J Clin Endocrinol Metab. Author manuscript; available in PMC 2010 November 22.

Published in final edited form as:
J Clin Endocrinol Metab. 2009 December ; 94(12): 5146–5154. doi:10.1210/jc.2009-1476.

Attenuated sex steroid receptor expression in Fallopian tube of women with ectopic pregnancy

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Abstract

Context—Sex steroid hormone receptor (SHR) dynamics are well-documented in human endometrium but have not been comprehensively studied in Fallopian tube (FT).

Objective—To compare expression patterns and hormonal regulation of SHR in FT with that described in endometrium, and determine whether SHR expression is altered in FT of women with ectopic pregnancy (EP).

Design—Tissue analysis and culture.

Patients or Other Participants—Women undergoing surgery for benign gynaecological conditions (n=14) and EP (n=6).

Interventions—Q-RT-PCR and immunohistochemistry were used to determine SHR mRNA expression and protein localization, respectively. SHR levels were measured in tubal explant cultures stimulated with estrogen and progestogen.

Results—ERα and ERβ mRNAs were constitutively expressed in FT during the menstrual cycle. PR-AB and PR-B mRNAs were decreased in mid-luteal compared to follicular phase. ERα, PR-AB and PR-B mRNAs were downregulated in human FT *in vitro* by treatment with progestogen. ERα, ERβ1, ERβ2, PR and AR proteins localised to cell nuclei of epithelium, stroma and smooth muscle of non-pregnant FT. In FT from women with EP, PR-B mRNA was decreased when compared to mid-luteal FT, and ERα protein was not detected.

Conclusions—SHR expression in FT is different from that observed in endometrium recovered at similar stages of the menstrual cycle and expression in FT from women with EP is also altered compared with normal FT. These data are an important benchmark for furthering understanding of normal human FT physiology, transcriptional changes in FT in response to progesterone, and disorders of FT function, such as EP.

Keywords

Estrogen receptor; progesterone receptor; androgen receptor; allopian tube; ectopic pregnancy

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Disclosure summary
The authors have nothing to disclose.

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Introduction

The female reproductive system is exposed to fluctuating levels of sex steroids, including estrogen and progesterone during the normal menstrual cycle. A number of studies have investigated the expression patterns of the estrogen (ER), progesterone (PR) and androgen (AR) receptors in the human endometrium and documented both cell-specific patterns of expression as well as hormone dependent changes during the menstrual cycle (reviewed in (1)). However, the expression of sex steroid hormone receptors has yet to be comprehensively studied in the normal human Fallopian tube or in the context of tubal pathologies, such as ectopic pregnancy.

Sex steroid-regulated changes in Fallopian tube gene expression and function likely contribute to successful embryo tubal transport and implantation. The aim of this study was to compare expression patterns and regulation of sex steroid hormone receptors in the Fallopian tube with that described in endometrium. We also hypothesised that their expression pattern would be altered in Fallopian tube of women with ectopic pregnancy.

Sex steroid hormone receptors belong to a superfamily of genes that function as ligand-activated transcription factors (2). They have a conserved arrangement of functional domains, the most important of which are the DNA-binding domain containing two zinc fingers and a ligand binding domain found towards the C-terminus of the protein.

Two ER genes have been identified: ERα (ESR1; (3)) and ERβ (ESR2; (4)). Splice variant isoforms of the latter are expressed in a variety of human tissues (5, 6). ERα and ERβ exhibit different functional properties *in vitro* and show distinct patterns of gene regulation (7, 8). At present, although expression of ERβ1 and ERβ2 has been detected in normal endometrium, their roles are still unclear (1). However, it has been suggested that both ERβ1 and ERβ2 may have an impact on ERα-mediated gene expression (9).

Expression of ER in the human Fallopian tube was first described prior to the discovery of ERβ (10, 11). ER was immunolocalised to epithelial cells in the ampullary and fimbrial sections of the Fallopian tube and was reputed to increase throughout the follicular phase before reaching a plateau in the luteal phase (11). The antibody used in the studies was a mouse monoclonal antibody raised against purified calf uterine estrogen receptor. Expression of ERβ was recently documented in the human Fallopian tube using a rabbit polyclonal antibody raised against the amino-terminus of ERβ but levels were not assessed at different phases of the menstrual cycle (12). Expression of ERα or ERβ has not been examined in Fallopian tube from women with ectopic pregnancy.

Human PR is expressed as two isoforms, PR-A (94 kDa) and PR-B (116 kDa) both encoded by a single gene (13). PR-A is a truncated form of PR-B and lacks 164 amino acids from the N-terminus. Although both PR-A and PR-B bind progesterone, selective physiological roles for the two isoforms of PR have been documented (14). Generally, PR-B is transcriptionally the more active of the two isoforms (15). Furthermore, PR-A can act as a dominant repressor of PR-B-dependent activation of progestin-sensitive reporter genes, and likewise inhibits the transcriptional activity of receptors for androgens, glucocorticoids and mineralocorticoids (16).

Expression of PR in the Fallopian tube was first characterised in the early 1990s (10, 11). Using antibodies that crossreact with both PR-A and PR-B, these studies showed that immunoexpression of epithelial PR expression was most intense in the follicular phase but that it was not detected in the late luteal phase (11). A general reduction in PR protein expression has also been reported in Fallopian tube from women with ectopic pregnancy using immunoscoring and a mouse monoclonal antibody (IgG1 clone PR88) raised against

purified human progesterone receptor (17). More recently, expression of mRNAs specific for PR-A and B have been studied in non-pregnant Fallopian tube. Regional tubal expression of PR-A and PR-B mRNA was shown to vary, but again this was not studied in relation to cycle phase (18).

Human AR is encoded by a single copy gene on the X chromosome and *in vivo* it binds either testosterone or 5α-dihydrotestosterone (19). AR expression has been documented in the non-pregnant human Fallopian tube by Western blot analysis using rabbit monoclonal antibodies which recognise full length AR proteins (20), but no quantitative or topographical studies have, to our knowledge, been performed.

In the current study, we performed a detailed analysis of expression of ERα, ERβ1, ERβ2, PR-A, PR-B and AR in the human Fallopian tube across the menstrual cycle and compared this with expression in Fallopian tubes of women with ectopic pregnancy. These *in vivo* observations were complemented and extended using an *in vitro* model.

Methods

Tissue collection

Ethical approval for this study was obtained from Lothian Research Ethics Committee (04/S1103/20). All women were aged 18-45 years. Written and informed consent was obtained from all patients before sample collection. Fallopian tube biopsies (all from ampullary region unless stated), endometrial biopsies (for histological dating) and sera (for measurement of circulating estradiol and progesterone concentrations for endocrine staging) were collected from women with regular menstrual cycles (21-35 days) undergoing gynecological procedures for benign conditions who had no previous history of ectopic pregnancy and had not taken any hormonal preparations in the three months prior to surgery (n=14; see Table 1). None of the gynaecological conditions listed have been reported to date to affect sex steroid hormone receptor expression in the endometrium so it was assumed that this would also be the case in the Fallopian tube. Fallopian tube was also obtained from women undergoing surgical management of tubal ectopic pregnancy (n=6; see Table 2). None of the women undergoing surgical management of ectopic pregnancy presented acutely with haemodynamic shock, and all required serial serum beta-HCG and ultrasound monitoring prior to diagnosis. Part of the Fallopian tube was (a) immersed in RNAlater™ (Ambion, Texas, USA) at 4°C overnight then flash frozen at −70°C; part of the Fallopian tube and the endometrial biopsies were (b) fixed in 10% neutral buffered formalin overnight at 4°C, stored in 70% ethanol, and wax embedded; or (c) collected into PBS for tissue culture. The endometrial biopsies underwent haematoxylin and eosin staining and dating by an expert histopathologist.

Quantitative RT-PCR

RNA was extracted from cells/tissues as detailed in the manufacturer's protocol (Qiagen, RNeasy mini kits). All samples were treated with DNase I (Qiagen) in order to remove any contaminating genomic DNA. Complementary DNA was synthesised from 400ng of total RNA in 20μl reaction volumes containing: 1×RT buffer, magnesium chloride, dNTPs, random hexamers, RNase inhibitor and Multiscribe reverse transcriptase (Applied Biosystems, Cheshire, UK). Reactions were incubated at 25°C for 20 min, 42°C for 60 min then 95°C for 5 min. Negative controls contained either template RNA but no reverse transcriptase (RT negative) or no template RNA (RT water). PCR reaction mixtures contained Taqman 2x Master-mix (1x; Applied Biosystems), forward and reverse primers (300nM; Eurogentec) and probe (200nM; Eurogentec) for ERα, ERβ, PR-AB, PR-B or AR, and forward and reverse primers and probe for ribosomal 18S (all 50nM; Applied

Biosystems). ERα, ERβ, PR-AB, PR-B and AR primers and probes were designed using Primer Express software and their sequences are shown in Table 3. Ribosomal 18S was used as a housekeeping gene. Negative control (water in place of cDNA) samples were included in each PCR run along with the RT negative and RT H_2O control samples described above. All samples were analysed in triplicate using the $2^{-\Delta\Delta Ct}$ method. PCR reactions were run on an ABI 7900 Sequence Detection System (Perkin-Elmer Applied Biosystems, USA).

Immunohistochemistry

Immunohistochemical localisation of ERα, ERβ1, ERβ2, PR and AR was performed on Fallopian tube sections using biotinylated secondary antibodies and peroxidase conjugated detection systems. Immunoreactivity was detected using the chromagen 3,3′-diaminobenzidine (DAB). In brief, tissue sections were dewaxed in xylene and rehydrated in descending grades of alcohol. Sections were subjected to antigen retrieval as described in Table 4 and then non-specific activity was blocked sequentially with 3% hydrogen peroxide (Sigma-Aldrich), avidin and biotin, and protein blocks. Sections were incubated overnight at 4°C with antibodies specific to either: ERα, ERβ1, ERβ2, PR and AR (see Table 4). Sections were subsequently incubated with biotinylated secondary antibodies (Vector Laboratories, Peterborough, UK) and HRP complex (ABC-Elite, Vector Laboratories) or Streptavidin (Dako, Cambridge, UK), then immunoreactivity detected using DAB (Vector Laboratories). Counterstaining was then performed with Harris' haemotoxylin and mounted in Pertex (Cellpath Technologies, Hemel Hempstead, UK).

Tissue culture

Tubal explant cultures were performed as previously described (21) using surgical tissue from the ampullary region (unless indicated otherwise) of non-pregnant Fallopian tubes collected across the menstrual cycle. Fallopian tube biopsies were cut into small pieces (2-3 mm), placed in a 12-well dish with the luminal epithelial surface facing upwards and cultured in RPMI containing 10% charcoal-stripped fetal-bovine serum at 37°C in 5% CO_2. After 24 hours in culture, the tissues (n=5) were exposed to ethanol (as a control), and either estradiol (E_2) (10nM), medroxyprogesterone acetate (MPA) (1μM), or a combination of 10nM E_2 and 1μM MPA, for a further 24 hours. Following treatments quantitative RT-PCR for ERα, ERβ1, ERβ2, PR-AB, PR-B and AR was performed as above.

Statistical analysis

Data were logarithmically transformed prior to statistical analysis. Significant difference was determined by one-way ANOVA and Tukey's posthoc analysis.

Results

Expression of PR-AB and PR-B mRNAs is significantly diminished in the mid-luteal phase of the menstrual cycle

Expression of ERα and ERβ2 mRNAs did not change according to phase of menstrual cycle (Fig. 1A and C). There was a trend for expression of ERβ1 mRNA to be higher in the mid-luteal compared to the follicular phase of the menstrual cycle although this was not statistically significant (Fig. 1B). Expression of PR-AB and PR-B mRNAs was significantly reduced in the mid-luteal phase compared to the follicular phase of the menstrual cycle ($p<0.05$, Fig. 1D and E). Tubal AR mRNA expression showed a similar, but non-significant trend (Fig. 1D).

Progestogen downregulates expression of ERα, PR-AB and PR-B mRNAs in the human Fallopian tube *in vitro*

Incubation of tubal explants for 24h in the presence of E2 alone had no impact on total concentrations of any of the SHR mRNAs (Fig. 2). Likewise there was no change in expression of ERβ1, ERβ2 or AR mRNAs after incubation with MPA alone or E2 plus MPA (Fig. 2B, C and F, respectively). In contrast expression of ERα, PR-AB and PR-B mRNAs was significantly downregulated after incubation with MPA alone or MPA plus E_2 for 24 hours ($p<0.01$) (Fig. 2A, D and E, respectively).

Expression of PR-B mRNA expression is significantly diminished in the Fallopian tube of women with ectopic pregnancy

Total concentrations of ERα, ERβ1, ERβ2, PR-AB, and AR mRNAs were not statistically different in the Fallopian tube of women with ectopic pregnancy and those recovered during the luteal phase of the cycle (Fig. 3A, B, C and D, respectively). However, expression of PR-B mRNA was significantly reduced in the Fallopian tubes of women with ectopic pregnancy ($p<0.05$) (Fig. 3E) compared to those from the mid-luteal phase.

ERα protein was not detected in the Fallopian tube of women with ectopic pregnancy

ERα protein was immunolocalised to the nuclei of the epithelial cells, stroma and smooth muscle of non-pregnant Fallopian tube from all phases of the menstrual cycle (representative images in Figs. 4A and 5A) but expression was not detected in women with ectopic pregnancy (Fig. 5B). ERβ1, ERβ2, PR and AR were all detected in all the nuclei within the epithelial layer and within approximately 50% of those within the stromal and smooth muscle layers in all the Fallopian tube biopsies (representative images in Fig. 4B-F and Fig. 5C-F). One full-length Fallopian tube from the mid-luteal phase was examined for regional expression of SHR proteins. Similar patterns of nuclear staining were detected in epithelial, stromal and smooth muscle cells using antibodies specific for ERα, ERβ1, ERβ2, PR and AR in sections from the isthmic, ampullary and infundibulo-fimbrial regions.

Discussion

To our knowledge, this is the first comprehensive description of the patterns of expression of ERα, ERβ1, ERβ2, PR-A, PR-B and AR in the human Fallopian tube at different stages of the menstrual cycle. We have also used an *in vitro* model system to investigate the impact of acute exposure to E_2 and/or MPA on expression of sex steroid hormone receptors in this tissue. In addition, we report on differences in sex steroid hormone receptor expression in Fallopian tube from women with ectopic pregnancy compared to non-pregnant Fallopian tube.

We demonstrate that total expression of PR-AB and PR-B mRNAs are significantly reduced in non-pregnant Fallopian tubes recovered during the mid-luteal phase of the cycle as compared with those obtained during during the follicular phase. Surprisingly, expression of ERα mRNA in the Fallopian tube remains constant across the menstrual cycle i.e. ERα is not downregulated in the mid luteal phase, when the tissue is exposed to peak levels of circulating progesterone.

Our data extend the findings of a study that examined expression of PR-B mRNA in the Fallopian tube during the mid-luteal phase but did not compare expression to other phases of the menstrual cycle (22). In the context of Fallopian tube function, particularly the ciliary and tubal smooth muscle activity essential for successful embryo-tubal transport and subsequent implantation in the uterus, the downregulation of PR is important (23). Progesterone is reported to have an inhibitory action on ciliary and tubal smooth muscle

activity because high progesterone levels in the luteal phase coincide with a reduced frequency of contractions and ciliary activity *in vitro* (24, 25). Information on the regulation of specific genes by progesterone in the oviduct is limited, largely due to limited access to human Fallopian tube cells. This contrasts with the data on the endometrium where as many as 571 genes (representing 131 biochemical pathways) have been shown to be progesterone-regulated (26).

However, our data suggest that expression of SHR in the Fallopian tube do not simply mirror the changes seen in the endometrium. For example, *in vivo* expression of PR, and ERα, is reduced in endometrium in the mid-secretory phase of the menstrual cycle under the influence of progesterone and seems to be closely connected to the onset of endometrial receptivity (27). We therefore used an *in vitro* system to confirm and extend the findings that we observed in the non-pregnant Fallopian tube during the menstrual cycle by examining the impact of E_2 and MPA on sex steroid hormone receptor expression. In these studies, we demonstrated downregulation of expression of PR-AB, PR-B and ERα mRNAs after treatment of Fallopian tube explants with MPA alone, and in combination with E_2.

There are many *in vivo* and *in vitro* studies which report results consistent with the downregulation in expression of PR-A, PR-B and ERα mRNAs after incubation with progestagens (reviewed in (1)). For example, several studies have shown that administration of the PR antagonist, mifepristone, to patients in the early secretory phase of the menstrual cycle prevents downregulation of PR and ERα expression in the mid secretory phase (28, 29). Progestogens attenuate the actions of E_2 in endometrium (30) and both PR-A and PR-B have been shown to inhibit ER transcriptional activity via a ligand-dependent mechanism in rat uterine cells (31). A more recent study has reported that PR-B overexpression in breast cancer cell lines reduces expression of ERα (32). This effect is dependent on recruitment of a corepressor transcriptional complex to a progesterone response element half site in the ERα promoter (32). *In vitro* studies have also shown that PR is downregulated by progestogen although this effect is cell-type dependent. In the T47D breast cancer cell line and in endometrial epithelial cells expression of both PR-A and PR-B is suppressed by progestogen treatment (10nM ORG 2058 and 0.2μm MPA, respectively) (33, 34). In contrast, PR-A and PR-B are upregulated in endometrial stromal cells by progestogen treatment (0.2μm MPA) (34). The differences seen in our *in vivo* compared to our *in vitro* findings in the Fallopian tube suggest that additional local factors may be involved in the regulation of ERα expression.

We also report reduced expression of PR-B mRNA in Fallopian tube from women with ectopic pregnancy compared to non-pregnant Fallopian tube from the mid-luteal phase of the menstrual cycle. It is not possible to collect Fallopian tube from women with intra-uterine pregnancies and so Fallopian tube collected from the mid luteal phase, when circulating progesterone levels are raised, provides the most appropriate control. Our findings support those of an earlier immunohistochemical study that could not detect PR protein expression in the Fallopian tube of women with ectopic pregnancy (17). It may also offer an explanation for the absence of adequate tubal decidualization observed with this condition (35). Mice deficient in PR fail to mount a decidual response and a recent study in humans has shown that PRs regulate distinct gene networks and cellular functions in decidualizing endometrium (36, 37).

In addition, we were unable to detect expression of ERα protein by immunohistochemistry in the Fallopian tube from women with ectopic pregnancy which was surprising as there was no difference in the total amount of ERα mRNA. Although this may reflect the limitations of our immunohistochemical approach, receptor protein was readily detected in tubes obtained during the normal cycle. Notably, ERα gene polymorphisms have been associated

with female infertility (38), and ERα has been shown to serve as a dominant regulator in Fallopian tube development in the rat (39). Furthermore, a recent study in mice has identified a molecular mechanism for ERα-mediated tubal protein synthesis and secretion that appears to be important for successful embryonic development (40). Further studies are required to determine whether ERα plays a critical role in Fallopian tube physiology and to establish whether reduced translation or enhanced degradation may contribute to reduced expression of protein.

In summary, we report that there are variations in sex steroid hormone receptor expression in the non-pregnant human Fallopian tube during the menstrual cycle, that there is *in vitro* regulation of some SHRs in the Fallopian tube by E_2 and MPA, and identified differences in expression in Fallopian tubes from women with ectopic pregnancy compared to the non-pregnant Fallopian tube. Sex steroid receptor dynamics and responsiveness to estrogen and progestogen have been demonstrated in many studies in the endometrium (reviewed in (1)). However, studies in the human Fallopian tube have been limited to date. Our data is therefore an important benchmark for furthering understanding of normal human Fallopian tube physiology, transcriptional changes in the Fallopian tube in response to progesterone, and the aetiology of disorders of Fallopian tube function, such as ectopic pregnancy.

Acknowledgments

The authors would like to thank Paula Lourenco for technical support; Catherine Cairns, Sharon McPherson and Catherine Murray for patient recruitment; and Ronnie Grant for graphical assistance and Sheila Milne for secretarial support.

Funding

This work was supported by an MRC Programme Grant (G0500047) (HODC), Wellbeing of Women (R40608) (AWH/HODC) and the Caledonian Research Foundation (AEK).

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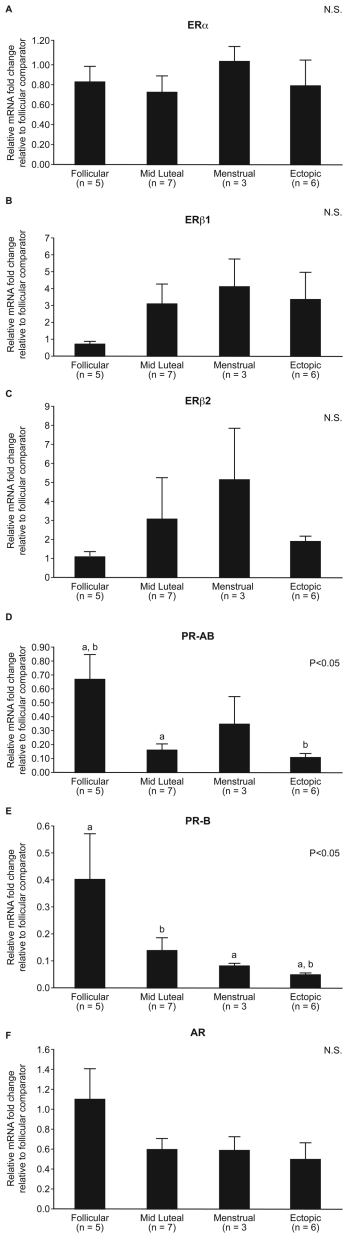

Fig. 1. Sex steroid hormone receptor mRNA expression in the human Fallopian tube across the menstrual cycle (follicular, mid-luteal and menstrual phases)

Expression of ERα (A), ERβ1 (B), ERβ2 (C), and AR (F) did not change according to phase of menstrual cycle. PR-AB (D) and PR-B (E) mRNA expression were significantly diminished in the luteal phase compared to expression in the follicular phase of the cycle ($p<0.05$).

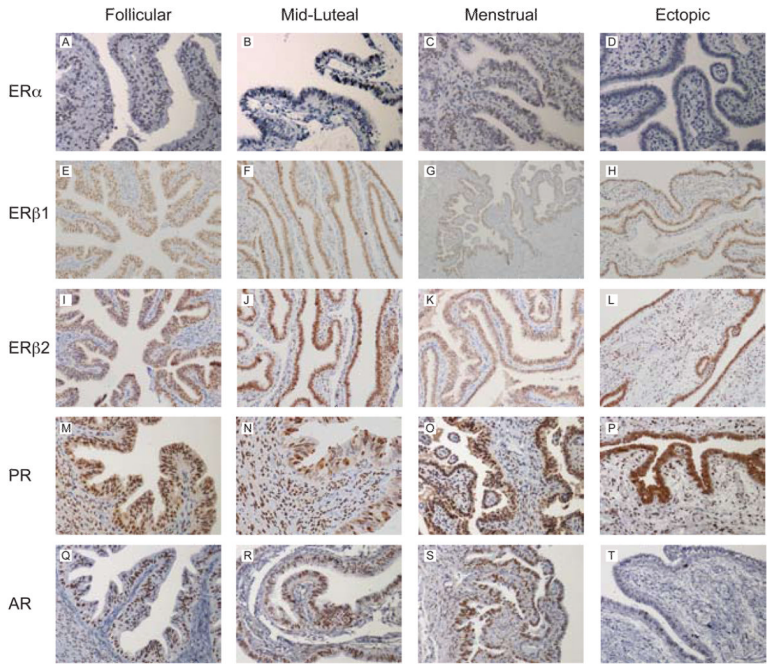

Fig. 2. Tubal explant culture with sex steroids

Stimulation of tubal explants (n=5) with estradiol (E_2) (10nM) and medroxyprogesterone acetate (MPA) (1μM) showed no change in ERβ1 (B), ERβ2 (C) and AR (F) mRNA expression after 24 hours. ERα (A), PRA-AB (D) and PR-B (E) mRNA expression was significantly downregulated after treatment with medroxyprogesterone acetate, and in combination with estradiol ($p<0.01$).

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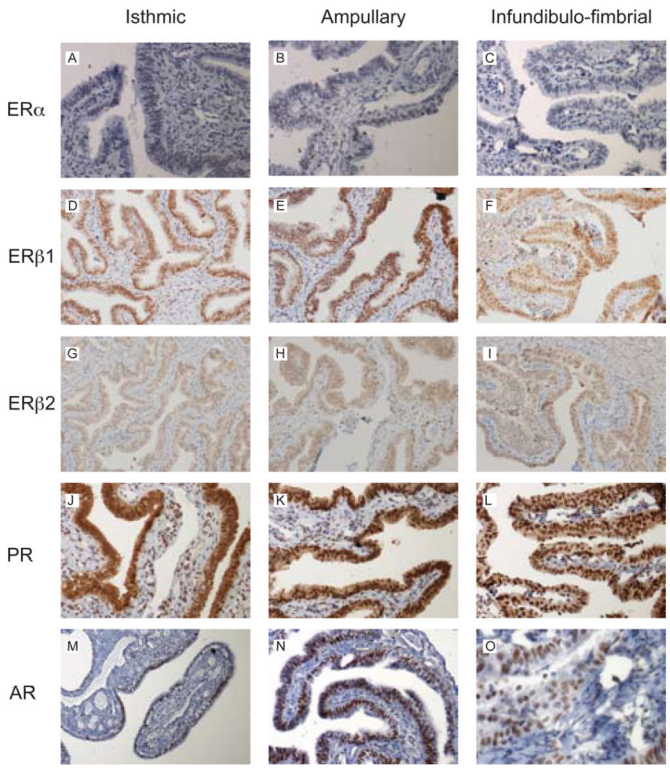

Fig. 3. Sex steroid hormone receptor mRNA expression in the human Fallopian of women with ectopic pregnancy

ERα (A), ERβ1 (B), ERβ2 (C), PR-AB (D) and AR (F). PR-B mRNA expression was significantly lower in the Fallopian tube of women with ectopic pregnancy compared to expression in the non-pregnant Fallopian tube in the mid-luteal phase of the cycle ($p<0.05$).

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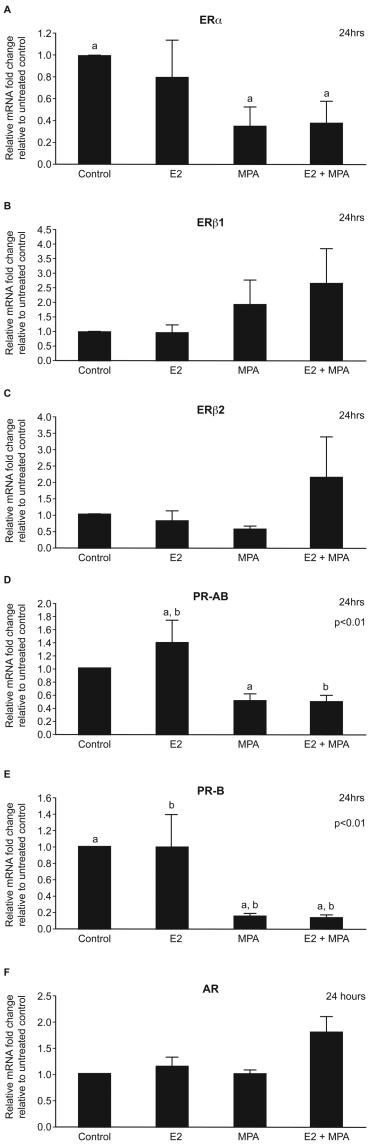

Fig. 4. Representative images of the immunolocalisation of sex steroid hormone receptor protein in the human Fallopian tube across the menstrual cycle

A) ERα expression in the follicular phase. B) ERβ1 expression in the mid-luteal phase. C) ERβ2 expression in the follicular phase. D) PR expression in the follicular phase. E) PR expression in the mid-luteal phase. F) AR expression in the follicular phase. Sex steroid hormone receptor protein was localised to the nuclei of all the epithelial cells, approximately 50% of the stromal cells and the smooth muscle in all biopsies of non-pregnant Fallopian tube at all stages of the menstrual cycle.

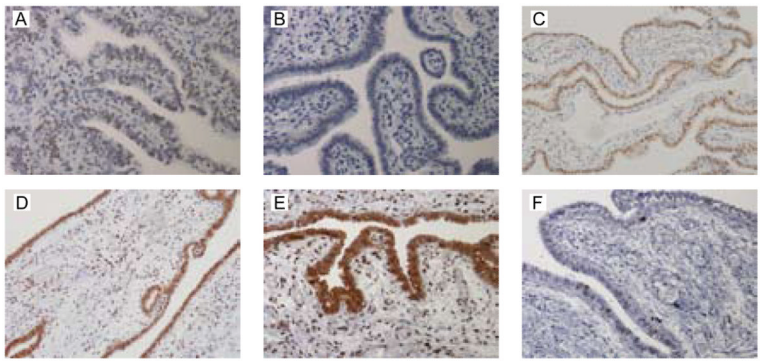

Fig. 5. Representative images of the immunolocalisation of sex steroid hormone receptor protein in Fallopian tube from women with ectopic pregnancy

A) ERα expression in the menstrual phase (control). B) ERα expression in women with ectopic pregnancy. ERβ1 (C), ERβ2 (D), PR (E), and AR (F) expression in women with ectopic pregnancy. There was no evidence of ERα protein expression in Fallopian tube obtained from women with ectopic pregnancy (compare A with B). However, ERβ1, ERβ2, PR, and AR immunolocalisation in Fallopian tube from women with ectopic pregnancy was similar to that observed for non-pregnant Fallopian tube.

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J Clin Endocrinol Metab. Author manuscript; available in PMC 2010 November 22.

Table 1

Demographics for Fallopian tube and endometrial biopsies from women undergoing surgery for benign gynaecological conditions.

	Age	Para	Cycle phase	Serum E_2 (pmol/L)	Serum Prog (nmol/L)	Endometrial histology	Surgery	Reason for surgery	Uterine pathology
1	41	2	Follicular	1022.87	0.81	Proliferative	TAH	HMB	Adenomyosis
2	37	2	Follicular	940.44	3.82	Proliferative	TAH	HMB, pelvic pain	Adenomyosis
3	44	3	Follicular	829.42	4.19	Proliferative	TAH	HMB, pelvic pain	Adenomyosis, fibroid
4	36	3	Follicular	770.63	5.16	Proliferative	TAH	HMB	No abnormality
5	44	2	Follicular	116.3	2.88	Proliferative	STAH	HMB, dysmenorrhoea	No abnormality
6	34	2	Mid luteal	471	62.65	Mid secretory	LAVH	HMB, dysmenorrhoea	Adenomyosis, fibroid
7	40	2	Mid luteal	242	53.1	Mid secretory	TAH	HMB, dysmenorrhoea	No abnormality
8	35	2	Mid luteal	424	76.9	Mid secretory	TAH	pelvic pain	No abnormality
9	38	3	Mid luteal	266	37.1	Mid secretory	TAH	HMB	No abnormality
10	32	1	Mid luteal	549.91	88	Mid secretory	TAH	Dysmenorrhoea	No abnormality
11	43	2	Mid luteal	201	24.55	Mid secretory	TAH	HMB, pelvic pain	Fibroid
12	40	1	Mid luteal	1633.0	54.38	Mid secretory	TAH	HMB, dysmenorrhoea	No abnormality
13	35	4	Menstrual	73	2.67	Menstrual	TAH	HMB, dysmenorrhoea	Adenomyosis
14	42	2	Menstrual	55	15.05	Menstrual	TAH	HMB	No abnormality

TAH = total abdominal hysterectomy

STAH = sub-total hysterectomy

LAVH = laparoscopically-assisted vaginal hysterectomy

HMB = heavy menstrual bleeding

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Table 2

Demographics of Fallopian tube biopsies for women undergoing surgery for ectopic pregnancy.

	Gestation (days)	hCG (IU/L)	Prog (nmol/L)
1	58	15956	67.43
2	50	487	39.21
3	59	2056	24.58
4	53	1854	63.07
5	52	2425	61.68
6	56	225	20.36

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Table 3

Sex steroid receptor primer/probe sequences used for Taqman RT-PCR.

Primer / Probe	Sequence	Position	Accession No
ERα forward	TGATTGGTCTCGTCTGGCG	1523–1541	NM_000125
ERα reverse	CATGCCCTCTACACATTTTCCC	1602–1624 (r)	NM_000125
ERα probe	TGCTCCTAACTTGCTCTTGGACAGGAACC	1572–1600	NM_000125
ERß1 forward	CCTGGCTAACCTCCTGATGCT	1459–1480	AB006590
ERß1 reverse	CCACATTTTTGCACTTCATGTTG	1529–1552 (r)	AB006590
ERß1 probe	AGATGTTCCATGCCCTTGTTACTCGCA	1499–1525 (r)	AB006590
ERß2 forward	ATCCATGCGCCTGGCTAAC	2628–2647	AB006589
ERß2 reverse	GAGTGTTTGAGAGGCCTTTTCTG	2684–2707 (r)	AB006589
ERß2 probe	TCCTGATGCTCCTGTCCCACGTCA	2648–2671	AB006589
PR-AB forward	CAGTGGGCGTTCCAAATGA	2151–2170	NM_000926
PR-AB reverse	TGGTGGAATCAACTGTATGTCTTGA	2209–2233(r)	NM_000926
PR-AB probe	AGCCAAGCCCTAAGCCAGAGATTCACTTT	2170–2199	NM_000926
PR-B forward	CGGACACCTTGCCTGAATT	1579-1595	NM_000926
PR-B reverse	CAGGGCCGAGGGAAGAGTAG	1626-1645	NM_000926
PR-B probe	CGGCCATACCT ATCTCCCTGG ACGG	1600-1624	NM_000926
AR forward	GTACCCTGGCGGCATGGT	951–1016	L29496
AR reverse	CCCATTTCGCTTTTGACACA	951–1016	L29496
AR probe	AGCAGAGTGCCCTATCCCAGTCCCA	951–1016	L29496

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Table 4

Antibodies used for immunohistochemistry.

Receptor	Antigen retrieval	Antibody diluent	Primary antibody	Secondary antibody	Negative control
ERα	Pressure cook in 0.01M sodium citrate pH6	Normal goat serum and 5% BSA	Mouse monoclonal antibody M7407 (Dako)	Biotinylated goat-anti-mouse	MIgG
ERβ1	Pressure cook in 0.01M sodium citrate pH6	Normal goat serum and 5% BSA	Mouse monoclonal IgG2a (Serotec)	Biotinylated goat-anti-mouse	Pre-absorbed peptide
ERβ2	Pressure cook in 0.01M sodium citrate pH6	Normal goat serum and 5% BSA	Mouse monoclonal (Serotec)	Biotinylated goat anti-mouse	Pre-absorbed peptide
PR (recognises both isoforms)	Pressure cook in 0.01M sodium citrate pH6	Normal goat serum and 5% BSA	Mouse monoclonal (Novatec)	Biotinylated goat-anti-mouse	MIgG
AR	Pressure cook in 0.05M glycine EDTA pH8	Tris Buffered Saline	Rabbit-anti-human AR (Santa Cruz)	Biotinylated goat-anti-rabbit	RIgG