11-deoxy prostaglandin $F_{2\alpha}$, a thromboxane A2 receptor agonist, partially alleviates embryo crowding in *Lpar3*$^{(-/-)}$ females

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Objective: To determine cyclooxygenase-derived prostanoid signaling in alleviating embryo crowding in the *Lpar3*$^{(-/-)}$ females.
Design: Experimental mouse model.
Setting: Research laboratories.
Animal(s): Wild-type, *Lpar3*$^{(+/-)}$, and *Lpar3*$^{(-/-)}$ mice.
Intervention(s): Intraperitoneal (IP) administration of prostaglandin E_2 (PGE_2), cPGI (a stable analogue of PGI_2), and 11-deoxy prostaglandin $F_{2\alpha}$ (11-deoxy $PGF_{2\alpha}$, a thromboxane A_2 receptor agonist) to preimplantation gestation day 3.5 *Lpar3*$^{(-/-)}$ females.
Main Outcome Measure(s): Implantation sites were detected by blue dye reaction and embryo spacing was determined by the distribution of the implantation sites along the uterine horns on gestation day 4.5; pregnancy outcome was measured by litter size at birth.
Result(s): Administration of PGE_2 + cPGI on gestation day 3.5 *Lpar3*$^{(-/-)}$ females restored on-time implantation but did not affect embryo spacing or the number of implantation sites detected on gestation day 4.5; PGE_2 + cPGI treatment increased litter size at birth. Administration of PGE_2 + cPGI + 11-deoxy $PGF_{2\alpha}$ on gestation day 3.5 *Lpar3*$^{(-/-)}$ females rescued on-time implantation, partially dispersed the clustered implantation sites normally observed in the *Lpar3*$^{(-/-)}$ females, increased the number of implantation sites detected on gestation day 4.5, and increased litter size at birth.
Conclusion(s): The thromboxane A_2 receptor agonist 11-deoxy $PGF_{2\alpha}$ can partially alleviate embryo crowding in the *Lpar3*$^{(-/-)}$ females and embryo crowding likely contributes to reduced litter size in the *Lpar3*$^{(-/-)}$ females. (Fertil Steril® 2012;97:757–63. ©2012 by American Society for Reproductive Medicine.)
Key Words: Lysophosphatidic acid (LPA) receptor 3, COX-derived prostanoids, prostanoid receptors, prostanoid receptor agonists, 11-deoxy $PGF_{2\alpha}$, embryo implantation, embryo spacing

Embryo spacing in polyovulating animals tends to be evenly spaced along each uterine horn, which may minimize early embryo mortality resulting from local overcrowding. Even embryo spacing is thought to be achieved through uterine myometrial contractions (1–3).

Lysophosphatidic acid (LPA) is an extracellular lipid mediator with myriad actions that include cell proliferation, survival, and migration. The LPA signals through six known G protein-coupled receptors (GPCRs), LPA_{1-6} (4, 5). The LPA signaling has been implicated in many aspects of female reproduction, such as fertilization, embryo development, ovum transportation in the oviduct, uterine contraction, embryo implantation, and pregnancy maintenance (reviewed in Refs. 6 and 7). The LPA_3-mediated signaling is specifically involved in two separate events: implantation timing and embryo spacing (8, 9). Deletion of *Lpar3* in mice results in delayed embryo implantation and aberrant embryo spacing, which is reflected by the crowding of implantation sites on the cervical half of a uterine horn (proximal uterine segment) and multiple embryos sharing a single placenta (8). *Lpar3*$^{(-/-)}$ uterine horns lose LPA_3 agonist-induced uterine contraction (9), supporting the theory that dysregulated myometrial activities may contribute to embryo crowding in the *Lpar3*$^{(-/-)}$ females. The LPA_3 has also been implicated in the aberrant embryo spacing induced by transient β_2-adrenoceptor activation (10).

Delayed embryo implantation and aberrant embryo spacing phenotypes in *Lpar3*$^{(-/-)}$ females have also been observed in rats and mice treated with indomethacin, a cyclooxygenase

Received July 25, 2011; revised November 18, 2011; accepted December 5, 2011; published online January 4, 2012.
X.Y. has nothing to disclose. H.D. has nothing to disclose. J.C. has nothing to disclose.
Supported by funding from the Office of the Vice President for Research at the University of Georgia and NIH R15HD066301, NIH R01HD065939 (X.Y.) and NIH R01HD050685 (J.C.).
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Fertility and Sterility® Vol. 97, No. 3, March 2012 0015-0282/$36.00
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doi:10.1016/j.fertnstert.2011.12.004

(COX) inhibitor (11–14), and *Pla2g4a*$^{(-/-)}$ mice deficient for cytosolic phospholipase $A_{2\alpha}$ ($cPLA_{2\alpha}$) (15). Both *Lpar3*$^{(-/-)}$ females and *Pla2g4a*$^{(-/-)}$ females have reduced uterine expression of COX-2, the rate-limiting enzyme for prostagnoid synthesis (8, 15). These observations reveal the importance of COX-derived prostanoid signaling in on-time embryo implantation and even embryo spacing (8, 11–15).

The COX-derived prostanoids include prostaglandins PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane A_2 (TxA_2), which activate their respective GPCRs, $DP_{1\text{-}2}$, $EP_{1\text{-}4}$, FP, IP, and TP (16, 17). These GPCRs are expressed in the uterus and mediate the effects of COX-derived prostanoids in myometrial activity (18, 19). EP_1, EP_3, FP, and TP have a contractile effect on the myometrium, whereas $DP_{1\text{-}2}$, EP_2, EP_4, and IP have a relaxant effect (16, 18, 20, 21). It has been demonstrated that PGE_2 and cPGI (a stable analogue of PGI_2) can restore normal implantation timing but fail to rescue embryo spacing defect in *Lpar3*$^{(-/-)}$ females and *Pla2g4a*$^{(-/-)}$ females (8, 15). It has not been determined whether any specific COX-derived prostanoid signaling affects embryo spacing. The PGE_2 can induce contractile (by EP_1 and EP_3) and relaxant (by EP_2 and EP_4) effects, whereas cPGI only induces relaxant (by IP) effects. Because they do not have an obvious effect on embryo spacing yet can rescue on-time implantation in *Lpar3*$^{(-/-)}$ females (8), it was hypothesized that COX-derived prostanoid(s) that can induce a contractile effect rather than a relaxant effect on the myometrium may alleviate embryo crowding in *Lpar3*$^{(-/-)}$ females.

MATERIALS AND METHODS

Animals

Wild-type, *Lpar3*$^{(+/-)}$, and *Lpar3*$^{(-/-)}$ mice (129/SvJ and C57BL/6 mixed background) were generated and genotyped, as described (8). The animals were housed on a 12-hour light/dark cycle (6:00 AM to 6:00 PM) at 23° ± 1°C with 30%–50% relative humidity. All methods used in this study were approved by the Animal Subjects Programs of The Scripps Research Institute and the University of Georgia and conform to National Institutes of Health guidelines and public law.

Mating, Administration of Prostanoid Receptor Agonists, Detection of Implantation Sites, Gestation Period, and Litter Size

Females were mated with fertile males and checked for a vaginal plug the next morning (midnight of mating night was designated as day 0, 00:00). Implantation sites were detected by intravenous injection of blue dye on gestation day 4.5 for the control mice (including wild-type and *Lpar3*$^{(+/-)}$, both of which have normal embryo implantation) (8), which was designated as group 1 in this study, and on gestation day 5.5 for the *Lpar3*$^{(-/-)}$ females (undetectable implantation sites on gestation day 4.5 due to delayed implantation) (8), which was designated as group 2. The previously reported treatment regimen was followed (8) to determine COX-derived prostanoid signaling in embryo spacing in *Lpar3*$^{(-/-)}$ females. Briefly, gestation day 3.5 *Lpar3*$^{(-/-)}$ females were intraperitoneally injected (at 10:00 and 18:00, respectively) with 100 μL of PGE_2 (5 μg) + cPGI (5 μg) in vehicle (10% ethanol with saline), which was designated as group 3, or 100 μL of PGE_2 (5 μg) + cPGI (5 μg) + 11-deoxy $PGF_{2\alpha}$ (5 μg) in vehicle, which was designated as group 4. Implantation sites were detected on gestation day 4.5, as previously described (8). The uterine horns without visible implantation site(s) were flushed with 1 × phosphate-buffered saline (PBS) to examine the presence of healthy-looking blastocyst(s), thus the pregnancy status. The nonpregnant mice without implantation sites and healthy-looking blastocysts were excluded in this study. The numbers of mice included in these four study groups were group 1 (n = 16), group 2 (n = 12), group 3 (n = 8), and group 4 (n = 13). Another set of animals from each of these four groups was used to determine the gestation period and litter size at birth. Only the animals who delivered pups were included. The numbers of mice were group 1 (n = 29), group 2 (n = 17), group 3 (n = 5), and group 4 (n = 12). In addition, there were also three other groups of *Lpar3*$^{(-/-)}$ females for the following treatments: a vehicle-injected *Lpar3*$^{(-/-)}$ group (n = 12), a (+)-fluprostenol (5 μg)-injected *Lpar3*$^{(-/-)}$ group (n = 5), and an 11-deoxy $PGF_{2\alpha}$ (5 μg)-injected *Lpar3*$^{(-/-)}$ group (n = 16) following the same protocol as the designated groups 3 and 4. Fluprostenol is a metabolically stable analogue of $PGF_{2\alpha}$ and an FP agonist. Because these three different treatments only partially rescued or failed to rescue on-time implantation detected on gestation day 4.5 (Fig. 1), these three groups were not included in the analysis of embryo spacing at gestation day 4.5. All the mice were dissected between 11:00 and 12:00 hours on gestation day 4.5 except group 2 (untreated *Lpar3*$^{(-/-)}$ females) on gestation day 5.5. All the prostanoid receptor agonists used in this study were purchased from Cayman Chemical Company.

Analysis of Embryo Spacing

Each uterus was placed on a weighing paper and stretched evenly to a comparable length for photographing. Embryo spacing was determined by analyzing the distribution of implantation sites along the uterine horns: 1) each uterine horn was divided into 100 even parts, from 0 at the ovary side to 100 at the cervix side, 0–50 and 51–100 were also assigned as distal and proximal uterine segments, respectively (Fig. 2A); 2) each blue band (implantation site) was located by a number, indicating its position on a uterine horn (Fig. 2); 3) the percentage of total implantation sites within the distal and the proximal uterine segments from the same group was then determined (Fig. 3A); 4) the mean localization (mean), the SD, and the coefficient of variation (CV = SD/mean) for each female with implantation sites were calculated (both SD and CV indicating the relative level of dispersion of implantation sites on the uterine horns); and 5) because each animal had one set of data from step 4, the average mean, SD, and CV in each group were then calculated (Fig. 3B–D). This procedure was used to calculate the relative distribution of implantation sites for all four studied groups: group 1, untreated gestation day 4.5 control; group 2, untreated gestation day 5.5 *Lpar3*$^{(-/-)}$ females; group 3, PGE_2 + cPGI-treated gestation day 4.5 *Lpar3*$^{(-/-)}$ females; and group 4, PGE_2 +

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