Differential expression and regulation of cyclooxygenases, prostaglandin E synthases and prostacyclin synthase in rat uterus during the peri-implantation period

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Abstract

It has been shown that both prostaglandin I₂ (PGI₂) and PGE₂ are essential for mouse implantation, whereas only PGE₂ is required for hamster implantation. To date, the expression and regulation of cyclooxygenase (COX) and prostaglandin E synthase (PGES), which are responsible for PGE₂ production, have not been reported in the rat. The aim of this study was to examine the expression pattern and regulation of COX-1, COX-2, membrane-associated PGES-1 (mPGES-1), mPGES-2 and cytosolic PGES (cPGES) in rat uterus during early pregnancy and pseudopregnancy, and under delayed implantation. At implantation site on day 6 of pregnancy, COX-1 immunostaining was highly visible in the luminal epithelium, and COX-2 immunostaining was clearly observed in the subluminal stroma. Both mPGES-1 mRNA and protein were only observed in the subluminal stroma surrounding the implanting blastocyst at the implantation site on day 6 of pregnancy, but were not seen in the inter-implantation site on day 6 of pregnancy and on day 6 of pseudopregnancy. Our data suggest that the presence of an active blastocyst is required for mPGES-1 expression at the implantation site. When pregnant rats on day 5 were treated with nimesulide for 24 h, mPGES-1 protein expression was completely inhibited. cPGES immunostaining was clearly observed in the luminal epithelium and subluminal stromal cells immediately surrounding the implanting blastocyst on day 6 of pregnancy. mPGES-2 immunostaining was clearly seen in the luminal epithelium at the implantation site. Additionally, immunostaining for prostaglandin I synthase (PGIS) was also strongly detected at the implantation site. In conclusion, our results indicate that PGE₂ and PGI₂ should have a very important role in rat implantation.

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Introduction

One of the first detectable signs of implantation is an increase in endometrial vascular permeability at the sites of blastocyst apposition and the initiation of the decidual cell reaction (Psychoyos 1986). It has been shown that prostaglandin E₂ (PGE₂) is able to restore the endometrial vascular permeability response and decidualization (Kennedy & Lukash 1982, Keys & Kennedy 1990, Hamilton & Kennedy 1994). Additionally, PGE₂ plays a major role in the hatching of mouse blastocysts (Baskar *et al.* 1981), is able to induce implantation of mouse blastocysts (Holmes & Gordashko 1980) and may also have immunomodulatory roles at the implantation site (Lala 1989).

Cyclooxygenase (COX) exists in two isoforms, COX-1 and COX-2, and can catalyze the bis-oxygenation of arachidonic acid leading to the production of PGG₂ and the reduction of 15-hydroperoxide of PGG₂ to form PGH₂ (Smith *et al.* 2000). COX-1 appears to be constitutively synthesized in many tissues (Seibert *et al.* 1994, Samet

et al. 1995). However, COX-2 expression is up-regulated in response to various pro-inflammatory or mitogenic agents (Hla & Neilson 1992, Smith *et al.* 1994). In mouse uterus during early pregnancy, COX-1 is present before implantation in the luminal epithelium and subepithelial stromal cells, whereas COX-2 is locally expressed in the uterus in the region surrounding the implanting blastocyst at the time of embryo attachment and persists until early on day 5 (Chakraborty *et al.* 1996). In gene knockout studies with COXs, COX-1-deficient female mice are fertile with specific parturition defects, whereas COX-2-deficient females are infertile with abnormalities in ovulation, fertilization, implantation and decidualization (Dinchuk *et al.* 1995, Langenbach *et al.* 1995, Lim *et al.* 1997). Recently, Wang *et al.* (2004a) reported that the reproductive abnormalities in COX-2-deficient mice depend upon genetic background.

PGE₂ can bind to and activate a set of functionally distinct cell surface receptors – EP₁, EP₂, EP₃

and EP4 – which are classified on the basis of their responses to various agonists and antagonists to PGE₂ (Negishi *et al.* 1995). PGE synthase (PGES) is a terminal prostanoid synthase and can enzymatically convert the cyclooxygenase product PGH₂ to PGE₂. There are four isoforms of PGES: membrane-associated PGES (mPGES)-1, mPGES-2, cytosolic PGES (cPGES) and glutathione S-transferase (GST) μ (Murakami & Kudo 2004). mPGES-1, a membrane-associated and inducible perinuclear enzyme with glutathione-dependent activity, is expressed in a variety of tissues including prostate, testis and small intestine (Jakobsson *et al.* 1999). mPGES-1 is preferentially coupled with the inducible COX-2 to promote delayed PGE₂ generation and, if COX-2 already exists in cells, can also regulate immediate PGE₂ generation (Murakami *et al.* 2000). mPGES-2, constitutively expressed in various cells and tissues, can be coupled with both COX-1 and COX-2, and may play a role in the production of the PGE₂ involved in both tissue homeostasis and disease (Murakami *et al.* 2003). cPGES is constitutively expressed in a wide variety of cells and tissues, and is predominantly linked with COX-1 to promote the immediate response, during which a relatively high concentration of arachidonic acid is released in a short period (Tanioka *et al.* 2000).

mPGES-1 mRNA and protein were highly expressed in the stroma immediately surrounding the blastocyst but not in the luminal epithelium in mouse uterus on day 5 of pregnancy. There was no mPGES-1 expression on day 5 of pseudopregnancy and under delayed implantation, suggesting that mPGES-1 expression at the implantation site requires the presence of an active blastocyst (Ni *et al.* 2002). mPGES-1 is also highly expressed at implantation sites in hamster uterus (Wang *et al.* 2004b). In mouse uterus, cPGES expression is at a high level at implantation sites and in the decidual cells (Ni *et al.* 2003). However, the expression pattern of mPGES-2 in the rodent uterus has yet to be examined.

Among various prostaglandins, PGE₂ and prostacyclin (PGI₂) have been considered as the primary candidates involved in implantation and decidualization in the mouse (Lim *et al.* 1999, Ni *et al.* 2002). In contrast, in the rat, PGE₂ but not PGI₂ is a key mediator of increased vascular permeability at the implantation site (Kennedy 1979). PGE₂ also plays a key role during hamster implantation (Wang *et al.* 2004b). To date, the expression and regulation of COX and PGES in rat uterus during early pregnancy have not been reported. The aim of this study was to examine the expression pattern and regulation of COX-1, COX-2, mPGES-1, mPGES-2, cPGES and prostacyclin synthase (PGIS) in rat uterus during early pregnancy.

Materials and Methods

Animals and treatments

Mature rats (Sprague–Dawley strain) were caged in a controlled environment (14 h light:10 h darkness). 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 (day 1 = the day of vaginal plug). Pregnancy on days 1–5 was confirmed by recovering embryos from the reproductive tracts. The implantation sites on day 6 were identified by i.v. injection of 1 ml of 1% Chicago blue (Sigma) in 0.85% sodium chloride. At least three rats were used in each stage or treatment in this study.

Pseudopregnancy was induced by caging adult females with vasectomized males and was confirmed by checking the vaginal plug (either in the vagina or on the cage floor) the next morning. The uteri were collected on days 1, 5 and 6 of pseudopregnancy respectively.

To induce delayed implantation, the pregnant rats on day 4 of pregnancy were treated with progesterone (5 mg/rat, s.c.; Sigma) and then ovariectomized at 0830–0900 h. Progesterone (5 mg/rat) was injected to maintain delayed implantation from days 5–7. The progesterone-primed delayed-implantation rats were treated with estradiol-17 β (250 ng/rat; Sigma) to terminate delayed implantation. The rats were killed by stunning and cervical dislocation to collect uteri at 24 and 36 h after estrogen treatment. The implantation sites were also identified by i.v. injection of Chicago blue solution. Delayed implantation was confirmed by flushing the blastocysts from the uterus.

Treatments with inhibitors

In order to examine the effects of nimesulide (a COX-2-specific inhibitor) on implantation, pregnant rats were treated with nimesulide (40 mg/kg in 0.2 ml DMSO; Cayman Chemical, Ann Arbor, MI, USA) on day 5 for 24 h or treated twice on both days 5 and 6 for 48 h. The rats were killed to collect uteri at 24 and 48 h respectively.

Immunohistochemistry

Rat uteri were immediately fixed in Bouin's solution for 24 h, dehydrated and embedded in paraffin wax. Sections (7 μ m) were cut, deparaffinized and rehydrated. Non-specific binding was blocked in 10% (v/v) normal horse serum in PBS at 37 °C for 1 h. The sections were incubated with rabbit anti-human mPGES-1, rabbit anti-human cPGES, rabbit anti-human mPGES-2, rabbit anti-human COX-2, rabbit anti-mouse PGIS (Cayman Chemical) or goat anti-human COX-1 (Santa Cruz Biotechnology, Inc., CA, USA) in 10% (v/v) horse serum at 4 °C overnight. The sections were then incubated with biotinylated goat anti-rabbit IgG or biotinylated rabbit anti-goat IgG followed by a streptavidin–alkaline phosphatase complex and Vector Red according to the manufacturer's protocol (Vectastain ABC-AP kit; Vector Laboratories, Burlingame, CA, USA). Vector Red was visualized as a red color. Endogenous alkaline phosphatase activity was inhibited by supplementing 1 mM levamisole (Sigma) into the Vector Red substrate solution. Moreover, the same concentration of

normal rabbit IgG or goat IgG was used in place of the corresponding primary antibody as a negative control. For rabbit anti-mPGES-1 antibody, a specific blocking peptide (Cayman Chemical) was preincubated with the primary antibody for 30 min and then incubated with sections. The sections were counter-stained with hematoxylin and mounted. The degree of immunostaining was assessed subjectively by blinded examination of the slides by two investigators.

In situ hybridization

A 427 bp (108–518 bp) mPGES-1 cDNA fragment was reverse transcribed and amplified with forward primer 5'-CGGAATTCACACTGCTGGTCATCAAG and reverse primer 5'-CGGGATCCTTCAGCTGCTGGTCACAG (Genbank accession number AB041997) with the total RNA from mouse uterus. In these primers, protection bases (CG) and an EcoRI site were added at the 5' end of the forward primer, and protection bases (CG) and a BamHI site at the 5' end of the reverse primer. The PCR fragment for mPGES-1 was recovered from the agarose gel and cloned into pGEM-3Zf (+) plasmid through EcoRI and BamHI sites respectively. The cloned mPGES-1 fragment was further verified by sequencing. These plasmids were linearized with appropriate enzymes for labeling. Digoxigenin (DIG)-labeled antisense or sense cRNA probes were transcribed *in vitro* using a DIG RNA labeling kit (T7 for sense, SP6 for antisense; Roche).

Uteri were cut into 4–6 mm pieces and flash frozen in liquid nitrogen. Frozen sections (10 μ m) were mounted on 3-aminopropyltriethoxy-silane (Sigma)-coated slides and fixed in 4% paraformaldehyde solution in PBS. The sections were washed in PBS twice, treated in 1% (v/v) Triton-100/PBS for 20 min and again washed three times in PBS. Following prehybridization washes in 50% (v/v) formamide and 5 $\times$ SSC (1 $\times$ SSC is 0.15 M sodium chloride, 0.015 M sodium citrate) at room temperature for 15 min, the sections were hybridized in the hybridization buffer (5 $\times$ SSC, 50% (v/v) formamide, 0.02% (w/v) BSA, 250 μ g/ml yeast tRNA, 10% (w/v) dextran sulfate, 1 μ g/ml denatured DIG-labeled antisense or sense RNA probe for mouse mPGES-1) at 55°C for 16 h. After hybridization, the sections were washed for stringency in 50% (v/v) formamide/5 $\times$ SSC at 55°C for 15 min, 50% (v/v) formamide/2 $\times$ SSC at 55°C for 30 min, 50% (v/v) formamide/0.2 $\times$ SSC at 55°C twice for 30 min each and 0.2 $\times$ SSC at room temperature for 5 min. After non-specific binding was blocked in 1% (w/v) block mix (Roche) for 1 h, the sections were incubated in sheep anti-DIG antibody conjugated to alkaline phosphatase (1:5000; Roche) in 1% block mix (Roche) overnight at 4°C. The signal was visualized with 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate and 0.4 mM nitroblue tetrazolium in the buffer containing 100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 50 mM MgCl₂. Endogenous alkaline phosphatase activity was inhibited with 2 mM levamisole

(Sigma). Sections were counter-stained with 1% (w/v) methyl green in 0.12 M glacial acetic acid and 0.08 M sodium acetate for 30 min.

Results

COX-1 immunostaining during early pregnancy

COX-1 immunostaining in the rat uterus during early pregnancy is shown in Fig. 1. There was a low level of COX-1 immunostaining in the luminal epithelium on days 1–3 of pregnancy (Fig. 1A). On days 4 and 5, COX-1 immunostaining reached a high level in the luminal epithelium (Fig. 1B). On day 6, COX-1 immunostaining was highly visible in the luminal epithelium and at a basal level in the subluminal stroma at the implantation site (Fig. 1C), whereas a slightly lower level of COX-1 immunostaining was detected in the luminal epithelium at the inter-implantation site (Fig. 1D). On day 7, a low level of COX-1 immunostaining was seen in the luminal epithelium and primary decidua (Fig. 1E). On days 8 and 9, a low level of COX-1 immunostaining was observed in the whole decidua (Fig. 1F).

During pseudopregnancy, COX-1 immunostaining was not seen in the uterus on day 1. However, a high level of COX-1 immunostaining was observed in the luminal epithelium on days 5 and 6 of pseudopregnancy (Fig. 1G and H).

COX-2 immunostaining in the rat uterus during early pregnancy

COX-2 immunostaining in the rat uterus during early pregnancy is shown in Fig. 2. There was no detectable COX-2 immunostaining in the uteri on days 1 and 2 of pregnancy (data not shown). On days 3 and 4, a low level of COX-2 immunostaining was located in the scattered population of subluminal stromal cells (Fig. 2A and B). In the morning of day 5, COX-2 immunostaining was strongly localized in the stromal cells underlying the luminal epithelium (Fig. 2C). At 2400 h on day 5 when the attachment reaction began, COX-2 immunostaining was also strongly detected in the subluminal stroma (Fig. 2D). At the implantation site on day 6, COX-2 immunostaining was detected in the stromal cells, but not in the primary decidua at the anti-mesometrial side (Fig. 2E). However, COX-2 immunostaining was strongly detected in the whole stromal cells near the lumen at the anti-mesometrial side at the inter-implantation site on day 6 (Fig. 2F). On day 7, COX-2 immunostaining was detected in both the secondary decidua and a layer of decidua cells underlying the luminal epithelium, but not in the rest of the primary decidua (Fig. 2G). On days 8 and 9, COX-2 immunostaining was weak in the stromal cells on the mesometrium side and highly visible in the connective tissues between the longitudinal and circular muscles, but not in the decidua (Fig. 2H).

Figure 1 COX-1 immunostaining in rat uteri during early pregnancy on days 1 (A), 5 (B), 6 (C, implantation site), 6 (D, inter-implantation site), 7 (E) and 9 (F). COX-1 immunostaining was also observed in the luminal epithelium in rat uteri on days 5 (G) and 6 (H) during pseudopregnancy. Abbreviations: de, deciduas; g, gland; le, luminal epithelium; lu, lumen; st, stroma. Arrows indicate embryos; scale bar, 60 μ m.

On day 5 of pseudopregnancy, COX-2 immunostaining was detected in the subluminal stroma at the anti-mesometrial side (Fig. 2I). However, no immunostaining was seen in the uterus on day 6 of pseudopregnancy (Fig. 2J).

mPGES-1 mRNA expression during early pregnancy

In situ hybridization of mPGES-1 mRNA is shown in Fig. 3. On days 1–5, there were no detectable mPGES-1 mRNA signals in the whole uteri (Fig. 3A). However, a strong level of mPGES-1 signal was observed in the subluminal stroma immediately surrounding the implanting blastocyst, but not in the luminal and glandular epithelia at 0800 h on day 6 (Fig. 3B). No equivalent signals were seen in the inter-implantation areas on day 6 (Fig. 3C). In addition, after the mPGES-1 sense probe was used in place of the antisense probe, there were no detectable signals in the uterus on day 6 (Fig. 3D). At 2000 and 2400 h on day 6, strong mPGES-1 signals were also observed in the subluminal stroma, similar to those at 0800 h on day 6 (Fig. 3E and F). On day 7, mPGES-1 signals were highly visible in the primary decidua (Fig. 3G). On days 8 and 9, mPGES-1

signals were strongly detected in the subluminal decidual cells at the mesometrial side (Fig. 3H).

mPGES-1 immunostaining during early pregnancy

The pattern of mPGES-1 immunostaining during early pregnancy is similar to that of mPGES-1 mRNA signals and is shown in Fig. 4. There was no detectable signal on days 1–5 (Fig. 4A). At 0800 h on day 6, mPGES-1 immunostaining was clearly observed in the subluminal stroma immediately surrounding the implanting blastocyst (Fig. 4B), whereas no signal was seen in the inter-implantation area (Fig. 4C). Compared with the morning, mPGES-1 immunostaining was detected in a slightly larger area of subluminal stroma at 2000 h on day 6 (Fig. 4D). After rabbit anti-human mPGES-1 antibody was preincubated with mPGES-1-blocking peptide, no signals were observed in the uterus (Fig. 4E). On day 7, mPGES-1 immunostaining was also highly visible in the subluminal decidual cells surrounding the embryo, but not on the mesometrial side (Fig. 4F). On day 8, mPGES-1 immunostaining was strongly detected in the primary decidua (Fig. 4G). On day 9, mPGES-1 immunostaining was strongly localized

Figure 2 COX-2 immunostaining in rat uteri during early pregnancy on days 3 (A), 4 (B), 5 (C), 5 (D, at 2400 h), 6 (E, implantation site), 6 (F, inter-implantation site), 7 (G) and 9 (H). During pseudopregnancy, COX-2 immunostaining was seen in the subluminal stromal cells on day 5 (I), but not in rat uterus on day 6 (J). Arrows indicate embryos; scale bar, 60 μ m.

in the primary decidua and the decidual area on the mesometrial side (Fig. 4H).

mPGES-1 expression during pseudopregnancy

From days 1 to 6 of pseudopregnancy, there were no detectable signals for both mPGES-1 mRNA and immunostaining in the uterus (data not shown).

mPGES-1 expression under delayed implantation and activation of implantation

Under delayed implantation, there were no detectable signals for either mPGES-1 mRNA or immunostaining (Fig. 5A and B). A low level of mPGES-1 mRNA and protein was

detected in the subluminal stroma immediately surrounding the implanted blastocysts 24 h after delayed implantation was terminated by estrogen treatment (Fig. 5C and D). When delayed implantation was terminated by estrogen treatment for 36 h, both mPGES-1 mRNA signals and immunostaining were strongly localized in the subluminal stroma immediately surrounding the implanted blastocysts (Fig. 5E and F), which were similar to the mPGES-1 expression on day 6 of pregnancy.

Effects of nimesulide on mPGES-1 expression at the implantation site

In order to examine the effects of nimesulide on mPGES-1 expression, pregnant rats on day 5 were treated once with

Figure 3 *In situ* hybridization of mPGES-1 mRNA in rat uteri during early pregnancy on days 5 (A), 6 (B, implantation site at 0800 h), 6 (C, inter-implantation site), 6 (E, implantation site at 2000 h), 6 (F, implantation site at 2400 h), 7 (G) and 9 (H). When the mPGES-1 sense probe was used in place of the antisense probe, there was no detectable signal in the uterus on day 6 of pregnancy (D). Arrows indicate embryos; scale bar, 60 μ m.

nimesulide for 24 h or treated twice on both days 5 and 6 for 48 h. In the control, both mPGES-1 mRNA signals and immunostaining were strongly detected in the subluminal stroma immediately surrounding the implanted blastocysts like the mPGES-1 expression on day 6 (Fig. 6A and B). When pregnant rats were treated with nimesulide for 24 h on day 5, although there were no detectable changes in mPGES-1 mRNA signals (Fig. 6C), mPGES-1 immunostaining was significantly reduced to an undetectable level (Fig. 6D). In the control on day 7, both mPGES-1 mRNA signals and immunostaining were highly visible in the subluminal primary decidual cells (Fig. 6E and F). After pregnant rats were treated with nimesulide for 48 h, the signals for both mPGES-1 mRNA and immunostaining were significantly reduced to a low level (Fig. 6G and H).

cPGES immunostaining during early pregnancy

There was no detectable cPGES immunostaining on days 1–5 of pregnancy (Fig. 7A and B). On day 6, cPGES

immunostaining was highly visible in the luminal epithelium and subluminal stroma surrounding the implanted blastocyst (Fig. 7C), but was not seen in the inter-implantation site (Fig. 7D). On day 7, cPGES immunostaining was mainly seen in the primary decidua (Fig. 7E). On day 8, cPGES immunostaining was detected in the whole decidual cells (Fig. 7F).

mPGES-2 immunostaining during early pregnancy

There was a high level of mPGES-2 immunostaining in the luminal epithelium and a basal level in the glandular epithelium on day 1 (Fig. 8A). On days 2 and 3, mPGES-2 immunostaining was at an intermediate level in both luminal and glandular epithelia and at a low level in the subluminal stroma (Fig. 8B). On days 4 and 5, a high level of mPGES-2 was seen in both luminal and glandular epithelia (Fig. 8C). Moreover, a similar pattern of mPGES-2 immunostaining was also seen on day 5 of pseudopregnancy (Fig. 8D). On day 6, mPGES-

Figure 4 mPGES-1 immunostaining in rat uteri during early pregnancy on days 5 (A), 6 (B, implantation site at 0800 h), 6 (C, inter-implantation site), 6 (D, implantation site at 2000 h), 7 (F), 8 (G) and 9 (H). After rabbit anti-human mPGES-1 antibody was preincubated with mPGES-1 blocking peptide, there was no detectable immunostaining in rat uterus at 2000 h on day 6 of pregnancy (E). Arrows indicate embryos; scale bar, 60 μ m.

2 immunostaining was at a high level in both luminal and glandular epithelia, and at a low level in the subluminal stroma at the implantation site (Fig. 8E), whereas mPGES-2 immunostaining was not seen in the inter-implantation site (Fig. 8F). However, there was only a low level of mPGES-2 immunostaining in both luminal and glandular epithelia on day 6 of pseudopregnancy (Fig. 8G). On days 7–9 of pregnancy, mPGES-2 immunostaining was highly visible in the decidual cells (Fig. 8H).

PGIS immunostaining in the rat uterus during early pregnancy

PGIS immunostaining was strongly detected in the myometrium on days 1–9 of pregnancy (Fig. 9A, B and H). There was no detectable PGIS immunostaining in the luminal and glandular epithelia, and in the subluminal stroma, on days 1–5 of pregnancy (Fig. 9A and B). PGIS immunostaining was strongly detected in the luminal and glandular epithelia, and the stroma surrounding the implanting blastocyst, on day 6 of pregnancy (Fig. 9C), whereas there was a weak PGIS immunostaining only in the luminal epithelium and the subluminal stroma in the inter-implantation area (Fig. 9D). Similarly, a low level of PGIS immunostaining was seen in the luminal and glandular epithelia on day 6

of pseudopregnancy (Fig. 9E). On day 7 of pregnancy, PGIS immunostaining was also highly seen in the myometrium and primary decidua (Fig. 9F). After rabbit normal IgG was used to replace rabbit anti-PGIS antibody, no immunostaining was seen in the uterus on day 7 of pregnancy (Fig. 9G). PGIS immunostaining was strongly detected in the decidual cells on days 8 and 9 of pregnancy (Fig. 9H).

Discussion

In this study, both mPGES-1 mRNA and protein were highly visible in the subluminal stroma surrounding the implanting blastocyst at the implantation site on day 6, but were not seen in the inter-implantation site on day 6 of pregnancy or in the rat uterus on day 6 of pseudopregnancy. Moreover, both mPGES-1 mRNA and protein expression were detected after delayed implantation was terminated by estrogen treatments and embryos implanted. These results are consistent with those found in the mouse uterus (Ni *et al.* 2002), but slightly different from results in the hamster (Wang *et al.* 2004b). In hamster uterus, mPGES-1 was highly expressed in both luminal epithelium and stromal cells around the implanting blastocyst on day 5, and mainly in primary decidual cells on day 6 (Wang *et al.* 2004b). However, mPGES-1 expression was not observed in the luminal epithelium in

Figure 5 *In situ* hybridization of mPGES-1 mRNA and mPGES-1 immunostaining in rat uterus under delayed implantation and after activation with estrogen treatment. Arrows indicate implanting blastocysts; scale bar, 60 μ m.

either rat or mouse uterus (Ni *et al.* 2002). Our data suggest that mPGES-1 expression in rat uterus required the presence of an active blastocyst during the peri-implantation period.

Because mPGES-1 is preferentially coupled with the inducible COX-2 to promote delayed PGE₂ generation (Murakami *et al.* 2000), we also examined COX-2 immunostaining in rat uterus. COX-2 immunostaining was strongly detected in the subluminal stromal cells on the morning of day 5, and also in the subluminal stromal cells surrounding the implanting blastocyst at midnight on day 5 of pregnancy. However, COX-2 immunostaining was seen in the secondary decidua but not in the primary decidua on the morning of day 6. Although the COX-2 protein was not co-localized with mPGES-1 protein in the subluminal stromal cells at implantation sites, it is possible that the high level of COX-2 protein in this location may produce enough PGH₂ for mPGES-1 to synthesize PGE₂ in a slightly later stage.

Nevertheless, mPGES-1-deficient mice are viable and fertile, and develop normally compared with wild-type controls. mPGES-1-deficient mice displayed a marked reduction in inflammatory responses compared with mPGES-1 wild-type mice in multiple assays (Trebino *et al.* 2003). In another study, mPGES-1-deficient mice did not exhibit any detectable abnormality in the reproduction system either (Uematsu *et al.* 2002); in this system another PGES might compensate for mPGES-1 deficiency. In our study, cPGES and mPGES-2 were also strongly detected at the implantation site. During embryo implantation, it is possible that both cPGES and mPGES-2 might compensate

Figure 6 Effects of nimesulide on mPGES-1 expression in pregnant rat uterus. Pregnant rats were treated with nimesulide (40 mg/kg in 0.2 ml DMSO) on day 5 for 24 h or were treated twice on both days 5 and 6 for 48 h. Arrows indicate implanting blastocysts; scale bar, 60 μ m.

Figure 7 cPGES immunostaining in rat uteri on days 1 (A), 5 (B), 6 (C, implantation site), 6 (D, inter-implantation site), 7 (E) and 8 (F) respectively. Arrows indicate embryos; scale bar, 60 μ m.

for the mPGES-1 deficiency. In the mouse, COX-2 compensation occurs in the absence of COX-1 (Reese *et al.* 1999). Moreover, COX-1 can replace specific functions of COX-2 *in vivo* in the context of genetic disparity (Wang *et al.* 2004a). This kind of functional compensation among PGES isoforms has yet to be identified. A better approach is to see what kind of phenotypes could be obtained after all three PGES isoforms are deleted.

When pregnant rats were treated with nimesulide for 24 h on day 5, mPGES-1 protein expression was completely inhibited although implantation sites were still visible and mPGES-1 mRNA was still highly expressed in the sub-luminal stromal cells, compared with control. Additionally, both mPGES-1 mRNA and protein expression was significantly inhibited by 48-h nimesulide treatment on day 5. Our results were consistent with previous studies with COX inhibitors. Several papers have shown that COX inhibitors had no significant effects on endometrial vascular permeability (Kennedy 1977, Phillips & Poyser 1981, Sookvanichsilp & Pulbutr 2002). However, COX-2 inhibitors could significantly reduce the uterine decidualization (Sookvanichsilp & Pulbutr 2002). We also found that the size of the implantation sites and decidualization were severely affected by nimesulide, although nimesulide had no effect on the number of implantation sites (J. Cong

et al., unpublished data). When infused into the lumen of sensitized rat uteri in which endogenous PG synthesis is inhibited, PGE₂ is able to restore the endometrial vascular permeability response and decidualization (Kennedy & Lukash 1982, Keys & Kennedy 1990, Hamilton & Kennedy 1994). In steroid-injected ovariectomized rats, following unilateral intrauterine instillation of sesame oil, administration of indomethacin inhibited the onset of the decidual cell reaction and had deleterious effects on the luminal epithelium. Inclusion of PGE₂ in the instillate accelerated stromal cell differentiation and overcame the inhibitory effect of indomethacin (Keys & Kennedy 1990). All of these data suggest that PGE₂ is essential for rat decidualization. Mice devoid of COX-1 exhibit parturition defects (Langenbach *et al.* 1995). COX-2-depleted mice showed implantation and decidualization defects during early pregnancy (Dinchuk *et al.* 1995, Lim *et al.* 1997), suggesting the involvement of COX-2-directed PG synthesis in the process of implantation. Furthermore, COX-2 is aberrantly expressed in the uterus surrounding the blastocyst in leukemia inhibitory factor (LIF)($-/-$) mice (Song *et al.* 2000). LIF has been shown to be essential for mouse implantation (Stewart *et al.* 1992).

In the rat uterus, cPGES immunostaining was not seen on days 1–5 of pregnancy. However, cPGES immu-

Figure 8 mPGES-2 immunostaining in rat uteri during early pregnancy on days 1 (A), 3 (B), 5 (C), 6 (E, implantation site), 6 (F, inter-implantation site) and 8 (H). During pseudopregnancy, mPGES-2 immunostaining was shown on days 5 (D) and 6 (G). Arrows indicate embryos; scale bar, 60 μ m.

nostaining was highly visible in the luminal epithelium and subluminal stromal cells immediately surrounding the implanting blastocyst on day 6 of pregnancy; this was slightly different from results in the mouse, where cPGES immunostaining was only detected in the luminal epithelium surrounding the implanting blastocyst, but not in the subluminal stroma at the implantation site on day 5 of pregnancy (Ni *et al.* 2003). Because cPGES is constitutively expressed in a wide variety of cells and tissues, and is predominantly linked with COX-1 to promote an immediate response (Tanioka *et al.* 2000), COX-1 immunostaining in the rat uterus during early pregnancy was examined. Interestingly, COX-1 immunostaining was strongly detected in the luminal epithelium and weakly so in the subluminal stroma at the implantation site on day 6 of pregnancy. The co-localization of COX-1 and cPGES in the luminal epithelium at the implantation site may suggest a role for PGE₂ production through this pathway. In human placenta, apoptotic early gestational syncytiotrophoblasts were heavily stained with cPGES, suggesting that cPGES could be involved in apoptosis or repair mechanisms (Meadows

et al. 2004). It is well documented that the rodent uterine epithelium around the embryo undergoes apoptosis in response to the presence of the blastocyst (Schlafke *et al.* 1985, Parr *et al.* 1987, Welsh & Enders 1991). cPGES may also be involved in apoptosis occurring at the implantation site.

mPGES-2 immunostaining was strongly detected in the luminal epithelium on days 1–5 of pregnancy. On day 6 of pregnancy, mPGES-2 immunostaining was highly visible in the luminal epithelium and weakly seen in the subluminal stromal cells and glandular epithelium at the implantation site. In several cell lines, mPGES-2 promoted PGE₂ production via both COX-1 and COX-2 in the immediate and delayed responses with modest COX-2 preference (Murakami *et al.* 2003). In this study, COX-1 and mPGES-2 were co-localized in the luminal epithelium at the implantation site, but mPGES-2 localization was very different from COX-2. It appears that mPGES-2 was mainly coupled with COX-1 to produce PGE₂ in the luminal epithelium at the implantation site because mPGES-2 immunostaining was not observed in the inter-implantation site.

Figure 9 PGIS immunostaining in rat uteri on days 1 (A), 5 (B), 6 (C), 6 (D, inter-implantation area), 7 (F) and 9 (H) of pregnancy. (E) PGIS immunostaining on day 6 of pseudopregnancy. After rabbit normal IgG was used to replace rabbit anti-PGIS antibody, no immunostaining was detected on day 7 of pregnancy (G). Abbreviation: m, myometrium. Scale bar, 60 μ m. Arrows indicate embryos.

In our study, PGIS was also strongly detected at the implantation site in rat uterus although there was a high level of PGES expression. It was estimated that the concentration of 6-keto-PGF 1α (a stable metabolite of PGI $_2$) in implantation sites on the evening of day 5 is at least 40-fold that in inter-implantation sites in rat uterus (Kennedy & Zamecnik 1978). In mouse uterus, PGI $_2$ is the most dominant prostaglandin followed by PGE $_2$, and is significantly higher at implantation sites than at inter-implantation sites (Lim *et al.* 1999). In contrast, Kennedy (1979) showed that, in the rat, PGE $_2$ but not PGI $_2$ is a key mediator of increased vascular permeability at the implantation site. In rat uterine homogenates during early pseudopregnancy and pregnancy, PGE $_2$ peaked on day 5 of pseudopregnancy and on day 5 of pregnancy. Indomethacin treatment of rats during early pregnancy caused a delay in implantation, a significant reduction in uterine weight, and a much reduced number or absence of implanted blastocysts in the uterus on day 9

(Phillips & Poyser 1981). In hamster, PGE $_2$, but not PGI $_2$, is the major PG at implantation sites where COX-2 and mPGES-1 – but not PGI – synthases are co-expressed. This elevated uterine PGE $_2$ at implantation sites may serve to initiate or amplify physiological signals required for specific aspects of the implantation process in hamsters (Wang *et al.* 2004b). In rhesus monkey uterus, the coupling of cPGES and COX-1 in the luminal epithelium may be responsible for the synthesis of PGE $_2$ in monkey endometrium, and the coupling of mPGES and COX-2 in the glandular epithelium may be of importance for preparing the receptive endometrium (Sun *et al.* 2004).

In conclusion, at the implantation site in rat uterus, COX-1, cPGES and mPGES-2 were highly expressed in the luminal epithelium surrounding the implanting blastocyst, whereas COX-2 and mPGES-1 were highly visible in the subluminal stromal cells surrounding the implanting blastocyst, suggesting that PGE $_2$ should be produced in

large amounts through COX-2/mPGES-1, COX-1/cPGES and/or COX-1/mPGES-2 pathways. Because PGIS is also strongly expressed at the implantation site in rat uterus, it is possible that both PGE2 and PGI2 may be essential for rat implantation.

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