Secretory phospholipase A2-X (Pla2g10) is a novel progesterone receptor target gene exclusively induced in uterine luminal epithelium for uterine receptivity in mice

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Abstract

**Background:** Aberration of estrogen (E_2) and/or progesterone (P_4) signaling pathways affects expression of their target genes, which may lead to failure of embryo implantation and following pregnancy. Although many target genes of progesterone receptors (PRs) have been identified in uterine stroma, only a few PR targets have been reported in the epithelium. Secretory phospholipase A_2-(PLA_2)-X, a member of the PLA_2 family that releases arachidonic acids for the synthesis of prostaglandins that are important for embryo implantation, is dysregulated in the endometrium of patients suffering from repeated implantation failure. However, it is not clear whether sPLA_2-X is directly regulated by ovarian steroid hormones for embryo implantation in the uterus.

**Result:** P_4 induced the Pla2g10 encoding of secretory PLA_2-X in the apical region of uterine LE of ovariectomized mice via PR in both time- and dose-dependent manners, whereas E_2 significantly inhibited it. This finding is consistent with the higher expression of Pla2g10 at the diestrus stage, when P_4 is elevated during the estrous cycle, and at P_4-treated delayed implantation. The level of Pla2g10 on day 4 of pregnancy (Day 4) was dramatically decreased on Day 5, when PRs are absent in the LE. Luciferase assays of mutagenesis in uterine epithelial cells demonstrated that four putative PR response elements in a Pla2g10 promoter region are transcriptionally active for Pla2g10. Intrauterine delivery of small interfering RNA for Pla2g10 on Day 3 significantly reduced the number of implantation sites, reinforcing the critical function(s) of Pla2g10 for uterine receptivity in mice.

**Conclusions:** Pla2g10 is a novel PR target gene whose expression is exclusively localized in the apical region of the uterine LE for uterine receptivity for embryo implantation in mice.

**Background**

It is well understood that prostaglandins (PGs) are critical for sequential events of female reproduction from ovulation to parturition [1-3]. PGs are generated from arachidonic acid (AA) by phospholipase A_2s (PLA_2s) followed by cyclooxygenases. PLA_2 enzymes are classified into two groups, cytosolic and secretory. Cytosolic PLA_2s (cPLA_2s), which are regulated by Ca^{2+}-dependent translocation and phosphorylation, have a preference for AA in membrane phospholipids and play an essential role in agonist-induced AA release. The cPLA_2α-derived AA is important for the PG synthesis that is required for on-time implantation [1]. Several secretory PLA_2s (sPLA_2s), including groups IIA, III, V, and X, are likely to be involved in AA release and subsequent eicosanoid production during inflammatory conditions [4]. Several sPLA_2s, as well as cPLA_2α, are spatiotemporally induced in mouse uterus for uterine receptivity [1]. It was previously reported that PLA2G10 encoding of sPLA_2-X is dysregulated in the endometrium of patients with repeated implantation failure (RIF) [5]. However, detailed mechanism(s) by which ovarian steroid hormones regulate expression of Pla2g10 in the uterus remain unanswered.
Ovarian steroid hormones, estrogen (E₂) and progesterone (P₄), orchestrate dynamic changes in the uterus during reproductive cycles [6-8]. These hormones act on uterine physiology mainly via their own nuclear receptors; namely, estrogen receptors and progesterone receptors (PRs), respectively [9, 10]. Sophisticated actions of these hormones on major uterine cell types, including various immune cells, are prerequisites for changing the uterine environment from the pre-receptive to the receptive phase for successful embryo implantation [11-13]. Desynchronized actions of these hormones may provide various causes of RIF. P₄ play critical roles for the establishment and maintenance of pregnancy by not only its endocrine but also immunological effects [14-16]. P₄–PR transcriptional network along with estrogen signaling promotes spatiotemporal regulation of various target genes for achieving uterine receptivity in the uterus [17]. Whereas most of the PR target genes are expressed in stromal cells, several genes including Amphiregulin (Areg), Indian hedgehog (Ihh), Calcitonin (Ct), GATA binding protein 2 (Gata2), and sex-determining region Y-related high-mobility group box 17 (Sox17) have been identified in the uterine epithelium to date [18-22]. Here we demonstrate that Pla2g10, one of dysregulated genes in the endometrium of patients with RIF, is a novel PR target gene that is exclusively induced in uterine luminal epithelium (LE) for uterine receptivity for embryo implantation in mice.

**Results**

**PLA2G10 dysregulated in human endometrium of patients with RIF is regulated by P₄**

Previously, we demonstrated that a group of genes, including PLA2G10, is dysregulated in the endometrium of patients with RIF [5]. Volcano plots and real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for endometrial samples demonstrate that PLA2G10 mRNAs were significantly down-regulated in the endometrium of patients with RIF at mid-luteal phase (Fig. 1a, b). To further investigate the underlying mechanism of dysregulated expression of PLA2G10 in the endometrium of patients with RIF, we examined steroid hormonal regulation of Pla2g10 in mouse uterus. Because P₄ is an essential steroid hormone to prepare embryo implantation in the uterus, it was first examined whether Pla2g10 expression is regulated in the uterus by P₄ using an ovariectomized (OVX) mouse model. P₄ was given to OVX mice whose uteri were collected at different time points (0, 3, 6, and 24 h) after hormone treatment (Fig. 1c, d). The RT-PCR and real-time RT-PCR results demonstrate that Pla2g10 expression was gradually increased by P₄ in a time-dependent manner, with the highest level at 24 h (Fig. 1d). Immunofluorescence staining shows that PLA2G10 is mainly localized in the apical region of LE cells in mouse uterus (Fig. 1c). These results suggest that Pla2g10 may be a novel P₄ target gene that is exclusively induced in the LE of the uterus.

**E₂ inhibits both basal and P₄-dependent expression of Pla2g10 in mouse uterus in a time-dependent manner**
To investigate the effects of E$_2$ on Pla2g10 expression in mouse uterus, E$_2$ with or without P$_4$ was given to OVX mice whose uteri were collected at different time points after hormone treatment(s). A single injection of E$_2$ significantly reduced basal levels of Pla2g10 mRNAs in mouse uterus of OVX mice (Fig. 1e). Furthermore, E$_2$ suppressed P$_4$-dependent induction of Pla2g10 at 3 and 6 h after hormone treatments, whereas the inhibitory action was no longer effective at 24 h (Fig. 1f). These results suggest that E$_2$ has inhibitory actions on basal and P$_4$-dependent expression of Pla2g10 in mouse uterus.

P$_4$ regulates Pla2g10 induction via its nuclear PR in a dose-dependent manner

To investigate whether Pla2g10 expression is regulated by P$_4$ in a dose-dependent manner, various concentrations (0.25 to 2 mg) of P$_4$ were given to OVX mice and Pla2g10 expression was evaluated 24 h after P$_4$ injection. RT-PCR and real-time RT-PCR analyses show Pla2g10 induction by P$_4$ in a dose-dependent manner, with a peak level in uterine samples with 2 mg (Fig. 2a). To determine whether P$_4$-induced Pla2g10 expression is mediated via nuclear PRs in mouse uterus, OVX mice were pretreated with a PR antagonist RU-486 30 min before P$_4$ injection. RU-486 pretreatment significantly abrogated P$_4$-dependent induction of Pla2g10 as well as Areg, a known P$_4$ target gene expressed in the LE of mouse uterus. These results suggest that P$_4$-dependent induction of Pla2g10 expression works through nuclear PR in the uterus (Fig. 2b, c).

Expression of Pla2g10 is elevated in P$_4$-dominant diestrus stage during estrous cycle

To further understand P$_4$-dependent regulation of Pla2g10 in the uterus, we examined its expression in the uterus at different stages of the estrous cycle during which the uterus undergoes cyclic hormonal changes. Consistent with hormone-dependent profiles of Pla2g10 expression, it was notably expressed in the diestrus stage when P$_4$ is dominant, but not in the proestrus and estrus stages when levels of E$_2$ are high (Fig. 2d, e). Accordingly, PLA2G10 was mainly detected in the LE of mouse uterus in the diestrus stage (Fig. 2e).

Pla2g10 expression coincides with PR in the LE for uterine receptivity for embryo implantation

During early pregnancy in mice, the uterus is influenced by P$_4$ from newly formed corpus lutea from day 3 of pregnancy (Day 3) onwards [6]. Thus, we examined expression patterns of Pla2g10 in mouse uterus during early pregnancy. Pla2g10 was highly expressed on Days 3 and 4, whereas it remained at basal levels on Days 1 and 2 when E$_2$ was dominant (Fig. 3a). Interestingly, P$_4$-dependent expression of Pla2g10 was significantly reduced in both implantation site (IS) and inter-IS on Day 5 (Fig. 3b). Similar
observation was made in the uterus on Days 4 and 5 of pseudopregnancy (data not shown). Considering that P₄ levels are similarly maintained on Days 4 and 5 [23, 24], downregulation of PLA2G10 in the LE on Day 5 may be associated with loss of PR in this compartment. In fact, it is interesting to observe that PR as well as and PLA2G10 is dramatically reduced in the LE irrespective of the implanting blastocyst on Day 5 (Fig. 3b) and day 5 of pseudopregnancy (data not shown).

**Pla2g10 is regulated by P₄-PR-dependent signaling in mouse uterus during delayed implantation**

To further evaluate whether the sudden decrease of the PLA2G10 expression in the LE on Day 5 is caused by loss of PR, we used an experimentally-induced delayed implantation model (DIM) in mice (Fig. 3c). PLA2G10 expression was maintained in a P₄-primed uterus at a state of dormancy (P₄ 24 h). However, 24 h after termination of delayed implantation with an injection of E₂ (P₄+E₂ 24 h), it disappeared in the LE in mouse uterus (Fig. 3d). The loss of PLA2G10 in the LE at P₄+E₂ 24 h coincided with loss of PR in the LE during DIM. Taken together, these results suggest that PLA2G10 expression exclusively depends on PR in mouse uterus during early pregnancy and DIM.

**Pla2g10 promoter has functional PREs**

To further understand the molecular mechanism(s) by which the P₄-PR signaling pathway regulates Pla2g10 expression at transcriptional levels, a series of luciferase assays with a proximal promoter region of Pla2g10 gene containing putative PR response elements (PREs) was performed in Ishikawa cells, human endometrial adenocarcinoma cells. *In silico* analyses via a PROMO program ([http://alggen.lsi.upc.es](http://alggen.lsi.upc.es)) suggest that four putative PREs were found in -840/+65 of the Pla2g10 promoter. These PREs were also validated by ChIP-seq analyses in a previous study [25] The luciferase activity of the Pla2g10 promoter was significantly increased when co-transfected with PRA or PRB expression vector along with P₄ (Fig. 4a). To determine which PRE is functionally critical for PR-dependent Pla2g10 transcription, four putative PREs in the Pla2g10 proximal promoter region (-840/+65) were mutated (Fig. 4b). All four mutations (mt) at -801/-793, -356/-350, -310/-304, and -290/-284 PREs at the Pla2g10 promoter showed about 40% reduced luciferase activity when co-transfected with PR(s) (Fig. 4c). These results indicate that Pla2g10 transcription is directly regulated by PR in the uterus.

**In vivo delivery of siRNA to knock-down Pla2g10 disturbs embryo implantation**

We next performed *in vivo* interference of Pla2g10 to investigate whether Pla2g10 contributes to embryo implantation in mouse uterus. Intrauterine injection of siRNA against Pla2g10 (siPla2g10) (100 pmol per uterine horn) on Day 3 caused significant knock-down of PLA2G10 expression on Day 4 (Fig. 5a). *In vivo*
interference of Pla2g10 expression with siPla2g10 in mouse uterus significantly decreased the number of IS on Day 6 (6.5 vs 2.5) compared to the control horns with negative control siRNA (siNC) (Fig. 5b, c). However, a few embryos successfully implanted in mouse uterus with siPla2g10. Gross histology and ALP staining for IS on Day 6 showed that implantation normally occurs in the uterus with siPla2g10 (Fig. 5d). Furthermore, ARG2 localization in the decidualizing stromal cells surrounding the implanted embryo on Day 6 was similarly observed between uteri with siPla2g10 and siNC (Fig. 5e), suggesting that the uterine environment could be locally disturbed, but not systemically altered in mouse uterus by siPla2g10. Collectively, these results suggest that P₄-PR signaling induces expression of Pla2g10 in LE, which participates in PG biosynthesis critical for uterine receptivity for embryo implantation in mice (Fig. 6).

**Discussion**

Pla2g10 is known as a Ca²⁺-dependent low molecular-weight enzyme (13 - 18 kDa) that is involved in biosynthesis of PGs, an important lipid mediator for embryo implantation [26, 27]. We previously demonstrated that mice deficient of Pla2g4a, a cytosolic form of PLA₂, have aberrant uterine spacing of embryos and deferred embryo implantation. The deferred implantation and fetal growth restriction in Pla2g4a deficient mice were significantly recovered by exogenous PG administration [1]. Subsequent studies have supported this notion that PLA₂-s-derived AA is important for PG synthesis that is crucial for on-time implantation [28-30]. In our previous study, PLA2G10 was identified as a dysregulated gene in microarray analyses in the endometrium of patients with RIF in whom P₄ signaling could be locally impaired [5]. In fact, Pla2g10 expression was gradually increased by P₄ in a dose-dependent manner (Fig. 1). In line with this result, a recent study shows that the role of PLA₂-s in acrosome reaction *in vitro* depends on P₄ concentration [31]. In this study, we clearly demonstrate that Pla2g10 is a novel PR target gene whose expression is exclusively induced in the LE in mouse uterus.

Molecular cross-talks between the blastocyst and the uterus induce growth factors, adhesion molecules, cytokines, and transcription factors to prepare uterine conditions for embryo implantation [6, 32-35]. P₄-PR-target gene networks are known to have critical functions for embryo implantation [8, 36, 37]. Most of PR target genes, such as Hoxa10 and Hand2, are expressed in stromal cells [8, 17]. Only several PR target genes, such as Areg, Ihh, CT, Gata2, and Sox17 have been identified in the epithelial compartment [18-22]. Areg is a well-known PR target gene whose expression is increased in the uterine epithelium in response to P₄ for uterine receptivity. With the onset of blastocyst attachment late on Day 4, Areg mRNA accumulated in the LE exclusively at the sites of blastocysts [18], which may compensate for the deficiency of HB-EGF around the time of embryo implantation in the uterus of HB-EGF knockout mice [38]. Very high levels of Ihh mRNA are seen in the luminal and glandular epithelia on Day 3 for preparing embryo implantation [19]. Consistent with these results, Pla2g10 is expressed in LE during the early pregnancy (Fig. 3). P₄ promotes expression of not only Pla2g10, a Ca²⁺-dependent enzyme, but also CT in uterine epithelium [20, 39]. Interestingly, CT leads to increased concentration of intracellular Ca²⁺, suggesting that the P₄-PR signaling, probably via CT induction, could regulate Pla2g10 expression and
functional activities for uterine receptivity for embryo implantation [20, 40]. *Gata2*, a P₄ target gene, is colocalized in the uterine epithelium during early pregnancy with PR, and promotes expression of Pgr gene but also regulates downstream progesterone responsive genes, such as *Sox17*, in conjunction with the PR [21, 22, 41]. We also found three putative GATA binding sites in nearby -801/-793 PRE of the *Pla2g10* promoter (data not shown). Thus, it is suggested that *Pla2g10* expression could be regulated in the uterine epithelium via PR-GATA2 dependent manner.

It is well-known that stromal PR is the major regulator of the expression of P₄ target genes and the ability of P₄ to inhibit E₂-induced epithelial cell proliferation [42]. However, a recent study shows that epithelial PR acts to inhibit E₂-induced epithelial proliferation and is essential for uterine development and function, suggesting the importance of epithelial PR for embryo implantation [43]. During early pregnancy, PR is transiently expressed in the epithelium just prior to embryo implantation [43, 44]. After embryo implantation occurs, PR expression in the epithelium rapidly decreases [44] whereas its expression in uterine stroma increases and persists throughout decidualization in mice [45]. Loss of PR expression in the uterine epithelium is crucial for luminal closure for embryo implantation [46]. A previous report suggests that E₂ down-regulates PR in uterine epithelium through paracrine actions mediated by stromal ERα [47]. This could support the notion that a rapid decrease of P₄-dependent *Pla2g10* expression in the epithelium in mouse uterus may be caused by reduction of epithelial PR (Fig. 3b). In fact, we found that expression of P₄-dependent *Pla2g10* was suppressed by E₂ in uteri of OVX mice (Fig. 1f). This notion is supported by the results that *Pla2g10* promoter has functional PREs (Fig. 4c) and expression of PLA2G10 is synchronized with that of PR in epithelial cells during early pregnancy (Fig. 3a, b). Although *Areg* is specifically induced in uterine epithelium surrounding the implanting blastocyst on Day 5, *Pla2g10* is not influenced by the presence of implanting blastocyst (Fig. 3b, d). This suggests that the molecular mechanism by which P₄-PR signaling regulates *Pla2g10* expression seems to be different from other PR target genes expressed in the epithelium in the mouse uterus during embryo implantation. Intrauterine delivery of siRNA has been performed to elucidate the function of genes on embryo implantation in mice [48-50]. In general, the *in vivo* action of delivered siRNAs partially inhibits expression levels of target genes and reduces the number of IS at the time of embryo implantation. Fig. 5 shows similar results that intrauterine delivery of siRNA for *Pla2g10* inhibited PLA2G10 expression in LE on Day 4 and reduced the number of IS on Day 6.

**Conclusion**

Collectively, this is the first report that *Pla2g10* is a novel P₄-PR target gene that is exclusively induced in LE to prepare uterine receptivity for embryo implantation in mice (Fig. 6). Further studies are needed to comprehensively understand molecular regulation of steroid hormone receptors on transcriptional activity of the *Pla2g10* promoter.

**Methods**
Animals

All animals were maintained and handled according to the policies approved by CHA University Institutional Animal Care and Use Committee (IACUC, approval number 170002). Eight-week-old adult ICR mice were provided by Orient Bio, Inc (Gapyeong, Gyeonggi, Korea).

Hormone treatments

To examine the actions of ovarian steroid hormones on expression of Pla2g10, adult female mice were OVX, rested for 14 days, and then appropriately treated with steroid hormones for each experiment performed in this study. Mice were sacrificed and their uterine horns were collected for real-time RT-PCR and/or immunofluorescence after ovarian steroid hormone treatment.

To investigate time-dependent actions of P₄ (Sigma-Aldrich, USA) and E₂ (17b-estradiol, Sigma-Aldrich) on the expression of Pla2g10 in mouse uterus, adult OVX mice were subcutaneously injected with P₄ (2 mg/mouse) or P₄ + E₂ (200 ng/mouse) and sacrificed at various time points (0, 3, 6, and 24 h) after injection. To examine the dose-dependent induction of Pla2g10 by P₄, mice were given a single injection of vehicle (sesame oil, 0.1 ml/mouse) or P₄ at various concentrations (0.25 to 2 mg). To analyze whether P₄ works through a nuclear PR for Pla2g10 expression in mouse uterus, adult OVX mice were pretreated with the PR antagonist RU-486 (1 mg/mouse, Sigma-Aldrich), 30 min before P₄ (2 mg/mouse) injection and then sacrificed 24 h later.

Preparation of uterine samples during early pregnancy

Uterine samples during early pregnancy were prepared as previously described [35]. Briefly, 8- to 10-week-old female mice were housed with proven fertile males for pregnancy. The next morning when the vaginal plug was found was considered as Day 1. Pregnant mice were sacrificed on various days of pregnancy, and their uteri were collected for real-time RT-PCR and/or immunofluorescence. IS in the morning (0900 h) of Day 5 and 6 were visualized by intravenous injection (0.1 ml/mouse) of Chicago sky blue 6B solution (1% in saline, Sigma-Aldrich). The IS were demarcated by discrete blue bands along the uterus. IS on Day 6 were collected and immediately frozen in liquid nitrogen for frozen section to perform histological analyses including immunofluorescence staining and alkaline phosphatase (ALP) activity assay.

To induce an experimentally-induced delayed implantation model in mice, pregnant ICR female mice were OVX at the morning of Day 4 and given P₄ (2 mg/mouse) from Day 5 to 7 as described previously [51]. To activate dormant blastocysts and initiate implantation, P₄-primed delayed implanting pregnant mice were injected with E₂ (25 ng/mouse) on Day 7. Mice were sacrificed 24 h after the last hormone injection, and IS were visualized using Chicago sky blue 6B solution.
RNA extraction, RT-PCR, and real-time RT-PCR

The experiment was performed as previously described [35]. Briefly, uteri (3-5 mice per each group) were collected and immediately frozen in liquid nitrogen. Then, total RNA was extracted individually using Trizol Reagent (Ambion, USA) according to manufacturer’s protocols. cDNA was synthesized from total RNA using M-MLV reverse transcriptase (Promega, USA) with random primers and oligo dT. Synthesized cDNA was utilized for PCR with specific primers at optimized cycles. Real-time RT-PCR was performed by monitoring real-time increases in the fluorescence of SYBR Green dye. Real-time RT-PCR was performed using the Realtime PCR detection system (Bio-Rad, USA) and iQ™SYBR® Green supermix (Bio-Rad). For comparison of transcript levels between samples, a standard curve of cycle thresholds for several serial dilutions of a cDNA sample was established and then used to calculate the relative abundance of each gene. Values were then normalized to the relative amounts of rPL7 cDNA. All PCR reactions were performed in duplicate.

Immunofluorescence staining

To determine the presence and cell-type specific localization of PLA2G10 after P₄ treatment, and during the estrous cycle and early pregnancy, uteri were fixed in 4% paraformaldehyde (PFA) and embedded in paraplast (Leica Biosystems, Germany). Uterine sections (5 µm) were deparaffinized, dehydrated, and subjected to antigen retrieval in 0.01 M sodium citrate buffer, pH 6.0, for 20 min. For immunofluorescence staining of ARG2 (Arginase 2), a marker for decidualization, frozen sections (13 µm) of IS on Day 6 were fixed in 4% PFA, washed in PBS, and permeabilized with 0.1% triton-X 100 in PBS. Non-specific staining was blocked using Protein Block Serum-Free (Dako, Denmark) for 1 h. Then, sections were incubated overnight with primary rabbit-anti-PLA2G10 antibody (1:100, Santa Cruz Biotechnology, USA) for PLA2G10 or primary rabbit-anti-ARG2 antibody (1:200, abcam, USA) for ARG2 at 4 °C, washed in phosphate-buffered saline (PBS), and incubated with Alexa Fluor 488 goat-anti-rabbit secondary antibody (1:1000, Invitrogen Corp., USA) for 1 h at room temperature. Sections were washed in PBS, counterstained with propidium iodide (PI, Sigma-Aldrich) for 20 min, and mounted for observation using a LSM880 confocal microscope (Carl Zeiss, Germany).

Hematoxylin & Eosin (H&E) staining and ALP activity assay

H&E staining and ALP activity assay were performed to evaluate gross histology of implanted embryos and decidualization in IS of the uterus with siPla2g10 on Day 6, respectively. Frozen sections (13 µm) were fixed in 4% PFA, washed in PBS, and either stained with hematoxylin (Cancer Diagnostics, USA) and eosin (Richard Allan Scientific, USA) or incubated with a 100mM Tris HCl buffer (pH 9.5) containing ALP
substrate working solution (Vector Laboratories, SK-5400, USA). Slides were counterstained with fast red and mounted to observe ALP activity under light microscopy.

Construction of expression and reporter vectors

A proximal region (-840 to +65) of \textit{Pla2g10} promoter (p) was amplified from mouse genomic DNA by PCR with Forward 1 (5'-GCT AGC GGT GGT TCC AAG GTT TCA CTC AG-3') and Reverse 1 (5'-CTC GAG GTC ACA GAG GTG GCC CAC AC -3') primers. The amplified \textit{Pla2g10}(p) was cloned into pGL4.10 vector (Promega) and named pGL4.10/Pla2g10(p)-840/+65. The vector was independently mutated at four PREs, namely -801/-794, -356/-349, -310/-303, and -290/-283 in \textit{Pla2g10}(p)-840/+65 using the EZ change™ Site-directed Mutagenesis Kit (Enzynomics, Inc., Korea). The four mutated PREs were named pGL4.10/Pla2g10(p)-801mt, pGL4.10/Pla2g10(p)-356mt, pGL4.10/Pla2g10(p)-310mt, and pGL4.10/Pla2g10(p)-290mt, respectively. PRA and PRB cDNAs were provided by Dr. J.W. Jeong (Michigan State University, MI, USA). The cDNAs were cloned into a pcDNA3.1 \textit{Nhel-Xhol} site and named pcDNA3.1/PRA and pcDNA3.1/PRB, respectively.

Transfection and luciferase assay

Ishikawa cells, human endometrial adenocarcinoma cells, were plated in 12-well plates with DMEM/F12 and 10% charcoal-stripped (CS)-FBS 24 h before transfection. pcDNA3.1, pcDNA3.1/PRA, or pcDNA3.1/PRB expression vectors were co-transfected with pGL4.10/Pla2g10(p)-840/+65, pGL4.10/Pla2g10(p)-801mt, pGL4.10/Pla2g10(p)-356mt, pGL4.10/Pla2g10(p)-310mt, or pGL4.10/Pla2g10(p)-290mt vectors, and a pRL-null vector that was used as an internal control for normalization by GenePORTER®3000 Transfection Reagent (Genlantis, USA). The medium was replaced with DMEM/F12 and 2% CS-FBS with 1 µM P_4 (Sigma-Aldrich) 4 h after transfection. Cells were harvested and analyzed for firefly and renilla luciferase activities using the Dual-Glo™ Luciferase Assay System (Promega) 24 h after transfection. Luminescence was measured with Synergy Mx™ (Bio Tek, Inc., USA).

\textit{In vivo} RNA interference of \textit{Pla2g10} in mouse uterus

Knock-down of \textit{Pla2g10} in mouse uterus was performed as previously described by Ruan \textit{et al.} with some modifications [48]. Briefly, 100 pmol siPla2g10 (BIONEER Corp., Korea; 5'-GAA CAA AUG CCA AGA ACU U-3') or siNC (BIONEER Corp.) were combined with 5 µl of lipofectamine 2000 in 10 µl of Opti-MEM. The solutions were injected into each uterine horn at 18:00 - 20:00 h on Day 3 for \textit{in vivo} RNA interference of \textit{Pla2g10} in mouse uterus.
Statistics

All values represent the mean ± standard deviation. The unpaired Student’s t-test was used for statistical evaluation. A p-value of less than 0.05 was considered statistically significant.

Declarations

Ethics approval and consent to participate

This study was approved by the Institution Review Board at CHA Bundang Medical Center, CHA University, before sample collection (IRB No 2011-01-001) and all women signed an informed consent form before participating in the study.

Consent for publication

The content of the manuscript has been approved by all the authors.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors’ Contributions

HRK and HS conceived and designed the experiments in the manuscript. HKP and SHP performed the formal analysis. HKP, SHP, ML, GRK, MP, SCY, YSK and HJL performed the experiments and analyzed
data. HKP, SHP, ML, GRK, MP, SCY, YSK and HJL performed the data visualization. HRK and HS supervised this study. HKP, SHP, HRK and HS wrote the original draft. HKP, SHP, HJL, HRK and HS reviewed and edited the manuscript. All authors read and approved the final manuscript.

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References

1. Song H, Lim H, Paria B, Matsumoto H, Swift L, Morrow J, et al. Cytosolic phospholipase A2alpha is crucial for 'on-time' embryo implantation that directs subsequent development. Development. 2002;129(12):2879-89.
2. Brown N, Morrow JD, Slaughter JC, Paria BC, Reese J. Restoration of on-time embryo implantation corrects the timing of parturition in cytosolic phospholipase A2 group IVA deficient mice. Biol Reprod. 2009;81(6):1131-8.
3. Niringiyumukiza JD, Cai H, Xiang W. Prostaglandin E2 involvement in mammalian female fertility: ovulation, fertilization, embryo development and early implantation. Reprod Biol Endocrin. 2018;16(1):43.
4. Murakami M, Yamamoto K, Miki Y, Murase R, Sato H, Taketomi Y. The Roles of the Secreted Phospholipase A2 Gene Family in Immunology. Adv Immunol. 2016;132:91-134.
5. Choi Y, Kim H-R, Lim EJ, Park M, Yoon JA, Kim YS, et al. Integrative analyses of uterine transcriptome and microRNAome reveal compromised LIF-STAT3 signaling and progesterone response in the endometrium of patients with recurrent/repeated implantation failure (RIF). PloS One. 2016;11(6):e0157696.
6. Wang H, Dey SK. Roadmap to embryo implantation: clues from mouse models. Nat Rev Genet. 2006;7(3):185-99.
7. Lim HJ, Wang H. Uterine disorders and pregnancy complications: insights from mouse models. J Clin Invest. 2010;120(4):1004-15.
8. Wu SP, Li R, DeMayo FJ. Progesterone Receptor Regulation of Uterine Adaptation for Pregnancy. Trends Endocrin Met. 2018;29(7):481-91.
9. Patel B, Elguero S, Thakore S, Dahoud W, Bedaiwy M, Mesiano S. Role of nuclear progesterone receptor isoforms in uterine pathophysiology. Hum Reprod Update. 2015;21(2):155-73.
10. Marquardt RM, Kim TH, Shin JH, Jeong JW. Progesterone and Estrogen Signaling in the Endometrium: What Goes Wrong in Endometriosis? Int J Mol Sci. 2019;20(15).
11. Cha J, Sun X, Dey SK. Mechanisms of implantation: strategies for successful pregnancy. Nat Med. 2012;18(12):1754-67.
12. Gnainsky Y, Dekel N, Granot I. Implantation: mutual activity of sex steroid hormones and the immune system guarantee the maternal-embryo interaction. Semin Reprod Med. 2014;32(5):337-45.

13. Fujiwara H, Ono M, Sato Y, Imakawa K, Lizuka T, Kagami K, et al. Promoting Roles of Embryonic Signals in Embryo Implantation and Placentaion in Cooperation with Endocrine and Immune Systems. Int J Mol Sci. 2020;21(5).

14. Arck P, Hansen PJ, Mulac Jericevic B, Piccinni MP, Szekeres-Bartho J. Progesterone during pregnancy: endocrine-immune cross talk in mammalian species and the role of stress. Am J Reprod Immunol. 2007;58(3):268-79.

15. Solano ME, Arck PC. Steroids, Pregnancy and Fetal Development. Front Immunol 2019;10:3017.

16. Piette PC. The Pharmacodynamics and Safety of Progesterone. Best Pract Res Clin OB. 2020.

17. Wetendorf M, DeMayo FJ. Progesterone receptor signaling in the initiation of pregnancy and preservation of a healthy uterus. Int J Dev Biol. 2014;58:95.

18. Das S, Chakraborty I, Paria B, Wang X, Plowman G, Dey S. Amphiregulin is an implantation-specific and progesterone-regulated gene in the mouse uterus. Mol Endocrinol. 1995;9(6):691-705.

19. Matsumoto H, Zhao X, Das SK, Hogan BL, Dey SK. Indian hedgehog as a progesterone-responsive factor mediating epithelial-mesenchymal interactions in the mouse uterus. Dev Biol. 2002;245(2):280-90.

20. Zhu L-J, Cullinan-Bove K, Polihronis M, Bagchi MK, Bagchi IC. Calcitonin is a progesterone-regulated marker that forecasts the receptive state of endometrium during implantation. Endocrinology. 1998;139(9):3923-34.

21. Rubel CA, Wu SP, Lin L, Wang T, Lanz RB, Li X, et al. A Gata2-Dependent Transcription Network Regulates Uterine Progesterone Responsiveness and Endometrial Function. Cell Rep. 2016;17(5):1414-25.

22. Wang X, Li X, Wang T, Wu SP, Jeong JW, Kim TH, et al. SOX17 regulates uterine epithelial-stromal cross-talk acting via a distal enhancer upstream of Ihh. Nat Commun. 2018;9(1):4421.

23. Young IR, Renfree MB, Mesiano S, Shaw G, Jenkin G, Smith R. The comparative physiology of parturition in mammals: hormones and parturition in mammals. In: Hormones and Reproduction of Vertebrates. Elsevier. 2011;95-116.

24. Milligan SR, Finn CA. Minimal progesterone support required for the maintenance of pregnancy in mice. Hum Reprod. 1997;12(3):602-7.

25. Rubel CA, Lanz RB, Kommagani R, Franco HL, Lydon JP, DeMayo FJ. Research resource: Genome-wide profiling of progesterone receptor binding in the mouse uterus. Mol Endocrinol. 2012;26(8):1428-42.

26. Murakami M, Lambeau G. Emerging roles of secreted phospholipase A(2) enzymes: an update. Biochimie. 2013;95(1):43-50.

27. Wang H, Dey SK. Lipid signaling in embryo implantation. Prostaglandins. 2005;77(1-4):84-102.
28. Ye X, Hama K, Contos JJ, Anliker B, Inoue A, Skinner MK, et al. LPA3-mediated lysophosphatidic acid signalling in embryo implantation and spacing. Nature. 2005;435(7038):104-8.
29. Hama K, Aoki J, Inoue A, Endo T, Amano T, Motoki R, et al. Embryo spacing and implantation timing are differentially regulated by LPA3-mediated lysophosphatidic acid signaling in mice. Biol Reprod. 2007;77(6):954-9.
30. Ye X, Diao H, Chun J. 11-deoxy prostaglandin F2α, a thromboxane A2 receptor agonist, partially alleviates embryo crowding in Lpar3 (−/−) females. Fertil Steril. 2012;97(3):757-63.
31. Nahed RA, Martinez G, Escoffier J, Yassin S, Karaouzène T, Horgandeur J-P, et al. Progesterone-induced acrosome exocytosis requires sequential involvement of calcium-independent phospholipase A2β (iPLA2β) and group X secreted phospholipase A2 (sPLA2). J Bio Chem. 2016;291(6):3076-89.
32. Lim HJ, Dey SK. HB-EGF: a unique mediator of embryo-uterine interactions during implantation. Exp Cell Res. 2009;315(4):619-26.
33. Green CJ, Fraser ST, Day ML. Insulin-like growth factor 1 increases apical fibronectin in blastocysts to increase blastocyst attachment to endometrial epithelial cells in vitro. Hum Reprod. 2015;30(2):284-98.
34. Rosario GX, Stewart CL. The Multifaceted Actions of Leukaemia Inhibitory Factor in Mediating Uterine Receptivity and Embryo Implantation. Am J Reprod Immunol. 2016;75(3):246-55.
35. Kim HR, Kim YS, Yoon JA, Yang SC, Park M, Seol DW, et al. Estrogen induces EGR1 to fine-tune its actions on uterine epithelium by controlling PR signaling for successful embryo implantation. FASEB J. 2018;32(3):1184-95.
36. Franco HL, Jeong JW, Tsai SY, Lydon JP, DeMayo FJ. In vivo analysis of progesterone receptor action in the uterus during embryo implantation. Semin Cell Dev Biol. 2008;19(2):178-86.
37. Large MJ, DeMayo FJ. The regulation of embryo implantation and endometrial decidualization by progesterone receptor signaling. Mol Cell Endocrinol. 2012;358(2):155-65.
38. Xie H, Wang H, Tranguch S, Iwamoto R, Mekada E, Demayo FJ, et al. Maternal heparin-binding-EGF deficiency limits pregnancy success in mice. P NatL Acad Sci USA. 2007;104(46):18315-20.
39. Achache H, Revel A. Endometrial receptivity markers, the journey to successful embryo implantation. Hum Reprod Update. 2006;12(6):731-46.
40. Cavagna M, Mantese JC. Biomarkers of endometrial receptivity—a review. Placenta. 2003;24 Suppl B:S39-47.
41. Rubel CA, Franco HL, Jeong J-W, Lydon JP, DeMayo FJ. GATA2 is expressed at critical times in the mouse uterus during pregnancy. Gene Expr Patterns. 2012;12(5-6):196-203.
42. Simon L, Spiewak KA, Ekman GC, Kim J, Lydon JP, Bagchi MK, et al. Stromal progesterone receptors mediate induction of Indian Hedgehog (IHH) in uterine epithelium and its downstream targets in uterine stroma. Endocrinology. 2009;150(8):3871-6.
43. Franco HL, Rubel CA, Large MJ, Wetendorf M, Fernandez-Valdivia R, Jeong JW, et al. Epithelial progesterone receptor exhibits pleiotropic roles in uterine development and function. FASEB J. 2012;26(3):1218-27.

44. Tan J, Paria BC, Dey SK, Das SK. Differential uterine expression of estrogen and progesterone receptors correlates with uterine preparation for implantation and decidualization in the mouse. Endocrinology. 1999;140(11):5310-21.

45. Croy BA, Yamada AT, DeMayo FJ, Adamson SL. The guide to investigation of mouse pregnancy: Academic Press. 2013.

46. Wetendorf M, Wu S-P, Wang X, Creighton CJ, Wang T, Lanz RB, et al. Decreased epithelial progesterone receptor A at the window of receptivity is required for preparation of the endometrium for embryo attachment. Biol Reprod. 2017;96(2):313-26.

47. Kurita T, Lee KJ, Cooke PS, Taylor JA, Lubahn DB, Cunha GR. Paracrine regulation of epithelial progesterone receptor by estradiol in the mouse female reproductive tract. Biol Reprod. 2000;62(4):821-30.

48. Ruan YC, Guo JH, Liu X, Zhang R, Tsang LL, Dong JD, et al. Activation of the epithelial Na+ channel triggers prostaglandin E(2) release and production required for embryo implantation. Nat Med. 2012;18(7):1112-7.

49. Chen JJ, Xiao ZJ, Meng X, Wang Y, Yu MK, Huang WQ, et al. MRP4 sustains Wnt/beta-catenin signaling for pregnancy, endometriosis and endometrial cancer. Theranostics. 2019;9(17):5049-64.

50. Zhang D, Yang Y, Liang C, Liu J, Wang H, Liu S, et al. poFUT1 promotes uterine angiogenesis and vascular remodeling via enhancing the O-fucosylation on uPA. Cell Death Dis. 2019;10(10):775.

51. Lee JE, Oh HA, Song H, Jun JH, Roh CR, Xie H, et al. Autophagy regulates embryonic survival during delayed implantation. Endocrinology. 2011;152(5):2067-75.