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ORIGINAL ARTICLE

Alterations in progesterone receptor membrane component 2 (PGRMC2) in the endometrium of macaques afflicted with advanced endometriosis

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ABSTRACT: The hormonally driven expression and cell-specific localization patterns of the progesterone receptor membrane components (PGRMC1 and PGRMC2) in the macaque endometrium during the menstrual cycle are unknown. Additionally, the expression and localization patterns of PGRMC1 and PGRMC2 in the secretory eutopic endometrium of primates afflicted with endometriosis are also unknown. Therefore, we used real-time PCR to quantify transcript expression levels of the PGRMCs in well-defined samples of endometrium collected from artificially cycled macaques during the menstrual cycle, and in the secretory phase endometrium of naturally cycling macaques afflicted with endometriosis. *In situ* hybridization and immunocytochemistry were used to localize PGRMC1 and PGRMC2 mRNA and protein, respectively. We compared the patterns of expression and localization of the PGRMCs with the expression and localization patterns of nuclear progesterone receptor (PGR). PGRMC1 and PGR were elevated during the proliferative phases of the cycle, and then declined to nearly undetectable levels during the late secretory phase of the cycle. Levels of PGRMC2 were lowest during the proliferative phases of the cycle and then increased markedly during the secretory phases. Strong staining for PGRMC2 was localized to the luminal and glandular epithelia during the secretory phases. When compared with artificially cycled disease-free animals, macaques with endometriosis exhibited no changes in the expression or localization patterns for PGR and PGRMC1 but exhibited strikingly reduced levels of PGRMC2 transcript and altered intracellular staining patterns for the PGRMC2 protein. Collectively, these results suggest that membrane-bound PGRMC2 may provide a pathway of action that could potentially mediate the non-genomic effects of progesterone on the glandular epithelia during the secretory phase of the cycle. Further, reduced levels of membrane-bound PGRMC2 may be associated with the progesterone insensitivity often observed in the endometrium of primates afflicted with endometriosis.

Key words: endometriosis / endometrium / menstrual cycle / PGRMC / progesterone

Introduction

Non-genomic progesterone receptors have been identified, including progesterone receptor membrane component 1 (PGRMC1) and PGRMC2 (Gerdes *et al.*, 1998). The PGRMC1 transcript is more widely distributed throughout tissues of the human body, and therefore PGRMC1 has garnered wide-spread attention across various biomedical disciplines, including reproductive biology. For example, PGRMC1 has been localized to the tissues of the male and female reproductive tracts, and PGRMC1 is expressed in the spermatozoa and oocyte (Losel *et al.*, 2008). PGRMC1 was first identified in the primate uterus by a gene-profiling study performed by the Giudice laboratory, who reported the down-regulation of endometrial PGRMC1 transcript during the mid-secretory phase in women (Kao *et al.*, 2002). A similar down-regulation of PGRMC1 transcript was subsequently observed on Days 21 and 23 of the secretory phase in the endometrium of artificially cycled macaques (Ace and Okulicz, 2004). A recent study used 2D PAGE and tandem mass spectrometry to show that the PGRMC1 protein is also reduced in the endometrium of women during the secretory phase of the cycle (Chen *et al.*, 2009). The PGRMC1 protein has been localized by immunocytochemistry (ICC) to the outer cell membranes of

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the syncytiotrophoblasts of the human placenta (Zhang *et al.*, 2008).

PGRMC1 binds multiple ligands and subsequently has several proposed physiological functions. PGRMC1 was originally identified as a progesterone-binding moiety, and PGRMC1 exhibits high-affinity (K_d = 11 nM) and low-affinity (K_d = 286 nM) binding sites for progestins (Meyer *et al.*, 1998). Several recent studies have demonstrated convincing evidence(s) that the protective influences by progesterone on cell survival, especially on cultured ovarian granulosa cells and human ovarian cancer cells, may be mediated directly through the PGRMC1 pathway (Peluso *et al.*, 2009a, b; Peluso *et al.*, 2010). PGRMC1 can also bind other steroids—androgens, corticoids and synthetic hormones (such as progestin R5020)—although the functionality of the majority of these receptor-ligand interactions remains unknown (recently reviewed in Rohe *et al.*, 2009). PGRMC1 has also been localized to the endoplasmic reticulum and Golgi apparatus, and therefore PGRMC1 likely participates (in some capacity) in cholesterol trafficking and sterol metabolism, possibly functioning as a 'sterol-sensing' protein (Gellersen *et al.*, 2009). Although PGRMC2 is structurally related to PGRMC1, established ligands and proposed physiological functions of PGRMC2 are lacking (reviewed in Cahill, 2007).

Strong evidences suggest that the majority of progesterone-mediated changes in the endometrium are facilitated directly via the nuclear progesterone receptor (PGR), but it has also become widely accepted that some progestational effects may be mediated via membrane-bound progesterone receptors (Fernandes *et al.*, 2005; Gellersen *et al.*, 2009). In this regard, the progestational effects on the stroma are likely facilitated through PGR localized to the stromal cells, where relatively high levels of PGR are maintained throughout the secretory phase of the cycle thereby permitting PGR to act as a transcription factor to directly regulate gene transcription (Slayden and Brenner, 2004). However, PGR is undetectable in the epithelial cells of the glands localized to the functionalis zone during the mid- to late secretory phase of the cycle, and therefore the pathway(s) regulating the progestational effects on the uterine epithelia are poorly understood (Slayden and Keator, 2007). Progesterone may act on the epithelia through paracrine 'progestomedins', factors that are secreted by the stromal cells that then bind to their receptors located on the adjacent epithelium (Bazer and Slayden, 2008), but unfortunately this progestomedin theory cannot explain how some of the progestational effects on the endometrium (such as glandular secretion and trafficking of the oxytocin receptor) occur in $PGR^{-/-}$ knockout mice and in several cell lines lacking PGR (reviewed in Losel *et al.*, 2003; Couse *et al.*, 2006; Pru, 2009). Collectively, the pathways through which progesterone facilitates changes throughout the entire primate endometrium, especially within the glandular epithelia, during the mid- to late secretory phase of the menstrual cycle are currently not clear (Bergeron, 2000; Hess *et al.*, 2006).

Paradoxically, levels of PGR are often normal or even slightly elevated in the eutopic endometrium of primates afflicted with endometriosis, but many of these subjects exhibit a blunted response to progesterone (i.e. 'progesterone resistance' or 'progesterone insensitivity'), thereby suggesting an alternative steroid-binding pathway may be directly impaired in animals exhibiting progesterone insensitivity (Hastings and Fazleabas, 2006). This blunted response to progesterone causes (among other things) increased secretion of the matrix metalloproteinases, increased levels of cytokines and other immodulatory molecules and decreased levels of trafficking proteins (such as IGF-binding proteins)—and collectively, this abnormal response (or lack of response) to progesterone diminishes endometrial receptivity and leads to an increased incidence of infertility (Young and Lessey, 2010). Several gene-profiling studies have established a long list of candidate genes and proteins differentially expressed in the endometrium of women with and without endometriosis (Burney *et al.*, 2007; Aghajanova *et al.*, 2011), but the mechanisms surrounding this disorder remain poorly understood. Although progesterone insensitivity and infertility are attributed to a diminished and/or altered response to progesterone, previous studies have not investigated changes in either PGRMC1 or PGRMC2 in the primate endometrium of women or macaques afflicted with endometriosis.

The primary aim of this study was to evaluate the effects of progesterone and estradiol (E_2) in artificially cycled female rhesus macaques to assess the hormonally driven changes in the transcript expression and cell-specific localization patterns of PGRMC1 and PGRMC2 in the primate endometrium. We chose the artificially cycled macaque for this study, first because the hormonal milieu is difficult to control in women, and secondly because we have previously demonstrated that artificially cycled macaques provide an excellent translational animal model to determine hormonally regulated changes in the primate uterus (reviewed in Brenner and Slayden, 1994, 2004; Slayden and Brenner, 2005). We also sought to examine the patterns of expression and localization of the PGRMCs and PGR in the endometrium of macaques afflicted with endometriosis during the secretory phase of the cycle, because both macaques and women with endometriosis experience an attenuated response to progesterone and infertility. We report novel data that demonstrates PGRMC1 and PGRMC2 are regulated by progesterone and E_2 in the endometrium, and that PGRMC2 is up-regulated in the epithelial cells of the functionalis glands during the secretory phase of the menstrual cycle. Markedly lower levels of PGRMC2 were detected in the endometrium of animals afflicted with endometriosis during the secretory phase of the menstrual cycle compared with disease-free animals. Collectively, these results suggest that hormonally driven changes in the PGRMC2 pathway may play an important functional role in the non-human primate endometrium during the secretory phase of the menstrual cycle.

Materials and Methods

Treatments

Non-human primate tissues were obtained during previous studies conducted at the Oregon National Primate Research Center (ONPRC). Animal care was supervised by the veterinary staff of the ONPRC Division of Animal Resources after review and approval by the ONPRC Animal Care and Use Committee. Oophorectomized rhesus macaques (*Macaca mulatta*) were treated with Silastic implants that release E_2 and E_2 plus progesterone to induce artificial menstrual cycles as described (Brenner and Slayden, 2004; Slayden and Brenner, 2004). Hormone levels of E_2 and progesterone for all animals used in this study are listed in Table I.

The endometrium from oophorectomized animals treated with no exogenous hormones for at least 3 months were utilized as baseline controls (referred to as 'no hormone'; $n = 7$). Endometrial tissues collected from artificially cycled macaques treated with E_2 were examined at the following

phases of the cycle: 2–3 days following progesterone withdrawal (menses; $n = 6$), 5–7 days following progesterone withdrawal (mid-proliferative; $n = 3$), 14 days after progesterone withdrawal (late proliferative; $n = 6$) and 28 days of E_2 alone (extended proliferative; $n = 11$). The endometrium was collected from animals treated with E_2 plus progesterone after 7 days (mid-secretory; $n = 4$) or 14 days (late secretory; $n = 17$) of treatment, respectively.

Endometrial samples were collected during the secretory phase (serum progesterone ≥ 2.0 ng/ml) of the natural cycle from macaques afflicted with advanced endometriosis ($n = 5$). Tissues from naturally cycling macaques were used for this analysis because monkeys afflicted with endometriosis are typically not diagnosed until the disorder has reached an advanced state. Additionally, these animals oftentimes present with endometriotic-cyst encapsulated ovaries, therefore ovariectomies are typically not performed on animals with advanced endometriosis at ONPRC. The animals in this study were diagnosed with endometriosis during physical examination, and this diagnosis was confirmed by conventional ultrasound and needle biopsy (e.g. aspiration of red and/or chocolate colored fluids). Of these five animals, two had large (> 4 cm) chocolate lesions, one had a large red lesion (> 5 cm), one presented with multiple white lesions and one animal exhibited white, red and chocolate lesions surrounding an extremely enlarged uterus (noted by the veterinarian staff as ~10 times normal).

Table I Mean concentration of plasma progesterone and estradiol (±SEM) in macaques utilized for this study.

Treatment (sample size)	Estradiol (pg/ml)	Progesterone (ng/ml)
No hormone (7)	12.5 ± 2.8	0.11 ± 0.02
Menses (6)	92.8 ± 15.8	0.18 ± 0.06
Mid-proliferative (3)	83.0 ± 30.9	0.23 ± 0.15
Late proliferative (6)	80.0 ± 12.2	0.16 ± 0.07
Extended proliferative (11)	99.6 ± 10.8	0.08 ± 0.03
Mid-secretory (4)	98.0 ± 16.1	6.33 ± 0.82
Late secretory (17)	92.4 ± 8.4	4.72 ± 0.60
Endometriosis (5)	33.6 ± 4.1	3.87 ± 0.92

All measurements were determined by radioimmunoassay at the Endocrine Services Technology Core laboratory located at the ONPRC campus

Tissue preparation

The uterus was bisected along the longitudinal axis from the fundus to cervix and then further divided into quarters. Full-thickness tissue sections (~5 mm) extending from the uterine lumen to the outer myometrium were taken from each quarter. Tissue blocks were embedded in OCT compound (Sakura Finetek, Torrance, CA, USA) and sectioned or fixed in 4% paraformaldehyde and subsequently embedded in paraffin following standard methods. The remaining endometrium was separated from the outer myometrium, weighed and frozen separately in liquid nitrogen for subsequent mRNA quantification.

Real-time TaqMan® PCR

Frozen samples of endometrial tissue were thawed in 10 volumes of TRIzol (Invitrogen, Carlsbad, CA, USA), and total RNA was isolated, purified and quality assessed as described (Keator *et al.*, 2011). High-quality RNA [260/280 ratio > 1.8; RIN > 6 (Schroeder *et al.*, 2006)] was reverse transcribed into first-strand cDNA using random hexamer primers (Promega, Madison, WI, USA) in the presence of Omniscript reagents (Qiagen, Valencia, CA, USA). Sequences for the PGR mRNA (XM_002799793), PGRMC1 mRNA (NM_001194918) and PGRMC2 mRNA (NM_006320) were uploaded into Primer Express software [Applied Biosystems (ABI), Foster City, CA, USA]. Primer Express was used to design optimal TaqMan primer and probe sequences for real-time PCR (listed in Table II). Primer and probe sequences were analyzed to confirm sequence specificity by using the following publicly available resources: (i) BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi), (ii) the NetAffyx Analysis Center macaque genome database (www.affymetrix.com) and (iii) the *Ensembl* website (www.ensembl.org). The

Table II Primer and probe sequences.

Gene	Sequence	Direction	Region	Application
PGRMC1	ccaggacccccgcatac	Se	365	qPCR
	tttggtcacgtcgaacacctt	AS	419	qPCR
	catggccatcaacg	Probe	383	qPCR
	tgtgcaccaagaacaaagga	Se	1036	ISH
	gtgagggcaaggacaacaat	AS	1284	ISH
	attgttgtccttgccctcac	Se	1265	ISH
	ggagagcaaacacctgttcc	AS	1541	ISH
PGRMC2[a]	gactgccgatgccagagaag	Se	244	qPCR
	aagcggcgagaagttttgg	AS	326	qPCR
	tccgttccaggaagac	Probe	298	qPCR
	gggcttcatcatcctgaaaa	Se	1925	ISH
	tccctgacaaagccaaaaac	AS	2270	ISH
	cagaggactggccacatttt	Se	1495	ISH
	Ttgaatgtttttcgcagcag	AS	1820	ISH
PGR	agcttcacttgtcatttgagtgaaa	Se	790	qPCR
	ccgaagatctcagatcccagtagt	AS	868	qPCR
	ctacaacccgaggcggctagtgctc	Probe	819	qPCR

Se, sense; AS, antisense; ISH, *in situ* hybridization; qPCR, real-time PCR; probe, TaqMan probe.
[a]Regions listed for PGRMC2 coincide with the known human mRNA sequence (accession no. NM_006320.4), see text for further details.

primer and probe sequences for PGRMC1 and PGRMC2 have been validated recently in the macaque ovary by comparing the values obtained from real-time PCR with microarray data (Bishop *et al.*, 2009; Bishop *et al.*, 2011). Methods for real-time PCR with TaqMan technology to assess the transcript levels of PGR, PGRMC1, PGRMC2 and the constitutively expressed RPL32 (housekeeping gene) in the macaque endometrium were performed as described recently (Keator *et al.*, 2011).

In situ hybridization

Methods for *In situ* hybridization (ISH) using [^{35}S]-UTP (1250 Ci/mmol; Perkin Elmer, Waltham, MA, USA) radio-labeled riboprobes (500 000 cpm) were performed as recently described (Keator *et al.*, 2011). The primers used to synthesize the single-stranded PGRMC1 and PGRMC2 riboprobes for ISH are listed in Table II. Cell-specific hybridization of radio-labeled antisense PGRMC1 and PGRMC2 probes was determined by comparing the amount of antisense hybridization to background. Background was defined as (i) the amount of sense probe hybridized to the same cells and (ii) the relative silver grain density observed over blank fields.

Immunocytochemistry

Localization of PGRMC1 and PGRMC2 proteins were evaluated on paraffin-embedded tissues. Serial 5-μm sections were cut and mounted on Superfrost Plus slides (Fisher Scientific), deparaffinized in xylene and rehydrated stepwise in descending ethanols. Antigen retrieval was performed by heating the sections in citrate buffer (pH 6.0) for 10 min in a pressure cooker. All reactions/incubations were performed at room temperature (~23°C) unless noted otherwise. Slides were treated with 3% hydrogen peroxide (in methanol) for 30 min to block endogenous peroxidases. Slides were incubated with normal serum (ABC-kit, Vector Labs, Burlingame, CA, USA) for 20 min to block non-specific IgG reactions. Sections were then incubated at 4°C overnight with a goat polyclonal antibody raised to detect PGRMC1 (8.0 μg/ml; ab48012; Abcam, Cambridge, MA, USA) or a mouse monoclonal antibody directed to PGRMC2 (2.5 μg/ml; H00010424-M04; Abnova, Walnut, CA, USA). Primary antibody was omitted from slides used as negative controls.

Detection of PGR was performed on microwave stabilized cryosections (5 μm). All incubations were performed at room temperature (~23°C) unless noted otherwise. Slides bearing the sections were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.3; Sigma) for 10 min. Slides were incubated with a phosphate-buffered solution containing glucose oxidase (1 U/ml), sodium azide (1 mM/l) and glucose (10 mM/l) to block endogenous peroxidase activity. Slides were then incubated with normal mouse serum (20 min) and treated with biotin blocking solution (Vector Labs) for 30 min. Slides were incubated at 4°C overnight with a primary monoclonal antibody directed against PGR (1 μg/ml; Ab-8; Thermo Fisher Scientific, Rockford, Ill). Omission of the primary antibody and incubation with timothy pollen were used as negative controls.

After overnight incubation with primary antibody, the following day all slides (paraffin-embedded and cryosections) were rinsed in PBS (containing 0.075% non-ionic detergent BRIJ), incubated with normal serum for 20 min and then incubated with the appropriate biotinylated secondary IgG for ~45 min. The slides were rinsed with PBS, reacted with avidin–biotin peroxidase reagents (for ~60 min) and rinsed in Tris buffer (pH 7.6). The slides were incubated in 0.025% diaminobenzidine to visualize antibody–antigen complexes. Sections were then stabilized by incubating the slides in 0.025% osmium tetroxide (for ~1 min). Slides were counter stained with hematoxylin, dehydrated in ascending ethanols, cleared through a series of xylene and then sealed under Permount.

Photomicrography

ICC and ISH slides were viewed on a Zeiss AxioImager A.1 microscope (Carl Zeiss, Inc., Oberkochen, Germany) with planapochromatic lenses. Digital photomicrographs were captured with a Leica DFC 480 camera (Leica, Wetzlar, Germany). All photomicrographic plates were background adjusted, cropped and subsequently annotated in Photoshop Creative Suite 4 (Adobe Systems, Seattle, WA, USA).

Statistical analysis

Real-time PCR expression data were log-transformed and then subjected to a one-way analysis of variance using the mixed model subroutine in SAS software (SAS Institute, Inc., 2008). Main effects due to hormonal treatment were considered significant at $P \leq 0.05$. All data are presented as the untransformed means ± SE.

Results

Changes in endometrial transcript levels

Figure 1 shows the hormonally induced changes in the transcript expression for PGR, PGRMC1 and PGRMC2 during the artificial menstrual cycle. Transcript levels for PGR, PGRMC1 and PGRMC2 among the mid-, late and the extended proliferative phases (excluding menses) were not significantly different, and therefore these proliferative phase values were combined. Compared with animals treated with no hormone, PGRMC1 and PGR transcripts were up-regulated ($P < 0.05$) by treatment with E_2 during the proliferative phase. Compared with no hormone controls, PGRMC2 was down-regulated ($P < 0.05$) during menses and the proliferative phases.

Compared with animals treated with E_2 during the proliferative phases of the cycle, PGR and PGRMC1 were down-regulated ($P < 0.05$) by treatment with E_2 plus progesterone during the mid- and late secretory phases of the cycle. Compared with no hormone controls, PGR and PGRMC1 were reduced ($P < 0.05$) in the late secretory phase. Compared with no hormone and the proliferative phase, PGRMC2 was up-regulated markedly ($P < 0.05$) during the mid-secretory phase of the cycle (i.e. 7 days of E_2 plus progesterone treatment). Levels of PGRMC2 then returned to baseline (i.e. no hormone level) during the late secretory phase of the cycle, but these levels remained higher ($P < 0.05$) than the levels observed during the proliferative phases.

Endometriosis alters expression of PGRMC2

The levels of PGRMC2 in animals with endometriosis were markedly lower ($P < 0.05$) when compared with the levels of PGRMC2 detected in the mid- and late secretory endometrium of artificially cycled disease-free animals (Fig. 2). Low levels of transcript expression for PGR and PGRMC1 were also observed in the secretory endometrium of animals with endometriosis (Supplementary data, Table SI). These transcript levels of PGR and PGRMC1 were not significantly different ($P > 0.05$) than the levels of PGR and PGRMC1 detected during either the mid- or late secretory phases of artificially cycled disease-free animals treated with E_2 plus progesterone, respectively.

Cell-specific localization of PGR

Localization for PGR protein (Fig. 3) illustrates the classic hormonally driven changes in PGR as reported previously. Strong staining for PGR

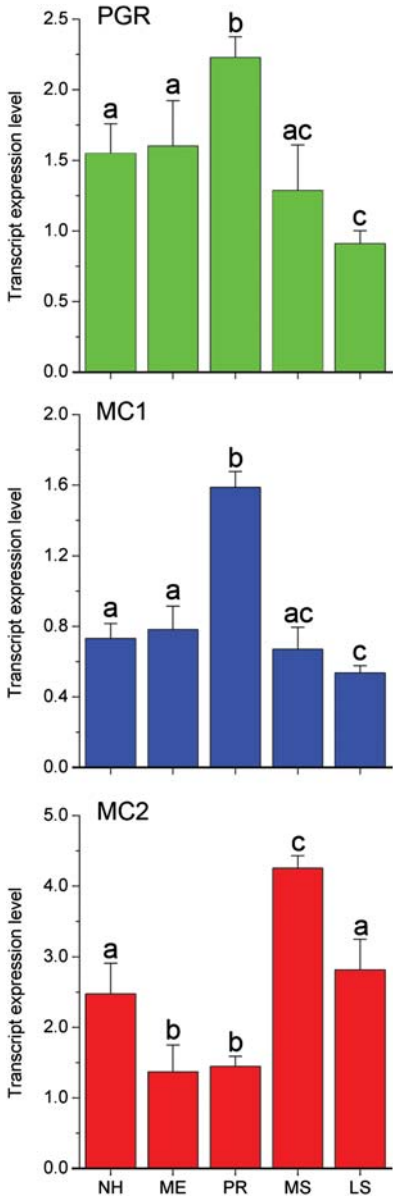

Figure 1 Expression of nuclear PGR, PGRMC 1 and 2, in the macaque endometrium as determined by real-time PCR. Different subscripts indicate significant differences ($P < 0.05$) between hormone treatments. NH, no hormone; ME, menses; PR, combined proliferative phases; MS, mid-secretory; LS, late secretory. See text for complete treatment descriptions.

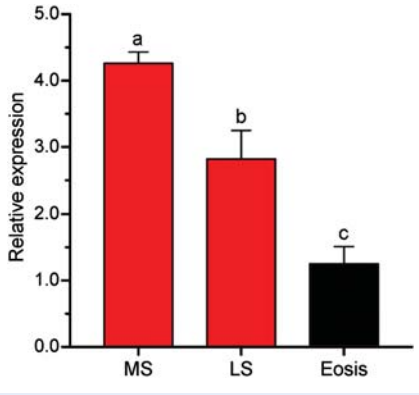

Figure 2 Expression of PGRMC2 is reduced in the endometrium of macaques afflicted with advanced endometriosis. Markedly reduced ($P < 0.05$) levels of PGRMC2 transcript were detected in the endometrium of naturally cycled macaques with endometriosis (Eosis; black bar) during the secretory phase compared with the endometrium of artificially cycled macaques (red bars) during the mid-secretory (MS) and late secretory (LS) phases of the menstrual cycle, respectively. Different letters denote significant differences between means.

was observed in the stroma during menses. Very strong staining for PGR was observed in the luminal epithelia, and in the glandular epithelia and stroma, throughout the entire endometrium during the proliferative phase. A noticeable decrease in staining intensity was observed in the luminal epithelium and in the epithelia of glands in the functionalis zone during the mid-secretory phase of the cycle, whereas moderate staining for PGR was retained in the stroma. During the late secretory phase, moderate staining for PGR was observed in the luminal epithelia, whereas staining for PGR was absent to weak in the functionalis glands and stroma. Moderate staining was retained in the epithelia of the basalis glands under the influence of progesterone during the mid- and late secretory phases, respectively. Staining for PGR was not observed in the vascular smooth muscle or endothelial cells of any vessels at any phase of the artificial menstrual cycle examined.

Cell-specific localization of PGRMC1

Hormonally induced patterns of localization for PGRMC1 transcript (Fig. 4) and protein (Fig. 5) were observed during the artificial menstrual cycle. The ISH probes specific for PGRMC1 transcript hybridized to stromal and epithelial cells of the endometrium at all phases of the artificial cycle examined. Low numbers of silver grains and only light staining for PGRMC1 protein was observed in the upper functionalis stroma and glands in animals treated with no hormone or during menses. An increase in silver grains and strong staining for the PGRMC1 protein was observed in the upper stroma, glandular epithelia and luminal epithelial cells during the proliferative phases examined. Staining for PGRMC1 was moderate in the glandular epithelia and stroma of the basalis zone of the proliferative phase endometrium. A marked decrease in hybridization for PGRMC1 mRNA was observed during the mid-secretory phase. After 7 days of progesterone, moderate staining was observed in the functionalis stroma and the luminal epithelium, whereas weak staining was observed in the epithelia of the functionalis glands, and weak detection of the PGRMC1 antigen was seen in the entire basalis zone. Tissues from the late secretory phase of the cycle exhibited very weak hybridization for PGRMC1 transcript throughout the entire endometrium, and positive staining for the PGRMC1 protein was confined exclusively to the basalis stroma, scattered cells in the functionalis stroma and to individual cells isolated within the very apical layer of the luminal epithelium.

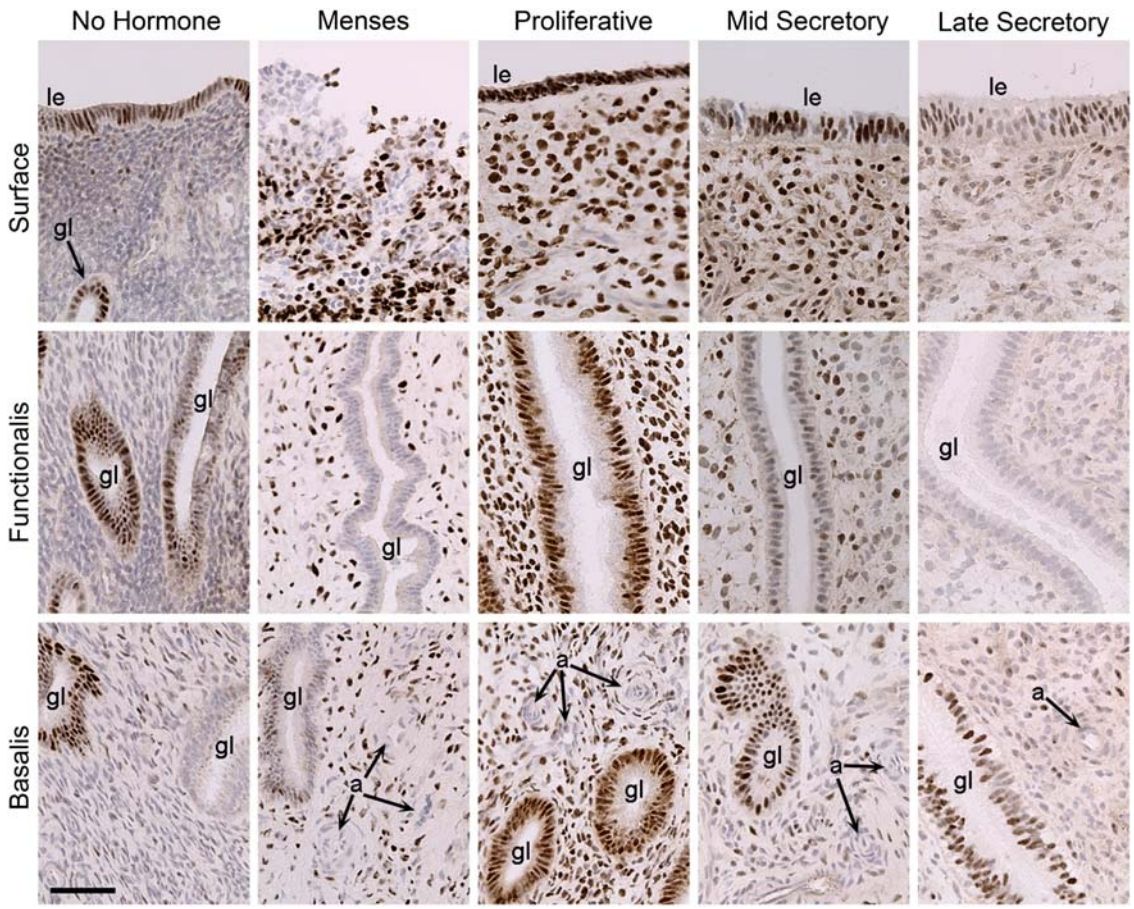

Figure 3 Localization of PGR throughout the artificial cycle. Strong nuclear staining for PGR was observed in the proliferative endometrium, with noticeable decreases in staining intensity in endometrial tissues collected from animals during the secretory phases of the cycle. Bar = 50 μm; a, artery; gl, gland; le, luminal epithelium.

Cell-specific localization of PGRMC2

Hybridization for the PGRMC2 transcript (Fig. 6) revealed localization patterns that paralleled similar cell-specific changes in staining intensity for the PGRMC2 protein (Fig. 7). PGRMC2 mRNA and protein were localized to the luminal epithelia, glandular epithelia and stroma at all phases of the cycle examined. Animals treated with no hormone exhibited strong hybridization (i.e. dense silver grains) and strong-to-moderate staining for PGRMC2 protein in epithelial and stroma cells, respectively, with no noticeable differences between the basalis and functionalis zones. During menses and the proliferative phases noticeably weaker hybridization for the PGRMC2 transcript was observed in the functionalis stroma, whereas a definitive decrease in staining intensity for the PGRMC2 protein was observed in the glandular epithelia and stroma of the functionalis and basalis zones, respectively. Dense clusters of silver grains were noted over the luminal epithelium, and over the glandular epithelia and stroma throughout the entire endometrium, during the mid-secretory phase of the cycle. After treatment with E_2+ progesterone for 14 days, fewer silver grains were observed over the stroma and the glandular epithelium, especially in those glands confined to the basalis zone. Strong-to-moderate staining for the PGRMC2 protein was retained in the luminal epithelia and in the epithelia of glands in the functionalis zone during the late secretory phase of the cycle. PGRMC2 was not localized to the vascular smooth muscle cells in any sections examined and very weak staining for the PGRMC2 protein was observed in the endothelial cells of vessels only in a very limited number (less than five total) of all slides examined (not shown).

Localization patterns of PGRMC2 are altered in animals afflicted with endometriosis

ICC for PGR, PGRMC1 and the PGRMC2 proteins in the endometrium of animals with endometriosis revealed changes in the staining intensity and pattern of localization for PGRMC2 (Fig. 8). The localization patterns of PGR and PGRMC1 in the endometrium of animals with endometriosis were very similar to the patterns of localization observed in artificially cycled disease-free animals (see Figs 3 and 5). Notably weaker staining for PGRMC2 was observed in animals with the disease compared with disease-free animals (see Fig. 7), and this weak staining intensity correlated with the reduced transcript level detected by real-time PCR.

Differences in the intracellular localization patterns of PGRMC2 in disease-free animals and macaques afflicted with endometriosis were

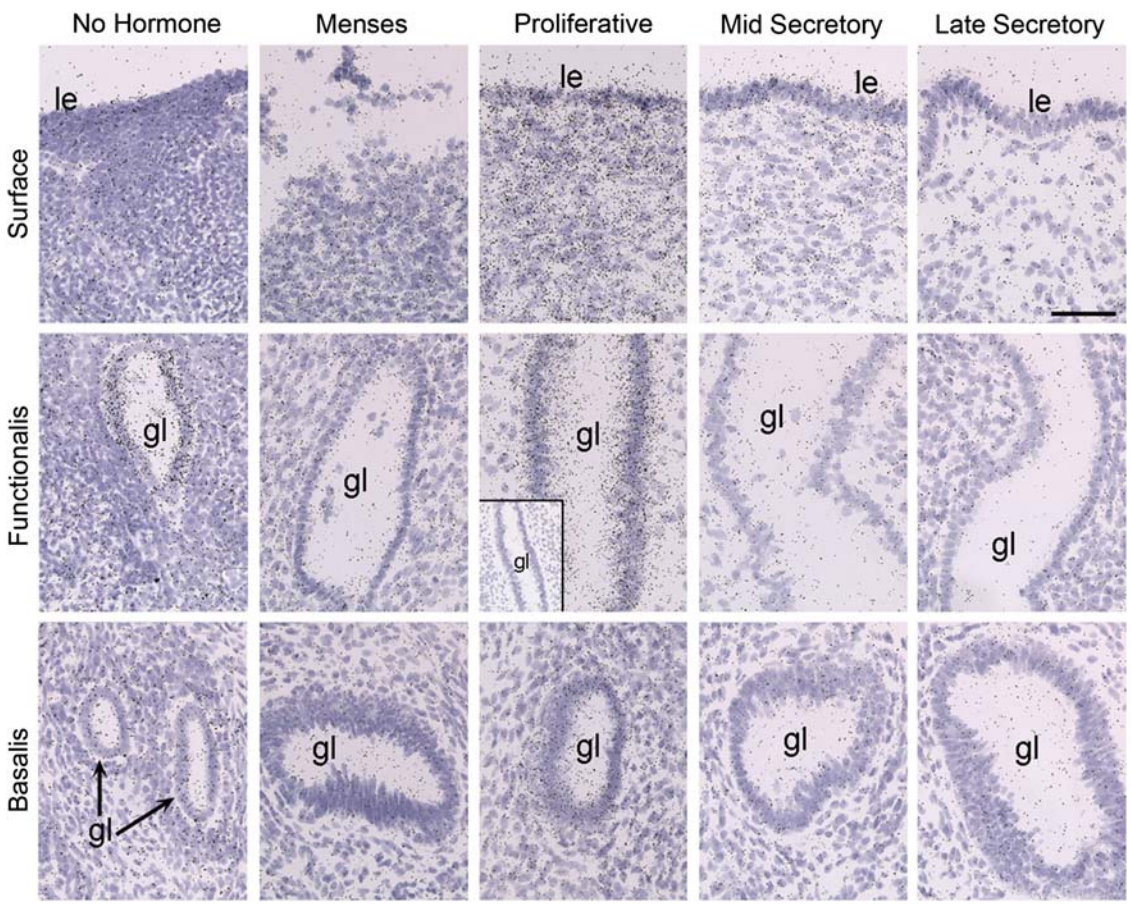

Figure 4 Cell-specific localization of PGRMC1 mRNA in the macaque endometrium. Dense silver grains were detected in the endometrial tissues collected from animals in the proliferative phase of the cycle (late proliferative sample shown). Bar = 50 μm; a, artery; gl, gland; le, luminal epithelium. Inset shows hybridization with the sense riboprobes (negative control).

revealed in oil-immersion high-powered microphotographs (Fig. 9). In disease-free animals, PGRMC2 was predominantly localized to the cytoplasm of the epithelial cells of both functionalis and basalis glands, respectively. In animals afflicted with endometriosis, PGRMC2 was localized exclusively to the nuclear envelope of functionalis and basalis epithelial cells, within the nucleolus and also found in cytoplasmic granular bodies (likely Golgi bodies) within the glandular epithelia.

Discussion

This is the first study to identify the hormonal regulation and the associated cell-specific localization changes of PGRMC1 and PGRMC2 in the macaque endometrium. Moreover, this is the first comparison of the localization patterns of PGR and the PGRMCs under common hormonal states. We report that treatment with E_2 plus progesterone significantly up-regulated PGRMC2 during the mid-secretory phase of the cycle, whereas the same treatment with progesterone down-regulated PGR and PGRMC1. Strong hybridization for the PGRMC2 transcript and staining for the PGRMC2 protein were retained in the glandular and luminal epithelia localized to the functionalis zone of the endometrium during the late secretory phase of the cycle, providing the first evidence that clearly demonstrates an increase in a putative progestin-binding moiety in the primate endometrium in the upper functionalis glands during the secretory phases of the cycle. Several studies have demonstrated a rapid non-genomic effect of progesterone on epithelial cells of multiple tissues, and the strong up-regulation of PGRMC2 localized within the endometrial epithelial cells—and not PGR localized to the adjacent stroma or the closely related PGRMC1—suggest a novel pathway of progesterone action on the glandular epithelium during the secretory phase of the menstrual cycle.

PGRMC2 was strikingly reduced in the secretory endometrium of naturally cycling macaques with endometriosis examined in this study, and to our knowledge, this is also the first evidence to demonstrate an altered expression of a putative membrane-bound progesterone receptor in the endometrium of primates afflicted with advanced endometriosis. The expression of endometrial PGRMC2 was reduced 50–75% in macaques with endometriosis, and additionally, the presence of endometriosis altered the intracellular localization of PGRMC2, as evidenced by stronger staining for the PGRMC2 protein around the nuclear envelope and in trafficking vesicles distributed throughout the cytoplasm in epithelial and stromal cells (see

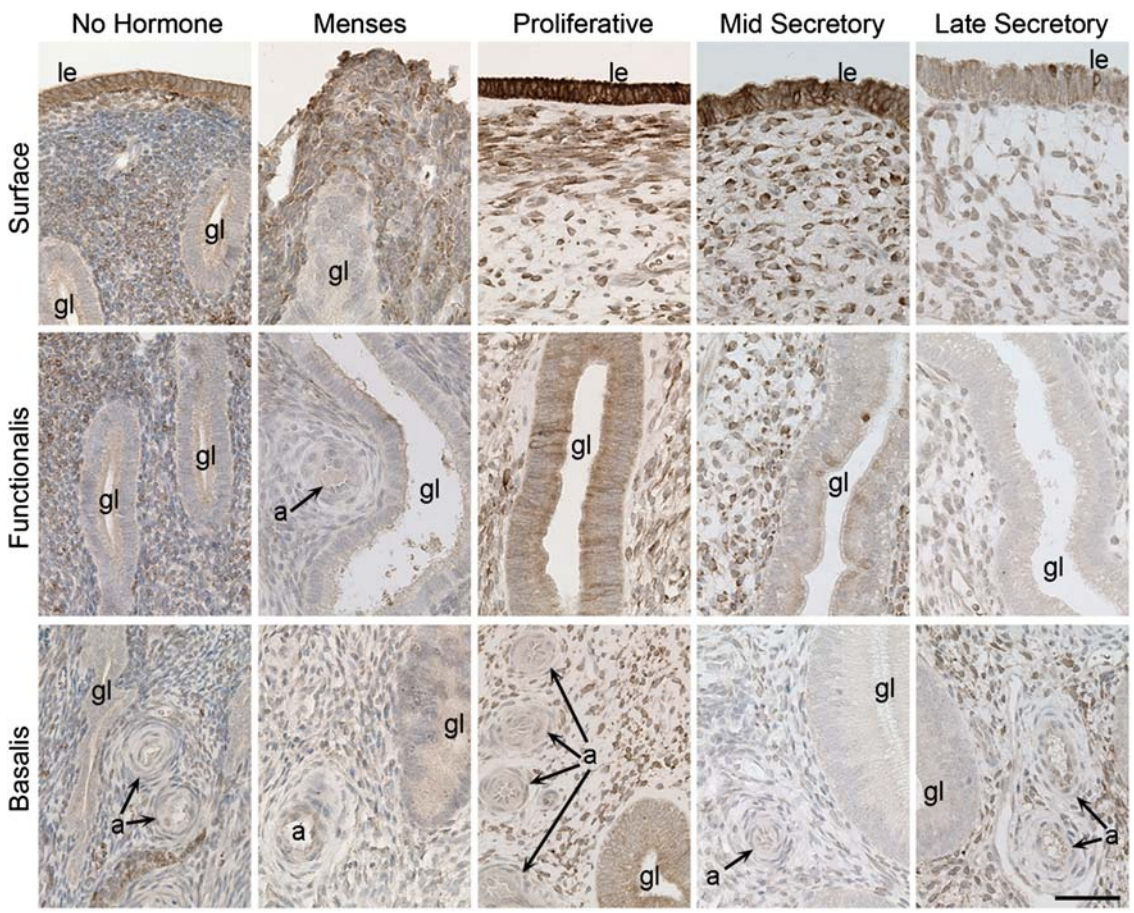

Figure 5 ICC for PGRMC1 protein in the macaque endometrium. The strongest staining intensity for the PGRMC1 antigen was observed in the proliferative phase endometrium paralleling the changes in PGRMC1 mRNA detected by ISH. Bar = 50 μm; a, artery; gl, gland; le, luminal epithelium.

Fig. 9). Thus, this increased staining intensity of intracellular PGRMC2 protein indicates that individuals with endometriosis may lack a PGRMC2 cell membrane-bound pathway. Albeit speculative, these results suggest that reductions in the levels of PGRMC2 and the altered intracellular localization of PGRMC2 may somehow be correlated with the abnormal endometrial function associated with individuals afflicted with endometriosis.

Endometriosis is a complex disease and presents with multiple etiologies, including increased levels of inflammatory biomolecules and an attenuated response to progesterone, abnormal conditions that have been linked with the infertility oftentimes experienced by women afflicted with endometriosis (Weiss *et al.*, 2009). This attenuated response to progesterone has been linked loosely to altered expression levels of the nuclear estrogen receptors (ERα and ERβ) and PGR (Hastings and Fazleabas, 2006). However, the expression and localization patterns of endometrial PGR (especially in the upper functionalis glands) are not abnormal in many women and non-human primates experiencing progesterone insensitivity, suggesting progesterone resistance may not be attributed to an abnormal expression of PGR (Fazleabas, 2010). On the other hand, if PGRMC2 facilitates any progestational effects within the endometrial glands, then abnormal expression and/or intracellular localization of PGRMC2 may contribute to the blunted response to progesterone leading to abnormal endometrial function and decreased fertility observed in subjects with endometriosis. We attest that the limited tissues collected from the small number of macaques used in this study represent a mere fraction of the populace afflicted with endometriosis and that the functional roles of PGRMC2 remain unknown, but nonetheless, these results promote the pursuit of future studies to aggressively investigate the significance of reduced levels of PGRMC2 in the endometrium of primates afflicted with endometriosis.

Throughout this study we maintained the viewpoint that PGRMC2 is a progesterone-binding moiety. These conclusions were drawn largely from the original identification of PGRMC2 as a progesterone-binding entity, and because of the progesterone-binding capabilities of the closely related PGRMC1. However, PGRMC2 has not been afforded the same attention as the closely related PGRMC1, and although PGRMC2 was originally described as a potential steroid-binding moiety of the plasma membrane (Gerdes *et al.*, 1998), the ligands that directly interact with PGRMC2 have not been determined. Additionally, due to a lack of reagents designed specifically against PGRMC2 (e.g. PGRMC2-specific agonists or antagonists), we were unable to perform physiological *in vivo* or *in vitro* experiments to complement the highly descriptive findings presented in this study. Thus, the actual physiological role(s) and functionality of PGRMC2 in the primate endometrium (and other tissues) remain unknown. Future

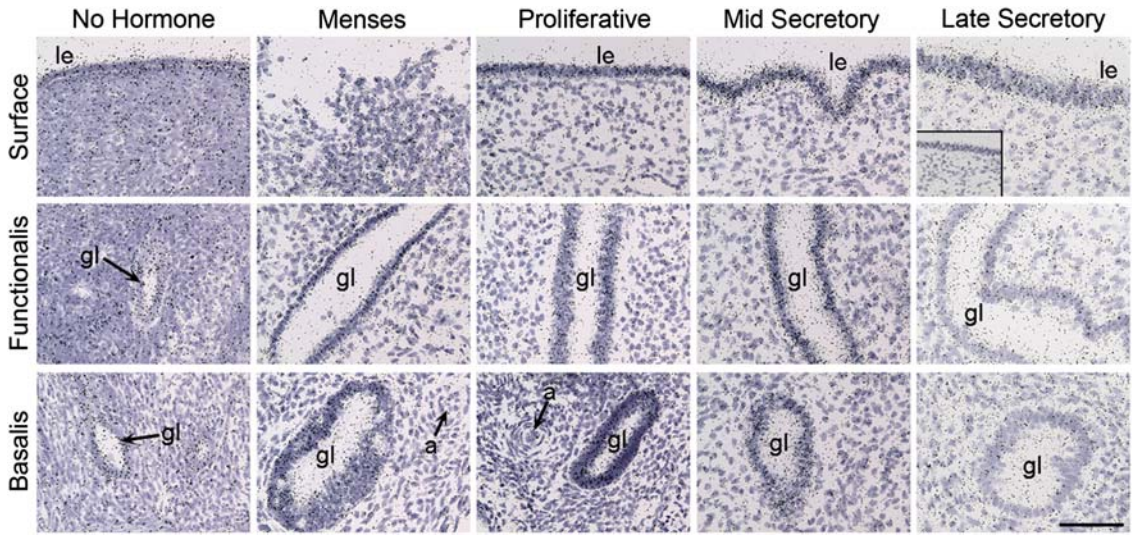

Figure 6 Brightfield photomicrographs showing the *in situ* hybridization for PGRMC2 in the macaque endometrium. Specific hybridizations to the antisense PGRMC2 riboprobes were observed at all timepoints examined, with increased numbers of silver grains detected in the epithelia of animals treated with no hormone and in the secretory phases of the cycle. Fewer silver grains were observed in animals during menses and during the proliferative phase. No hybridizations were observed with the sense riboprobes (negative control) on a section of secretory endometrium. Bar = 50 μm; a, artery; gl, gland; le, luminal epithelium. Secretory samples are from the late secretory phase.

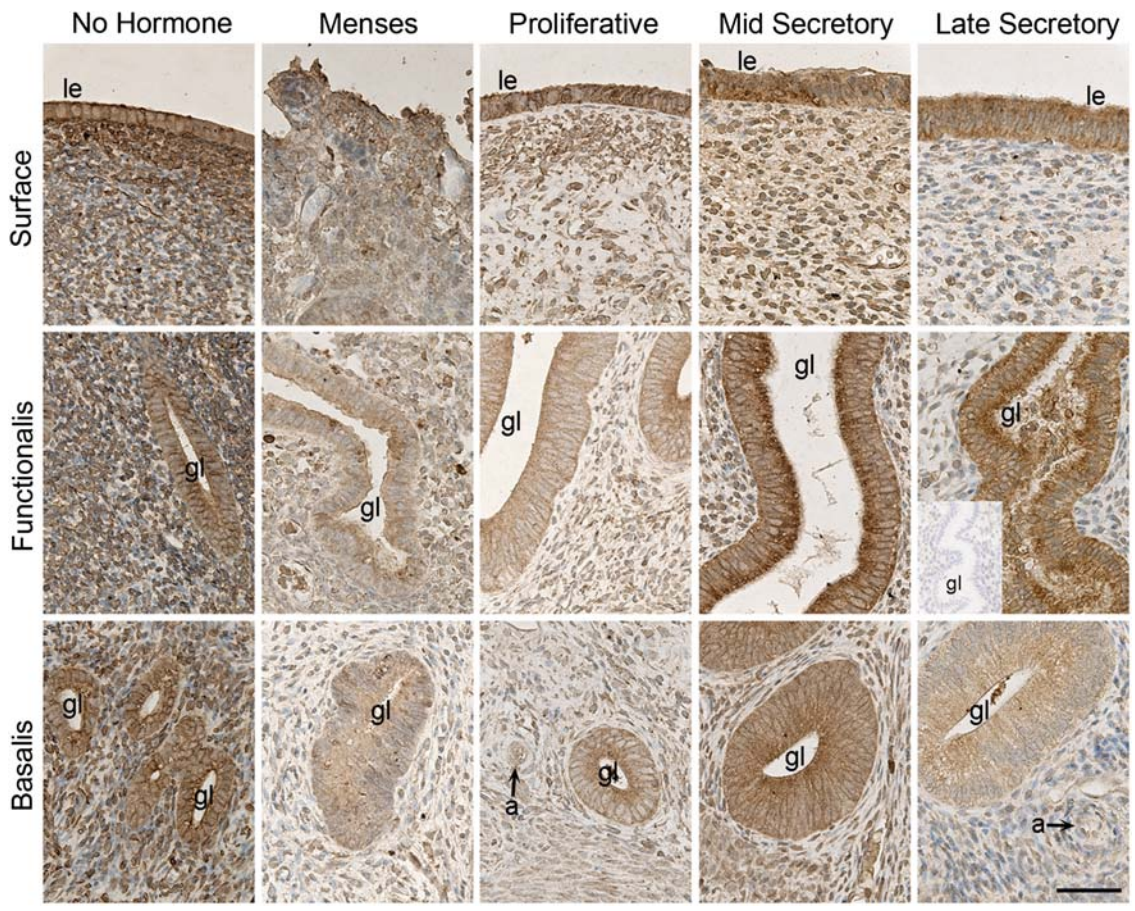

Figure 7 Staining for PGRMC2 protein in the macaque endometrium. Strong staining was observed in the luminal epithelia throughout the cycle. Changes in staining intensity were apparent in the glandular epithelia, particularly in glands of the functionalis zone. Bar = 50 μm; a, artery; gl, gland; le, luminal epithelium. Inset shows omission of the primary antibody; extended proliferative phase sample shown.

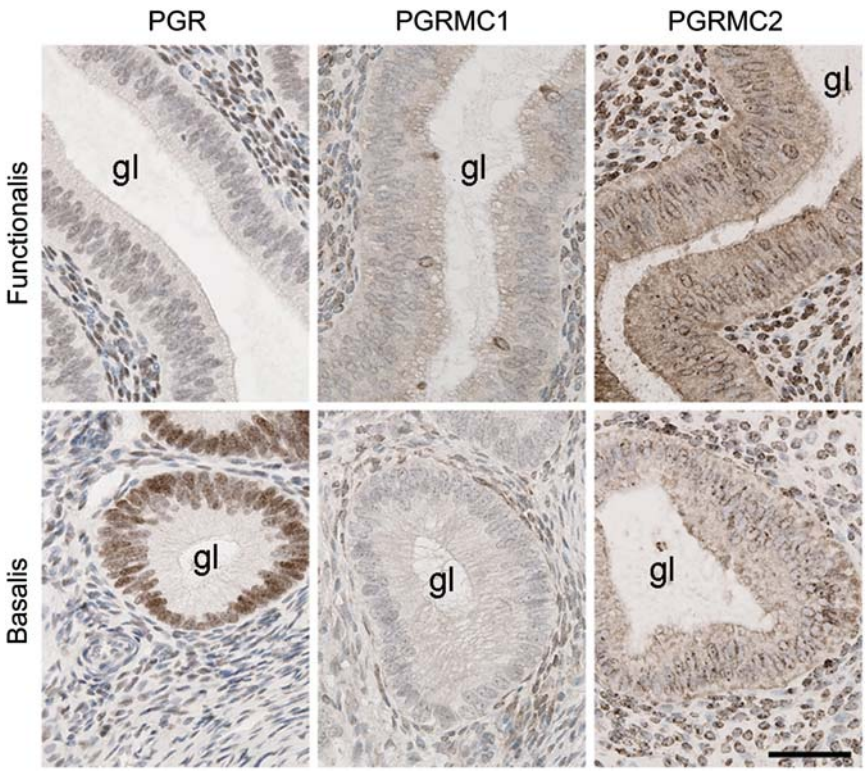

Figure 8 Endometriosis alters PGRMC2 in the macaque endometrium. Weak staining for PGR, PGRMC1 and PGRMC2 was observed during the secretory phase in the endometrium of macaques afflicted with endometriosis. Bar = 50 μm; gl, gland.

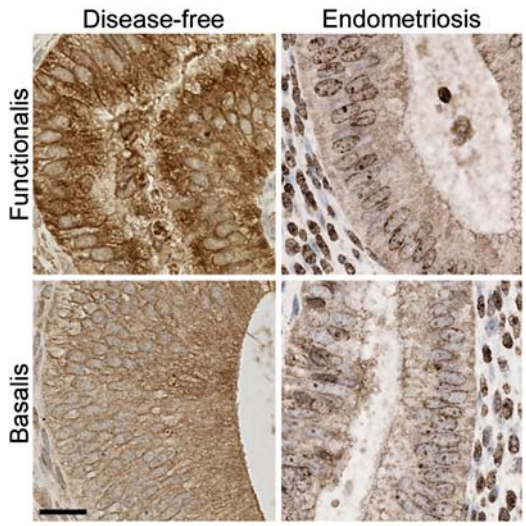

Figure 9 Endometriosis alters the intracellular localization of PGRMC2 in the macaque endometrium during the secretory phase of the cycle. High-powered microphotographs revealed PGRMC2 localized to the nuclear envelope, nucleolus and cytoplasmic granules in the glandular epithelia in the endometria of animals afflicted with endometriosis. This pattern of intracellular staining was markedly different compared with the patterns of PGRMC2 staining observed in the endometrium of disease-free animals. Bar = 20 μm.

studies are required to elucidate the true physiological role(s) for PGRMC2 in the primate endometrium.

We propose that the high levels of PGRMC1 may suppress apoptosis in the functionalis zone of the primate endometrium during the proliferative phase of the cycle. The highest levels of PGRMC1 were observed during the proliferative phase of the cycle, when cell proliferation peaks in the stroma and functionalis glands in the primate endometrium (Narkar *et al.*, 2006). PGRMC1 inhibits apoptosis in the granulosa cells of follicles, and the PGRMC1 pathway also regulates the viability and survival of cultured granulosa cells and several lines of immortalized ovarian cancer cells (Peluso *et al.*, 2009a, b; Peluso *et al.*, 2010; Peluso, 2011). Additionally, PGRMC1 levels are lowest, and the rate of apoptosis is greatest (Narkar *et al.*, 2006; Rodriguez *et al.*, 2008), during the late secretory phase of the cycle. Moreover, although PGRMC1 binds progestins, PGRMC1 also interacts with other endogenous steroids, synthetic hormones (e.g. R5020) and intracellular components (Gellersen *et al.*, 2009; Rohe *et al.*, 2009); therefore, it is both possible and plausible to speculate that the PGRMC1 pathway may be directly involved in cellular function in the endometrium during the proliferative phase of the cycle. Collectively, the largest changes in the mitotic index (i.e. mitotic and apoptotic ratios) occur in the functionalis zone of the primate endometrium (Brenner *et al.*, 2003), and those cells exhibited the greatest fluctuations in levels of PGRMC1.

The hormonal regulation of PGRMC1 and PGRMC2 appears to be complex. The patterns of expression collectively suggest that E_2 may stimulate the expression of PGRMC1 (and PGR) and inhibit expression

of PGRMC2 during the proliferative phase of the cycle. However, PGRMC1 was recently suggested to inhibit the expression of the PGRMC2 transcript in proliferating cancer cells *in vitro* (Ahmed *et al.*, 2010), and therefore the high levels of PGRMC1 may inhibit PGRMC2 expression in the primate endometrium during the proliferative phase of the cycle. Moreover, the inverse patterns of PGRMC1 and PGRMC2 expression in the macaque endometrium—especially in the glandular epithelia—also indicate that high levels of PGRMC2 may occur due to the marked reduction in PGRMC1 during the secretory phase of the cycle. Further, the reductions in PGRMC1 and increases in PGRMC2 during the secretory phase of the cycle appear to be mediated predominantly by progesterone. However, these changes occurred in the secretory phase of artificially cycled animals that exhibit constant levels of E_2, therefore future work examining the putative role of estrogen during the secretary phase of the cycle is required to fully understand the mechanisms regulating expression of the PGRMCs. Collectively, further *in vivo* experiments beyond the scope of the present study using specific PGRMC1 agonists and antagonists that have been developed recently for wide-spread usage (Ahmed *et al.*, 2010), and similar reagents designed specifically to interact with the PGRMC2 protein that are not yet available, will be required to elucidate the functional role of the PGRMCs in the primate endometrium.

In conclusion, treatment with E_2 and E_2 plus progesterone induced dynamic changes in the expression and localization patterns of PGRMC1 and PGRMC2 during the artificial menstrual cycle in the primate endometrium. The functional roles for the PGRMCs are still not known, but the up-regulation of PGRMC2 during the secretory phase of the cycle suggests a putative direct pathway for progesterone action in the endometrial glands of the functionalis zone. Reduced levels and abnormal intracellular localization patterns of PGRMC2 in the endometrium of animals afflicted with endometriosis suggest that alterations in membrane-bound PGRMC2 may contribute to the phenomenon of progesterone resistance. Changes in the levels of PGRMC1 may participate and/or regulate endometrial cell survival (mitosis and apoptosis) throughout the menstrual cycle. In sum, this study has revealed hormonally driven changes in the expression and localization patterns of PGRMC1 and PGRMC2, and therefore provides a strong foundation to further examine the putative functional roles for these progestin-binding moieties in the primate endometrium during the different phases of the menstrual cycle.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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Authors' roles

C.S.K. and O.D.S. constructed the study design. C.S.K and K.M. performed laboratory procedures, and C.S.K ran the statistical analyses. All authors contributed to the drafting of the manuscript.

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Conflict of interest

None declared.

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