Activation of peroxisome proliferators-activated receptor δ (PPARδ) promotes blastocyst hatching in mice

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ABSTRACT: Prostaglandins participate in a variety of female reproductive processes, including ovulation, fertilization, embryo implantation and parturition. In particular, maternal prostacyclin (PGI2) is critical for embryo implantation and the action of PGI2 is not mediated via its G-protein-coupled membrane receptor, IP, but its nuclear receptor, peroxisome-proliferator-activated receptor δ (PPARδ). Recently, several studies have shown that PGI2 enhances blastocyst development and/or hatching rate in vitro, and subsequently implantation and live birth rates in mice. However, the mechanism by which PGI2 improves preimplantation embryo development in vitro remains unclear. Using molecular, pharmacologic and genetic approaches, we show that PGI2-induced PPARδ activation accelerates blastocyst hatching in mice. mRNAs for PPARδ, retinoid X receptor (heterodimeric partners of PPARδ) and PGI2 synthase (PGIS) are temporally induced after zygotic gene activation, and their expression reaches maximum levels at the blastocyst stage, suggesting that functional complex of PPARδ can be formed in the blastocyst. Caraprostacyclin (a stable analogue of PGI2) and GW501516 (a PPARδ selective agonist) significantly accelerated blastocyst hatching but did not increase total cell number of cultured blastocysts. Whereas US1605 (a PGIS inhibitor) interfered with blastocyst hatching, GW501516 restored US1605-induced retarded hatching. In contrast to the improvement of blastocyst hatching by PPARδ agonists, PPAR antagonists significantly inhibited blastocyst hatching. Furthermore, deletion of PPARδ at early stages of preimplantation mouse embryos caused delay of blastocyst hatching, but did not impair blastocyst development. Taken together, PGI2-induced PPARδ activation accelerates blastocyst hatching in mice.

Key words: PPARδ activation / blastocyst / hatching / PPARδ conditional knock-out mice

Introduction

Prostaglandins (PGs) participate in a variety of cellular functions, including modulation of vascular tone, cell proliferation, apoptosis and differentiation (Lim and Dey, 2000, 2002; Dey et al., 2004). Cyclooxygenases (COX-1 and COX-2) are the rate-limiting enzymes in the synthesis of PGs by mediating the conversion of arachidonic acid into PGH2, which is then converted to various PGs by specific synthases. Multiple reproductive failures in COX-2-deficient female mice have demonstrated that PGs are critical for various female reproductive processes, including ovulation, fertilization and embryo implantation (Lim et al., 1997). In particular, maternally derived prostacyclin (PGI2) is an essential factor for embryo implantation in mice. While the action of PGI2 is generally mediated via its G-protein-coupled membrane receptor, IP, critical roles of PGI2 in embryo implantation and decidualization are mediated via its nuclear receptor, peroxisome proliferator-activated receptor δ (PPARδ) (Lim et al., 1999; Ding et al., 2003).

PPAR family of transcription factors (PPARα, PPARγ and PPARδ) belongs to the nuclear hormone superfamily (Huang, 2008). The identification of PPAR as a receptor for liver peroxisome proliferators stimulated extensive investigations regarding the role of these receptors in lipid metabolism and homeostasis. Transcriptional activation of target genes by PPARs depends on ligand-induced heterodimerization with a retinoid X receptor (RXR) and recruitment of several cofactors that are shared by various nuclear hormone receptors (Lim and Dey, 2002; Lim et al., 2004; Huang, 2008), implicating the complex signalling networks of PPARs. Three PPAR isoforms exhibit different expression patterns and ligand dependency. PPARα is highly expressed in the liver and is implicated in lipid homeostasis. PPARγ is mainly expressed in white adipose tissue and is implicated in adipocyte differentiation. PPARδ participates in many biological...
processes, including lipid and glucose metabolism, wound healing, tumorigenesis and inflammation.

Several recent studies have demonstrated that PGI₂ is the most abundant PG produced by oviducal epithelial cells in humans and mice, and facilitates blastocyst development and hatching of in vitro cultured mouse embryos (Huang et al., 2002, 2004a). Human embryos co-cultured with homologous oviductal cells had higher hatching rates. Oviduct epithelial cells express enzymes crucial to PGI₂ synthesis, COX-1, COX-2 and PGI₂ synthase (PGIS) in humans and mice, and can produce a large amount of PGI₂ when incubated with excess arachidonic acid (Huang et al., 2002, 2004a). In addition, PGI₂ seems to enhance implantation and live birth potentials of in vitro cultured embryos (Huang et al., 2004b). A recent study has suggested that PGI₂-mediated PPAR activation is critical to cell proliferation of preimplantation embryos, and PPAR deficiency leads to impaired cell proliferation and subsequently delayed blastocyst development and hatching (Huang et al., 2007b). In contrast, another report has shown that PGI₂ does not increase the cell numbers of in vitro cultured blastocysts, while it enhances blastocyst development and hatching (Liu et al., 2006). Thus, it still remains unclear whether PGI₂-induced PPARg activation facilitates cell proliferation, leading to advances of blastocyst development and hatching. In this paper, using genetic, pharmacologic and molecular approaches, we demonstrate that ligand-induced activation of PPARg facilitates blastocyst hatching without a significant change in embryonic cell number.

Materials and Methods

Collection of eggs and preimplantation embryos

Four-week-old C57BL6 or ICR female mice were bred with adult male mice (Orient Bio, Seongnam, Korea). Mice were housed with the lights off for 12 h daily and fed ad libitum. Superovulation was induced by a standard hormone treatment using an intraperitoneal injection of 5 IU pregnant mare’s serum gonadotrophin (Sigma, St. Louis, MO, USA) followed by 5 IU human chorionic gonadotrophin (Sigma) 48 h later. After mating overnight with fertile males, females were inspected for vaginal plugs the next morning and sacrificed at appropriate times for each experiment. Two-cell embryos and blastocysts were harvested from oviducts and uteri, respectively and cultured in M16 medium supplemented with 0.1% bovine serum albumin (BSA; M16 + BSA). For embryo RNA extraction, embryos at a variety of developmental stages were collected, washed and stored in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) until use.

RNA extraction and RT–PCR

Total RNA was isolated from 20 pooled embryos using TRIzol reagent according to the manufacturer’s protocol. One microgram glycogen was added before RNA extraction to enhance the isolation efficiency of embryo RNA. For the external reference, rabbit α-globin mRNA was also added before RNA extraction. Total RNA was pelleted by centrifugation, washed using 1 ml of 75% ethanol and dissolved in RNase-free water. Total RNA isolated from embryos at various developmental stages was subjected to RT using Moloney murine leukaemia virus (MMLV) reverse transcriptase (Roche Applied Science, Indianapolis, IN, USA) for cDNA synthesis. RNA was denaturated at 95°C for 5 min and reverse transcriptase was added at 42°C for 60 min in 20 μl reaction mixture consisting of 1X RNA PCR buffer, 1 mM dNTPs, 2.5 μM oligo dT primer, 1 unit/μl RNasin ribonuclease inhibitor and 2.5 unit/μl MMLV reverse transcriptase. After the reaction was conducted at 95°C for 5 min, samples were stored at −70°C until the PCR. One microlitre of RT product (1 equivalent of single embryo) was used for RT–PCR with specific primers at optimized cycles (MyCycler, Bio-Rad Laboratories, Hercules, CA, USA). The primer sequences and size of the amplicons are listed in Table I.

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Culture of preimplantation mouse embryos with PPARδ agonists, PPAR antagonists or a PGIS inhibitor

Embryos were collected in HEPES-buffered M2 medium and cultured in M16 + BSA under mineral oil at 37°C in a humidified atmosphere of 5% CO2 in air. Embryos collected at 2-cell or blastocyst stage were cultured in drops of M16 with various concentrations of carbaprostacyclin (cPGI; a stable analogue of PGI2, Cayman, Ann Arbor, MI, USA), GW501516 (PPARδ selective agonist, MERCK, Darmstadt, Germany), PPAR antagonists, GW9662 (Sigma) and T0070907 (Cayman) and/or a PGIS inhibitor (US 605, Cayman). Blastocyst development and hatching were evaluated at post hCG 96 and 144 h, respectively.

Generation of PPARδ-deficient preimplantation embryos with Cre-ERTM

PPARδ conditional knock-out [(PPARδ (ck/ck))] mice were kindly provided by Dr Ron Evans (Salk Institute, San Diego, CA, USA). PPARδ(ck/ck) mice (Barak et al., 2002) were bred with Cre-ERTM transgenic mice (Hayashi and McMahon, 2002) to maintain a colony of tamoxifen-induced PPARδ knock-out mice. Offspring were genotyped by PCR with genomic DNA and appropriate primers specific for PPARδ conditional knock-out construct (Barak et al., 2002) and Cre-ERTM transgene (Hayashi and McMahon, 2002), respectively.

Cre-ERTM transgenic mice ubiquitously express a fusion protein between Cre and a mutated form of the ligand-binding domain of the estrogen receptor (Cre-ERTM) that renders Cre activity tamoxifen inducible, allowing for conditional deletion of genes at a variety of developmental stages (Hayashi and McMahon, 2002). ER TM prevents binding of its natural ligand (17β-estradiol) at normal physiological concentrations, but renders the ER TM domain responsive to tamoxifen.

To produce embryos deficient of PPARδ during early embryogenesis, PPARδ(ck/ck):Cre-ERTM female mice were superovulated and bred with the same genotyped males. Collected two-cell-staged embryos were cultured in M16 + BSA with tamoxifen to induce temporal deletion of PPARδ. Tamoxifen was initially dissolved in ethanol and diluted with culture media to appropriate concentrations used in this study. Developmental rates and hatching rates of embryos derived from PPARδ(ck/ck):Cre-ERTM x PPARδ(ck/ck):Cre-ERTM and from wildtype x wildtype with or without tamoxifen were examined at post hCG 96 and 144 h, respectively.

Statistics

Fisher’s exact test or student’s t-test was performed to examine statistical significance (*, P < 0.05; **, P < 0.01).

Results

PPARδ and its heterodimeric partners are temporally expressed during preimplantation embryo development

Using RT–PCR, we have examined whether PPARδ and other members of PPAR family (PPARα and PPARγ) are expressed in preimplantation mouse embryos. PPARδ is predominantly expressed among three members of PPAR family (Fig. 1A). PPARδ mRNA was detected at the pronuclear stage, suggesting that there is maternal PPARδ mRNA. While PPARδ expression from 8-cell stage onwards was predominantly induced at the blastocyst stage, PPARα and PPARγ are not expressed in preimplantation embryos after zygotic gene activation.

To function as a receptor for PGI2, PPARδ has to recruit one of RXRs as its heterodimeric partner. Thus, we have examined temporal expression patterns of RXR family members (α, β, γ) during preimplantation embryo development (Fig. 1B). Among three RXR members, RXRα was predominantly expressed and its expression significantly increased at the blastocyst stage. RXRβ had expression patterns similar to that of RXRα at much lower levels and RXRγ mRNA levels were very low to undetectable. Furthermore, we examined temporal expression patterns of PGIS and IP, membrane receptor for PGI2 during preimplantation embryo development (Fig. 1C). PGIS mRNA was expressed from morula stage and its level increased at the blastocyst stage. Whereas PPARδ was highly expressed at the blastocyst stage, IP expression was very low or undetectable. Collectively, these results suggest that PPARδ-RXR transcriptional complex could be formed to mediate PGI2 actions during preimplantation embryo development.

cPGI and a PPARδ selective agonist significantly accelerates blastocyst hatching in vitro

To examine whether PGI2-PPARδ-RXR complex facilitates blastocyst formation and subsequent hatching in mouse embryos, superovulated 2-cell stage embryos were cultured with a stable analogue of PGI2,
cPGI or a selective PPAR\(\delta\) agonist, GW501516. While the blastocyst formation rate of embryos cultured with cPGI did not reach statistical significance, both cPGI and GW501516 increased blastocyst formation rates 48 h later (Day 4) (Fig. 2A). Furthermore, cPGI and GW501516 clearly accelerated blastocyst hatching 96 h after treatment (Day 6) (Fig. 2B), suggesting that PGI\(_2\) action on blastocyst hatching is mediated via its nuclear receptor, PPAR\(\delta\) in mice. Since blastocyst hatching is influenced by the cell number of blastocyst (Montag et al. 2000), total cell number of blastocysts cultured with either cPGI or GW501516 were counted at 96 h after treatment. Whereas cPGI and GW501516 accelerated blastocyst hatching, neither cPGI nor GW501516 significantly increased total cell number of blastocysts (Fig. 2C and D). These results suggested that accelerated blastocyst hatching is not directly associated with the cell number of blastocysts in mice.

**Activation of PPAR\(\delta\) reverses inhibitory effects of a PGIS inhibitor, U51605 on blastocyst hatching**

To examine whether activation of PPAR\(\delta\) signalling pathway is critical for blastocyst hatching in mice, blastocysts were cultured with U51605, a PGIS inhibitor or with U51605 + GW501516, and then their hatching was evaluated from 48 to 96 h later. U51605 significantly interfered with initiation of blastocyst hatching (Fig. 3) although it did not affect blastocyst development of 2-cell embryos (data not shown). Addition of GW501516 effectively reversed inhibitory effects of U51605 on blastocyst hatching, suggesting that the function of PGI\(_2\) on blastocyst hatching is mediated via its nuclear receptor PPAR\(\delta\).

**PPAR antagonists interfere with blastocyst hatching in mice**

To further examine whether advanced blastocyst hatching is associated with PPAR\(\delta\) activation, Day 4 blastocysts were collected and cultured with PPAR antagonists for 48 h. Since PPAR\(\delta\)-specific antagonist is not commercially available, and PPAR\(\alpha\) and PPAR\(\gamma\) are not expressed in preimplantation embryos (Fig. 1A), PPAR antagonists, GW9662 and T0070907, which have more potent inhibitory effects on PPAR\(\gamma\) and/or PPAR\(\alpha\) were used to interfere with blastocyst hatching. Hatching rates of blastocysts treated with either GW9662 or T0070907 were significantly reduced 48 h after treatment (Fig. 4A). Whereas a few blastocysts failed to initiate hatching in control, many blastocysts treated with either GW9662 or

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**Figure 2** Activation of PPAR\(\delta\) accelerates blastocyst hatching, but not blastocyst development. (A and B) Graphs of blastocyst development rates (A) and hatching rates (B) of 2-cell embryos cultured for 48 and 96 h with PPAR\(\delta\) agonists (cPGI or GW501516), respectively. Three independent experiments were performed and similar results were obtained (total number of embryos in each group, \(n = 51–55\)). (C) Total cell number of Day 6 blastocysts after 96 h culture with PPAR\(\delta\) agonists (cPGI or GW501516). (D) Representative images of Hoechst-stained Day 6 blastocysts in (C). *indicates \(P < 0.05\) and the graph in C presents mean ± SD.
T0070907 did not show any sign of hatching and were entirely encased by zona pellucida (arrowheads in Fig. 4). Total cell number of blastocysts cultured with GW9662 or T0070907 was similar to that of control blastocysts (Fig. 4C and D).

**PPARδ deletion at preimplantation stages delays blastocyst hatching**

To examine the distinct roles of PPARδ for preimplantation embryo development and blastocyst hatching in vitro, we have established a mouse model that we can delete PPARδ at the 2-cell stage. Two-cell stage embryos derived from PPARδ(ck/ck):Cre-ER<sup>TM</sup> × PPARδ(ck/ck):Cre-ER<sup>TM</sup> were cultured in media containing tamoxifen for activation of Cre-ER. PCR with blastocyst genomic DNA (Fig. 5A) showed that ~75% blastocysts derived from this experimental setting were PPARδ-deficient. Thus, we considered the embryos derived from PPARδ(ck/ck):Cre-ER<sup>TM</sup> × PPARδ(ck/ck):Cre-ER<sup>TM</sup> as PPARδ(ck/ck):Cre-ER<sup>TM</sup> thereafter. Below the level of 80 μM tamoxifen, there were no apparent cytotoxic effects on preimplantation embryo development of 2-cell embryos treated with tamoxifen with respect to blastocyst formation, total cell number of blastocysts and blastocyst hatching in vitro (data not shown). To examine direct roles of PPARδ on blastocyst formation, blastocyst hatching and total cell number of blastocysts, PPARδ(ck/ck) and PPARδ(ck/ck):Cre-ER<sup>TM</sup> embryos at 2-cell stage were collected and cultured with or without tamoxifen (80 μM).

Tamoxifen treatment did not have any significant effects on total cell number of both wildtype and PPARδ(ck/ck):Cre-ER<sup>TM</sup> blastocysts (data not shown). With respect to blastocyst development, PPARδ(ck/ck):Cre-ER<sup>TM</sup> embryos developed to the blastocyst similar to PPARδ(ck/ck) embryos regardless of the presence of tamoxifen (Fig. 5B). In contrast to the blastocyst development, blastocyst hatching was significantly delayed in PPARδ(ck/ck):Cre-ER<sup>TM</sup> embryos cultured with tamoxifen (Fig. 5C). In addition, complete hatching rate of PPARδ(ck/ck):Cre-ER<sup>TM</sup> embryos treated with tamoxifen was significantly reduced (data not shown). On the other hand, the total cell...
number of tamoxifen-treated PPARδ deficient blastocysts were not different from that of PPARδ(ck/ck) blastocysts (Fig. 5D). Collectively, deletion of PPARδ at early preimplantation stages leads to delayed blastocyst hatching without significant changes in cell number.

Discussion

PGs are well known to be critical for multiple events of female reproductive events from ovulation to parturition (Lim et al., 1997, 1999; Reese et al., 1999, 2001; Lim and Dey, 2000; Dey et al., 2004; Wang et al., 2007). Previously, we have shown that among PGs, COX-2-derived PGI2 mediates blastocyst implantation via its nuclear receptor, PPARδ in mice (Lim et al., 1999). Here, we demonstrated that PGI2 facilitates blastocyst development and hatching via its nuclear receptor PPARδ without a significant change in the total cell number in mice. Our works collectively suggested that the activation of PPARδ is important for blastocyst hatching in the embryo as well as blastocyst implantation in the uterus in mice.

Our RT–PCR results showed that PPARα and PPARγ are not expressed in preimplantation mouse embryos after zygotic gene activation. It is consistent with the results of a previous work that selective agonists for PPARα and PPARγ do not have any effects on blastocyst development and hatching in mice (Huang et al., 2007b). Since PPARα and PPARγ are not expressed in preimplantation embryos, PPAR antagonists that have more potency to inhibit PPARα and PPARγ than PPARδ were used in this study, and they effectively interfered with blastocyst hatching. To regulate its target genes important for blastocyst development and hatching, PPARδ is required to form a heterodimer with one of RXRs (Lim et al., 1999; Lim and Dey, 2000; Huang, 2008). We demonstrate that RXRs, especially RXRα, as well as PPARδ, are expressed in preimplantation mouse embryos with a peak level at the blastocyst stage, suggesting that they may regulate the expression of target genes important for preimplantation embryo development. In fact, good quality embryos express significantly higher levels of RXR mRNA transcripts than bad quality embryos in bovine preimplantation embryos (Mamo et al., 2005). Collectively, concomitant expression of PPARδ, RXRs and PGIS at the blastocyst stage suggested that PGI2-induced PPARδ activation is involved in blastocyst hatching in mice.

It has been proposed that blastocyst hatching is mediated by two major factors: a crucial increase of cell number in embryos (Montag et al., 2000) and the digestion of zona pellucida by proteolytic enzymes (Sawada et al., 1990). There have been many reports that proteolytic processes with a variety of proteases are involved in blastocyst hatching although it still remains unclear. The cysteine proteases, cathepsin L and P, are expressed in blastocyst and functionally involved in blastocyst hatching in golden hamster (Sireesha et al., 2008). Two implantation serine proteases, ISP1 and ISP2, and other proteases such as plasminogen activators and matrix metallo-proteases have been proposed to play a role in the hatching process (Seshagiri et al., 2003; Aflalo et al., 2005; Sharma et al., 2006). Barak et al. suggested that imbalance in extracellular matrix

![Figure 5](image-url)

**Figure 5** Conditional deletion of PPARδ in early preimplantation embryos delays blastocyst hatching. (A) An example of PPARδ genotyping PCR for blastocysts cultured with or without tamoxifen (TM). (B) Blastocyst developmental rates of 2-cell embryos (Day 2) cultured with or without TM on Day 4. (C and D) A graph for % blastocyst without any sign of hatching on Day 6 (C) and total cell number (D) of Day 6 blastocyst cultured with or without TM. Three independent experiments were performed and similar results were obtained (total number of embryos in each group, n = 51–55). **indicates P < 0.01 and the graph in D represents mean ± SD.
remodelling caused by either insufficient or excessive proteolysis in PPARδ knockout post-implantation embryos could cause the breakdown of the placenta-decidual interface (Barak et al., 2002). This implicates that PGI2-induced PPARδ activation may regulate the expression of a number of proteases that could be involved in proteolytic process of blastocyst hatching. PPARδ agonist induces a massive differentiation of trophoblast giant cells via Akt phosphorylation during placenta development (Nadra et al., 2006). Interestingly, inhibition of PI3K/Akt signalling pathway resulted in a significant delay in blastocyst hatching (Riley et al., 2005). Further investigation to understand downstream pathways of PPARδ in blastocyst hatching will be required for the clinical application of PPARδ selective agonists to improve human IVF cycles suffering from hatching problems such as zona hardening.

Using multiple approaches including a conditional knock out model, we demonstrated that PPARδ activation facilitates blastocyst hatching without significant changes in cell numbers in mice. However, a recent study using conventional PPARδ knockout embryos has shown that PPARδ deficiency causes severely impaired cell proliferation, subsequently leading to poor blastocyst development and delayed hatching (Huang et al., 2007b). This discrepancy in phenotypes of two different PPARδ knockout mouse models could be due to the maternal effects of PPARδ deficiency. In fact, quality improvement of oocyte matured in vitro enhances blastocyst development and hatching in mice (Yeo et al., 2008). There are many evidences that maternal deficiency of growth factors, cytokines and transcription factors results in suboptimal or poor quality of oocyte and subsequent embryo development (Panic et al., 2006; Huang et al., 2007a; Sun et al., 2008; Yeo et al., 2008). For example, the different development potentials of IP(+/-) embryos derived from IP(-/-) oocytes or IP(+-/-) sperm support this rationale. Whereas IP(+/-) embryos derived from IP(-/-) sperm develop as competently as IP(+/-+) embryos, IP(+/-) embryos derived from IP(-/-) oocytes showed retarded early embryo development (Huang et al., 2007a). It clearly suggested that lagged development of IP(+/-) embryos from IP(-/-) oocytes but not IP(+/-) sperm could be originated from defective oocyte development caused by IP deficiency in oocytes and/or somatic cells during follicle development.

Thus, PPARδ deficiency in oocyte and/or somatic cells in developing follicles could affect the quality of oocytes, possibly resulting in impaired cell proliferation in the embryo. Such defects in proliferation and/or survival of blastomeres in the embryo may negatively influence following embryo development and blastocyst hatching in conventional PPARδ knock-out embryos. Indeed, blastocyst hatching in vitro is dependent on a sufficiently high number of embryonic cells (Montag et al., 2000). In the present study using PPARδ conditional knock out model in which PPARδ is deleted from 2-cell stage, we could deviate this phenomenon and provide the evidence that PGI2-induced PPARδ activation is important for blastocyst hatching, but does not significantly influence cell proliferation and/or apoptosis in preimplantation mouse embryos. Collectively, these two PPARδ mouse models suggest that PPARδ is critical for oocyte quality with subsequent embryo development and blastocyst hatching for implantation.

**Authors’ roles**

H.J.K. carried out the majority of the experiments and drafted the manuscript. S.J.H. and J.A.Y. performed other experiments. J.H.J., H.J.L. and T.K.Y. were involved in the study design, data collection and interpretation. H.S. received the fund for the project, planned and supervised the experiments and wrote the paper.

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**References**


