Oestrogen and progesterone receptors in the marmoset endometrium: changes during the ovulatory cycle, early pregnancy and after inhibition of vascular endothelial growth factor, GnRH or ovariectomy

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Abstract

Marmosets are widely used, but detailed studies on localisation of endometrial oestrogen receptors α and β (ER α and ER β), and the progesterone receptor (PR) are lacking. These receptors were localised and semi-quantitatively analysed throughout the ovulatory cycle, weeks 2, 3 and 4 of pregnancy and after treatment with GnRH antagonist, vascular endothelial growth factor (VEGF) Trap or ovariectomy. The PR in epithelial cells increased markedly between the mid- and late proliferative phases before declining in the mid-secretory phase and pregnancy. PR in stromal cells was present throughout the cycle and levels were maintained in pregnancy. ER α was present at the mid-proliferative phase and increased in glands at the late proliferative and early secretory phases, before declining at the late secretory phase and week 4 of pregnancy. Stromal ER α showed a similar trend, but decreased earlier, by the mid-secretory phase. ER β was highly expressed in epithelial cells throughout the cycle and in pregnancy. In stroma, increases in ER β expression were observed at the late proliferative phase with the staining index decreasing by half as the secretory phase progressed and in pregnancy. GnRH antagonist, VEGF Trap or ovariectomy caused significant reductions in PR and ER β expression, but not in ER α when compared with the late proliferative phase of the normal cycle. Endothelial cells expressed ER β , but not ER α or PR. It is concluded that the steroid receptor profile in the marmoset endometrium is generally similar to the human and should provide a useful model for studies on hormonal manipulation of the endometrium.

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Introduction

Cellular proliferation and function in the endometrium, including cyclical menstruation and establishment of pregnancy, are regulated by steroid hormones, in particular oestrogen and progesterone. Disorders of the endometrium constitute a major health problem and manipulation of endometrial steroid receptors are involved in development of novel therapies (Jabbour *et al.* 2006). Insights into the regulation of steroid receptors in women have been obtained following treatment *in vivo*, using receptor antagonists such as mifepristone (Critchley *et al.* 2003, Milne *et al.* 2005), but such studies are constrained and the investigation of normal uterine physiology and the response to novel agents that affect it requires the use of animal models. The principal experimental non-human primates for studies of hormone action in the endometrium have been macaques (Brenner & Slayden 1994, 2004) and baboons (Albrecht & Pepe 2003), but their use is restricted

and alternative species are being sought (Carroll *et al.* 2007). The common marmoset (*Callithrix jacchus*) is widely used in reproductive research (Fraser & Lunn 1999, Abbott *et al.* 2003, Einspanier *et al.* 2006b). Its advantages are that it breeds throughout the year with high fecundity so that there are many self-sustaining colonies. Housing is relatively easy and economical because of its small size, which also makes it efficient for studying effects of valuable compounds with limited availability.

The marmoset uterus is of the simplex type as in the human. However, it cannot be assumed that the regulation of the uterus in this non-menstruating New World primate will be similar to the human, or the macaques (Old World primates) for that matter (Abbott *et al.* 2003). While the length of the ovulatory cycle is similar, the follicular phase is shorter at 8 days and the luteal phase is correspondingly longer being in the region of 20 days. Implantation takes place 11 days after ovulation, as opposed to days 7 and 8 in humans and

macaques (Enders 2000, Niklaus *et al.* 2001) and depth of implantation is superficial (Niklaus *et al.* 2001, Wulff *et al.* 2003). Furthermore, levels of total and free plasma steroids, including oestradiol and progesterone, are exceptionally high (Harlow *et al.* 1983, Smith & Fraser 1991). This is a result of end organ resistance mediated by two factors. First, overexpression and higher affinity of an intracellular cytosolic-binding protein, the immunophilin FK-binding protein 51, that specifically impairs binding to receptors and contributes to steroid hormone resistance, at least to glucocorticoid and progesterone (Hubler *et al.* 2003, Westberry *et al.* 2006). In addition, the steroid receptors are of low binding affinity (Westberry *et al.* 2006). In early pregnancy, the corpus luteum secretes plasma progesterone at levels similar to the mid-luteal phase, rather than rising further as in macaques and humans (Smith & Fraser 1991, Rowe *et al.* 2002).

The aim of the current study was to establish the localisation and changes in oestrogen receptors α and β 1 (ER α and ER β) and the progesterone receptor (PR) during the normal cycle, the implantation period, early pregnancy and after hormone manipulation *in vivo*. Antibodies that specifically identify these receptors in the marmoset reproductive tract have been identified and characterised (Saunders *et al.* 2000, McKinnell *et al.* 2001, Sierens *et al.* 2004). Although some information on the localisation of these receptors in marmoset endometrium has been reported, these observations have been limited to a small number of animals (Niklaus *et al.* 2001, Sierens *et al.* 2004) and disagree with earlier results (Kholkute *et al.* 2000), suggesting an absence of expression in epithelial cells. The detailed evaluations in the current study was made possible as a result of collecting tissue from experiments primarily aimed at elucidating the changes in angiogenesis occurring in the ovary during the normal cycle (Dickson & Fraser 2000), early pregnancy (Rowe *et al.* 2002) and after inhibiting gonadotrophin releasing hormone (GnRH; Taylor *et al.* 2004) or vascular endothelial growth factor (VEGF; Wulff *et al.* 2001, 2002). To compare these treatments, which suppress plasma oestradiol and prevent the ovulatory rise in progesterone, with the complete removal of ovarian steroids, marmosets were ovariectomised at the same stage of the cycle as the GnRH and VEGF antagonist treatments were initiated.

Material and Methods

Animals and treatments

The studies were approved by the local Primate Ethical Committee and carried out under Project Licences PPL 60/2472 and PPL 60/3250 granted by the UK Home Office. Adult female marmosets (*C. jacchus*), weighing between 350 and 450 g, were housed with a younger sister or pre-pubertal female. Ovulation was determined

by measuring plasma progesterone profiles in samples collected on Monday, Wednesday and Friday. Plasma progesterone levels are <32 nmol/l during the follicular phase in the marmoset. Ovulation, designated luteal day 0, was defined as the day preceding a rise in progesterone concentrations above 32 nmol/l followed by a progressive increase in progesterone (Harlow *et al.* 1983, 1984, Gilchrist *et al.* 2001). There is some variation in the length of the luteal phase in the marmoset and together with the relatively short follicular phase it makes it difficult to collect tissue to constitute an early proliferative phase group. It was decided therefore, to synchronise the proliferative phase by inducing luteolysis beginning on days 14–16 post-ovulation. This method of synchronising follicular recruitment is followed by follicle selection on cycle day 5 and ovulation around day 10 (Summers *et al.* 1985, Gilchrist *et al.* 2001), periods which were classified as mid- and late proliferative phases respectively. To synchronise the timing of the proliferative phase, marmosets were injected with 1 μ g prostaglandin F_{2 α} analogue (cloprostenol, Planate, Coopers Animal Health Ltd, Crewe, UK), intramuscularly to induce luteolysis (Summers *et al.* 1985, Gilchrist *et al.* 2001). Secretory phase tissue was collected either from animals that had been synchronised with prostaglandin or from spontaneous cycles over the periods, early secretory (days 2–4 post-ovulation), mid-secretory (days 12–14 post-ovulation) or late secretory phase (days 19–21 post-ovulation). To study changes during very early pregnancy, marmosets were treated with prostaglandin analogue as described above and a fertile male introduced. The stage of pregnancy was calculated taking day 0 as estimated day of ovulation. Uteri were collected at weeks 2, 3 and 4 of pregnancy where the presence of a trophoblast had been confirmed by serial sections as published before (Rowe *et al.* 2002, 2003).

A second experiment addressed the effects of ovariectomy or inhibiting GnRH or VEGF throughout the proliferative phase. Marmosets were treated with a GnRH antagonist or VEGF Trap starting between luteal days 14–16 (Wulff *et al.* 2002, Taylor *et al.* 2004, 2006). To block the action of VEGF, we employed the VEGF Trap, a recombinant chimeric protein comprising portions of the extracellular domains of the human VEGF receptors VEGFR1 and VEGFR2 expressed in sequence with the Fc portion of human immunoglobulin (Holash *et al.* 2002). The VEGF Trap binds all isoforms of VEGF-A, and placental growth factor, but does not bind VEGF B or C. VEGF Trap was administered as a single injection of 25 mg/kg s.c. To block the GnRH receptor, the GnRH antagonist, Teverelix, [N-Ac-D-Nal¹, D-pCl-Phe², D-Pal³, D-(Hci)⁶, Lys(iPr)⁸, D-Ala¹⁰]GnRH (Europetides, Argenteuil, France) dissolved in water to a concentration of 10 mg/ml was used. To provide a slow-release depot Teverelix was administered at a single dose of 12 mg/kg, injected subcutaneously (Taylor

et al. 2004). In addition, to determine the effects of total withdrawal of ovarian steroids, four marmosets were ovariectomised following sedation with ketamine hydrochloride (Parke-Davis Veterinary, Pontypool, Gwent, UK), anaesthesia by Saffan (Alphaxalone/Alpadalone, Schering-Plough Animal Health, Welwyn Garden City, UK) and maintenance of analgesia by buprenorphine (Alstoe Animal Health, Melton Mowbray, UK) i.m. Ovariectomy was carried out between days 14 and 16 of the luteal phase, and uteri collected 10 days later; the same schedule as for the VEGF Trap and GnRH antagonist treatments. These treatments were continued for 10 days and results from these groups were compared with the late proliferative phase which corresponds to 10 days post-luteolysis. Five animals were studied for each group throughout the cycle and pregnancy and four for each treatment group.

At the allotted stage, animals were sedated using 100 µl ketamine hydrochloride (Parke-Davis Veterinary) and killed with an i.v. injection of 400 µl Euthetal (sodium pentobarbitone, Rhone Merieux, Harlow, Essex, UK). After cardiac exsanguination, the uterus was removed immediately, weighed and fixed in 4% neutral buffered formalin for 24 h before being transferred into 70% ethanol, dehydrated and embedded in paraffin according to standard procedures. The uteri were placed sagittally, serially sectioned and tissue sections (5 µm) placed onto superfrost slides (Merck Poole, Dorset, UK). Sections were cut so that most of the fundal region of the uterus was observed. Whole uterine sections, in duplicate, were mounted on the microscope slides. All specimens included in the study met the stage of the cycle confirmed by ovarian morphology.

Immunocytochemistry

Paraffin wax was removed by immersion in Xylene (Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK). The sections were rehydrated by a decreasing concentration of ethanol and washed in tap water. To localise PR, antigen retrieval was performed by boiling sections in 0.01 M citrate buffer, pH 6, for 5 min at high pressure setting 2 in a Tefal Clypso pressure cooker (Tefal, Essex, UK). Slides were then left for 20 min in hot buffer and washed in water. The endogenous peroxidase activity was blocked by immersing all sections in 3% (v/v) H₂O₂ in methanol (both from BDH Laboratory Supplies, Poole, UK) for 30 min followed by two 5-min washes in Tris-buffered saline (TBS; 0.05 mol/l Tris (pH 7.4), NaCl 9 g/l). To prevent non-specific binding of the biotinylated second antibody, sections were blocked with normal porcine serum (NPS; Diagnostic Services, Carlisle, UK) diluted in a ratio of 1:5 in TBS containing 5% (w/v) BSA (Sigma) for 30 min. In addition, an avidin-biotin block was performed using an Avidin-Biotin blocking kit (Vector Laboratories Inc., Peterborough, UK) according to the manufacturer's specifications.

The sections were then incubated overnight at 4 °C in immunostaining chambers (Shandon, coverplate Anatomical International, Runcorn, UK) with rabbit polyclonal anti-PR antibody (PR (C-19): sc-538, diluted in a ratio of 1:500) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). After three washes in TBS, the sections were incubated with a secondary antibody, swine anti-rabbit biotinylated (diluted in a ratio of 1:500; E0353, DAKO, Ely, UK) diluted in NPS for 30 min, followed by three washes with TBS. The sections were incubated in ABC HRP (DAKO, K0355) for 30 min. All sections were washed three times in TBS and immunostaining was developed using Liquid DAB + (Dako) until staining was optimal. After about 5 min, the reaction was stopped by immersing sections in tap water. The sections were counterstained with haematoxylin, dehydrated in a graded ethanol series, cleared in xylene and a cover slip applied using Pertex mounting medium (CellPath plc, Hemel Hempstead, UK).

The protocol for localisation of ER α was similar with a few minor differences. The serum block was a normal rabbit serum (NRS, diluted in a ratio of 1:5 in TBS containing 5% (w/v) BSA (Sigma) and the secondary antibody was rabbit anti-mouse biotinylated (RAMB, diluted in a ratio of 1:500 in NRS, Dako, E0464). Avidin diluted in NRS was added to the sections and incubated for 30 min at room temperature. The sections were washed three times with TBS. Biotin diluted in NRS was added to the sections and incubated for 20 min followed by three washes with TBS. The mouse monoclonal ER α antibody (NCL-ER-6F11/2, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) diluted in a ratio of 1:50 in NRS, and used previously (McKinnell *et al.* 2001), was added to the sections which were incubated overnight at 4 °C. Day 2 of the protocol was identical to that performed for PR.

The protocol to localise ER β was similar to the one for ER α , but antigen retrieval was performed using 0.01 M glycine/EDTA buffer (pH 3.5). After blocking with NRS, the sections were incubated overnight with the mouse anti-human ER β 1 (MCA1974S, clone PPG5/10, Serotec, Düsseldorf, Germany; Sierens *et al.* 2004), diluted in a ratio of 1:60. Day 2 of the protocol was identical to that performed for ER α , using the same secondary antibody. To determine the specificity of the antibodies, the primary antibody was replaced with appropriate rabbit immunoglobulin G (IgG; Dako), or mouse IgG (Vector) at the same concentration as the primary antibody.

Analysis of receptor staining

Immunocytochemistry staining was analysed by using a microscope (AxioStar; Zeiss, Jena, Germany). Both authors independently examined the uterus systematically while being blinded to the status of the animal from which the tissue was obtained. The scores

(see below) were assigned by each examiner. These were generally in excellent agreement. Where differences in score arose, the specimen was checked so that agreement could be reached. The mean values from the two scorers were taken for the final result. Each specimen was morphologically evaluated by division into six anatomic zones: (1) the luminal epithelium, (2) the stroma adjacent to the luminal epithelium, (3) glands of the upper zone, or functionalis (4) the stroma in the functionalis region, (5) the glands of the lower zone, or basalis and (6) the stroma in the basalis region. The functionalis glands were delineated by their oval shape, while the basalis glands tended towards being longitudinal (Rowe *et al.* 2004). The division of the glands into a single zone in the basal layer, rather than two layers as observed in higher primates has been established in previous studies (Rune *et al.* 1992). In addition, by weeks 3 and 4 of pregnancy, decidualised cells were clearly recognisable.

Each zone was given an intensity score and a proportional score to provide a staining index. The intensity score reflected the intensity of the brown positive staining in the cell nuclei on a scale of 0 (no staining), 1 (light), 2 (moderate) or 3 (strong), while the proportional score reflected the percentage of cell nuclei that stained positive in the different groups of cells. The percentage score was converted to the scale 0 to 3 and added to the intensity score for each observation to produce a 'staining index'. For example, if 50% of cells were stained strongly, the intensity score would be 1.5 + 3. Thus, the maximum intensity score was 6. Values for each group were calculated as means and standard error. In general, changes in percentage score were similar to those in staining intensity. The method was a simplified adaptation of the HSCORE (Murray *et al.* 1999). In the latter method, account is also taken of variations in staining intensity within the percentage of cells showing positive staining. As the intensity was fairly uniform within a compartment in individual sections we omitted this step in our calculations.

After examination, representative sections were photographed using a Provis microscope (Olympus Optical, London, UK) fitted with a digital camera (DC S330; Eastman Kodak). The captured images were transferred to a computer (G4; Apple Computer Inc., Cupertino, CA, USA) and compiled using Photoshop 5.0 (Adobe Systems Inc).

Steroid assays

Oestradiol concentrations in serum collected by exsanguination were determined by an ELISA assay described previously (Taylor *et al.* 2004), all samples being run in a single assay. Plasma progesterone concentrations in routine samples to determine cycle stage were determined by an ELISA assay as previously described (Wulff *et al.* 2001).

Statistical analysis

The significance of differences between means for plasma hormone concentrations were determined by ANOVA followed by Bonferroni's post-test with $P < 0.05$ being considered significant. Differences in staining index between the groups was performed by using the non-parametric Mann-Whitney *U*-test, $P < 0.05$ being considered to reflect a significant difference from the control group or between groups.

Results

Plasma concentrations of oestradiol and progesterone were as expected from stage of the cycle (Fig. 1). Oestradiol rose significantly ($P < 0.001$) from the mid-proliferative phase to the late proliferative peak before falling significantly ($P < 0.01$) at the early secretory phase; levels during pregnancy were similar to the mid-secretory phase. Ovariectomy, GnRH antagonist and VEGF Trap treatment all significantly reduced ($P < 0.001$) the oestradiol levels when compared with the late proliferative phase. Progesterone concentrations were elevated during the secretory phase and maintained during early pregnancy.

For each receptor, photomicrographs are depicted for the stages which demonstrated the predominant changes in staining index, namely, mid-proliferative, early secretory, late secretory and week 4 of pregnancy. The staining index for each receptor in the basalis glands, functionalis glands, stroma in the basalis region and stroma in the functionalis region is shown for all stages in the accompanying histograms. Since staining indices for the luminal epithelium and stroma next to the luminal epithelium changed in a manner similar to that of the functionalis glands and stroma next to these glands, data for the former parameters are not shown.

PR in all epithelial compartments increased ($P < 0.01$) between the mid-proliferative phase and late proliferative phase, most markedly in the functionalis (Fig. 2). After the early secretory phase, a significant decline in PR occurred during the remainder of the cycle and pregnancy with the luminal epithelium being most affected, followed by the functionalis glands. The glands of the basalis region, especially those nearest to the myometrium retained receptor for the longest, not becoming significantly reduced until the late secretory phase ($P < 0.05$), and disappearing by week 4 of pregnancy ($P < 0.01$).

In contrast to the glands, PR expression was present in stroma at the mid-proliferative phase. In the stroma basalis region, PR tended to be low, but was only significantly lower ($P < 0.01$) than maximal values at the late secretory phase. A consistent significantly lower ($P < 0.05$) expression was observed at 2 and 3 weeks of pregnancy. In the stromal functionalis, PR was highly expressed and was largely unaffected by stage, except at

Figure 1 Plasma concentrations of (A) oestradiol and (B) progesterone at the time of collection of the uterus in marmosets at the mid-proliferative (MP), late proliferative (LP), early, mid- and late secretory phases (ES, MS, LS) of the ovulatory cycle, at weeks 2, 3 and 4 of pregnancy (P 2, 3 and 4) and after treatment with VEGF Trap, GnRH antagonist, or ovariectomy initiated at the late luteal phase. Values are means $\pm$ S.E.M. Columns with different letters are significantly different.

the late secretory phase which was significantly lower ($P < 0.05$) than the proliferative phase values. The large decidualised cells observed by weeks 3 and 4 of pregnancy were negative; however, small cells present in the decidualised zone, presumably fibroblasts, were strongly stained for PR. In some sections, in particular in pregnancy, and after reduction in oestradiol, a weak cytoplasmic stain was noted. This was ignored for the purposes of quantification.

After treatment with VEGF Trap, GnRH antagonist or ovariectomy, the late proliferative rise in PR in epithelial cells was significantly inhibited ($P < 0.05$) in all compartments (Fig. 3) when compared with the late proliferative phase control. Similar inhibitory effects were observed in the stromal zones.

ER α expression in the basalis and functionalis glands increased from the mid- to late proliferative phase ($P < 0.01$ and $P < 0.05$ respectively) as the cycle progressed to the early secretory phase, and then significantly declined ($P < 0.05$) at the late secretory phase (Fig. 4) when residual expression was largely in the epithelial cells of basalis glands that were nearest to the myometrium. Expression of ER α also declined as pregnancy progressed, most markedly in the functionalis

glands with weeks 2, 3 and 4 being significantly lower ($P < 0.05$) than the proliferative and early secretory phases and with week 4 also being lower ($P < 0.01$) than the mid-secretory phase. In basalis glands, the decline in ER α in the late secretory phase was less marked. By 3 weeks of pregnancy, basalis gland expression was significantly lower ($P < 0.05$) than the late proliferative and early secretory phases, and by 4 weeks expression had declined ($P < 0.01$) further. In the stromal basalis zone, expression was highest during the proliferative phase, declining markedly ($P < 0.01$) by the early-secretory phase, with further significant ($P < 0.05$) reductions by the mid-secretory phase. An absent or very low expression was maintained during early pregnancy. In the stromal functionalis, a significant increase ($P < 0.01$) in expression occurred between the mid- and late proliferative phases but declined by the early secretory phase ($P < 0.05$). Expression was maintained at secretory phase levels at weeks 3 and 4 of pregnancy. ER α was not detected in decidual cells.

After treatment with VEGF Trap, GnRH antagonist or ovariectomy, expression of ER α was maintained in all epithelial layers, there being no significant differences to late proliferative controls in any parameter except a small rise ($P < 0.05$) in staining index in basalis glands after GnRH antagonist (Fig. 5). Similarly, stromal staining was largely unaltered, except for a significant decline in expression ($P < 0.05$) in the ovariectomised animals in the functionalis area.

ER β was present throughout the endometrium and staining index for ER β in the epithelium was less responsive to stage of the reproductive cycle than ER α (Fig. 6). In the basalis glands, staining index was maintained throughout the cycle with a slightly reduced expression ($P < 0.05$) at weeks 3 and 4 of pregnancy. In the epithelium of the functionalis glands, staining index showed a small increase ($P < 0.05$) between the early proliferative and mid-secretory phase and was maintained during pregnancy. In the stroma between all compartments, ER β expression significantly increased ($P < 0.05$) between the mid- and late proliferative phases. After this peak, levels significantly declined ($P < 0.01$) again to the late secretory phase, with levels being largely maintained at secretory phase values in pregnancy. The large decidualised cells observed by weeks 3 and 4 of pregnancy were strongly stained for ER β (Fig. 6).

After treatment with VEGF Trap, GnRH antagonist or ovariectomy, ER β in the basalis glands was not significantly altered but in the functionalis glands was significantly lower; VEGF Trap and ovariectomy ($P < 0.01$), and GnRH antagonist ($P < 0.05$; Fig. 7). In the stroma next to the lumen and the functionalis zone, all three treatments caused a significant suppression ($P < 0.01$) in the staining index, while in the stroma of the basalis zone this effect was significant ($P < 0.05$) only for VEGF Trap.

Figure 2 Immunohistochemical localisation of PR (brown) in sections of marmoset endometrium counterstained with haematoxylin (blue) from (A–C) mid-proliferative phase (MP), (D–F) early secretory phase (ES), (G–I) late-secretory phase (LS) and (J–L) week 4 of pregnancy (P4). The top row is of low magnification to show distribution throughout the endometrium. High power magnifications of the epithelial and stromal cells of basalis and functionalis regions are shown in the middle and bottom panels respectively. Basalis glands (BG), functionalis glands (FG), stroma in functionalis zone (FG) and stroma of basalis zone (SB) are identified in the left-hand panels. Changes in staining intensity are shown in (M–P). Grey columns show data for the normal cycle, black show values at weeks 2 (P2), 3 (P3) and 4 (P4) of pregnancy. Top panel, scale bar = 200 μ m; middle and lower panels, scale bar = 20 μ m. Values are means $\pm$ S.E.M. Panels with different letters are significantly different.

A closer examination of the patterns of staining in the stroma revealed that neither PR nor ER α was expressed in endothelial cells, while a proportion of perivascular cells was positively stained. In contrast, ER β was expressed in the majority of endothelial cells in addition to a proportion of the surrounding stroma (Fig. 8). This arrangement was maintained in the large maternal vessels (Fig. 8). Changes in the intensity of ER β in endothelial cells after VEGF Trap, ovariectomy or GnRH antagonist appeared similar to the other stromal populations (not shown).

Pseudostratification was common in glands during the secretory phase, especially in the basalis zone. This is best illustrated in the sections shown in Fig. 4 D,G,E and H. The magnitude of pseudostratification appeared to increase as the secretory period progressed. By the late secretory phase, pseudostratification was also observed in the luminal epithelium (not shown).

Discussion

This is the first detailed analysis of the localisation of ER and PR in the marmoset endometrium. We demonstrate how synchronisation of the cycle by treatment with a luteolysis-inducing prostaglandin allows collection of tissue at precise stages. Despite the marmoset having distinct differences in some of the mechanisms in the way the ovarian hormones are processed at the receptor level, the pattern of steroid receptor localisation is generally similar to that observed in women and for other non-human primates studied to date.

The most dramatic changes during the cycle were observed for PR, which increased markedly in all epithelial compartments between the mid-proliferative phase to the late proliferative phase. Staining index declined after the early secretory phase with the luminal epithelium and the functionalis glands being most

Figure 3 Immunohistochemical localisation of PR (brown) in sections of endometrium from a control marmoset in the late proliferative phase and after treatment with VEGF Trap. Changes in staining index for PR

affected. The increase in PR in epithelium from the mid- to late proliferative phase is a consequence of oestrogen stimulation, while its decline in the glands in the secretory phase is attributed to the exposure to progesterone (Jabbour *et al.* 2006). A relative resistance to PR decline in the basalis epithelium is also observed in baboons (Hild-Petito *et al.* 1992). The maintenance of PR in the stroma during the secretory phase, in contrast to its reduction in the glands, is also found in women, macaques and baboon (e.g. Hild-Petito *et al.* 1992, Jabbour *et al.* 2006).

ER α increased in the glands and stroma during the proliferative phase. Like PR, ER α declined as the secretory phase progressed, presumably also as a result of exposure to high levels of progesterone. In contrast to PR, ER α also declined markedly in the stroma by the mid-secretory phase. This is similar to macaques (Brenner & Slayden 1994) and women (Lecce *et al.* 2001). ER β was consistently highly expressed throughout the cycle in the epithelial cells and contrasted with ER α in maintaining a high staining index throughout the secretory phase. However, in stroma, expression gradually declined as the secretory phase progressed in a pattern similar to ER α , implying a negative regulation of ER β by progesterone in stroma but not in epithelial cells. Although we found ER β to be highly expressed in the endometrium, as in the human and macaque (Crithcley *et al.* 2001) it has been suggested that it has a minor function in this tissue when compared with ER α (Harris 2007). These conclusions are based largely on studies in the ER β knockout mouse and its potential role in the primate endometrium must be determined by use specific of ER α and ER β antagonists.

In pregnancy, PR-staining index declined rapidly in the functionalis glands, slowly in basal glands and had disappeared by pregnancy week 4. PR expression was maintained in the stroma during pregnancy, and at week 4 was significantly higher in the functionalis zone than the late secretory phase. Thus, despite the high plasma levels of oestradiol, the PR-staining index in epithelial cells declines during pregnancy suggesting the down-regulation of the receptor is related to the duration of exposure to progesterone. These findings are similar to those in the baboon (Hild-Petito *et al.* 1992). ER α expression steadily declined in the glands as pregnancy progressed. Thus, contrasted with the stroma where it was absent at 2 weeks but recovered thereafter. Recently, RT-PCR analysis of uterine expression of ER α and PR from marmosets in the mid-secretory phase and pregnancy revealed no apparent differences in mRNA

after treatment with VEGF Trap, GnRH antagonist or ovariectomy for 10 days (open column) when compared with late proliferative controls (shaded columns). $N=4$ per group. Other details as provided in Fig. 2. Top panel, scale bar = 200 μ m; middle and lower panels, scale bar = 20 μ m. Values are means $\pm$ S.E.M. Panels with different letters are significantly different.

Figure 4 Immunohistochemical localisation of ER α (brown) in sections of marmoset endometrium counterstained with haematoxylin (blue) from (A–C) mid-proliferative phase (MP), (D–F) early secretory phase (ES), (G–I) late secretory phase (LS) and (J–L) week 4 of pregnancy (P4). The top row is of low magnification to show distribution throughout the endometrium. High power magnifications of the basalis and the functionalis regions are shown in the middle and bottom panels respectively. Basalis glands (BG), functionalis glands (FG), stroma in functionalis zone (FG) and stroma of basalis zone (SB) are identified in the left-hand panels. Changes in staining intensity are shown in (M–P). Grey columns show data for the normal cycle, black show values at weeks 2 (P2), 3 (P3) and 4 (P4) of pregnancy. Top panel, scale bar = 200 μ m; middle and lower panels, scale bar = 20 μ m. Values are means $\pm$ S.E.M. Panels with different letters are significantly different.

(Einspanier *et al.* 2006b). The presence of ER β in the decidua in the absence of ER α agrees with observations in the human (Milne *et al.* 2005), while the numbers of decidualised cells staining for PR in the marmoset appears to be less.

To obtain further insight into the role of oestradiol in stimulating these changes, ovariectomy, GnRH antagonist or VEGF Trap treatment was carried out at the late secretory phase to remove or suppress the secretion of ovarian hormones. Endometrium was studied 10 days later, equivalent to the late proliferative phase in controls in which luteal function had been terminated by prostaglandin treatment 10 days previously. Oestrogen is responsible for endometrial proliferation and induces its own receptor (Jabbour *et al.* 2006), but despite deprivation of ovarian oestradiol, the staining index for ER α was similar to the oestradiol-stimulated late proliferative levels in all areas. It should be noted that

at the end of the 10-day period of oestradiol deprivation, ER α -staining index was higher than at the time of onset of the treatments at the late secretory phase. The only site where ER α was decreased was the stroma in the functionalis area in the ovariectomised group. In contrast, the induction of expression of PR in both epithelial cells and stroma was prevented by these treatments. These observations confirm the rise in PR and require the follicular phase rise in oestrogen. However, they show that the rise in ER α does not have this dependence. Thus, it appears to be driven either by progesterone withdrawal or requires only residual amounts of oestrogen that may remain available from the circulation or by enzyme conversion with the endometrium during the 10-day period following ovariectomy.

That a reduction in plasma progesterone has an important role in stimulating expression of ER α , is also

Figure 5 Immunohistochemical localisation of ER α (brown) in sections of endometrium from a control marmoset in the late proliferative phase

implied by the fact that, while expression is very low in the functionalis glands at the progesterone-dominated late secretory phase, ER α is more rapidly restored than that of PR, being present by the mid-proliferative phase in intact animals, while PR expression was minimal. The reduction in PR at the end of the cycle removes the progesterone block to ER α synthesis and leads to ER α expression by the onset of the subsequent proliferative phase, followed by PR expression. The presence of ER α expression on one hand, associated with a decline in PR expression on the other after ovariectomy, is similar to findings in the atrophic endometrium of post-menopausal women (Snijders *et al.* 1992) and the ovariectomised baboon (Hild-Petito *et al.* 1992). It should also be considered that these treatments result in a less extensive endometrium than is observed under cyclic conditions. It seems that the remaining population of cells may be particularly resistant in terms of maintaining ER α expression.

In contrast to ER α , the staining index for ER β was reduced in the epithelium of the functionalis and in the stroma by all three treatments while VEGF Trap also resulted in a decline in ER β in the luminal epithelium. Although not as markedly suppressed as the PR, these results provide evidence that ER β expression also appears to be oestrogen dependent over short time periods.

Since PR expression requires oestrogen stimulation, the prevention of the rise in PR by the treatments suggests that this requires a higher level of oestrogen than does the maintenance of the ERs. The suppression in PR was most profound in the endometrium overall in the ovariectomised marmosets suggesting that the higher residual levels of oestradiol present after VEGF Trap and GnRH antagonist treatment afforded a degree of stimulation of the PR.

Oestradiol and progesterone are considered to be important in the regulation of the endometrial vasculature, although their exact roles have yet to be defined (Nayak & Brenner 2002, Jabbour *et al.* 2006). A distinctive feature of endometrial endothelial cells of the marmoset was the preferential staining for ER β over PR or ER α at all stages of the cycle and during pregnancy. This phenomenon has been described in women (Critchley *et al.* 2001, Lecce *et al.* 2001) and macaques (Brenner & Slayden 2004), and has been confirmed by studies on human microvascular endometrial endothelial cell (HEEC) cultures (Krikun *et al.* 2005). This suggests a role for oestrogen directly upon endometrial

and after treatment with VEGF Trap. Staining index for ER α after treatment with VEGF Trap, GnRH antagonist or ovariectomy for 10 days (open columns) when compared with late proliferative controls (shaded columns). Staining is largely retained despite treatments. Top panel, scale bar=200 μ m; middle and lower panels, scale bar=20 μ m. Values are means $\pm$ S.E.M. Panels with different letters are significantly different.

Figure 6 Immunohistochemical localisation of ER β (brown) in sections of marmoset endometrium counterstained with haematoxylin (blue) from (A–C) mid-proliferative phase, (D–F) early secretory phase, (G–I) late-secretory phase and (J–L) week 4 of pregnancy. The top row is a low magnification to show distribution throughout the endometrium. High power magnifications of the basalis region and functionalis region are shown in the middle and bottom panels respectively. Basalis glands (BG), functionalis glands (FG), stroma in functionalis zone (FG) and stroma of basalis zone (SB) are identified in the left-hand panels. Changes in staining intensity are shown in (M–P). Grey columns show data for the normal cycle, black columns show values at weeks 2 (P2), 3 (P3) and 4 (P4) of pregnancy. Top panel, scale bar = 200 μ m; middle and lower panels, scale bar = 20 μ m. Values are means $\pm$ S.E.M. Panels with different letters are significantly different.

endothelial cells influencing angiogenesis and vascular permeability. Ovariectomy, GnRH antagonist or VEGF Trap treatment all caused a decline in expression of ER β in the stroma and as higher staining intensity in endothelial cells when compared with other cells in the stroma after these treatments was not apparent it suggests that the expression of endothelial ER β receptor is oestrogen-dependent. Although VEGF is the principal angiogenic factor which acts upon endothelial cells (Ferrara 2004), VEGF Trap treatment did not appear to result in preferential suppression of the endothelial ER β receptor, suggesting that the effect observed was the result of reduction in oestradiol stimulation. The extent to which oestrogen action on the endothelium is primarily mediated via VEGF remains to be established.

In addition to inhibition of VEGF leading to a reduced ovarian steroid stimulation to the endometrium, this angiogenic growth factor also has direct actions on

endometrium, which are currently being explored. VEGF is synthesised in both the stroma and epithelial cells (Rowe *et al.* 2003). The principal actions are likely to be on endometrial endothelial cells, mediated by VEGF receptors (Nayak & Brenner 2002, Rowe *et al.* 2003). A link between oestrogen action on endometrial endothelial cells and expression of VEGF, other angiogenic factors or their receptors has yet to be established as addition of oestrogen to HEEC cultures failed to alter their expression levels (Krikun *et al.* 2005).

With respect to previous studies on location of steroid receptors in the marmoset uterus, the finding of highest levels of ER α in stroma during the late proliferative phase agrees with Kholkute *et al.* (2000) but as these authors failed to detect expression of either ER α or PR in the epithelial cells or detect ER α during the secretory phase in either stroma or glands, it would seem that the antibodies used or technical conditions were sub-optimal. In a

Figure 7 Immunohistochemical localisation of ER β (brown) in sections of endometrium from a control marmoset in the late proliferative phase and after treatment with VEGF Trap. Changes in staining index for ER β after treatment with VEGF Trap, GnRH antagonist or ovariectomy for

subsequent study, localisation of ER α and PR in epithelium of the marmoset was established (Narkar *et al.* 2006). However, the occurrence of high levels of PR in glands during the early proliferative and late secretory phases and low levels in the stroma contrast with the current report, as does the finding of a reduction in ER α in the late proliferative phase. No quantitative Statistical analysis was performed and there was insufficient detail for direct comparison with the current report.

With respect to differences between the pattern of ER and PR in the marmoset endometrium, to those in other primates, the low level of epithelial PR at the mid-proliferative phase is lower than the human, while the decline in PR during the secretory phase appears to lag behind (Snijders *et al.* 1992). These differences may reflect the longer period to implantation (11 days) in the marmoset and the longer (20-day) luteal phase. In early pregnancy, the marmoset seems to maintain a level of ER α expression that is not observed in baboon endometrium (Hild-Petito *et al.* 1992). Pseudostratification was common in glands of the marmoset during the secretory phase, especially in the basalis zone, a phenomenon reported in the vervet monkey (Carroll *et al.* 2007) but not noted in macaques or human. We have observed continued proliferation of epithelial cells in the marmoset in the early secretory phase (H M Fraser, unpublished observations) and this may account for the build up in cell layers. Another difference was the apparent absence of decidualisation of the endometrium in the normal cycle although the phenomenon was readily apparent in early pregnancy. The absence of decidualisation in the absence of pregnancy constitutes a significant difference to the human, macaques and baboon (Dunn *et al.* 2003), and is perhaps related to the more superficial nature of implantation in the marmoset. Finally, ER β expression has also been reported in natural killer cells in the human endometrium (Henderson *et al.* 2003), but we have been unable to detect this cell type in the marmoset endometrium.

In conclusion, the localisation of ER and PR in the marmoset endometrium, reveals a predominantly similar profile to the human. The studies using VEGF Trap and GnRH antagonist are examples of situations in which the effects of a novel compound that could impact on endometrial function may be explored. For example, the localisation of ER β receptors on endothelial cells of the marmoset endometrium should provide a valid model system in which their role in regulation of the uterine vasculature may be investigated. Use of the marmoset as a non-human primate model in female reproduction continues to expand; recently, it has been demonstrated that endometriosis can be induced in this species

10 days (open column) when compared with late proliferative controls (shaded columns). Top panel, scale bar=200 μ m; middle and lower panels, scale bar=20 μ m. Values are means $\pm$ S.E.M. Panels with different letters are significantly different.

Figure 8 Areas of stroma from marmosets showing localisation of (A and D) PR, (B and E) ER α and (C and F) ER β in early secretory phase endometrium of blood vessels (top panel) and in large maternal vessels at week 3 of pregnancy. Note the absence of PR and ER α from the endothelial cells (arrows) (E) lining the blood vessels which are strongly stained for ER β . Top panel, scale bar = 100 μ m; bottom panel, scale bar = 20 μ m.

(Einspanier *et al.* 2006a) and novel embryonic stem cell lines have been derived from this species (Sasaki *et al.* 2005) taking advantage of the benefits of the special features of its reproductive cycle.

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