induce labour (Jouppila et al., 1976), and in monkeys the onset of parturition can be delayed by prostaglandin synthesis (Novy et al., 1974). Concentrations of prostaglands in peripheral plasma, amniotic fluid and tissues increase at labour. The prostaglandin F_2 α one of the most abundant prostaglandins, is particularly involved in reproductive functions such as ovulation, luteolysis and parturition (Goldberg and Ramwell, 1975). Sugimoto et al. (1997) developed the mice lacking the prostaglandin F receptor. These mice do not deliver fetuses at term, and continue their pregnancy, although they are normal in other aspects of reproduction physiology. No abnormalities were found in the weight or histology of placentae at this stage in prostaglandin F_2 α receptor-deficient mice. Therefore, this FP-deficient mouse provides a good animal model for studying of the throughout pregnancy, including postterm pregnancy.

The purpose of this study was to investigate the distribution and incidence of apoptosis in the placenta and decidua of pregnant mice lacking the prostaglandin F receptor.

MATERIALS AND METHODS

All animals were maintained in accordance with the institutional guidelines for care and use of laboratory animals. Mice were housed under standard conditions of light (12 h light : 12 h darkness) and temperature (23 ± 1°C). Mice were given free access to a nutritionally balanced diet and tap water.

Prostaglandin F_2 α (FP) receptor-deficient mice were obtained as described previously (Sugimoto et al., 1997). Normal adult female mice with the +/− genotype bred in our animal facility were mated with either FP-deficient male mice or male mice with the +/− genotype, and the resulting female FP-deficient mice and wild type mice were used in the present experiment. The mice genotypes were identified by polymerase chain reaction.

The day a copulation plug was found was considered to be day 0 of pregnancy. Placentae were removed from fetuses on day 10–23 of pregnancy. At the same time, the number of pups, survival, weight, and amniotic fluid were recorded. Three pregnant mice were used for each pregnant day. At least three placentas from different mice for each day of pregnancy were fixed in a 10 per cent formaldehyde neutral buffer solution and embedded in paraffin. Five sections were cut for each sample. One section was stained with hematoxylin and eosin, and the others were used to detect apoptosis. For three other mice for each day of pregnancy, the decidua was isolated by gently separating the placenta and myometrial regions to count the decidua and assess placental weight. The tissues were flash frozen, and stored at −80°C until processed for analysis of DNA fragmentation.

DNA fragmentation assay

The placental and decidual tissues dissected were frozen immediately in liquid nitrogen. Each sample was homogenized in 300–500 μl of lysis buffer (100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 200 mM NaCl, 0.2 per cent SDS, pH 8.0, 0.5 per cent Triton X-100) and 10 μl proteinase K (10 mg/ml) followed by overnight incubation at 37°C. The samples were then incubated with 5 μl of RNase A (10 mg/ml) for 30 min at 37°C, and DNA was extracted with isopropanol. DNA was precipitated with 70 per cent ethanol, air dried, suspended in a TE buffer, incubated at 37°C for 60 min, and left overnight at 4°C DNA (15 μg from each samples) was electrophoretically separated on a 1.8 per cent agarose gel. The gel was stained with 0.02 per cent ethidium bromide to visualize the DNA fragmentation and photographed.

In situ detection of DNA nicking

Detection of fragmentation was performed using the terminal deoxynucleotidyl transferase-mediated dUDP nick end-labelling (TUNEL) technique. Briefly, consecutive sections (5 μM) from formalin-fixed, paraffin-embedded tissue blocks were placed on coated slides for use in a molecular biological-histochemical system. The TUNEL procedure was performed using an Apop Tag kit (Oncor, Gaithersburg, MD, USA) according to the manufacturer's instructions. The tissue sections were deparaffinized and protein was digested with 20 μg/ml proteinase K for 15 min at room temperature. The quenching endogenous peroxidase activity was used with 3 per cent H_2O_2 in phosphate-buffered saline. After washing with PBS, an equilibration buffer was applied directly to the specimen. A terminal deoxynucleotidyl transferase (TdT) enzyme and dUTP-digoxigenin were added and incubated at 37°C for 1 h in a humidified chamber. The reaction was then stopped with a stop/wash buffer supplied with the kit, and the slides were incubated with an anti-digoxigenin-peroxidase solution for 30 min at room temperature, colorized with DAB/H_2O_2, and counterstained with methyl green. Negative controls were processed with labelled dUTP in the absence of the TdT enzyme. Sections of normal rodent mammary gland were used as positive controls. The apoptotic index was obtained by dividing the number of apoptotic cells by the total number of cells counted and multiplying the result by 100.

The significance tests used were the Mann–Whitney U test and the Student's *t*-test. A P value <0.05 was accepted for significant differences between the groups.

RESULTS

To identify the difference in reproduction between wild and FP-deficient mice, we detected the number of pups, survival, weight, and amniotic fluid from 30 mice. We find no differences in the number of pups, weight or amniotic fluid before day 18 of pregnancy. Wild-type mice delivered normal fetuses at term (day 18 or 19), but FP-deficient mice were unable to deliver fetuses. Table 1 showed the changes between the wild type and FP-deficient mice at day 18 of pregnancy.

Table 1. The characteristics between wild type and FP-deficient mice at day 18 of pregnancy

	Wild type mice (n=6)	FP-deficient mice (n=5)	Statistical significance
Maternal mice (week)	12±16	12±16	NS
Parity	0	0	NS
Number of pup	8.3±1.7	7.5±1.5	NS
Weight of pup (g)	1.18±0.12	1.19±0.11	NS
Weight of amniotic fluid (g)	0.102±0.006	0.098±0.007	NS
Weight of placenta (g)	0.107±0.007	0.096±0.006	NS
Weight of decidua (g)	0.026±0.004	0.024±0.003	NS

All values were expressed as means ± s.d. or number; NS, non-significant.

To confirm the changes in the decidua and placenta in FP-deficient mice as gestation progressed, we observed the decidua and placenta weights on the indicated gestational days. As shown in [Figure 1(A, B)], the decidua and placenta weights increased before day 14 and 18, and then decreased with gestational day. After day 19, the amniotic fluid decreased significantly, and the fetuses died gradually in the uterus. Sixty-five per cent of pups died by day 22 of pregnancy. All fetuses died in the uterus by day 23 of pregnancy with no amniotic fluid. The body weights of surviving fetuses increased with gestational day [Figure 1(C)].

To determine whether apoptosis occurred in the mouse placenta and decidua, DNA fragmentation was performed in the placental and decidual tissue. DNA fragments from the placental and decidual tissue can be detected by the 'DNA ladder' on agarose gel electrophoresis (Figure 2). DNA fragmentation was always more pronounced in decidual tissue than placenta on any day of pregnancy. As shown in Figure 2, DNA laddering was not significantly different between wild type and FP-deficient mice and more extensive in day 22 than day 18 in the placental and decidual tissue.

Apoptosis has also been demonstrated in various cell types within placental tissue by the TUNEL method. The similar TUNEL staining results were found in placenta and decidua of FP-deficient mice compared with that wild type mice (Figure 3). In the placenta, most TUNEL-positive cells were found in the trophoblast and stromal cells [Figure 4(A–D)]. A higher intensity of apoptotic cells was in the decidual basalis [Figure 4(E–H)]; however, the apoptotic cells were not uniformly distributed. The main area was the centre of the decidual basalis, and was decreased toward the margin of placenta. The more TUNEL-positive cell of decidua scattered at decidual area near the side villous trophoblast compare with the side uterus.

An increased TUNEL-positive staining in syncytiotrophoblast surface was found in the placenta at postterm, compared with those at term [Figure 4(I–L)]. However, the extent of staining was different at different placentas from the same day 22 of pregnancy or, even same mouse.

The index of TUNEL-positive cells were not significantly different in placenta and decidua of FP-deficient mice compared with that of wild type mice before day 18 of pregnancy.

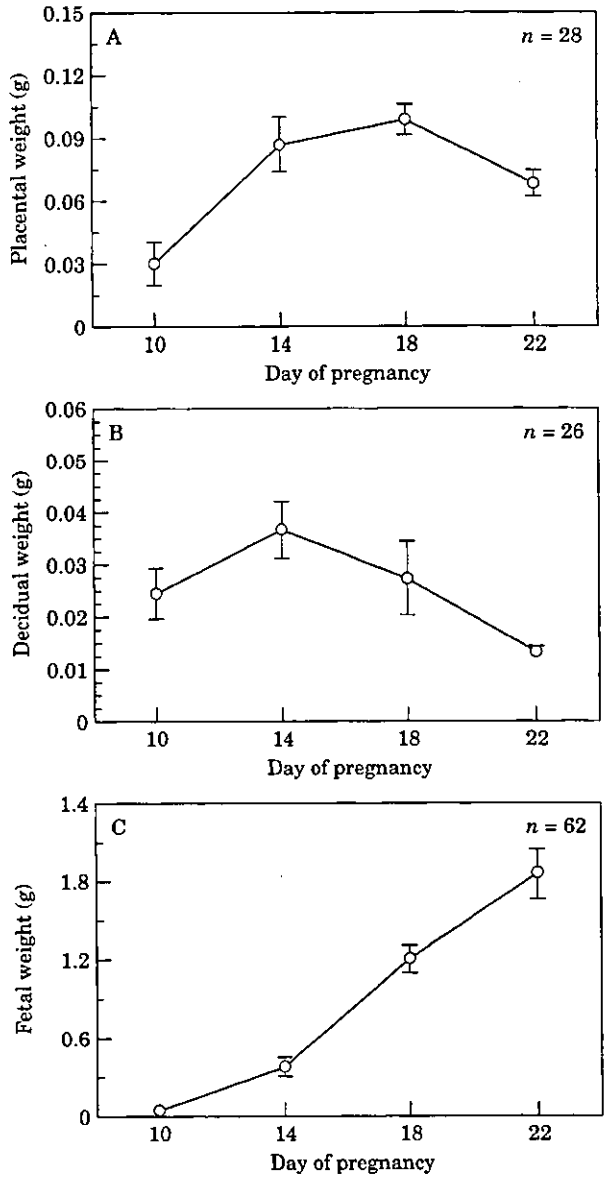


Figure 1. Changes of placental (A) decidual (B), and fetal weight (C) in FP-deficient mice with day of pregnancy.

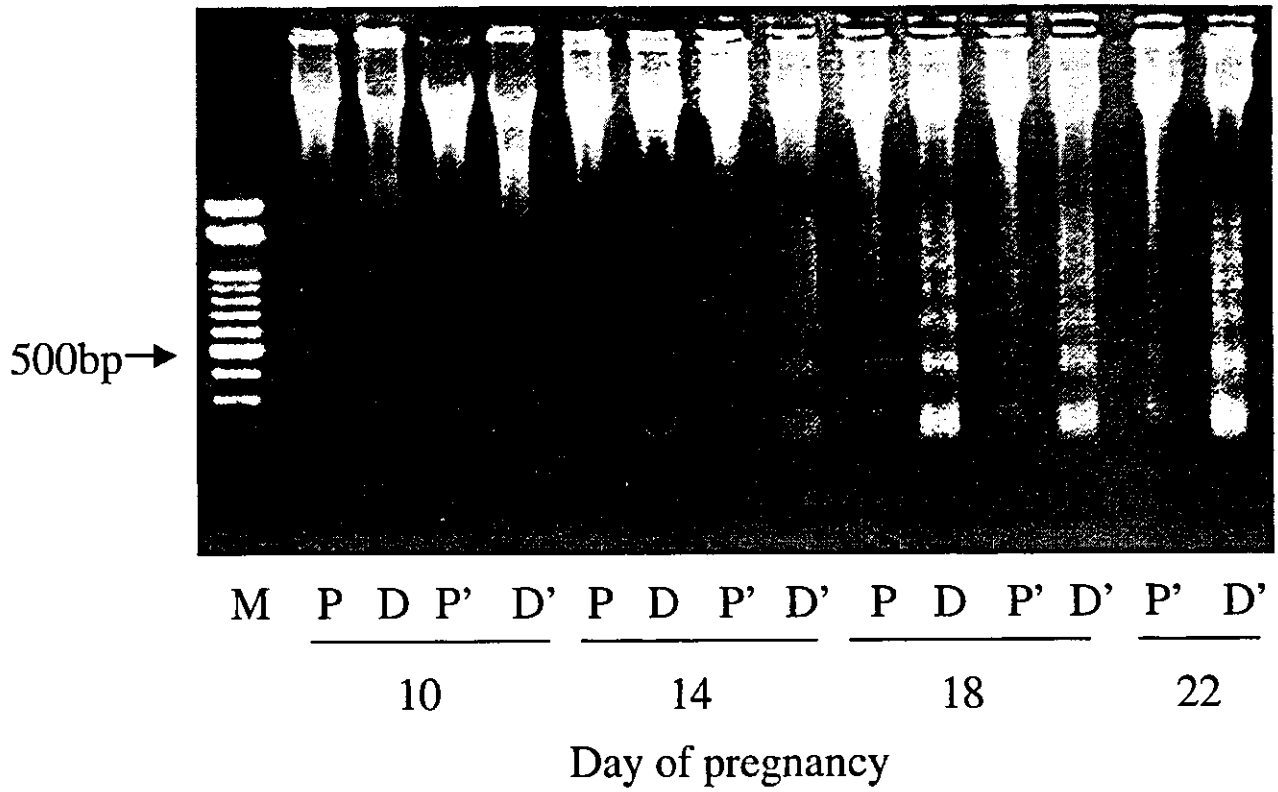


Figure 2. Electrophoretic analysis of DNA fragmentation in placental and decidual tissue at day 10 and 22 of pregnancy. Equal amounts DNA (15 μl) were electrophoretically separated on 1.8 per cent agarose gels and stained with ethidium bromide. A 100-bp DNA ladder was used to reference the molecular size. P, Placenta of wild type mice; D, decidua of wild type mice; P', placenta of FP-deficient mice; D', decidua of FP-deficient mice, M, molecular marker.

The number of TUNEL-positive cell in two type increased as gestation progressed toward term. Especially, it was prominent in the placentae and deciduae (24 h before pups died) at day 22 of pregnancy compared with those at day 18 of pregnancy (Table 2). The TUNEL positive cells in the decidual were higher in number than those in the placenta on the indicated day of pregnancy.

DISCUSSION

As pregnancy progresses towards term, the placenta appears to age or degenerate. Although this does not produce any functional deficiency, but rather placental maturation and the function reaches a peak. Postterm pregnancy is considered to be a stage of gestation with progressive placental functional failure. However, there are no specific gross or histological placental changes, which are indicative of insufficient placental function (Vorherr, 1975). The common placental lesions in post-term pregnancy are similar to the findings of normal-term placentae, which show typical features of senescence. Recently, apoptosis has been proposed as a mechanism in placental development and aging (Smith and Baker, 1999b).

Our study demonstrated that the development of pups was not completely consistent with the increased weight of the placenta or decidua. It is not surprising that the weight of the placenta increased with gestational day before day 18. The increase in placental weight is in agreement with the increase in fetal weight. Placental growth is suggestive of an increase in efficiency of transport and exchange to meet an increasing metabolic requirement of the fetus. Decidual weight increased with gestational age until day 14. This early decrease in the decidual weight was thought to be the beginning of decidual regression. This change may contribute to the preparation and placental separation from the uterus. After day 18, the decrease in both placental and decidual weights represented the placental senescence.

The TUNEL assay is one of the most frequently used methods to detect apoptosis. This method appeared to be a more sensitive approach for demonstrating apoptosis because of its ability to detect early biochemical changes occurring in apoptotic cells before morphological features became apparent (Chan et al., 1998, 1999; Smith et al., 1997b). However, TUNEL labelling alone may not be sufficient to determine whether PCD is occurring. It has been reported that several factors can produce a false-positive signal in TUNEL assays (Pulkkanen et al., 2000; Stahelin et al., 1998). Therefore, we combined the TUNEL method and a DNA fragmentation assay in our study. The present results showed that the findings using the TUNEL method were similar to those of the DNA fragmentation assay.

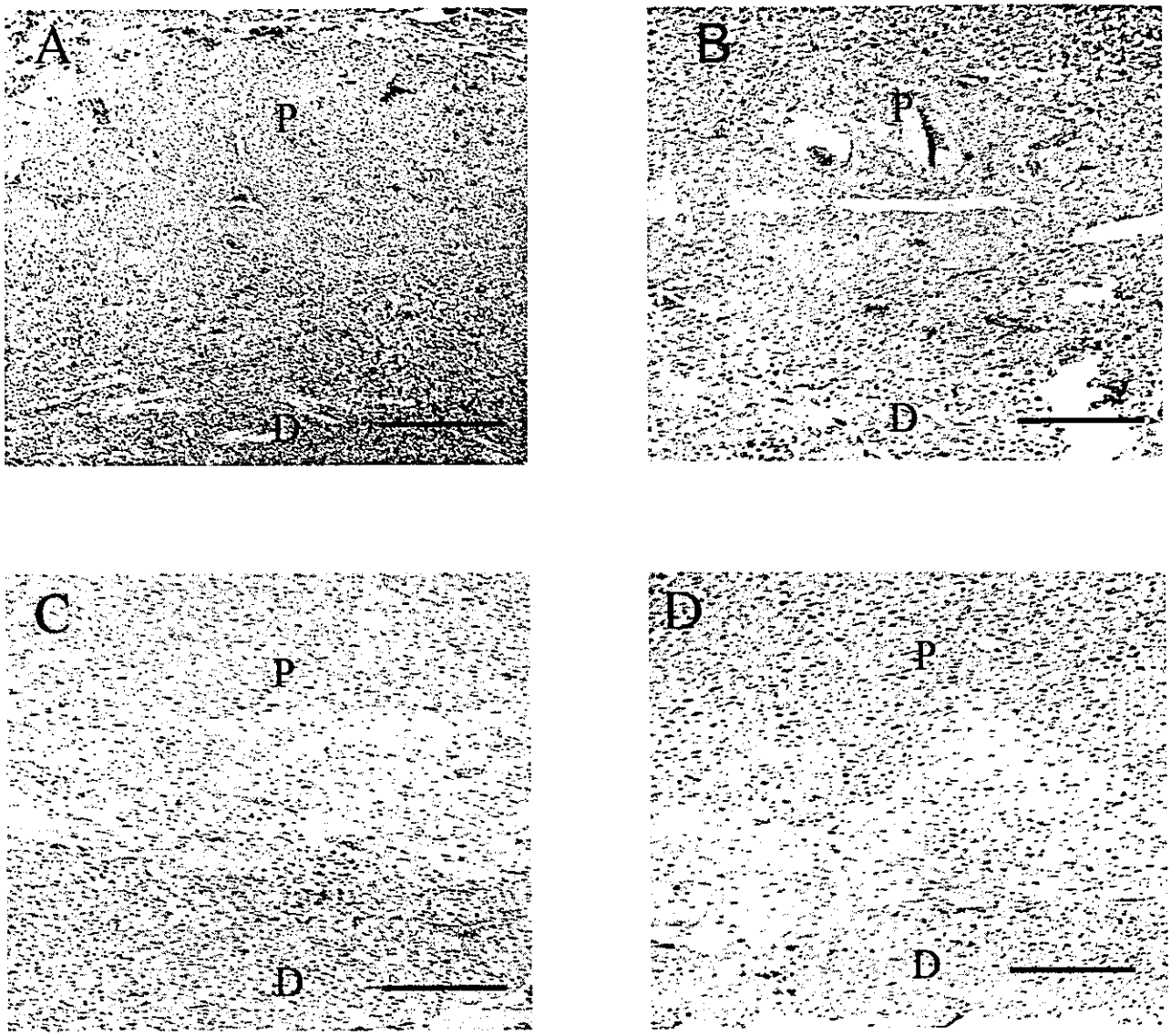


Figure 3. TUNEL staining in placentas and deciduas of the wild type (A, C) and FP-deficient mice (B, D) at day 10 (A, B) and 18 (C, D) of pregnancy. Dark-brown staining indicates a positive reaction. Tissues were counterstained with 1 per cent methyl green. P, Placenta; D, decidua. Bar=250 μm

This study showed a similar degree of apoptosis in the placentas of FP-deficient mice compare with that of normal wild type mice before day 18 of pregnancy. There was no difference in the number of pups, weight and amniotic fluid before day of pregnancy between two type mice. No abnormalities were observed in the weight of placenta and decidua in FP-deficient mice compare with that of wild type mice. The plasma concentration of estradiol were not significantly different between the two type of mice during the pregnancy (Sugimoto et al., 1997; Tsuboi et al., 2000). The plasma concentration of progesterone of wild type mice decreased rapidly in late pregnancy, FP-deficient mice did not show a decrease. These results suggested that failure of parturition in the FP-deficient mice may be due to the persistent production of progesterone. Recent studies have demonstrated that apoptosis was not affected by the level of human chorionic gonadotropin (Kokawa et al., 1988a), progesterone in rat (Gu et al., 1994) and hamster (Chen et al., 2001). Therefore, it is likely that apoptosis may be regulated by a similar mechanism in the placenta of FP-deficient mice compare with that of wild type mice. FP-deficient mouse is a more ideal animal model for studying the changes of apoptosis in development and aging of placenta throughout pregnancy, especially postterm pregnancy.

Our study showed more extensive apoptosis in the decidual tissue than in the placental tissue on each day of pregnancy. The decidua is thought to provide nutrition to the developing embryo, protect the embryo from the immunologic responses of the mother, and regulate trophoblast invasion into the uterine stroma. Several studies demonstrated that apoptosis occurs in the human decidua in the first trimester. They suggested that apoptosis had a critical role in embryonic

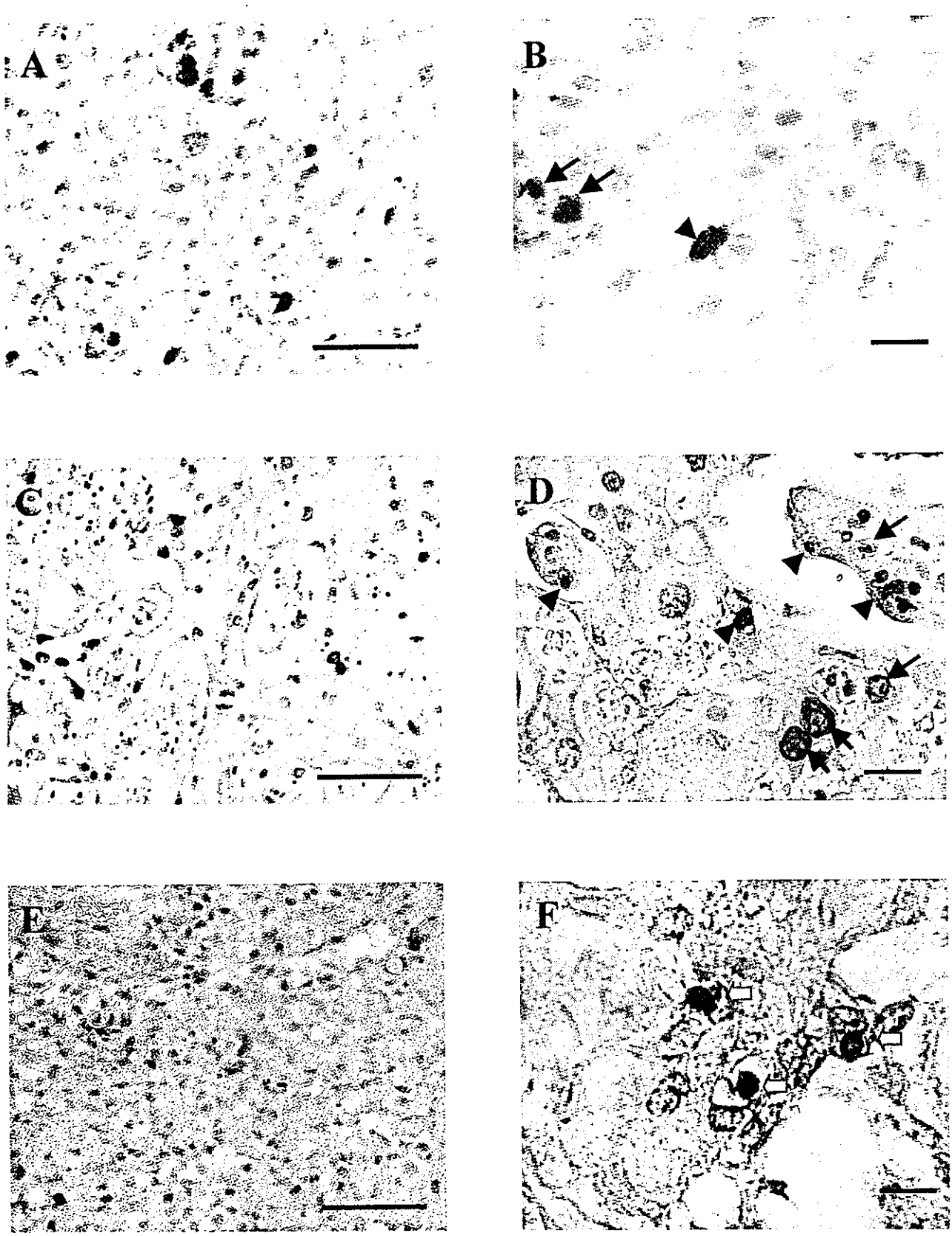
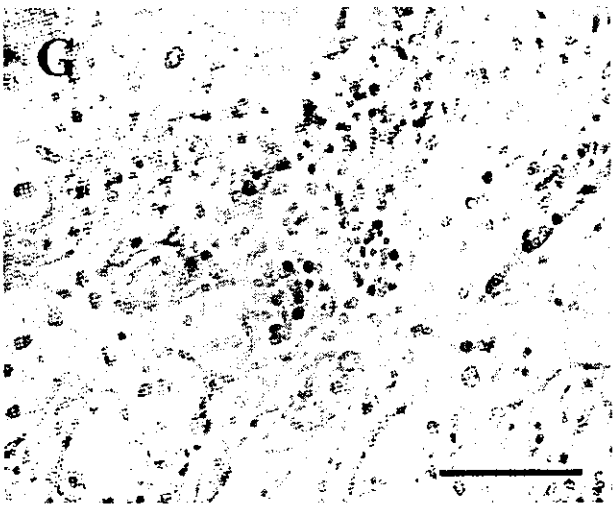


Figure 4. TUNEL staining in placentae and deciduae of the FP-deficient mice. Dark-brown staining indicates a positive reaction. Tissues were counterstained with 1 per cent methyl green. TUNEL-positive cells in trophoblast (arrowheads) and stromal (arrows) at day 14 (A, B) and 22 (C, D) of pregnancy. (E, F) TUNEL-positive cells in decidual tissues at day 18 of pregnancy, showing numerous apoptotic cells. (G, H) TUNEL-positive cells in decidual tissues (hollow arrow) at day 22 of pregnancy, the number of apoptotic cells was significantly increased. The increased TUNEL-positive staining in syncytiotrophoblast surface (arrows) was seen on day 22 of pregnancy (I, J) compare with the placenta on day 18 (K, L). Bars=50 μm (A, C, E, G, I, K); 13 μm (B, D, F, H, J, L).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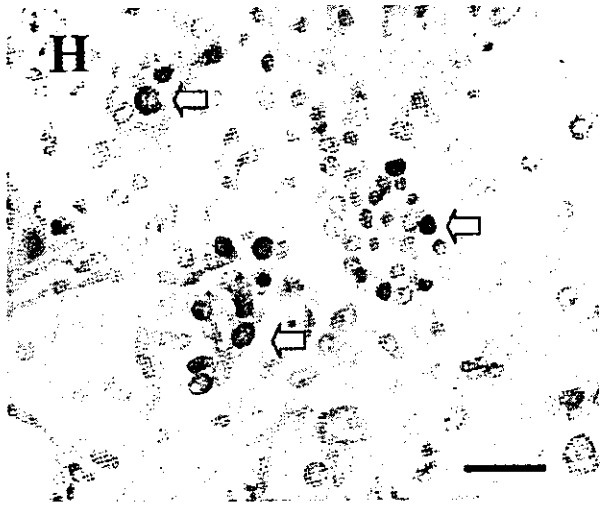
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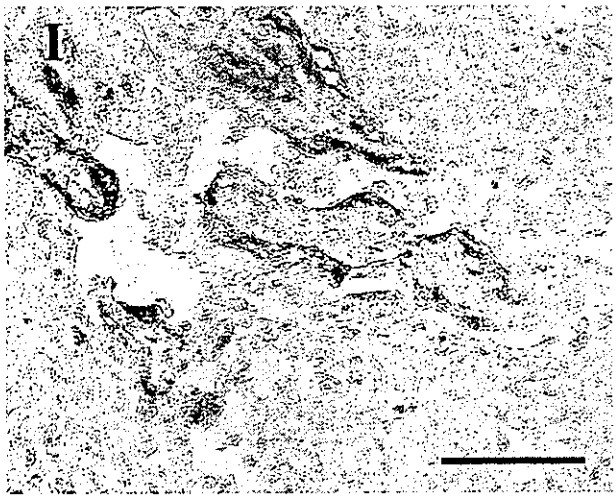
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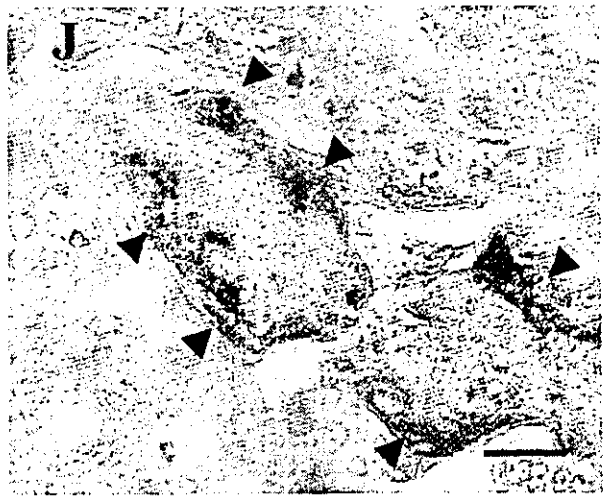
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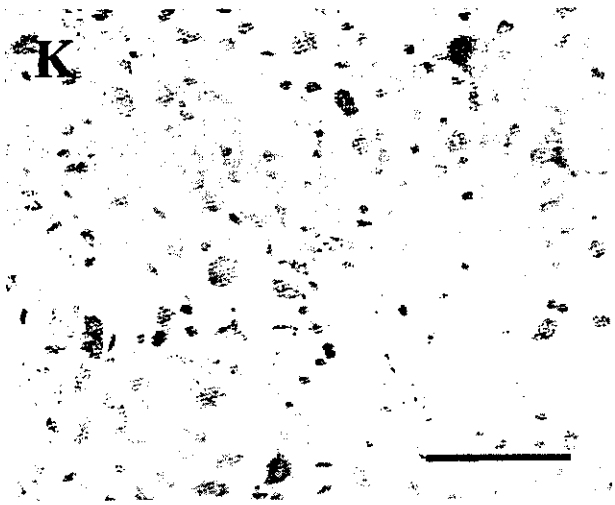
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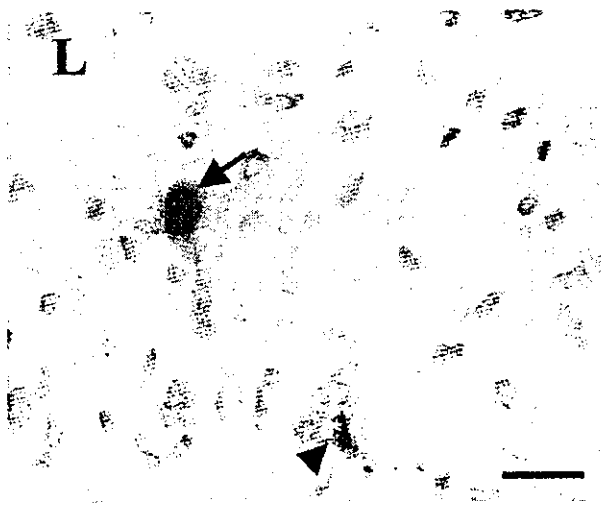
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Table 2. Incidence of apoptosis positive cells with TUNEL method (per cent)

Day of gestation	Wild type mice			FP-deficient mice			Statistical significance
	n	Placenta	Decidua	*n*	Placenta	Decidua	
10	6	0.61±0.12	2.24±0.25	5	0.54±0.10	2.15±0.23	NS
14	7	0.98±0.21	3.78±0.36	8	0.96±0.19	3.89±0.37	NS
18	12	1.94±0.38*	6.12±0.79*	10	1.80±0.82*	5.94±0.54*	NS
22				6	4.08±0.51**	9.82±0.98**	

Data were expressed as means ± s.d. or number.
NS, No significant differences of wild type and FP-deficient mice at same day of pregnancy.
*$P<0.05$ Significant differences from day 10 of pregnancy.
**$P<0.05$ Significant differences from day 18 of pregnancy.

development and wastage (Hammer and Dohr, 1999; Jerzak et al., 1998; Kokawa et al., 1998a). Unfortunately, these authors did not differentiate the incidences of apoptosis within the different tissues of human placentae. To the best of our knowledge, no study of apoptosis in decidual tissue at term placenta has been reported. We also found that apoptotic cells are not uniformly distributed in the decidual tissue. The main area was the centre of the decidual basalis, where blastocyst implantation takes place. Decidualization in the rat has been associated with the progressive induction of apoptosis in decidual cells (Gu et al., 1994; Piacentini and Autuori, 1994). To our knowledge, this is the first demonstration of the greater higher rate of apoptosis in the decidua throughout gestation. Decidual apoptosis may play an important role in the regulation of placental growth and aging throughout pregnancy.

In the human placenta, whether the apoptosis index increases or decreases during normal gestation is still controversial. Smith et al., 1997b) found an increase in the apoptosis index in the whole placenta during gestation, while Chan et al. (1998, 1999) found a decrease. These studies did not differentiate between the various tissues in the placenta. Our study showed that apoptotic cells of the mouse placentae increased with gestational day not only in the placental, but also in the decidual tissue. The number of apoptotic cells in the mouse placentas was higher than that from human placentas according to Smith's results. Similar to Axt's study (Axt et al., 1999b), apoptosis was predominantly detected in the trophoblasts or stromal cells with a lower incidence in the endothelial cells. The increasing placental apoptosis with gestational age suggest that apoptosis may be a normal placental aging process.

In the present study, we demonstrated a significant increase in the mouse apoptosis in the placenta and decidual tissue from post-term placentae in comparison to those from the term placentae. This is similar to previous studies in human post-term pregnancy, who identified a significant increase of apoptosis in placenta of the post-term pregnancy compared with the term pregnancy (Axt et al., 1999b; Smith and Baker, 1999). As mentioned in our results, we also showed that more TUNEL-positive staining in syncytiotrophoblast layer was found in the post-term placenta, compared with those in the term placenta. This can be explained by the detected apoptosis occurring in post-term mouse placentae (day 22), in which the fetus soon dies within 24 h. Some studies have demonstrated that enhanced apoptosis in syncytriotrophoblast is associated with abnormal pregnancies such as first trimester abortion and ectopic pregnancies (Kokawa et al., 1998a,b). The syncytriotrophoblast apoptosis has also been suggested to precede the breaks in the trophoblast covering villi and the loss in the integrity of the syncytium (Nelson, 1996). Ejima et al., 2000) found that the number of TUNEL-positive cells increased more in mouse placentae of an inflammatory placental dysfunction model than that in the normal mouse placentae. We consider that the detected increase in apoptosis and the stronger staining in syncytiotrophoblast layer in mouse placentas are related to intrauterine fetal death, which results in placental dysfunction. We are hoping to investigate that the relationship between apoptosis at post-term pregnancy and fetal death in detail.

In conclusion, the placental and decidual apoptosis may play an important role in placental development and aging. The increased apoptosis expression in mouse term placentas may be related to the normal physiological function of apoptosis. On the other hand, further increased apoptosis in post-term pregnancies indicates the possible role of pathological processes.

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Possible coupling of prostaglandin E receptor EP_1 to TRP5 expressed in *Xenopus laevis* oocytes☆

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Abstract

We previously reported that the prostaglandin E_2 (PGE_2) receptor subtype EP_1 is coupled to intracellular Ca^{2+} mobilization in CHO cells, which is dependent on extracellular Ca^{2+} in a pertussis toxin-insensitive manner [H. Katoh, et al., Biochim. Biophys. Acta 1244 (1995) 41–48]. However, it remains unknown about the signal transduction involved in this response. To investigate the mechanism regulating Ca^{2+} mobilization mediated by EP_1 receptors in detail, we performed a series of experiments using the *Xenopus laevis* oocyte expression system and found that endogenous G_q and/or G_{11}, and not G_{i1} is involved in the Ca^{2+} mobilization induced by PGE_2. We further investigated the receptor-activated Ca^{2+} channel (RACC)-related response by introducing mRNA for mouse transient receptor potential 5 (TRP5), a possible candidate for the RACC, and found effective coupling between them. These results suggest that the EP_1 receptors induce Ca^{2+} mobilization via G_q and/or G_{11} and Ca^{2+} influx via TRP.

Keywords: EP_1; Ca^{2+} mobilization; Transient receptor potential; Receptor-activated Ca^{2+} channel

Prostaglandin E_2 (PGE_2) exerts various physiological and pathophysiological actions in a variety of tissues and cells [1,2]. These activities of PGE_2 are mediated by specific G protein-coupled receptors, which have been classified on the basis of pharmacological experiments into four subtypes, EP_1, EP_2, EP_3, and EP_4 according to their responsiveness to selective agonists and antagonists. Cloning of cDNA for these receptors has revealed that each EP subtype has a unique structure and is coupled to a different signaling pathway [3].

The EP_1 subtype is expressed in the papillary collecting ducts of the kidney [4], the smooth muscle of the muscularis mucosa in the gastrointestinal tracts [5], and the sensory neurons of the dorsal root ganglion [6]. The localization of EP_1 demonstrated by these investigations coincides well with the PGE_2-induced physiological responses via the EP_1 subtype, such as the inhibition of Na^+ and water reabsorption in the collecting ducts [7,8], pain perception, blood pressure maintenance [9], colon carcinogenesis [10], and induction of allodynia [11]. However, the mechanism of EP_1-mediated signal transduction in these tissues and cells remains largely unknown.

We previously cloned the cDNA for mouse EP_1 and demonstrated that this receptor mediates intracellular Ca^{2+} mobilization in a CHO cell expression system [12]. We also demonstrated using CHO cells stably expressing the mouse EP_1 receptor (CHO-EP_1) that Ca^{2+} mobilization mediated by the EP_1 receptor is induced by two distinct pathways; one is a transient release from an internal Ca^{2+} store, which is sensitive to a PLC inhibitor, U73122, and the other is a Ca^{2+} influx from external space, which is resistant to U73122 [13]. Although we showed that Ca^{2+} mobilization via EP_1 receptors is pertussis toxin-insensitive, direct evidence has not been obtained to identify the G proteins coupled to the EP_1 receptors nor the plasma membrane Ca^{2+} channels re-

☆ *Abbreviations:* ACh, acetylcholine; IP_3, inositol 1,4,5-triphosphate; PG, prostaglandin; PLC, phospholipase C; RACC, receptor-activated Ca^{2+} channel; TRP, transient receptor potential.

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sponsible for the Ca^{2+} influx. The EP_1 mediated Ca^{2+} influx in CHO-EP1 cells indicates the presence of receptor-activated Ca^{2+} channel (RACC)-related responses, which have been recognized to have physiological significance in a variety of tissues and cells [14]. A number of *Drosophila* transient receptor potential (TRP) homologs have been identified as likely candidates for RACCs [15]. Since the original *Drosophila* TRP and TRP-like channels are involved in light induced signal transduction via G protein-mediated activation of PLC, the mammalian homologs have been considered as candidates for Ca^{2+} channels activated by G protein-coupled receptors [15].

To investigate the mechanism regulating Ca^{2+} mobilization mediated by the EP_1 receptors in detail, we performed a series of experiments using the *Xenopus laevis* oocyte expression system. This system is useful for investigating the involvement of G proteins in Ca^{2+} mobilization, since the expression of some endogenous G protein α subunits such as $G\alpha_{i1}$, $G\alpha_q$, and $G\alpha_{11}$ have found to be effectively suppressed by the application of specific antisense oligodeoxynucleotides [16]. We investigated the RACC-related response in this system by introducing mRNA for mTRP5, a possible candidate for the RACC activated by EP_1, and found effective coupling between them.

Materials and methods

Materials. Mouse TRP5 cDNA (pCI-neo-mTRP5) was kindly provided by Dr. Y. Mori (Okazaki National Research Institute, Japan) [17]. In vitro transcription using the plasmid mTRP5/pGEMHE was performed as described previously [18]. Plasmid pSPM10 carrying the porcine muscarinic M_1 receptor [19] was linearized by *Xba*I and used as the template for in vitro transcription using SP6 RNA polymerase. Mouse EP_1 receptor cDNA [12] was subcloned into the *Eco*RI site of pGEM-5Zf (Promega, Madison, WI) containing the 3′-non-coding region of the *X. laevis* β-globin gene derived from pGEMHE (pGEM-5HE), linearized by *Eco*RV, and used as the template for in vitro transcription using SP6 RNA polymerase. Antisense oligodeoxynucleotides (ODNs) to the *X. laevis* $G\alpha_{i1}$ protein (XAG_{i1}, 5′-CCC ATG GCG ACG GTT CTC CG-3′), $G\alpha_q$ protein (XAG_q, 5′-GTC ATG CCT CCT TGA CTA GT-3′), and $G\alpha_{11}$ protein (XAG_{11}, 5′-GTC ATC CCT TCC CCC CGG CA-3′) were designed to span the start codons of the published cDNA sequences for these *X. laevis* G proteins [16,20]. All chemicals used in this study were commercial products of reagent grade.

Preparation of RNA-injected oocytes. Preparation of RNA-injected *X. laevis* oocytes was performed as described previously [18]. Healthy-looking oocytes were injected in Ca^{2+}-free modified Barth's saline with 50 nl sterile solution containing mRNA for mTRP5 together with M_1 or EP_1 receptor mRNA (13.3 ng each/oocyte) and incubated further for 2–3 days. In knock down experiments of the G proteins, oocytes were injected with one of the antisense ODNs to the *X. laevis* G proteins (50 ng/oocyte) on the day after mRNA injection.

Electrophysiological recordings. Oocytes were voltage-clamped at a holding potential of −80 mV in Ca^{2+}-free frog Ringer (FR) solution (115 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes, and 1 mM EGTA, pH 7.4, with NaOH) with two intracellular glass electrodes (1–2 MΩ with 3 M KCl) connected to an OC-725C amplifier (Warner Instruments, Hamden, CT). The current and voltage outputs were monitored using a flat pen recorder and a MacLab A/D converter with Scope software (ADInstruments Pty, Castle Hill, Australia) as described previously [18]. For the detection of Ca^{2+} influx, the perfusion line was switched for 30 s to Ca^{2+}-containing FR solution (1 mM EGTA was substituted to 2 mM $CaCl_2$). ACh and PGE_2 were dissolved in Ca^{2+}-free FR to 100 and 1 μM, respectively, and applied similarly by perfusion. The amplitude of current trace was measured as

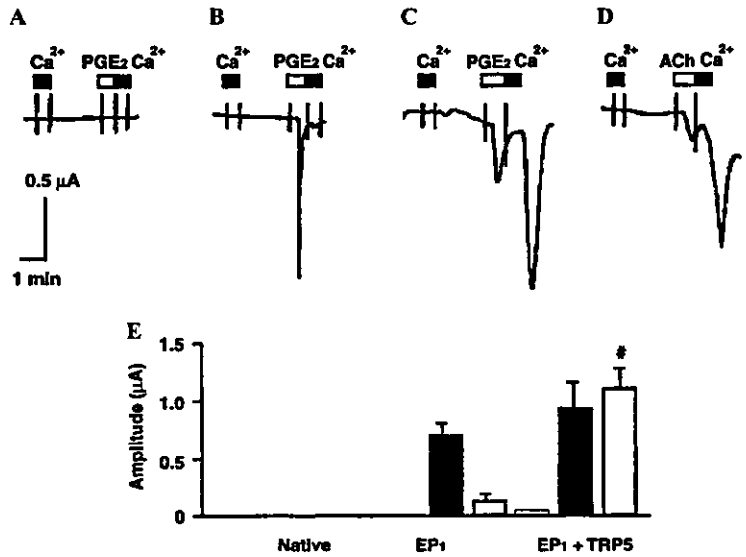

Fig. 1. Activation of mTRP5 channels after stimulation of the co-expressed prostaglandin E receptor EP_1 subtype with PGE_2. (A)–(D) Whole-cell current traces were recorded at a holding potential of −80 mV in Ca^{2+}-free FR from non-injected (A), mEP_1 mRNA-injected (B), mTRP5 + mEP_1 mRNA-injected (C) and mTRP5 + M_1 mRNA-injected (D) oocytes. Two millimolar Ca^{2+}-containing FR (closed bars), Ca^{2+}-free PGE_2 or ACh solution (PGE_2; 1 μM or ACh; 100 μM, open bars) was applied for 1 min as indicated by the bars. (E) Average (±SEM, $n = 6$) amplitudes of the current responses of oocytes are presented of the first application of Ca^{2+}-containing FR (gray columns), the application of 1 μM PGE_2 in Ca^{2+}-free FR (closed columns), and the subsequent application of Ca^{2+}-containing FR (open columns). $^{\#}P < 0.05$ versus native PGE_2-Ca^{2+}.

the maximal deflection from the baseline to the peak of response. Values are shown as means ± SEM. Statistical significance was evaluated by the Student's *t* test.

Results

Mouse PGE receptor subtype EP_1 has the potential to couple to TRP5 in the X. laevis oocyte expression system

To evaluate mEP_1-mediated receptor-activated Ca^{2+} channel (RACC)-related responses, we used the *X. laevis* oocyte expression system co-injected with mouse TRP5 (mTRP5) mRNA, which has been found not to be involved in store operated Ca^{2+} channel (SOC) responses in this system [18]. We recorded the whole cell current from oocytes perfused with Ca^{2+}-free FR and treated with 2 mM Ca^{2+} containing FR for 30 s before and after stimulation of the co-expressed mEP_1 receptors with PGE_2 (1 μM). We confirmed by monitoring the *I–V* relationship of the oocytes co-expressing mTRP5 and M_1 or mEP_1 receptors that the Ca^{2+}-evoked response after Ach or PGE_2 is mediated by an increase in the Ca^{2+}-activated Cl^- channel current, which is characteristic of a Ca^{2+} influx into oocytes [21] (data not shown).

In native, non-injected oocytes, no current response was evoked when recording solution was switched from Ca^{2+}-free FR to 2 mM Ca^{2+}-containing FR, or to Ca^{2+}-free FR containing 1 μM PGE_2 (Fig. 1A). In oocytes injected with mRNA for the mEP_1 receptor alone, application of PGE_2 in Ca^{2+}-free solution evoked a transient inward current (Fig. 1B). Although switching of the recording solution to Ca^{2+}-containing FR did not evoke a current response before PGE_2-infusion, a small Ca^{2+}-induced current was observed after the infusion, which reflects an endogenous RACC-related response. In oocytes co-injected with mRNAs for the mTRP5 and mEP_1 receptors, application of Ca^{2+} after PGE_2 stimulation evoked a significantly large inward current response, which was similar to the response observed in the oocytes co-injected with mRNAs for the mTRP5 and M_1 receptors (Figs. 1C and D).

Mixed antisense oligodeoxynucleotides to X. laevis $G\alpha_q$ and $G\alpha_{11}$ inhibited Ca^{2+} release induced by PGE_2 but not the opening of mTRP5 channels

To determine potential endogenous Gα proteins involved in the Ca^{2+} influx mediated by mTRP5, we performed a series of experiments using antisense oligodeoxynucleotides to Gα proteins (XAG) in the oocytes co-injected with mRNAs for mTRP5 and mEP_1 receptors. Compared with untreated oocytes (Fig. 2A), oocytes preloaded with XAG for G_{i1} (XAG_{i1}) showed no inhibitory effect on the responses to PGE_2 or on the mTRP5-mediated inward current evoked after PGE_2

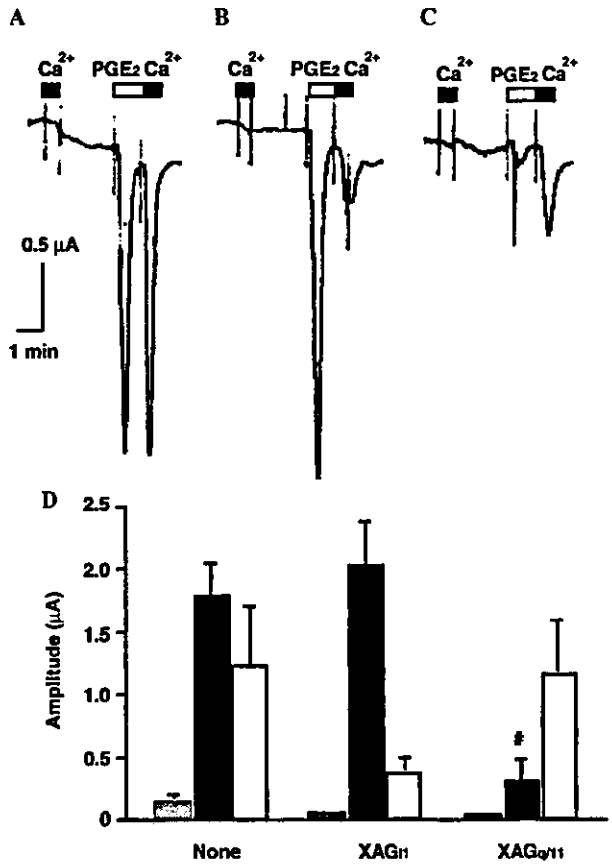

Fig. 2. Effects of preinjection of antisense oligodeoxynucleotides to the *X. laevis* G proteins on Ca^{2+} mobilization induced by PGE_2. Representative current traces were recorded from oocytes co-expressing the mTRP5 and mEP_1 receptors and preinjected without (A) or with 50 ng anti-G_{i1} antisense oligodeoxynucleotides (ODNs), XAG_{i1} (B) or 50 ng anti-$G_{q/11}$ mixed antisense ODNs, $XAG_{q/11}$ (C). Two millimolar Ca^{2+}-containing FR (closed bars) or Ca^{2+}-free PGE_2 solution (1 μM, open bars) was applied for 30 s as indicated by the bars. (D) Average (± SEM, $n = 4$) amplitudes of the current responses are presented of the first application of Ca^{2+}-containing FR (gray columns), the application of 1 μM PGE_2 in Ca^{2+}-free FR (closed columns), and the subsequent application of Ca^{2+} containing FR (open columns). $^{\#}P < 0.05$ versus no PGE_2.

infusion (Fig. 2B). In oocytes preloaded with mixed XAG for G_q and G_{11} ($XAG_{q/11}$), PGE_2 evoked a significantly small current response by itself, whereas the mTRP5-mediated current response after PGE_2 infusion was intact (Fig. 2C). The suppressive effect of each XAG on endogenous Gα proteins was confirmed by immunoblot analyses using specific antibodies (data not shown).

Discussion

We have previously demonstrated that mEP_1 receptors induce intracellular Ca^{2+} mobilization via an unidentified pertussis toxin-insensitive G protein(s) in

CHO cells stably expressing EP_1 cDNA (CHO-EP_1) [13]. In oocytes injected with mRNA for mEP_1, infusion of PGE_2 induced a small current response followed by a small but detectable RACC-related response, both of which were not detected in native oocytes (Figs. 1A and B). Since among the four mEP receptors only the mEP_1 and mEP_3 receptors are known to mediate Ca^{2+} mobilization, these observations indicate that native oocytes do not express either of these receptors endogenously. By application of Ca^{2+} after PGE_2 stimulation, a large inward current response was obtained in oocytes co-injected with mRNAs for the mTRP5 and mEP_1 receptors (Fig. 1C), indicating that PGE_2 stimulation of mEP_1 may be coupled to the TRP5-mediated Ca^{2+} influx. The RACC-related response to PGE_2-mEP_1 stimulation was roughly similar to the response to Ach-M_1 receptor stimulation (Fig. 1D).

In previous experiments, we showed that mEP_1-stimulated Ca^{2+} mobilization in mEP_1-expressed CHO cells may be mediated by a PT-insensitive G protein species [12,13]. The current antisense experiments in oocytes revealed that mEP_1-mediated Ca^{2+} mobilization induced by PGE_2 is significantly suppressed by $XAG_{q/11}$ but not by XAG_{i1} (Fig. 2), indicating the involvement of $G_{q/11}$. We could not further determine which of the endogenous Gα proteins, $G\alpha_q$ or $G\alpha_{11}$, is involved in mEP_1-mediated Ca^{2+} mobilization, since it is impossible to distinguish between these two Gα proteins using each XAG in the current system, as described previously [18].

Our previous observation in CHO-mEP_1 cells that a PLC inhibitor, U73122, completely suppressed Ca^{2+} release from the intracellular store, but did not suppress the sustained phase of Ca^{2+} influx from extracellular space, indicated the presence of a unique RACC-related response in CHO-mEP_1 cells, since RACC-related responses are generally believed to require PLC activation [15]. However, it remains largely unknown as to how RACCs are activated, and the requirement for store depletion and inositol-1,3,4-trisphosphate production for the activation of each TRP remains controversial [15]. In this study, we investigated the possible coupling of mEP_1 to mTRP5 since the coupling mechanism between mTRP5 and another G protein-coupled receptor, the muscarinic M_1 receptor, has been intensively studied in the same oocyte expression system [18]. In oocytes co-injected with mRNAs for mTRP5 and mEP_1, an enhanced RACC-related response was observed (Figs. 1 and 2), indicating the possible coupling between EP_1 and mTRP5 as well as M_1. However, the RACC-related response mediated by mEP_1 was not significantly impaired by $XAG_{q/11}$, although that by M_1 was (Fig. 2 and [18]). This result suggests that the endogenous G_q and/or G_{11} protein, which is essential for Ca^{2+} mobilization induced by PGE_2, may not be required for the RACC-related response mediated by mEP_1. However, it is also possible that this discrepancy may be due to the different efficiencies of mEP_1-coupled G_q and/or G_{11} to activate Ca^{2+} mobilization from the internal store and Ca^{2+} influx from external space, since the mixed antisense oligodeoxynucleotides were not able to completely abolish the expression of each Gα protein. Singer-Lahat et al. [22,23] demonstrated evidence suggesting that RACC-related responses can be activated by direct interaction with a trimeric G protein, using CHO cells and A9 fibroblasts expressing muscarinic M_3 receptors. In our oocyte system, a very low level of Gα protein expression may be able to maintain the direct RACC-related responses. Further analyses regarding the mechanism of mTRP5 activation such as PLC activation in our system are required to clarify this point.

In summary, we demonstrated that EP_1 receptors have the potential to induce Ca^{2+} mobilization via $G_{q/11}$ and to couple to mTRP5 in the oocyte expression system.

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Role of prostanoids on female reproduction revealed by receptor-deficient mice

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Abstract

Prostanoids consisting of prostaglandins (PGs) and thromboxane, are synthesized from arachidonic acid by cyclooxygenases (COXs) acting as a rate-limiting enzyme. Prostanoids exert a variety of biological and pathophysiological actions in the body, which are mediated by specific receptors. Prostanoid receptors are classified into 8 types and subtypes. Pharmacological actions of prostanoids have been analysed by the effects of COX inhibitors, nonsteroidal anti-inflammatory drugs and of the in vivo and in vitro actions of agonists and antagonists. One of the crucial actions of prostanoids is on the female reproductive processes such as ovulation, fertilization, luteolysis and uterine contraction. Recently, cDNA cloning of prostanoid receptors and generation of each

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receptor-deficient mice have established and enabled us to analyse which type of PG and which receptor subtype are crucial for each reporductive process. Hence we review our recent study on the significance of prostanoids in the female reproductive system, which revealed by each receptor-deficient mice. Among prostanoid receptors, only $PGF_{2\alpha}$ receptor (FP)- and PGE_2 receptor subtype EP_2- deficient mice showed the failure of partutrition and litter size, respectively. These studies indicated that luteolytic action of $PGF_{2\alpha}$ is essential for induction of parturition in late pregnancy, and that COX-2 in the myometrium at term is responsible for producing uterotonic PGs. On the other hand, EP_2-deficient mice with a normal labor decreased litter size on the basis of impaired expansion of cumulus cells in early pregnancy. In this period, inducible COX-2 affects the processes of ovulation and fertilization. Recent studies on COX-2 deficient mice showed multiple reproductive failures during pregnancy. These results are useful in not only developing novel drugs in female reproductive area but also understanding and overcoming harmful reproductive side effects of conventional and novel drugs in non-reproductive areas.

Introduction

Prostaniods consisting of prostaglandins (PGs) and thromboxane (TX) are synthesized via the cyclooxygenase (COX) pathway from arachidonic acid which was released from phospholipids by phospholipase (Fig.1). They are relased outside the cells and act as local short-range hormones via cognate receptors on plasma membranes. These receptors are classified into five basic types, termed DP, EP, FP, IP and TP, on the basis of their sensitivity to the five primary prostanoids, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2, respectively [1]. Furthermore, EP is subdivided into four subtypes, EP_1, EP_2, EP_3, and EP_4, on the basis of their response to synthetic agonists and antagonists. Prostanoids exert a

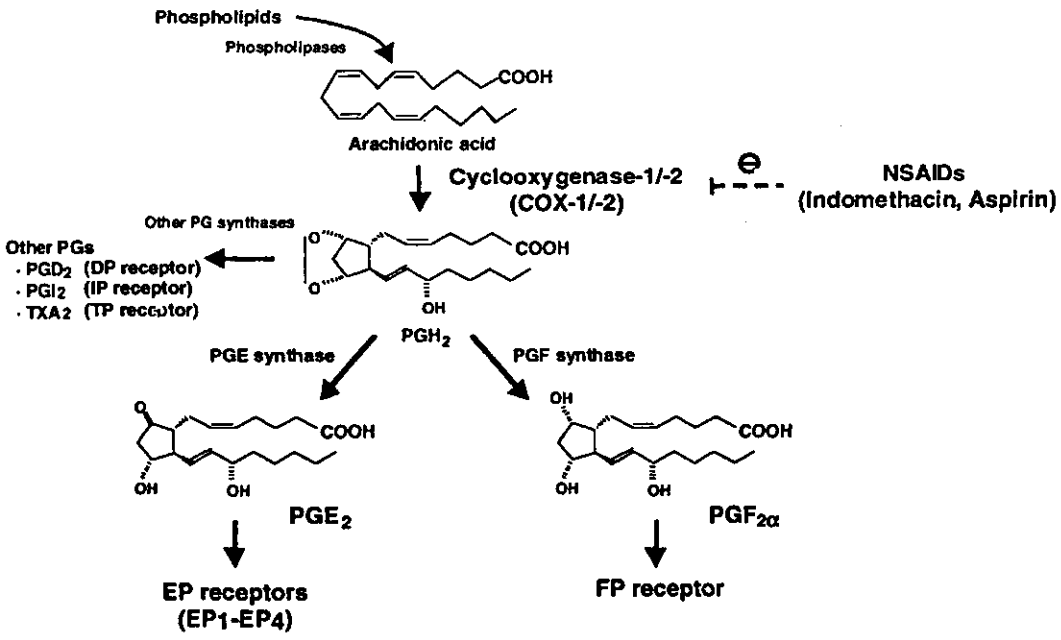

Figure 1. Biosynthetic pathway of prostanoids

wide variety of physiological and pathophysiological actions in the whole body [2]. Classicially crucial role of prostanoids on several pathological processes such as fever generation and pain modulation have been well recognized on the basis of the pharmacological effects of nonsterodical anti-inflammatory drugs (NSAIDs) such as indomethacin and aspirin, which inhibit prostanoid production via activity of COXs[3]. Furthermore, prostanoids have been considered to be important facotrs mediating several processes in female reproduction such as ovulation, fertilization, decidualization and parturition[4]. Indeed NSAIDs are known to inhibit ovulation and parturition as their side effects. In recent years, cDNA cloning of these synthesizing enzymes and receptors of prostanoids have enabled us to generate each gene-deficient mice [5]. A number of these mice are reported to exhibit abnormalities in female reproduction, including decreased litter size, deley or loss of parturition, or complete infertility (Table 1). These results clearly indicate that endogenous prostanoids are essential for some aspects in female reproduction. In this review, we will focus the roles of prostanoids on the physiology of female reproduction on the basis of analyses of phenotypes of prostanoid receptor-deficient mice.

Table 1. Litter size and parturition in prostanoid-related gene knockout mice

Gene	Litter size	Parturition	Reference
$cPLA_2$	reduced	delayed	[6, 7]
COX-1	normal	delayed	[8]
COX-2	(infertile)	(infertile)	[9]
FP	normal*	lost	[10]
EP_2	reduced	normal	[11-13]

*, Number of pups upon ovariectomy-induced parturition.

Luteolysis and parturition

The ovarian cycle consists of follicular phase, ovulation, and luteal phase. After the follicle ovulates, the granulosa cells differentiate into the luteal cells. The most important function of the corpus luteum is the secretion of progesterone (P_4), which is essential for the maintenance of pregnancy. Therefore, functional luteoloysis can be difined as the cessation of the production of P_4 in the corpus luteum. The life span of the corpus luteum is known to be prolonged by hysterectomy in domestic animals, suggesting the presence of a luteolytic factors (termed as luteolysin) derived from the uterus, and $PGF_{2\alpha}$ could be accepted as this luteolysin [14, 15]. In humans, the administration of $PGF_{2\alpha}$ is known to induce luteolysis, whereas hysterectomy has no effects on the life span of the corpus luteum. This suggests that luteolytic $PGF_{2\alpha}$ could be produced not only in the uterus but presumably in the ovary [16]. Nonetheless $PGF_{2\alpha}$ has luteolytic activity in widespread species. However, FP-deficient mice have normal length of estrous cycle [10], although FP gene is abundantly expressed in the corpus luteum of normal estrous cycle[17]. This

result implies that $PGF_{2\alpha}$ is not essential for the luteolysis in the normal estrous cycle at least in the mouse. In contrast, FP-deficient mice do not show the normal decline in the serum P_4 at term, indicating that endogenous $PGF_{2\alpha}$ is essential for the luteolysis in late pregnancy (Fig.2). Because P_4 withdrawal in late pregnancy initiates the parturition process in rodents, FP-deficient mice are not able to deliver any fetuses, but parturition is able to be restored at 20 hours after the treatment of ovariectomy. It has been reported that the mice deficient in cytosolic phospholipase A_2 ($cPLA_2$) or COX-1 also exhibit impaired luteolysis and delayed parturition (Table 1). Thus the combination of these isozymes would be responsible for the synthesis of luteolytic $PGF_{2\alpha}$ in late pregnancy. Indeed the increase in uterine $PGF_{2\alpha}$ content at term is lost in COX-1-deficient mice, and the administration of $PGF_{2\alpha}$ restores successful parturition [8]. In wild-type mice, COX-1 mRNA, which is dominantly localized in endometrial epithelial cells, progressively increased between days 15 and 17 of pregnancy, reaching maximal levels on day 17. Thereafter COX-1 mRNA level began to decrease during parturition on day 20 of pregnancy. In contrast, COX-1 mRNA is kept at high levels even on day 20 without parturition in FP-deficient mice [18]. This result suggests that fall of serum P_4 could down-regulate COX-1 as a negative feed back system.

The most recognized function of PGs in parturition is the direct stimulatory effect on uterine smooth muscle [4]. $PGF_{2\alpha}$ and PGE_2 are supposed to be physiological stimulants of myometrium during parturition, because these PGs are abundantly produced in intrauterine tissues during parturition[19] and NSAIDs have labor-delaying effect [20]. However, parturition can be restored after ovariectomy in FP-deficient mice, and the mice deficient in any other prostanoid receptors including PGE receptor, subtypes EP_1-EP_4, do not exhibit impaired parturition. Thus single deletion of either of 8 prostanoid receptors has no effects on uterine contraction during parturition, and they may compensate each other.

While the role of COX-1 in late pregnancy is able to be examined by COX-1 deficient mice, it is impossible to analyze late pregnant COX-2-deficient mice due to their impairment in early pregnancy. However, COX-2 may be responsible for the synthesis of uterotonic PGs, because strong expression of COX- 2 is induced in myometrium during parturition in wild-type mice (Fig. 2). Indeed this expression is not observed at term in FP-deficient mice, which lack parturition [18]. Furthermore when FP-deficient mice are subjected to ovariectomy, COX-2 expression is again induced with concomitant recovery of parturition. Although COX-2 is reported to be dominantly expressed in chorion and amnion in humans, COX-2 expression level in these tissues upon spontaneous parturition is higher than that upon cesarean section, indicating that uterotonic PGs are produced via COX-2 [21, 22]. NSAIDs have been found effective in delaying delivery in clinical trials, but the adverse side effects on the fetal ductus arteriosus have limited the use of such treatment in preterm labor [23]. Considering that the severe effect on fetal ductus arteriosus appears only after genetic inactivation of both of COX-1 and COX-2 in mice [24], COX-2 specific inhibitor has the potential for the inhibition of labor with less side effects than conventional NSAIDs [25]. Indeed COX-2 inhibitor is reported to delay LPS-induced preterm parturition [26]. However, Reese *et al.* have reported that COX-2 inhibitor delays murine natural parturition only at high doses. Thus the role of COX-2 during parturition should be addressed more carefully.

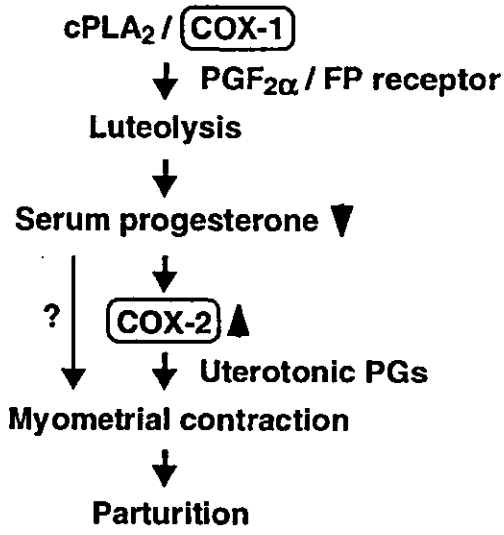

Figure 2. Role of PG pathway in murine parturition

Ovulation and fertilization

Ovulation is triggered when mature antral follicles are stimulated with luteinzing hormone (LH) released from the pituitary. NSAIDs are reported to inhibit LH release from the pituitary [27]. This is because PGE_2 is involved in the release of LH-releasing hormone in the hypothalmus [28]. However, the mice lacking either COX-1 or COX-2 did not have adnormality in the serum LH level, suggesting that neither of COX isozymes is essential for LH release [29]. In the ovary, NSAIDs inhibited the ovulation process induced by the simulation of LH, and this inhibition was recovered by the administration of PGE_2 or $PGF_{2\alpha}$, indicating that these PGs are important mediators of ovulation [30]. These PGs may be synthesized by the COX-2, since this isozyme was transiently induced in granulosa cells in the follicle upon the stimulation of gonadotropin [31]. Recently COX-2 deficient mice are reported to exhibit the phenotype of infertility due to the impaired ovulation as well as the defects in fertilization, implantation, and decidualization [9]. On the other hand, COX-1 deficient mice have no abnormality in their litter size [8] (Table 1). The arachidonic acid required for the formation of PGs in the ovulation process may be released by the action of $cPLA_2$, because inhibition of $cPLA_2$, because inhibition of $cPLA_2$ resulted in the decrease in both of the intraovarian PGE_2 content and the ovulation number [32]. Furthermore a reduction of litter size was reported as the phenotype of $cPLA_2$ deificient mice [6,7].

Among 8 prostanoid receptor-deficient mice, only EP_2-deficient mice have shown the phentotype of decreased litter size [11-13]. The ovulation number and the fertilization rate of these mice are 80% and 20% of those of wild-type mice, respectively. Thus PGE_2 contributes the process of ovulation and fertilization at least in part via EP_2 receptor. The defects of ovulation and fertilization in EP_2-deficient mice are suggested to result from the impaired function of cumulus cells [11]. Cumulus cells existing around the ovum have important roles on ovulation, transition to oviduct and maturation of ovum, and

fertilization [33]. They induce EP_2 gene during the course of ovulation and fertiliation in response to gonadotropin [11], and secrete extracellular matrix to exert cumulus expansion. PGE_2 has been shown to mimic the action of gonadotropin to exhibit cumlus expansion *in vitro* [34]. However, the cumulus cells of EP_2- deficient mice do not expand upon stimulation of PGE_2, while they are able to expand in response to gonadotropin. Furthermore the cumulus cells of cumulus oocyte complex removed from the oviduct of EP_2-deficient mice exhibit incomplete expansion *in vivo*. These observations suggest that the important role of PGE_2/EP_2 system on cumulus expansion (Fig. 3). Thus agonist of EP_2 receptor in association with gonadotropin might become an effective inducer of ovulation and fertilization.

As described above, COX-2 deficient mice show impaired implantation of wild-type blastocysts [9], suggesting that PGs are an important mediator of implantation process. The observation that EP_2 gene is transiently up-regulated in luminal epithelial cells during the peri-implantation period has suggested the involvement of EP_2 receptor with this process [35, 36]. However, no abnormalities are found in the implantation of wild-type blastocysts in EP_2-deficient mice [11]. This might be due to the compensation of EP_4 receptor because both of EP_2 and EP_4 are expressed in luminal epithelial cells during implantation and coupled to the stimulation of cyclic AMP. Alternatively PG might dominantly act through PPARδ to exet roles on implantation because the impairment of implantation in COX-2 deificient mice are recovered by the administration of PGI_2 analogue or PPARδ agonist [37]. However, further investigation such as the analysis of PPARδ-deficient mice may be required because the dose of PPARδ against used in the recovery of COX-2 deificient mice is relatively high.

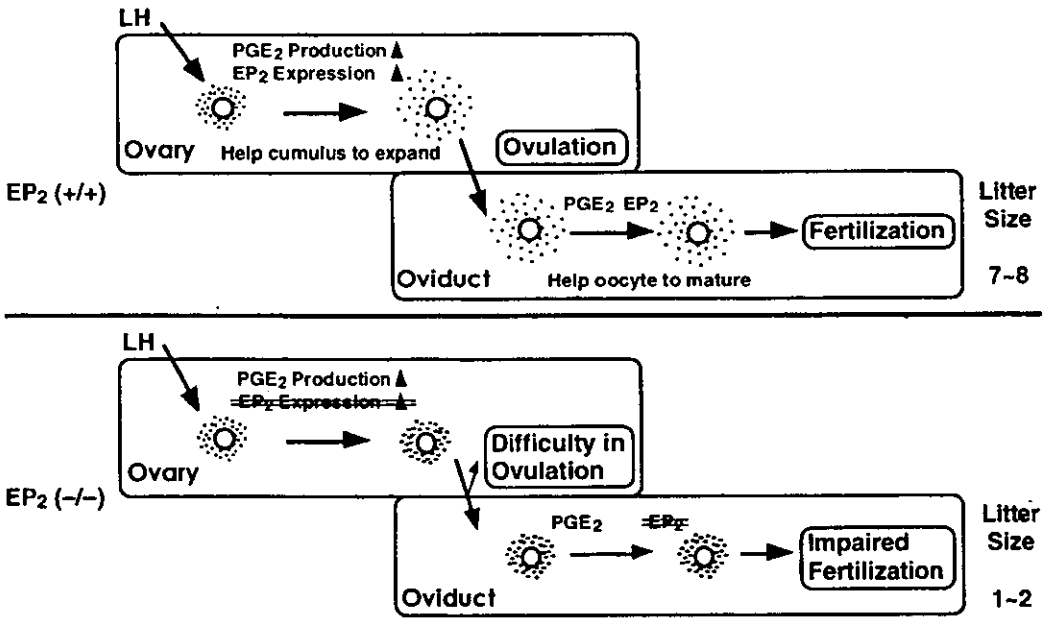

Figure 3. Role of EP_2 receptor in ovulation and fertilization

Perspective

Recent analyses of prostanoid receptor-defcidient mice serve a lot of insights about roles of prostanoids and their receptors on various areas of physiology [5]. In this communication, we reviewed the roles of prostanoids in the physiology of female reproduction. These knowledge would be useful in not only developing new drug therapies in female reproduction but also addressing side effects on female reproduction of the new or conventional drugs in non-reproductive area. Indeed, long use of conventional NSAIDs could be a cause of infertility [38]. Given that role of each PG receptor would be clarified and specific agonists and/or antagonists developed, such side effects may be overcome in the near future by manipulating PG-mediated actions selectively.

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