Embryo Spacing and Implantation Timing Are Differentially Regulated by LPA3-Mediated Lysophosphatidic Acid Signaling in Mice¹

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ABSTRACT

In polytocous animals, blastocysts are evenly distributed along each uterine horn and implant. The molecular mechanisms underlying these precise events remain elusive. We recently showed that lysophosphatidic acid (LPA) has critical roles in the establishment of early pregnancy by affecting embryo spacing and subsequent implantation through its receptor, LPA3. Targeted deletion of Lpa3 in mice resulted in delayed implantation and embryo crowding, which is associated with a dramatic decrease in the prostaglandins and prostaglandin-endoperoxide synthase 2 expression levels. Exogenous administration of prostaglandins rescued the delayed implantation but did not rescue the defects in embryo spacing, suggesting the role of prostaglandins in implantation downstream of LPA3 signaling. In the present study, to know how LPA3 signaling regulates the embryo spacing, we determined the time course distribution of blastocysts during the preimplantation period. In wild-type (WT) uteri, blastocysts were distributed evenly along the uterine horns at Embryonic Day 3.8 (E3.8), whereas in the Lpa3-deficient uteri, they were clustered in the vicinity of the cervix, suggesting that the mislocalization and resulting crowding of the embryos are the cause of the delayed implantation. However, embryos transferred singly into E2.5 pseudopregnant Lpa3-deficient uterine horns still showed delayed implantation but on-time implantation in WT uteri, indicating that embryo spacing and implantation timing are two segregated events. We also found that an LPA3-specific agonist induced rapid uterine contraction in WT mice but not in Lpa3-deficient mice. Because the uterine contraction is critical for embryo spacing, our results suggest

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that LPA3 signaling controls embryo spacing via uterine contraction around E3.5.

embryo spacing, female reproductive tract, growth factors, implantation, LPA3, lysophosphatidic acid, signal transduction, uterus

INTRODUCTION

Implantation is a series of processes that are regulated by various kinds of signaling pathways between the embryo and the uterus during the initial period of gestation. Implantation consists of positioning, attachment, and invasion of the embryo (blastocyst) in the uterus [1-3]. In many polytocous species (species that have many offspring in a single birth), the embryos are distributed evenly rather than randomly along the uterus. Preformed implantation sites could have been a possible explanation for the even distribution of blastocysts, although this hypothesis is inconsistent with the finding that the implantation sites are equidistant along the uterine horn, irrespective of the number of blastocysts transferred into the uterus [4]. In rabbits, blastocysts were found to enter the uterus at Embryonic Day 3 (E3.0), move rapidly from E3.0 to E5.0, move slowly and align equidistantly around E6.0, and finally implant around E7.0 [4]. Thus, it is likely that there are at least two events for the completion of implantation. First, blastocysts align equidistantly along the uterus (spacing), and then they implant (implantation).

Several previous reports gave some insight into the molecular mechanisms regulating the spacing and the implantation. Administration of relaxin (a potent inhibitor of myometrial activity) in rats was found to disrupt the normal distribution of the blastocysts before implantation [5, 6]. In addition, the frequency of myometrial contractions in pregnant rats was significantly higher in the immediate preimplantation period than in the rest of the postimplantation period [7]. These observations indicated that myometrial activity in the uterus is at least partly responsible for proper embryo spacing. Several bioactive molecules have been found to regulate the implantation through their corresponding cellular receptors. These include ovarian steroid hormones, prostaglandins (PGs), and lysophosphatidic acid (LPA). Administration of a progesterone receptor antagonist to pregnant mice prevented implantation without affecting the development of blastocysts [8]. In addition, implantation was found to be defective in mice and rats treated with indomethacin, an inhibitor of PG-endoperoxide synthase (PTGS), a critical enzyme for PG biosynthesis, as well as in PTGS2-deficient mice [9-11]. Consistent with these

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PTGS studies, implantation was found to fail in cytosolic phospholipase A_2 alpha (PLA2G4A) knockout mice [12]. PLA2G4A is a major provider of arachidonic acid for the PTGS system in PG synthesis. These results suggest a pivotal role of ovarian steroid hormones and PGs in implantation.

Among the above-mentioned bioactive molecules that are possibly involved in early implantation events, LPA (1- or 2acyl-LPA) may also be important, because it influences both the spacing and the implantation. LPA is a simple phospholipid that mediates multiple cellular processes both in vivo and in vitro through at least five G protein-coupled receptors specific to LPA (LPA1/EDG2/vzg-1, LPA2/EDG4, LPA3/EDG7, LPA4/GPR23, and LPA5/GPR92) [13-17]. The in vitro actions include platelet aggregation, cell migration, cell proliferation, and cytoskeletal reorganization [18]. LPA also stimulates ovum transport and maturation of oocytes, suggesting that it has roles in female reproductive organs [19, 20]. Experiments with LPA receptor knockout mice showed that LPA signaling has very important roles in both physiological and pathological conditions, such as neural development (LPA1), neuropathic pain (LPA1), diarrhea (LPA2), and implantation (LPA3) [21-24].

In Lpa3-deficient uteri, implantation sites, which are normally detectable at E4.5, were completely absent at E4.5 but detectable at E5.5 (delayed implantation). In addition, the implantation sites in $Lpa3^{-/-}$ uteri were unevenly distributed [24]. As a result, $Lpa3^{-/-}$ female mice showed significantly reduced litter sizes, which could be attributed to the delayed implantation and the altered embryo spacing [24]. Interestingly, around E3.5, Lpa3 expression is up-regulated transiently by the action of an ovarian hormone, progesterone, which has critical roles in the establishment of early pregnancy, including implantation [25]. In addition, the expression of PTGS-2, as well as of PG levels, was up-regulated around E3.5, which was not observed in Lpa3-deficient uteri. Administration of PGs (prostaglandin E₂ [PGE₂] and carbaprostacyclin [cPGI], a stable analog of prostacyclin) into E3.5 Lpa3-deficient females could restore the delayed implantation [24]. Thus, it is likely that PGs are the effectors of the progesterone-regulated LPA3 signaling. However, PGE₂ and cPGI administration did not correct the embryo crowding in Lpa3-deficient females [24], indicating that embryo spacing is regulated differentially from embryo implantation downstream of LPA3-mediated signaling. In the present study, to obtain insights into the mechanism underlying embryo spacing, we examined the role of LPA3 signaling in embryo spacing and uterine contraction in the preimplantation period.

MATERIALS AND METHODS

Mice

The *Lpa3* gene was originally disrupted by homologous recombination, as described previously [24]. Heterozygous mice were intercrossed, and the resulting *Lpa3^{+/+}* or *Lpa3^{-/-}* mice were used for analysis. All mice used in the present study were of mixed background (129/SvJ and C57BL/6J). Mice were bred and maintained at the Animal Care Facility in the Graduate School of Pharmaceutical Sciences, the University of Tokyo, under specific pathogen-free conditions in accordance with institutional guidelines.

Measurements of Embryo Distribution

Wild-type (WT) or *Lpa3*-deficient mice were mated with fertile WT males (E0.5 = vaginal plug, 1200 h). On E3.5 (1200 h), E3.8 (1900 h), or E4.5 (1200 h), mice were i.v. perfused with 4% paraformaldehyde for fixation. Then, the whole uterus was carefully excised and embedded in paraffin wax. Longitudinal sequential sections were stained with hematoxylin and eosin to identify the blastocysts. The distance between each blastocyst was calculated by National Institutes of Health image software.

Blastocysts and Transfer into Uterus

Females were mated with vasectomized males to induce pseudopregnancy. On E2.5, the eight-cell embryos were collected from pregnant WT mice and cultured in KSOM medium (Millipore, Billerica, MA) overnight and then transferred to the uteri of pseudopregnant recipients on E2.5. Implantation sites on E4.5 were localized by i.v. injection of Evans blue dye (200 μ l, 1% in 1× PBS; Sigma, St. Louis, MO) [26]. For PG treatment, *Lpa3*-deficient female mice were i.p. administrated with vehicle (10% EtOH with saline) or PGs (5 μ g of PGE, and 5 μ g of cPGI) at 1000 and 1800 h on E3.5.

Measurements of Uterine Contraction

The uteri from WT or *Lpa3*-deficient mice on E3.5 were removed carefully to avoid excessive stretching; each was placed in a Petri dish containing Krebs solution (118 mM NaCl, 4.5 mM KCl, 1.0 mM MgSO₂, 1.0 mM KH₂PO₄, 25 mM NaHCO₃, 1.8 mM CaCl₂, and 6.0 mM glucose), and excessive tissue was removed. The mechanical activity of the uterine samples was recorded continuously under isometric conditions. Each sample was connected to a force displacement transducer (model TB-612T; Nihon Kohden Co., Ltd., Tokyo, Japan) coupled to a multichannel amplifier (model MEG-6108; Nihon Kohden), and mounted in a 10-ml organ bath with Krebs solution suffused with 95% O₂:5% CO₂ at 37°C. Each sample was allowed to equilibrate for 30 min under an initial load of 1.0 g. The myometrial contractility was recorded before and after the addition of 10 μ M T13, an LPA3-selective agonist [27], and later 10 μ M acetylcholine.

Statistical Analysis

Results are expressed as means \pm SD. The data were analyzed with the Student *t*-test. P < 0.05 was considered significant.

RESULTS

Aberrant Embryo Spacing During Peri-Implantation Period in Lpa3-Deficient Mice

In mice, embryos in the late morula stage or early blastocyst stage enter the uterus around E3.0. The blastocysts begin to interact with the uterine luminal epithelium around E3.5 and implant around E4.0. To pinpoint the time when the blastocysts are distributed along the uterine horn, we determined the locations of blastocysts along the uterine horns during E3.0-E3.8. The axial transverse and sequential hematoxylin and eosin-stained uterine sections from both WT and $Lpa3^{-/-}$ mice during E3.0-E3.8 were examined (Fig. 1a). The presence and locations of blastocysts along the uterine horns were determined under microscopy (Fig. 1a). The location is expressed as the percentage of the uterine length from the uterotubal junction (Fig. 1b), and the degree of equidistance is shown as a coefficient of variation, which is the mean of the distances between blastocysts in a horn divided into the standard deviation (Fig. 1c). On E3.0, most blastocysts were located in the vicinity of the uterotubal junction both in WT and $Lpa3^{-/-}$ uteri (data not shown). On E3.5, most of the blastocysts were distributed evenly in most of the WT uteri, although some of them were still present in clusters (Fig. 1, a-c). By contrast, most blastocysts in $Lpa3^{-/-}$ uteri clustered in the vicinity of the uterotubal junction, although some were found near the cervix (Fig. 1, a-c). On E3.8, blastocysts were evenly distributed in almost all uteri in WT mice, whereas they were not evenly distributed in $Lpa3^{-/-}$ uteri (Fig. 1, a-c). The cluster of blastocysts was similar to the implantation sites seen at E5.5 in $Lpa3^{-/-}$ uteri [24]. These data demonstrate that LPA3-mediated signaling is required at least for embryo spacing during the preimplantation period and that embryo spacing occurs before embryo implantation.

LPA3-Mediated Signaling on Uterine Contraction

Myometrial activity is important for both the transport and spacing of the preimplantation embryos [4–6]. Thus, we

HAMA ET AL.



FIG. 1. Distribution of embryos in *Lpa3*-deficient uteri is uneven. **a**) Sequential histopathological pictures of longitudinal section with blastocysts for each time point. Arrowheads point to the blastocyst location. Representative experiment was shown for each time. Bar = 5 mm. **b**) Schematic localization of embryos along WT (wild-type, labeled *Lpa3^{+/+}*) and *Lpa3*-deficient (*Lpa3^{-/-}*) uterine horns at E3.5 and E3.8. Data were obtained from the axial transverse and sequential hematoxylin and eosin-stained sections. The location of blastocysts is represented by the percentage of uterine length from the uterotubal junction. **c**) The degree of equidistance is expressed as a coefficient of evaluation, which is the mean of the distances between blastocysts in a horn divided into the standard deviation.

**p<0.05

0

E3.5

E3.8

E4.5



FIG. 2. LPA3-specific agonist, T13, induced uterine contraction through LPA3. Uterine contractile activity upon 10 μ M T13 (*) or 10 μ M acetylcholine (**) treatment in isolated E3.5 WT or $Lpa3^{-/-}$ uteri. Resting applied tension was 1.0 g. At least four independent experiments were performed, and a representative experiment is shown.

examined the effects of LPA3-mediated signaling on myometrial activity with T13 [27]. T13 induced a potent contractile response on isolated E3.5 WT uteri (Fig. 2). However, this contractile response was completely absent in isolated E3.5 *Lpa3*-deficient uteri (Fig. 2). The E3.5 *Lpa3*-deficient uteri contracted normally in response to acetylcholine, a potent inducer of uterine contraction (Fig. 2), indicating that the *Lpa3*deficient uteri lose LPA3 agonist-induced contraction but retain uterine myometrial contractility per se. These data raise the possibility that LPA3 signaling regulates embryo spacing through uterine contraction during the preimplantation period.

Effect of Embryonic Crowding on Implantation Timing

We show that embryo spacing occurs prior to embryo implantation (Fig. 1b). Lpa3-deficient females have defects in both embryo spacing and implantation timing [24]. These results raise the possibility that embryo crowding is responsible for the delayed implantation in the Lpa3-deficient uterus. We previously showed that multiple embryos (three to four in each uterine horn) transferred into pseudopregnant $Lpa3^{-/-}$ uteri resulted in delayed implantation and uneven distribution of implantation sites at E5.5 [24]. To eliminate any potential influence of embryo crowding on implantation, we transferred a single WT embryo into each E2.5 WT or Lpa3-deficient pseudopregnant uterine horn. One implantation site could be consistently detected in 9 of 10 transferred uterine horns in WT mice. By contrast, no implantation site was ever observed in $Lpa3^{-/-}$ mice (Fig. 3), whereas the transferred embryo, which was collected by flushing each uterine horn on E4.5, was found to be well developed and had already hatched (data not shown). These results clearly demonstrate that the singly-transferred embryos still had delayed implantation in the Lpa3-deficient uteri but on-time implantation in WT uteri (Fig. 3), indicating that delayed implantation in Lpa3-deficient uteri is an event independent of embryo crowding. Furthermore, this aberrant implantation timing was rescued by the exogenous administration of PGE₂ and cPGI into E3.5 Lpa3-deficient females (data not shown). These data show that embryo spacing and implantation timing are two segregated events, meaning that LPA3-mediated signaling controls embryo spacing around E3.5 and independently regulates the implantation timing thereafter.

DISCUSSION

Our recent study demonstrated a critical role of LPA3mediated LPA signaling in embryo implantation. Targeted deletion of *Lpa3* was shown to cause delayed implantation, altered embryo spacing, significantly reduced litter size in female mice, and activation of the PG synthetic pathway, and the resulting PGs were shown to be involved in the implantation process downstream of LPA3 signaling [24]. However, it is unclear whether LPA3-mediated LPA signaling regulates implantation timing and embryo spacing independently or whether it controls one event that subsequently influences the other. In the present study, we have further demonstrated that LPA3-mediated LPA signaling regulates implantation timing and embryo spacing independently (Fig. 4).



FIG. 3. Embryo crowding is not the cause for delayed implantation in $Lpa3^{-/-}$ uteri. A single blastocyst was injected into a pseudopregnant uterine horn in both WT and $Lpa3^{-/-}$ mice at E2.5. Detection of singly-transferred embryos in WT and $Lpa3^{-}$ deficient uteri at E4.5 was performed by blue dye injection. Red arrowheads show the implantation sites. Ten experiments were performed for both WT and $Lpa3^{-/-}$ mice, and representative experiments are shown. Bar = 5 mm.



FIG. 4. LPA3-mediated signaling regulates both embryo spacing and implantation independently. Proposed model of LPA3-mediated signaling in embryo spacing and implantation. LPA3-mediated signaling can stimulate uterine contraction and regulate embryo spacing around E3.5 through unknown mechanisms. It also determines implantation timing thereafter via PTGS2-derived prostaglandins (PGs).

Previous studies have demonstrated that myometrial contraction is critical for proper embryonic spacing. In the present study, we showed that LPA induced uterine myometrial contraction via LPA3 because an LPA3-specific agonist, T13, induced uterine myometrial contraction in WT uteri but not in Lpa3-deficient uteri. Thus, it might be speculated that LPA3-mediated signaling regulates embryo spacing through its effect on uterine contraction. We recently showed that Lpa3 expression in uteri dramatically changes during the estrus cycle and is up-regulated at E2.5-3.5 [24, 25]. In addition, the Lpa3 expression in uteri is dependent on progesterone, an ovarian hormone that has a critical role in the establishment of early pregnancy, including implantation. Recent findings indicate that uterus activity is controlled by steroid hormones, including progesterone [28]. Thus, progesterone may regulate Lpa3 expression, thereby contributing to uterus function during early pregnancy. At present, the molecular mechanism by which LPA3 activation leads to uterine contraction remains unknown (Fig. 4). PG, which induces the contraction of smooth muscle, can be a favoring factor in LPA3-mediated uterine contraction (Fig. 4), because it has already been shown to be essential in embryo spacing [9-12]. Further studies are necessary to identify the linkage of LPA3 and PG signaling in embryo implantation.

A problem with treatments for infertility by assisted reproductive technologies (ART) is that the implantation rate is poor [29]. Moreover, certain abnormal pregnancies, such as ectopic pregnancy and placenta previa, are associated with the mislocation of zygotes in the uterus. It is possible that these abnormal pregnancies are due to attenuated LPA3 signaling. LPA3 is a potential therapeutic target that promotes implantation in ART and that prevents such abnormal pregnancies.

In conclusion, we showed that LPA3 signaling regulates the uterine contraction, which can control the embryo spacing. Thus, in the establishment of early pregnancy, LPA3 signaling regulates two events: the embryo implantation mediated by PG signaling and the embryo spacing induced by uterine contraction.

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