PGRMC1+ cells
(l)
Strong
Moderate
Weak

Figure 2: PGRMC1 immunolabeling in the human endometrium along the menstrual cycle.
PGRMC1 was immunolocalized in endometrium collected during the early **(a, b)**, mid **(c)** and late **(d, k)** proliferative phase or during the early **(e)**, mid **(f-i)** and late **(j, l)** secretory phase. **(a-j)** Immunofluorescence of PGRMC1 (in green) and epithelial marker E-cadherin (in white). The nuclei were stained in blue. Scale bar = 200µm. **(k,l)** Original PGRMC1 immunolabeling (in white), corresponding spatial plots (in green) and quantification after modelization of whole tissue sections and virtual cutting of the tissue into 500-µm-thick slices. Each point of spatial plot represents a modelized cell and the level of green reflects the intensity of the PGRMC1 signal for each cell. PGRMC1 signal was quantified according to the intensity threshold (low, medium and high), the cell type and the depth in the endometrium. Values are presented as a percentage of the total number of stromal or epithelial cells within each virtual tissue slice. Panels **k** and **l** are presented as representative images and correspond to panels **d** and **j**, respectively.

Modelization (illustrative spatial plots in Figure 2k and l) combined with a virtual cutting of the tissue into 500-µm-thick slices, and followed by a quantification of the whole tissue section (see Figure S1 and corresponding text in Material and Methods) allowed to objectify this suspicion. More specifically, this analysis (Figure 2k-l and Figure S2) allowed to consider separately the percentage of cells immunolabeled for PGRMC1 and the intensity of the PGRMC1 signal per positive cell (strong, moderate or weak, see levels of green in Figure 2k-l and Figure S2). We found that the proportion of cells immunostained for PGRMC1 is indeed significantly higher in the proliferative endometrium (Figure 2k, Figure S2a-c and i-k, and Table III) than in the secretory endometrium (Figure 2l, Figure S2d-h and l-p, and Table III).

Table III: Quantification of PGRMC1 immunolabeling.

% of cells (mean ± SEM)	Proliferative phase		Secretory phase	
	Stroma	Epithelium	Stroma	Epithelium
Positive (total)	94.3 ± 2.1 ****	91.5 ± 4.8 ****	38.7 ± 4.6 ###	20.6 ± 3.4
Strong	31.9 ± 9.2 **	13.8 ± 4.6	7.7 ± 2.7	0.9 ± 0.4
Moderate	25.3 ± 5.0	23.2 ± 3.6 **	10.6 ± 2.2	4.1 ± 1.4
Weak	37.1 ± 7.4 *	54.5 ± 7.0 ****	20.4 ± 3.0	15.6 ± 3.0

Significant differences between cycle phases for the same cell compartment are indicated by * and significant differences between cell compartments within the same phase are indicated by #. Statistical test: Anova two-way and post-hoc Tukey. Only statistically significant differences are shown. * $p < 0.05$, ** $p < 0.01$, ***/### $p < 0.001$, **** $p < 0.0001$.

Moreover, the proportion of cells showing a strong or moderate signal was significantly higher during the proliferative than the secretory phase, both in the stroma and the epithelium. Increased PGRMC1 labeling in upper endometrial layers during the proliferative phase is essentially due to stronger labeling per positive cell rather than larger percentage of positive cells (Figure S2a-c and i-k). This gradient is less frequent in secretory endometrium (see Figure S2d, e, l and m) since global PGRMC1 expression is decreased during this phase.

3.3 *PGRMC1* mRNA concentrations also vary with tissue depth in the human endometrium

To gain further insight in PGRMC1 expression and determine whether spatiotemporal distribution of *PGRMC1* mRNA concentrations reflects protein immunolabeling, *in situ* hybridization analysis was performed using the RNAscope technique in parallel with immunostaining of the PGRMC1 protein. Given the variability observed between the expression profiles of the different samples, the 7 analyzed samples are presented (Figure S3).

In immunofluorescence images, *PGRMC1* mRNA labeling (in red in Figure S3) appears punctiform and cytoplasmic. Enlarged images (Figure S3c and g) suggest that *PGRMC1* mRNA localization is frequently correlated with a protein signal (in green) within the same cell, especially in areas with a high percentage of immunolabeled cells.

Analysis by modelization (Figure S3), identification of virtual tissue slices and signal quantification in whole tissue sections (Figure S4) was applied simultaneously to mRNA and protein.

Although quantification of *PGRMC1* mRNA did not take into account the intensity of the signal per cell, the values are in good agreement with immunolabeling and support both stronger expression during the proliferative phase and increased expression of PGRMC1 in upper layers of the endometrium. However, in the basalis, the percentage of cells positive for *PGRMC1* mRNA was systematically higher in the epithelium than in the stroma during both cycle phases.

3.4 Cyclical variation of *PGRMC1* mRNA levels is similar in the endometrium of patients with endometriosis/adenomyosis and control patients

We extended our study to analyze the expression of PGRMC1 in the functional layer of endometrium samples from patients with endometriosis or adenomyosis. Quantification of *PGRMC1* mRNA by RT-qPCR shows cyclical variations identical to those measured in patients not affected by these pathologies: an increase during the proliferative phase and a decrease during the secretory phase (Figure

3). Interestingly, no significant difference in the expression of *PGRMC1* was observed between the pathological groups and the control group, for any of the three phases of the menstrual cycle.

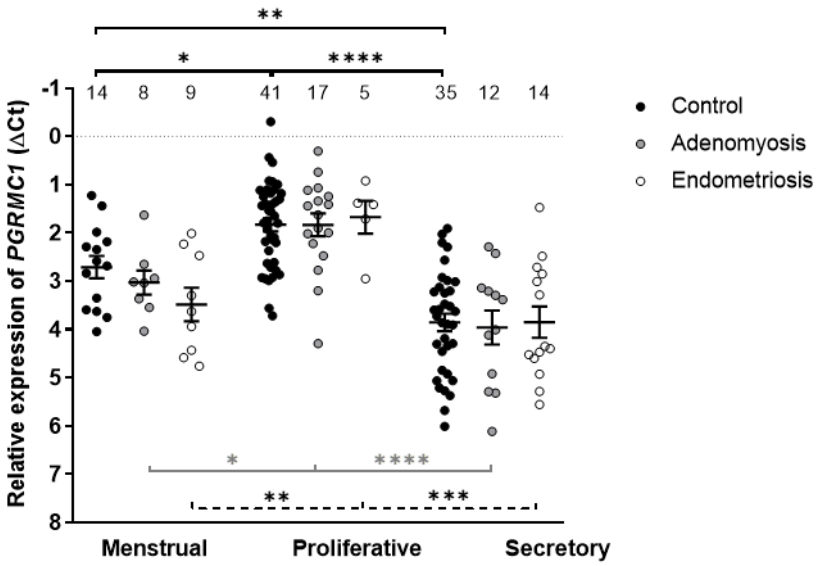

Figure 3: *PGRMC1* mRNA levels vary during the menstrual cycle in the endometrium of patients with endometriosis/adenomyosis.
Relative concentrations of *PGRMC1* mRNA in the functional layer of endometrial samples from patients with endometriosis (in white) or adenomyosis (in grey) collected during menstrual, proliferative or secretory phase were measured by RT-qPCR and normalized according to HKGs. Values are presented as dots for individual values (ΔCt), and as means ± SEM and compared to samples from control patients (values from Figure 1, in black). The number of samples is indicated at the top of the graph. Statistical test: Anova two-way and post-hoc Tukey. Tests were performed to compare phases in the same pathology group or to compare pathology groups in the same phase. Only statistically significant differences are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. No statistical difference was found between pathology groups from identical phases.

3.5 PGRMC1 immunolabeling is decreased in the endometrium of patients with endometriosis/adenomyosis

The pattern of PGRMC1 immunostaining in the endometrium of patients with adenomyosis and/or endometriosis (Figures 4 and S5 and Table IV) are similar to those of patients in the control group (Figures 2 and S2 and Table III): the PGRMC1 signal (i) is mainly stromal, (ii) is broadly more abundant during the proliferative phase than during the secretory phase, and (iii) decreases from the uppermost layer of the endometrium to the basal layer. However, comparison of PGRMC1 immunolabeling quantification between patients with or without endometriosis/adenomyosis showed that the percentage of cells positive for PGRMC1 labeling during the proliferative phase was significantly reduced in the endometrium of patients with endometriosis/adenomyosis compared to control samples (Figure 5a). This was essentially due to a reduction in the proportion of cells with strong (Figure 5b) and moderate (Figure 5c) signal. During the secretory phase, the global percentage of positive cells was similar in both patient groups (Figure 5a) but we noticed a trend for a (not significant) decrease in the percentage of cells with strong and moderate signal in samples of patients with endometriosis/adenomyosis (Figure 5b and c), and consequently a significant increase in the percentage of cells with weak signal (Figure 5d).

PGRMC1 labeling appeared essentially stromal in immunofluorescence images of control and endometriosis/adenomyosis patients (Figures 2 and 4). However, paired comparison between corresponding stroma and epithelium within tissues slices did not reach significance neither for the percentage of positive cells nor for signal intensity, except during the secretory phase for the percentage of positive cells in control patients. This suggests that visual predominance of stromal PGRMC1 signal essentially reflects the abundance of stromal cells in the tissue, rather than a specific PGRMC1 enrichment in these cells.

Table IV: Quantification of PGRMC1 immunolabeling in the human endometrium of patients with endometriosis/adenomyosis.

% of cells (mean ± SEM)	Proliferative phase		Secretory phase	
	Stroma	Epithelium	Stroma	Epithelium
Positive (total)	59.2 ± 6.9 **	61.6 ± 6.1 ****	34.7 ± 6.1	31.3 ± 4.7
Strong	1.1 ± 0.7	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
Moderate	4.3 ± 2.4	3.1 ± 2.2	2.3 ± 1.0	0.4 ± 0.2
Weak	53.8 ± 6.4 **	58.5 ± 5.7 ****	32.3 ± 5.5	30.9 ± 4.7

Differences between cycle phases for the same cell compartment. Statistical test: Anova two-way and post-hoc Tukey. Only statistically significant differences are shown. ** $p < 0.01$, **** $p < 0.0001$.

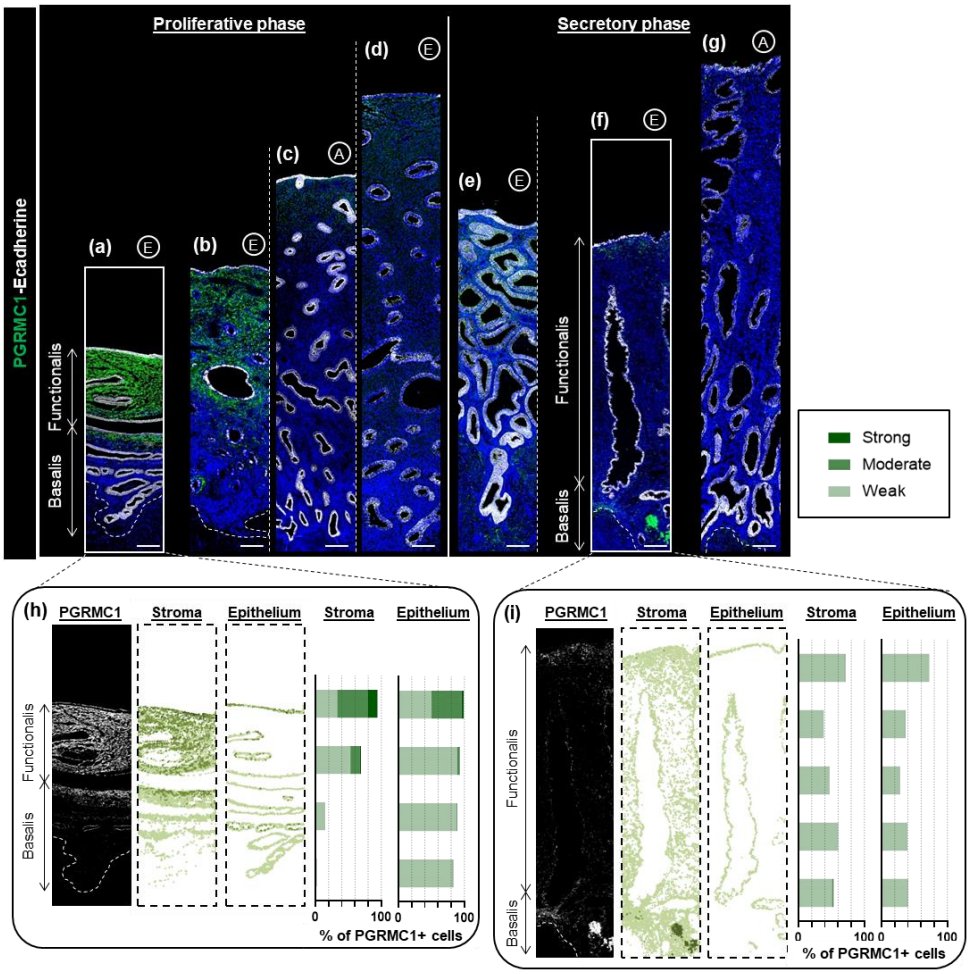
PGRMC1-Ecadherine
Proliferative phase
Secretory phase
(a) E
(b) E
(c) A
(d) E
(e) E
(f) E
(g) A
Functionalis
Basalis
Strong
Moderate
Weak
(h)
PGRMC1
Stroma
Epithelium
Stroma
Epithelium
Functionalis
Basalis
0
100
0
100
% of PGRMC1+ cells
(i)
PGRMC1
Stroma
Epithelium
Stroma
Epithelium
Functionalis
Basalis
0
100
0
100
% of PGRMC1+ cells

Figure 4: PGRMC1 immunolabeling in the human endometrium of patients with endometriosis/adenomyosis.
PGRMC1 was immunolocalized in endometrium of patients with endometriosis (E) or adenomyosis (A), collected during the early **(a, h)**, mid **(b, c)** and late **(d)** proliferative phase or during the early **(e)** and mid **(f, g, i)** secretory phase. **(a-g)** Immunofluorescence of PGRMC1 (in green) and epithelial marker E-cadherin (in white). The nuclei were stained in blue. Scale bar = 200μm. **(h and i)** Original PGRMC1 immunolabeling (in white), corresponding spatial plots (in green) and quantification after modelization of whole tissue sections and virtual cutting of the tissue into 500-μm-thick slices. Each point of spatial plot represents a modelized cell and the level of green reflects the intensity of the PGRMC1 signal for each cell. PGRMC1 signal was quantified according to the intensity threshold (low, medium and high), the cell type and the depth in the endometrium. Values are presented as a percentage of the total number of stromal or epithelial cells within each virtual tissue slice. Panels **h** and **i** are presented as representative images and correspond to panels **a** and **f**, respectively).

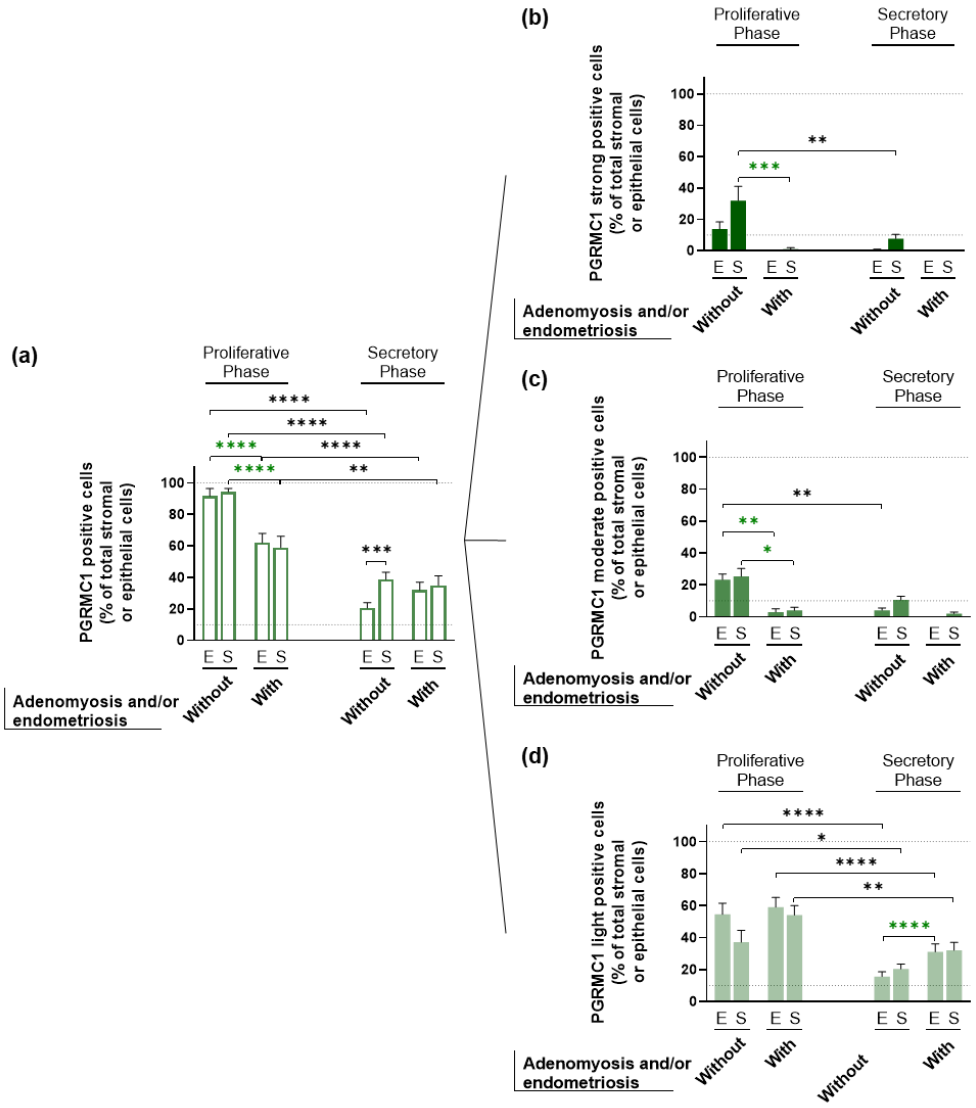
(a)
Proliferative Phase
Secretory Phase
PGRMC1 positive cells (% of total stromal or epithelial cells)
E S
Without
With
Adenomyosis and/or endometriosis
(b)
PGRMC1 strong positive cells (% of total stromal or epithelial cells)
(c)
PGRMC1 moderate positive cells (% of total stromal or epithelial cells)
(d)
PGRMC1 light positive cells (% of total stromal or epithelial cells)

Figure 5: Comparison of endometrial PGRMC1 immunolabeling between control patients and patients with endometriosis/adenomyosis.
Quantification results of PGRMC1 immunolabeling in endometrium from patients with endometriosis and/or adenomyosis (values derived from Figure 8 and Table IV) were compared to patients without endometriosis/adenomyosis (values derived from Figure 3 and Table III). The PGRMC1 signal was quantified in each virtual tissue slice according to the intensity threshold and the cell type (epithelial, E or stromal, S). Values are presented as mean ± SEM of the percentage of the total number of stromal or epithelial cells. In **a**, percentage of positive cells. In **b-d**, percentage of cells with strong, moderate or weak immunolabeling, respectively. Statistical test: Anova two-way and post-hoc Tukey. Only statistically significant differences are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

4. Discussion & Conclusion

In this study, we investigated the spatiotemporal profile of PGRMC1 expression in the human endometrium along the menstrual cycle and compared endometria from patients without or with endometriosis/adenomyosis. In summary, we identify temporal and spatial variations. PGRMC1 expression progressively increases during the proliferative phase and decreases during the secretory phase. In addition, expression in endometria often displays a gradient from the surface to the basal layer, mostly during the proliferative phase.

Quantitative RT-PCR was used to monitor changes in global concentration of *PGRMC1* mRNA in the functional layer. Our results are in agreement with and extend microarray and qPCR data (Bunch et al., 2014; Burney et al., 2007; Talbi et al., 2006) reporting increased levels (by 2-3 fold in average) of *PGRMC1* mRNA during the proliferative phase by comparison with the secretory phase in the endometrium of patients with no detected endometriosis/adenomyosis. The larger number of samples in our study allowed us to distinguish subphases (early/mid/late) at reveal that: (1) mean *PGRMC1* mRNA concentration is minimal during the late secretory phase and then (2) progressively increases during the menstrual and proliferative phase, (3) to peak during the late proliferative phase and (4) to progressively decrease during the secretory phase. Moreover, a similar cyclical variation was measured in the functional layer of the eutopic endometrium from patient with

endometriosis or adenomyosis, with no significant difference when comparing the 3 patient groups (control/endometriosis/adenomyosis) for each of the 3 cycle phases. To the best of our knowledge, this is the first analysis of PGRMC1 expression in the endometrium of patients with adenomyosis. Expression in endometriosis was addressed in microarray data ((Burney et al., 2007) and Gene Expression Omnibus entry GSE6364). These showed no difference in *PGRMC1* mRNA quantification between control and endometriosis patients during the proliferative phase (19841 +/- 2061 vs 19555 +/- 1614 arbitrary units, respectively) or the secretory phase (9926 +/- 4198 vs 8298 +/- 1970 arbitrary units, respectively). In support of our data, other teams using macaque experimental models reproducing the menstrual cycle and endometriosis concluded that *PGRMC1* mRNA levels during the [estradiol and progesterone]-primed pseudo-secretory phase were lower than during the [estradiol]-primed pseudo-proliferative phase (Ace & Okulicz, 2004; Christopher S. Keator, Mah, Lawson, & Slayden, 2009) but were not significantly different between animals without or with artificial endometriosis (C. S. Keator et al., 2012).

A major part of our work was dedicated to investigating spatial modulation of PGRMC1 expression, both at the mRNA and protein levels. For this purpose, we turned to high resolution whole slide scanning of sections representing the entire thickness of the tissue (from the luminal epithelium towards the interface between the basalis and the myometrium). Concomitant distinction between epithelial and stromal cells combined with cell modelization followed by virtual cutting of the sample in slices, and quantification of the fluorescent signals allowed us to provide a detailed atlas of PGRMC1 expression according to cell type, tissue depth and strength of the protein signal. The results of this study are however influenced by the inclusion and exclusion criteria of the patients. Variations could therefore be observed by broadening these criteria, in particular by analyzing samples from younger patients (age less than 40 years old).

Since the initial observation that endometrial PGMRC1 protein was more abundant during the mid-proliferative than during the mid-secretory phase (J. I. Chen et al., 2009), other teams have investigated and commented on PGRMC1 immunolabeling in the human

endometrium but these studies relied on less or non-quantitative techniques and were essentially based on analysis of limited tissue areas. Our findings thus confirm these studies and provide more detailed spatiotemporal information.

First, PGRMC1 immunolabeling was reported to be more abundant in stromal cells compared with glandular and luminal epithelium (Bunch et al., 2014). Direct comparison of values for stromal and epithelial PGRMC1 immunolabeling in corresponding tissue compartments (Figure S2 and Table III) shows that the predominance of the stromal PGRMC1 signal essentially results from the surface covered by the stroma by comparison with epithelial structures, rather than from differences in the percentage of positive cells between the two tissue compartments.

Second, to the best of our knowledge, our study shows for the first time that the intensity of PGRMC1 immunolabeling is maximal, in most samples, within the superficial part of the functional layer, both in the stromal and in epithelial cells. PGRMC1 immunolabeling then progressively decreases with depth within the functional layer, and in the basalis, by reduction either of the total amount of positive cells and/or of the intensity of the signal. This gradient is much more pronounced in proliferative endometria than in secretory endometria in which immunolabeling globally decreases. Changes in percentage of cells positive for *PGRMC1* mRNA roughly follow a similar pattern. Our findings support observations in the macaque model showing that PGRMC1 mRNA and protein were more abundant in the functionalis than in the basalis (C. S. Keator et al., 2012). We further show that expression is not homogenous throughout the whole thickness of the functionalis suggesting that spatial regulation of PGRMC1 expression is not limited to differences between the functionalis and the basalis.

Occasional divergences between RNA and protein data within a same virtual tissue slide could result from the use of two different techniques targeting two different molecular products. Such discrepancies are in agreement with a recent article stating that the concordance rate between results obtained by RNAscope and immunolabeling varies between 58.7% and 95.3% (Atout, Shurrab, &

Loveridge, 2021). However, additional biological hypotheses can be formulated, such as a different half-life for the mRNA and the protein, or post-transcriptional regulatory mechanisms.

Variation of PGRMC1 protein and mRNA amounts along the menstrual cycle suggests that PGRMC1 expression is increased by estradiol and repressed by progesterone (combined with estradiol) in the human endometrium. This hypothesis is supported by fluctuations of endometrial PGRMC1 in *in vivo* models mimicking the menstrual cycle in the macaque (C. S. Keator et al., 2012) or using low-dose intrauterine administration of the progestin levonorgestrel in patients with abnormal uterine bleeding, resulting in substantial decrease in PGRMC1 expression after 6 months (Sletten et al., 2020). Be that as it may, fluctuating concentration of the steroid hormones may explain the temporal control of PGRMC1 expression, but additional levels of regulation responsible for spatial adjustment remain to be identified.

A previous study concluded that levels of *PGRMC1* mRNA and of PGRMC1 immunolabeling in stromal cells were reduced during the secretory phase in patients with endometriosis by comparison with control patients (Bunch et al., 2014). Such differences were not evidenced by our analysis. Although differences in technical approaches could be involved, it is important to note that we used the same primers for the quantitative PCR. The discrepancy could result from the heterogeneity between patients. Indeed, firstly, by using a larger panel of patients for our qPCR analysis, we noticed large interpatient variations within each phase of the cycle, both in patients with endometriosis/adenomyosis and in control patients. Secondly, interpatient variations were also evidenced by the immunolabeling, especially for the percentage of labeled cells during the secretory phase. Finally, we highlight differences in PGRMC1 expression according to tissue depth while the localization of the sections analyzed was not specified in the other study.

The major novelty of our work is to highlight the spatial adjustments of PGRMC1 expression, underscoring the importance to precisely consider local amounts of PGRMC1 when investigating its potential functions. Endometrial roles of PGRMC1 remain largely unknown but

two major topics have been approached: proliferation and decidualization. Experiments involving xenografts containing Ishikawa endometrial adenocarcinoma cells suggest that PGRMC1 supports cell growth (Friel et al., 2015). Indeed, PGRMC1 depletion resulted in reduced cell growth in the grafts. This is consistent with the spatiotemporal pattern of PGRMC1 expression evidenced in the present study, which could be related with cell proliferation. Decidualization (differentiation of endometrial stromal cells) and successful fertility seem to require appropriate amounts of PGRMC1. Indeed, reduced amounts of PGRMC1 during the secretory phase were required for receptivity (Salsano et al., 2017) but insufficient amounts were linked to miscarriage (Lyzikova et al., 2020). The peak of PGRMC1 expression in the proliferative phase would support a role as a mediator of estrogenic rather than progestin activity. Moreover, its preferential localization around the surface of the functional layer could be consistent with a stimulatory function of PGRMC1 on endometrial cell proliferation. It could therefore be hypothesized that PGRMC1 influences endometrial receptivity by participating in the molecular mechanisms necessary to prepare the endometrium for embryonic implantation. Although a decrease in PGMRC1 protein was observed in the endometrium of patients with endometriosis, most of these patients had at least one successful pregnancy. Additional work is therefore needed to elucidate the precise requirement and contribution of PGRMC1 in fertility.

In conclusion, we show that endometrial PGRMC1 expression is controlled both in time, with maximal expression in the late proliferative phase as reported before, and in space, with stronger expression occurring in the outermost layer of the endometrium to progressively decrease towards the basalis. PGRMC1 is expressed quite similarly in the stroma and in the epithelial structures, but appears essentially stromal due to predominance of this tissue. Although ovarian steroids may be suspected to control the global temporal variation of PGRMC1 expression, their action is not straightforward and further work is required to identify the additional levels of regulation responsible for local adjustment. This is particularly important in the light of recent studies highlighting crucial

roles of PGRMC1 and emphasizing the correlation between adequate PGRMC1 amounts and fertility (Lyzikova et al., 2020; Salsano et al., 2017).

Supplementary Materials:
Figure S1: Schematic representation of PGRMC1 signal quantification by Halo software.
Figure S2: Quantification of PGRMC1 immunolabeling.
Figure S3: Co-labeling of PGRMC1 protein and PGRMC1 mRNA in the cycling human endometrium.
Figure S4: Quantification of PGRMC1 mRNA evidenced by *in situ* hybridization.
Figure S5: Quantification of PGRMC1 immunolabeling in the human endometrium of patients with endometriosis/adenomyosis.

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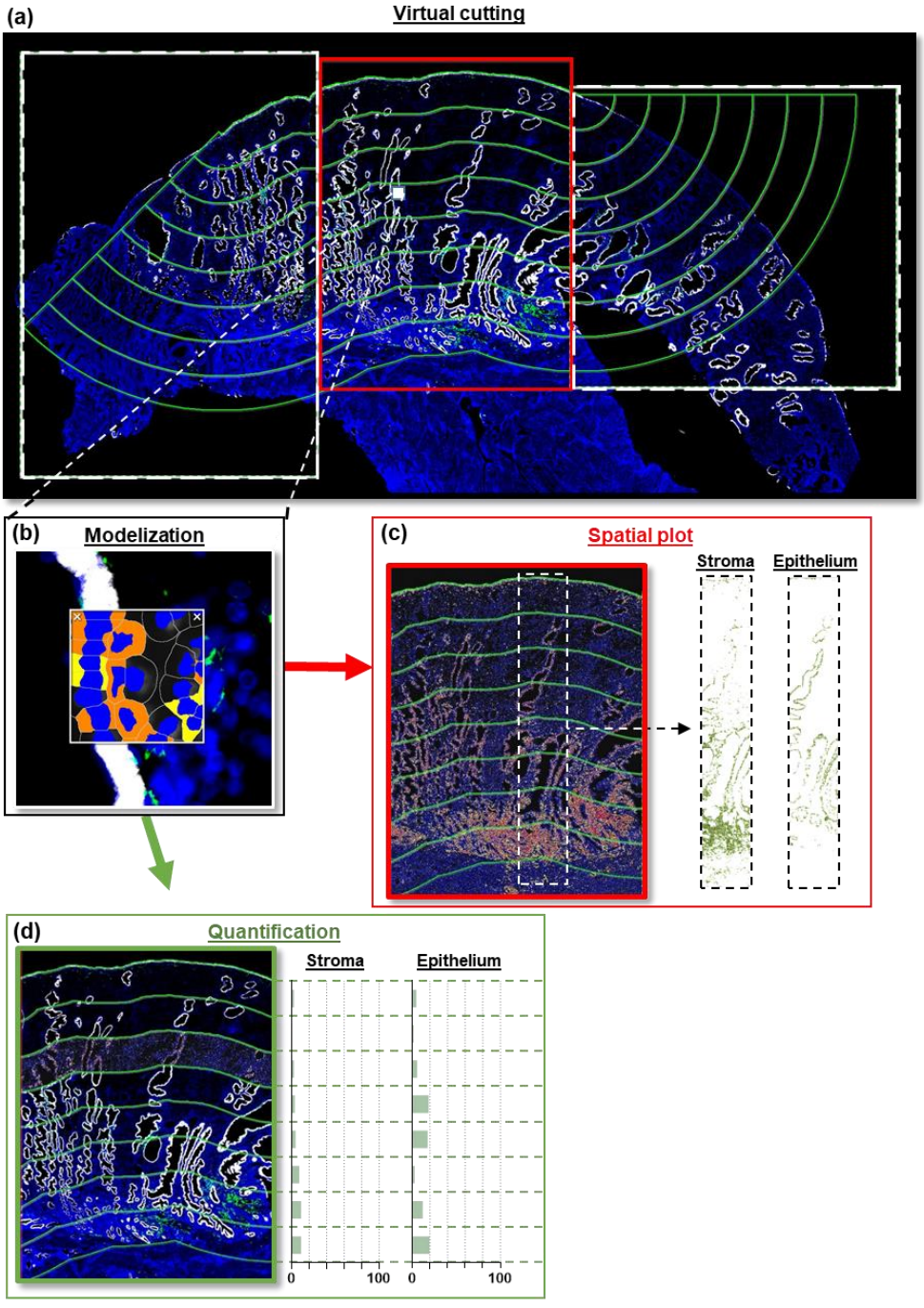
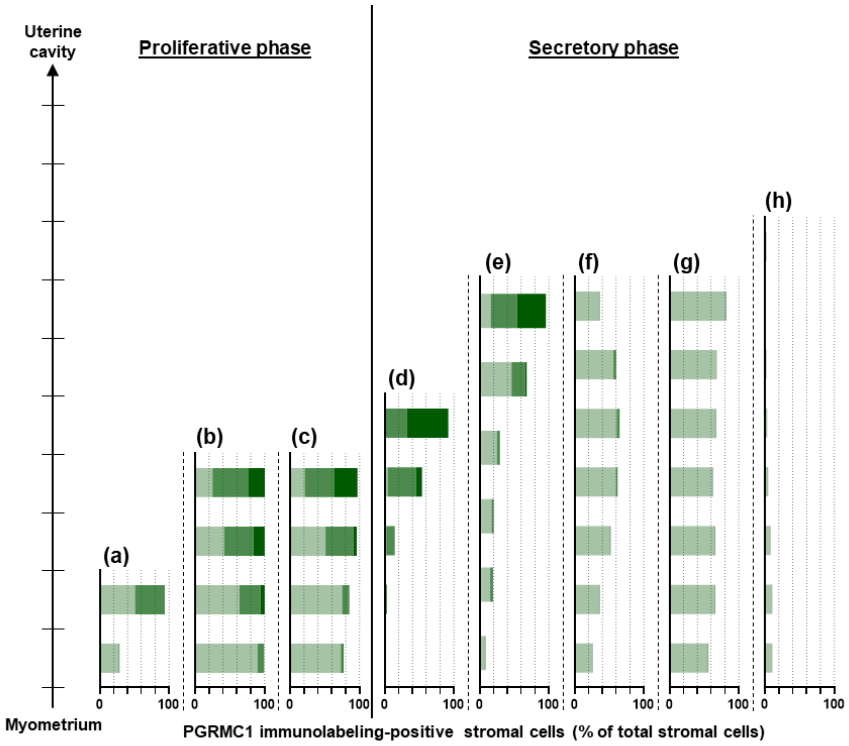

(a)
Virtual cutting
(b)
Modelization
(c)
Spatial plot
Stroma
Epithelium
(d)
Quantification
Stroma
Epithelium
0
100
0
100

Figure S1: Schematic representation of PGRMC1 signal quantification by Halo software.
(a) Selection of included (boxed in red) or excluded (boxed in white) tissue areas and virtual cutting (in green) of tissue slices. **(b)** Modelization of endometrial cells and categorization of endometrial cells according to the PGRMC1 signal intensity (transparent, negative cell; yellow, light intensity; orange, moderate intensity; red, strong intensity). **(c)** Spatial plot of a selected tissue section, with distinction between stromal and epithelial cells. Each point represents a modelized cell and the level of green reflects the intensity of the PGRMC1 signal for each cell. **(d)** Quantification of the modelized endometrial cells for separate virtual tissue slices within the whole included area of the sample. PGRMC1 signal was plotted according to the cell type (stromal or epithelial), the depth in the endometrium and with different shades of green to represent intensity thresholds (low, medium and high). Values are presented as a percentage of the total number of stromal or epithelial cells within each virtual tissue slice.


Uterine cavity
Proliferative phase
Secretory phase
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
0
100
Myometrium
PGRMC1 immunolabeling-positive stromal cells (% of total stromal cells)

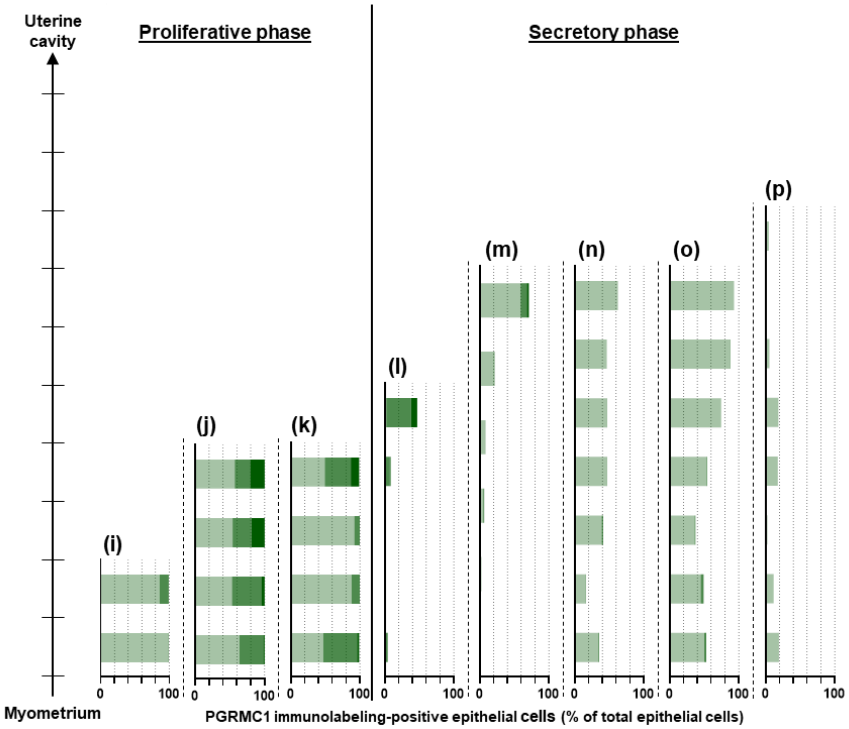

Uterine cavity
Proliferative phase
Secretory phase
(i)
(j)
(k)
(l)
(m)
(n)
(o)
(p)
0
100
Myometrium
PGRMC1 immunolabeling-positive epithelial cells (% of total epithelial cells)

Figure S2: Quantification of PGRMC1 immunolabeling.
Quantification results after modelization of whole tissue sections and virtual cutting of the tissue into 500-µm-thick slices. Endometrial samples are identical and presented in the same sequence as partial illustrations in Figure 2. PGRMC1 signal was quantified according to the intensity threshold (low, medium and high), the cell type (stromal, **a-h** or epithelial, **i-p**) and the depth in the endometrium. Values are presented as a percentage of the total number of stromal or epithelial cells within each virtual tissue slice.

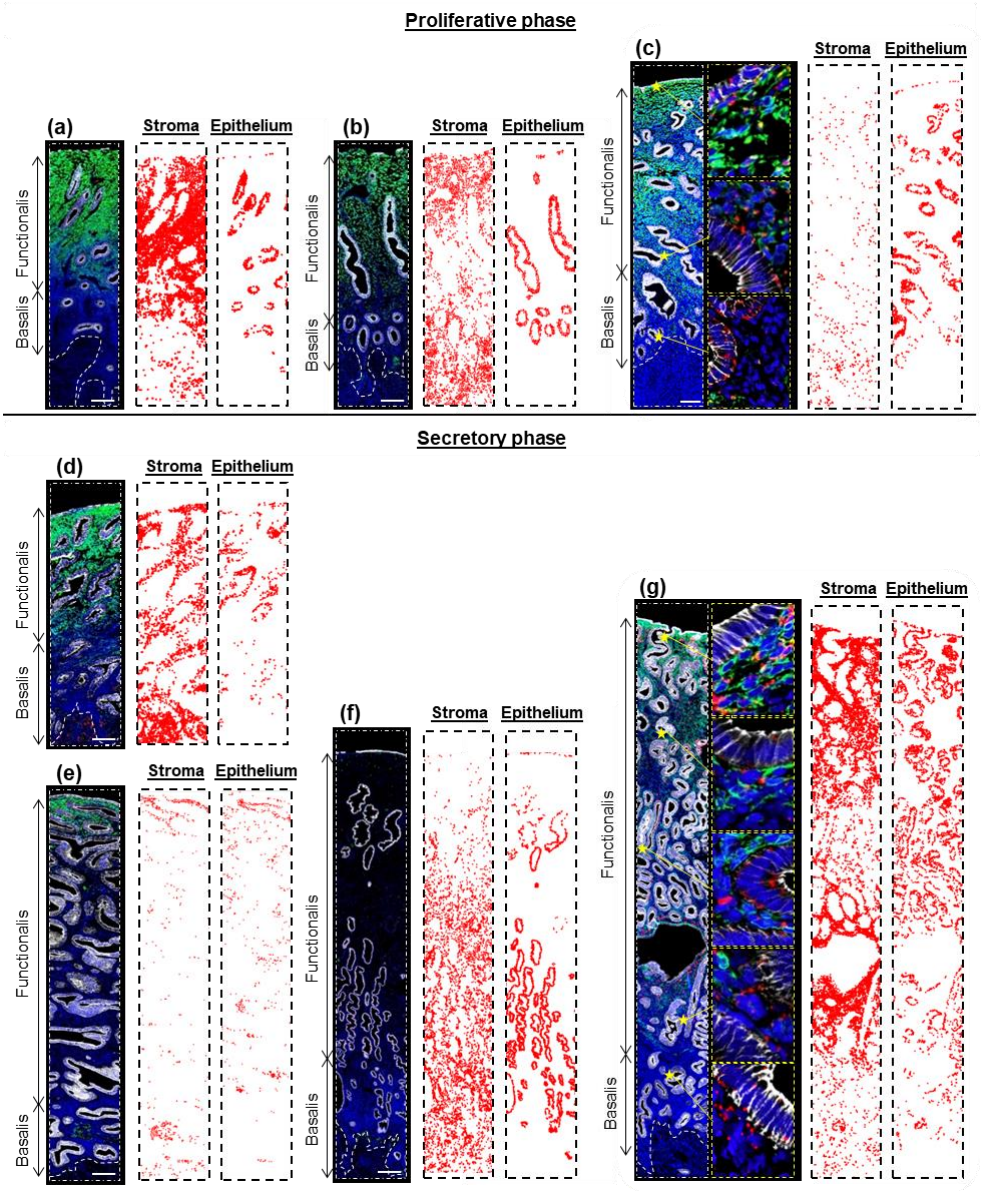

Proliferative phase
(a)
Stroma
Epithelium
Functionalis
Basalis
(b)
Stroma
Epithelium
Functionalis
Basalis
(c)
Stroma
Epithelium
Functionalis
Basalis
Secretory phase
(d)
Stroma
Epithelium
Functionalis
Basalis
(e)
Stroma
Epithelium
Functionalis
Basalis
(f)
Stroma
Epithelium
Functionalis
Basalis
(g)
Stroma
Epithelium
Functionalis
Basalis

Figure S3: Co-labeling of PGRMC1 protein and *PGRMC1* mRNA in the cycling human endometrium.

The *PGRMC1* mRNA was localized by *in situ* hybridization simultaneously with PGRMC1 immunolabeling in a selection of tissue sections shown in Figure 2. Panels **a-c** correspond to Figure 2**b-d** respectively (proliferative endometria) and panels **d-g** correspond to Figure 2**e**, **f**, **h** and **j** respectively (secretory endometria). Each panel presents, on the left, an image (on dark background) of the co-labeling (protein in green, mRNA in red and E-cadherin in white). The nuclei were stained in blue. In panels **c** and **g**, selected areas indicated by yellow stars are shown with magnification. Scale bar = 200µm, or 20µm for the enlargements. In each panel, images on white background present spatial plots of *PGRMC1* mRNA signal after modelization, with a distinction for stromal and epithelial cells. Each point represents a modelized cell.

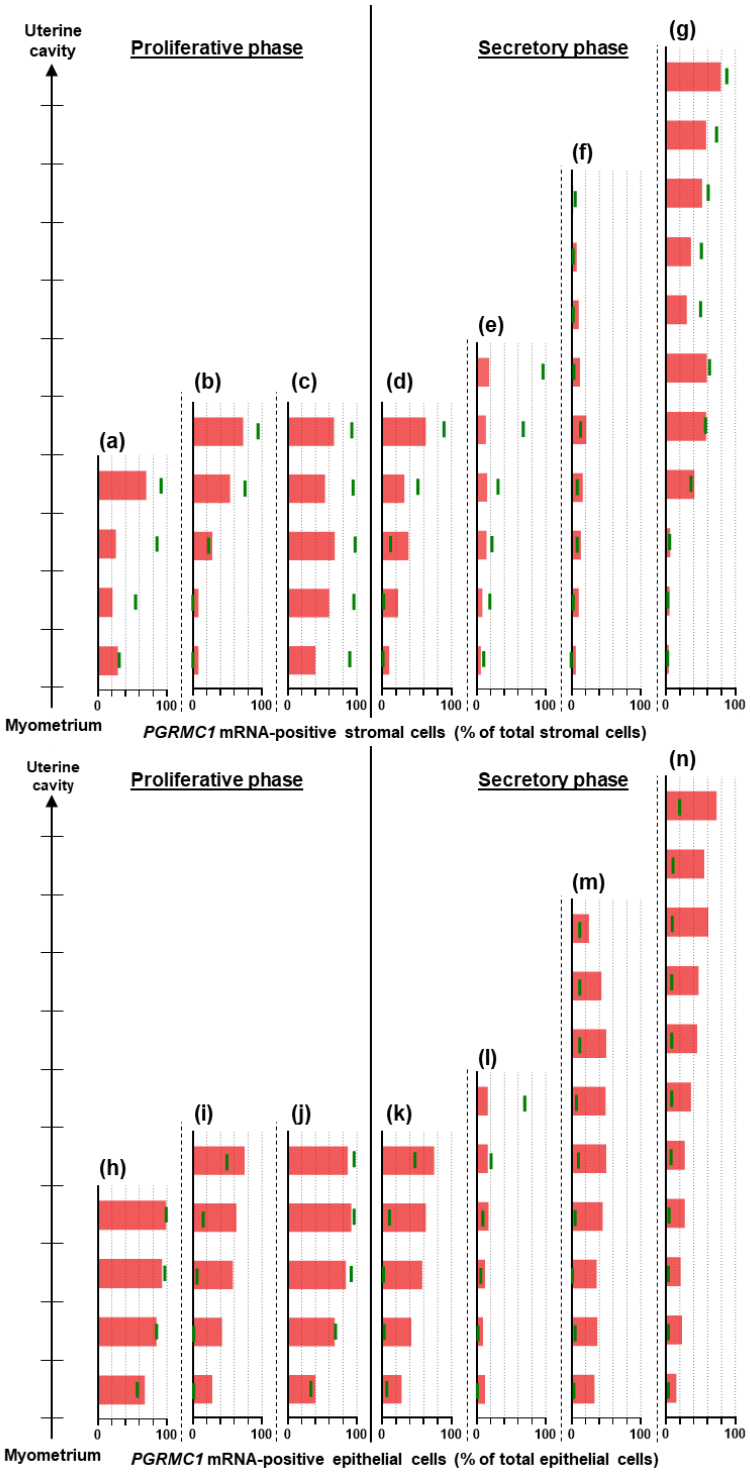

Uterine cavity
Proliferative phase
Secretory phase
(a)
(b)
(c)
(d)
(e)
(f)
(g)
0
100
Myometrium
PGRMC1 mRNA-positive stromal cells (% of total stromal cells)
Uterine cavity
Proliferative phase
Secretory phase
(h)
(i)
(j)
(k)
(l)
(m)
(n)
Myometrium
PGRMC1 mRNA-positive epithelial cells (% of total epithelial cells)

Figure S4: Quantification of *PGRMC1* mRNA evidenced by *in situ* hybridization. Quantification results after modelization of whole tissue sections and virtual cutting of the tissue into 500-µm-thick slices. Endometrial samples are identical and presented in the same sequence as partial illustrations in Figure 4. Cells positive for *PGRMC1* mRNA (in red) were quantified according to the cell type (stromal or epithelial) and the depth in the endometrium. Values are presented as a percentage of the total number of stromal or epithelial cells within each virtual tissue slice. For the sake of comparison, cells positive for PGRMC1 immunolabeling were quantified simultaneously and values are presented as green lines.

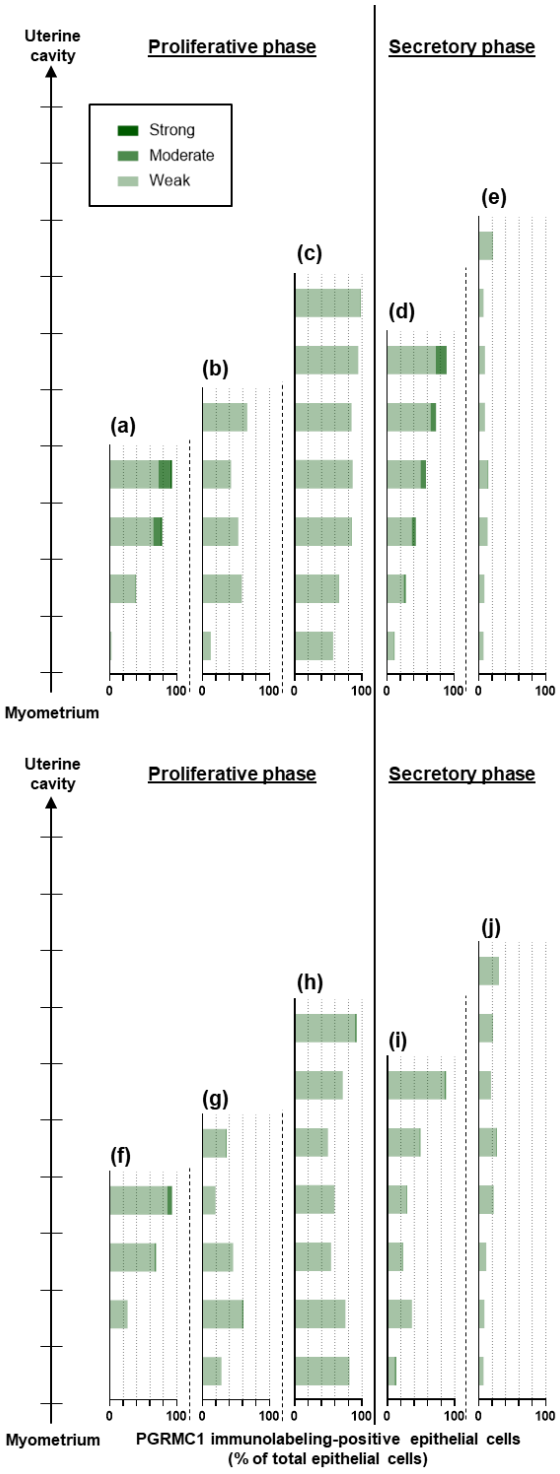

Uterine cavity
Proliferative phase
Secretory phase
Strong
Moderate
Weak
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
0
100
Myometrium
PGRMC1 immunolabeling-positive epithelial cells
(% of total epithelial cells)

Figure S5: Quantification of PGRMC1 immunolabeling in the human endometrium of patients with endometriosis/adenomyosis.
Quantification results after modelization of whole tissue sections and virtual cutting of the tissue into 500-µm-thick slices. Endometrial samples are identical and presented in the same sequence as partial illustrations in Figure 4. PGRMC1 signal was quantified according to the intensity threshold (low, medium and high), the cell type (stromal, **a-e** or epithelial, **f-j**) and the depth in the endometrium. Values are presented as a percentage of the total number of stromal or epithelial cells within each virtual tissue slice.

ii. Additional descriptive analyses

1. Characterization of PGRMC1 localization in the basal layer of the human endometrium

For the experiments investigating fluctuations of *PGRMC1* mRNA amounts by RT-qPCR in the cycling endometrium, only the functional layer of the endometrium was collected from the hysterectomy specimen because it is easily removed by simple scraping and because it usually represents the tissue part with the most drastic cyclical changes. On the other hand, sampling for histological sections can be performed in order to collect the full thickness of the tissue, from the surface epithelium to the myometrium, thereby allowing to specifically focus on PGRMC1 expression in the basal layer.

Consistent with the gradient in the intensity of the PGRMC1 signal from the uterine cavity to the myometrium, PGRMC1 expression in the basal layer was low compared to the functional layer. However, especially in the proliferative phase, some basal glands presented a strong PGRMC1 immunolabeling compared to the surrounding stroma (Figure 18). No link between the presence of this occasional expression pattern and age, absence/presence of treatment or clinical features of the patients concerned could be established from these data.

We hypothesized that PGRMC1 was overexpressed in epithelial stem cells located in the basal layer. Co-immunolabeling of PGRMC1 with the epithelial stem cell markers SSEA-1 and N-Cadherin was therefore considered (Salamonsen et al., 2021). However, at this point of the work in progress, the procedure still requires some technical adjustments. Nevertheless, the potential preferential expression of PGRMC1 in basal epithelial stem cells represents an interesting and still unconsidered perspective for the role of PGRMC1 in the endometrium.

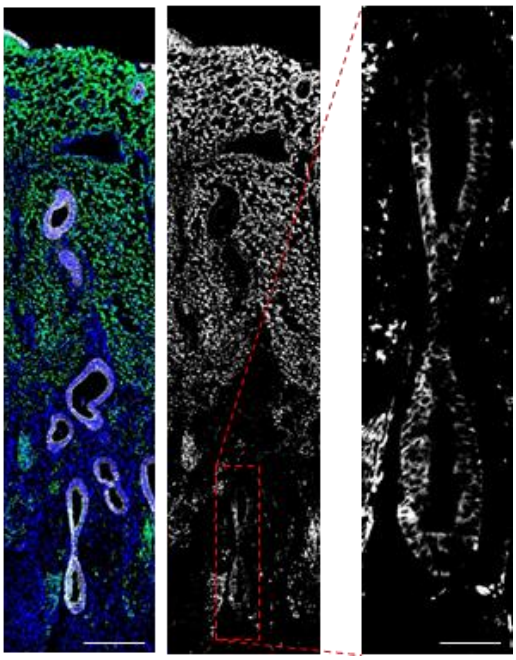


Figure 18: Immunolabeling of PGRMC1 in basalis of human endometrium.
Immunofluorescence of PGRMC1 (in green or white in negative image) and epithelial marker E-cadherin (in white). The nuclei were stained in blue. A field with representative PGRMC1 signal occasionally observed in the basalis epithelium is selected (red dotted delineation) and shown with magnification. Scale bar = 200µm or 50µm for the enlargement.

2. Characterization of the expression and tissue localization of PGRMC1 in endometriosis lesions

The progression of the study of the mRNA amounts, protein abundance and localization of PGRMC1 in endometriosis lesions faced several difficulties.

Firstly, collecting a sample of eutopic endometrium at the time of lesion removal would have allowed precise assessment of the cycle phase and direct comparison between eutopic and ectopic endometrium. However, simultaneous eutopic endometrial biopsy was usually not ethically justified and was only performed for a limited number of patients.

Secondly, because hormonal treatments could substantially modify PGRMC1 expression, ideal samples would originate from untreated patients.

Finally, since PGRMC1 can be expressed in lesion-hosting tissue, precise distinction between the lesion and the local tissue is required. Markers commonly used to identify endometrial stromal cells such as vimentin and CD10 were tested in several categories of endometriosis lesions (ovarian and DIE), in order to assess their specificity towards endometrial stroma. However, neither vimentin nor CD10 has proven to be sufficiently reliable to be used to discriminate endometriotic stroma from host tissue. Consequently, we chose to work without discriminating markers and to restrict our study to peritoneal lesion samples where the proportion of non-endometriotic tissue is estimated to be negligible.

We obtained from the UCLouvain Biobank two samples of peritoneal endometriosis lesions from patients without progestin treatment, coupled with simultaneous samples of corresponding eutopic endometrium. One patient was in proliferative phase and the

other in secretory phase (Figure 19b and c). A comparative analysis of the paired eutopic and ectopic endometria was performed (Figure 19a, b).

In both ectopic samples, PGRMC1 immunolabeling presents some intra-tissue variability. In some fields, PGRMC1 protein appears more localized in the epithelium while in others, the signal intensity seems more intense in the stroma. Nevertheless, at the scale of a whole section, the expression profile is essentially characterized by a predominant epithelial PGRMC1 signal (Figure 19b and c).

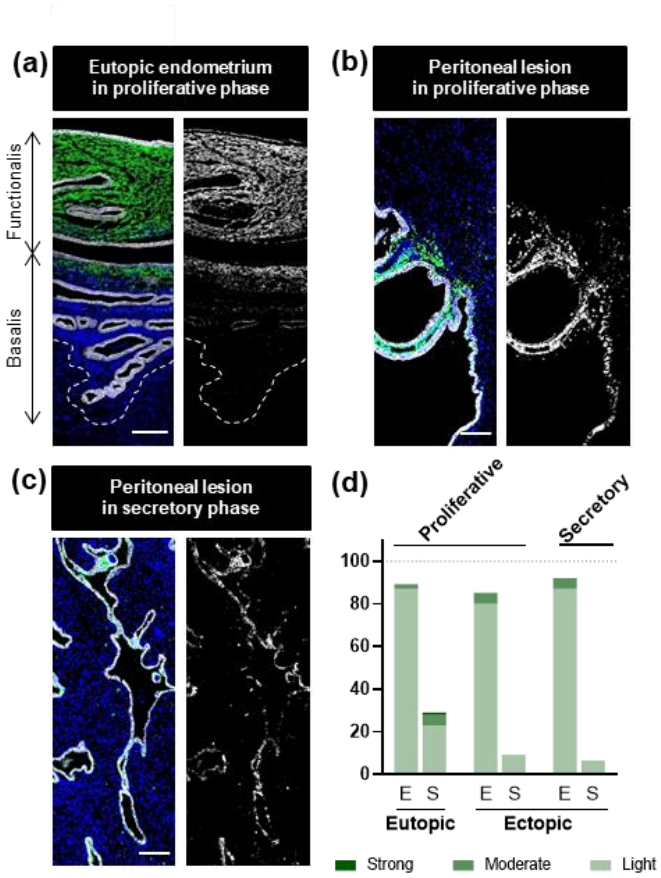


Figure 19: Immunolabeling of PGRMC1 in peritoneal lesion and one associated eutopic endometrium.

(a, b, c) Immunofluorescence of PGRMC1 (in green or white in negative image) and epithelial marker E-cadherin (in white) in associated eutopic **(a)** and ectopic **(b)** endometrium from patient in proliferative phase and in peritoneal lesion in second patient in secretory phase **(c)**. The nuclei were stained in blue. Scale bar = 200µm. **(d)** The PGRMC1 signal was quantified according to the intensity threshold and the cell type (epithelial, E or stromal, S). Values are presented as mean of the percentage of the total number of stromal or epithelial cells.

The signal was further analyzed through modelization and was quantified by the same procedure as for patients without endometriosis (see section **III.a**.*i;* Material & Methods) with the help of the Halo software (Figure 19d). PGRMC1 immunostaining was both more intense and proportionally more represented in the epithelium of peritoneal lesions than in the stroma. Surprisingly, the tissue localization and the proportion of cells expressing PGRMC1 were equivalent in the two samples although they corresponded to a proliferative and a secretory phases. Finally, in comparison with the eutopic endometrium, the intensity of the PGRMC1 signal per cell was higher in the ectopic epithelium and the signal intensity as well as the percentage of cells expressing PGRMC1 was lower in the ectopic stroma.

B. ESTRADIOL AND/OR PROGESTERONE INITIATE TEMPORAL VARIATIONS IN *PGRMC1* EXPRESSION IN THE HUMAN ENDOMETRIUM

The similarity between the variations in PGRMC1 expression and those in blood estradiol and progesterone concentrations during the menstrual cycle prompted us to hypothesize that endometrial PGRMC1 expression was timely regulated by estradiol and/or progesterone.

Two previous studies have shown or indicated some direct correlation between hormone intake and PGRMC1 expression in the endometrium:

- Exogenous administration of estradiol stimulated and progesterone repressed PGRMC1 expression in the endometrium of ovariectomized macaques (Christopher S. Keator et al., 2009).
- The progestin levonorgestrel repressed PGRMC1 expression in the endometrium of patients subjected to 6 months of treatment with an intrauterine device (Sletten et al., 2020).

In order to address the question of the potential hormonal regulation of PGRMC1 expression during the menstrual cycle and decipher the underlying mechanisms and signaling pathways, I measured the variations of PGRMC1 expression in a mouse model of human endometrial xenograft mimicking physiological hormonal fluctuations (**III.**B.*ii*) and in endometrial cell culture (**III.**B.*iii*). However, these two approaches were only undertaken after additional descriptive analyses in the human endometrium, aiming to correlate PGRMC1 expression and/or localization with hormone receptors whose regulatory mechanisms are better described (**III.**B.*i*).

i. PGRMC1 and progesterone receptors in the human endometrium

To identify potential mechanisms of co-regulation of PGRMC1 expression with other progesterone receptors present in the human endometrium, the expression and tissue localization of nPR (*PGR* gene), mPRα (*PAQR7* gene), and PGRMC2 were determined by RT-qPCR (Figure 20) and immunofluorescence (Figures 21, 22, and 23), respectively, during the menstrual cycle.

1. Expression of progesterone receptors in the human endometrium

Our RT-qPCR results show that the expression of *PGRMC2* and *PAQR7* did not vary during the menstrual cycle (Figure 20a). On the opposite, the variations of *PGR* expression during the cycle shared some similarities with those of *PGRMC1*. Indeed, *PGR* expression increased from the menstrual phase to the proliferative phase and then decreased from the proliferative phase to the secretory phase. Although *PGR* mRNA levels in the menstrual and secretory phases were found to be equivalent while *PGRMC1* expression was lower in the secretory phase, a possible correlation between the expression of these two genes was identified (Figure 20b). Overall, these results suggest that *PGRMC1* and *PGR* may share regulatory mechanisms of their endometrial expression, at least with respect to the temporal dimension (phases of the menstrual cycle).

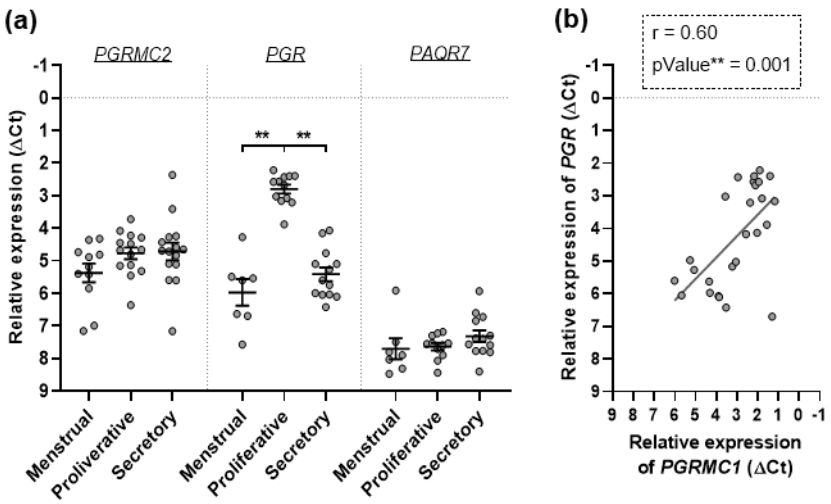


Figure 20: Relative expression of *PGRMC2*, *PGR* and *PAQR7* in human endometrium.

(a) Relative concentrations of *PGRMC2*, *PGR* and *PAQR7* mRNA in functional layer of endometrium collected during the menstrual, proliferative or secretory phase were measured by RT-qPCR, normalized according to HKGs and are presented as dots for individual values (ΔCt), and as means ± SEM. Statistical test: Kruskal-Wallis with Dunn's post-hoc, only statistically significant differences are shown. ** pValue < 0.01 **(b)** Pearson correlation test between *PGRMC1* and *PGR* expressions in the same samples from patients in proliferative and secretory phases exposed in **(a)**. The results were presented as dots for individual values (ΔCt) with the simple linear regression.

2. Immunolocalization of progesterone receptors in human endometrium

By immunofluorescence, nPR, mPRα, and PGRMC2 proteins were co-labeled with PGRMC1 protein, respectively, in human endometrium in the proliferative and secretory phases, in a selection of previously referenced samples (Materials & methods **III.a.***i.*).

In agreement with RT-qPCR results, immunolabeling of mPRα and PGRMC2 showed no variation with cycle phase nor depth in the tissue (results not presented).

In contrast, nPR immunostaining varied during the menstrual cycle and according to tissue compartment: the signal appeared more intense in proliferative phase than in secretory phase. Moreover, it was more intense in the epithelium than in the stroma during the proliferative phase (Figure 21).

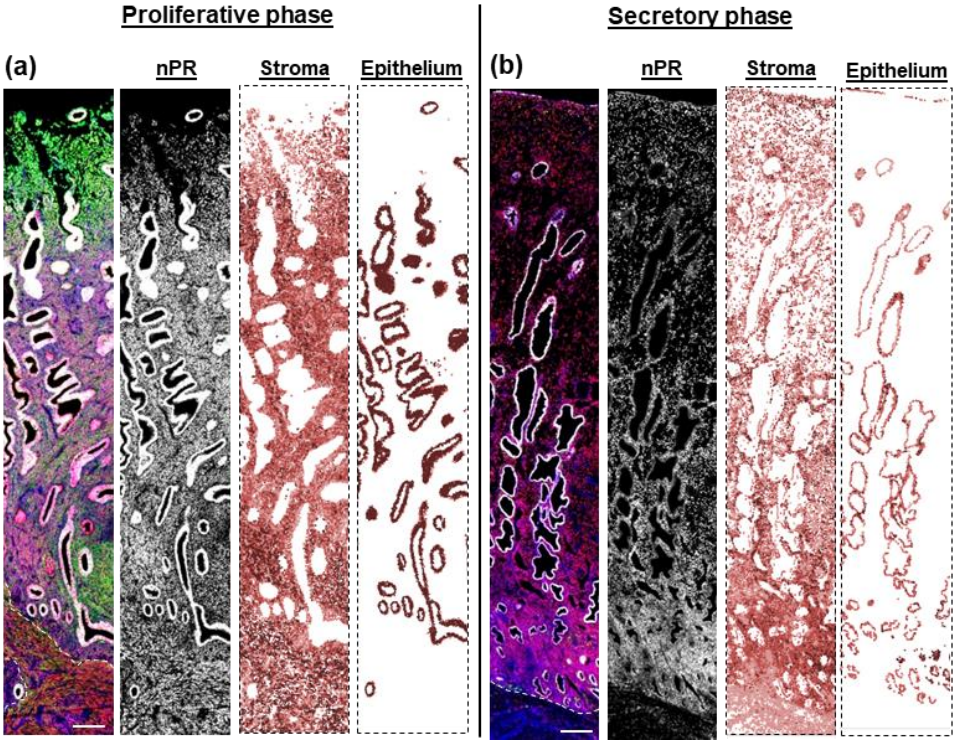


Figure 21: Co-immunolocalization of PGRMC1 and nPR in human endometrium. PGRMC1 (in green), nPR (in red or in white in negative images) and epithelial marker E-cadherin (in white) were co-immunolocalized in endometrium collected during the proliferative **(a)** or secretory **(b)** phase. The nuclei were stained in blue. Scale bar = 200µm. Corresponding spatial plots (in red) of cells expressing nPR, with distinction for stromal and epithelial cells are presented. Each point represents a modelized cell and the level of red reflects the intensity of the nPR signal for each cell.

Interestingly, a gradient of nPR signal intensity was detected as a function of tissue depth in the epithelium of the secretory endometrium (Figure 22). However, in contrast to PGRMC1 labeling, epithelial nPR expression increases progressively from the surface epithelium to the basal glands.

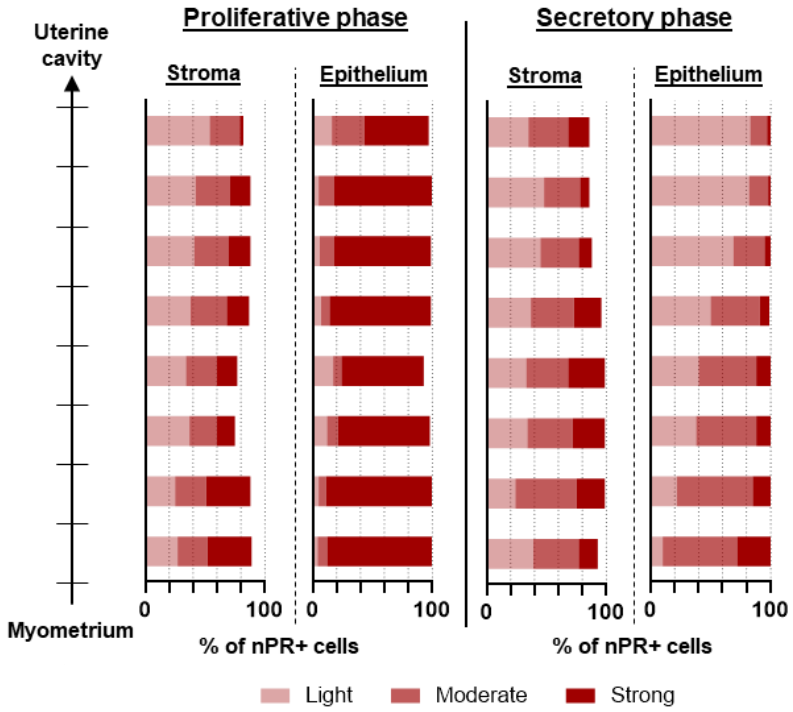


Figure 22: Quantification of nPR immunolabeling.
Quantification results of cells expressing nPR after modelization of whole tissue sections and virtual cutting of the tissue into 500-µm-thick slices. Endometrial samples are identical and presented in the same sequence as partial illustrations in Figure 21. nPR signal was quantified according to the intensity threshold (low, medium and high), the cell type (stromal or epithelial) and the depth in the endometrium. Values are presented as a percentage of the total number of stromal or epithelial cells within each virtual tissue slice.

On the other hand, the quantification of the nPR signal of two endometrial samples in proliferative phase and four samples in secretory phase shows that the percentage of cells immunolabelled for nPR was very high during both phases and highlights a higher proportion of intensely labelled epithelial cells during the proliferative phase (Figure 23).

Finally, double immunolabeling suggests that almost all cells expressing PGRMC1 also expressed the nPR protein (Figure 23). This proportion was also significantly higher in the epithelium than in the proliferative stroma and significantly higher in the proliferative stroma than in the secretory stroma. However, the lower proportion of cells immunolabeled for PGRMC1 in the secretory phase (see **III.a.***i.*; Figure 9) most likely accounts for the differences observed between proliferative and secretory phases.

Therefore, the similarities in expression and localization of PGRMC1 and nPR proteins during the menstrual cycle support the hypothesis of hormonal regulation by estradiol and/or progesterone. Furthermore, the presence of a gradient of nPR expression according to the depth of the endometrium in the secretory epithelium suggests a potential progesterone-mediated repression of PGRMC1 expression by nPR.

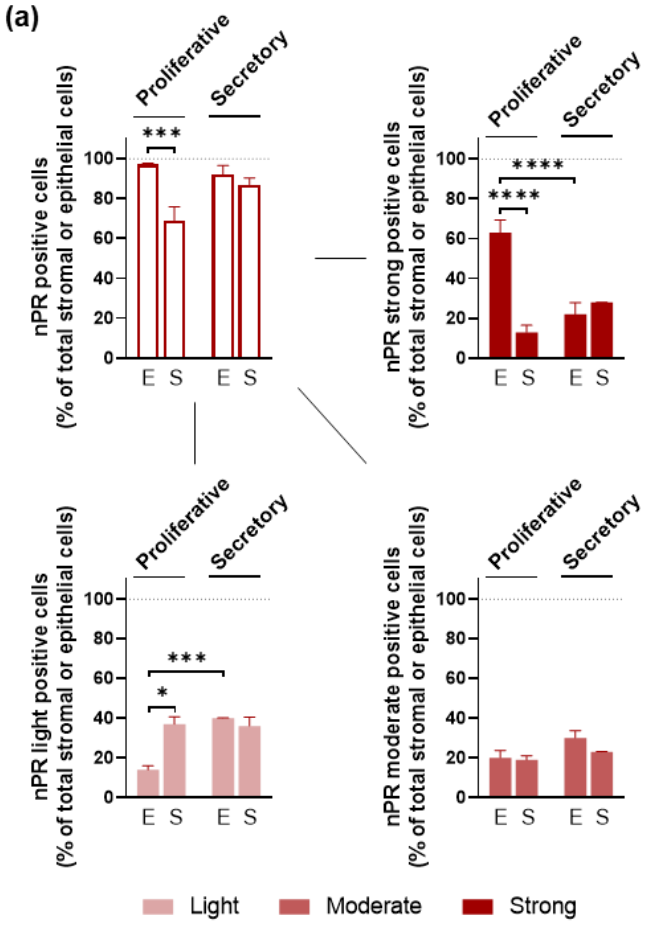

(a)
Proliferative
Secretory
nPR positive cells
(% of total stromal or epithelial cells)
nPR strong positive cells
(% of total stromal or epithelial cells)
nPR light positive cells
(% of total stromal or epithelial cells)
nPR moderate positive cells
(% of total stromal or epithelial cells)
E S E S
Light
Moderate
Strong
(b)
PGRMC1 and nPR positive cells
(% of PGRMC1 positive
stromal or epithelial cells)

Figure 23: Quantification of nPR and PGRMC1 co-immunolabeling.
(a) Comparison of endometrial nPR immunolabeling between samples from patients in proliferative (n=2) and secretory phase (n=4). As previously described, the nPR signal was quantified in each virtual tissue slice according to the intensity threshold (low, medium and high) and the cell type (epithelial, E or stromal, S). Values are presented as mean ± SEM of the percentage of the total number of stromal or epithelial cells. **(b)** Proportion of endometrial cells co-expressing PGRMC1 and nPR in samples from patients in proliferative (n=2) and secretory phase (n=4). The co-labeled cells were quantified in each virtual tissue slice according to the cell type (epithelial, E or stromal, S). Values are presented as mean ± SEM of the percentage of the stromal or epithelial cells expressing PGRMC1. Statistical test: Anova two-way and post-hoc Tukey. Only statistically significant differences are shown. * $p < 0.05$, ** $p< 0.01$, *** $p< 0.001$, **** $p < 0.0001$.

ii. Study of hormonal regulation of PGRMC1 expression in a mouse model of human endometrial xenografts

In order to establish a direct correlation between hormonal supply of estradiol and/or progesterone, and PGRMC1 expression, we measured *PGRMC1* mRNA amounts in a human endometrial xenograft model used in the laboratory (Coudyzer et al., 2015).

1. Experimental model

In this model, human endometrial explants are grafted subcutaneously into the flank of previously ovariectomized SCID mice (Figure 24a). Pellets delivering estradiol and progesterone are also implanted in the back of the mice at the time of grafting.

After 21 days of graft establishment under these conditions (estradiol and progesterone), removal of the hormonal pellets causes pseudo-menstruation of the grafts characterized by induction of matrix metalloproteinases (notably *MMP-3* and *MMP-1*) and degradation of the extracellular matrix which can be monitored by type III fibrillar collagen immunostaining (Figure 24b-c). After 4 days of pseudo-menstruation, the implantation of a new hormonal pellet delivering estradiol or two hormonal pellets delivering estradiol and progesterone inhibits the production of MMPs and induces regeneration of the extracellular matrix (Figure 24d and e).

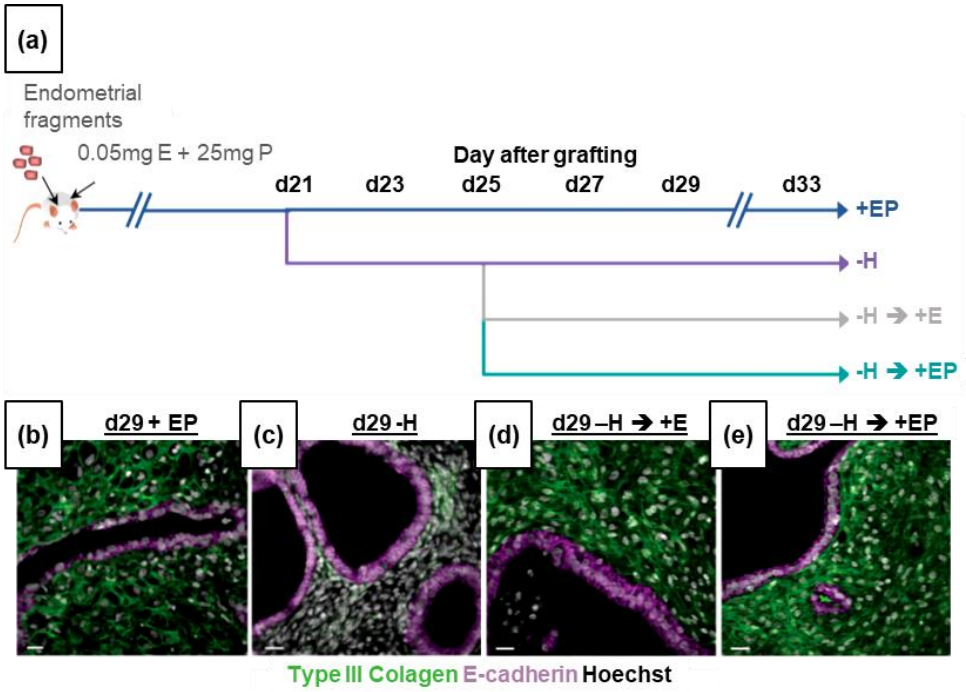


Figure 24: Experimental protocol and immunolocalization of Type III collagen in murine model of human endometrial xenograft.
(a) Experimental protocol. Human endometrium was grafted into SCID mice and estradiol and progesterone pellet (+EP) were implanted simultaneously (at day d0). Mice selected for our study were sacrificed at d27, d29 or d33 after hormone withdrawal at d21 (-H) and insertion of new E (-H, +E) or EP (-H, +EP) pellet at d25 **(b-e)** Immunolocalization of type III collagen fibers (in green) and epithelial marker E-cadherin (in pink) on sections from xenografted in the indicated conditions. The nuclei were stained in white. Scale bar: 20µm. *Adapted from (Coudyzer et al., 2015)*

Taking advantage of a collection of xenograft mRNAs available in the laboratory, we measured the concentration of *PGRMC1* mRNA in samples from the collection and compared it with the mRNA of *PGR*, *MMP-1* and *MMP-3*. Four mRNA samples were used for each of the following three conditions:

- After 6 days of hormone withdrawal (mice sacrificed at d27; -H).

- After 4 days of hormone withdrawal followed by 4-8 days of estradiol delivery through new pellet reinsertion (mice sacrificed at d29 or d33; -H, +E).

- After 4 days of hormone withdrawal followed by 4 days of estradiol and progesterone delivery through new pellets reinsertion (mice sacrificed at d29; -H, +EP).

2. Influence of estradiol and progesterone on *PGRMC1* expression

As expected, *PGR* mRNA concentration (Figure 25a) increased significantly upon addition of the estradiol-delivering pellet after 4 days of pseudo-menstruation by hormone withdrawal (-H, +E vs. -H), confirming the inducing role of estradiol. In contrast, the addition of pellets delivering estradiol and progesterone led to intermediate levels of *PGR* mRNA, confirming the repressive role of progesterone (-H, +EP vs -H, +E). Regarding the expression of *MMP-3* and *MMP-1* (Figure 25b and c), the addition of ovarian steroids, estradiol with or without progesterone, caused a reduction (significant or not, probably due to the small number of samples) in their expression.

No significant difference was observed between these three conditions for *PGRMC1* mRNA concentration (Figure 25d). However, compared to samples obtained after 6 days of hormone withdrawal, the *PGRMC1* mRNA concentration was ~2-fold higher in 3 out of 4 samples after new estradiol supply (-H, +E vs. -H) and all samples after new estradiol and progesterone supply contained lower *PGRMC1* mRNA concentrations than these three samples with estradiol (-H, +EP vs. -H, +E). In other words, only 1 sample out of 4 with a new estradiol pellet had a surprisingly lower *PGRMC1* mRNA concentration than the values of the four samples after hormone withdrawal.

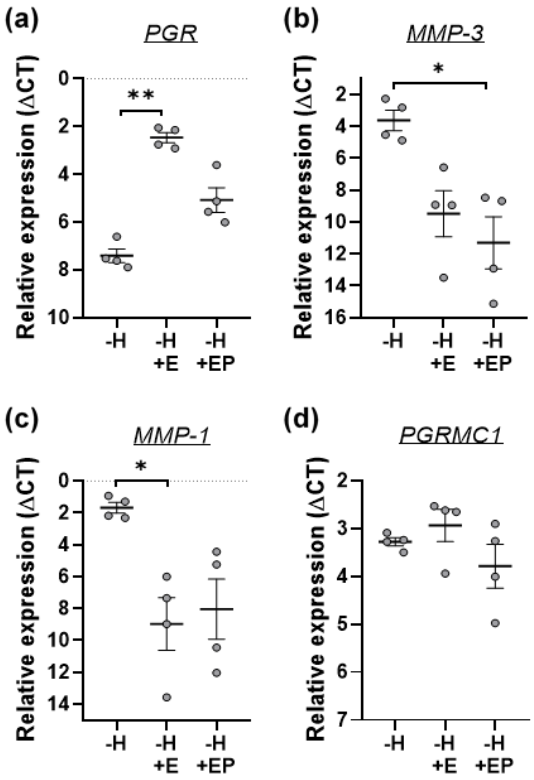


Figure 25: Relative expression of *PGRMC1*, *PGR*, *MMP-3* and *MMP-1* in a murine model of human endometrial xenografts in response to estradiol and/or progesterone re-exposure.

Human xenografted endometrial tissue was collected from mice after 6 days of pellet removal (-H), after 4 days of pellet removal and 4-8 days of estradiol pellet re-implantation (-H, +E) or after 4 days of pellet removal and 4 days of estradiol and progesterone re-implantation (-H, +EP). Relative concentrations of *PGR* **(a)**, *MMP-3* **(b)**, *MMP-1* **(c)** and *PGRMC1* **(d)** mRNA were measured by RT-qPCR, normalized according to HKGs and are presented as dots for individual values (ΔCt), and as means ± SEM. Statistical test: Kruskal-Wallis with Dunn's post-hoc, only statistically significant differences are shown. * pValue < 0.05 ** pValue < 0.01

These results provide mixed but encouraging information. Indeed, they do not confirm a clear hormonal regulation, as observed for *PGR* mRNA in this model, but they do not invalidate the hypothesis of an inducing role of estradiol and/or a repressive role of progesterone. Nevertheless, the number of xenograft samples available was too small to conclude.

At this point, it was decided to turn to cell culture because, besides being a more accessible experimental model, it would offer the advantage to allow paired comparison between experimental conditions (with or without hormones) unlike the animal model.

iii. Study of hormonal regulation of PGRMC1 expression in endometrial stromal cell cultures

During my thesis, many experiments were performed to establish a causal link between hormonal input and *PGRMC1* expression in human endometrial cell cultures. The initial experimental conditions were setup based on the literature and the laboratory's expertise in this field but the expression of *PGRMC1* was not modified, neither by estradiol nor by progesterone.

Therefore, multiple experimental features were adjusted, offering various choices (cell lines or primary cultures, epithelial or stromal cells, mono- or co-culture, low or high hormonal concentrations, progesterone or other progestins, variable incubation times, composition of the culture medium, ...). However, these changes did not modify *PGRMC1* expression which remained unsensitive to hormone addition in all conditions tested. These attempts are therefore not presented in detail. Only results obtained with primary stromal cells subjected for 72h to physiological concentrations of estradiol alone or estradiol combined with progesterone or the progestin MPA are presented to illustrate this part of my experimental work.

1. Experimental model

Preparations of endometrial cells in primary culture were obtained using a conventional procedure consisting of moderate proteolysis of the tissue followed by sequential filtration (Figure 26). A filtration through 300 µm diameter pores allows the retention and removal of insufficiently digested biomaterial, while a filtration through 30 µm pores then allows the separation of individualized stromal cells from epithelial structures preserved as fragments.

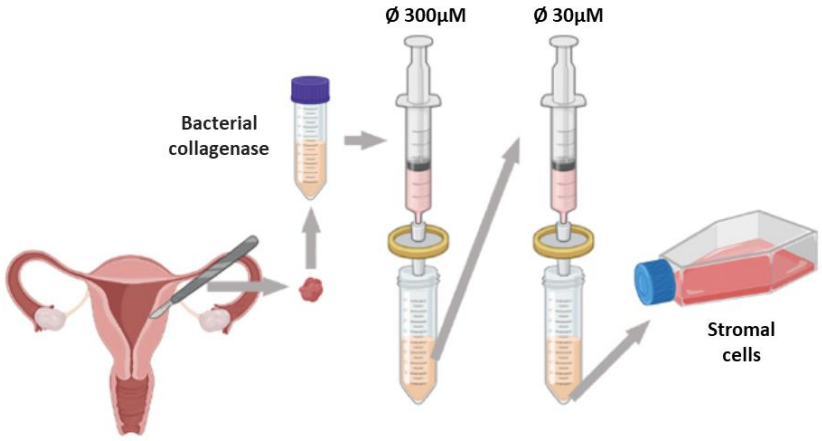


Figure 26: Experimental protocol of human endometrial primary cell culture.

In agreement with the decrease of the stromal signal observed in eutopic endometrium and endometriosis lesions, a particular interest was given to the repressive effect of progesterone on PGRMC1 expression in endometrial stromal cells. Only endometrial stromal cells were used for the following experiments.

The validity of the primary stromal cultures was assessed based on several criteria: (i) the purity of the primary stromal cultures, (ii) the expression of hormone receptors and (iii) the ability to respond to a hormonal stimulus.

(i) The proportion of stromal cells was systematically established by vimentin immunostaining (Figure 27). Only preparations with a proportion of vimentin-expressing cells greater than 95% were retained.

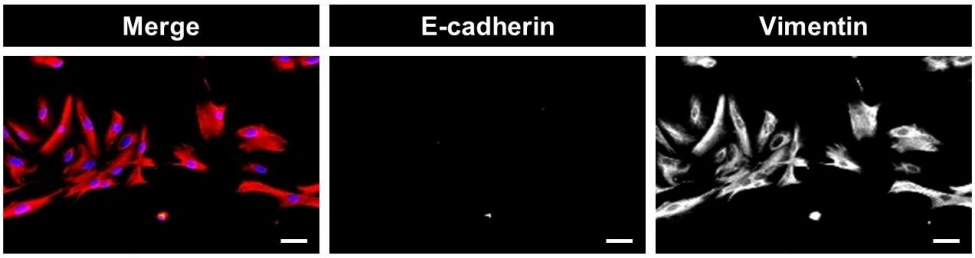


Figure 27: Evaluation of the purity of isolated primary stromal cells.
Immunolabeling of epithelial marker E-cadherin (in green or white in negative image) and stromal marker vimentin (in red or white in negative image) in primary stromal cells. The nuclei were stained in blue. Scale bar = 50μm.

(ii) The presence of nuclear receptors ER*α* and nPRs was punctually confirmed by immunostaining (Figure 28). The ER*α* signal appeared less noticeable in primary stromal cultures compared to progesterone receptors. However, it is unclear whether this was due to the quality of the antibody used or to a lower expression of ER*α* in our cultures.

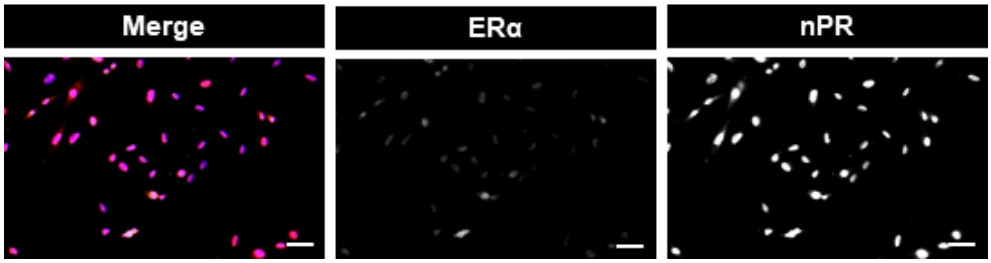


Figure 28: Evaluation of steroid receptors, ER*α* and nPRs expression in primary stromal cell culture.
Immunolabeling of ER*α* (in green or white in negative image) and nPR (in red or white in negative image) in primary stromal cells. The nuclei were stained in blue. Scale bar = 50μm.

(iii) Cell capacity to respond to hormones was estimated by RT-qPCR measurement of the expression of genes previously identified in the literature as being likely to vary according to hormonal supply. Therefore, the expression of the *PGR*, *MMP-1* and *MMP-3* genes was simultaneously measured together with that of *PGRMC1*.

2. Influence of estradiol on *PGRMC1* expression in primary stromal cell culture

The role of estradiol in the hormonal regulation of *PGRMC1* expression was assessed by comparing the expression levels measured in primary stromal cultures grown in the presence of 1nM estradiol or in the presence of the corresponding concentration of 2-hydroxypropyl β-cyclodextrin (HP-βCD) used to solubilize estradiol.

Compared with the control condition (HP-βCD), a significant increase in *PGR* expression was observed (Figure 29a). However, the magnitude of this increase was less than 2-fold in 3 out of the 9 samples tested and the *PGR* mRNA concentration was strongly increased in only one of them (by ~7.3x). *PGRMC1* expression also appeared to be stimulated by estradiol in this same sample but remained stable in the other primary endometrial stromal cultures (Figure 29b).

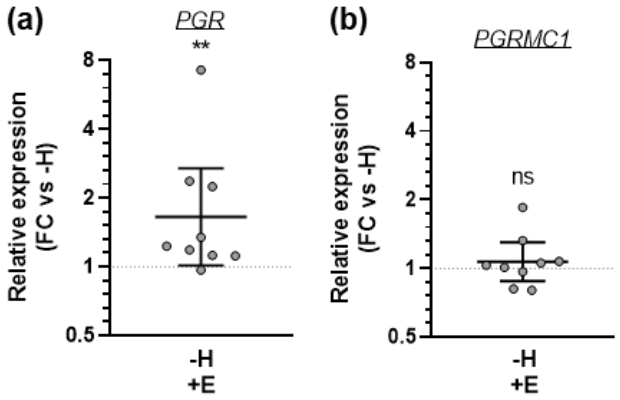


Figure 29: Evaluation of the effects of estradiol addition in primary stromal cell culture.

Primary stromal cells were incubated for 72h with estradiol (1nM; E) or with controls, HP-βCD in same concentration. Relative concentrations *PGR* **(a)** and *PGRMC1* **(b)** were measured by RT-qPCR and normalized according to HKGs. The values were compared to HP-βCD values and presented as individual Fold Changes (FC) in log2 scale and as geometric mean with geometric SD. Statistical test: Wilcoxon paired test, not significant (ns), ** $p < 0.01$.

3. Influence of progesterone and progestin MPA on PGRMC1 expression in primary stromal cell culture

In order to estimate the influence of progesterone and MPA on *PGRMC1* expression in primary stromal cultures, the expression levels measured in primary stromal cells cultured in the presence of 1nM estradiol combined with 100nM progesterone or 100 nM MPA were compared with those obtained in primary stromal cells cultured with estradiol alone.

HP-βCD and DMSO were used to solubilize progesterone and MPA, respectively. I first checked that addition of HP-βCD or DMSO at concentrations corresponding to progestin controls add not effect on *PGRMC1* expression (Figure 30). The results confirmed that the condition with 1nM estradiol could be used as a reference.

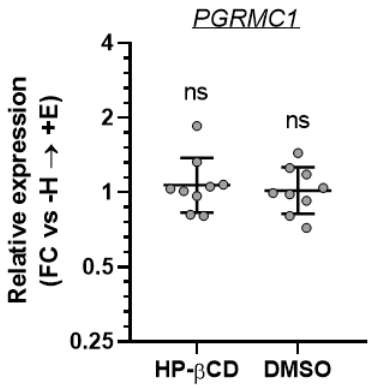


Figure 30: *PGRMC1* expression does not vary in endometrial stromal cells cultured with estradiol, HP-βCD or DMSO.
Primary stromal cells were incubated for 72h with estradiol (1nM; E) or with respective controls, HP-βCD or DMSO in appropriate concentrations. Relative concentrations of *PGRMC1* was measured by RT-qPCR and normalized according to HKGs. The values were compared to E values and presented as individual Fold Changes (FC) in log2 scale and as geometric mean with geometric SD. Statistical test: Wilcoxon paired test, not significant (ns).

By comparison with the control condition (1nM E), our results indicate that incubation of primary stromal cultures with 1nM estradiol combined with 100nM MPA significantly decreased the expression of *MMP-3* and *MMP-1* but not that of *PGR* (Figure 31a-c). However, expression of these three genes was not modified by addition of 100nM progesterone with 1nM estradiol (Figure 31a-c). Furthermore, it is important to note that the magnitude (~2-fold on average) of MMP repression remained below what was previously observed (Figure 25c-d) in the xenograft model developed in our laboratory (Coudyzer et al., 2015).

Compared to the control experimental condition (E), no significant change in *PGRMC1* expression was observed in primary stromal cells subjected to 1nM estradiol combined with 100nM progesterone or MPA (Figure 31d).

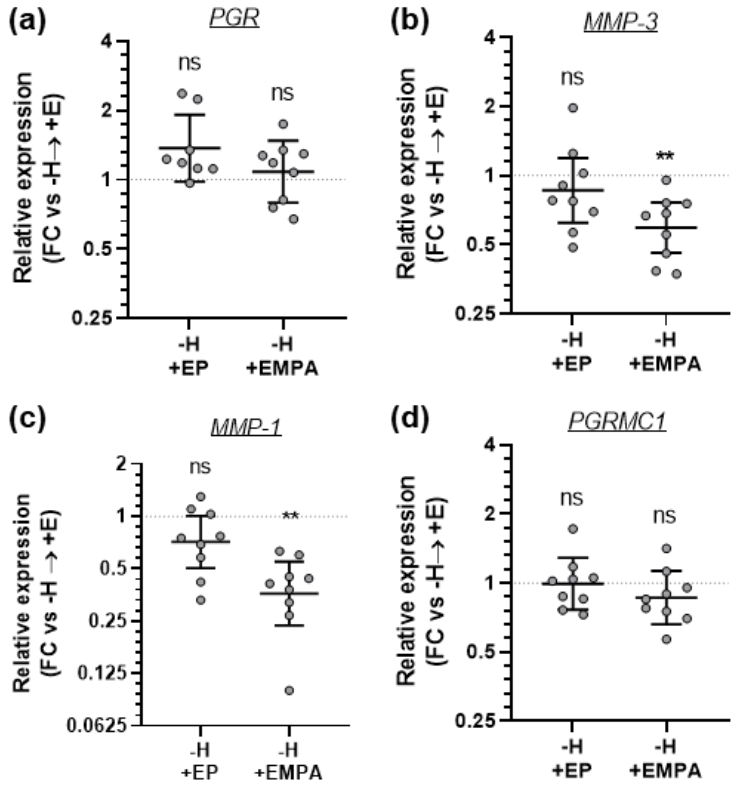


Figure 31: Evaluation of the effects of progesterone or MPA addition in primary stromal cell culture.

Primary stromal cells were incubated for 72h with estradiol (1nM; E), with estradiol and MPA progestin (1nM-100nM; E-MPA) or with respective controls, HP-βCD or DMSO in appropriate concentrations. Relative concentrations of *PGR* **(a)**, *MMP-1* and *MMP-3* **(a)** and *PGRMC1* **(c)** were measured by RT-qPCR and normalized according to HKGs. The values were compared to E values, used as experimental control, and presented as individual Fold Changes (FC) in log2 scale and as geometric mean with geometric SD. Statistical test: Wilcoxon paired test, not significant (ns), ** $p < 0.01$.

4. Influence of 8Br-cAMP on *PGRMC1* expression

The secretory phase of the menstrual cycle is induced in response to progesterone production in combination with estradiol. However, during the secretory phase, endometrial stromal fibroblasts undergo a particular modification, pre-decidualization, which can be mimicked in cell culture by adding cAMP simultaneously with the supply of estradiol and progesterone. We therefore hypothesized that cAMP could contribute to the regulation of *PGRMC1* expression in endometrial stromal cells.

To test this hypothesis, our primary stromal cultures were subjected for 72h to 0.5mM 8Br-cAMP in combination with 1nM estradiol and 100nM progesterone. In addition, an experimental condition in which only 8Br-AMPc was added to the culture medium was performed simultaneously in 2 experiments.

Cell decidualization was estimated by measuring the expression of the decidualization marker, *Prolactin*, as well as by morphological evaluation. In agreement with the literature, 8Br-AMPc increased *Prolactin* expression already in the absence of estradiol and progesterone and even more so in their presence (Figure 32a and b). Moreover, morphological analysis allowed identification of primary stromal cells with a decidualized appearance in the presence of estradiol and progesterone and/or 8Br-AMPc (Figure 32c).

Surprisingly, RT-qPCR analysis of *PGRMC1* expression indicated that the addition of 0.5mM 8Br-AMPc significantly stimulated *PGRMC1* expression (by just under 2x on average) in primary stromal cultures (Figure 32d). In contrast, the simultaneous addition of estradiol and progesterone did not appear to influence this effect (Figure 32e). These results suggest that cAMP, involved in stromal cell decidualization, is not responsible for the decrease of PGRMC1 expression during the secretory phase.

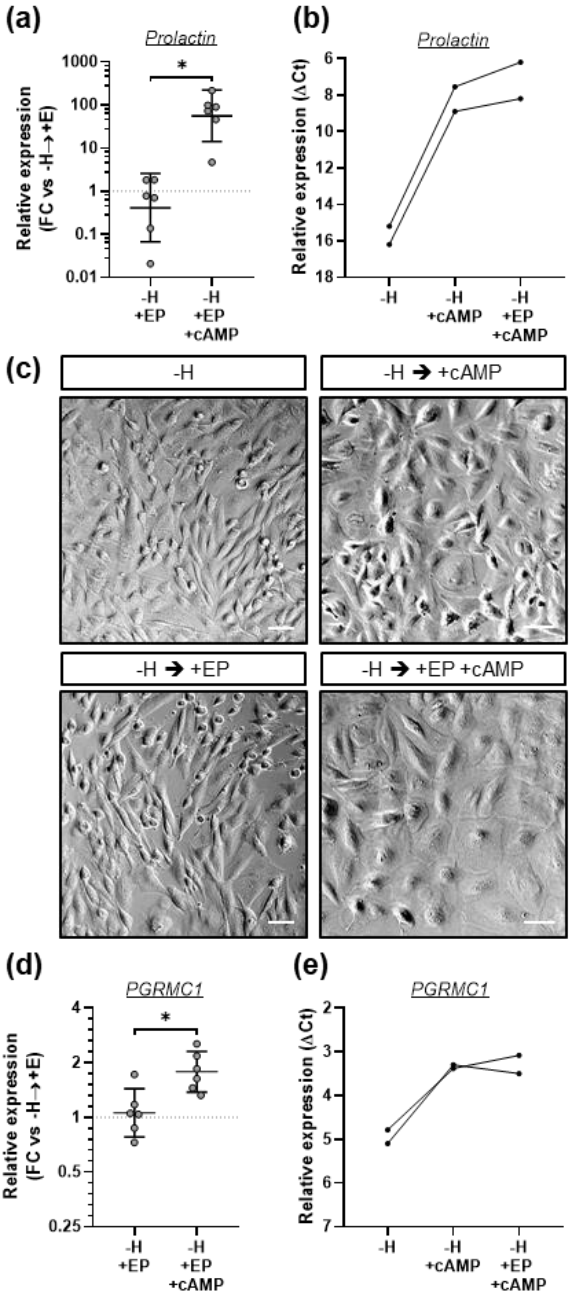

(a)
Prolactin
Relative expression (FC vs -H→+E)
1000
100
10
1
0.1
0.01
*
-H +EP
-H +EP +cAMP
(b)
Prolactin
Relative expression (ΔCt)
6
8
10
12
14
16
18
-H
-H +cAMP
-H +EP +cAMP
(c)
-H
-H ➔ +cAMP
-H ➔ +EP
-H ➔ +EP +cAMP
(d)
PGRMC1
Relative expression (FC vs -H→+E)
4
2
1
0.5
0.25
*
-H +EP
-H +EP +cAMP
(e)
PGRMC1
Relative expression (ΔCt)
2
3
4
5
6
7
-H
-H +cAMP
-H +EP +cAMP

Figure 32: Relative expression of *PGRMC1* in primary endometrial cells incubated with 8Br-cAMP combined or not with estradiol and progesterone.
Primary stromal cells incubated for 72h with 8Br-cAMP (0.5mM; +cAMP) combined or not with estradiol and progesterone (1nM-100nM; -H ➔ +EP) or with respective controls, without addition (-H) or with estradiol (1nM; -H ➔ +E). **(a-b; d-e)** Relative concentrations of *Prolactin* **(a,b)** and *PGRMC1* **(d,e)** were measured by RT-qPCR and normalized according to HKGs. **(a,d)** The values were compared to E values, used as experimental control, and presented as individual Fold Changes (FC) in log2 scale and as geometric mean with geometric SD. Statistical test: Wilcoxon paired test, not significant. * $p < 0.05$. **(b, e)** Due to the limited samples analyzed, the results are presented as paired dots for individual values (ΔCt) and no statistical analysis was performed. **(c)** Comparative morphometric analysis performed in primary stromal cells incubated in these different hormone conditions. Scale bar = 50µm.

C. ROLE(S) OF PGRMC1 IN THE ENDOMETRIUM

In parallel with the analyses aimed to establish the mechanisms of regulation of PGRMC1 expression, several experiments were conducted to define PGRMC1 functions in the human endometrium.

In order to evaluate the consequences of the decrease in PGRMC1 expression previously observed in eutopic and ectopic endometrial stroma on tissue functions, the major part of the experiments was performed in culture of endometrial stromal cells.

i. PGRMC1 and endometrial tissue remodeling

In accordance with the laboratory's research topic, a particular attention was given to the identification of potential functions of PGRMC1 related to endometrial tissue remodeling. Therefore, the role of PGRMC1 in endometrial cell proliferation and in the regulation of the expression of *MMP-3* and *MMP-1*, two MMPs that have a major role in cyclic endometrial remodeling, was briefly specifically investigated before turning to a broad-spectrum identification of potential PGRMC1 targets by RNA sequencing.

1. PGRMC1 modulates endometrial stromal cell proliferation

Although the role of PGRMC1 in cell proliferation has already been discussed in the literature, particularly in the context of cancer, it remains very poorly documented for the endometrium.

Consequently, descriptive (Figure 33, 34 and 35) and functional (Figure 36) experiments were performed to establish an indirect or direct correlation between PGRMC1 and the proliferation marker Ki67.

First, co-immunolabeling of Ki67 and PGRMC1 proteins was performed in two endometrial samples from patients in proliferative phase and in two samples in secretory phase (Figure 33). These

samples were selected among those presented previously (see Materials & methods **III.A**.*i*.). Quantification of epithelial and stromal cells expressing one and/or the other protein was then performed as described in the same section. The intensity of the Ki67 signal, however, was not taken into account in the quantification analyses.

Immunofluorescence results suggest that the Ki67 signal is mainly localized in the epithelium in proliferative phase. But more importantly, it seems to be more present in the most superficial layer of the endometrium to progressively decrease towards the basal layer, like the PGRMC1 signal. The enlargement of several fields from the surface epithelium to the basal layer highlighted that most of the cells expressing Ki67 were also immunolabeled for PGRMC1, particularly in the proliferative phase.

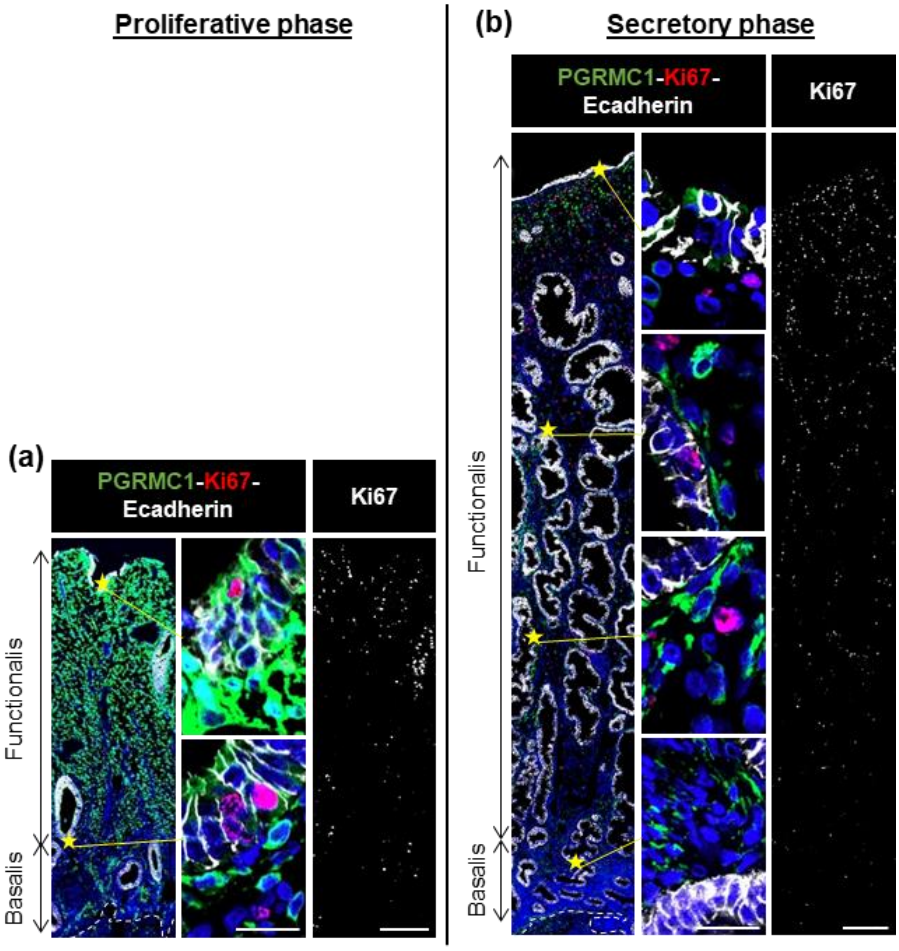


Figure 33: Co-immunolabeling of PGRMC1 and Ki67 in human proliferative and secretory endometrium.

Immunolabeling of PGRMC1 (in green), Ki67 (in red or white in negative images) and E-cadherin (in white) in endometrium from patients without endometriosis/adenomyosis, in proliferative **(a)** or secretory phase **(b)**. The nuclei were stained in blue. Scale bar = 200µm, 20µm for the enlargements. The localization of each magnified field in the depth of the endometrium is indicated by a star.

Modelling and quantitative analysis of cells immunolabelled for PGRMC1 and/or Ki67 performed with the help of the Halo software further support these observations. An intensity gradient is indeed visible from the most superficial layer of the endometrium to the basal layer. It is, moreover, much more perceptible in the epithelium in proliferative phase, when the proportion of cells expressing Ki67 is the highest (Figure 34).

Moreover, in the proliferating sample, the proportion of epithelial cells expressing Ki67 decreased throughout the functional layer from the surface epithelium and increased again in the basal layer. This rebound of positive epithelial cells in the basal layer was previously observed in the same proliferative sample for the PGRMC1 signal (see **III.A**.*i*.; Figure 3m), further emphasizing the matching between Ki67 and PGRMC1 expression profiles.

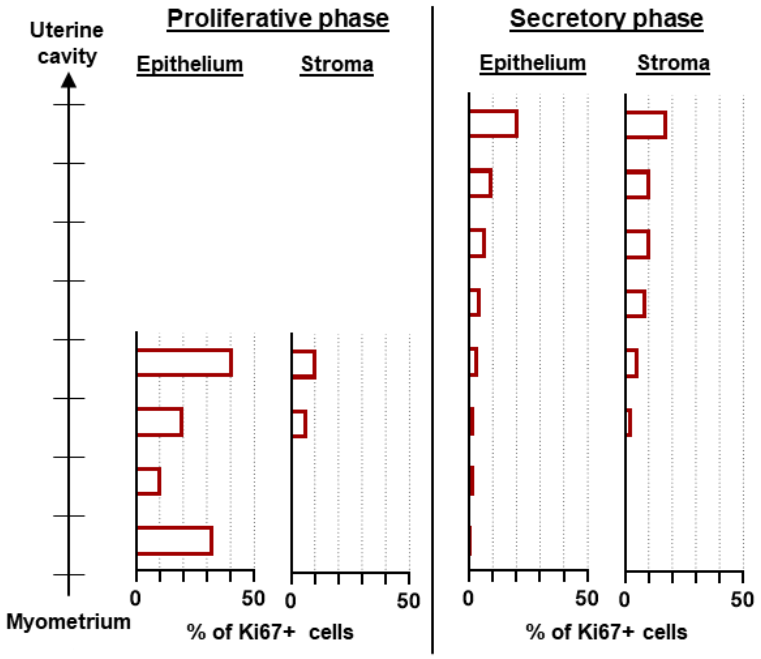


Figure 34: Quantification of Ki67 immunolabeling.
Quantification results of cells expressing Ki67 after modelization of whole tissue sections and virtual cutting of the tissue into 500-µm-thick slices. Endometrial samples are identical and presented in the same sequence as partial illustrations in Figure 33. Ki67 signal was quantified according to the cell type (stromal or epithelial) and the depth in the endometrium. Values are presented as a percentage of the total number of stromal or epithelial cells within each virtual tissue slice.

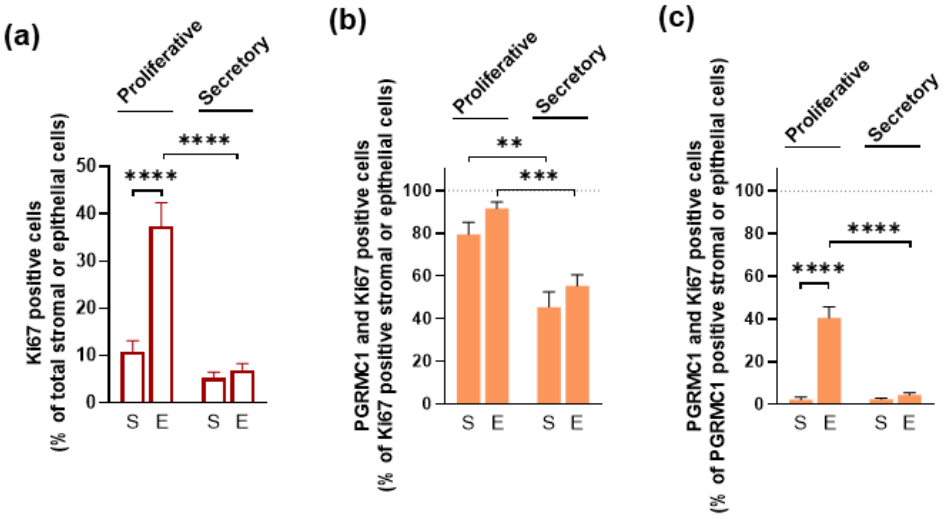


Figure 35: Quantification of Ki67 and PGRMC1 co-immunolabeling.
(a) Comparison of endometrial Ki67 immunolabeling between samples from patients in proliferative (n=2) and secretory phase (n=2). As in **a**, the Ki67 signal was quantified in each virtual tissue slice according to the cell type (epithelial, E or stromal, S). Values are presented as mean ± SEM of the percentage of the total number of stromal or epithelial cells. **(b, c)** Proportion of endometrial cells co-expressing PGRMC1 and Ki67 in samples from patients in proliferative (n=2) and secretory phase (n=2). The co-labeled cells were quantified in each virtual tissue slice according to the cell type (stromal, S or epithelial, E). Values are presented as mean ± SEM of the percentage of the stromal or epithelial cells expressing Ki67 **(b)** or PGRMC1 **(c)**. Statistical test: Anova two-way and post-hoc Tukey. Only statistically significant differences are shown. **** $p < 0.0001$.

Our results also indicate that the proportion of Ki67-expressing cells was significantly higher in the proliferative phase than in the secretory phase and higher in the epithelium than in the stroma in the proliferative phase (Figure 35a). Finally, quantitative analysis of cells co-immunolabeled with PGRMC1 and Ki67 pinpointed that regardless of the cycle phase and cell type, almost all cells that express Ki67 co-express PGRMC1 in proliferative endometrium (Figure 35b). The reverse was not true because the values were limited by the proportion of cells immunolabeled for Ki67 (Figure 35c).

These preliminary descriptive analyses thus highlight a parallelism in the respective expression profiles of PGRMC1 and Ki67 in human endometrium and suggest a correlation, particularly during the proliferative phase.

In a second step, experiments were performed in cell culture in order to determine the origin of this correlation and to identify the potential influence of PGRMC1 on endometrial cell proliferation (Figure 36).

Primary endometrial stromal cells were transfected with siRNA-CTL or siRNA-PGRMC1, previously validated in two endometrial cell lines (see **III.B**.*ii*.). Because the literature supports that PGRMC1 has functions independent of hormonal input and because we wished to identify functions potentially altered by PGRMC1 depletion in the stroma of progestin-resistant endometriosis lesions, we chose to include a hormone-free condition in our cultures. Transfected cells were then incubated without (-H) or with estradiol (+E) to mimic the proliferative phase. After 48h of culture, cell proliferation rates were measured using a colorimetric assay (see Materials & methods). The consequences of PGRMC1 repression on cell proliferation were then assessed by comparing the cell viability rates obtained in the two experimental conditions.

Measurement of *PGRMC1* expression by RT-qPCR demonstrated the efficacy of siRNA-PGRMC1: an average 10x decrease in *PGRMC1* mRNA levels was observed in cells transfected with siRNA-PGRMC1 compared to those transfected with siRNA-CTL (Figure 36a).

In hormone-free condition (Figure 36b), the results obtained in primary stromal cultures from 8 separate patients suggest that *PGRMC1* repression decreases the cell population by an average of

10% compared to the control condition (siCTL). However, it is important to note that the proliferation rates measured after 48h of culture were low even in the control condition.

In comparison with transfected cells incubated without hormone, an average 30% increase in proliferation rate was observed in primary stromal cells incubated with estradiol and siCTL (Figure 36c). Downregulation of PGRMC1 slightly increased (<30%) the cell population of 3 out of 4 primary stromal cell cultures in the presence of estradiol (Figure 36d).

Analysis of more samples is still needed to determine a potential role for PGRMC1 in endometrial stromal cell proliferation. The magnitude of the observed effects is still negligible and may reflect an experimental artifact rather than a true impact of PGRMC1 on cell proliferation. Repeating these experiments in a cell type with a higher proliferation rate would allow to identify a larger difference between the experimental conditions.

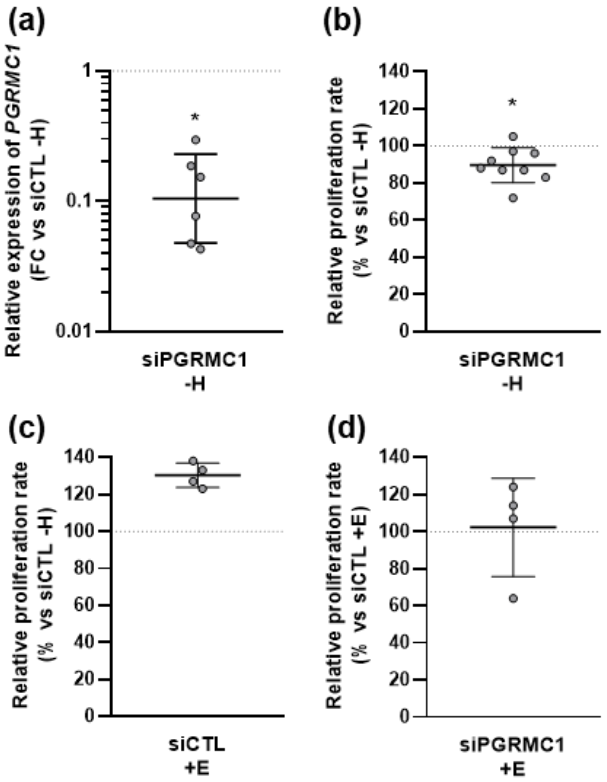


Figure 36: siRNA-PGRMC1 regulates the proliferation of primary endometrial stromal cells.

Primary endometrial stromal cells were treated with 10nM of siRNA-PGRMC1 s21310 (siPGRMC1) or control siRNA-CTL (siCTL) and incubated without (-H; **a, b**) or with 10nM estradiol (+E; **c, d**) during 48h (n=4-8). **(a)** Relative concentrations of *PGRMC1* was measured by RT-qPCR and normalized according to HKGs. The values were compared to siCTL values, and presented as individual Fold Changes (FC) in log10 scale and as geometric mean with geometric SD. **(b-d)** Colorimetric assay of cell viability. Absorbance values were compared to siCTL controls and ratios were expressed as % of cell viability and as mean with SD. **(a, b)** Statistical test: Wilcoxon paired test, not significant. * $p < 0.05$. **(c, d)** Due to the limited number of samples analyzed, no statistical analysis was performed.

2. PGRMC1, a repressor of *MMP-1* and *MMP-3*

For this part of the project, the experimental strategy was largely inspired by a publication on which my initial research project was partially based (Allen et al., 2015). Indeed, in this publication, the authors determined, in a human trophoblastic cell culture model, that the repression of *MMP-9* by the progestin MPA is dependent on PGRMC1 and, consequently, they suggested a potential role of PGRMC1 in the remodeling of the endometrial extracellular matrix necessary for embryonic implantation. Therefore, one of my first objectives for the functional topic was to determine whether PGRMC1 was involved in the progesterone regulation of the expression of MMPs crucial for cyclic endometrial remodeling.

Most MMPs are expressed in the human endometrium (Goffin et al., 2003; Vassilev et al., 2005). But, MMP-3, MMP-1, MMP-8 and MMP-12 are the MMPs whose expression varies most during the menstrual cycle in response to blood levels of ovarian steroids (Gaide Chevronnay et al., 2012; Henriet P, 2016). In cultured stromal cells, the repression of MMP-3 and MMP-1 expression by progesterone, and to a lesser extent MMP-2 and MMP-9, has also been demonstrated (Osteen et al., 1994; Salamonsen, Butt, Hammond, Garcia, & Zhang, 1997; Schatz, Papp, Toth-Pal, & Lockwood, 1994). Consequently, we chose to evaluate the potential repressive effect of progesterone mediated by PGRMC1 on the expression of *MMP-3* and *MMP-1* (Figures 37 and 38).

Based on (Allen et al., 2015), PGRMC1 expression was decreased using siRNA in an endometrial stromal cell line (T-HESC) before being subjected to a series of successive culture conditions aimed at stimulating (IL-1α) or repressing (MPA) the expression of *MMP-3* and *MMP-1*. Additionally, a culture condition in which a transcription inhibitor was added (actinomycin D) was performed to identify the

source of the potential expression variations. Finally, a second complementary approach aiming at decreasing the activity of PGRMC1 was jointly performed. It was based on the use of a commercial small molecule, AG-205, which was referenced as a specific PGRMC1 inhibitor. More technical details are presented in the "Materials & Methods" section.

The quality of the experimental strategies and culture conditions was respectively assessed (i) by measuring the variations in *PGRMC1* expression between invalidated/inhibited conditions and appropriate controls (Figures 37a and 38a) for each culture condition and (ii) by measuring the variations in *MMP-3* and *MMP-1* expression between each culture condition of the controls (siCTL or DMSO) and the control culture condition (/).

Our results demonstrate that, for each culture condition, PGRMC1 expression was significantly decreased by siRNA-PGRMC1 but not by AG-205 when compared with the appropriate control conditions (Figures 37a and 38a). Furthermore, with the exception of Figure 37b, the expression of *MMP-1* and *MMP-3* was significantly higher than in the control culture condition (/) in the presence of IL-1α, and returned to expression levels similar to the control condition when MPA was added (Figures 37c and 38b and c). Although no significant difference was detected, a similar trend of *MMP-1* expression in stromal cells transfected with siCTL was observed (Figure 37b). Consequently, and with the exception of those presented in Figure 37b, the results obtained were considered reliable.

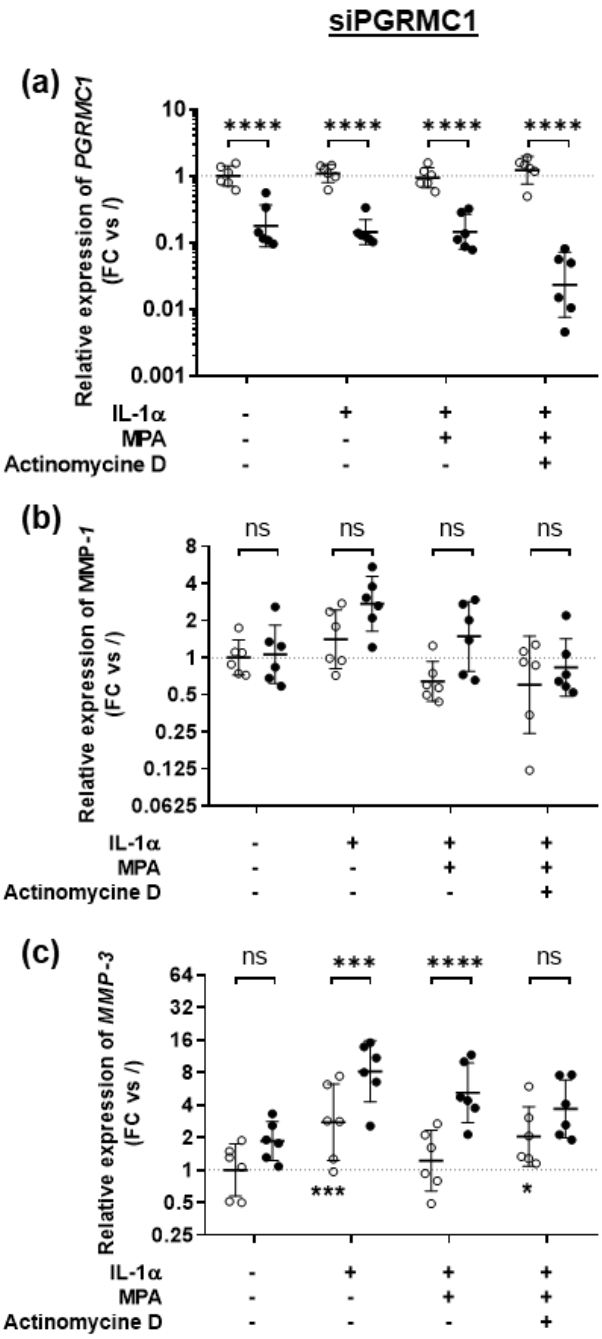

siPGRMC1
(a)
Relative expression of PGRMC1 (FC vs /)
10
1
0.1
0.01
0.001
****
****
****
****
IL-1α - + + +
MPA - - + +
Actinomycine D - - - +
(b)
Relative expression of MMP-1 (FC vs /)
8
4
2
1
0.5
0.25
0.125
0.0625
ns
ns
ns
ns
IL-1α - + + +
MPA - - + +
Actinomycine D - - - +
(c)
Relative expression of MMP-3 (FC vs /)
64
32
16
8
4
2
1
0.5
0.25
ns
***
****
ns
***
*
IL-1α - + + +
MPA - - + +
Actinomycine D - - - +

Figure 37: Evaluation of the decrease of PGRMC1 expression on *MMP-3* and *MMP-1* expression in T-HESC cultures.
T-HESC cells were treated with 10nM of siRNA-PGRMC1 s21310 (siPGRMC1; in black) or control siRNA-CTL (siCT; in white) and cultured with or without IL-1α, MPA and/or actinomycin D. Relative concentrations of *PGRMC1* **(a)**, *MMP-1* **(b)** and *MMP-3* **(c)** were measured by RT-qPCR and normalized according to HKGs. The values were compared to control values (/), and presented as individual Fold Changes (FC) in log10 **(a)** log2 scale **(b, c)** and as mean with SD. Statistical test: Anova two-way and post-hoc Tukey. Upper chart statistics: vs siCTL; Lower chart statistics: vs /. Not significant (ns), *** $p < 0.001$, **** $p < 0.0001$.

By similitude with MMP-9 in (Allen et al., 2015), when PGRMC1 expression or activity was reduced, *MMP-3* and/or *MMP-1* expression was significantly increased in the presence of IL-1α and MPA (Figures 37c and 38b and c). On the other hand, the addition of complementary experimental conditions showed that this increase in expression could be observed both in the presence and absence of MPA. This suggested that the repressive effect of PGRMC1 on the expression of these genes was not dependent on the intake of progestin and that it was therefore in no way the relay of any progestin activity in this specific context. Moreover, the expression levels of *MMP-3* and *MMP-1* remained stable under the conditions of cultures including actinomycin D, suggesting that the previously observed increases in expression were not due to an increase in the stability of the corresponding mRNAs but rather to a stimulation of their respective expression.

Finally, the expression of *MMP-3* and *MMP-1* was increased more when PGRMC1 was inhibited with the inhibitor AG-205 than when its expression was decreased by the transfection with an siRNA. This difference in the magnitude of the observed effects was then tentatively attributed to the specific characteristics of each of the two experimental strategies: we suspected that AG-205 was more efficient by directly preventing protein activity (whatever its amount) whereas the siRNA had to sufficiently reduce the expression.

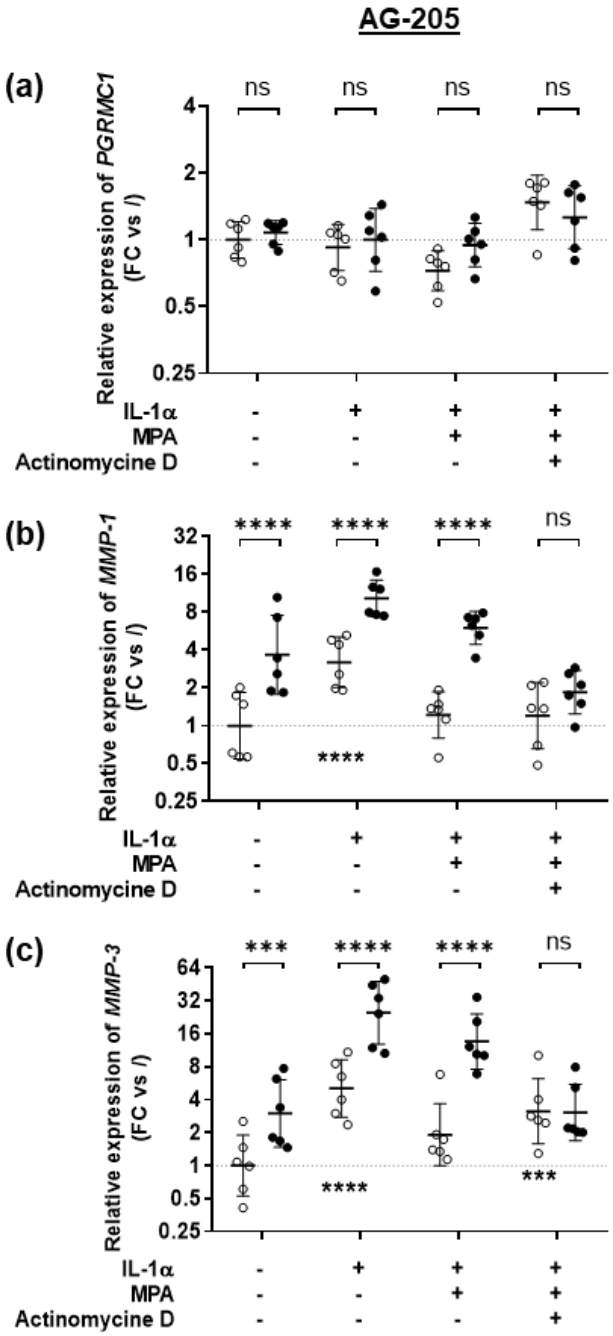

AG-205
(a)
Relative expression of PGRMC1 (FC vs /)
ns
ns
ns
ns
4
2
1
0.5
0.25
IL-1α - + + +
MPA - - + +
Actinomycine D - - - +
(b)
Relative expression of MMP-1 (FC vs /)
****
****
****
ns
****
32
16
8
4
2
1
0.5
0.25
IL-1α - + + +
MPA - - + +
Actinomycine D - - - +
(c)
Relative expression of MMP-3 (FC vs /)
***
****
****
ns
****
***
64
32
16
8
4
2
1
0.5
0.25
IL-1α - + + +
MPA - - + +
Actinomycine D - - - +

Figure 38: Evaluation of the decrease of PGRMC1 activity on *MMP-3* and *MMP-1* expression in T-HESC cultures.
T-HESC cells were incubated with 15µM AG-205 (in black) or DMSO (in white) control and cultured with or without IL-1α, MPA and/or actinomycin D. Relative concentrations of *PGRMC1* **(a)**, *MMP-1* **(b)** and *MMP-3* **(c)** were measured by RT-qPCR and normalized according to HKGs. The values were compared to control values (/), and presented as individual Fold Changes (FC) in log2 scale and as mean with SD. Statistical test: Anova two-way and post-hoc Tukey. Upper chart statistics: vs DMSO; Lower chart statistics: vs /. Not significant (ns), *** $p < 0.001$, **** $p < 0.0001$.

Encouraged by these preliminary results, the investigation of the potential roles of PGRMC1 in the endometrium was pursued by identifying all the targets of PGRMC1 by transcriptomic comparison. This analysis was performed simultaneously in the endometrial stromal line (T-HESC) as well as in an endometrial epithelial cell line (HEC-1A). To promote reproducible and statistically significant results, we setup the experimental approach using the AG-205 inhibitor.

ii. *"AG-205 Upregulates Enzymes Involved in Cholesterol Biosynthesis and Steroidogenesis in Human Endometrial Cells Independently of PGRMC1 and Related MAPR Proteins"*

This section describes the experimental work that was performed to identify genes downstream of PGRMC1 activity through transcriptomic comparison. Interference with PGRMC1 activity was achieved either through addition of AG-205 or by transfection with a specific siRNA.

The rather unexpected data are presented as formatted in the resulting publication in Biomolecules (accepted and published in October 2021).

Considering the number of readings and downloads for this article, it was selected by the editors as a "Title Story" on the homepage of the Biomolecules website (in January 2022).

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AG-205 Upregulates Enzymes Involved in Cholesterol Biosynthesis and Steroidogenesis in Human Endometrial Cells Independently of PGRMC1 and Related MAPR Proteins

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Abstract: An inappropriate response to progestogens in the human endometrium can result in fertility issues and jeopardize progestin-based treatments against pathologies such as endometriosis. PGRMC1 can mediate progesterone response in the breast and ovaries but its endometrial functions remain unknown. AG-205 is an alleged PGRMC1 inhibitor but its specificity was recently questioned. We added AG-205 in the cultures of two endometrial cell lines and performed a transcriptomic comparison. AG-205 significantly increased expression of genes coding enzymes of the cholesterol biosynthetic pathway or of steroidogenesis. However, these observations were not reproduced with cells transfected with siRNA against PGRMC1 or its related proteins (MAPRs). Furthermore, AG-205 retained its ability to increase expression of selected target genes even when expression of PGRMC1 or all MAPRs was concomitantly downregulated, indicating that neither PGRMC1 nor any MAPR is required to mediate AG-205 effect. In conclusion, although AG-205 has attractive effects encouraging its use to develop therapeutic strategies, for instance

against breast cancer, our study delivers two important warning messages. First, AG-205 is not specific for PGRMC1 or other MAPRs and its mechanisms of action remain unclear. Second, due to its effects on genes involved in steroidogenesis, its use may increase the risk for endometrial pathologies resulting from imbalanced hormones concentrations.

Keywords: PGRMC1; endometrium; AG-205; cholesterol; steroidogenesis

1. Introduction

An appropriate response to progesterone is crucial for the physiology of female reproductive organs. In addition to the well-documented nuclear progesterone receptors, two other families of proteins have been the subject of growing interest for their potential to mediate progesterone response: the class II progestin and adipoQ receptors (PAQRs, also called mPRs) and the membrane-associated progesterone receptors (MAPRs). The latter family contains four members sharing a cytochrome b5-like heme-binding domain on which their bioactivity depends (Kimura et al., 2012): progesterone receptor membrane component-1 and -2 (PGRMC1 and PGRMC2), neudesin (encoded by the *NENF* gene, for *neudesin neurotrophic factor*) and neuferricin (encoded by the *CYB5D2* gene for *cytochrome b5 domain containing 2*): among MAPRs, PGRMC1 received the greatest attention because it is suspected to participate in, or control, a large range of biological functions. including cell proliferation, apoptosis, steroid metabolism, lipid metabolism, membrane trafficking, angiogenesis, and progesterone response. Moreover, PGRMC1 overexpression is considered to play roles in carcinogenesis (Cahill, Jazayeri, Catalano, et al., 2016). PGRMC1 is also required for optimal fertility. Indeed, in zebrafish, a double knockout of *PGRMC1* and *PGRMC2* resulted in decreased fertility due to a reduction in ovulation and a downregulation of the nuclear progesterone receptor protein (X. J. Wu & Zhu, 2020). Moreover, a conditional *Pgrmc1* knockout in a mouse uterus led to the appearance of multiple endometrial cysts and a decrease in the number of offspring (McCallum et al., 2016). PGRMC1 was also shown to favour human trophoblastic cell implantation (Allen et al., 2015).

A significant proportion of the studies investigating PGRMC1 have relied on the use of AG-205 (PubChem entry 1202545, CAS 1375078-57-1, UNII-02137X034H, IUPAC: 1-((4aR,9bS)-2,8-Dimethyl-

3,4,4a,9b-tetrahydro-1H-pyrido(4,3-b)indol-5-yl)-2-(1-(4-chlorophenyl)tetrazol-5-yl)sulfanyl-ethanone). AG-205 is a small molecule (Figure S1) commercialized by major biotech companies as a PGRMC1 inhibitor / ligand, although evidence is lacking to support this assumption. AG-205 was initially identified as one of four aromatic molecules able to bind the *Arabidopsis thaliana* AtMAPR2 (Yoshitani et al., 2005) aka AtMP3 (UniProt entry Q9SK39). Similar to the four MAPR proteins, AtMAPR2/AtMP3 contains a cytochrome b5-like heme-binding domain and, more precisely, two key tyrosine residues (positions 107 and 113 in PGRMC1) required for heme binding (Min et al., 2005). Because the addition of AG-205 to purified PGRMC1 modified the spectroscopic properties of the PGRMC1-heme complex and induced dissociation of heme-dependent PGRMC1 homodimers, it was assumed that PGRMC1 was the human orthologue of yeast AtMAPR2/AtMP3 (Ahmed, Rohe, Twist, Mattingly, & Craven, 2010). It is not clear whether the ability of AG-205 to alter the spectrometric properties of the other MAPRs was tested. Moreover, comparison of the AtMAPR2/AtMP3 protein sequence with that of the 4 human MAPRs (entry O00264 for PGRMC1, O15173 for PGRMC2, Q9UMX5 for neudesin and Q8WUJ1 for neuferricin), using the Clustal Omega Multiple Sequence Alignment tool from EMBL-EBI does not support a closer homology between AtMAPR2/AtMP3 and PGRMC1 than with the three other MAPRs.

In the ovary and the breast, the addition of AG-205 promoted apoptosis (Pedroza et al., 2020; Will, Liu, & Peluso, 2017) modified regulation of the cell cycle (Aizen & Thomas, 2015; Pedroza et al., 2020; Terzaghi et al., 2016) and reduced cell migration and invasion capacities (Pedroza et al., 2020). As a consequence, AG-205 was patented for its therapeutic potential against breast cancer (Rolf Joseph Craven, 2017). However, to the best of our knowledge, AG-205 is only used for research purposes, and although PGRMC1 was proposed to be an important regulator of essential pathways in the breast and ovary, much less is known about its endometrial functions and mechanisms of action. In the human endometrium, progesterone is a critical inducer of modifications occurring to favour blastocyst implantation and pregnancy, including decidualization, i.e., a specific differentiation of the endometrial stromal cells. PGRMC1 is expressed in the human endometrium and its potential contribution to decidualization was recently reported (Salsano et al., 2021).

Interestingly, the addition of AG-205 to endometrial stromal cells undergoing artificial decidualization in response to progesterone (combined with estradiol) upregulated expression of genes related to metabolism, molecular transport and hormonal biosynthesis. However, it is unclear whether these changes required progesterone. Indeed, direct PGRMC1 binding to progesterone remains highly debated. Alternatively, the presence of potential SH2- and SH3-binding domains strongly suggests that PGRMC1 can act as a "hub" protein, connecting multiple partners (Cahill & Medlock, 2017) to activate molecular pathways that are—directly or indirectly—sensitive to progesterone. Moreover, and more importantly, two recent publications have strongly challenged AG-205 specificity towards PGRMC1 (and PGRMC2). Firstly, knocking out *PGRMC1* and/or *PGRMC2* expression did not alter the ability of AG-205 to induce the formation of large endosomes in CHO-K1 and HeLa cells (Wang-Eckhardt & Eckhardt, 2020). Secondly, and through a more direct approach, no binding activity of AG-205 to apo- or heme-dimerized PGRMC1 was observed by isothermal titration calorimetry analysis (Kabe et al., 2021).

In the present study, we first used a transcriptomic approach to identify biological processes and individual genes impacted by the addition of AG-205 in two endometrial cells lines cultured in the absence of progesterone. We then compared these transcriptomes with those derived from the same endometrial cells transfected with siRNAs directed against *PGRMC1* or against the four MAPRs. In both cell lines, the addition of AG205 increased expression of genes involved in sterol biosynthesis and steroidogenesis, as previously reported, but this effect was independent of the presence of progesterone and of the 4 MAPRs.

2. Materials and Methods

2.1. Cell lines and Cell Culture

Two human endometrial cell lines were used for the experiments: the Telomerase-immortalised Human Endometrial Stromal Cell line (T-HESC, ATCC CRL-4003) derived from fibroblast-like cells obtained from an adult patient with myomas (Krikun et al., 2004), and the Human Endometrial Cancer One A cell line (HEC-1A, ATCC HTB-112)

derived from epithelial-like cells isolated from a patient with stage 1A endometrial adenocarcinoma (Kuramoto, Tamura, & Notake, 1972).

Cells were grown in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12; Gibco, ThermoFisher Scientific, Merelbeke, Belgium), supplemented with 10% Fetal Bovine Serum (FBS), 100U/mL penicillin, 100µg/mL streptomycin (ThermoFisher Scientific) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Chemical Compounds

AG-205 (Sigma, Saint-Louis, MO, USA) was diluted in dimethyl sulfoxide (DMSO) to prepare a 15 mM (1000×) stock solution.

2.3. Cell Viability Assay

The optimization of the final concentration and the incubation time of AG-205 was carried out with the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Leiden, The Netherlands) according to the manufacturer's recommendations. Briefly, cells were seeded in 96-well plates (2.10^4 cells/mL) and grown in DMEM/F12, without phenol red nor antibiotics, supplemented with 10% FBS. After 48 h incubation, medium was changed after supplementation with indicated concentrations of AG-205 or corresponding DMSO concentration as control. Cells were incubated for 24 h, 32 h or 48 h before the addition of 20 µL/well of CellTiter 96® AQueous One Solution Reagent containing a tetrazolium compound (MTS). After 1–4 h incubation at 37 °C, the quantity of formazan (a bio-reduced colored product of MTS directly proportional to the number of living cells) was measured at 490nm absorbance.

2.4. Inhibition Techniques (siRNA Transfection or AG-205 Addition)

siRNA-mediated gene silencing was performed by transient transfection with Lipofectamine RNAiMax (Invitrogen, Waltham, MA, USA) according to the manufacturer's recommendations.

Cells (2.10^4 cells/mL) were transfected with final 10 nM pre-designed Silencer siRNA(s) or negative control (Table S1, Supplementary Materials) and cultured in DMEM/F12, without phenol red nor antibiotics, supplemented with 10% FBS. At the same time, additional wells were prepared and cultured with the same medium (without transfection) for the AG-205 inhibition strategy. After 48h incubation, the medium of all wells was changed and 15 µM

of AG-205 or 0.1% DMSO, as negative control, was added to the appropriate wells for an additional 32 h.

2.5. RNA Extraction

After incubation, cells were lysed with TRIzol Reagent (Ambion, Huntingdon-Cambridgeshire, United Kingdom), vortexed for 10 s and incubated for 10 min at room temperature. The lysates were vortexed during 30 s and 20 ng of tRNA were added to each sample to stabilize the RNA. After homogenization, 200 µL of chloroform was added. The mixtures were vortexed for 30 s and incubated for 15 min at room temperature. After centrifugation for 15 min at 12,000× *g* and 4 °C, upper aqueous layers were collected. Samples were supplemented with 200 µL isopropanol 100%, vortexed for 30 s and stored for at least 1h at −80 °C to precipitate the RNAs. After a 30 s centrifugation at 12,000× *g* and 4 °C, the RNA pellet was washed with cold ethanol 70%, dried at room temperature and resuspended in autoclaved H_2O_d. For RNA sequencing, an additional step was performed to remove excess DNA with the TURBO DNA-free kit (Invitrogen) according to manufacturer's recommendations except that a longer centrifugation step (3 min) was carried out.

2.6. Quantitative Real-Time PCR

For all samples, the RNA concentrations were evaluated using Nanodrop ND-8000 spectrophotometer. Then, 500 ng total RNA were used for reverse transcription using SuperScript III Reverse Transcriptase kit (Invitrogen), according to the manufacturer's recommendations.

Primers for amplification of *PGRMC1* were previously published (Bunch et al., 2014). All other specific oligonucleotides were designed in our laboratory (Table S2) and their amplification efficiencies were checked before use. The real-time PCR amplifications were performed with the KAPA SYBR FAST qPCR Master Mix (2X), 0.25µM primers (forward and reverse) and 15ng cDNA. Two HouseKeeping Gene, RPL13a and β-actin were selected based on their stability of expression under our experimental conditions.

2.7. RNA Sequencing

Total RNA was isolated from three independent cell culture experiments. The RNA quantities and qualities were evaluated using

a Nanodrop ND-8000 spectrophotometer and an Agilent Bioanalyzer, respectively. RNA library preparation was performed using a polyA selection method. RNA sequencing was performed using the Illumina HiSeq system in a 2 × 150-bp configuration (single index, per lane) by GENEWIZ. RNA sequencing data of cells cultured with AG-205 are stored under GEO accession number GSE174305. RNA sequencing of cells cultured with the siRNA-PGRMC1 (s21310) were only used for the purpose of comparison and will be commented on in detail in another publication.

2.8. Bioinformatics Analysis Workflow

Read quality control was performed using FastQC software v0.11.8 (Andrews, 2010). Low quality reads were trimmed, and adapters were removed using Trimmomatic software v0.38 (Bolger, Lohse, & Usadel, 2014). Reads were aligned using HISAT2 software v2.1.0 (Kim, Paggi, Park, Bennett, & Salzberg, 2019) on GRCh38 genome. Gene counts were generated using featureCounts software from subread-2.0.0 (Liao, Smyth, & Shi, 2014) and Ensembl Homo_sapiens.GRCh38.94.gtf annotation file. Differential expression analyses were done using DESeq2 v1.30 (Love, Huber, & Anders, 2014), on R version 4.0.3. Genes with adjusted *p*-value < 0.05 and absolute log2 fold-change > 1 were considered as differentially expressed. Over Representation Analysis was done with clusterProfiler v3.16.1 bioconductor package.

2.9. Immunolabeling

Immunocytofluorescence was performed with cells cultured on glass coverslips. After appropriate incubation, cells were washed in PBS before fixation for 10 min in 4% paraformaldehyde. They were washed 5 times for 5 min in PBS and permeabilized for 15 min in PBS with 0.1% Triton X-100. Nonspecific binding sites were blocked for 1h at room temperature with PBS, 0.3% Triton X-100, 5% normal goat serum before incubating coverslips overnight at 4 °C with 1/200 anti-PGRMC1 primary antibody (D6M5M, cat no. 13856; Bioké, Leiden, The Nederlands). The next day, cells were washed 3 times for 5 min in PBS and incubated with 1/1000 secondary antibody (Goat anti-rabbit IgG, Alexa 488; Life Technologies, Merelbeke, Belgium) for 1h30 at room temperature. Nuclei were counterstained with Hoechst (BisBenzimide H33342, 1µg/mL; Sigma) during the incubation with the secondary

antibody. Fluorescence was observed with a Cell Observer Spinning Disk (COSD) confocal microscope (Zeiss). Signals were analysed and quantified with the image analysis platform HALO (Indica Labs, Albuquerque, NM, USA).

2.10. Cell Fractionation and Western Blot Analysis

Cells incubated with AG-205 or DMSO control were washed with PBS, lysed with cytoplasmic lysis buffer (50 mM Tris, 0.1% Nonidet P-40 (Igepal CA-630), supplemented with Complete protease inhibitor cocktail (1 tablet for 50 mL; Roche/Boehringer, Brussels, Belgium)) and incubated for 30 min on ice. The samples were centrifuged for 10 min at 14,000× *g* and 4 °C to separate cytoplasmic (supernatant) and nuclear (pellet) proteins. The pellets were washed 3 times before the addition of nuclear lysis buffer (0.1% SDS, 1% sodium deoxycholate, 0.5% NP-40 (Tergitol), supplemented with Complete protease inhibitor cocktail (1 tablet for 50 mL)) and incubation for 30 min under intense shaking. To complete lysis, the homogenates were successively passed through a 16G and a 30G syringe and sonicated. The nuclear fraction was isolated (supernatant) after a last centrifugation step.

Cells transfected with siRNA-PGRMC1, or siRNA-control were washed with PBS and lysed with RIPA buffer (50 mM HEPES, 50 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 0.5% octylglucoside, supplemented with Complete protease inhibitor cocktail (1 tablet for 50 mL)).

All lysates were sonicated, and the protein concentration was measured by the bicinchoninic acid (BCA) method. When necessary, samples were concentrated with Amicon Ultra-0.5 Centrifugal Filter Units (MerckMillipore, Overijse, Belgium), according to the manufacturer's recommendations. Next, sample buffer 5X (0.25M Tris-HCl, 10% SDS, 20% Glycerol, 0.005% Bromophenol Blue, 5mM DTT, pH 6.8) was added to each sample and the homogenates were heated for 5 min at 100 °C and centrifuged for 5 min at 14000 g. Samples were prepared to ensure corresponding amounts of biomaterial in compared conditions (DMSO versus AG-205; siCTRL vs siPGRMC1). Proteins were separated by SDS-PAGE (Running Buffer: Tris 0.025M, glycine 0.192M, SDS 0.1%) on a 12% polyacrylamide gel and transferred to a PVDF membrane (Perkin-Elmer, Zaventem, Belgium). Membranes were blocked for 2h at room temperature with Tris Buffer Saline (TBS: 20mM Tris-HCL, 0.5M NaCl, pH 7.5), supplemented with

0.05% Tween-20 (TBST) and 5% non-fat milk, to avoid non-specific binding, before incubation overnight at 4 °C with the primary anti-PGRMC1 antibodies (D6M5M) diluted at 1:1000 in TBST, 5% bovine serum albumin (BSA).

After 3 washes of 10 min in TBST, blots were incubated for 1h at room temperature with horseradish peroxidase-conjugated secondary antibodies (cat no. 050M4834; Sigma; or cat no. G21040; Life Technologies) diluted in TBST, 5% BSA. They were washed 3 times in TBST and once in TBS. Immunoreactive bands were revealed by chemiluminesence (SuperSignalTM West Femto Maximum Sensitivity Substrate; ThermoFisher Scientific) using the Fusion Solo S (Vilber Lourmat, Collegien, France).

For controls of equal loading, membranes were placed in a stripping buffer for 15′ (ReBlot Plus Strong Antibody Stripping Solution (10x); MerckMillipore), and re-probed with a primary anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody (catalog no. AM4300; Ambion, Huntingdon-Cambridgeshire, United Kingdom, as cytoplasmic control) diluted at 1:8000 in TBST, 5% non-fat milk and/or with a primary anti-histone H3 antibody (catalog no. Ab1791; Abcam, Amsterdam, The Nederlands, as a nuclear control) diluted at 1:10000 in TBST, 5% BSA.

2.11. Statistical Analysis

All statistical analyses were performed with GraphPad Prism 8.4.3. Analyses of RT-qPCR data were performed on paired ΔCt values (i.e., within the same experiment). The outliers were identified and excluded using the Grubbs' test.

3. Results

3.1. Setup of AG-205 Use in Endometrial Cell Culture

Our experiments were performed in two human endometrial cell lines, T-HESC from fibroblastic origin (Krikun et al., 2004) and HEC-1A from epithelial origin (Kuramoto et al., 1972). Since AG-205 was used at quite high concentrations in previous publications (in the micromolar range), we first determined the effect of AG-205 addition on the viability of the two endometrial cell lines. HEC-1A and T-HESC cells were cultured for 24, 32 or 48 h in the presence of AG-205 (or DMSO as control) and cell viability was measured by colorimetric assay at the end of the culture (Figure S2, Supplementary Materials). In both cell lines, AG-205 had no effect on cell viability for up to 48h when added at 5 or 15 μM. In contrast, cell viability was significantly reduced after 32 and 48 h upon addition of 30 μM AG-205, and at all time points upon addition of 45 μM AG-205. We therefore decided to perform all subsequent experiments with 15 μM AG-205 for 32 h.

To better understand the mechanisms of action of AG-205 on PGRMC1 in both endometrial cell lines, we next evaluated the effect of AG-205 addition on PGRMC1 expression. In both cell lines, the addition of AG-205 had no significant effect on *PGRMC1* mRNA levels (Figure 1a,d). Because AG-205 was previously shown to trigger PGRMC1 translocation towards the nucleus (Will et al., 2017), we also assessed PGRMC1 protein expression and subcellular localization by immunofluorescence, and by western blotting following cell fractionation. In both cells lines, PGRMC1 was essentially present in the cytoplasm and barely or not detected in the nucleus, and AG-205 addition had no detectable effect (Figure 1b,c,e,f).

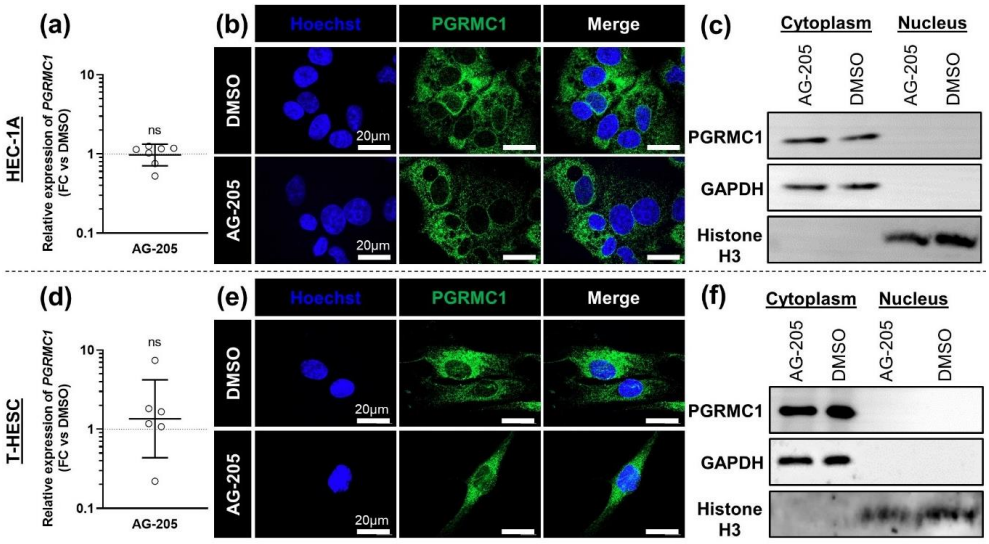

Figure 1. AG-205 does not modify expression of PGRMC1. Relative expression, immunolocalization and immunoblotting of PGRMC1 in HEC-1A (**a–c**) and T-HESC (**d–f**) cell lines treated with 15µM AG-205 or control DMSO during 32h. (**a**,**d**) Relative expression of *PGRMC1* was measured by RT-qPCR, normalized, compared to corresponding DMSO controls and is presented as individual fold changes (FC) in log scale and as geometric mean with geometric SD (n = 6–7). Statistical test: Wilcoxon paired test, not significant (ns). (**b**,**e**) The localization of PGRMC1 protein (in green) was assessed by immunocytofluorescence and nuclei were stained in blue (n = 10–12). (**c**,**f**) For both culture conditions (AG-205 and DMSO), PGRMC1 (24 kDa) was immunoblotted in equal amounts of cytoplasmic biomaterial and in equal amounts of nuclear biomaterial (n = 3). Immunoblotted histone H3 (17 kDa) and GAPDH (36 kDa) were used as loading controls for the nuclear and cytoplasmic fractions, respectively.

3.2. Effects of AG-205 in Endometrial Cell Culture

We next carried out an RNA sequencing-mediated transcriptomic comparison of cells cultured in the presence of AG-205 or with control DMSO. Volcano plots (Figure 2a,b) highlighted genes that were significantly upregulated by AG-205 addition. Interestingly, the top five Gene Ontology (GO) terms over-represented in this comparison were related to cholesterol / steroid metabolism (Table S3 and Figure S3, Supplementary Materials). More precisely, most genes coding for enzymes involved in cholesterol biosynthesis were upregulated in both cell lines (Figure 2c). The 50 genes that are most differentially expressed in both cell lines are listed in Table S4 (Supplementary Materials). This observation was attractive because previous studies have suggested a link between PGRMC1 and sterol metabolism (Asperger et al., 2020; Hand et al., 2003; Hughes et al., 2007; Mallory et al., 2005; Suchanek et al., 2005). Due to the large number of enzymes upregulated upon AG-205 addition, we hypothesized that this effect was more likely to result from modulation of one common regulator (or regulating pathway) rather than modulation of all individual genes. In this regard, insulin-induced gene 1 protein (INSIG1) stood out for several reasons. Firstly, INSIG1 is a well-known sterol regulator able to modulate several enzymatic steps in sterol metabolism (see Discussion). Secondly, INSIG1 was previously shown to directly interact with PGRMC1, although sensitivity of this interaction towards AG-205 was not addressed (Suchanek et al., 2005). Thirdly, in our transcriptomic analysis, expression of *INSIG1* was strongly upregulated in both cell lines in response to AG-205. We therefore selected three genes for further experiments: *INSIG1* and two strongly upregulated enzymes of the cholesterol biosynthesis pathway, sterol C4-methyl oxidase *MSMO1* and 17β-hydroxysteroid dehydrogenase-7 *HSD17B7*, both involved in the conversion of lanosterol to cholesterol. Upregulation of the expression of these three genes upon AG-205 addition was confirmed by RT-qPCR analysis of additional cell cultures (Figure 2d,e).

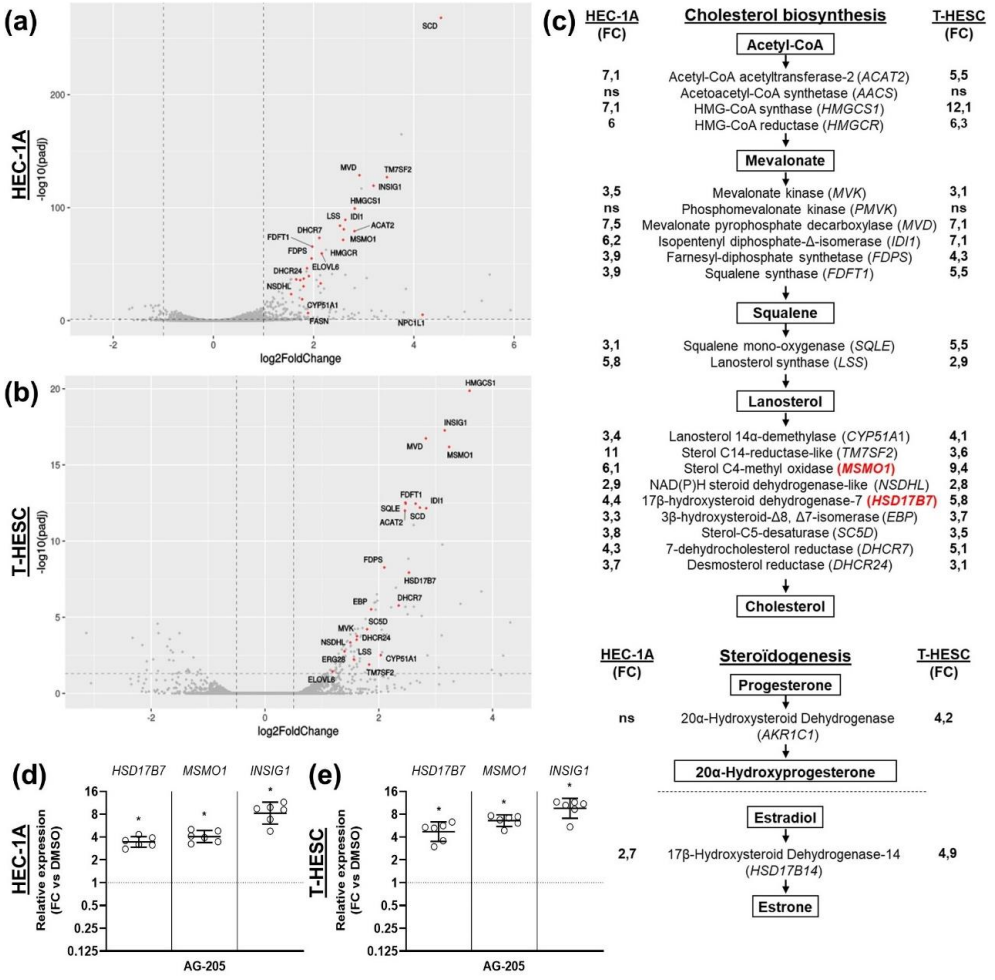
(a)
HEC-1A
-log10(padj)
log2FoldChange
SCD
MVD
TM7SF2
INSIG1
HMGCS1
LSS
IDI1
ACAT2
DHCR7
FDFT1
MSMO1
FDPS
HMGCR
ELOVL6
DHCR24
NSDHL
CYP51A1
FASN
NPC1L1
(b)
T-HESC
-log10(padj)
log2FoldChange
HMGCS1
INSIG1
MVD
MSMO1
FDFT1
IDI1
SQLE
SCD
ACAT2
FDPS
HSD17B7
EBP
DHCR7
SC5D
MVK
NSDHL
DHCR24
LSS
CYP51A1
ERG25
TM7SF2
ELOVL6
(c)
HEC-1A (FC)
Cholesterol biosynthesis
T-HESC (FC)
Acetyl-CoA
7,1 Acetyl-CoA acetyltransferase-2 (ACAT2) 5,5
ns Acetoacetyl-CoA synthetase (AACS) ns
7,1 HMG-CoA synthase (HMGCS1) 12,1
6 HMG-CoA reductase (HMGCR) 6,3
Mevalonate
3,5 Mevalonate kinase (MVK) 3,1
ns Phosphomevalonate kinase (PMVK) ns
7,5 Mevalonate pyrophosphate decarboxylase (MVD) 7,1
6,2 Isopentenyl diphosphate-Δ-isomerase (IDI1) 7,1
3,9 Farnesyl-diphosphate synthetase (FDPS) 4,3
3,9 Squalene synthase (FDFT1) 5,5
Squalene
3,1 Squalene mono-oxygenase (SQLE) 5,5
5,8 Lanosterol synthase (LSS) 2,9
Lanosterol
3,4 Lanosterol 14α-demethylase (CYP51A1) 4,1
11 Sterol C14-reductase-like (TM7SF2) 3,6
6,1 Sterol C4-methyl oxidase (MSMO1) 9,4
2,9 NAD(P)H steroid dehydrogenase-like (NSDHL) 2,8
4,4 17β-hydroxysteroid dehydrogenase-7 (HSD17B7) 5,8
3,3 3β-hydroxysteroid-Δ8, Δ7-isomerase (EBP) 3,7
3,8 Sterol-C5-desaturase (SC5D) 3,5
4,3 7-dehydrocholesterol reductase (DHCR7) 5,1
3,7 Desmosterol reductase (DHCR24) 3,1
Cholesterol
HEC-1A (FC)
Steroïdogenesis
T-HESC (FC)
Progesterone
ns 20α-Hydroxysteroid Dehydrogenase (AKR1C1) 4,2
20α-Hydroxyprogesterone
Estradiol
2,7 17β-Hydroxysteroid Dehydrogenase-14 (HSD17B14) 4,9
Estrone
(d)
HEC-1A
Relative expression (FC vs DMSO)
HSD17B7
MSMO1
INSIG1
AG-205
(e)
T-HESC
Relative expression (FC vs DMSO)
HSD17B7
MSMO1
INSIG1
AG-205

Figure 2. AG-205 increases RNA concentration of enzymes involved in sterol biosynthesis. RNA sequencing was used to compare transcriptomes of HEC-1A (**a**,**c**) or T-HESC cells (**b**,**c**) incubated for 32 h with 15μM AG-205 or control DMSO. (**a**,**b**) Volcano plots for HEC-1A (**a**) and T-HESC (**b**) cells were generated with Over Representation Analysis (ORA). Genes from the first GO term (GO:0016126) differentially expressed upon AG-205 addition (adjusted p value < 0.05 and a |log2 fold change| > 1) are represented by red dots. Only Top30 genes are labelled. (**c**) Synthetic representation of enzymes involved in cholesterol biosynthesis and steroidogenesis. Significant expression increases measured upon AG-205 addition by comparison with corresponding DMSO control are indicated as fold changes (FC) at left for HEC-1A and at right for T-HESC cells. (**d**,**e**) Relative expression of *HSD17B7*, *MSMO1* and *INSIG1* was measured by RT-qPCR in other cell samples, normalized, compared to corresponding control DMSO values, and is presented as individual fold changes (FC) in log2 scale and as geometric means with geometric SD (n = 6). Statistical test: Wilcoxon paired test, not significant (ns), * p < 0.05.

3.3. Effects of AG-205 Are Not Mimicked by Downregulation of PGRMC1 Expression

To directly address the contribution of PGRMC1 in the upregulation of these genes, we repeated the experiments with cells transfected with a commercial siRNA directed against *PGRMC1* mRNA (s21310). We first checked its effect on PGRMC1 expression at the mRNA and protein levels. As expected, 80h after transfection, *PGRMC1* mRNA concentration was reduced in both cell lines (Figure 3a,d). Coherently, the PGRMC1 signal by immunofluorescence (Figure 3b,e) and immunoblotting (Figure 3c,f) were strongly reduced in both cell lines.

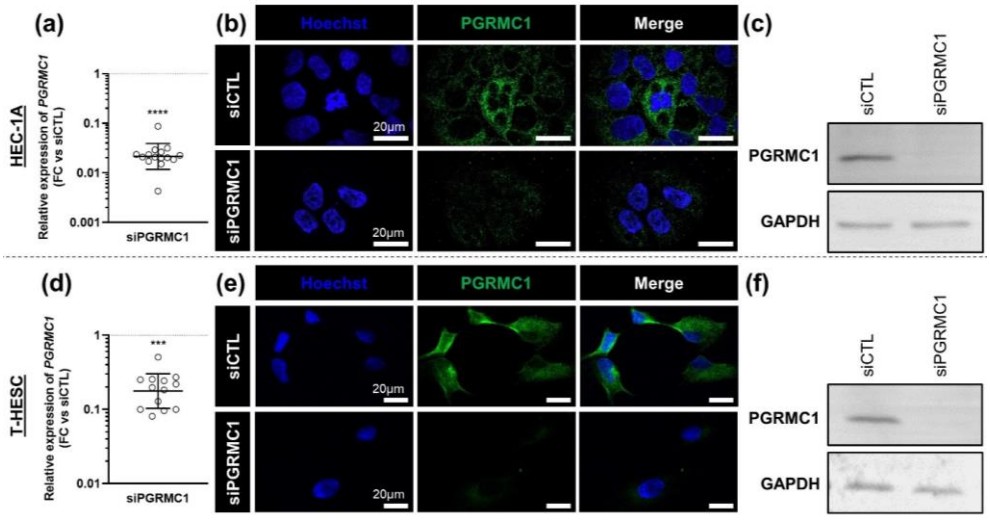

Figure 3. siRNA-PGRMC1 decreases expression of PGRMC1. Relative expression, immunolocalization and immunoblotting of PGRMC1 in HEC-1A (**a**–**c**) and T-HESC (**d**–**f**) cell lines treated with 10nM of siRNA-PGRMC1 s21310 (siPGRMC1) or control siRNA-CTL (siCTL) during 80h. (**a**,**d**) Relative expression of *PGRMC1* was measured by RT-qPCR, normalized, compared to corresponding siCTL values and is presented as individual fold changes (FC) in log scale and as geometric means with geometric SD (n = 13–15). Statistical test: Wilcoxon paired test, not significant (ns), *** $p < 0.001$, **** $p < 0.0001$. (**b**,**e**) Immunocytofluorescence of PGRMC1 protein (in green) and nuclear staining (Hoechst, in blue) (n = 4). (**c**,**f**) Immunoblot of PGRMC1 (24 kDa). GAPDH (36 kDa) was used as loading control (n = 3).

PGRMC1 downregulation did not reproduce the effect of AG-205 addition. Indeed, expression of the three selected genes was not significantly modified, or even slightly reduced, in cells transfected with the siRNA-PGRMC1 (Figure 4a, b).

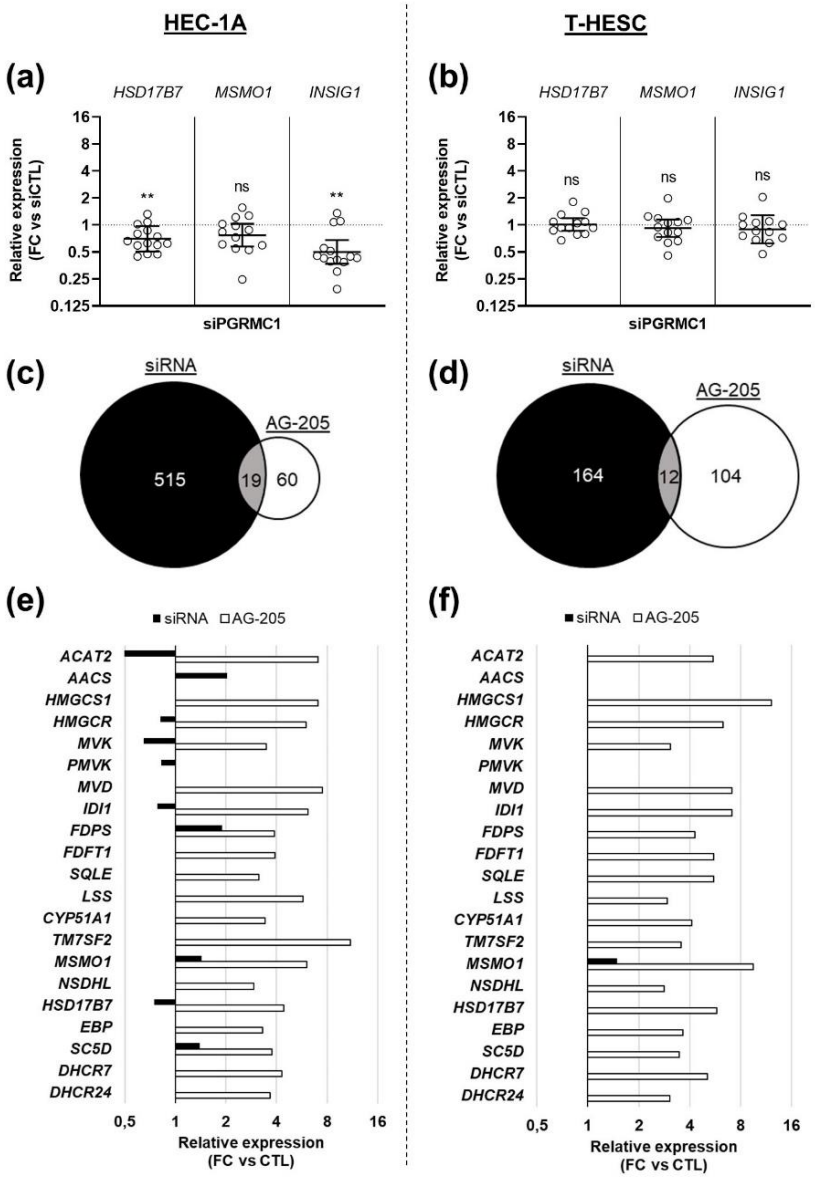

Figure 4. siRNA-mediated down-regulation of PGRMC1 expression does not mimic the effect of AG-205. HEC-1A (**a**,**c**,**e**) or T-HESC (**b**,**d**,**f**) cells were incubated for 80h with 10nM siRNA-PGRMC1 s21310 (siPGRMC1) or control siRNA-CTL (siCTL). (**a**,**b**) Relative expression of *HSD17B7*, *MSMO1* and *INSIG1* was measured by RT-qPCR (n = 13-14), normalized, compared to corresponding siCTL values and is presented as individual fold changes (FC) in log2 scale and as geometric means with geometric SD. Statistical test: Wilcoxon paired test, not significant (ns), ** $p < 0.01$. (**c**,**d**) Transcriptomes of siPGRMC1 and siCTL-transfected cells were established by RNA sequencing (n = 3) and analyzed by over-representation analysis (ORA). Results were compared with those previously obtained with AG-205 addition. Figures present the number of GO terms differentially expressed upon siPGRMC1 transfection (in black), AG-205 addition (in white) and in common (in grey). (**e**,**f**) The effect of siPGRMC1 (in black) on expression of genes represented in Figure 2c is compared to the effect of AG-205 (in white), with values retrieved from Figure 2c for direct visual comparison. Expression variations measured by comparison with corresponding control (siCTL or DMSO) are indicated as fold changes (FC).

To enlarge the scope of the comparison between the two experimental strategies (AG-205 or siRNA), we performed a new transcriptomic analysis to compare cells transfected with the siRNA-PGRMC1 or with a control siRNA. The top five most significantly enriched GO terms were totally different, in both cell lines, from those obtained with AG-205 (Table S5, Supplementary Materials). When comparing all transcriptomes together, only few GO terms were common to both strategies for each cell line (19 GOs for HEC-1A cells, Figure 4c and Table S6 (Supplementary Materials); and 12 GOs for T-HESC cells, Figure 4d and Table S7, Supplementary Materials). In contrast, several GOs were only identified in one analysis (515 GOs with the siRNA and 60 GOs with AG-205 in HEC-1A cells, Figure 4c; 164 GOs with the siRNA and 104 genes with AG-205 in T-HESC cells, Figure 4d). Most strikingly, the upregulation of genes involved in cholesterol biosynthesis was not mimicked in cells transfected with the siRNA (Figure 4e,f).

Although accurate *PGRMC1* downregulation by the s21310 siRNA was confirmed by our validation experiments (Figure 3), we decided to transfect cells with another PGRMC1-specific siRNA. The sequence of this second siRNA-PGRMC1 (18248) was directed towards exon 1 of *PGRMC1*, a sequence common to the two isoforms of *PGRMC1* predicted in silico, whereas the siRNA-PGRMC1 s21310 was targeting exon 2 (present in only one isoform). *PGRMC1* mRNA concentration

was reduced in both cell lines by siRNA 18248, although more moderately than with the first siRNA (Figure 5a,c). In agreement with data obtained with siRNA s21310, expression of the three selected genes was not modified in cells transfected with siRNA 18248, except for a marginal increase (<1.5 fold) of *HSD17B7* and *INSIG1* expression in HEC-1A cells but not in T-HESC cells (Figure 5b,d).

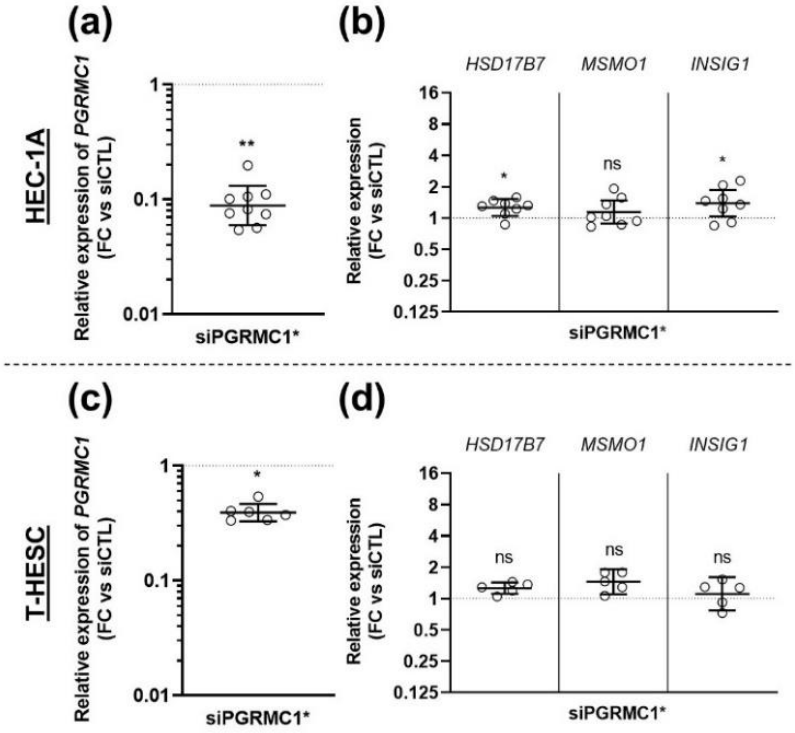

Figure 5. Down-regulation of *PGRMC1* expression by another siRNA does not mimic the effect of AG-205 on target genes. HEC-1A (**a**,**b**) and T-HESC (**c**,**d**) cells were incubated with 10 nM siRNA-PGRMC1 18248 (siPGRMC1*) or control siRNA-CTL (siCTL) during 72 h. Relative expression of *PGRMC1* (**a**,**c**), *HSD17B7*, *MSMO1* and *INSIG1* (**b**,**d**) was measured by RT-qPCR, normalized, compared to siCTL values and is presented as individual fold changes (FC) in log or log2 scale and as geometric means with geometric SD (n = 5–8). Statistical test: Wilcoxon paired test, not significant (ns), * $p < 0.05$, ** $p < 0.01$.

3.4. Effects of AG-205 Are Independent of PGRMC1

In order to further challenge the PGRMC1-dependency of AG-205 effects, we questioned whether the effects of AG-205 could be maintained after *PGRMC1* down-tuning. To this aim, the two endometrial cell lines were transfected with siRNA-PGRMC1 (s21310) or siRNA-CTL and incubated with AG-205 or its DMSO control (Figure 6). As expected, the expression of *PGRMC1* was significantly reduced in cells transfected with siRNA-PGRMC1 (Figure 6a,c). Since the MAPR family contains three other members, PGRMC2, neudesin (*NENF*) and neuferricin (*CYB5D2*), we also measured the effects of siRNA-PGRMC1 on their expression. Although *NENF* and *CYB5D2* expression was unaffected, *PGRMC2* expression was slightly, but significantly, upregulated in cells from both cell lines transfected with siRNA-PGRMC1.

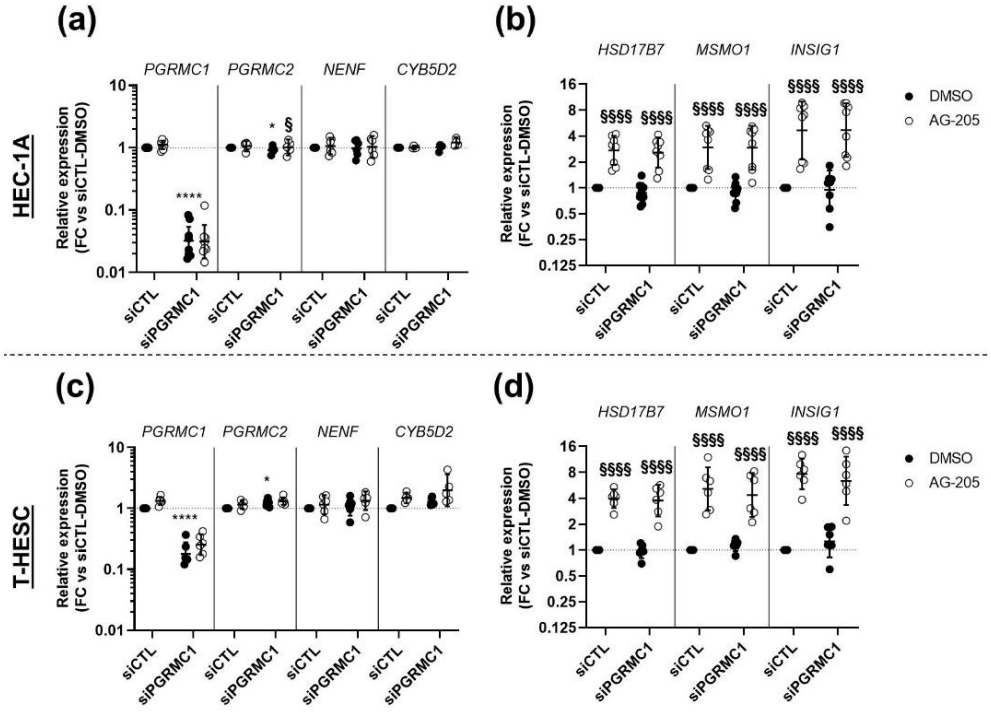

Figure 6. Effect of AG-205 on expression of selected target genes is not modified upon combined siRNA-mediated down-regulation of *PGRMC1*. HEC-1A (**a**,**b**) or T-HESC cells (**c**,**d**) were transfected with 10nM siRNA-PGRMC1 s21310 (siPGRMC1) or control siRNA-CTL (siCTL) during 48 h and then further incubated for 32 h with 15 μM AG-205 or DMSO. Relative expression of *PGRMC1, PGRMC2, NENF* and *CYB5D2* (**a**,**c**) and *HSD17B7, MSMO1* and *INSIG1* (**b**,**d**) was measured by RT-qPCR (n = 6–8), normalized, compared to dual control values (siCTL with DMSO) and is presented as individual fold changes (FC) in log or log2 scale and as geometric means with geometric SD. Statistical test: Anova-two ways with post-hoc Tukey. Statistically significant differences resulting from siPGRMC1 transfection or AG205 addition are indicated by * or §, respectively: */§ $p < 0.05$, ****/§§§§ $p < 0.0001$.

In agreement with our previous experiments, expression of the three selected genes was increased upon AG-205 addition in HEC-1A cells (mean increase ~3 to 4 fold) and in T-HESC cells (mean increase ~4 to 6 fold). Most importantly, this effect was perfectly identical whether cells were transfected with the siRNA-control or the siRNA-PGRMC1, indicating that the presence of PGRMC1 was not required to mediate AG-205 response (Figure 6b,d).

3.5. Effects of AG-205 Are Independent of All Four MAPRs

We next hypothesized that AG-205 was potentially able to interact with another MAPR rather than with PGRMC1. We therefore investigated whether the effects of AG-205 could be reproduced by transfecting cells with a siRNA against one of the three other MAPR genes (Figure 7), and whether the effects of AG-205 would be maintained or not upon simultaneous down-tuning of all four MAPRs (Figure 8).

In the first set of experiments, we transfected HEC-1A cells (Figure 7a,b) and T-HESC cells (Figure 7c,d) with one siRNA directed against *PGRMC1*, *PGRMC2*, *NENF* or *CYB5D2*. At the end of the culture, we first ensured that concentration of the targeted MAPR mRNA was significantly reduced. We also measured expression of the other MAPRs to identify potential compensation on their expression (Figure 7a,c). In both cell lines, each siRNA elicited downregulation of its target by at least 3-fold by comparison with the control siRNA. Globally, the siRNAs had no, or marginal, effects on the expression of the other MAPR genes. Only a very limited (<1.3-fold mean) but significant upregulation was measured for some combinations, as indicated in the figure. Then, we tested the effect of these siRNAs on expression of the three selected genes (Figure 7b,d) and measured some significant changes, but they were marginal by comparison with the effects of AG-205. The siRNA against *PGRMC2* induced a ~1.28-fold upregulation of *INSIG1* in HEC-1A cells. Surprisingly, the siRNA against *NENF* induced downregulation of the three genes in HEC-1A cells (~1.31-fold for *HSD17B7*; ~1.19-fold for *MSMO1* and ~1.32-fold for *INSIG1*) and upregulation in T-HESC cells (~1.32-fold for *HSD17B7*; ~1.48-fold for *MSMO1* and ~1.28-fold for *INSIG1*). Similarly, the siRNA against *CYB5D2* had opposite effects on some genes in both cell lines: it induced a ~1.23-fold downregulation of *HSD17B7* in HEC-1A cells and upregulation of *MSMO1* (~1.15-fold) and *INSIG1* (~1.27-fold) in T-HESC cells. In summary, transfection with any of the three other MAPR-targeting siRNAs did not reproduce the magnitude of the effects of AG-205.

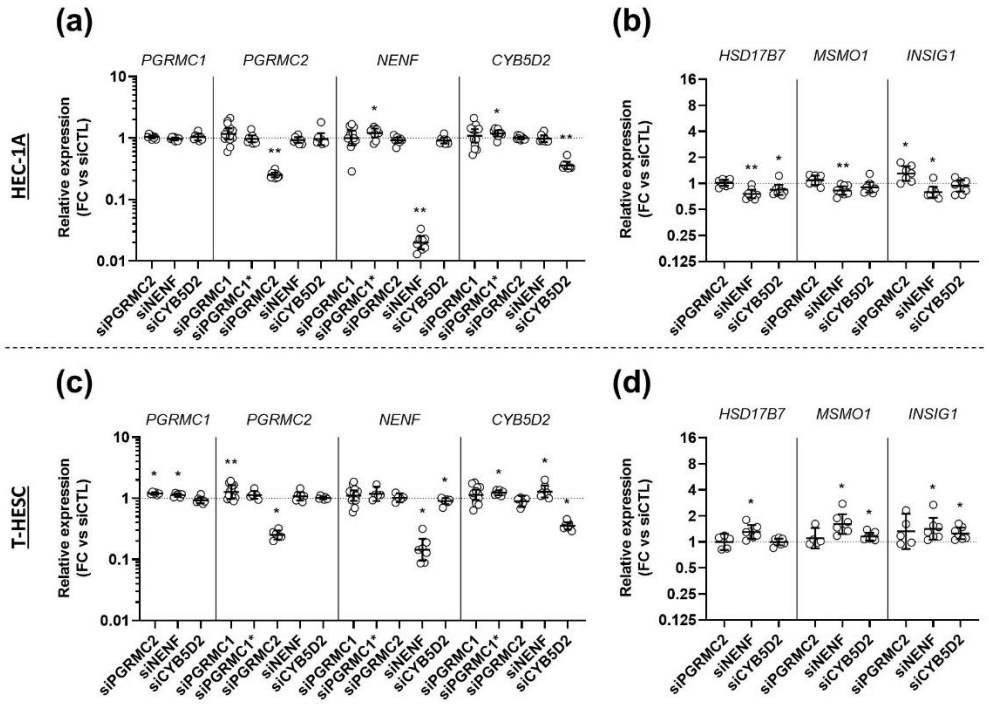

Figure 7. siRNA-mediated down-regulation of *PGRMC2, NENF* or *CYB5D2* expression does not mimic the effect of AG-205 on target genes. HEC-1A (**a**,**b**) and T-HESC (**c**,**d**) cells were incubated with 10nM siRNA-PGRMC1 s21310 (siPGRMC1), siRNA-PGRMC1 18248 (siPGRMC1*), siRNA-PGRMC2 (siPGRMC2), siRNA-NENF (siNENF), siRNA-CYB5D2 (siCYB5D2) or control siRNA-CTL (siCTL) during 72h (n = 4–8). Relative expression of *PGRMC1, PGRMC2, NENF* and *CYB5D2* (**a**,**c**) and *HSD17B7, MSMO1* and *INSIG1* (**b**,**d**) was measured by RT-qPCR, normalized, compared to siRNA-CTL values and is presented as individual fold changes (FC) in log or log2 scale and as geometric means with geometric SD. Statistical test: Wilcoxon paired test, * $p < 0.05$, ** $p < 0.01$. Only differences statistically significant by comparison with the control condition (siCTL) are indicated.

In the second series of experiments, in an approach similar to that used in Figure 6, we transfected cells with the siRNA control or with a mixture of the 5 siRNAs, simultaneously targeting all four MAPR mRNAs (including the 2 siRNAs against *PGRMC1*) before adding AG-205 or control DMSO. The efficiency of the simultaneous downregulation of *PGRMC1*, *PGRMC2*, *NENF* and *CYB5D2* was verified by measuring the expression of these genes in HEC-1A (Figure 8a) and T-HESC (Figure 8c). In the two cell lines, a significant decrease

in expression of all four genes was observed in cells transfected with the mixture of siRNAs by comparison with cells transfected with the siRNA-CTL, both in the presence of AG-205 or control DMSO.

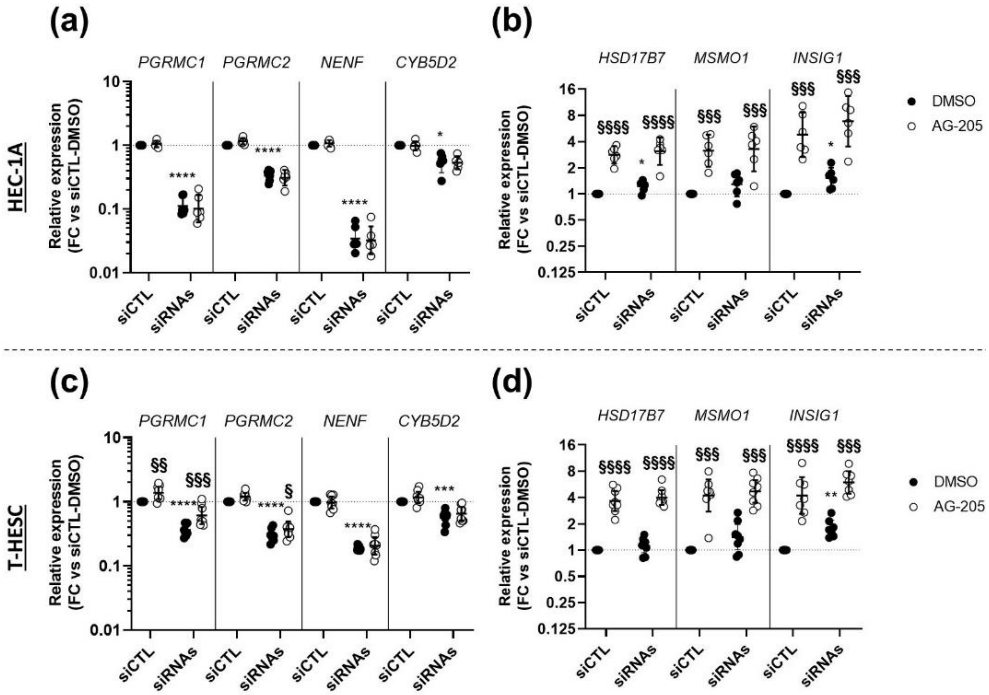

Figure 8. Effect of AG-205 on expression of selected target genes is not modified upon combined siRNA-mediated down-regulation of *PGRMC1*, *PGRMC2*, *NENF* and *CYB5D2* expression. HEC-1A (**a**,**b**) or T-HESC cells (**c**,**d**) were transfected with a combination of 2 nM siRNA-PGRMC1 s21310, 2nM siRNA-PGRMC1 18248, 2nM siRNA-PGRMC2, 2 nM siRNA-NENF and 2 nM siRNA-CYB5D2 (siRNAs) or 6 pmol control siRNA-CTL (siCTL) during 48 h. They were then further incubated for 32 h with 15 µM AG-205 or DMSO. Relative expression of *PGRMC1*, *PGRMC2*, *NENF* and *CYB5D2* (**a**,**c**) and *HSD17B7*, *MSMO1* and *INSIG1* (**b**,**d**) was measured by RT-qPCR (n = 6–8), normalized, compared to dual control values (siCTL with DMSO) and is presented as individual fold changes (FC) in log or log2 scale and as geometric means with geometric SD. Statistical test: Anova-two ways with post-hoc Tukey. Statistically significant differences resulting from siPGRMC1 transfection or AG205 addition are indicated by * or §, respectively: */§ $p < 0.05$, **/§§ $p < 0.01$, ***/§§§ $p < 0.001$, ****/§§§§ $p < 0.0001$.

The addition of AG-205 had no effect on the expression of the MAPR genes (comparison between black and white symbols in Figure 8a,c), except for two minor changes in T-HESC cells: for *PGRMC1* with both siRNA-CTL and anti-MAPR siRNA mixture, and for *PGRMC2* with anti-MAPR siRNA mixture only. The siRNA mixture also induced a slight increase in *HSD17B7* in HEC-1A cells and in *INSIG1* in both cell lines (Figure 8b,d).

As expected, AG-205 significantly increased the expression of the three selected genes *HSD17B7*, *MSMO1* and *INSIG1* in the two endometrial cell lines (Figure 8b,d) and, most importantly, this effect was maintained with a similar extent in cells transfected with the siRNA mixture against all four MAPR genes. Altogether, our results indicate that neither PGRMC1, nor any of the three other MAPRs is involved in AG-205-mediated increase in the expression of genes involved in the cholesterol biosynthesis and steroidogenesis pathways in endometrial cells.

4. Discussion

In the present study, we compared the effects of AG-205 addition and *PGRMC1* downregulation in the culture of endometrial cell lines.

Before turning to transcriptomic analysis, we optimized AG-205 concentration and incubation time, and addressed its potential effects on cell viability and PGRMC1 expression and subcellular localization. AG-205 was rarely added alone in cell culture medium in other studies because it was essentially used to address PGRMC1 contribution to the effect of another inducer. However, it was previously shown that cell viability is reduced in various cell types with AG-205 concentrations above 20 μM: reduction by about 40% and 60% in MDA-MB-231 breast cancer cells at 20 μM and 40 μM AG-205, respectively (Ahmed, 2010); reduction by about 25%, 42% and 50% after 24 h in lung cancer-derived stem cells at 25 μM, 50 μM and 100 μM AG-205, respectively (Hampton, Stewart, Napier, Claudio, & Craven, 2015). This is fully compatible with our measures of cell viability in both endometrial cells lines and supports our choice to further use 15μM AG-205. Throughout our experiments, AG-205 had, in general, no effect on the expression of *PGRMC1* or any other MAPR, although a marginal increase in *PGRMC1* expression was occasionally measured. Moreover, 15 μM AG-205 did not allow detection of increased

PGRMC1 nuclear localization, unlike previously reported in human ovarian cells with 50 μM AG-205 (Will et al., 2017).

In both tested cells lines—T-HESC cells from fibroblastic origin and HEC-1A from epithelial origin—the most striking effect of AG-205 highlighted by our transcriptomic analyses was increased mRNA concentration of several enzymes involved in cholesterol biosynthesis, the sterol-sensitive regulator *INSIG1* and specific enzymes involved in steroidogenesis. Our results are in global agreement with the reported effects of AG-205 in the culture of primary stromal cells induced to decidualize in response to combined estradiol and progesterone (Salsano et al., 2021). However, these effects were produced in the absence of progesterone, suggesting that they are not relevant to decidualization, and, most importantly, they were not mimicked by siRNA-mediated down-regulation of *PGRMC1* or any other related MAPR (*PGRMC2*, *NENF* or *CYB5D2*). Most strikingly, the upregulation of three illustrative genes in response to AG-205 addition was fully preserved when cells were concomitantly transfected by siRNA against *PGRMC1* or all four MAPRs. We thus show for the first time that changes in expression of this set of genes in endometrial cells in response to AG-205 addition are not mediated and do not depend on PGRMC1 or any other MAPR.

However, our study does not rule out that AG-205 could (in)directly interfere with molecular mechanisms involving PGRMC1 to explain previous publications. For instance, AG-205 was recently shown to influence PGRMC1 interactions with the actin cytoskeleton in MIA PaCa-2 cells (Teakel et al., 2020). Moreover, in some studies, the downregulation of *PGRMC1* expression generated effects similar to those induced by AG-205 addition. For instance, EGFR is known to form complexes with PGRMC1, in a PGRMC1 dimer-dependent manner (Ahmed et al., 2010; Kabe et al., 2016) and both experimental strategies (siRNA and AG-205) led to reduced EGFR levels in breast cancer cells (Ahmed et al., 2010). Unfortunately, additional and more specific approaches were not used in all publications reporting effects of AG-205, and their conclusions about PGRMC1 involvement should therefore be considered with great caution. This is, for instance, illustrated with another study focusing on a link between PGRMC1, EGFR and estradiol (Aizen & Thomas, 2015). The addition of AG-205 blocked the inhibitory effect of estradiol on zebrafish oocyte maturation, but the contribution of PGRMC1 to this mechanism and

whether AG-205 did not modify estradiol concentration by modulating expression of metabolic enzymes remain to be confirmed. This would be extremely instructive since a link between PGRMC1, estradiol/estradiol receptor and breast cancer progression was evidenced in other reports that relied on downregulation (Pedroza et al., 2021) or overexpression (Asperger et al., 2020) of PGRMC1, and are therefore not challenged by our study.

These and other examples clearly highlight that, although additional work is required to address its actual mechanisms of action, AG-205 should no longer be referred to, and commercialized as a specific inhibitor/ligand of PGRMC1, and studies based on the use of AG-205 should be replicated with an alternative approach specifically targeting PGRMC1, even when PGRMC1 downregulation is not considered an appropriate choice. For instance, progesterone is known to inhibit GnRH neurons and this effect was abolished by the concomitant addition of AG-205, but not by mifepristone, a progesterone antagonist acting on the canonical nuclear progesterone receptor (Bashour & Wray, 2012). The authors concluded that progesterone was therefore acting through PGRMC1 as an alternative progesterone receptor. Unfortunately, experiments with siRNA were disregarded because of heterogeneity in the GnRH neuron population. Potential changes in progesterone concentration were not monitored after AG-205 addition, and the precise contribution of PGRMC1 remains unclear.

Despite its lack of specificity towards PGRMC1, AG-205 may remain attractive for its anti-mitotic, anti-migratory and anti-invasive activities (Pedroza et al., 2020) which stimulated hopes of therapeutic applications, as illustrated by the patent targeting breast cancer (Rolf Joseph Craven, 2017). However, our data clearly highlight potential detrimental endometrial side-effects, by indicating that its use could modulate endometrial concentration of estradiol and progesterone through local metabolism/intracrinology (D. A. Gibson, Simitsidellis, Collins, & Saunders, 2018). Endometrial pathologies result from a hormonal imbalance between estradiol and progesterone. For instance, endometriosis is characterized by localisation of endometrial tissue at ectopic sites outside the uterus. Progesterone and synthetic progestins are efficiently used to inhibit estrogen-dependent progression of the lesions. Unfortunately, in one-third of the patients, progestogens fail to limit the expansion of the lesion (Jacques Donnez & Dolmans, 2021;

Reis et al., 2020). A decrease in expression of the nuclear progesterone receptors in stromal ectopic cells was established (McKinnon et al., 2018), but not systematically observed (Smuc et al., 2009) and, therefore, is not sufficient to explain progesterone resistance. The contribution of mPRs and MAPRs is currently investigated. However, our results suggest that therapeutic administration of AG-205 to women (for instance against breast cancer) could lead to increased estradiol concentration and/or decreased progesterone concentration, thereby favoring endometriosis development and progression, and increasing the risk of endometrial hyperplasia and cancer development.

In conclusion, although AG-205 may seem attractive for the development of new therapeutic strategies due to its various effects, in particular against cancer progression, it should no longer be considered as a PGRMC1 inhibitor and its precise mechanisms of action and potential detrimental side-effects in medical use should be carefully investigated and documented in the future.

Supplementary Materials: The following are available online at https://figshare.com/s/256ad96e895af6eaab9c [42], Table S1: siRNAs used for siRNA-mediated down-regulation, Table S2: Sequences of primers for specific amplification by quantitative real-time PCR, Table S3: Top5 GO terms identified by over-representation analysis (ORA) in HEC-1A and T-HESC cells cultured in the presence or absence (DMSO) of AG-205. Terms are sorted by adjusted p value (padj), Table S4: Top50 genes differentially expressed upon AG-205 addition in HEC-1A and T-HESC cells. Genes are sorted by adjusted p value (padj), Table S5: Top5 GO terms identified by over-representation analysis (ORA) in HEC-1A and T-HESC cells transfected with siPGRMC1 or siCTL. Terms are sorted by adjusted p value (padj), Table S6: Common GO terms identified by over-representation analysis (ORA) in transcriptomes of HEC-1A cells upon siPGRMC1 transfection or AG-205 addition, Table S7: Common GO terms identified by over-representation analysis (ORA) in transcriptomes of T-HESC cells upon siPGRMC1 transfection or AG-205 addition, Figure S1: Chemical structure of AG-205, Figure S2: Impact of AG-205 on cell viability, Figure S3: Ancestor chart of top5 GO terms identified by over-representation analysis (ORA) in HEC-1A and T-HESC cells cultured in the presence or absence (DMSO) of AG-205.

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Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: RNA sequencing data of cells cultured with AG-205 are stored in NCBI under GEO accession number GSE174305. RNA sequencing of cells cultured with the siRNA-PGRMC1 (s21310) was only used for the purpose of comparison and will be commented in detail in another publication. The transcriptomes of siRNA-transfected cells are only available from the corresponding author on reasonable request. The Supplementary Materials are available online in FigShare at https://figshare.com/s/256ad96e895af6eaab9c, accessed on 26th May 2021 (Charlotte Thieffry, 2021).

Conflicts of Interest: The authors declare no conflict of interest.

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Table S1. siRNAs used for siRNA-mediated down-regulation

Target mRNA	Targeted exon	Company	siRNA ID
PGRMC1	2	Ambion, ThermoFisher Scientific	S21310
PGRMC1	1	Ambion, ThermoFisher Scientific	18248
PGRMC2	3	Ambion, ThermoFisher Scientific	S706
NENF	4	Ambion, ThermoFisher Scientific	S26745
CYB5D2	1	Ambion, ThermoFisher Scientific	S42830

Table S2. 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′
NENF	Forward	5′-TGGCAGTGAAGGGAGTGGTGTTTG-3′
	Reverse	5′-CCGTAGTGTCATGGGTGAGGTCTG-3′
CYB5D2	Forward	5′-GACCGGGGACTGTTCTGAAG-3′
	Reverse	5′-TAGAACCGTCCTGTCACCCT-3′
RPL13a	Forward	5′-GGCTAAACAGGTACTGCTGG-3′
	Reverse	5′-GGTTGGTGTTCATCCGCTTG-3′
HSD17B7	Forward	5′-ATGGACCTAGATGAAGACACTGC-3′
	Reverse	5′-ATAGTGACCCTAATGTGCTTTTCC-3′
MSMO-1	Forward	5′-GGCTAGGGCATGTTTCTTGC-3′
	Reverse	5′-ACTTTCGCCAGCCTATCACC-3′
INSIG-1	Forward	5′-GTGGGGAACATAGGACGACA-3′
	Reverse	5′-TTGTCACTCAAGTCTGAACCAG-3′

Table S3. Top5 GO terms identified by over-representation analysis (ORA) in HEC-1A and T-HESC cells cultured in the presence or absence (DMSO) of AG-205. Terms are sorted by adjusted p value (padj)

	HEC-1A			T-HESC		
#	GO ID	Description	padj	GO ID	Description	padj
1	GO:0016126	sterol biosynthetic process	2.92e-24	GO:0016126	sterol biosynthetic process	1.16e-25
2	GO:0016125	sterol metabolic process	3.43e-22	GO:0016125	sterol metabolic process	7.54e-24
3	GO:0006694	steroid biosynthetic process	4.96e-19	GO:0046165	alcohol biosynthetic process	2.75e-22
4	GO:0008202	steroid metabolic process	1.41e-18	GO:1901615	organic hydroxy compound metabolic process	2.28e-21
5	GO:0046165	alcohol biosynthetic process	3.48e-16	GO:0006694	steroid biosynthetic process	3.51e-21

Table S4. Top50 genes differentially expressed upon AG-205 addition in HEC-1A and T-HESC cells. Genes are sorted by adjusted p value (padj)

	HEC-1A			T-HESC		
#	Gene	FC	padj	Gene	FC	padj
1	*SCD*	23,3	9,72e-269	*HMGCS1*	12,1	1,36e-20
2	*ACSS2*	13,5	1,27e-165	*INSIG1*	8,9	5,40e-18
3	*MVD*	7,5	2,16e-129	*MVD*	7,1	1,82e-17
4	*TM7SF2*	11,0	1,18e-127	*MSMO1*	9,4	6,62e-17
5	*INSIG1*	9,2	3,76e-120	*FDFT1*	5,5	2,97e-13
6	*PCSK9*	7,7	1,43e-117	*HMGCR*	6,3	3,48e-13
7	*HMGCS1*	7,1	6,34e-100	*SQLE*	5,5	3,48e-13
8	*IDI1*	6,2	6,48e-90	*SCD*	6,6	6,37e-13
9	*LSS*	5,8	1,01e-84	*IDI1*	7,1	7,03e-13
10	*MSMO1*	6,1	1,51e-81	*ACAT2*	5,5	1,00e-12
11	*ACAT2*	7,1	6,66e-80	*BHLHe40*	6,1	8,88e-12
12	*DHCR7*	4,3	6,04e-74	*DLL4*	8,7	1,69e-10
13	*HMGCR*	6,0	3,57e-72	*GDF15*	5,7	1,46e-09
14	*FDFT1*	3,9	3,45e-66	*FDPS*	4,3	5,41e-09
15	*ACSL1*	4,8	2,30e-63	*HSD17B7*	5,8	1,18e-08
16	*ELOVL6*	4,5	3,68e-60	*HSD17B14*	4,9	1,15e-07
17	*FDPS*	3,9	9,53e-56	*FABP3*	13,9	2,05e-07
18	*DHCR24*	3,7	4,61e-47	*SAT1*	3,9	3,11e-07
19	*LDLR*	3,6	3,41e-44	*FBXO32*	5,7	3,56e-07
20	*STARD4*	5,2	5,62e-42	*LDLR*	3,9	8,13e-07
21	*HSPA6*	45,6	1,87e-41	*FOLR3*	3,8	1,04e-06
22	*LIPG*	6,2	3,30e-41	*STARD4*	3,8	1,11e-06
23	*LPIN1*	4,4	7,09e-41	*EGR2*	10,9	1,32e-06
24	*SC5D*	3,8	3,87e-40	*DHCR7*	5,1	1,71e-06
25	*SLC2A6*	8,3	2,17e-38	*MCTP1*	6,1	2,06e-06
26	*ERG28*	3,5	7,52e-38	*C11orf96*	5,5	2,06e-06
27	*TMEM97*	2,9	2,00e-37	*MMP3*	4,7	2,24e-06
28	*SQLE*	3,1	3,34e-37	*EBP*	3,7	3,04e-06
29	*SFN*	3,2	3,70e-37	*MTSS1*	3,9	3,19e-06
30	*NEU1*	2,9	4,84e-37	*STC1*	5,7	6,42e-06
31	*SAT1*	4,2	9,83e-37	*CXCL2*	6,7	8,22e-06
32	*EBP*	3,3	1,47e-36	*QPRT*	3,4	1,29e-05
33	*SPP1*	2,6	6,06e-34	*CRYM*	19,8	2,20e-05
34	*QPRT*	2,9	9,14e-34	*GPRC5A*	0,3	2,65e-05
35	*HSD17B7*	4,4	1,16e-33	*NeU1*	3,3	5,07e-05
36	*MVK*	3,5	4,60e-31	*GLM*	3,1	5,75e-05
37	*PADI3*	4,3	4,68e-31	*TPI2*	3,0	5,98e-05
38	*SNAI3-AS1*	7,1	1,39e-29	*SC5D*	3,5	6,17e-05
39	*LCP1*	4,6	5,40e-29	*NPC1*	4,1	6,67e-05
40	*CDKN1A*	14,3	8,72e-29	*LINC02844*	0,1	1,08e-04
41	*PCYT2*	3,3	1,03e-27	*OSR1*	0,3	1,11e-04
42	*UCA1*	2,5	2,18e-26	*ALDH1A1*	0,4	1,11e-04
43	*TNFSF9*	4,4	2,43e-25	*SCIN*	3,0	1,22e-04
44	*GDA*	2,6	4,64e-25	*NCR3LG1*	4,4	1,35e-04
45	*NSDHL*	2,9	3,83e-24	*PCYT2*	2,8	1,35e-04
46	*CLDN4*	2,3	2,43e-21	*RUSC1-AS1*	4,2	1,36e-04
47	*AL109615.3*	3,3	7,24e-21	*PCSK9*	8,6	1,56e-04
48	*LPCAT1*	2,6	2,18e-20	*GPNMB*	3,2	1,61e-04
49	*BHLHE40*	2,3	5,73e-20	*MVK*	3,1	1,74e-04
50	*CYP51A1*	3,4	1,26e-19	*MMAB*	3,2	1,96e-04

Genes of interest involved in sterol biosynthesis process

Table S5. Top5 GO terms identified by over-representation analysis (ORA) in HEC-1A and T-HESC cells transfected with siPGRMC1 or siCTL. Terms are sorted by adjusted p value (padj)

	HEC-1A			T-HESC		
#	GO ID	Description	padj	GO ID	Description	padj
1	GO:0048514	blood vessel morphogenesis	9.04e-8	GO:0070848	response to growth factor	2.02e-5
2	GO:0001944	vasculature development	9.04e-8	GO:0043062	extracellular structure organization	9.28e-5
3	GO:0042330	taxis	9.48e-8	GO:0090287	regulation of cellular response to growth factor stimulus	4.41e-4
4	GO:0045596	negative regulation of cell differentiation	5.18e-7	GO:0006954	inflammatory response	6.69e-4
5	GO:0030155	regulation of cell adhesion	8.88e-7	GO:0008285	negative regulation of cell population proliferation	6.69e-4

Table S6. Common GO terms identified by over-representation analysis (ORA) in transcriptomes of HEC-1A cells upon siPGRMC1 transfection or AG-205 addition

#	GO ID	Description	padj_siRNA	padj_AG-205
1	GO:0009611	response to wounding	1.91e-5	1.44e-2
2	GO:0048871	multicellular organismal homeostasis	5.55e-5	9.69e-4
3	GO:0055088	lipid homeostasis	1.57e-3	1.31e-2
4	GO:1903034	regulation of response to wounding	1.86e-3	1.96e-3
5	GO:0002062	chondrocyte differentiation	1.93e-3	4.51e-2
6	GO:0050819	negative regulation of coagulation	1.98e-3	4.38e-2
7	GO:0051216	cartilage development	2.19e-3	4.13e-2
8	GO:0061041	regulation of wound healing	2.39e-3	1.04e-2
9	GO:0019216	regulation of lipid metabolic process	3.00e-3	1.24e-9
10	GO:0001501	skeletal system development	3.24e-3	4.38e-2
11	GO:0032102	negative regulation of response to external stimulus	3.41e-3	2.75e-5
12	GO:1903035	negative regulation of response to wounding	5.16e-3	2.96e-4
13	GO:0031099	regeneration	6.81e-3	4.92e-3
14	GO:0042730	fibrinolysis	9.05e-3	5.66e-3
15	GO:0051704	multi-organism process	1.29e-2	1.73e-2
16	GO:0010771	negative regulation of cell morphogenesis involved in differentiation	1.38e-2	3.90e-2
17	GO:0061045	negative regulation of wound healing	1.95e-2	4.69e-3
18	GO:0031639	plasminogen activation	2.98e-2	4.56e-2
19	GO:0031638	zymogen activation	4.08e-2	1.73e-2

Table S7. Common GO terms identified by over-representation analysis (ORA) in transcriptomes of T-HESC cells upon siPGRMC1 transfection or AG-205 addition

#	GO ID	Description	padj_siRNA	padj_AG-205
1	GO:0006954	inflammatory response	6.69e-4	1.02e-2
2	GO:0008285	negative regulation of cell population proliferation	6.69e-4	1.64e-2
3	GO:0048514	blood vessel morphogenesis	1.63e-3	9.19e-3
4	GO:0051272	positive regulation of cellular component movement	1.50e-2	1.47e-2
5	GO:0051051	negative regulation of transport	2.11e-2	2.25e-2
6	GO:0036315	cellular response to sterol	2.23e-2	3.85e-2
7	GO:0055088	lipid homeostasis	2.95e-2	7.48e-4
8	GO:0060537	muscle tissue development	3.06e-2	2.71e-2
9	GO:0042330	taxis	3.17e-2	3.27e-2
10	GO:0048568	embryonic organ development	3.39e-2	2.33e-2
11	GO:0055092	sterol homeostasis	3.87e-2	5.43e-6
12	GO:0015850	organic hydroxy compound transport	4.95e-2	2.05e-2

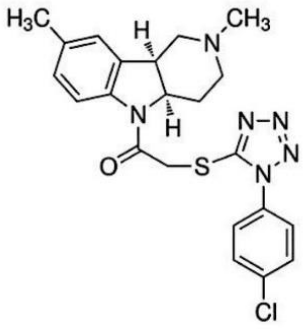

Figure S1. Chemical structure of AG-205.

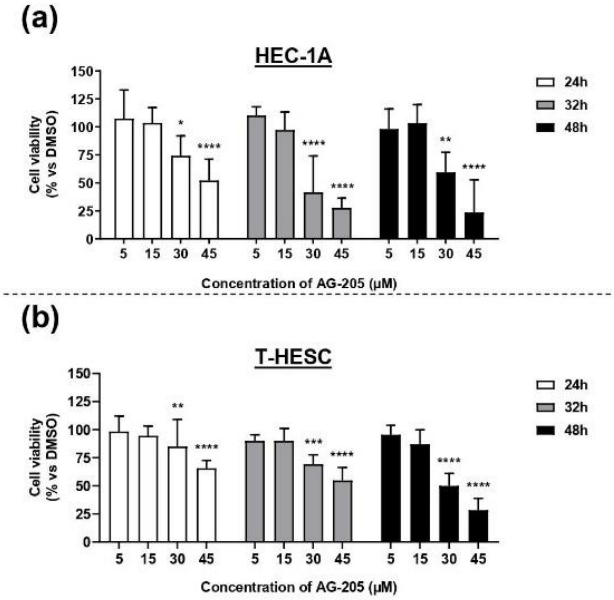

Figure S2. Impact of AG-205 on cell viability. Colorimetric assay of cell viability in HEC-1A (**a**) and T-HESC (**b**) cells after incubation for 24h (in white), 32h (in gray) or 48h (in black) with indicated concentrations of AG-205 or control DMSO (n=6). Absorbance values for cultures with AG-205 were compared to respective DMSO controls and ratios were expressed as % of cell viability and as mean with SD. Statistical test: Anova-two ways with post-hoc Tukey, * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$, **** $p< 0.0001$.

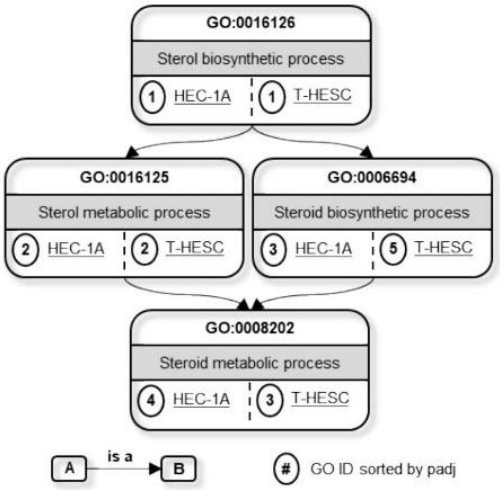

Figure S3. Ancestor chart of top5 GO terms identified by over-representation analysis (ORA) in HEC-1A and T-HESC cells cultured in the presence or absence (DMSO) of AG-205.

IV. DISCUSSION & PERSPECTIVES

The experimental work performed during my PhD has led to a series of original observations:

- PGRMC1 expression is regulated in space and time in the human endometrium along the menstrual cycle;
- PGRMC1 protein abundance appears to be decreased in the eutopic endometrium and in stromal cells of lesions in patients with endometriosis;
- The cyclic variations of this expression suggest a hormonal regulation which is supported by preliminary experiments in xenografts but could not be confirmed in cell culture;
- cAMP increases *PGRMC1* expression by stromal cells in primary culture;
- PGRMC1 and Ki-67 expression follow a similar gradient in the cycling endometrium, depending on the depth within the tissue, suggesting a link between PGRMC1 and control of endometrial cell proliferation;
- PGRMC1 represses the expression of *MMP-3* and potentially *MMP-1*;
- When added in endometrial cell culture, AG-205 is responsible for effects that are not dependent on PGRMC1 or other MAPRs, clearly indicating that AG-205 should no longer be used/commercialized as a specific PGRMC1 inhibitor/ligand.

The critical analysis of these results allows to identify paths for future experiments.

A. **PGRMC1** EXPRESSION IN HUMAN ENDOMETRIUM AND ENDOMETRIOSIS/ADENOMYOSIS

Hypotheses about endometrial PGRMC1 regulation (see III.**B.**) and functions (see III.**C.**) can be formulated based on the in-depth characterization of PGRMC1 mRNA and protein expression in the human endometrium.

i. Endometrium from patients without adenomyosis/endometriosis

The expression of PGRMC1 varies during the menstrual cycle: it increases progressively until the end of the proliferative phase and then decreases drastically until the end of the secretory phase. Moreover, PGRMC1 expression appears heterogeneous according to the depth within the endometrium and defines a gradient whose intensity decreases from the surface epithelium to the myometrium. Moreover, PGRMC1 seems to be occasionally overexpressed in basal glands.

A major contribution of my work is to highlight that, due to the expression gradient, previous publications reporting on endometrial PGRMC1 expression may be biased because they were based on crude tissue homogenates or on selected fields without mentioning their precise localization.

However, despite the original contributions of our detailed analysis, my conclusions about PGRMC1 expression might pertain only to the samples that were analyzed, based on the inclusion/exclusion criteria that were selected. In particular, we excluded samples from patients who were postmenopausal, underwent hormonal treatment within the last 3 months, showed clinical signs of endometrial cancer or endometritis, or whose cycle phase could not be determined unambiguously. As a consequence, most patients selected in our study for PGRMC1 immunolocalization

analysis were unintentionally over 40 years of age. Although most of the patients selected had successful pregnancies, the receptivity of the endometrium may depend on age. It is therefore not excluded that the localization pattern of PGRMC1 could show some discrepancies in the endometrium of younger, more fertile patients.

ii. The adenomyotic and/or endometriotic endometrium

The same cyclic and spatial expression features are observed in the **uterine/eutopic endometrium** of patients with or without adenomyosis/endometriosis. However, quantitative analyses of the PGRMC1 signal allowed us to identify a potential decrease of PGRMC1 in the eutopic endometrium compared to the endometrium of patients without endometriosis, particularly in the stroma in proliferative phase. These observations need to be corroborated with more samples, e.g. to allow to distinguish the samples according to the type of endometriosis which could potentially be a source of variability. Moreover, combination with additional techniques could strengthen the observations. For example, the distribution of the PGRMC1 between stroma and glands could be confirmed by western blotting performed with homogenates of filter-separated endometrial stroma and glands, according to the procedure used for preparation of primary cultures.

The expression and localization of PGRMC1 had not yet been investigated in **endometriosis lesions**. In our study, we evaluated its localization and expression by quantitative analysis of the protein signal and identified a decrease of the ectopic stromal signal in comparison with the corresponding eutopic endometrium during the proliferative phase. Moreover, the cycle phase of the patient had surprisingly no clear influence on the expression and localization of PGRMC1 in the peritoneal lesions as it is the case in the eutopic

endometrium. In both proliferative and secretory phases, PGRMC1 expression was proportionally predominant in the epithelium.

Although further analysis is clearly needed, our results suggest that the regulatory mechanisms of PGRMC1 expression in the endometriotic tissues may be disturbed, especially in the stroma. Based on the functions of PGRMC1 (see III.**C.**), its differential expression with control patients could participate in lesion development or in progesterone resistance. About this last point, complementary analysis of PGRMC1 expression in samples from patients with progesterone resistance could potentially corroborate these results. Nevertheless, the constant difficulty in obtaining enough adequate human samples may encourage to consider alternative murine models.

Although the mouse model has always been rejected as unsuitable for the study of tissue remodeling and menstruation, new mouse models of endometriosis have recently been evaluated and appreciated (Burns et al., 2021). The selection of the mouse model of endometriosis requires careful consideration of genetic background, hormonal cyclicity, immunocompetency, lesion detection strategy and a spontaneous endometrial attachment. Obviously, the ideal model does not exist. However, the most recent ones have incorporated innovative technical approaches such as the use of non-oophorectomized mice and the detection of endometrial lesions by *in vivo* fluorescent imaging to overcome the unique challenges posed by murine anatomy and physiology.

In addition, a new mouse species was recently discovered: the Spiny mouse (*Acomys cahirinus*). Spiny mouse is the only rodent capable of having a natural menstrual cycle. Still under investigation, cellular and genetic mechanisms underlying spontaneous decidualization, ovulation, cyclical endometrial shedding and

regeneration similar to those in humans have been observed (Bellofiore, Rana, Dickinson, Temple-Smith, & Evans, 2018; Seifert & Temple-Smith, 2022). Currently, the relevance of the Spiny mouse as a model of endometriosis has not yet been investigated but it was described as appropriate murine model of human fertility disorders such as implantation failure (McKenna, Bellofiore, Dimitriadis, & Temple-Smith, 2021). As such, it may represent a valuable emerging mouse model for the physiological and pathological study of PGRMC1 in the endometrium.

B. REGULATION OF PGRMC1

The spatiotemporal variations of PGRMC1 expression in the cycling human endometrium prompted us to hypothesize regulatory mechanisms.

i. Hormonal regulation

The contribution of the ovarian steroids was considered to explain the cyclic regulation of PGRMC1 expression.

1. Influence of ovarian steroids on PGRMC1 expression: a critical analysis of the experimental models

The estrogenic and progesterone influence on *PGRMC1* expression was investigated using two experimental models: **cultured endometrial cells** and **xenografts**. Since each of these models has its own advantages and disadvantages, it was important to assess their relevance to our research question.

Cell cultures are undoubtedly a simple and versatile model to set up and use to investigate regulatory mechanisms and signaling pathways. Therefore, we had great hope in cell culture not only for deciphering PGRMC1 regulatory mechanisms but also for the identification of its endometrial functions. However, the cell model also has its limitations. In cultured endometrial cells, estradiol and progesterone failed to modify *PGRMC1* expression, whatever the experimental conditions tested. The cell ability to respond to estradiol and/or progesterone was therefore questioned and addressed by monitoring the expression of control genes. However, despite the presence of ERα and nPRs in our cells (see Figure 28), expression of control genes did not vary as expected. In most samples, *PGR* expression was only weakly stimulated by estradiol, and progesterone failed to repress the expression of *PGR*, *MMP-3*, and *MMP-1*. Only MPA slightly decreased the expression of *MMP-3* and *MMP-1*.

Complementary experiments were performed in the laboratory to test other progesterone-responsive genes, such as Dickkopf-1 (*DKK-1*) which was expected to be stimulated by progesterone or progestin (Tulac et al., 2006). These preliminary results only showed a small increase in *DKK-1* expression (Figure 38) upon progesterone or MPA addition in combination with estradiol, by comparison with estradiol alone. Of note, *DKK-1* expression appeared surprisingly high in most samples cultured only with estradiol.

Altogether, the experiments focused on control genes for hormonal response did not provide unambiguous responses but suggested that mechanisms and signaling pathways stimulated by progesterone could be (partially) altered in our preparations of primary stromal cells.

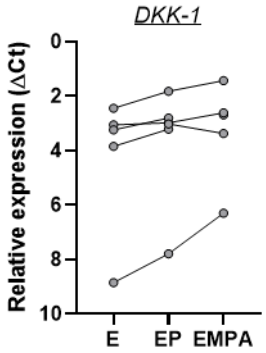

Figure 38: Evaluation of progesterone responsiveness of primary stromal cell culture.
Primary stromal cells incubated for 72h with estradiol (1nM; E), with estradiol and progesterone (1nM-100nM; EP), with estradiol and MPA progestin (1nM-100nM; EMPA) or with respective controls, HP-βCD or DMSO in appropriate concentrations. Relative concentrations of *DKK-1* were measured by RT-qPCR and normalized according to HKGs. Due to the limited samples analyzed, the results are presented as paired dots for individual values (ΔCt) and no statistical analysis was performed.

To rule out the possibility that nPR was constitutively active even in the absence of ligand, nPR expression was decreased in stromal cell culture by using a *PGR*-targeting siRNA and *PGRMC1* expression was measured in response to estradiol addition (Figure 39a). Despite a 2- to 3-cycle decrease in *PGR* expression (Figure 39b), only a slight increase (<1 cycle) in *PGRMC1* expression was observed. Confirmation of the decrease in nPR protein abundance is required but these preliminary results suggest that *PGR* repression is not sufficient to restore a satisfactory estrogenic response in our stromal cell cultures.

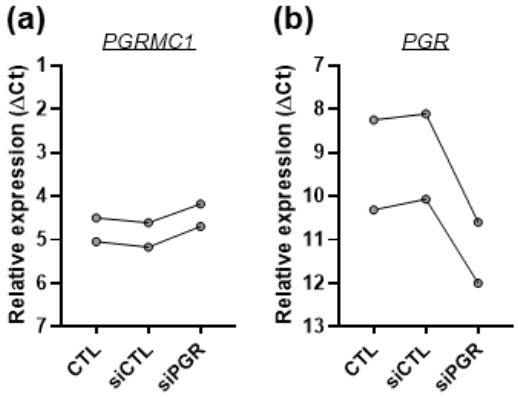

Figure 39: siRNA-mediated down-regulation of *PGR* does not modify substantially *PGRMC1* expression in primary stromal cells incubated with estradiol.

Primary stromal cells were transfected or not (CTL) with siRNA-*PGR* (siPGR) or siRNA-control (siCTL) and incubated for 72h with estradiol (1nM; E). Relative concentrations of *PGRMC1* **(a)** and *PGR* **(b)** were measured by RT-qPCR and normalized according to HKGs. Due to the limited samples analyzed, the results are presented as paired dots for individual values (ΔCt) and no statistical analysis was performed.

The lack of response to estradiol and/or progesterone therefore remains to be explained. Dependency between both hormonal responses should be considered. Indeed, failure of the model to reproduce upregulation of *PGRMC1* expression by estradiol could suffice to explain lack of repression by progestins. Since the

immunofluorescence signal was weak, the amount of the ERα receptor in the stromal cells remains to be confirmed by another technique such as western blotting. Alternatively, artificially increasing ERα expression by transfection of a plasmid carrying the *ESR1* gene could be sufficient to restore an estrogenic and, subsequently, progestational cellular response.

The identification of the molecular reasons explaining failure of hormonal response by cultured cells is an attracting challenge to allow future use of this experimental model, due to its numerous advantages. However, one must remain pragmatic, and finding an explanation may not be insufficient to solve the issue with reasonable time and effort. In this case, turning to alternative experimental models would be necessary.

In xenografts, my preliminary results demonstrate that the model allows hormonal modulation of the expression of *PGR* and the control genes *MMP-3* and *MMP-1*. Moreover, the expression levels of *PGR* measured in samples cultured in the presence of estradiol with or without progesterone remain consistent (difference < 1ΔCt on average) with those measured in endometrial samples from patients in proliferative and secretory phase respectively.

Therefore, human endometrial xenografts could be relevant if the mechanisms and signaling pathways involved in the hormonal regulation of *PGR* are sufficient to regulate *PGRMC1* expression.

In xenografts, *PGRMC1* expression levels measured in samples from mice without hormonal treatment or in mice implanted with pellets delivering estradiol and progesterone remained consistent (difference < 1ΔCt) with those measured in endometrial samples from patients in the menstrual and secretory phases, respectively. But, the *PGRMC1* expression values measured in xenograft samples from mice

implanted with estradiol pellets (~3ΔCt on average) remained lower than those observed in endometrium samples from patients in the proliferative phase (~1.8ΔCt on average).

It is possible that analysis of a larger number of samples would reduce this difference. However, the *PGR* expression values, obtained in the different xenograft groups, are all consistent with those measured in endometrial samples from patients in all respective cycle phases.

From the simplest model (cell culture) to the most complex (xenograft), there are however several other intermediate experimental models:

(i) **3D models** of reconstructed endometrium. Organoid describes 3D structures that resemble organs or tissues *ex vivo* in a supportive hydrogel droplet, replacing the extracellular matrix of the originating tissue. Organoids of human endometrial epithelial cells have already been generated and can be cultured long-term. These models have demonstrated morphological and functional characteristics similar to those of the endometrial epithelium *in vivo* in response to estradiol, progesterone and several signals of stromal origin (Alzamil, Nikolakopoulou, & Turco, 2021; Boretto et al., 2017; Turco et al., 2017). Organoids could represent a good model for the *in vitro* study of PGRMC1 in the endometrial epithelium. However, it is important to note that currently developed endometrial organoids are composed exclusively of epithelial cells and therefore do not include any stromal, vascular or immune components. A new model of co-culture of these organoids with primary endometrial stromal cells is under development. Although promising, this new model, named assembloid, shows signs of spontaneous tissue lysis, probably related to MMPs (Rawlings, Makwana, Taylor, et al., 2021; Rawlings,

Makwana, Tryfonos, & Lucas, 2021). The use of pharmacological agents to limit their activity is under study.

(ii) Human endometrial **explants**. Pilot experiments were performed in a human endometrial explant model. These endometrial fragments were cultured for 72h in the presence or absence of estradiol and/or progesterone. Experience was reproduced twice but, like primary stromal cultures, *PGRMC1* expression remained stable under all hormonal conditions (results not shown). Although physiologically complex, the explant model cannot be used beyond 72h of culture. The expression and activity of MMPs becomes uncontrollable and causes tissue lysis of the explant. This model could therefore successfully reproduce a modulation of PGRMC1 expression if it does not require a long hormonal impregnation.

Finally, a complementary approach has been initiated to investigate a potential link between ovarian steroids and local control of *PGRMC1* expression, by spatially correlating estradiol and progesterone levels with PGRMC1 protein amounts in endometrial **tissue sections**. To this aim, in collaboration with Arnaud Delcorte's group (Institute of Condensed Matter and Nanosciences at UCLouvain), intracellular estradiol and progesterone levels will be measured *in situ* by secondary ion mass spectrometry (SIMS), a metabolic imaging analysis at the single-cell scale (Gilmore, Heiles, & Pieterse, 2019; Moshkunov et al., 2021). In this way, we hope to find a gradient of estradiol and progesterone concentration according to the depth of the endometrium consistent with PGRMC1 expression: in favor of estradiol on the surface and/or in favor of progesterone near the basal. Endometrial sections are currently being analyzed to set up the procedure.

2. Hypotheses of regulatory mechanisms: the role of hormone receptors

The expression and localization of classical estradiol (ERα and ERβ) and progesterone (PR-A and PR-B) receptors have already been described in the human endometrium during the menstrual cycle.

Their distribution according to the depth of the endometrium is, on the other hand, less documented and their potential co-expression with PGRMC1 in endometrial cells was never addressed. Therefore, in order to establish a potential co-regulation with nPRs, I performed co-immunolabeling of PGRMC1 and nPRs in samples of human endometrium collected during the different phases of the menstrual cycle.

With the exception of the menstrual phase, expression of *PGR* varies similarly to *PGRMC1* during the menstrual cycle. However, my preliminary results suggest that, unlike PGRMC1, epithelial nPR expression increases progressively from the surface epithelium to the basal glands. These disparities between the expression and localization profiles of nPRs and PGRMC1 suggest that the hormonal regulation of nPRs as described in the literature is insufficient to explain the spatiotemporal variations of PGRMC1. Therefore, if there is hormonal regulation of PGRMC1, the receptors and signaling pathways involved could differ from those regulating nPRs expression. My experiments did not include distinction between PR-A and PR-B isoforms. Therefore, we cannot exclude a possible parallelism between the expression and localization of PGRMC1 and one of these two isoforms. Further analysis specifically targeting the PR-B isoform would be informative.

On the other hand, and although the proportion of cells co-expressing PGRMC1 and nPR is high, it is also conceivable that the repression of PGRMC1 expression by progesterone during the secretory phase depends on nPR. Experiments can be designed to directly identify a correlation between the presence of one of the steroid receptors and the expression of *PGRMC1* within the same cell by combining RNAscope *in situ* hybridization with immunostaining.

According to the literature, the expression of ERα and ERβ is homogeneous according to the depth of the endometrium (Bombail, MacPherson, Critchley, & Saunders, 2008). Therefore, the hypothesis PGRMC1 expression is directly induced through classical estradiol receptors is not favored. Additional mechanisms, responsible for local adjustment, could involve other estradiol receptors such as the membrane receptor GPER.

Expression pattern of GPER in the endometrium and in endometriosis appears partially consistent with that of PGRMC1. Indeed, during the menstrual cycle, they are both mainly expressed during the proliferative phase and progressively decrease during the secretory phase (Kolkova et al., 2010). In contrast, GPER expression appears increased in eutopic and ectopic endometrial epithelium (Plante et al., 2012; Yuguchi et al., 2013) while PGRMC1 protein expression tends to decrease in the endometriotic stroma. Interestingly, GPER can regulate gene expression through the cAMP, ERK, PI3K pathway (Yu et al., 2022). Currently, cAMP is the only factor that we have identified as capable of stimulating *PGRMC1* expression. Therefore, it is possible that GPER expression is altered in cultured endometrial stromal cells but that the addition of cAMP restores the inducing effect of estradiol on *PGRMC1* expression through the ERK, PI3K signaling pathway. Experiments aiming to define GPER expression levels *in vitro* by RT-qPCR, immunocytofluorescence and/or western blotting could confirm or refute this hypothesis.

ii. Regulation by immune cells

In the human endometrium, a negative correlation between PGRMC1 expression and the presence of endometrial immune cells was found. Indeed, the proportion and composition of immune cells varies in the endometrium during the menstrual cycle but is minimal during the proliferative phase (Jain, Chodankar, Maybin, & Critchley, 2022), when PGRMC1 expression is at its maximum. Conversely, we observed a reduced PGRMC1 expression in endometriotic patients, a condition characterized as a chronic inflammatory disease.

In the xenograft SCID mice model, estradiol fails to stimulate *PGRMC1* expression at levels similar to those observed in the proliferating human endometrium. Although the implants may contain the inflammatory or immune cell populations that were present at the time of transplantation, infiltration by circulating murine B and T cells is impossible in these immunodeficient mice.

It is therefore plausible that B and T cells participate in the stimulation of PGRMC1 expression in the endometrium while other immune cells, such as NK cells which are mostly represented in the secretory phase and in endometriosis, repress its expression. An *in vitro* co-culture model of adherent endometrial cells and immune cells in suspension would allow to test this hypothesis.

iii. Regulation by cell proliferation

The expression profile of Ki67 in the human endometrium describes a gradient according to the depth of the endometrium similar to that observed for PGRMC1, suggesting that PGRMC1 is expressed specifically in proliferating endometrial cells. Furthermore, the role of cAMP in the regulation of cell proliferation initiated by steroid hormones is well established and could contribute to the increased expression of PGRMC1 in cultured primary endometrial stromal cells observed in the presence of cAMP (Stork & Schmitt, 2002).

Identification of cells co-expressing *PGRMC1* mRNA and Ki67 protein by simultaneous RNAscope *in situ* hybridization and immunostaining would help in testing this hypothesis. In addition, expression of *PGRMC1* could be monitored in cells in which proliferation is impaired in vitro, for example by adding an inhibitor such as mitomycin to the culture medium.

iv. Spatial expression gradient: hypotheses of local regulatory mechanisms

The spatiotemporal regulation of PGRMC1 expression seems complex and considering a simple mechanism of stimulation-repression of its expression in the endometrium may not be sufficient. Three hypotheses for local regulation of PGRMC1 expression in the endometrium should be considered.

First, in the hypothesis of hormonal regulation, local production of ovarian steroids by intracrinology in endometrial cells was recently suggested (D. A. Gibson et al., 2018; Douglas A Gibson, Simitsidellis, Collins, & Saunders, 2020; Huhtinen et al., 2012). The levels of estradiol and progesterone effective in the endometrium do not only depend on cyclic variations in blood hormone concentrations but also on the balance between the tissue expression of enzymes involved in their synthesis and those responsible for their metabolism. Currently, the expression of these enzymes in the endometrium has not been characterized according to the depth of the tissue but a differential expression of these enzymes according to the tissue compartment, the cycle phase and/or the pathological context has already been identified for some of them and could support local fluctuations of PGRMC1 expression. For example, HSD17B2, which catalyzes the transformation of estradiol into its inactive compound, is specifically expressed in glandular epithelial cells and is markedly increased in the secretory phase (Maentausta et al., 1991) and in the eutopic endometrium of women with endometriosis and/or adenomyosis (Kitawaki et al., 2000). A better knowledge of the endometrial expression profiles of all the enzymes involved in the biosynthesis/metabolization pathway of steroid hormones could be relevant in understanding the mechanisms of spatial regulation of PGRMC1 expression in the endometrium and endometriosis.

On the other hand, the fact that no significant difference in *PGRMC1* expression was detected between the endometrium of patients with or without endometriosis by RT-qPCR, whereas a decrease in its protein abundance was suggested by immunostaining in the eutopic endometrium, could indicate the existence of post-transcriptional regulatory mechanisms.

Finally, it is possible that *PGRMC1* expression is dependent on complementary regulatory mechanisms such as miRNAs. To date, the miRNAs miR-181a, miRNA-139-5p, and miRNA-98 have been shown to repress *PGRMC1* expression (Panda et al., 2012, Wendler et al., 2011, Zhao et al., 2014). Their respective expression profile in the human endometrium according to tissue depth and during the menstrual cycle has not yet been determined but it could also participate in the regulation of *PGRMC1* expression in endometrium and endometriosis.

C. POTENTIAL FUNCTIONS OF PGRMC1 IN ENDOMETRIUM AND ENDOMETRIOSIS/ADENOMYOSIS

I initiated experiments aimed at the identification of PGRMC1 functions in endometrial stromal cells.

At present, our results suggest a potential role for PGRMC1 as a regulator of cell proliferation and as a repressor of *MMP-3* and *MMP-1* expression and further analysis is obviously needed to confirm and investigate the underlying mechanisms. Nevertheless, the main objective was to first focus on targeted potential roles of PGRMC1 in the human endometrium, and more particularly in tissue remodeling, before considering a broad-spectrum identification of its potential targets by RNA sequencing. This part of the global project about PGRMC1 in the host laboratory was later transferred to another PhD student, Marie Van Wynendaele who further analyzed transcriptomes of primary stromal cells rather than endometrial cell lines, after their transfection with a specific siRNA-PGRMC1 or a control siRNA.

On the other hand, some features that might be important for PGRMC1 function were not included in our preliminary study but will be investigated in future experiments. They comprise: (i) the influence of progestin input, (ii) the role of PGRMC2 as a potential partner and/or protein capable of a possible compensation, and (iii) the impact of post-translational modifications on PGRMC1 functions in the endometrium. Accordingly, a PGRMC2-specific siRNA and several plasmid constructs containing sequences of PGRMC1 mutated for one or more post-translational modification sites have already been prepared.

i. Endometrial remodeling

1. PGRMC1, a regulator of cell proliferation

In order to obtain results more relevant to physiological mechanisms, proliferation assays were performed with primary stromal cells. We observed that repression of PGRMC1 expression induces a decrease in cell number when stromal cells were incubated without hormone but also an increase in stromal cell number when cultured with estradiol. These results corroborate those of other teams stipulating that the activity of PGRMC1 in cell cycle control is dependent on hormonal input (Bai et al., 2021; Cai et al., 2021; Lodde & Peluso, 2011; Pedroza et al., 2021) and suggest that it differs according to the phase of the menstrual cycle. PGRMC1 could promote stromal cell proliferation during the menstrual phase and limit estradiol-dependent tissue expansion in the proliferative phase, and particularly on the endometrial surface where it is most expressed.

It is currently suggested that PGRMC1 regulates cell proliferation by modulating the mitotic spindle stability and cycle entry (Lodde & Peluso, 2011). The molecular mechanisms involved in the control of the cell cycle by PGRMC1 remain unclear. But, a study recently related that the Y180-phosphorylated form of PGRMC1 was able to repress proteins involved in the G1 checkpoint regulation (Thejer, Adhikary, Teakel, et al., 2020). So, it is possible to envisage that, during the menstrual cycle, hormonal intake could indirectly modulate the capacity of PGRMC1 to control the endometrial cell cycle by modifying its structure. However, this hypothesis requires further investigation.

Indeed, it is important to note that the magnitude of the effects observed on cell proliferation following PGRMC1 repression in primary stromal cells remains modest (~10%). Performing the same

proliferation experiments with cell cultures of greater proliferative potential to demonstrate potentially more drastic and significant effects is the most immediate prospect. In this respect, the endometrial epithelial cell lines, HEC-1A and Ishikawa, derived from uterine adenocarcinoma and available in the laboratory, could be used.

2. PGRMC1, a repressor of *MMP-3* and *MMP-1*

Like Allen et al., we identified a repressive role of PGRMC1 on MMP expression (Allen et al., 2015). Indeed, *MMP-3* and *MMP-1* expression was stimulated when *PGRMC1* was repressed by an siRNA in the endometrial stromal line T-HESC. However, by adding complementary control culture conditions, we demonstrated for the first time that this repressive effect was transcriptional and independent of the progesterone supply. However, some authors suggest that progestin compounds can modulate PGRMC1 functions through phosphorylation (Bai et al., 2021). A proportion of phosphorylated forms of PGRMC1 initially higher in T-HESC cultures than in the cytotrophoblastic cells used by Allen et al. could potentially explain our divergent conclusions about the influence of progesterone on the repressive effect of PGRMC1 towards MMPs expression.

In complement, further analysis by zymography to establish the influence of PGRMC1 on MMP activity and demonstration of these same effects in primary cultures would be relevant. Furthermore, it is possible that the transcriptional activity of PGRMC1 towards *MMP-3* and *MMP-1* depends on its nuclear localization. To investigate this mechanism, the use of mutant plasmid constructs that prevent nuclear translocation of PGRMC1 (Phospho-site and/or Sumo-site mutations) is being considered (Sabbir, 2019).

ii. Potential influence of PGRMC1 on endometrial physiological mechanisms and pathogenesis of endometriosis

As a membrane "receptor" for progesterone, the ability of PGRMC1 to transmit a progestin signal was studied mainly by other teams. However, the expression profile of PGRMC1 during the menstrual cycle suggests rather a major functional activity during the estradiol-dependent proliferative phase.

Although our study contributes to a better understanding of PGRMC1 expression and function in the endometrium and adenomyosis /endometriosis, our current knowledge does not yet allow to define with precision the role(s) of PGRMC1 in the endometrial tissue. We can only speculate on its physiological function, pathological activity and potential therapeutic end-point.

1. Physiological roles

Through different processes, PGRMC1 could participate in the physiological balance of the endometrium necessary for its **fertility** function. Indeed, the cyclical and spatial expression pattern of PGRMC1 suggest a potential role of the protein in the progressive preparation of an environment conducive to embryo implantation.

On the one hand, and although analyses addressing the co-expression of stem epithelial markers are needed, (i) the identification of some basal glands overexpressing PGRMC1, (ii) the increase of its expression between the secretory and the menstrual phase and (iii) its pro-proliferative activity in the primary stromal cells incubated without hormone could indicate its involvement in the repair and/or tissue regeneration of the endometrium during the menstrual and proliferative phases. Moreover, like progesterone in the secretory phase, PGRMC1 could play a complementary role in endometrial tissue remodeling by preventing its degradation during the

proliferative phase by repressing *MMP-3* and *MMP-1* and by participating in the induction of menstruation at the end of the secretory phase when its expression is the lowest.

On the other hand, the role of PGRMC1 in the decidualization process of stromal cells, conducive to embryo implantation, was already suspected by Salsano *et al.,* (Salsano et al., 2020; Salsano et al., 2021; Salsano et al., 2017). The strong decrease in PGRMC1 expression between the end of the proliferative phase and the beginning of the secretory phase observed in the human endometrium is in disagreement with their hypothesis. But, considering that stromal cells in culture resemble more decidualized than pre-decidualized cells after the addition of cAMP, the increase in *PGRMC1* expression could make sense and suggest that PGRMC1 participates in the maintenance of endometrial tissue integrity during pregnancy. The role of PGRMC1 in decidualization is investigated by our laboratory in primary stromal cultures transfected with siRNA-PGRMC1. Co-labeling of PGRMC1 and decidualization markers, such as prolactin and laminin, in endometrial sections from patients in the secretory phase is also considered.

2. Roles in endometriosis

Due to its decreased expression in eutopic endometrium and ectopic peritoneal stromal cells, PGRMC1 may contribute to **subfertility** observed in some patients, **inflammation-related pain**, and/or the **development of endometriosis lesions**.

First, fertility resulting from the preparation of the endometrium for embryo implantation requires a perfect balance in the hormonal response of the tissue. The decrease in fertility of endometriotic patients resulting from the decreased expression of PGRMC1 in the eutopic endometrium could be suspected. But the endometriotic samples analyzed were found to be from patients with at least one

successful pregnancy. Further analysis to define its expression in the eutopic endometrium of subfertile patients would be relevant. Nevertheless, based on our preliminary results, PGRMC1 cannot be considered as a reliable marker of human fertility.

In the same way as the decrease in nPRs (Jacques Donnez & Dolmans, 2021), the reduced expression of PGRMC1 in the ectopic stroma could participate in the progesterone resistance at the origin of the frequent failure of progestin treatments for endometriosis.

On the one hand, PGRMC1 is suspected to prevent nuclear translocation of NF-κB (Peluso et al., 2019). Accordingly, depletion of PGRMC1 could initiate activation of the NF-κB pathway and, consequently, inflammation and pain.

On the other hand, its activity could potentially be modified by the oxidative stress present in endometriosis lesions by promoting heme-staking dimer formation and heme-binding related functions of PGRMC1 including chemoresistance, limitation of apoptosis, EGFR-dependent proliferation, and transformation of lanosterol to cholesterol, a precursor of steroidogenesis (J. Donnez et al., 2016; Friel et al., 2015; Hughes et al., 2007; Kabe et al., 2018; Kabe et al., 2016).

The regulation of *MMP-3* and *MMP-1* expression by PGRMC1 could represent a local regulation mechanism of their activity involved in the migration and invasion of ectopic endometrial cells required for the development of endometriosis lesions. Experiments scheduled in the laboratory will aim to clarify the involvement of PGRMC1 in cell migration, and more precisely in the formation of invadopodia, in the endometrial epithelial cell line HEC-1A. Indeed, invadopodia are invaginations of the plasma membrane constituted by an actin cytoskeleton mainly observed in epithelial cells and whose role in the local degradation of the extracellular matrix necessary for cell

migration has already been mentioned (Mgrditchian, Sakalauskaite, Müller, Hoffmann, & Thomas, 2021). Cholesterol enrichment is also suspected in the plasma membrane of invadopodia. Due to its interaction with actin filament-associated proteins, its role in the regulation of MMPs and in cholesterol biosynthesis, PGRMC1 therefore appears to be of interest in the process of invadopodia formation (McGuire et al., 2021; Thejer, Adhikary, Kaur, et al., 2020).

Finally, the majority epithelial expression pattern of PGRMC1 in endometriosis lesions can also be occasionally observed in basalis of the eutopic endometrium. According to the literature, the basal glands have a stemming character and participate in the regeneration of the endometrium (Cousins et al., 2021). Therefore, these pattern similarities might suggest that in addition to a crucial role of PGRMC1 in the pathogenesis of endometriosis, it might also represent evidence that endometrial stem cells can be at the origin of the development of peritoneal lesions. But this hypothesis requires further investigation.

D. ABOUT THE IMPORTANCE OF GOOD TOOLS – OR "ONLY TRUST YOURSELF"

By conducting a pilot project in the laboratory, I regularly had to make choices among specific aims and strategies, largely depending on available experimental tools and past publications from other laboratories (Bunch et al., 2014; Peluso, 2013; Peluso et al., 2006; Peluso et al., 2008). In retrospect, progression of my project was deeply penalized by inadequate commercialized molecular tools and biased or erroneous literature.

Although widely referenced by the most specialized authors working on PGRMC1, the "Prestige"-grade anti-PGRMC1 antibody (REF: HPA002877, Sigma-Aldrich) and the AG-205 inhibitor, among others, drastically jeopardized my work. I have learned the hard way that it is crucial to start any experimental work by validating all key products, even when they are marketed as "high quality and specific", "validated" and/or referenced in multiple publications. In particular, the availability of the AG-205 "specific inhibitor" was supposed to represent a major advantage for the identification of PGRMC1 functions and mechanisms of action. Fortunately, I was able to valorize the very disappointing results in a publication aimed to alert the scientific community about caution for its usage.

However, if further experiments demonstrate that increasing the expression levels of enzymes involved in the cholesterol biosynthetic pathway results in a significant increase in cholesterol levels, an alternative therapeutic use of AG-205 in hypocholesterolemia could be considered. Hypocholesterolemia affects 2-5% of the population and is more a symptom of an underlying pathology, such as cancer, liver failure or depression, than a pathology. However, it is a risk factor for stroke. Similarly, the use of AG-205 to limit excessive expression of *MMP-3* and/or *MMP-1* may also be beneficial in patients with

dysfunctional menstrual bleeding. Further analysis to define the target(s) of AG-205, its integrated functional impact and to develop a specific tissue/cellular addressing mechanism is nevertheless necessary beforehand given its potentially harmful activity on other organs, such as the endometrium.

V. MATERIALS & METHODS

This section contains all the technical information related to the additional experiments that were neither published nor submitted before the writing of the thesis.

Tissue collection

The study was approved by the Ethical Committee of the Université Catholique de Louvain (2017/10JUL/362). Tissue samples were provided by the Biobank of Saint-Luc University Clinics as surgical leftovers from patients operated for clinical reasons independent from the study. Biomaterial was obtained by biopsy during laparoscopy or following hysterectomy from patients with spontaneous menstrual cycle (<50 years old) and without progestin treatment for the last 3 months. Samples of eutopic endometrium were separated in three groups: patients with endometriosis, patients with adenomyosis and control patients without endometriosis, adenomyosis, uterine cancer or endometritis. For each sample, the cycle phase was determined by an expert anatomo-pathologist. Tissue for histological examination was fixed in 4% paraformaldehyde and embedded in paraffin whereas tissue for RNA analysis was placed into RNA lysis buffer (SV total RNA isolation system; Promega) at -80°C until RNA extraction. The rest of the tissue was used for the primary culture.

Primary cells, cell lines

The selection of the functional layer of the endometrium, the tissue dissociation, the separation of stromal and epithelial cells by filtration and the primary cells culture were performed as previously described (Pretto et al., 2008). The first cell passages were privileged for the hormone experiments.

The human stromal endometrial cell line used for the experiments, the Telomerase-immortalised Human Endometrial Stromal Cell line (T-HESC, ATCC CRL-4003), is derived from fibroblast-like cells obtained from an adult patient with myomas (Krikun et al., 2004).

Cell culture, hormones and cAMP incubation

Cells were grown in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12; Gibco, ThermoFisher Scientific), classically supplemented with 10% Fetal Bovine Serum (FBS), 100U/mL penicillin, 100µg/mL streptomycin (ThermoFisher Scientific) and 1nM E2 in a humidified atmosphere of 5% CO2 at 37°C.

For the hormone experiments, 17β-estradiol-2-hydroxypropyl-β-cyclodextrin (E; Sigma) and progesterone-2-hydroxypropyl-β-cyclodextrin (P; Sigma) were solved in DMEM/F12, no phenol red (Gibco, ThermoFisher Scientific). The experimental progestin reference, MPA (Cayman) were diluted in 100% dimethyl sulfoxide (DMSO;) to achieve the stock concentration at 1mM.

Cells were previously seeded (2.10^4 cells/mL) and cultured for 24h in DMEM/F12, no phenol red, with 10% FBS, 100U/mL penicillin, 100µg/mL streptomycin, without E. Next, medium was supplemented with hormones (1nM E and/or 100nM P or MPA) with or without 0.5mM cAMP, or with 100nM 2-hydroxypropyl-β-cyclodextrin (HP-βCD; Sigma) or 0.01% DMSO as negative controls. The culture media were changed every 24h to maintain desired hormone concentrations. The lysates were recovered after 72h of hormone incubations.

Inhibition strategies

Two inhibition strategies were used in a unique inhibition protocol: siRNA-mediating gene silencing (Table I) and the using of an inhibitor, AG-205. Transient transfection was performed with Lipofectamine RNAiMax (Invitrogen) according to manufacturer's recommendations. The validation of efficiency and specificity of siRNA-PGRMC1 towards the others MAPRs as well as the optimization of the final concentration and the incubation time of AG-205 inhibitor were carried out in endometrial T-HESC and HEC-1A cell lines (Thieffry et al., 2021). The inhibitors AG-205 and actinomycin D were solved in 100% DMSO at 15mM and 0,8mM respectively.

Table I. siRNAs used for siRNA-mediated down-regulation.

Target mRNA	Targeted exon	Company	siRNA ID
PGRMC1	2	Ambion, ThermoFisher Scientific	S21310
PGR	4, 5	Ambion, ThermoFisher Scientific	S10416

For the MMPs experiments (Figure 40), T-HESC (2.10^4 cells/mL) were transfected with final 6pmol pre-designed Silencer siRNA-PGRMC1 or negative control (Table I). Transfected cells were cultured in DMEM/F12, no phenol red, with 10% FBS, 1nM E, without antibiotics. At the same time, additional wells were prepared with 2.10^4 cells/mL and cultured under the same conditions for the second inhibition strategy. After 48h incubation, medium of all the wells was changed and 15µM of AG-205 inhibitor or 0.1% DMSO, as negative control, were added to the appropriate wells. Two hours later, 100nM MPA and/or 0,8µM actinomycin D or the same concentration of negative control DMSO were added to culture medium. 2ng of

cytokine IL-1α was added 6hours later to induce the MMPs expression and the lysates were collected after 24hours incubation.

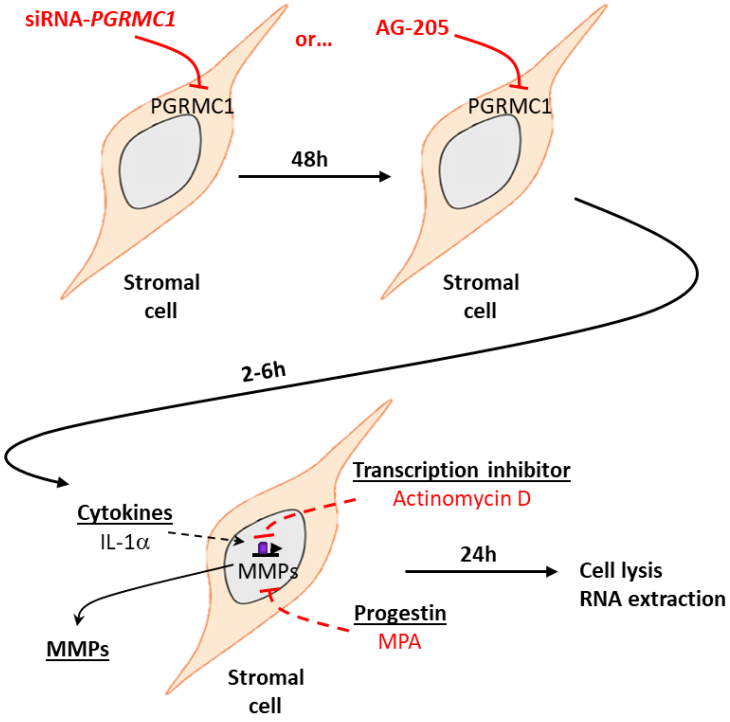

Figure 40: Experimental protocol used for the MMPs experiments.

Cell viability assay

The optimization of the final concentration and the incubation time of AG-205 was carried out with the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's recommendations. Briefly, cells were seeded in 96-well plates (2.10^4 cells/mL) and grown in DMEM/F12, without phenol red nor antibiotics, supplemented with 10% FBS. After 48h incubation, medium was changed after supplementation with indicated concentrations of AG-205 or corresponding DMSO concentration as control. Cells were incubated for 24h, 32h or 48h before addition of 20µL/well of CellTiter 96® AQueous One Solution Reagent containing

a tetrazolium compound (MTS). After 1-4h incubation at 37°C, the quantity of formazan (a bioreduced colored product of MTS directly proportional to the number of living cells) was measured at 490nm absorbance.

Immunolabeling

Immunofluorescence (IF) was performed on paraffin-embedded tissue samples cut in serial sections of 6µm. After dewaxing in Histosafe, antigen retrieval was performed in citrate Buffer for 75 min at 100°C and tissue sections were permeabilized for 15 min in phosphate-buffered saline (PBS), 0.3% Triton X-100. Nonspecific binding sites were blocked for 1h at RT with PBS, 0.3% Triton X-100, 10% bovine serum albumin (BSA) and 3% milk before incubating the slides overnight at 4°C with the primary antibody (Table II). The next day, tissue samples were washed 3 times for 5 min in PBS, 0.1% Triton X-100, and incubated with adequate combination of secondary antibodies (Table II) for 60 min at RT.

Immunocytofluorescence (ICF) was performed with cells cultured on glass coverslips. After appropriate incubation, cells were washed in PBS before fixation for 10 min in 4% paraformaldehyde. They were washed 5 times for 5 min in PBS and permeabilized for 15 min in PBS with 0.1% Triton X-100. Nonspecific binding sites were blocked for 1h at room temperature with PBS, 0.3% Triton X-100, 5% normal goat serum before incubating coverslips overnight at 4°C with primary antibodies (Table II). The next day, cells were washed 3 times for 5 min in PBS and incubated with 1/1000 secondary antibody (Table II) for 1h30 at room temperature.

Nuclei were counterstained with Hoechst (BisBenzimide H33342, 1µg/mL, Sigma) during the incubation with the secondary antibody.

Table II. Primary and secondary antibodies used for immunolabeling.

Primary antibodies	Isotype	Company	Commercial reference	Dilution
Anti-PGRMC1 (D6M5M)	Rabbit mAb	Cell Signaling	13856	1/250 (IF) 1/200 (ICF)
Anti-E-cadherin (Clone 36)	Mouse IgG2a mAb	BD Transduction Laboratories	610182	1/1000 (IF) 1/500 (ICF)
Anti-Vimentin (Clone V9)	Mouse IgG1 mAb	Santa Cruz	Sc-6260	1/100 (ICF)
Anti-nPR (Clone F-4)	Mouse IgG1, mAb	Santa Cruz	Sc-166169	1/250 (IF) 1/100 (ICF)
Anti-ERα (Clone F-10)	Mouse IgG2a, mAb	Santa Cruz	Sc-8002	1/100 (ICF)
Anti-Ki67 (Clone B56)	Mouse IgG1, mAb	BD Pharmingen	556003	1/250 (IF)
Secondary antibodies	**Isotype**	**Company**	**Commercial reference**	**Dilution**
Anti-IgG Rabbit, Alexa 488	Goat	ThermoFisher	A11034	1/1000
Anti-IgG2a Mouse, Alexa 568	Goat	ThermoFisher	A21134	1/1000
Anti-IgG1 Mouse, Alexa 647	Goat	ThermoFisher	A21240	1/1000

Microscopy and signal quantification

Morphological analysis was performed by Widefield Microscopy while fluorescence was observed with a Cell Observer Spinning Disk (COSD) confocal microscope (Zeiss) and/or with a Pannoramic P250 Flash III slide scanner (3DHistech, Budapest, Hungary).

The signals were analyzed and quantified with the image analysis platform HALO software (Indica Labs, Albuquerque, NM, USA) as previously described (see Materials & Methods section in **III.A.***i*.).

RNA extraction

After incubation, cells were lysed with TRIzol Reagent (Ambion, Life Technologies), vortexed for 10 s and incubated for 10 min at room temperature. The lysates were vortexed during 30 s and 20ng of tRNA were added to each sample to stabilize the RNA. After homogenization, 200µL of chloroform was added. The mixtures were vortexed for 30 s and incubated for 15 min at room temperature. After centrifugation for 15 min at 12,000g and 4°C, upper aqueous layers were collected. Samples were supplemented with 200µL isopropanol 100%, vortexed for 30 s and stored for at least 1h at -80°C to precipitate the RNAs. After a 30 s centrifugation at 12,000g and 4°C, the RNA pellet was washed with cold ethanol 70%, dried at room temperature and resuspended in autoclaved H_2O_d. For RNA sequencing, an additional step was performed to remove excess DNA with the TURBO DNA-free kit (Invitrogen) according to manufacturer's recommendations except that a longer centrifugation step (3 min) was carried out.

Quantitative Real-Time PCR

For all samples, the RNA concentrations were evaluated using Nanodrop ND-8000 spectrophotometer. 500ng total RNA were used for reverse transcription using SuperScript III Reverse Transcriptase kit (Invitrogen) according to manufacturer's recommendations.

Primers for amplification of *PGRMC1, MMP-*3 and *MMP-1* (Table III) were previously published and their amplification efficiencies were checked before use (Bunch et al., 2014; Coudyzer et al., 2013; Thieffry et al., 2021). The real-time PCR amplifications were performed with the KAPA SYBR FAST qPCR Master Mix (2X), 0.25µM primers (forward and reverse) and 15ng cDNA. The HouseKeeping Genes, *RPL13*a and *β-actin* were selected based on their stability of expression under our experimental conditions.

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

Target	Primers	Sequence
PGRMC1	Forward	5′-TGACCTTTCTGACCTCACTGC-3′
	Reverse	5′-GCCCACGTGATGATACTTGA-3′
Prolactin	Forward	5′-TGCTGCTGCTGGTGTCAAAC-3′
	Reverse	5′-TTGGGCTTGCTCCTTGTCTT-3′
MMP-3	Forward	5′-TGTATCTTGCCGGTCATTT-3′
	Reverse	5′-TGTGACAAGGTGCAAGCT-3′
MMP-1	Forward	5′-GCAAACACATCTGACCTACAGG-3′
	Reverse	5′-TCTCAATGGCATGGTCCAC-3′
DKK-1	Forward	5′-GGGAATTACTGCAAAAATGGAATA-3′
	Reverse	5′-ATGACCGGAGACAAACAGAAC-3′
RPL13a	Forward	5′-GGCTAAACAGGTACTGCTGG-3′
	Reverse	5′-GGTTGGTGTTCATCCGCTTG-3′
β-actin	Forward	5′-AGCCTCGCCTTTGCCGA-3′
	Reverse	5′-TCACGCCCTGGTGCCTG-3′

Statistical analysis

All statistical analyses were performed with GraphPad Prism 8.4.3.

Analyses of RT-qPCR data were performed on ΔCt values and the outliers were identified and excluded using the Grubbs' test.

For the quantification of PGRMC1 immunolabeling, statistical comparisons were performed using values for virtual slices rather than for the whole endometria to account for differences in tissue thickness between endometrial samples (essentially due to cycle phases).

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