

# Differential expression of prostaglandin E receptor subtype EP2 in rat uterus during early pregnancy

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**Summary.** PGE<sub>2</sub> is essential for mammalian female reproduction. This study was to examine the expression of EP2 gene in the rat uterus during early pregnancy, delayed implantation and artificial decidualization by *in situ* hybridization and immunohistochemistry. There was no detectable EP2 mRNA expression in the uterus from days 1 to 4 of pregnancy (day 1 = day of vaginal sperm). A low level of EP2 immunostaining was observed in the luminal and glandular epithelium from days 1 to 4 of pregnancy. Both EP2 mRNA and protein expression were highly detected in the luminal epithelium at implantation sites on day 6 of pregnancy. EP2 expression decreased from day 7 of pregnancy and was undetectable on days 8 and 9 of pregnancy. After delayed implantation was terminated by estrogen treatment and the embryo implanted, both EP2 mRNA and protein expression were strongly observed in the luminal epithelium at the implantation site. There was no detectable EP2 expression in both control and decidualized uteri. In conclusion, these data suggest that EP2 expression at implantation site may play an important role during embryo implantation in rats.

**Key words:** Prostaglandin, EP2, Rat, Uterus, Pregnancy

## Introduction

Prostaglandins (PGs) consist of PGE<sub>2</sub>, PGF<sub>2α</sub>, PGD<sub>2</sub>, PGI<sub>2</sub> and TXA<sub>2</sub> (Breyer et al., 2000). PGs are involved in various functions, including the modulation of vascular responses, cell proliferation and differentiation. PGE<sub>2</sub> is produced through three sequential enzymatic reactions: release of arachidonic acid (AA) from membrane glycerophospholipids by phospholipase A<sub>2</sub> (PLA<sub>2</sub>), conversion of AA to the unstable intermediate PGH<sub>2</sub> by cyclooxygenase (COX), and enzymatic conversion of PGH<sub>2</sub> to PGE<sub>2</sub> by prostaglandin E synthase (PGES) (Murakami et al.,

2002). PGE<sub>2</sub> receptors are classified into four subtypes, EP1, EP2, EP3 and EP4 according to their different affinities for some relatively selective agonists (Sugimoto et al., 2000). EP1 is coupled to Ca<sup>2+</sup> mobilization, but both EP2 and EP4 subtypes are coupled to the stimulation of adenylyl cyclase via Gs. On the contrary, EP3 is coupled to Gi, which inhibits adenylyl cyclase activity (Negishi et al., 1995).

PGs are shown to be important for implantation and decidualization in laboratory rodents (Kennedy, 1994; Lim et al., 1999). PGE<sub>2</sub> is able to induce implantation of mouse blastocysts (Holmes and Gordashko, 1980). Among various PGs, PGE<sub>2</sub> and prostacyclin (PGI<sub>2</sub>) have been considered as a primary candidate involved in implantation and decidualization in the rodents (Lim et al., 1999). EP2 mRNA expression was exclusively localized in the luminal epithelium of mouse uterus primarily during the peri-implantation period (Lim and Dey, 1997). In the rat, PGI<sub>2</sub> and PGE<sub>2</sub> are higher in implantation sites than in the surrounding uterus (Kennedy, 1977; Kennedy and Zamecnik, 1978). EP2 expression was also examined in the pseudopregnant rat uterus (Papay and Kennedy, 2000).

However, the expression and regulation of the EP2 gene during the peri-implantation period in other species are still unknown. The aim of the present study was to examine the expression and regulation of EP2 in the rat uterus during early pregnancy. *In situ* hybridization and immunohistochemistry were used to examine the expression of the EP2 gene in the rat uterus during early pregnancy, delayed implantation and artificial decidualization.

## Materials and methods

### *Animals and treatments*

Mature rats (Sprague-Dawley strain) were caged in a controlled environment with a 14 hr light: 10 hr dark cycle. All animal procedures were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University. Adult females were mated with fertile males of the same strain to induce

pregnancy. Pregnancy was confirmed by examining the spermatozoa in vaginal smear (day 1 = day of vaginal sperm positive). Pregnancy on days 1-5 was confirmed by flushing embryos from the reproductive tracts. The implantation sites on days 6-7 were identified by intravenous injection of 1 ml of 1% (w/v) Chicago blue solution in 0.85% (w/v) NaCl 5 min before the rats were sacrificed. At least 3 rats were used in each stage or treatment in this study.

To induce delayed implantation, the pregnant rats on day 4 of pregnancy were treated with progesterone (5 mg/rat, s.c.) and then ovariectomized at 0830-0900 h. Progesterone (5 mg/rat) was injected to maintain delayed implantation from days 5-7. Estradiol-17 $\beta$  (250 ng/rat) was given to the progesterone-primed delayed-implantation rats to terminate delayed implantation. The rats were killed by stunning and cervical dislocation to collect uteri 24 h after estrogen treatment. The implantation sites were identified by tail intravenous injection of 1 ml of 1% (w/v) Chicago blue solution 5 min before the rats were sacrificed.

In order to get artificial decidualization, mature female rats were ovariectomized and sensitized for the decidual cell reaction 5 days later according to the procedures described previously (Kennedy and Ross, 1997). Some of the rats were given bilateral intrauterine injection of 100  $\mu$ l of sesame oil at 1200-1300 on the equivalent of day 5 of pseudopregnancy. The animals that did not receive the intrauterine injection served as non-stimulated controls (Orlando-Mathur et al., 1996). Treated rats were killed 6 days after the intrauterine injection of oil.

#### Western blot

Rat liver, kidney and uterus on day 4 of pregnancy were homogenized in pre-cooled homogenization buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl, 1% Triton X-100, 0.25% sodium deoxycholate, 20 mg/ml phenylmethanesulfonyl fluoride) and centrifuged at 12,000 g and 4°C for 10 min. The supernatants were quantified for protein concentration, followed by SDS-PAGE and electron transfer onto nitrocellulose membrane. After being blocked in 5% nonfat milk in PBST (0.1% Tween-20 in PBS) at 4°C overnight, the membranes were incubated with rabbit anti-human EP2 antibody (1:800, Cayman Chemical, Ann Arbor, MI, USA) for 1 h. Following 3 washes in 5% nonfat milk in PBST, the membranes were incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase for 1 h. After 3 washes in 5% nonfat milk in PBST, 2 washes in PBST, and 2 washes in PBS, the membranes were incubated with ECL reagents, exposed and developed according to the manufacturer's protocol (Amersham Pharmacia Biotech UK Limited, England, UK).

#### Immunohistochemistry

Rat uteri were immediately fixed in Bouin's solution

for 24 h and embedded in paraffin wax. Sections (7  $\mu$ m) were cut, deparaffinized and rehydrated. Antigen retrieval was performed by incubating the sections in 0.1 mM EDTA (pH 9.0) at 88°C water bath for 10 min and by cooling naturally at room temperature for 20 min. Nonspecific binding was blocked in 10% (v/v) normal horse serum in PBS for 1 h. The sections were incubated with rabbit anti-human EP2 polyclonal antibody in 10% (v/v) horse serum (1:100, Cayman) for 1 h overnight at 4°C. This antibody was shown to have a cross-reaction with rat EP2 according to the instructions from the company. The sections were then incubated with biotinylated goat anti-rabbit antibody followed by an avidin-alkaline phosphatase complex and Vector Red (Vectastain ABC-AP kit, Vector Laboratories, Burlingame, CA, USA). Vector Red was visualized as a red color. Normal rabbit IgG was used to replace the primary antibodies for a negative control. Endogenous alkaline phosphatase activity was inhibited by supplementing 1 mM levamisole (Sigma) into Vector Red substrate solution. The sections were counterstained with hematoxylin and mounted. The degree of staining was assessed subjectively by blinded examination of the slides by two investigators.

#### In situ hybridization

The following primers were designed based on the mouse cDNA sequence (GenBank accession number D50589), forward primer 5'-GATGCTCATGCTCTTCGC and reverse primer: 5'-GAAGTCCGACAACAGAGG (from 523 to 1143 bp). A 621 bp fragment was obtained by using these primers to amplify the pcDNA3 plasmid containing rat EP2 cDNA (Kindly provided by Dr. Kathy McCusker from Merck Frosst Canada Inc., Canada). The amplified fragment (621 bp) of rat EP2 was recovered from the agarose gel and cloned into pGEM-T plasmid (pGEM-T Vector System 1, Promega). These plasmids were further amplified with SP6 and T7 primers for labeling. Digoxigenin (DIG)-labeled antisense or sense cRNA probes were transcribed in vitro using a DIG RNA labeling kit (Roche Diagnostics GmbH, Mannheim, Germany).

Uteri were cut into 4-6 mm pieces and flash frozen in liquid nitrogen. Frozen sections (10  $\mu$ m) were mounted on 3-aminopropyltriethoxy-silane (Sigma)-coated slides and fixed in 4% paraformaldehyde solution in PBS. In situ hybridization was performed as previously described (Ni et al., 2002). Briefly, the sections were incubated in the hybridization buffer (5 X SSC, 50% formamide, 0.02% BSA, 250  $\mu$ g/ml yeast tRNA, 10% dextran sulfate, 1  $\mu$ g/ml denatured DIG-labeled antisense or sense RNA probe for rat EP2) at 55°C or 16 h. After hybridization, the sections were washed in 50% formamide/5 X SSC, 50% formamide/2 X SSC, and 50% formamide/0.2 X SSC at 55°C twice for 30 min each, respectively. After blocking in 1% block mix (Roche) for 1 h, the sections were incubated in sheep anti-DIG antibody conjugated with alkaline

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phosphatase (1:5000, Roche) in 1% block mix overnight at 4°C. The signal was visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. Endogenous alkaline phosphatase activity was inhibited with 2 mM levamisole (Sigma). All of the sections were counter-stained with 1% methyl green.

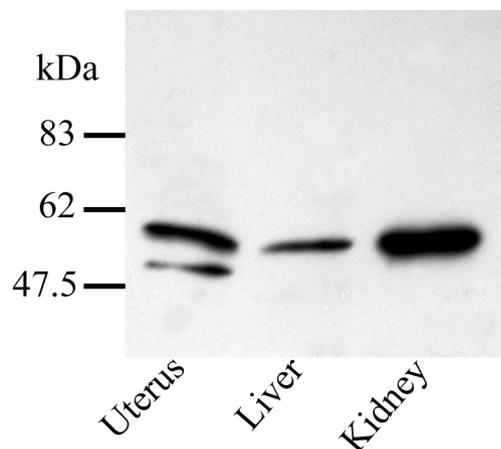
### Results

#### Western blot analysis

Western blot analysis was performed to detect EP2 protein in rat liver, kidney and uterus on day 4 of pregnancy. A band at 53 kDa was seen in rat liver and kidney. However, two bands were seen in the uterine sample on day 4 of pregnancy, one at ~53 kDa, and another one at ~50 kDa (Fig. 1). When rabbit anti-human EP2 antibody was replaced with normal rabbit IgG, the corresponding bands were not detected (data not shown).

#### EP2 mRNA expression in rat uterus during early pregnancy

There were no detectable EP2 mRNA signals in the uteri from days 1 to 4 of pregnancy (Fig. 2A,B). A basal level of EP2 mRNA expression was seen in the luminal epithelium on day 5 of pregnancy. EP2 mRNA expression was strongly localized in the luminal epithelium at the implantation site on day 6 of pregnancy (Fig. 2C), while no signal was seen at inter-implantation areas. When DIG-labeled EP2 anti-sense probe was replaced by DIG-labeled EP2 sense probe, there was no detectable signal at the implantation site on day 6 of pregnancy (Fig. 2D). On day 7 of pregnancy, EP2 mRNA signals were observed in the luminal epithelium



**Fig. 1.** Western blot analysis of EP2 protein in rat tissues. A band at 53 kDa was seen in rat liver and kidney. However, two bands were seen in the uterine sample on day 4 of pregnancy, one at ~53 kDa, and another one at ~50 kDa.

(Fig. 2E). However, there was no detectable EP2 mRNA expression in the uteri from days 8 and 9 of pregnancy (Fig. 2F,G). EP2 mRNA expression in the luminal epithelium of rat uterus during early pregnancy was summarized in Table 1.

#### EP2 immunostaining in rat uteri during early pregnancy

There was a low level of EP2 immunostaining in the luminal and glandular epithelium on days 1 and 2 of pregnancy (Fig. 3A). EP2 immunostaining slightly increased in the luminal and glandular epithelium on days 3-5 of pregnancy (Fig. 3B). At the implantation site on day 6, a strong EP2 immunostaining was observed in the luminal epithelium surrounding the implanting blastocyst, while a low level of EP2 immunostaining was seen in other areas of luminal epithelium (Fig. 3C). After rabbit anti-human EP2 antibody was replaced by normal rabbit IgG, there was no detectable immunostaining at the implantation site on day 6 of pregnancy (Fig. 3D). From days 7 to 9 of pregnancy, there was no detectable EP2 immunostaining in the uteri (Fig. 3E-G). EP2 protein expression in the luminal epithelium of rat uterus during early pregnancy was summarized in Table 1.

#### EP2 expression in the uterus under delayed implantation and activation

There was no detectable EP2 mRNA signal in the uterus under delayed implantation (Fig. 4A). After delayed implantation was terminated by estrogen treatment and embryo implanted, EP2 mRNA signal was observed in the luminal epithelium at the implantation site (Fig. 4B). Under delayed implantation, EP2 immunostaining was strongly detected in the luminal and glandular epithelia (Fig. 4C). After delayed implantation was terminated by estrogen treatment and the embryo implanted, a strong level of EP2 immunostaining was only detected in the luminal epithelium (Fig. 4D).

#### EP2 expression under artificial decidualization

There was no detectable EP2 mRNA signal in the uterine horn for control (Fig. 4E). After decidualization was artificially induced, EP2 mRNA signal was still not

**Table 1.** EP2 expression in the luminal epithelium of rat uterus during early pregnancy\*

	DAYS OF PREGNANCY									
	1	2	3	4	5	6(IS)	6(NIS)	7	8	9
EP2 mRNA	-	-	-	-	-	++	-	+	-	-
EP2 Protein	+	+	+	+	+	+++	+	-	-	-

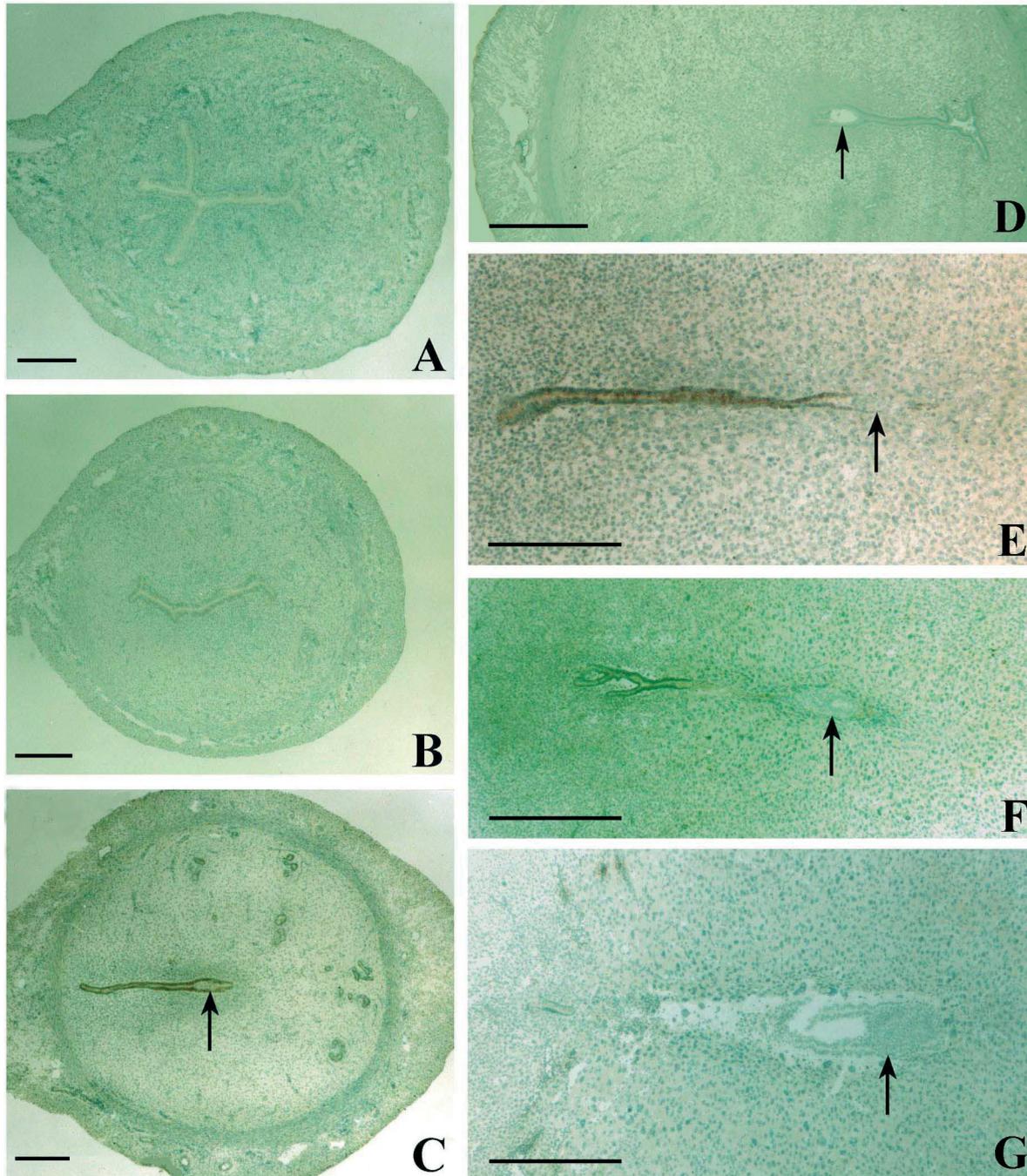
IS, implantation site; NIS, non-implantation site.

seen in the uterus (Fig. 4F). Although there was a basal level of EP2 immunostaining in the luminal epithelium in the control uterine horn (Fig. 4G), no EP2 immunostaining was seen in the uterus undergoing artificial decidualization (Fig. 4H).

**Discussion**

In our study, a band at ~53 kDa was seen in rat liver,

kidney and uterus, but a band at ~50 kDa was detected only in the rat uterus. Because there were no detectable bands after rabbit anti-human EP2 was replaced with normal rabbit IgG, this antibody should be specific for EP2. Additionally, rabbit anti-human EP2 antibody was shown to have a cross-reaction with rat EP2 protein by the supplier (Cayman). There are two transcripts of EP2 mRNA, uterine type and macrophage type. The macrophage type EP2 mRNA was expressed in various

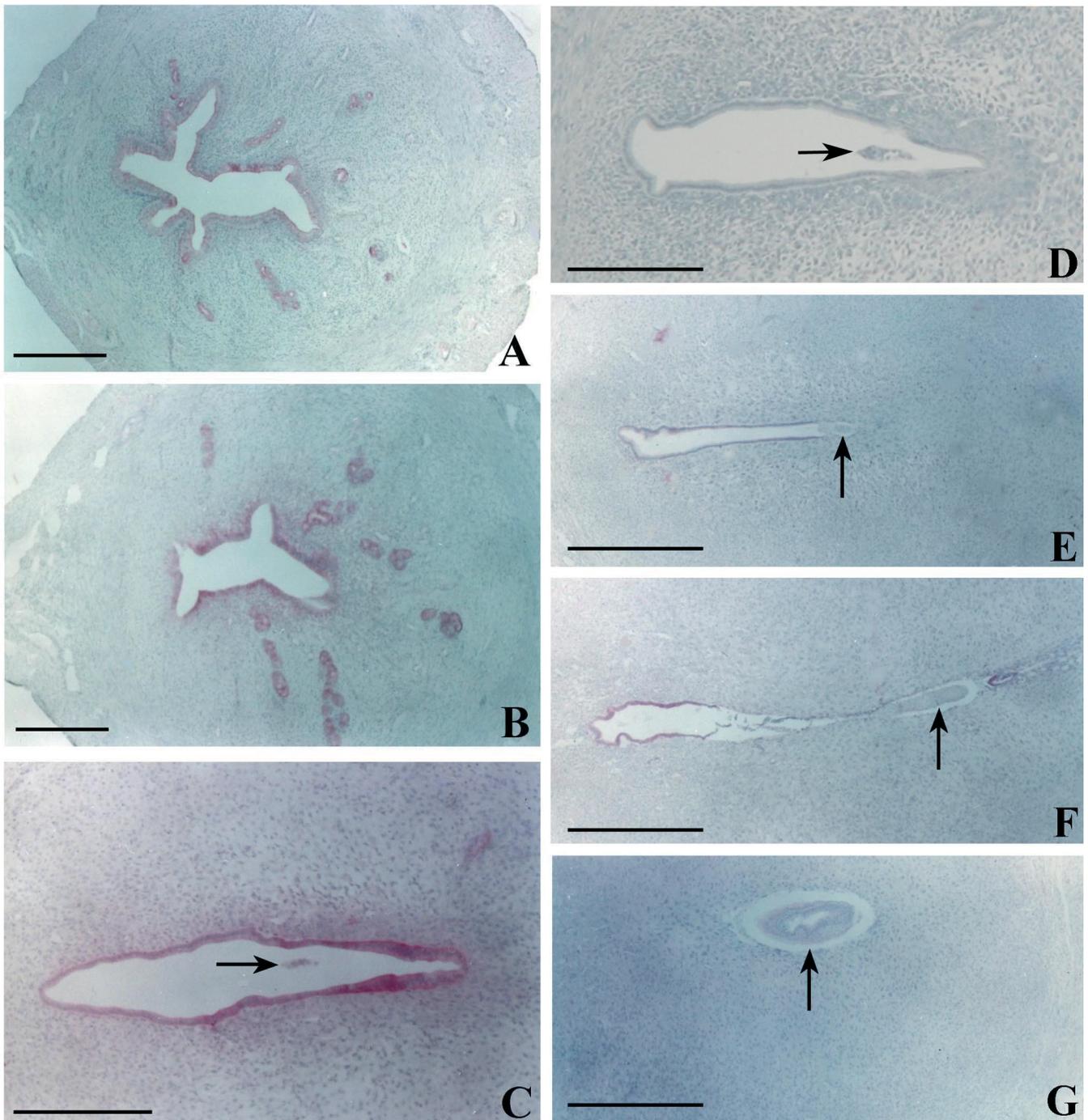


**Fig. 2.** In situ hybridization of EP2 mRNA in rat uterus on days 1 (A), 4 (B), 6 (C), 7 (E), 8 (F), and 9 (G) during early pregnancy. There was a strong level of EP2 mRNA signals in the luminal epithelium on days 6 (C) and 7 (E) of pregnancy. After EP2 antisense probe was replaced by EP2 sense probe, no signals were detected in the uterus on day 6 (D). Arrow: Embryos. Bar: 30  $\mu$ m.

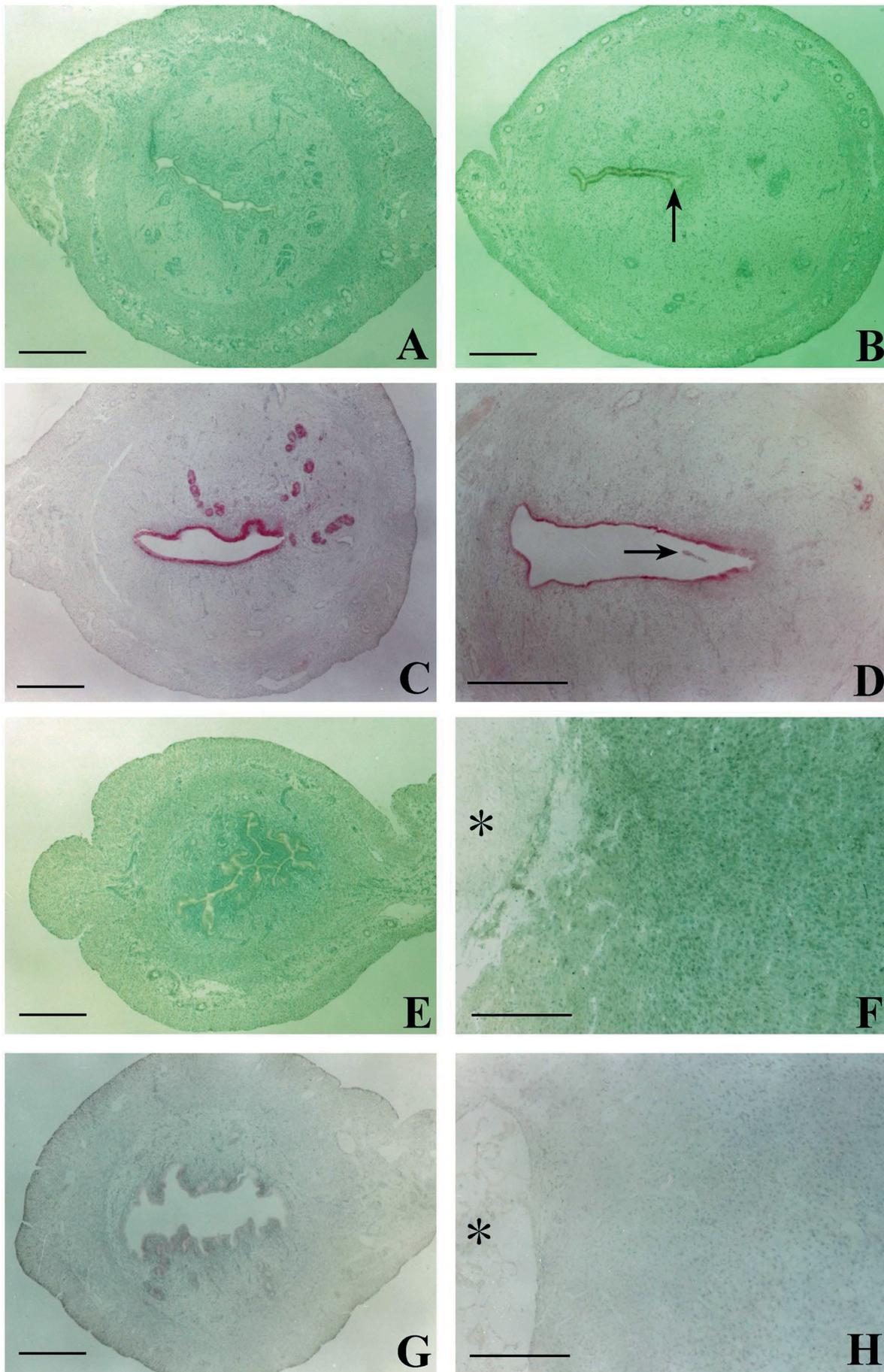
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tissues, whereas the uterine EP2 mRNA was found only in the uterus (Katsuyama et al., 1998). The band at ~53 kDa may be equivalent to macrophage type EP2, and the band at ~50 kDa may be uterine EP2.

There were apparent discrepancies between the in situ hybridization and immunohistochemical data in our study. Although there were no detectable EP2 mRNA signals in the uteri on days 3 and 4 of pregnancy, and



**Fig. 3.** Immunostaining of EP2 protein in rat uterus on days 1 (A), 4 (B), 6 (C), 7 (E), 8 (F), and 9 (G) during early pregnancy. EP2 immunostaining was strongly detected in the luminal epithelium on day 6 of pregnancy (C). However, no immunostaining was seen after rabbit anti-EP2 IgG was replaced by normal rabbit IgG (D). Arrow: Embryos. Bar: 25  $\mu$ m.



**Fig. 4.** EP2 expression in rat uterus under delayed implantation and artificial decidualization. Under delayed implantation, no EP2 mRNA signal was seen (A), while EP2 immunostaining was strongly seen in the luminal and glandular epithelia (C). After delayed implantation was terminated by estrogen treatment and the embryo implanted, both EP2 mRNA (B) and immunostaining (D) were evidently observed in the luminal epithelium. There was no detectable EP2 mRNA signal in the control uterus (E) and decidualized uterus (F). A basal level of EP2 immunostaining was seen in the luminal epithelium of control uterus (G), while no EP2 immunostaining was seen in the decidualized uterus (H). Arrow: implanting blastocyst; \*: uterine lumen. Bar: 25  $\mu$ m.

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under delayed implantation, EP2 immunostaining was obviously seen in the luminal and glandular epithelium under these conditions. These discrepancies suggest that the immunostaining method for detecting EP2 protein may be more sensitive than in situ hybridization method for showing EP2 mRNA. Furthermore, EP2 mRNA expression was indeed demonstrated in mouse uteri from days 1 to 4 of pregnancy and under delayed implantation (Lim and Dey, 1997).

EP2 expression in the rat uterus during early pregnancy was examined in this study. Both EP2 mRNA and immunostaining were highly expressed in the luminal epithelium at implantation sites on day 6 of pregnancy, but not detected or at a low level at inter-implantation sites. In the mouse, EP2 mRNA is expressed exclusively in the luminal epithelium primarily on day 4 (the day of implantation) and day 5 (early implantation) of pregnancy (Lim and Dey, 1997). In the pseudopregnant rat uterus, no specific EP2 expression were detected in any cell type on day 4 of pseudopregnancy, while strong signals were detected specifically in the luminal epithelium by day 5 of pseudopregnancy (Papay and Kennedy, 2000). There was no detectable EP2 mRNA signal in the rat uterus under delayed implantation, but EP2 mRNA signal was observed in the luminal epithelium at the implantation site after activation. However, EP2 immunostaining in the luminal epithelium was detected in the uteri both under delayed implantation and after activation. In the mouse, EP2 mRNA was present in the luminal epithelium under delayed implantation and the expression was further enhanced regardless of the location of the blastocysts after reinitiation of implantation (Lim and Dey, 1997). Because EP2 expression was detected in the mouse and rat uteri under the progesterone-maintained delayed implantation, these data may also suggest that EP2 expression in rodent uteri may be under the regulation of progesterone and estrogen. Indeed, in the adult ovariectomized mice, estrogen down-regulated the basal levels of EP2 mRNA, whereas progesterone up-regulated its levels in the luminal epithelium. The up-regulation of EP2 mRNA levels by progesterone was further augmented by estrogen treatment (Lim and Dey, 1997). A tissue-specific potential progesterone response element was found in the 2 kb segment containing the immediate 5'-flanking and 5'-noncoding regions of the EP2 gene in the pseudopregnant mouse uterus, which is very different from macrophage, suggesting that EP2 gene expression in the macrophage and uterus may be under the control of distinct mechanisms involving alternative promoters (Katsuyama et al., 1998).

EP2 is highly expressed in the luminal epithelium of rat and mouse uteri during the peri-implantation period, suggesting that EP2 could be a potential mediator of PGE<sub>2</sub> actions in regulating luminal epithelial differentiation. Previous studies have shown that a de-epithelialized stroma fails to decidualize after application of a decidual stimulus, suggesting that the stimulus

must transduce a "decidual" signal via the luminal epithelium (Lejeune et al., 1981). PGE<sub>2</sub> is able to restore the endometrial vascular permeability response and decidualization when infused into the lumen of sensitized rat uteri in which endogenous PG synthesis is inhibited (Hamilton and Kennedy, 1994). PGE<sub>2</sub> also exerts an immunomodulation that helps to prevent rejection of the conceptus (Lala, 1990). Interferon- $\tau$  (IFN- $\tau$ ) is the recognized pregnancy recognition signal produced by the embryo in ruminants, and in the bovine it is produced maximally between days 15 and 19 of pregnancy (Bartol et al., 1985). In bovine uterus, IFN- $\tau$  increases EP2 in endometrial stroma and in smooth muscle cells of myometrium (Arosh et al., 2004).

In a study, EP2-deficient females yielded significantly fewer implantation sites when mated to either wild-type or EP2 null males as compared with the wild-type females (Breyer et al., 2000). However, Hizaki et al. (1999) found that there was no difference in the ability of the mouse uterus to support implantation in EP2-deficient females. It is possible that PGE<sub>2</sub> may act via an alternate receptor such as EP4 receptor to prevent implantation defects in the EP2-deficient females because both EP2 and EP4 subtypes are coupled to the stimulation of adenylyl cyclase via Gs (Negishi et al., 1995). In the rat, PGI<sub>2</sub> and PGE<sub>2</sub> are higher in implantation sites than in the surrounding uterus (Kennedy, 1977; Kennedy and Zamecnik, 1978). The level of PGI<sub>2</sub> at mouse implantation sites was the highest followed by PGE<sub>2</sub> and other prostanoids. In addition, the level of PGE<sub>2</sub> was significantly higher at the implantation sites compared with the interimplantation sites (Lim et al., 1999). PGE<sub>2</sub> has a major role for inducing implantation of mouse blastocysts (Holmes and Gordashko, 1980). There are four isoforms of PGES for the enzymatic conversion of PGH<sub>2</sub> into PGE<sub>2</sub>, including membrane-associated PGES (mPGES)-1, mPGES-2, cytosolic PGES (cPGES) and GSTm (Murakami and Kudo, 2004). Both mPGES-1 and cPGES were highly expressed at implantation sites in the mouse uterus (Ni et al., 2002, 2003). mPGES-1 is also highly expressed at implantation sites in hamster uterus (Wang et al., 2004). These data suggest that PGE<sub>2</sub> produced in implantation sites through the enzymatic conversion of PGES may play an important role in implantation by acting through EP2 receptor. However, COX-2-derived PGI<sub>2</sub> is also shown to be the primary PG that is essential for implantation (Lim et al., 1999). It is possible that implantation is mediated by both PGE<sub>2</sub> and PGI<sub>2</sub>.

In conclusion, both EP2 mRNA and protein expressions were strongly observed in the luminal epithelium at the implantation site, suggesting that EP2 may play an important role during embryo implantation in rats.

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