

Original Article

Prostaglandin F receptor expression in intrauterine tissues of pregnant rats

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In this investigation, we studied the expression and localization of rat prostaglandin F (FP) receptor in uterine tissues of rats on gestational Days 10, 15, 18, 20, 21, 21.5 and postpartal Days 1 and 3 using Western blotting analysis, real-time PCR, and immunohistochemistry. A high level of immunoreactivity was observed on gestational Days 20, 21, and 21.5 with the most significant signals found on Day 20. FP receptor protein was expressed starting on gestational Day 15, and a fluctuating unsteady increase was observed until delivery. Uterine FP receptor mRNA levels were low between Days 10 and 18 of gestation ($p < 0.05$). The transcript level increased significantly on Day 20 and peaked on Day 21.5 just before labor ($p < 0.05$). There was a positive correlation between FP receptor mRNA expression and serum estradiol levels ($r_s = 0.78$; $p < 0.01$) along with serum estradiol/progesterone ratios ($r_s = 0.79$; $p < 0.01$). In summary, we observed an increase FP receptor expression in rat uterus with advancing gestation, a marked elevation of expression at term, and a concomitant decrease during the postpartum period. These findings indicate a role for uterine FP receptors in the mediation of uterine contractility at term.

Keywords: immunohistochemistry, pregnancy, prostaglandin F receptor, rat, uterus

Introduction

The principal site of action for prostaglandins (PGs) during labor is the myometrium, where these factors modulate contractility via specific receptors [1,4,6,21].

Activation of the myometrium involves increased expression of genes that permit the myometrium to contract during labor. These myometrial (or uterine) activation proteins (UAPs) include the prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin E_2 (PGE_2), and prostaglandin F (FP) receptors; prostaglandin E receptor 1e4 (EP1e4), oxytocin receptor, and PG endoperoxide H synthase-2 [15]. Uterine stimulators include oxytocin and prostaglandins [15].

PGs (especially $PGF_{2\alpha}$) produced by intrauterine tissues associated with pregnancy (amion, chorion, placenta, decidua, and myometrium) play important roles in all physiological processes of parturition. Parturition includes five separate but integrated physiological events: membrane rupture, cervical dilatation, myometrial contractility, placental separation, and uterine involution. Increasing evidence supports a role for PGs in each of these events [4,11,16]. The objective of this study was to evaluate the expression and localization of the FP receptor, a significant UAP, in uterine tissues of rats during mid to late pregnancy and postpartum (PP) using Western blotting analysis, real-time polymerase chain reaction (RT-PCR), and immunohistochemistry with specific antibodies against the FP receptor.

Materials and Methods

Animals

Forty-eight adult female Sprague Dawley rats weighing 200~250 g were purchased from Harlan Laboratories (The Netherlands). Animals were maintained with a 12-h light/dark schedule, and received standard experimental

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rat diet (Korkuteli Food Industry, Turkey) and water *ad libitum*. The rats were randomly divided into eight groups (six animals per group) and the uterine horns were excised, and pups and placentas were discarded at 09 : 00 h on Day 10, 15, 18, 20, or 21 of gestation; Day 21.5 (at labor), or the 1st or 3rd day postpartum (PP1 or PP3). The day of mating was designated as Day 1 of pregnancy with delivery occurring between 21 : 00 h on Day 21 and 09 : 00 h on Day 22. Samples from the antimesometrial region of one uterine horn were immersion fixed in neutral 4% formaldehyde for immunohistochemical analysis or snap frozen in liquid nitrogen and stored at -80°C until real-time quantitative reverse transcription PCR (qRT-PCR) examination of gene expression and Western blot analysis were conducted. mRNA levels were measured with an RT-PCR assay. Venous blood samples (1.0 mL) were obtained from the right atrium prior to surgery on Day 10, 15, 18, or 21 of gestation, PP1, or PP3. The samples were stored at -20°C for estradiol and progesterone measurements. All procedures involving the use of animals were approved by the Gazi University Animal Experiments Local Ethics Committee (approval no: 08.16; Turkey).

Immunohistochemical analysis

A peroxidase anti-peroxidase (PAP) immunocytochemical staining technique was used to analyze tissue samples. For immunohistochemical analysis uterine horn were immersion fixed in neutral 4% formaldehyde and then dehydrated in a series of graded alcohols (50, 60, 70, 80, 90 and 100% ethanol; Merck, Germany). After passing through xylene (Merck) and then the tissue were embedded in paraffin (Shandon, Thermo Fisher Scientific, USA). For this procedure, serial paraffin sections were cut with microtome (Leica, Germany) at a thickness of $4\sim 5\ \mu\text{m}$ and placed onto poly-L-lysine-coated slides (Gerhard Menzel, Germany). After deparaffinization in xylene and rehydration with reduced ethanol series (80, 90, 100%; Merck) endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide in absolute methanol for 10 min. After washing in phosphate buffered saline, the sections were incubated with 10% nonimmune serum (Cat. no: 85-9043, blocking solution, Histostain-Plus Kit, horseradish-peroxidase [HRP], broad spectrum; Invitrogen, USA) for 10 min to eliminate nonspecific staining. The tissues were then incubated with goat polyclonal antibodies (diluted to ratio 1 : 100) against the FP receptor (PGF_{2 α} R, N-18, antibody; Santa Cruz Biotechnology, USA) for 6 h and exposed to a biotinylated secondary antibody (diluted to ratio 1 : 100, Cat. no: 85-9043, Histostain-Plus Kit, HRP, broad spectrum second antibody; Invitrogen) for 10 min. Immunoreactivity was detected with an HRP-streptavidin complex (Cat. no: 85-9043, HRP-Streptavidin, Histostain-Plus Kit, HRP, broad spectrum; Invitrogen) using Diaminobenzidine

Histostain-Plus Kit chromogen (broad spectrum; Invitrogen) as a marker. The sections were counterstained with Mayer's hematoxylin (for 2 min, dehydrated with ethanol series 60, 70, 80, 90, 96, 100%) and after passing xylene (Merck) for 2 times. Slides were mounted with entellan (Merck). For the negative controls, the primary antibody was replaced with PBS. For the immunoglobulin G (IgG) control, 10% nonspecific rabbit serum (Cat.no: 85-9043, blocking solution, Histostain-Plus Kit, HRP, broad spectrum; Invitrogen) was used instead of the primary antibody. All incubation steps were carried out at room temperature in a humidified chamber. Immunohistochemical staining results were obtained using an image processing and analysis system (QWin image analysis tool kit; Leica Microsystems, Germany) linked to a Leica DM4000 light microscope. The staining intensity was scored as negative (-), weak (+), moderate (++), or strong (+++).

Western blot analysis

Proteins extracted from uterine tissues were subjected to Western blot analysis. Equivalent masses (0.3 g) of tissue were taken from the uterus. The tissue samples were homogenized using a mechanical homogenizer (IKA, Germany) in lysis buffer (1% NP-40 (Sigma, USA), 50 mmol/L Tris [pH 7.5], 50 mmol/L EDTA (Sigma), 1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1 mmol/L PMSF (Sigma), 10 $\mu\text{g}/\text{mL}$ aprotinin (Sigma), and 1 $\mu\text{g}/\text{mL}$ leupeptin (Sigma) with a 2 $\mu\text{g}/\text{mL}$ protease inhibitor cocktail (Sigma). The homogenates were centrifuged at 14,000 g for 10 min at 4°C in a microcentrifuge (Rotina 35R; Hettich, UK), and the supernatants were taken for analysis.

Protein concentration of the samples was determined using the bicinchoninic acid method (BCA protein assay kit; Pierce, USA) with bovine serum albumin (BSA; Pierce) as a standard. Next, 50 μg of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Pierce) using an electroblotter (OWL VEP-2; Thermo Fischer Scientific) at 350 mA for 2 h. The membrane was blocked using Tris-buffered saline containing 0.1% Tween-20 (TBST) with 5% nonfat dry milk (Cell Signaling Technology, USA) at 4°C for 1 h. The blocked membranes were incubated overnight at 4°C with primary antibodies against PGF_{2 α} R (anti-goat, N-18 1 : 1000; Santa Cruz Biotechnology) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; anti-rabbit, 1 : 1000; Sigma) diluted in TBS containing 5% nonfat milk powder. GAPDH was used as a loading control. The membranes were washed three times for 10 min each in TBST (20 mM Tris-HCl, 0.5 M NaCl, 0.05% Tween-20, pH 8.0) and then incubated with the HRP-conjugated anti-rabbit IgG secondary antibody (1

: 5000 dilution; Santa Cruz Biotechnology) for 2 h at room temperature. The membrane was then incubated with chemiluminescence substrate (ECL Western Blotting Substrate; Pierce) for 5 min and the proteins were detected with an enhanced chemiluminescence system (Amersham, USA). Chemiluminescent signals were captured by X-ray film (Pierce) imaging.

RNA extraction and reverse transcription

Total RNA was isolated from uterine tissues using a Trizol reagent system (peqGOLD TriFast™; PEQLAB, Germany). To avoid DNA contamination, we made some modifications to the manufacturers' instructions. The RNA pellets were treated with approximately 1~5 U RNase-free DNase (DNaseI; Roche Diagnostics, Germany) per µg RNA and incubated at 37°C for 30 min before being washed with 70% ethanol to prevent DNA contamination. After the ethanol was removed by air-drying, the RNA pellets were dissolved in 10~30 µL of RNase- and DNase-free water (Sigma). Isolated RNA was kept at -80°C. The yield and quality of RNA from each sample was determined by measuring the absorbance at 260 and 280 nm using a Nanodrop spectrophotometer (NanoDrop ND-1000; Thermo Fisher Scientific). Equal amounts of RNA were used for reverse transcription. Complementary DNA (cDNA) was reverse transcribed from 1 µg of total RNA using a Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics) according to the manufacturer's instructions. This process was performed in a PCR Sprint thermocycler (Thermo Hybaid, Germany).

qRT-PCR analysis

FP mRNA expression was measured using qRT-PCR with a LightCycler instrument (Roche Diagnostics). Beta-actin (ACTB), a housekeeping gene, was used to normalize FP mRNA expression levels. Probes and intron spanning primers for each assay were designed using the Universal Probe Library (UPL) Assay Design Center (Roche Applied Science, Germany). Gene-specific primer sequences (exon-exon junctions to allow discrimination between cDNA and genomic DNA) and UPL numbers are provided in Table 1.

A volume of 2 µL of cDNA product was subjected to real-time PCR in a 10 µL total reaction mixture containing 2.5 µL of TaqMan Universal PCR Master Mix (Roche Diagnostics), 0.5 µL sense and antisense primers, 0.1 µL

probe and template cDNA. The reaction mixture was prepared in a 96-well plate (LightCycler 480 Multiwell Plates 96; Roche Applied Science) and a qRT-PCR was performed using a LightCycler 480 instrument (Roche Diagnostics). After a single step of initial denaturation at 95°C for 10 min, followed by 55 cycles of 10 sec at 95°C, 20 sec at 65°C and 1 sec at 60°C for annealing and 30 sec at 40°C for cooling. Negative control reactions lacked template cDNA. Each sample was analyzed in triplicate. In order to determine amplification efficiencies of the target genes, standard curves were constructed for the samples used in a series of dilutions for analyzing both the gene of interest (GOI) and the housekeeping gene (ACTB). GOI (FP) and ACTB amplification efficiencies were approximately equal. The results were analyzed with LightCycler Software version 3.5 (Roche Diagnostics). FP gene expression analysis was performed using the Relative Expression Software Tool 2005 (REST; Qiagen, the Netherlands). Significant differences in mRNA levels were analyzed by a pairwise fixed reallocation randomization test as a statistical model included in the REST developed for group-wise comparison and statistical analysis of relative expression results.

Measurement of hormone levels

Serum estradiol and progesterone levels were measured with an enzyme-linked immunosorbent assay (ELISA) sandwich method (Cayman Chemical, USA). Monoclonal antibodies specific for rat estradiol and progesterone were used to precoat 96-well plates. Each plate contained a minimum of two blanks, two nonspecific binding wells, two maximum binding wells and an eight point standard curve run in duplicate. 50 µL of serum sample was added per well. Each sample was assayed at a minimum of two dilutions. Each dilution was assayed in duplicate. Standard marker dilution factors were between 6.6-4,000 pg/mL and 7.4-1,000 pg/mL for estradiol and progesterone, respectively. The samples and standards were evaluated according to the manufacturer's instructions. Absorbance at 450 nm was recorded using an ELISA plate reader (model 680 Microplate Reader; Bio-Rad Laboratories, USA).

Statistical analysis

All data were analyzed using SPSS statistical software (ver. 15.0 for Windows; SPSS, USA). Significant

Table 1. Gene-specific primer sequences and probe numbers

Gene	Forward primer	Reverse primer	UPL probe number
ACTB	5' CCCGCGAGTACAACCTTCT 3'	5' CGTCATCCATGGCGAACT 3'	17
PGF2α receptor	5' GAGATTTAGACGGAAGTCGAAGG 3'	5' GTGATCACCAGGCCACTAGC 3'	112

differences in mRNA expression were analyzed with a pairwise fixed reallocation randomization test as a statistical model included in the REST software developed for group-wise comparison and statistical analysis of relative expression results. The p values < 0.05 were considered significant.

Results

Immunohistochemical and Western blot analyses

An immunoreaction specific for the FP receptor was observed in all specimens studied. The FP receptor was detected by immunohistochemistry in cells and apical cell membranes in the superficial and glandular epithelium, connective tissue cells in the lamina propria, and vascular endothelial and smooth muscle cells. In addition, FP receptor expression was predominantly localized to myocytes in both the circular and longitudinal layers of the myometrium. A marked change in overall staining intensity was observed according to gestational age (Table 2). Overall immunoreactivity was lowest on Day 10 and increased on Days 15 and 18. A high level of immunoreactivity was observed on Days 20, 21, and 21.5 with the strongest signals found on Day 20 followed by a lower degree of reactivity during the postpartum period. Involvement of the vascular endothelial and smooth muscle cells was first observed on Day 20. Moderate immunostaining on Days 20, 21, and 21.5 was followed by weak staining on PP1 and PP3. On Days 20, 21, and 21.5, strong signals in both the circular and longitudinal layers of the myometrium were observed. In contrast, stronger staining was observed in the outer longitudinal myometrial layer compared to the inner circular muscle layer on previous days of gestation and the postpartum period (Fig. 1).

Western blot analyses demonstrated that no FP receptor protein was expressed on the 10th day of gestation. However, the protein was expressed starting on the 15th day of gestation, and a fluctuating unsteady increase was

observed until delivery. The greatest FP receptor protein levels were observed during the prenatal period (Day 21.5; Fig. 2). During the postnatal period, protein expression decreased steadily.

qRT-PCR analysis findings

Uterine FP receptor mRNA levels were low between Days 10 and 18 of gestation ($p < 0.05$). The level of FP transcript increased significantly on Day 20 and peaked on Day 21.5 just before labor ($p < 0.05$). After delivery, FP receptor mRNA levels fell rapidly through PP1 and PP3 compared to Day 21.5 ($p < 0.05$; Fig. 3). The present study demonstrated that FP receptor mRNA expression was increased in the rat uterus during pregnancy, reached maximal levels during labor, and declined postpartum. These results may indicate a role for the PGF2 α receptor in

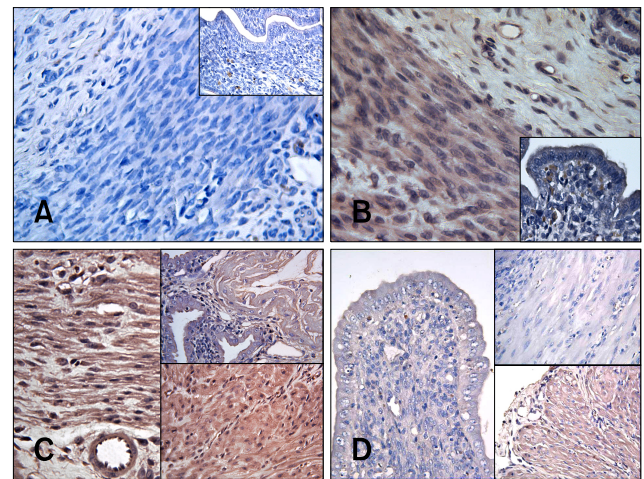


Fig. 1. Myometrial samples from Day 10 (A), Day 15 (B), and Day 21 (C) of gestation as well as postpartum Day 3 (PP3; D) showing FP receptor immunostaining in the endometrial epithelium, connective tissue of the lamina propria, and vascular endothelial and smooth muscle cells. Immunoperoxidase and Hematoxylin stain, $\times 400$.

Table 2. Prostaglandin F receptor distribution and staining intensity in pregnant rat uteri

Groups	SEE	GEE	CT	VESM	LM	CM
Day 10 (n = 6)	+	+	++	–	+	–
Day 15 (n = 6)	++	++	++	–	++	+
Day 18 (n = 6)	+++	++	++	–	++	+
Day 20 (n = 6)	+++	+++	+++	+++	+++	+++
Day 21 (n = 6)	+++	+++	++	++	+++	+++
Day 21.5 (n = 6)	+++	+++	++	++	+++	+++
PP1 (n = 6)	++	+	+	+	+	+
PP3 (n = 6)	+	+	+	+	+	+

SEE: superficial endometrial epithelium, GEE: glandular endometrial epithelium, CT: connective tissue in the lamina propria, VESM: vascular endothelial and smooth muscle cells, LM: longitudinal myometrium, CM: circular myometrium.

the final cervical ripening during gestation and parturition.

Hormonal analysis

Serum progesterone levels increased from Day 10 through Day 18. These levels declined after Day 18 through PP3. Serum estradiol levels increased gradually from Day 10

through Day 21 and declined during the postpartal period (Fig. 4). Post-hoc tests revealed significant differences between Day 18~21; Day 21-PP1, and PP1-PP3 ($p < 0.01$). There was a positive correlation between FP receptor mRNA levels and serum estradiol concentrations ($r_s = 0.78$; $p < 0.01$) and serum estradiol/progesterone ratios ($r_s = 0.79$; $p < 0.01$). In contrast, serum progesterone levels (Fig. 5) were negatively correlated with FP receptor mRNA expression ($r_s = -0.44$; $p < 0.01$).

Discussion

The PGs are recognized as the ‘triggers’ of labor [5] because the myometrium contracts in response to exogenous PGs both *in vivo* and *in vitro* [7,19], PG synthetic enzymes and levels in tissues and fluids increase before or at the time of labor, and inhibitors of PG synthesis delay birth and prolong pregnancy [15]. The current study clearly

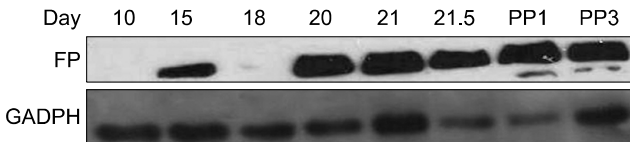


Fig. 2. Western blots showing FP receptor expression in intrauterine tissues (gestation Day 10, 15, 18, 20, 21, and 21.5 as well as PP1 and PP3).

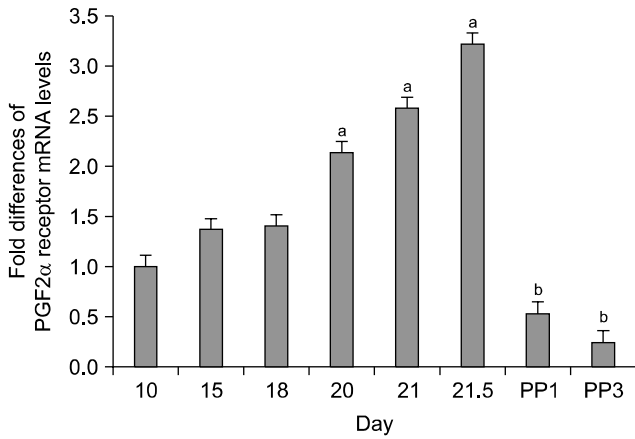


Fig. 3. Relative mRNA expression of the PGF2α receptor. Bars represent PGF2α receptor mRNA expression levels normalized to that of ACTB and relative to 10th day using the REST and 2^{-ΔΔCT}. An asterisk indicates that the value was significantly different from the 10th day of gestation ($p < 0.05$). An asterisk indicates that the value was significantly different from the 21.5th day of gestation ($p < 0.05$).

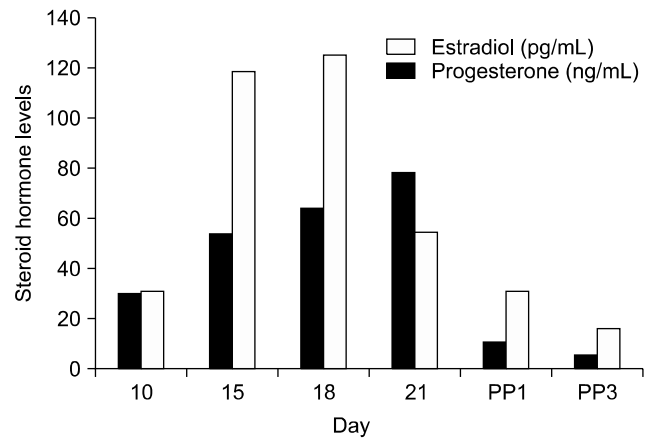


Fig. 4. Estradiol and progesterone levels in rats during late pregnancy and postpartum.

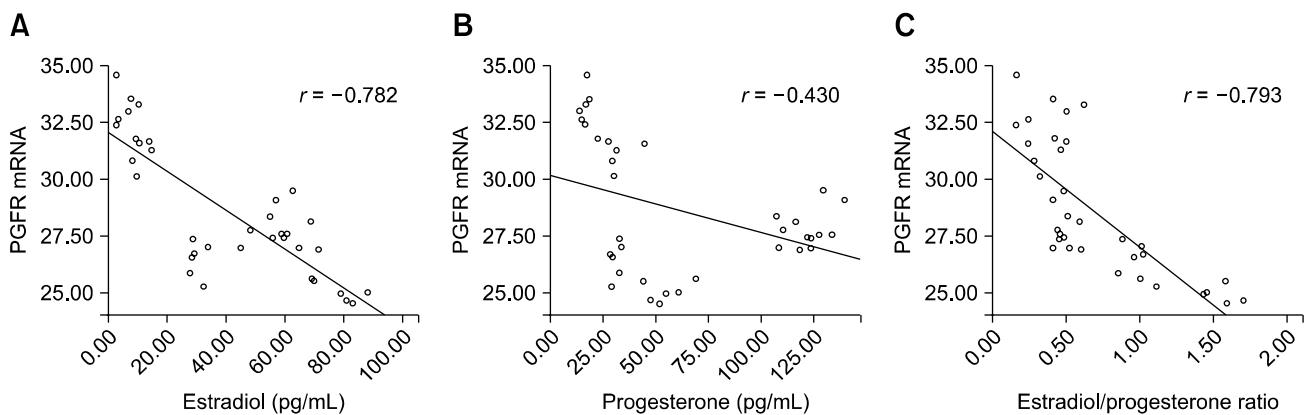


Fig. 5. Correlations between steroid hormone levels and FP receptor mRNA expression.

demonstrated that the FP receptor mRNA levels in rat uterus were low between Days 10 and 18 of gestation, significantly increased on Day 20, peaked during parturition, and fell rapidly after delivery. This pattern of FP receptor expression is similar to those previously reported for the myometrium [4,17] and entire uterus [2]. Responsiveness of the rat myometrium to PGF₂ α increases from Day 18 throughout the latter part of gestation [7]. Increases in FP receptor expression were reported to increase sensitivity of the myometrium to PGF₂ α action at term, even in the absence of increased PGF₂ α concentrations [4]. Accordingly, the results of the present investigation support the hypothesis that an increase in myometrial expression of the FP receptor is an important component of labor initiation.

Prostaglandin F₂ α exerts well-known effects on myometrial contractility and inhibits labor [11]. Parturition consists of five separate but integrated physiological events: myometrial contractility, membrane rupture, cervical dilatation, placental separation, and uterine involution [16]. Increasing evidence supports a role for PGs in each of these events [15,16]. Recently, FP receptor protein was identified in human amnion epithelium and mesoderm, chorion trophoblasts, and decidua by immunohistochemistry, and it was suggested that the induction of FP receptor expression at term may facilitate the contribution of the decidua to parturition [12]. In the present study, immunostaining intensity scores for the FP receptor observed in the endometrial epithelium, connective tissue of the lamina propria, and vascular endothelial and smooth muscle cells significantly increased from mid-pregnancy to term and decreased during the postpartal period. These observations serve as histological evidence indicating a physiological function of endometrial FP in rat uterus. Additionally, the results also support the recent concept that pregnant rat endometrium is an important regulator of myometrial contractility.

Interestingly, we observed a difference in immunostaining intensity between two muscular layers of myometrium on Day 10, 15, and 18 of gestation and during the postpartum period with stronger signals in the outer longitudinal muscles compared to the inner circular muscles. The myometrium is the muscular part of the uterus that is responsible for generating the force necessary to expel the fetus at term. It is composed of three well-defined layers. The circumferentially oriented smooth muscle cells of the circular muscle in the myometrium surround the endometrium. The smooth muscle cells of the outer longitudinal muscle layer of the myometrium align along the long axis of the uterus. These muscle layers exhibit distinct phenotypes during pregnancy. Differential production of prostaglandins [9] as well as different responses to stretch, noradrenaline, and estrogen stimulation in circular versus longitudinal muscle have been reported [10,13]. Thompson *et al.* [22] described

certain characteristic differences between the responses of circular and longitudinal muscles to PGF₂ α . Using contraction recordings, these authors demonstrated that the maximum response to PGF₂ α in longitudinal muscles was not different on Days 15 and 21 of gestation, and the half maximal effective concentration (EC₅₀) for PGF₂ α did not change in longitudinal muscles between Days 15 and 21. However, the magnitude of both the threshold and maximum responses of the circular muscles progressively increased between Days 15 and 21, and the EC₅₀ for circular muscles declined between Days 17 and 21. The functional significance of the differences between circular and longitudinal muscles is not entirely clear. It has been suggested that weak, high-frequency contractions of the circular layer and localized, infrequent contractions of the longitudinal layer during gestation prevent movement of the fetuses toward the cervix [13]. Observing strong immunoreactivity in both muscle layers on Day 20, 21, and 21.5 in our study may indicate differential FP expression in circular and longitudinal myometrial muscles in pregnant rats. Functional studies testing this hypothesis are lacking. Al-Matubsi *et al.* [1] did not observe any marked change in staining intensity with gestational age or labor. However, this study was conducted in rats on gestation Days 16~22.

Few data have been published on the regulation of FP receptor expression in the uterus during pregnancy [14]. FP receptor mRNA expression in the rat uterus appears to be related to changes in ovarian steroid concentrations [17]. Progesterone is the hormone responsible for maintaining uterine smooth muscle quiescence during pregnancy. Progesterone levels decline after Day 19 of pregnancy followed by simultaneous increases in the levels of estradiol and prostaglandin F after Day 20 [18]. In accordance with previous reports [11,21], serum progesterone levels increased from Day 10 through Day 18 followed by a decline after Day 18 through PP3 in the current study. Serum estradiol levels increased gradually from Day 10 through Day 21 and declined during the postpartal period. FP receptor mRNA levels were positively correlated with serum estradiol concentrations and serum estradiol/progesterone ratios. On the other hand, serum progesterone levels were negatively correlated with FP receptor mRNA expression. Similarly, Dong and Yallampalli [8] observed antagonistic effects of ovarian steroids on FP expression. Progesterone appears to block the stimulatory effects of estrogen on FP receptor expression. Increases in myometrial FP receptor mRNA levels have always been correlated with a decline in progesterone concentrations whether occurring naturally before the onset of term labor or artificially induced by ovariectomy [3,8]. Furthermore, exogenous progesterone successfully inhibits increases in the level of myometrial FP receptor mRNA associated with labor whether at term or during premature induction by ovariectomy [8]. The mechanisms underlying the inhibitory

effect of progesterone on myometrial FP receptor mRNA expression are largely unknown [17]. In contrast, FP receptor levels are increased by estradiol in pregnant and nonpregnant rat uterus [8].

In conclusion, our findings support the speculation that increased myometrial expression of the FP receptor is an important component of labor initiation. In addition, the ratio of estrogens to progesterone levels changes at term. These results suggested that steroid hormone-induced changes in PGF receptor expression may mediate the effects of steroid hormones on uterine contractility.

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