Stage-specific Integration of Maternal and Embryonic Peroxisome Proliferator-activated Receptor δ Signaling Is Critical to Pregnancy Success

Received for publication, August 8, 2007, and in revised form, September 21, 2007 Published, JBC Papers in Press, October 26, 2007 DOI 10.1074/jbc.M706577200

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Successful pregnancy depends on well coordinated developmental events involving both maternal and embryonic components. Although a host of signaling pathways participate in implantation, decidualization, and placentation, whether there is a common molecular link that coordinates these processes remains unknown. By exploiting genetic, molecular, pharmacological, and physiological approaches, we show here that the nuclear transcription factor peroxisome proliferator-activated receptor (PPAR) δ plays a central role at various stages of pregnancy, whereas maternal PPARδ is critical to implantation and decidualization, and embryonic PPARδ is vital for placentation. Using trophoblast stem cells, we further elucidate that a reciprocal relationship between PPARδ-AKT and leukemia inhibitory factor-STAT3 signaling pathways serves as a cell lineage sensor to direct trophoblast cell fates during placentation. This novel finding of stage-specific integration of maternal and embryonic PPARδ signaling provides evidence that PPARδ is a molecular link that coordinates implantation, decidualization, and placentation crucial to pregnancy success. This study is clinically relevant because deferral of on time implantation leads to spontaneous pregnancy loss, and defective trophoblast invasion is one cause of preeclampsia in humans.

Implantation, decidualization, and placentation are critical steps for successful pregnancy in most mammals, including humans. Although various signaling pathways play important roles in these processes (1–4), whether there is a common molecular link that coordinates them involving both uterine and embryonic components remains unknown.

Implantation is normal only if it occurs within a limited time period termed window implantation when the blastocyst is implantation-competent and the uterus is receptive (3). We have shown previously that compromised prostaglandin (PG)6 signaling resulting from either cytosolic phospholipase 2α (cPLA2α) or cyclooxygenase-2 (COX-2) deficiency causes deferral of on time implantation that creates adverse ripple effects impacting decidualization, placentation, and fetal well being (5, 6). We also had circumstantial evidence that prostacyclin (PGI1) derived from the cPLA2α-COX-2 axis plays a role during early pregnancy via activation of peroxisome proliferator-activated receptor δ (PPARδ, also known as PPARβ) (7). These studies led us to speculate that PPARδ serves as a molecular link coordinating various critical steps during pregnancy.

PPARδ belongs to the PPAR family, which includes two other members PPARα and PPARγ. They are ligand-dependent transcription factors and heterodimerize with retinoic acid X receptors for functional activation (8). PPARδ participates in many biological processes, including lipid and glucose metabolism (9, 10), epidermal maturation and wound healing (11, 12), muscle development and function (13–15), tumorigenesis (16–18), and inflammation (19, 20).

Combining multiple approaches, we show here that although uterine PPARδ is essential for normal implantation and decidualization, embryonic PPARδ via coordinated interaction with AKT and leukemia inhibitory factor (LIF)-STAT3 signaling is vital for specifying trophoblast cell differentiation during placentation.

EXPERIMENTAL PROCEDURES

Reagents—Antibodies to anti-phospho-p38 MAPK (Thr-180/Tyr-182), total p38 MAPK, phospho-extracellular signal-regulated kinase (ERK) 1/2 (Thr-202/Tyr-204), total ERK1/2, phospho-AKT (also known as protein kinase B) (Ser-473), total

Pharmacological tools and cell lines are described in detail in the experimental procedures. The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

6 The abbreviations used are: PG, prostaglandin; ANG, angiopoietin; CDX2, caudal-related homeobox 2; COX-2, cyclooxygenase-2; cPLA2α, cytosolic phospholipase 2α; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; HAND1, heart and neural crest derivatives expressed transcript 1; LIF, leukemia inhibitory factor; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; PGI2, prostacyclin; PL-I, placental lactogen-I; PPAR, peroxisome proliferator-activated receptor; STAT, signal transducer and activator of transcription; TGCG, trophoblast giant cell; TPBP/4311, trophoblast-specific protein α; TS cells, trophoblast stem cells; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor; WT, wild type; TRITC, tetramethylrhodamine isothiocyanate; IP, prostaglandin I receptor; EP, prostaglandin E receptor.

8 This work was supported in part by National Institutes of Health Grants HD12304, DA05668, P01-CA-77839 (to S. K. Dey), ES07814, HD37830 (to S. K. Dey), and HD050315 (to H. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The Journal of Biological Chemistry Vol. 282, No. 52, pp. 37770–37782, December 28, 2007 © 2007 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
AKT, phospho-signal transducer and activator of transcription 3 (STAT3) (Tyr-705), or total STAT3 were obtained from Cell Signaling; anti-cytokeratin was from DAKO Diagnostics; anti-caudal-related homeobox 2 (CDX2) was from BioGenex; and anti-PPARδ, vascular cell adhesion molecule-1 (VCAM-1), or suppressor of cytokine signaling 3 (SOCS3) were from Santa Cruz Biotechnology. COX-2 antibody was custom-made as described previously (21). Peptide corresponding to amino acids 563–577 of the mouse mature COX-2 protein was used to produce the rabbit antiserum to COX-2, and no cross-reactivity with COX-1 was detected. Biotin-conjugated BS-I Isolectin B4, fibroblast growth factor 4 (FGF4), heparin, AH-6809, and prostaglandin F2α were purchased from Sigma. LIF was obtained from Amersham Biosciences. Invitrogen. ECL kit for Western blot was purchased from Amersham Biosciences.

Animals—PPARδ null mice originally established on a C57BL/6J/Sv129 background (22) were back-crossed with CD1 mice for 10 generations. FLK1lacZ+/− mice were originally generated on a C57BL/6J/Sv129 background (23) and underwent backcrossings with CD1 mice for 10 generations. PPARδ−/−/FLK1lacZ+/− mice were generated by crossing PPARδ−/− males with FLK1lacZ+/− females on CD1 background. All mice used were housed in the Institutional Animal Care Facility according to National Institutes of Health and institutional guidelines for laboratory animals.

Ovulation and Fertilization—To examine ovulation and fertilization, wild-type (WT) or PPARδ−/− mice were bred with fertile males with same genotypes, respectively. Mice were killed on day 2 of pregnancy and oviducts were flushed with Whitten’s medium to recover eggs and embryos. Their morphology was examined under a dissecting microscope (5).

Implantation and Decidualization—Implantation sites on day 4 midnight and days 5 and 6 midmorning of pregnancy were visualized by intravenous injections of Chicago Blue dye solution (24). Uteri of mice without blue bands were flushed with Whitten’s medium to recover unimplanted blastocysts. To experimentally induce artificial decidualization, pseudopregnant WT or PPARδ−/− mice received intraluminal infusion of sesame oil (25 μl) in one uterine horn on day 4 and were killed 4 days later. Uterine weights of infused and noninfused (control) horns were recorded, and fold increases in uterine weights served as an index of decidualization (25).

Reciprocal Blastocyst Transfer—Day 4 WT or PPARδ−/− blastocysts were transferred into uteri of WT or PPARδ−/− pseudopregnant recipients on day 4 (24), and recipients were killed on the morning of days 5 and 6 to examine implantation by the blue dye method. Other recipients were sacrificed on day 10 for placentation analysis.

Immunostaining and Lectin Histochemistry—Immunofluorescence staining of PPARδ in preimplantation embryos was performed as described by us (26). Fluorescence signals were viewed under a Zeiss LSM 510 confocal laser microscope. Immunolocalization of PPARδ, COX-2, phospho-AKT, phospho-STAT3, cytokeratin, VCAM-1, PI-1, SOCS3, and CDX2 was performed in 10% neutral-buffered formalin or Bouin’s fixed and paraffin-embedded sections of uteri and placentas using a Histostain-SP kit as described (21). To examine the maternal vasculature of the labyrinth zone, we performed BS-I Isolectin binding assay, which identifies the cell-surface carbohydrate structure of trophoblast cells lining maternal blood spaces within the labyrinth. Staining was done by the procedure as described (27). Immunohistological and lectin binding analysis were performed on at least 4–6 different implantation sites or placentas at each developmental stage obtained from three different mice.

Prostaglandin Assays—PGs were quantitated using gas chromatography/negative ion chemical ionization mass spectrometric assays as described previously (7). PGI2 was measured as its stable metabolite 6-keto-PGF1α.

In Situ and Northern Hybridization—In situ and Northern hybridizations were performed as described previously (28). Antisense 35S-labeled or 32P-labeled cRNA probes were generated using appropriate polymerases from mouse-specific cDNAs. Northern hybridized bands were quantified using a personal densitometer (GE Healthcare).

LacZ Staining—LacZ staining in frozen sections was performed as described previously by us (29).

Trophoblast Stem (TS) Cell Culture—WT or PPARδ−/− TS cell lines were generated as reported previously (30). Cells were maintained in a proliferative state in media containing 70% embryonic mouse fibroblast cells conditioned medium, 30% TS cell medium, FGF4 (25 ng/ml), and heparin (1 μg/ml). To induce TS cell differentiation, cells were cultured in medium free of sera, FGF4, and heparin for 2–6 days with medium changes every day.

Western Blotting—Protein extraction and Western blotting were performed as described previously (31). Antibodies to total and phospho-ERK1/2, p38, AKT, or STAT3 were used. Bands were visualized using an ECL kit and quantitated using a personal densitometer (GE Healthcare).

Statistical Analysis—Comparison of means was performed using a one-tailed Student’s t test. Data are shown as means ± S.E.

RESULTS

PPARδ Deficiency Limits Term Pregnancy Success—To delineate PPARδ functions in pregnancy, we first analyzed pregnancy outcome in PPARδ−/− mice on CD1 background. Although homozygous null females give birth to viable pups, the litter size is significantly smaller compared with WT females (Fig. 1A). To search for the cause of reduced litter size, we examined early pregnancy events in PPARδ−/− mice.

Ovulation and Fertilization Are Normal in PPARδ−/− Mice—To examine ovulation and fertilization status in PPARδ−/− mice, we recorded the number of ovulated eggs and fertilized two-cell embryos on day 2 of pregnancy. Normal ovulation and fertilization were noted in PPARδ−/− females mated with null males. As shown in Fig. 1B, all null females ovulated with comparable numbers of ova as WT females. The yield of two-cell embryos among ovulated eggs determined the fertilization rate. The number of two-cell embryos was similar in both WT and null females (Fig. 1C). Because PPARδ is dispensable for normal
ovulation or fertilization, we continued our search for the cause of reduced fertility post-fertilization in PPARδ−/− mice.

PPARδ Deficiency Defers On Time Implantation—Using immunofluorescence, we first examined PPARδ expression in preimplantation embryos, and we noted that PPARδ is localized in the nucleus at all stages of embryos spanning 1-cell zygotes to blastocysts (Fig. 1D). This observation suggests that PPARδ signaling could be important during early pregnancy.

Thus, we next compared implantation status in WT and mutant mice.

As shown in Fig. 1, E and F, whereas 45 and 100% of WT mice showed distinct implantation sites at 2000 and 2400 h on day 4, respectively, none of the PPARδ−/− females showed implantation even at 0100 h on day 5 as examined by the blue dye method. Unimplanted blastocysts with normal morphology were recovered (Fig. 1G), indicating their normal development. However, over 90% (10 of 11) of null mice showed blue bands when examined at 0800 h on day 5, suggesting deferred implantation in PPARδ−/− females. This result provides the first genetic evidence that PPARδ is critical for timely onset of implantation.

Maternal PPARδ Is Crucial for Normal Implantation—To ascertain the relative contribution of uterine versus embryonic PPARδ in implantation, we performed reciprocal blastocyst transfer experiments. Day 4 WT or PPARδ−/− blastocysts were transferred into PPARδ−/− or WT recipients on day 4 of pseudopenancy. As shown in Table 1, WT blastocysts implanted normally after transfer into WT recipients (65 and 66% on days 5 and 6, respectively). In contrast, WT blastocysts transferred into PPARδ−/− recipients had considerably reduced implantation rate when examined on day 5; only 21% of transferred blastocysts showed implantation in 6 of 11 mutant recipients. But when examined on day 6, all mice showed implantation with an average of 44% implantation sites, reinforcing that implantation is deferred in the absence of maternal PPARδ. Reduced implantation rates were not observed when PPARδ−/− blastocysts were transferred into WT uteri; 60% of blastocysts transferred showed implantation in all mice examined on days 5 and 6. Collectively, the results show that maternal, but not embryonic, PPARδ is a major contributor to on time implantation.

PGE2 via EP2/4 Receptors Compensates for the Loss of PPARδ during Implantation—Observations of deferred implantation, but not its complete failure, in PPARδ−/− mice suggested that alternative pathways by PGI2 or other PGs may partially compensate for the loss of PPARδ during implantation. To address this issue, we first measured PG levels and found that PGI2 (measured as 6-keto-PGF2α) levels are highest followed by PGE2 (measured as PGF2α) levels in WT and PPARδ−/− mice as examined on day 2 of pregnancy. Numbers within the bars in B indicate the number of mice with 2-cell embryos, total number of mice with ovulation, and those in C indicate the number of mice with 2-cell embryos. D, immunofluorescent nuclear PPARδ is detected in preimplantation embryos. ICM, inner cell mass; Tr, trophoderm. Bar, 50 μm. E, genetic loss of PPARδ defers the window of implantation. Numbers within the bar indicate the number of mice with blue bands/mice examined. F, representative photographs of uteri with or without implantation (blue bands) in WT or PPARδ−/− mice. G, recovered unimplanted blastocysts from PPARδ−/− mice showing normal morphology. Bar, 50 μm.
TABLE 1
Implantation of blastocysts transferred into pseudopregnant WT or PPARδ−/− mice
Day 4 WT or PPARδ−/− blastocysts were transferred into PPARδ−/− or WT recipients on day 4 of pseudopregnancy. Recipients were sacrificed on days 5 and 6 to examine the number of implantation sites (IS) by the blue dye method. Uteri without IS were flushed with Whitten’s medium to recover any unimplanted blastocysts. NA indicates not applicable.

| Genotypes | Recipients | Day of sacrifice | No. of blastocysts transferred | No. of recipients | No. of mice with IS (%) | No. of IS (%) | No. of blastocysts recovered |
|-----------|------------|------------------|-------------------------------|------------------|------------------------|--------------|-----------------------------|
| +/+       | +/+        | 5                | 96                            | 7                | 7 (100)                | 62 (65)      | NA                          |
| +/+       | −/−        | 6                | 76                            | 6                | 6 (100)                | 50 (66)      | NA                          |
| −/−       | +/+        | 5                | 172                           | 11               | 6 (55)                 | 36 (21)*     | 29                          |
| −/−       | +/+        | 6                | 144                           | 9                | 9 (100)                | 64 (44)      | NA                          |
| −/−       | +/+        | 5                | 72                            | 6                | 6 (100)                | 49 (68)      | NA                          |
| −/−       | +/+        | 6                | 56                            | 5                | 5 (100)                | 36 (64)      | NA                          |

*p < 0.05 (Student’s t test).

FIGURE 2. Pharmacological silencing of PGE2 signaling exacerbates the deleterious effects of loss of PPARδ on implantation. A, PGI2 and PGE2 are the major PGs produced in day 5 implantation sites of WT and PPARδ−/− mice. No significant differences in uterine PG levels were noted between WT and null mice (p > 0.05, n = 5–6; Student’s t test). PGI2 was measured as 6-keto-PGF1α. Data are means ± S.E. B and C, expression of IP and EP2/4 receptors in WT and PPARδ−/− peri-implantation uteri. In situ hybridization analysis shows that IP is expressed in a subpopulation of stromal cells just underneath the circular muscle layer and in interstitial cells within the myometrium on days 4 and 5 in PPARδ−/− mice, whereas its expression remains low in WT uteri. EP2 and EP4 transcripts are comparable in peri-implantation uteri of WT and null mice. Arrows indicate the location of implanting blastocysts. ls, lumen; myo, myometrium; s, stroma. Bar, 200 μm. D, co-treatments with EP2- and EP4-selective antagonists, AH-6809 plus AH-23848, further reduce implantation in PPARδ−/− mice. Numbers within the bar indicate the number of mice with blue bands/mice examined. Bl, blastocysts; Veh, vehicle.

absence of PPARδ. We found that the expression of these receptor subtypes is comparable in both WT and PPARδ−/− uteri. EP2 was primarily expressed in the receptive luminal epithelium on day 4 and EP3 in the luminal epithelium surrounding the implanting blastocyst on day 5 (Fig. 2C), suggesting their potential roles in implantation in null females. In contrast, EP1 is expressed at a very low to undetectable level, and EP3 is primarily expressed in mesometrial stromal cells on day 5, suggesting their minimal, if any, involvement in blastocyst attachment. These results would imply that PGE2, via EP1/2 receptors, partially offsets the loss of PPARδ during implantation. Thus, we asked if pharmacological inhibition of EP2/4 receptors would further deteriorate implantation in PPARδ−/− mice. Indeed, whereas coadministration of AH-6809 (EP1/2 antagonist) with AH-23848 (EP4 antagonist) at 5 mg each/kg body weight on day 4 failed to inhibit implantation in WT mice, the same treatment remarkably blocked implantation in PPARδ−/− mice when examined on day 5 (Fig. 2D). This finding points toward a compensatory contribution of PGE2 to implantation in the absence of PPARδ.

Decidualization Is Compromised in PPARδ−/− Mice—Because PPARδ is highly expressed in stromal cells with the onset and progression of decidualization (7), we speculated that PPARδ deficiency would deter normal decidual development. Indeed, overall decidual growth, as assessed by weights of implantation sites, was substantially reduced in PPARδ−/− females compared with WT females on days 5–8 of pregnancy (Fig. 3A). Reduced decidual responses were also seen in experimentally induced decidualization by intraluminal oil infusion in day 4 pseudopregnant PPARδ−/− in the absence of embryo (Fig. 3B), providing evidence that defective decidualization is because of uterine loss of PPARδ. The reduced decidual growth was correlated with an aberrant pattern and levels of ERK1/2 and p38

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MAPK phosphorylation in the stroma during blastocyst- and oil-induced decidualization (Fig. 4, C–F). These molecules are known to participate in decidualization (31). These findings again suggest that maternal PPARδ is critical for normal implantation and decidualization.

PPARδ Deficiency Confers Abnormal Angiogenesis in the Decidua Basalis—By day 7 of pregnancy, the antimesometrial mesometrial oriented decidua slows down growth but undergoes tissue remodeling. The antimesometrial decidua capsularis progressively degenerates creating room for the growing embryo, whereas the mesometrial decidua basalis undergoes angiogenesis forming a vascularized zone that brings maternal and fetal blood vessels in close proximity to form a functional placenta (1). This timely decidual remodeling is well correlated with shifting of PPARδ expression from the antimesometrial decidua to the mesometrial decidua basalis and to the trophoblast sprouting from the ectoplacental cone (supplemental Fig. S1).

Using Flk1lacZ+/− and PPARδ−/−/Flk1lacZ+/− mice that express β-galactosidase as a read out for Flk-1 promoter activity in endothelial cells (23), we observed an abnormal pattern of angiogenesis in the decidua basalis of PPARδ−/− females. As shown in Fig. 4A, the density of lacZ-stained blood vessels was comparable in mesometrial decidua in both WT and null mice on day 8, but the vessel density progressively decreased in WT mesometrial decidua as opposed to sustained higher density in metrial gland and heightened ANG-2 expression in the decidua basalis (Fig. 4, E and F), indicating that their expression is independent of PPARδ signaling during this period. Collectively, the results suggest that PPARδ is required for appropriate expressions of COX-2 and VEGF for normal angiogenesis during decidual basalis development.

PPARδ Deficiency Disrupts Normal Placentation—Because normal implantation and decidualization is important for subsequent embryonic growth and placentalization (3), observations of defective implantation and decidual growth in PPARδ−/− mice led us to explore whether these early defects affected the process of placentalization in null mice. As expected, impaired fetoplacental development was noted in PPARδ−/− mice with significant anomalies in ectoplacental trophoblast development and chorioallantoic fusion on day 10 of pregnancy. For example, cytokeratin immunolocalization showed shallow trophoblast invasion into the decidua basalis (Fig. 5A). Although the fusion of the chorion and allantois occurred regardless of the presence of PPARδ with normal expression of VCAM-1 required for chorioallantoic attachment (34, 35), the process was developmentally behind for about 24 h (Fig. 5A). In fact, chorioallantoic villous branching in day 11 null placentas was comparable with that seen in day 10 WT placentas. Because the ectoplacental cone, but not the chorion and allantois, expresses PPARδ on day 8 (supplemental Fig. S1), this delay in chorioallantoic attachment and villous branching in null placentas
is perhaps due a secondary effect imposed by deferred implantation and defective decidual growth.

To characterize placental defects in the absence of PPARδ, we examined the expression of heart and neural crest derivatives expressed transcript 1 (HAND1) and achaete-scute complex homolog-like 2 (Ascl2, also known as MASH2), known to have stimulatory and repressive roles in specifying giant and spongiotrophoblast cell fates (36–39). As shown in Fig. 5B, HAND1 and MASH2 were normally expressed on day 10 and day 11 PPARδ−/− placenta in WT mice, indicating that PPARδ signaling does not influence HAND1 and MASH2 expression in differentiating trophoblast cells during placentation.

To better define which subtypes of trophoblast cells abnormally developed from diploid trophoblast progenitor cells in ectoplacental cones of PPARδ−/− placenta, we examined the expression of PL-1, a giant cell marker, and trophoblast-specific protein α (TPBP, also known as 4311), a spongiotrophoblast cell marker (40). PL-1 was expressed in a deferred manner in days 10 and 11 mutant placentas, but TPBP was expressed aberrantly at lower levels (Fig. 5C), indicating an impaired spongiotrophoblast development in the absence of PPARδ.

We then performed reciprocal embryo transfer experiments to further sort out the maternal versus embryonic contribution to observed spongiotrophoblast defects in PPARδ−/− mice. Although WT trophoblast giant and spongy cells developed normally with correct expression of PL-1 and TPBP in WT recipients on day 10, PPARδ−/− blastocysts developing in WT recipients showed impaired ectoplacental development again with reduced TPBP-positive spongiotrophoblasts (Fig. 5D). This impairment was not observed for WT embryos in mutant mothers, although less PL-1- and TPBP-expressing cells were noted (Fig. 5D), perhaps because of the deferred implantation in mutant recipients.

We next examined the consequences of compromised ectoplacental development from PPARδ deficiency on midgestational embryo-uterine development. As shown in supplemental Fig. S2, about 42% of implantation sites showed signs of resorption with intraluminal hemorrhage and retarded embryonic growth as examined on day 14 of pregnancy. To delineate causes for these defects, we analyzed trophoblast development in those surviving mutant placentas with viable embryos. We found that attenuated spongiotrophoblast development in the absence of PPARδ continued through day 14 with a marked increase in secondary trophoblast giant cells. For example, whereas WT placentas showed a discontinuous layer of giant cells, multiple layers of giant cells were present in day 14 null placentas (Fig. 6A). Increased numbers of trophoblast giant cells perhaps occurred at the expense of spongiotrophoblast development, because the TPBP-expressing spongy junctional zone was considerably reduced in PPARδ−/− placentas (Fig. 6B). It was striking to see that some giant cells in mutant placentas retained TPBP expression (Fig. 6B).

Furthermore, consistent with defective trophoblast cell invasion in day 10 null placentas, a shallow invasion of trophoblast cells into the decidual basalis was observed on day 14. For example, TPBP- and CDX2-positive glycogen trophoblasts in WT placentas migrated into the interstitium of the decidua basalis even beyond the boundaries of cytokeratin-positive endovascular giant cell invasion but not in null placentas (Fig. 6, A–C). In fact, the number of glycogen trophoblast cells was reduced in the absence of PPARδ. In addition, endovascular giant cells, which expressed high levels of COX-2 in WT placentas, were poorly developed, leaving an intact endothelial layer overlying maternal blood vessels in PPARδ−/− placentas (Fig. 6D). This defective invasion of both endovascular giant and glycogen spongy cells in null mutant females appears to be associated with failure of adaptation and close apposition of the decidual blood space with fetoplacental components. On the other hand, the maternal-fetal vasculature within the labyrinth zone in WT and mutant placentas showed normal development with a similar pattern of Isolectin B4 binding (Fig. 7A), a marker for trophoblast cells lining maternal blood spaces (27). Using Flk1lacZ+/− and PPARδ−/−/Flk1lacZ+/− mice, we also observed a comparable density of lacZ-stained fetal blood vessels (Fig. 7B), suggesting normal labyrinth vascular network in the absence of PPARδ. Collectively, the results support our argument that the loss of PPARδ shifts trophoblast cell differentia-
expression in trophoblast giant cells with reduced levels in the spongy layer as opposed to its higher expression in giant and diploid spongiosotrophoblasts in WT placentas (Fig. 8D). These results suggest that STAT3 signaling continuously promotes trophoblast giant cell formation from diploid spongiosotrophoblasts in the absence of PPARδ.

To test that a balance between PPARδ-AKT and LIF-STAT3 signaling directs normal spongiosotrophoblast differentiation into glyco gen and giant cells during placentation, we utilized TS cells in culture. We first analyzed PPARδ expression in differentiating WT TS cells by Northern blotting. As shown in Fig. 9, A and B, although PPARδ expression was low in proliferating TS cells, its expression was up-regulated if cultured under differentiation conditions for 2–4 days. However, PPARδ expression declined in cultured TS cells on day 6 with terminal differentiation (Fig. 9, A and B). Interestingly, GW501516, a selective PPARδ agonist (10), but not LIF, up-regulated PPARδ expression in differentiating TS cells (Fig. 9, A and B). This auto-induction of PPARδ expression by its ligand indicates a potential role for this signaling axis in influencing trophoblast cell fate during differentiation.

We next examined HAND1 and MASH2 expression in differentiating WT and null TS cells in culture. Consistent with our in vivo findings, the loss of PPARδ had little impact on HAND1 and MASH2 expression in TS cells undergoing default differentiation (Fig. 9, C and D), suggesting that PPARδ is not essential for normal HAND1 and MASH2 expression in trophoblasts during placentation development.

To further explore cell-specific defects from the loss of PPARδ, we analyzed PL-1 and TPBP expression in cultured TS cells. The aberrant elevation of PL-1 and TPBP expression in PPARδ−/− TS cells during default differentiation (Fig. 10, A−C) provides evidence that PPARδ signaling is required for normal trophoblast cell differentiation. Regarding higher levels of TPBP expression in differentiating null TS cells, it is possible that PPARδ deficiency facilitates trophoblast progenitor cell differentiation into giant cells via a TPBP-positive stage as observed in vivo (Fig. 6B). However, upon activation of PPARδ by the selective agonist GW501516, we observed that this agonist substantially elevated the expression of both TPBP and PL-1 in WT but not in null cells (Fig. 10, A−C), suggesting that activation of PPARδ also promotes TS cell differentiation. This paradoxical observation of increased trophoblast cell differentiation either by silencing or activating PPARδ supports the concept that an appropri-
PPARδ in Implantation, Decidualization, and Placentation

FIGURE 6. PPARδ deficiency derails normal trophoblast function in mature placentas. A, immunolocalization of cytokeratin (CK) showing shallow trophoblast invasion into the decidua with increased trophoblast giant cell population in day 14 PPARδ−/− placentas. B, in situ hybridization of TPBP mRNA expression revealing reduced spongy cell formation in the absence of PPARδ. Interestingly, some null giant cells sustain TPBP expression indicating that giant cell transformation transit through a TPBP-positive stage. C, CDX2 expression specifies the differentiation of TPBP-positive (β) glycogen trophoblast cells. D, COX-2 proteins are prominently detected in invasive endovascular trophoblast cells in WT, but not in mutant placentas, suggesting the role of COX-2-PG-PPARδ signaling axis in facilitating trophoblast invasion into the maternal decidua. All figures are representative sections obtained from 3 to 5 placentas analyzed in two independent experiments. Bv, blood vessel; Dec, deciduum; GlyT, glycogen trophoblast cells; Lb, labyrinth zone; SpT, diploid spongiotrophoblast; TGC, trophoblast giant cell. Bar, 200 μm.

FIGURE 7. Maternal-fetal vasculature is comparable in WT and PPARδ−/− labyrinth zone. A, BS-1 Isolectin B4 staining revealing normal pattern of trophoblast cells lining the maternal blood spaces in WT and null labyrinth zones. B, LacZ-stained fetal blood vessels showing normal development of fetal circulatory network in the absence of PPARδ. Placentas (5–6 each) from three individual mice were subjected to Isolectin binding assay and LacZ staining; representative pictures are shown. f, fetal blood vessels; m, maternal blood space. Bar, 200 μm.

Maternal-fetal vasculature is comparable in WT and PPARδ−/− labyrinth zone. A, BS-1 Isolectin B4 staining revealing normal pattern of trophoblast cells lining the maternal blood spaces in WT and null labyrinth zones. B, LacZ-stained fetal blood vessels showing normal development of fetal circulatory network in the absence of PPARδ. Placentas (5–6 each) from three individual mice were subjected to Isolectin binding assay and LacZ staining; representative pictures are shown. f, fetal blood vessels; m, maternal blood space. Bar, 200 μm.

Recent evidence suggests that a short deferral of implantation timing adversely affects subsequent developmental processes in the face of compromised PG signaling resulting from either cPLA2α, COX-2, or LPA3 null mutation (5, 6, 25, 47). However, it remained elusive regarding the underlying mechanism and the molecular link between the critical steps. Our present investigation provides evidence that PPARδ serves as a molecular link that coordinates multiple signaling pathways in a stage-specific manner for the success of pregnancy. The genetic loss of PPARδ signaling does not influence ovulation, fertilization, and preimplantation development. In contrast, although maternal PPARδ signaling is essential for normal implantation and decidualization, embryonic PPARδ is required for normal placentation.

DISCUSSION

Successful implantation and the establishment of chorioallantoic placentas are gateways to successful pregnancy. Recent evidence suggests that a short deferral of implantation timing adversely affects subsequent developmental processes in the face of compromised PG signaling resulting from either cPLA2α, COX-2, or LPA3 null mutation (5, 6, 25, 47). However, it remained elusive regarding the underlying mechanism and the molecular link between the critical steps. Our present investigation provides evidence that PPARδ serves as a molecular link that coordinates multiple signaling pathways in a stage-specific manner for the success of pregnancy. The genetic loss of PPARδ signaling does not influence ovulation, fertilization, and preimplantation development. In contrast, although maternal PPARδ signaling is essential for normal implantation and decidualization, embryonic PPARδ is required for normal placentation.

Maternally regulated PPARδ signaling is critical to normal trophoblast cell development.

To search for the cause of increased giant cell population at the expense of spongiotrophoblasts in the absence of PPARδ in vivo, we examined the effect of LIF on TS cell differentiation in culture. We noted that LIF significantly promoted WT TS cell differentiation to giant cells with similar high expression levels of PL-I as in PPARδ null cells. The most striking observation was the significant reduction in TPBP expression in PPARδ−/− TS cells when treated with LIF during differentiation (Fig. 10, A–C). This extensive giant cell formation from PPARδ−/− TS cells at the expense of TPBP-expressing spongy cells in response to LIF recapitulates in vivo defects in PPARδ−/− placentas, suggesting that a balance between PPARδ and LIF signaling is important for normal trophoblast cell development.

To provide further insights into the mechanism that differentially couples PPARδ with LIF signaling, we examined the effects of GW501516 and LIF on AKT and STAT3 phosphorylation during TS cell differentiation. AS shown in Fig. 11, A–D, whereas GW501516 activated AKT signaling in WT differentiating TS cells, LIF induced STAT3 phosphorylation in both WT and PPARδ−/− TS cells. Collectively, these results provide evidence that a coordinated interaction between the PPARδ-AKT and LIF-STAT3 signaling pathways is important for normal spongiotrophoblast and giant cell differentiation.
PPARα is Essential for On Time Implantation—PGs are involved in a variety of physiological and pathological processes, including multiple steps during pregnancy. Although PGE$_2$ via its membrane EP receptors is critical for normal ovulation and fertilization (25, 48, 49), PGI$_2$ plays an important role in implantation and decidualization (7, 50). In the absence of appreciable levels of uterine IP receptor and adverse reproductive phenotypes in IP$^{-/-}$ mutant mice during pregnancy (7, 51), we speculated and later provided pharmacological evidence that PGI$_2$ impacts implantation via its nuclear target PPARα (7). Our present observation of deferral of the implantation window in the presence of normal ovulation, fertilization, and preimplantation embryo development in CD1 PPARα$^{-/-}$ mice provides the first genetic evidence that PGI$_2$-PPARα signaling is physiologically relevant for early pregnancy events. More importantly, we found that PGE$_2$, via its EP$_{2/4}$ receptors plays a complementary and important role in implantation because pharmacological silencing of EP$_{2/4}$ receptors exacerbates the incidence of implantation failure in PPARα$^{-/-}$ females. This observation is consistent with our earlier finding that PGE$_2$ improves implantation in COX-2-deficient mice when coadministered with cPGI$_2$, a stable analog of PGI$_2$ (7). The physiological function of uterine PPARα in implantation is unique among the PPAR family, because PPARγ and PPARδ are expressed at very low levels in the uterus during implantation (7), and female mice lacking PPARα have no apparent reproductive defects (52).

PPARδ Ensures Normal Angiogenesis during Decidua Basalis Development—It is not clearly understood how PPARδ deficiency leads to abnormal tissue remodeling and angiogenesis during the development of decidual basalis prior to the onset of fetoplacentation. One may argue that aberrant uterine angiogenesis is a consequence, not a cause, of compromised decidual growth in PPARδ$^{-/-}$ mice. VEGF and angiopoietins participate in ovarian and uterine angiogenesis in a yin-yang manner, whereas ANG-2 stimulates vessel sprouting by blocking the sta-

![Image](image-url)
bilizing signaling of ANG-1 in the presence of VEGF, it antagonizes ANG-1 signaling in the absence of VEGF, contributing to vessel regression (50, 53). Because ANG-1 expression is low to undetectable in the decidual basalis, it is conceivable that declining VEGF expression in the face of normal ANG-2 expression from days 8 to 10 leads to progressive vessel regression in mesometrial decidua during normal pregnancy. In the absence of PPARδ, an aberrantly sustained VEGF expression with unaltered ANG-2 expression causes abnormal angiogenesis with increased vessel leakage because of the lack of vessel stabilization normally driven by ANG-1. Potential roles of PGE$_2$ in decidual angiogenesis may also not be excluded because EP$_3$ receptors are expressed in mesometrial decidua (54). The ability of PGE$_2$ to up-regulate COX-2 (48) suggests that PGE$_2$ generated by this auto-induction loop maintains VEGF expression in the absence of PPARδ, contributing to abnormal angiogenesis in the decidua basalis.

## FIGURE 9. **Northern hybridization of PPARδ, PL-I, and TPBP mRNAs in TS cells.**

A and B, GW501516 (GW), a selective PPARδ agonist (20 nM), but not LIF (10 ng/ml), induces PPARδ mRNA expression in differentiating WT TS cells in culture. C and D, HAND1 and MASH2 mRNA expressions are comparable in WT and PPARδ/−/− TS cells during differentiation in culture. Bar diagrams in B and D show relative levels of target mRNA with reference to ribosomal protein L7 (rPL7) mRNA after densitometric scanning of autoradiograms. rPL7 is a housekeeping gene and was used to confirm RNA integrity and loading.

## FIGURE 10. **GW501516 and LIF induce differential expression of PL-I and TPBP mRNAs in differentiating WT and PPARδ/−/− TS cells in culture.** PPARδ deficiency facilitates TS cell differentiation into PL-I-positive giant cells (A and B) and TPBP-expressing spongiotrophoblasts (see Veh groups) (A and C). Interestingly, activation of PPARδ signaling by GW501516 induces WT TS cell differentiation with enhanced expression of PL-I and TPBP (see GW groups) (A and C). In contrast, although LIF induces both giant cell and spongy cell differentiation of WT TS cells, it primarily directs PPARδ/−/− TS cell differentiation into giant cells with reduced TPBP expression (see LIF groups) (A and C). mRNAs were analyzed by Northern hybridization. Bar diagrams in B and C show relative levels of target mRNAs relative to rPL7 mRNA after densitometric scanning of autoradiograms. rPL7 was used to confirm RNA integrity and loading.
In addition to reduced spongiotrophoblast formation, we also observed a remarkably decreased volume of glycogen trophoblast cells in placentas of PPARδ null females. Although the origin of glycogen cells remains unknown, it is thought that these cells are derived from spongiotrophoblasts, because they express the spongiotrophoblast-specific gene TPBP and appear to arise within the spongy layer after diploid spongiotrophoblasts (57, 58). Recent evidence suggests that AKT signaling is essential for glycogen trophoblast cell transformation, because AKT1-mutant placentas exhibit near complete loss of glycogen-containing cells in the spongy layer (59). Interestingly, we observed that although AKT phosphorylation predominantly occurs in the diploid spongiotrophoblast but not in glycogen cells in wild-type placentas, phospho-AKT is largely reduced in the absence of PPARδ. This observation suggests that an appropriate PPARδ-AKT signaling facilitates diploid spongiotrophoblast cell proliferation and further transformation into glycogen trophoblast cells, the total cell number of which increases by 80-fold during midgestational development (57). These findings together with our observation of accelerated giant cell formation with the loss of PPARδ led us to suggest that PPARδ functions as a cell lineage sensor regulating normal trophoblast differentiation into spongy cells but not giant cells. If this is so, it still needs to be addressed whether the spongiotrophoblast-to-giant cell transformation in the absence of PPARδ is a default pathway or influenced by other signaling mechanisms.

In this regard, we show that LIF-STAT3 signaling is functionally intact in diploid spongiotrophoblasts regardless of the PPARδ status. Moreover, we revealed that genetic ablation of PPARδ down-regulates the expression of SOCS3, a natural repressor of LIFR/gp130 signaling, in spongiotrophoblasts, which in turn favors LIF-STAT3-driven giant cell differentiation. Similar observation of increased giant cell formation at the expense of spongiotrophoblasts in SOCS3 mutant mice (42) further supports our contention that tightly regulated coordination between the PPARδ-AKT and LIF-STAT3 signaling pathways appropriately allocates spongiotrophoblast cell differentiation into glycogen trophoblast and giant cells.

Peters et al. (60) in 2000 reported that PPARδ mutant mice do not exhibit much embryonic lethality, suggesting that PPARδ does not play any important role in embryonic survival in this mutant mouse line. In 2002, Evans and co-workers (61) first reported placental dysfunction leading to embryonic lethality of PPARδ−/− homozygotes on Sv129/Jae or C57BL/6J background, but they did not offer any explanation for the underlying mechanism of this defect. During the course of our investigation in characterizing maternal versus embryonic roles of PPARδ in implantation, decidualization, and placentation, Desvergne and co-worker (22) also reported placental defects with impaired trophoblast giant cell development in homozygous conceptuses from cross-breeding of PPARδ heterozygous mice on C57BL/6J/Sv129 mixed genetic background. This study also reported that the TPBP-expressing spongy cell population was significantly reduced in homozygous placentas arising from crossings of homozygous mutants (22). In this study, however, no explanation was provided regarding the discrepancy of different results seen in PPARδ null placentas from heterogeneous versus homozygous crossings. Moreover, this study provided no clues as to the contribution of maternal PPARδ during various steps of pregnancy. However, their observations in PPARδ−/− placentas arising from homozygous mutant females are consistent with our present findings of reduced spongiotrophoblast cell population in PPARδ−/− placentas. Although the exact reasons for observed discrepancies for PPARδ null placental phenotypes remain elusive, contributions of the genetic background to the observed differences cannot be excluded. In fact, the extent of PPARδ homozygous embryonic mortality resulting from placental deficiency following heterozygous matings is dependent upon the genetic makeup of
mice (62). Similar observations are noted for other gene mutant mouse lines (63, 64). Nonetheless, this study presents a comprehensive and cohesive story for how this PPARδ signaling coordinates various pathways for guiding maternal and embryonic interactions for the success of pregnancy.

Acknowledgments—We thank Janet Rossant, James C. Cross, and Michael J. Soares for reagents and helpful discussion; Ronghua Yuan and Yong Guo for breeding of mutant mice; and Jason D. Morrow for prostaglandin measurements.

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