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## Evidence of prokineticin dysregulation in Fallopian tube from women with ectopic pregnancy

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### Abstract

**Objective**—To demonstrate expression and regulation of prokineticins (PROKs) and their receptors (PROKRs) in Fallopian tube (FT) from non-pregnant women and women with ectopic pregnancy (EP).

**Design**—Tissue analysis.

**Setting**—Large UK teaching hospital

**Patients**—Women undergoing hysterectomy for benign gynecological conditions (n=15) and surgery for EP (n=16).

**Interventions**—Quantitative RT-PCR and immunohistochemistry were used to determine FT PROK/PROKR mRNA expression and protein localization, respectively. PROK/PROKR levels were measured in tubal explant cultures stimulated with estrogen and progesterone.

**Main outcome measures**—Differential expression of PROK and PROKR.

**Results**—FT PROK2 and PROKR1 mRNA levels were upregulated during the progesterone-dominant mid-luteal phase of the menstrual cycle. Increased PROKR1 expression was observed in tubal explant cultures treated with medroxyprogesterone acetate. PROK and PROKR proteins were localized to the epithelium and smooth muscle layers of the FT. PROKR1 and PROKR2 mRNA levels were lower in FT from women with EP compared to non-pregnant FT from the mid-luteal phase.

**Conclusion**—These data suggest a potential role for PROKs in FT function. PROKs are known to affect smooth muscle contraction in the gut. Dysregulated PROK expression in FT could affect FT smooth muscle contractility and embryo-tubal transport, providing a potential cause for EP.

### Keywords

Fallopian tube; ectopic pregnancy; menstrual cycle; prokineticin; smooth muscle contractility

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## INTRODUCTION

Ectopic pregnancy remains a considerable cause of morbidity and occasional mortality (1). One in 80 pregnancies is ectopic and over 98% implant in the Fallopian tube (1;2). The exact mechanism leading to tubal implantation is unknown. However, given that the human embryo appears to have the ability to implant on any given epithelial surface (3), it is likely that ectopic pregnancy is the result of embryo retention in the Fallopian tube due to Fallopian tube dysfunction. Transport of the pre-implantation embryo through the Fallopian tube is accomplished, in part, by smooth muscle contraction (4;5).

The prokineticins, PROK1 and PROK2, have angiogenic actions but are primarily known for their function as regulators of specific and potent contractions of smooth muscle (6). They are the cognate ligands for two closely homologous G protein-coupled receptors, PROK receptor (PROKR) 1 and PROKR2, through which either PROK can signal (6). PROKs were initially reported to be expressed in the gastrointestinal tract, where they were shown directly to stimulate contraction of the ileum longitudinal muscle of guinea pigs (7). However, the opposing effect of relaxation through a nitric oxide-mediated mechanism has also been reported recently in the proximal colon in mice (8). In addition, it has been reported that PROK2 has no effect on fore-stomach or colon contraction (9), suggesting that the intracellular milieu in different tissues results in differential coupling and different phenotypic effects.

PROKs and their receptors are expressed in the ovary, uterus and in various tissues of pregnancy (10;11). PROK1 is expressed in the uterine epithelium, as well as the smooth muscle cells of the myometrium, with maximum expression at the time of implantation in the progesterone dominant mid-secretory phase (11). PROK1 is expressed in much greater levels than PROK2. PROK2 and the PROK receptors are also expressed in the various cellular compartments of the uterus but they do not show a temporal variation across the menstrual cycle (11), suggesting a regulatory role for PROK1 and a more permissive role for PROK2 in uterine function. Furthermore, it has been hypothesized that PROK1 facilitates successful intrauterine embryo implantation through induction of leukemia inducible factor (LIF) expression (12), which has been shown to promote adhesion of trophoblast cells to extracellular matrix proteins, in vitro.

It is therefore plausible that the PROKs and their receptors could regulate smooth muscle contraction in the human Fallopian tube facilitating controlled and timely embryo transport into the uterine cavity, and that attenuated expression could lead to embryo retention and ectopic pregnancy. However, PROK/PROKR expression has to our knowledge never been demonstrated in human Fallopian tube. In this study, we report expression and tissue localization of the PROKs and PROKR in human Fallopian tube, their regulation by progesterone and show that PROKR expression is dysregulated in ectopic pregnancy.

## MATERIALS AND METHODS

### Collection of human tissues

Ethical approval for this study was obtained from the Lothian Research Ethics Committee (04/S1103/20) and informed written consent was obtained from all patients. Fallopian tube tissues from the ampullary region of the Fallopian tube were collected at the time of hysterectomy (n = 15) or during surgical management of tubal pregnancy (n = 16). Women were between 18 and 45 years of age (mean age for hysterectomy patients was  $40 \pm 4$  and mean age for tubal ectopic pregnancy patients was  $31 \pm 5$ ). Samples were prepared by either a) short-term storage in phosphate-buffered saline (PBS) prior to explant culture, b) immersion in RNAlater™ (Ambion, Texas, USA) at 4 °C overnight and then flash frozen at

–80 ° for RNA extraction or c) fixation in 4% neutral-buffered formalin overnight at 4 °C followed by storage in 70% ethanol, and embedding in paraffin wax for immunohistochemical staining. The menstrual cycle phase of each patient at the time of hysterectomy was determined using the following parameters: 1) last menstrual period confirmed by patient, b) histological examination and staging of an endometrial biopsy taken with the Fallopian tube by a gynecological pathologist and c) measurement of estradiol and progesterone levels in serum of patients. The clinical data corresponding to each sample are listed in Table 1. None of the gynaecological conditions listed have been reported to date to affect PROK/PROKR expression in the endometrium so it was assumed that they would have no effect on expression in the Fallopian tube. Fallopian tubes removed during surgical management for ectopic pregnancy were all from the ampullary region of the Fallopian tube. Gestational age, human chorionic gonadotropin (hCG) and progesterone levels of each ectopic pregnancy are listed in Table 1.

### Hormonal treatment of Fallopian tube explants

Fallopian tube tissue culture was performed, in triplicate, using tissue from three different patients. Two of the Fallopian tubes were from the follicular phase of the menstrual cycle and one was from the late-luteal phase. Each tissue was cut into small pieces (2-3 mm) which were placed in each well of a 12-well dish and cultured in RPMI containing 10% charcoal-stripped fetal-bovine serum at 37 °C in 5% CO<sub>2</sub>. PROK expression in the endometrium has previously been demonstrated to be upregulated by progesterone after 24 hours (11). Explants were therefore treated with 10 nM estradiol (Sigma-Aldrich, Dorset, UK), 1 μM medroxyprogesterone acetate (MPA) (Sigma-Aldrich, Dorset, UK) (both in ethanol) and a combination of the two steroids for 24 hours according to this protocol. Explants were also treated with ethanol to control for the steroid diluent. After 24 hours, media was removed and tissues were collected for RNA extraction.

### RNA Extraction and cDNA preparation

Total RNA was extracted from tissues using the RNeasy kit (Qiagen, West Sussex, UK) according to the manufacturer's protocol. RNA concentrations were determined using a Nanodrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). 200 ng of RNA was reverse transcribed into cDNA in a 10 μL reaction using random hexamers (Applied Biosystems, Foster City, CA, USA).

### Quantitative RT-PCR

Taqman RT-PCR was used to quantify transcript levels. Reactions were performed using an ABI Prism 7900 (Applied Biosystems, Foster City, CA, USA) using standard conditions. Previously validated primer and FAM (6-carboxyfluorescein) labeled probe sequences are listed in Supplementary Table 1. 1 μL of cDNA (from above) was used per 25 μL qRT-PCR reaction and all reactions were performed in triplicate. Gene expression was normalized to RNA loading using primers and VIC™ labeled probe (Applied Biosystems, Foster City, CA, USA) for ribosomal 18S as an internal standard. Using the 2-ΔΔCt method, mRNA expression results were normalized against 18S and expressed as relative to a positive RNA standard (cDNA from a single mid-secretory endometrial tissue) which was included in all reactions.

### Immunohistochemistry

Standard immunohistochemistry protocols were used (11). All antibodies were diluted in normal goat serum: PROK1 rabbit polyclonal serum antibody (Phoenix Pharmaceuticals, Belmont CA, USA), 1000-fold; PROKR1 rabbit polyclonal antibody (IgG purified) (MBL International Corporation, Woburn, MA, USA), 250-fold; PROK2 rabbit polyclonal

antibody (IgG purified) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 50-fold; PROKR2 rabbit polyclonal antibody (IgG purified) (MBL International corporation, Woburn, MA, USA), 67-fold and smooth muscle actin mouse monoclonal antibody (Sigma-Aldrich, Dorset, UK), 5000-fold. Rabbit serum diluted 1000-fold in normal goat serum was used as a negative control for PROK1 staining. Rabbit IgG diluted 50-fold in normal goat serum was used as a negative control for PROK2, PROKR1 and PROKR2 staining. Mouse IgG diluted 5000-fold in normal goat serum was used as a negative control for smooth muscle actin staining. Staining was reviewed by an expert gynecological pathologist.

### Statistical Analysis

Statistical analyses were performed with GraphPad Prism (version 5.0, GraphPad Software, La Jolla, CA, USA). Analysis of the differences in PROK and PROKR levels in mid-luteal phase Fallopian tubes and in Fallopian tubes from ectopic pregnancies was performed using a Mann-Whitney test. Samples for which q-RT-PCR found undetermined levels of transcript were removed from the analysis. For the analysis of PROK and PROKR levels in hormonally treated explants, a One-Way ANOVA was performed followed by Dunnett's post-hoc analysis.

## RESULTS

### **PROK and PROKR mRNA levels in Fallopian tube obtained throughout the menstrual cycle and from women with ectopic pregnancy**

*PROK1*, *PROK2*, *PROKR1*, and *PROKR2* mRNA levels were measured in Fallopian tubes from the follicular and mid-luteal phases of the menstrual cycle and in Fallopian tubes from women with ectopic pregnancies using Taqman q-RT-PCR. *PROK1* and *PROKR1* levels were relatively lower compared with *PROK2* and *PROKR2* levels in all Fallopian tube tissue samples compared (Figure 1). *PROK1* levels did not vary across the menstrual cycle (Figure 1a). *PROK2* levels were higher in the mid-luteal phase compared to the follicular phase of the menstrual cycle (Figure 1c;  $p < 0.05$ ). *PROKR1* levels were significantly higher in the mid-luteal phase of the menstrual cycle compared to the follicular phase (Figure 1e;  $p < 0.05$ ). *PROKR1* levels were also significantly lower in Fallopian tubes from ectopic pregnancies compared to levels in Fallopian tubes from the mid-luteal phase of the menstrual cycle (Figure 1f;  $p < 0.05$ ). *PROKR2* levels did not vary across the menstrual cycle but were significantly lower in Fallopian tubes from ectopic pregnancies compared to levels in Fallopian tubes from the mid-luteal phase of the menstrual cycle (Figure 1h;  $p < 0.01$ ). Due to ethical constraints, 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.

### **Hormonal regulation of PROK and PROKR mRNA in human Fallopian tube explants**

*PROK1*, *PROK2* and *PROKR2* mRNA levels were not influenced by hormonal treatment of human Fallopian tube explants (Figure 2). *PROKR1* levels were significantly upregulated by MPA treatment (Figure 2c;  $p < 0.01$ ) and combined estrogen and MPA treatment (Figure 2c;  $p < 0.05$ ). Estrogen treatment alone did not alter the expression of any of the genes analyzed. We also compared PROKR levels in Fallopian tube from women with low serum hCG levels ( $< 1000$  IU/L) with PROK levels in Fallopian tube from women with high serum hCG levels ( $> 1000$  IU/L) and found no statistically significant differences in expression between these two groups (data not shown).

## Immunohistochemical localization of PROK and PROKR protein in human Fallopian tube

PROK1, PROK2, PROKR1 and PROKR2 proteins were found to immunolocalize to the epithelium of the Fallopian tube (Figure 3 a, c, e, g). These proteins were also found in the smooth muscle layer as confirmed by colocalization of PROKs and PROKRs with smooth muscle actin in serial tissue sections (Figure 3 b, d, f, h). In Fallopian tubes collected from women with ectopic pregnancies, the PROKs and PROKRs showed similar localization to that in sections from non-pregnant women (Figure 4).

## DISCUSSION

To our knowledge this is the first comprehensive description of *PROK* and *PROKR* expression in human Fallopian tube at different phases of the menstrual cycle. We have also used an *in vitro* model system to investigate the impact of acute exposure to estradiol and/or MPA on expression of PROK and PROKR expression in this tissue. In addition, we report on altered PROKR expression in Fallopian tube from women with ectopic pregnancy compared to non-pregnant Fallopian tube.

*PROK2* and *PROKR1* mRNA are more highly expressed in Fallopian tube from the progesterone-dominant, mid-luteal phase of the menstrual cycle compared with the follicular phase, suggesting that tubal PROK2 and PROKR1 are regulated by progesterone. This is also supported by our *in vitro* data which shows that tubal *PROKR1* mRNA is significantly upregulated by treatment with MPA and a combination of estradiol and MPA. *PROK2* mRNA expression was not significantly upregulated by MPA treatment *in vitro*, suggesting that factors, in addition to progesterone, may control *PROK2* expression *in vivo*.

In contrast to the endometrium we have shown that tubal *PROK2* and *PROKR2* mRNA levels are relatively higher compared to *PROK1* and *PROKR1* levels, suggesting that PROK2 may be the dominant PROK active in the Fallopian tube in the mid-luteal phase. PROK2 has previously been shown to signal through PROKR1 (13). Thus, it is possible that, in the Fallopian tube, PROK2 may be signaling through PROKR1 whereas in the endometrium, PROK1 has been shown to signal through PROKR1 (14).

We have also demonstrated that *PROKR* mRNA levels are lower in Fallopian tube from ectopic pregnancies than in mid-luteal phase Fallopian tube collected from non-pregnant women, and that PROK/PROKR protein is localized to the smooth muscle of the Fallopian tube. This adds support to our hypothesis that the control of Fallopian tube smooth muscle contractility and embryo transport could be regulated by PROK and PROKR expression.

It is plausible that reduced PROK expression in Fallopian tube from women with ectopic pregnancy could contribute to impaired smooth muscle contractility and dysregulated embryo tubal transport through the cyclo-oxygenases, COX-1/COX-2, and subsequent production of prostaglandins. PROK1 has been shown to upregulate cyclo-oxygenase-2 (COX-2) levels in the endometrium (14). COX-2 is a member of the cyclo-oxygenase family of enzymes responsible for the conversion of arachidonic acid into prostaglandin, and has been shown to play a role in implantation in mice (15). The human Fallopian tube is known to express COX-1 and COX-2 (16) and prostaglandins have recently been shown to increase the contractility of smooth muscle in the Fallopian tube (17).

Nevertheless, the reduced PROK1 levels we observed in Fallopian tubes from women with ectopic pregnancies may simply be the result of lower maternal serum beta-human chorionic gonadotropin (hCG) levels observed in ectopic pregnancy. hCG increases PROK1 expression in endometrium (12), and generally, ectopic pregnancies have lower hCG levels than normal intrauterine pregnancies (18;19). However, when we compared PROKR levels



in Fallopian tube from women with low serum hCG levels (< 1000 IU/L) with PROK levels in Fallopian tube from women with high serum hCG levels (> 1000 IU/L), we found no statistically significant differences in expression between these two groups. The signaling downstream of PROKR1 in the Fallopian tube therefore warrants further investigation.

In summary, we show that PROK and PROKR are expressed in the epithelium and smooth muscle of human Fallopian tube and have demonstrated that PROK and PROKR expression is regulated by the hormonal changes that occur during the menstrual cycle. Our finding of altered PROKR expression in Fallopian tube from women with ectopic pregnancy suggests that PROKs may be important in tubal wall smooth muscle contractility and successful embryo transport.

### Capsule

Embryo retention in human Fallopian tube is thought to lead to ectopic pregnancy. Attenuated prokineticin expression may affect embryo-tubal transport and lead to retention of embryos in the Fallopian tube.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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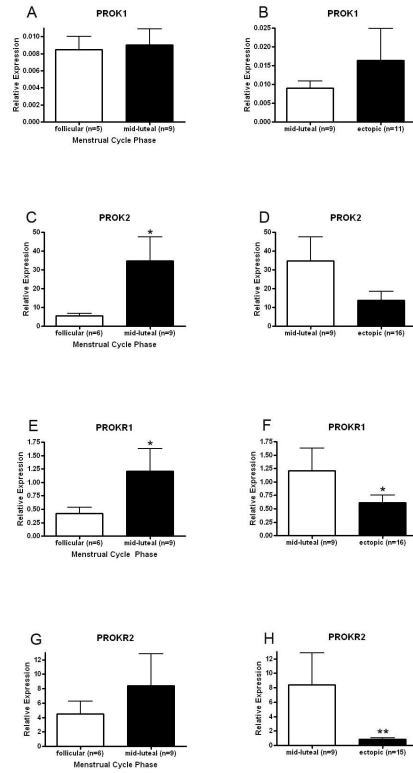
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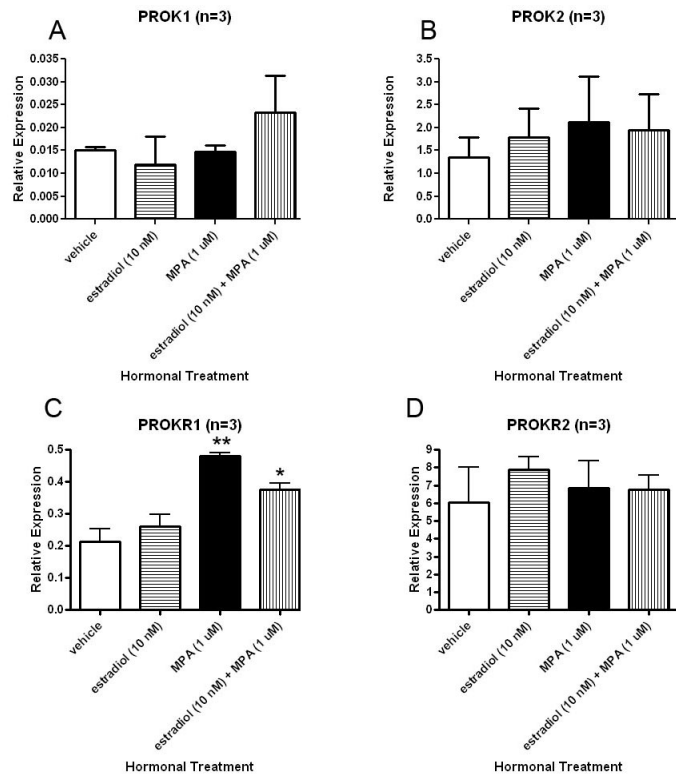
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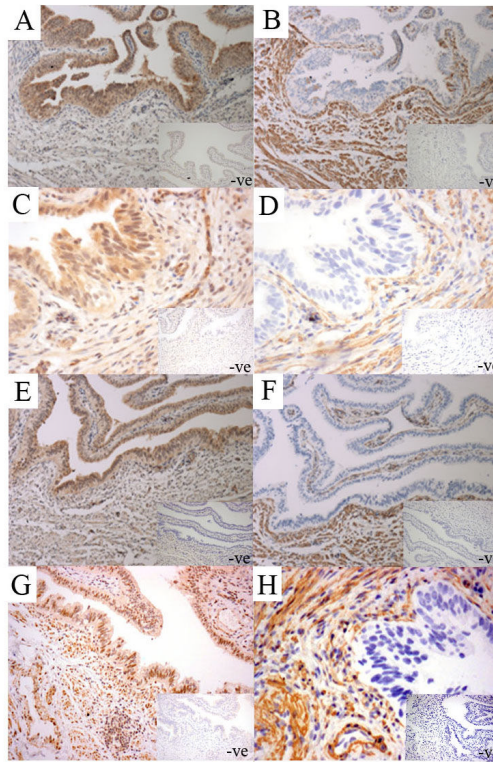


**Figure 1. PROK and PROKR mRNA expression in non-pregnant human Fallopian tube obtained across the menstrual cycle and in Fallopian tube from women with ectopic pregnancies** Taqman RT-PCR was used to quantify *PROK1* (A), *PROK2* (B), *PROKR1* (C) and *PROKR2* (D) transcript levels in Fallopian tube from the follicular and mid-luteal stages of the menstrual cycle as well as in Fallopian tube from women with ectopic pregnancies. Levels of each gene are expressed relative to the expression of the particular gene in a single mid-secretory endometrial sample which was quantified using the same primers and probes. Statistical analysis was performed using the Mann-Whitney test. *PROK1* (A) levels were not significantly different between the follicular and mid-luteal phases, or between mid-luteal phase tissue and tubal ectopic tissue (B). Five follicular Fallopian tube samples were included in the analysis of *PROK1* expression because *PROK1* did not amplify in sample 2. Eleven ectopic Fallopian tube samples were included in the analysis of *PROK1* expression because *PROK1* transcript did not amplify in samples 16, 19, 23, 25 or 27. *PROK2* (C) levels were significantly higher in mid-luteal phase compared to the follicular phase ( $p < 0.05$ ) and were no different between mid-luteal phase and ectopic Fallopian tube (D). *PROKR1* (E) levels were also significantly higher in the mid-luteal phase compared with the follicular phase ( $p < 0.05$ ) and were significantly lower in Fallopian tube from women with ectopic pregnancy compared to mid-luteal phase Fallopian tube ( $p < 0.05$ ) (F). *PROKR2* (G) levels were not significantly different between the follicular and mid-luteal phases, but were found significantly lower in Fallopian tube from women with ectopic pregnancy compared to mid-luteal phase Fallopian tube ( $p < 0.01$ ) (H). \*  $p < 0.05$ , \*\*  $p < 0.01$ .



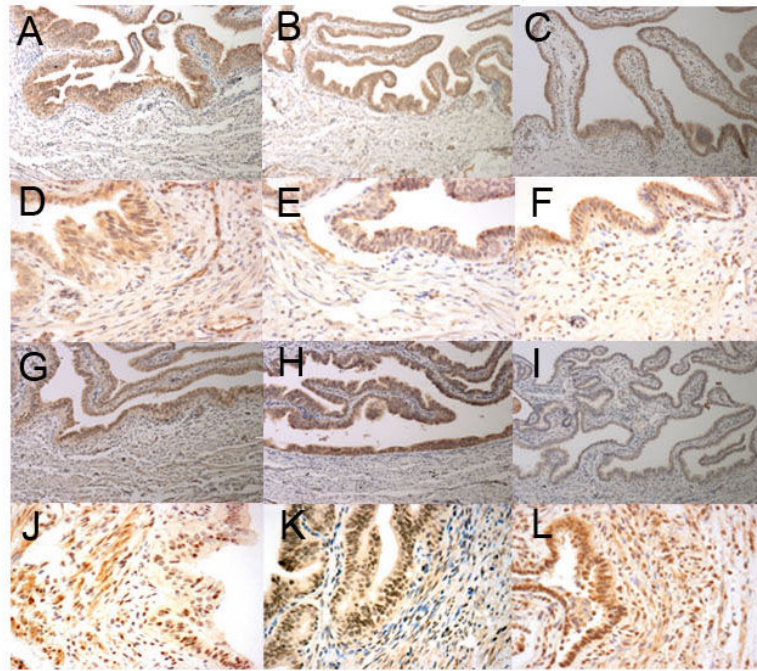
**Figure 2. Hormonal regulation of PROK and PROKR in Fallopian tube explants**

Fallopian tube explants from three different patients (n=3) were cultured with ethanol, 10 nM estradiol, 10 μM medroxyprogesterone acetate (MPA) or a combination of the two steroids for 24 h. RNA was extracted from the explants and cDNA was prepared. Taqman RT-PCR was used to analyze *PROK/PROKR* expression in the treated tissues. Statistical analysis was performed using one-way ANOVA and Dunnett's post-hoc analysis. *PROK1* (A) showed no change in expression following hormonal stimulation. *PROK2* (B) showed no change in expression following hormonal stimulation, *PROKR1* (C) was significantly increased with MPA treatment ( $p < 0.01$ ) and estrogen plus MPA treatment ( $p < 0.05$ ). *PROKR2* (D) levels did not change following hormonal treatment. \*\*  $p < 0.01$ , \*  $p < 0.05$ .



**Figure 3. Immunohistochemical localization of PROK and PROKR protein in Fallopian tube and colocalization with smooth muscle actin**

Immunohistochemistry was performed to PROK1, PROK2, PROKR1 and PROKR2 protein localization in human Fallopian tube tissue sections. Colocalization with smooth muscle actin in serial sections was also performed. All proteins were localized to the cytoplasm of the epithelium and to the smooth muscle layers (PROK1 – A, PROK2 – C, PROKR1 - E, PROKR2 – G). Localization of the proteins to the smooth muscle layer was confirmed by colocalization with smooth muscle actin in serial sections (B, D, F, H). Staining with rabbit serum (for PROK1), rabbit IgG (for PROK2, PROKR1 and PROKR2) and mouse IgG (for smooth muscle actin) were performed as negative controls and are shown for each stain. For antibody dilutions see the materials and methods.



**Figure 4. Immunohistochemical localization of PROK and PROKR in Fallopian tube obtained across the menstrual cycle and from women with ectopic pregnancies**

Immunohistochemistry was performed to localize PROK1, PROK2, PROKR1 and PROKR2 in Fallopian tube sections from the follicular and mid-luteal phases of the menstrual cycle, as well as in Fallopian tubes from ectopic pregnancies. A) PROK1, follicular, B) PROK1, mid-luteal, C) PROK1, ectopic, D) PROK2, follicular, E) PROK2, mid-luteal, F) PROK2, ectopic, G) PROKR1, follicular, H) PROKR1, mid-luteal, I) PROKR1, ectopic, J) PROKR2, follicular, K) PROKR2, mid-luteal, L) PROKR2, ectopic.

**Table 1 A**

Clinical information for Fallopian tube samples across the menstrual cycle

Sample	cycle phase	serum estrogen (pmol/L)	serum progesterone (nmol/L)	reason for surgery	uterine pathology
1	follicular	1022.87	0.81	HMB	adenomyosis
2	follicular	940.44	3.82	HMB, PP	adenomyosis
3	follicular	829.42	4.19	HMB, PP	adenomyosis, fibroid
4	follicular	770.63	5.16	HMB	no abnormality
5	follicular	116.3	2.88	HMB, PP	no abnormality
6	follicular	55.0	2.2	HMB, PP	adenomyosis, fibroids
7	mid-luteal	549.91	88	dysmen	no abnormality
8	mid-luteal	242	53.1	HMB, dysmen	no abnormality
9	mid-luteal	201	24.55	HMB, PP	fibroid
10	mid-luteal	399.8	80.45	HMB, dysmen	adenomyosis, fibroid, polyp
11	mid-luteal	1633.0	54.38	HMB, dysmen	no abnormality
12	mid-luteal	424	76.9	PP	no abnormality
13	mid-luteal	266	37.1	HMB	no abnormality
14	mid-luteal	331	83.5	HMB	no abnormality
15	mid-luteal	242.9	38.1	HMB	no abnormality

HMB= heavy menstrual bleeding, dysmen = dysmenorrhoea, PP = pelvic pain

**Table I B**

Clinical information for Fallopian tube samples from ectopic pregnancies

Sample no.	gestation length (days)	serum hCG (IU/L)	serum progesterone (nmol/L)
16	58	15956	67.43
17	50	487	39.21
18	59	2056	24.58
19	53	1854	63.07
20	52	2425	61.68
21	56	225	20.36
22	64	1383	24.35
23	78	1204	134.70
24	56	203	19.77
25	49	502	30.54
26	64	5981	158.13
27	44	508	7.06
28	44	12161	10.64
29	46	10285	31.70
30	52	1082	23.92