CORRIGENDUM

Cytosolic phospholipase $A_2\alpha$ deficiency is crucial for 'on-time' embryo implantation that directs subsequent development

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Cytosolic phospholipase $A_2\alpha$ deficiency is crucial for 'on-time' embryo implantation that directs subsequent development

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SUMMARY

Cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) is a major provider of arachidonic acid (AA) for the cyclooxygenase (COX) system for the biosynthesis of prostaglandins (PGs). Female mice with the null mutation for *Pla2g4a* ($cPLA_2\alpha$) produce small litters and often exhibit pregnancy failures, although the cause(s) of these defects remains elusive. We show that the initiation of implantation is temporarily deferred in *Pla2g4a*$^{-/-}$ mice, shifting the normal 'window' of implantation and leading to retarded feto-placental development without apparent defects in decidual growth. Furthermore, $cPLA_2\alpha$ deficiency results in aberrant uterine spacing of embryos. The deferred implantation and deranged gestational development in *Pla2g4a*$^{-/-}$ mice were significantly improved by exogenous PG administration. The results provide evidence that $cPLA_2\alpha$-derived AA is important for PG synthesis required for on-time implantation. This study in *Pla2g4a*$^{-/-}$ mice, together with the results of differential blastocyst transfers in wild-type mice provides the first evidence for a novel concept that a short delay in the initial attachment reaction creates a ripple effect propagating developmental anomalies during the subsequent course of pregnancy.

Key words: $cPLA_2\alpha$, implantation, uterus, embryo, pregnancy

INTRODUCTION

Embryo implantation in the uterus is one of the most crucial steps in mammalian embryo development. It requires both prior preparation of the receptive uterus and activation of the blastocyst, and is initiated by the attachment of the blastocyst trophectoderm to the uterine luminal epithelium between 22:00 and 24:00 hours on day 4 of pregnancy in mice (Das et al., 1994). Concurrently with the attachment reaction, an increased endometrial vascular permeability becomes evident at the site of implantation. This event is followed by localized endometrial decidualization with luminal epithelial apoptosis and subsequent invasion of the trophoblast through the basement membrane into the stroma (Dey, 1996). Coordinated interactions between ovarian estrogen and progesterone (P_4) prime the uterus for implantation. For example, removal of preimplantation ovarian estrogen secretion in mice by ovariectomy results in blastocyst dormancy and prevents the attachment reaction. Dormant blastocysts can be activated to implant in the P_4-primed uterus by a single injection of estrogen (Paria et al., 1993a; Paria et al., 1993b). The molecular signals that regulate implantation are of considerable clinical relevance as understanding the nature of these signals should lead to strategies for correcting implantation failures and pregnancy losses.

Prostaglandins (PGs) are implicated in various female reproductive functions (Lim et al., 1997; Lim et al., 1999a). PGs are generated from arachidonic acid (AA) by phospholipase A_2s (PLA_2s) followed by cyclooxygenases (COX). PLA_2 plays crucial roles in diverse cellular functions, including phospholipid metabolism, immune functions and signal transduction by generating bioactive lipid mediators (Gijon and Leslie, 1999; Valentin and Lambeau, 2000). Once activated by a variety of stimuli, PLA_2 hydrolyzes the ester bonds of fatty acids at the *sn*-2 position of phospholipids, producing free fatty acids and lysophospholipids. The mammalian PLA_2 superfamily consists of four major subfamilies that include cytosolic ($cPLA_2$), secretory ($sPLA_2$), Ca^{2+}-independent ($iPLA_2$) and platelet-activating factor (PAF) acetylhydrolase. While $cPLA_2$ and $sPLA_2$ participate in various cellular functions by generating free fatty acids, including AA, $iPLA_2$ and PAF acetylhydrolase primarily contribute to membrane remodeling and attenuation of PAF bioactivity, respectively (Murakami et al., 2000). PLA_2-derived AA gives rise to various lipid mediators, including PGs, leukotrienes, thromboxanes and endocannabinoids. These mediators via various signaling

pathways exert a wide range of cellular functions (Clark et al., 1995; Mechoulam et al., 1998; Serhan and Oliw, 2001). Among the PLA_2 superfamily members, $cPLA_2$ is a key regulator of eicosanoid biosynthesis, because it selectively releases AA (Clark et al., 1995). It lacks sequence similarity to other members of the PLA_2 superfamily, suggesting a unique role of $cPLA_2$. Recently, two other $cPLA_2$ isoforms, $cPLA_2\beta$ and $cPLA_2\gamma$, have been identified in the human EST database (Underwood et al., 1998; Song et al., 1999), assigning a new name for $cPLA_2$ as $cPLA_2\alpha$, although they, unlike $cPLA_2\alpha$, do not show substrate preference for AA (Song et al., 1999).

As a major source of AA for PG synthesis, $cPLA_2\alpha$ undergoes Ca^{2+}-dependent translocation to the perinuclear and endoplasmic reticular (ER) membranes, the sites of COX enzymes (Clark et al., 1995; Murakami et al., 2000). To date, more than ten PLA_2 and two COX isoforms (COX1 and COX2) have been identified in mammals (Smith and DeWitt, 1996; Six and Dennis, 2000; Valentin and Lambeau, 2000), indicating their differential roles in distinct biological responses to various stimuli. For example, in mast cells, $sPLA_2$ and $cPLA_2\alpha$ are functionally coupled to COX1 and COX2, and participate in early and late PGD_2 synthesis, respectively (Reddy and Herschman, 1997).

Owing to their vasoactive, mitogenic and differentiating properties, PGs are implicated in ovulation and implantation (Lim et al., 1997). Recent genetic evidence points towards essential functions of enzymes responsible for PG biosynthesis in reproduction. Consistent with our previous report of uterine induction of COX-2 at the site of blastocyst implantation (Chakraborty et al., 1996), mice with null mutation for *Ptgs2*, which encodes COX2, show multiple female reproductive failures, such as defective ovulation, fertilization, implantation and decidualization (Lim et al., 1997). By contrast, female mice with null mutation for *Ptgs1*, which encodes COX1, are fertile with limited parturition defects (Langenbach et al., 1995). Studies on *Ptgs2*$^{-/-}$ mice further showed that while prostacyclin (PGI_2) plays a major role in implantation, PGE_2 plays a complementary role in this process (Lim et al., 1999a).

Of the many PLA_2s, $cPLA_2$ ($cPLA_2\alpha$) is known to couple functionally to COX2 in specific cell types (Reddy and Herschman, 1997; Takano et al., 2000), and mice with null mutation for *Pla2g4a*, which encodes $cPLA_2\alpha$, have small litters with presumed parturition defects and often show pregnancy failures (Bonventre et al., 1997; Uozumi et al., 1997). However, the underlying cause of this reduced fertility still remains unknown. Using *Pla2g4a* mutant mice and differential blastocyst transfers in wild-type mice, we show here that a short deferral of the normal 'window' of implantation results in severe progressive developmental anomalies during the course of pregnancy. In addition, $cPLA_2\alpha$ deficiency results in abnormal uterine spacing of embryos.

MATERIALS AND METHODS

Mice

The disruption of the *Pla2g4a* gene was originally achieved in J1 ES cells by homologous recombination as described (Bonventre et al., 1997). Genotyping was by PCR analysis of genomic DNA. All of the mice used were housed in the Animal Care Facility at the University of Kansas Medical Center, according to NIH and institutional guidelines for laboratory animals.

Ovulation and fertilization

To examine ovulation and fertilization, wild-type or *Pla2g4a*$^{-/-}$ mice were bred with fertile males with same genotypes, respectively. The morning of finding a vaginal plug was designated day 1 of pregnancy. Mice were killed on day 2 of pregnancy and oviducts were flushed with Whitten's medium to recovery eggs and embryos. Their morphology was examined under a dissecting microscope. In vitro maturation and fertilization were performed with partial zona pellucida dissection as previously described by us (Matsumoto et al., 2001).

Implantation and decidualization

Implantation sites on days 5 and 6 of pregnancy were visualized by an intravenous injection of Chicago Blue dye solution (Paria et al., 1993b). The uteri of mice with a few or without implantation site were flushed with Whitten's medium to recover unimplanted blastocysts. Recovered embryos were observed under a dissecting microscope.

For reversal experiments, PGE_2 and cPGI (Cayman Chemical, Ann Arbor, MI) were prepared in 10% ethanol/90% saline. Injections (PGE_2 + cPGI, 5 μg each/mouse, i.p.) were given at 10:00 and 18:00 hours on day 4 of pregnancy. The control mice received the vehicle. Implantation sites were recorded on day 5 morning (10:00 hours) by the blue dye method.

To induce artificial decidualization, pseudopregnant wild-type or *Pla2g4a*$^{-/-}$ mice received intraluminal infusion of sesame oil (25 μl) in one uterine horn on day 4 or day 5 (10:00 hours) and were killed 4 days later. Uterine weights of the infused and non-infused (control) horns were recorded and the fold increases in uterine weights were used as an index of decidualization (Lim et al., 1997).

Blastocyst transfer

Mating females with vasectomized males induced pseudopregnancy. Day 4 wild-type or *Pla2g4a*$^{-/-}$ blastocysts were transferred into the uteri of wild-type or *Pla2g4a*$^{-/-}$ pseudopregnant recipients on day 4 (Paria et al., 1993b). The number of implantation sites was recorded by the blue dye method on day 5 morning (10:00 hours) (Paria et al., 1993b). To examine pregnancy outcome of implantation beyond the normal 'window' of uterine receptivity, day 4 wild-type blastocysts were transferred into wild-type pseudopregnant recipients on day 4 or day 5 of pseudopregnancy. Recipients were examined for subsequent developmental events on day 12 or observed for delivery of pups at term.

Gross observation and histological examination of implantation sites and embryos

Day 12 implantation sites and embryos were examined using the protocol as previously described with some modification (Hogan et al., 1994). Isolated day 12 implantation sites were weighed individually, fixed in 10% formalin overnight and dissected to isolate embryos. Isolated embryos were weighed individually and their images were captured to examine the size and gross morphology. For histological examination, paraffin wax-embedded sections of day 12 implantation sites were stained with Eosin and Hematoxylin.

Measurement of PGs

PGs were quantitated using gas chromatography/negative ion chemical ionization mass spectrometric assays as described previously (DuBois et al., 1994).

RT-PCR analysis

RT-PCR analyses were performed using gene-specific primers by following a protocol previously described (Paria et al., 1993a).

Hybridization probes

Sense or antisense ^{35}S-labeled cRNA probes were generated using appropriate polymerases from cDNAs to *Pla2g4a*, *Pla2g2d*, *Pla2g2e*, *Pla2g10*, *Lif*, *Hoxa10*, *Hegfl*, *Areg*, *Ptgs1* and *Ptgs2* for in situ

hybridization as previously described (Das et al., 1994). Mouse cDNAs to *Pla2g4a*, *Pla2g2d*, *Pla2g2e* and *Pla2g10* were generated by RT-PCR cloning using TOPO cloning kit (Invitrogen, Carlsbad, CA).

In situ hybridization

In situ hybridization was performed as previously described (Das et al., 1994; Song et al., 2000). Frozen sections (12 μm) were mounted onto poly-L-lysine-coated slides, fixed in cold 4% paraformaldehyde solution in PBS, acetylated and hybridized at 45°C for 4 hours in hybridization buffer containing the ^{35}S-labeled antisense cRNA probes. After hybridization, the sections were treated with RNase A (20 μg/ml) at 37°C for 20 minutes. RNase A-resistant hybrids were detected by autoradiography. Sections hybridized with the sense probes served as negative controls.

RESULTS

Ovulation and fertilization are modestly reduced in *Pla2g4a*$^{-/-}$ mice

To determine the cause(s) of reduced fertility in *Pla2g4a*$^{-/-}$ female mice, we examined in detail the reproductive phenotypes of these females during pregnancy. There is evidence that genetic background of mice contributes to different phenotypes (Threadgill et al., 1995). Furthermore, a number of inbred mouse strains including C57BL/6J and 129/Sv have a natural null mutation in *Pla2g2a* encoding sPLA$_2$-IIA (Kennedy et al., 1995; MacPhee et al., 1995). Thus, defective reproduction in *Pla2g4a*$^{-/-}$ mice on C57BL/6J background may reflect deficiency of both cPLA$_2\alpha$ and sPLA$_2$-IIA. By contrast, the outbred CD1 mice with larger litter sizes have variable genotype (+/+, +/–, –/–) of *Pla2g2a* mutation (Kennedy et al., 1995). Therefore, to study the reproductive events in more detail, we introduced cPLA$_2\alpha$ deficiency in CD1 mice by crossing with C57BL/6J *Pla2g4a*$^{-/-}$ mice. The results on these two strains are described.

C57BL/6J

To examine the ovulation and fertilization status in *Pla2g4a*$^{-/-}$ mice, we counted the number of ovulated eggs and fertilized 2-cell embryos on day 2 of pregnancy. All of the wild-type (n=13) and *Pla2g4a*$^{-/-}$ (n=10) mice ovulated (Fig. 1A). A slight, but statistically insignificant, reduction in the number of ovulated eggs was noted in *Pla2g4a*$^{-/-}$ mice. A modest reduction in the fertilization rate in mutant mice was also noted. This modest reduction was not due to defective sperm functions in *Pla2g4a*$^{-/-}$ males, as assessed by in vitro fertilization and cross-breeding experiments (data not shown). Thus, subsequent experiments used wild-type and *Pla2g4a*$^{-/-}$ females mated with males of the same genotypes.

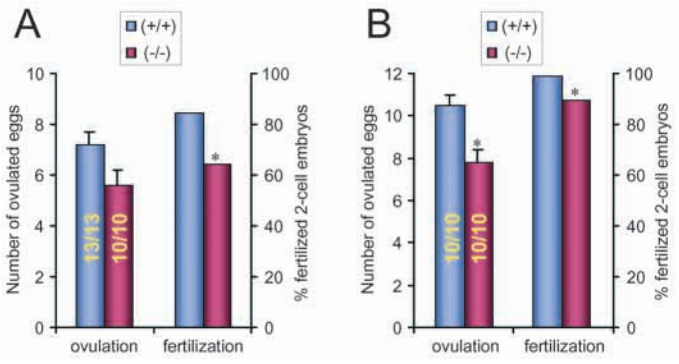

Fig. 1. Ovulation and fertilization rates in *Pla2g4a*$^{-/-}$ mice. The rate of ovulation and fertilization in wild-type and *Pla2g4a*$^{-/-}$ mice was examined on day 2 of pregnancy on C57BL/6J (A) and CD1 (B) genetic backgrounds. The numbers within the bars indicate the number of mice with ovulation/total number of mice. Results of ovulation are mean±s.e.m. Statistical significance was evaluated using unpaired t-test and χ^2-test, respectively (*P<0.01).

CD1

Our next objective was to determine if these phenotypes were retained in *Pla2g4a*$^{-/-}$ mice on the CD1 background. While ovulation and fertilization rates were increased in both wild-type and *Pla2g4a*$^{-/-}$ mice, their profiles were similar to C57BL/6J mice (Fig. 1B). These results suggested that cPLA$_2\alpha$ has a modest role in ovulation and fertilization, but not to the extent that was observed for COX2 deficient mice showing profound defects in these processes (Lim et al., 1997).

Normal 'window' of implantation is altered in *Pla2g4a*$^{-/-}$ mice

Although ovulation and fertilization were somewhat reduced in *Pla2g4a*$^{-/-}$ mice on C57BL/6J or CD1 background, these reduced rates cannot fully account for the reduced litter size observed in these mice (Bonventre et al., 1997; Uozumi et al., 1997). Furthermore, frequent pregnancy failure in plug-positive *Pla2g4a*$^{-/-}$ mice (Bonventre et al., 1997) also suggests uterine defects between fertilization and parturition. We thus investigated whether cPLA$_2\alpha$ deficiency impedes implantation and decidualization in mice on these backgrounds. Increased vascular permeability at the site of blastocyst implantation was recorded on day 5 of pregnancy by the blue dye method (Paria et al., 1993b).

C57BL/6J

While an average of approx. nine implantation sites (9.4±0.4/mouse) were observed in all of the eight wild-type mice, only about three implantation sites (2.6±0.6/mouse) were detected in nine out of 10 *Pla2g4a*$^{-/-}$ mice on day 5 (09:00 hours) of pregnancy. The reduced number of implantation sites was not due to compromised ovulation and fertilization in *Pla2g4a*$^{-/-}$ mice for two reasons. First, the few implantation sites that were detected in *Pla2g4a*$^{-/-}$ mice on day 5 of pregnancy showed a very weak blue reaction, suggesting defective vascular permeability changes during the attachment reaction. Second, the number of blastocysts recovered from these *Pla2g4a*$^{-/-}$ mice was more than the visible implantation sites, suggesting that these blastocysts failed to initiate the attachment reaction.

CD-1

Implantation defects were more prominent in *Pla2g4a*$^{-/-}$ mice on this background. On day 5, an average of less than two implantation sites (1.8±0.3/mouse) was detected in four out of 12 *Pla2g4a*$^{-/-}$ mice examined (Fig. 2A). Not only was the number of implantation sites remarkably low, but also a large number of unimplanted blastocysts was recovered from all 12 mice after flushing their uteri (Fig. 2B). Of the 61 blastocysts recovered, 55 of them were zona free and only six blastocysts

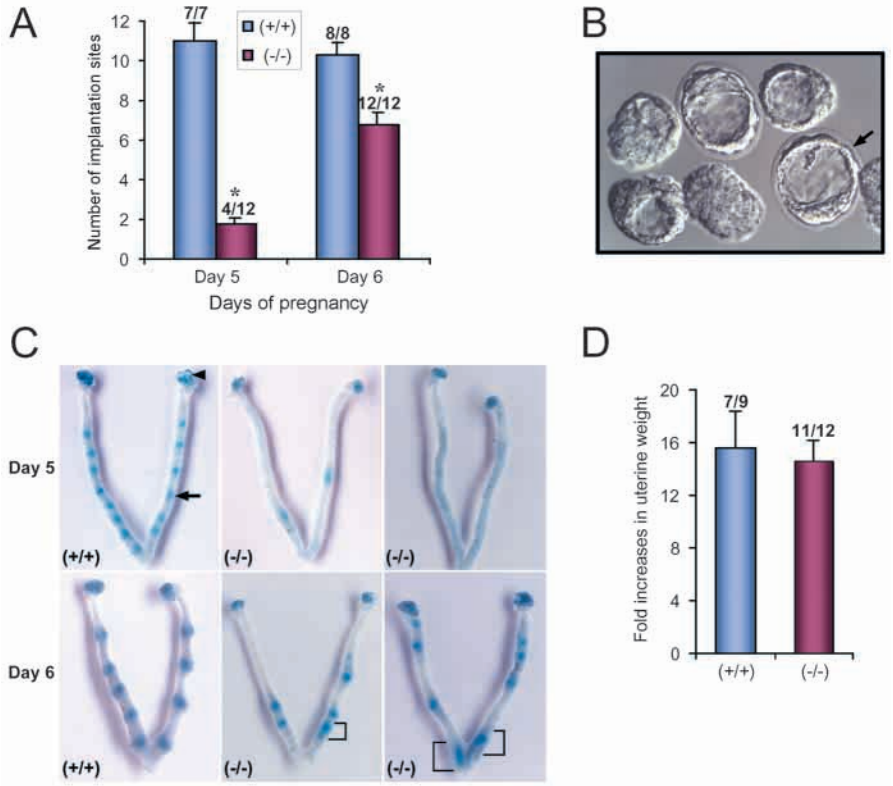

Fig. 2. Implantation and decidualization in *Pla2g4a*$^{-/-}$ mice. (A) The number of implantation sites was examined on days 5 and 6 of pregnancy in wild-type and *Pla2g4a*$^{-/-}$ mice by the blue dye method. The numbers above the bars indicate the number of mice with implantation sites/total number of mice (unpaired *t*-test, **P*<0.001). The uteri of mice with a few or without implantation sites were flushed to recover unimplanted blastocysts. The mice without implantation sites or blastocysts were excluded from the experiments. (B) A representative photomicrograph of blastocysts recovered from *Pla2g4a*$^{-/-}$ mice on day 5 of pregnancy (1000 hours) is shown. Note blastocysts with (arrow) or without zona pellucida. (C) Representative photographs of uteri with implantation sites (blue bands) on days 5 and 6. Note very few or no implantation sites on day 5, but unevenly spaced implantation sites on day 6 in *Pla2g4a*$^{-/-}$ mice. Arrowhead and arrow indicate ovary and implantation site, respectively. Brackets indicate crowding of implantation sites. (D) Decidualization. Wild-type or *Pla2g4a*$^{-/-}$ mice received intraluminal oil infusion on day 4 of pseudopregnancy. On day 8, uterine weights were recorded. Fold increases denote comparison of weights between infused and non-infused uterine horns. The numbers above the bars indicate the number of responding mice/total number of mice. No significant difference in decidualization was noted between wild-type and *Pla2g4a*$^{-/-}$ mice (unpaired *t*-test; *P*>0.05).

were zona encased. Morphological appearance of these blastocysts apparently looked normal. Similar to C57BL/6J *Pla2g4a*$^{-/-}$ mice, the permeability changes at the implantation sites were also poor (Fig. 2C, top). By contrast, an average of 11 distinct implantation sites was detected in seven out of seven wild-type mice examined (Fig. 2A,C).

It has long been held that implantation in rodents occurs only for a limited period (~24 hours) defined as the 'window' of receptivity for implantation (Paria et al., 1993b; Dey, 1996). In mice, the uterus becomes receptive on day 4 of pregnancy with the initiation of the attachment reaction around midnight (Paria et al., 1993b; Das et al., 1994; Dey, 1996). We surmised that unimplanted blastocysts that we observed in *Pla2g4a*$^{-/-}$ uteri on day 5 could implant beyond the normal 'window' of implantation. As shown in Fig. 2A, all of the *Pla2g4a*$^{-/-}$ mice (12/12) showed distinct implantation sites (6.8±0.6) on day 6 of pregnancy, providing evidence that implantation had occurred beyond the normal 'window'. Similar results were obtained using C57BL/6J mice (data not shown). Collectively, these results establish that implantation occurs beyond the normal 'window' of implantation in *Pla2g4a*$^{-/-}$ mice, suggesting that uterine and/or blastocyst cPLA$_2\alpha$ is crucial to the initial attachment reaction.

Experimentally induced decidualization is normal in *Pla2g4a*$^{-/-}$ mice

The blastocyst attachment reaction is followed by extensive stromal cell proliferation and differentiation into decidual cells. Decidualization can also be induced experimentally in pseudopregnant or steroid hormonally prepared uteri by intraluminal oil infusion (Lim et al., 1997). Although the attachment reaction between the blastocyst and uterine luminal epithelium is deferred in the absence of cPLA$_2\alpha$, whether it is also crucial for decidualization is not known. Thus, we examined decidualization in *Pla2g4a*$^{-/-}$ mice by intraluminal oil infusion on day 4 of pseudopregnancy (Fig. 2D). The results show that 11 out of 12 *Pla2g4a*$^{-/-}$ mice had similar decidual response as the wild-type mice with respect to increased uterine weight (14.5±1.6-fold versus 15.6±2.7-fold). Taken together, the results suggest that the initial attachment reaction is perturbed in *Pla2g4a*$^{-/-}$ mice, but not the ability of the uterine stroma for decidualization. This is a novel finding that has not been observed in many other mutant mice with peri-implantation defects (Benson et al., 1996; Lim et al., 1997; Robb et al., 1998).

Pla2g4a expression follows dynamic changes in *Ptgs1* and *Ptgs2* expression in the uterus during implantation

As *Pla2g4a*$^{-/-}$ mice show implantation defects, it is possible that the *Pla2g4a* gene has a cell-specific expression pattern relevant to implantation. We compared the expression of *Pla2g4a* with *Ptgs1* (COX1) and *Ptgs2* (COX2) during implantation by in situ hybridization. As shown in Fig. 3A, *Pla2g4a* is expressed in the uterine epithelia on day 4 of pregnancy in a pattern similar to that of *Ptgs1* (Chakraborty et al., 1996). With the initiation and progression of implantation, the pattern of *Pla2g4a* expression was similar to that of *Ptgs2* (COX2) on days 5-8 of pregnancy. *Pla2g4a* was expressed in stromal cells surrounding the implanting blastocyst on day 5

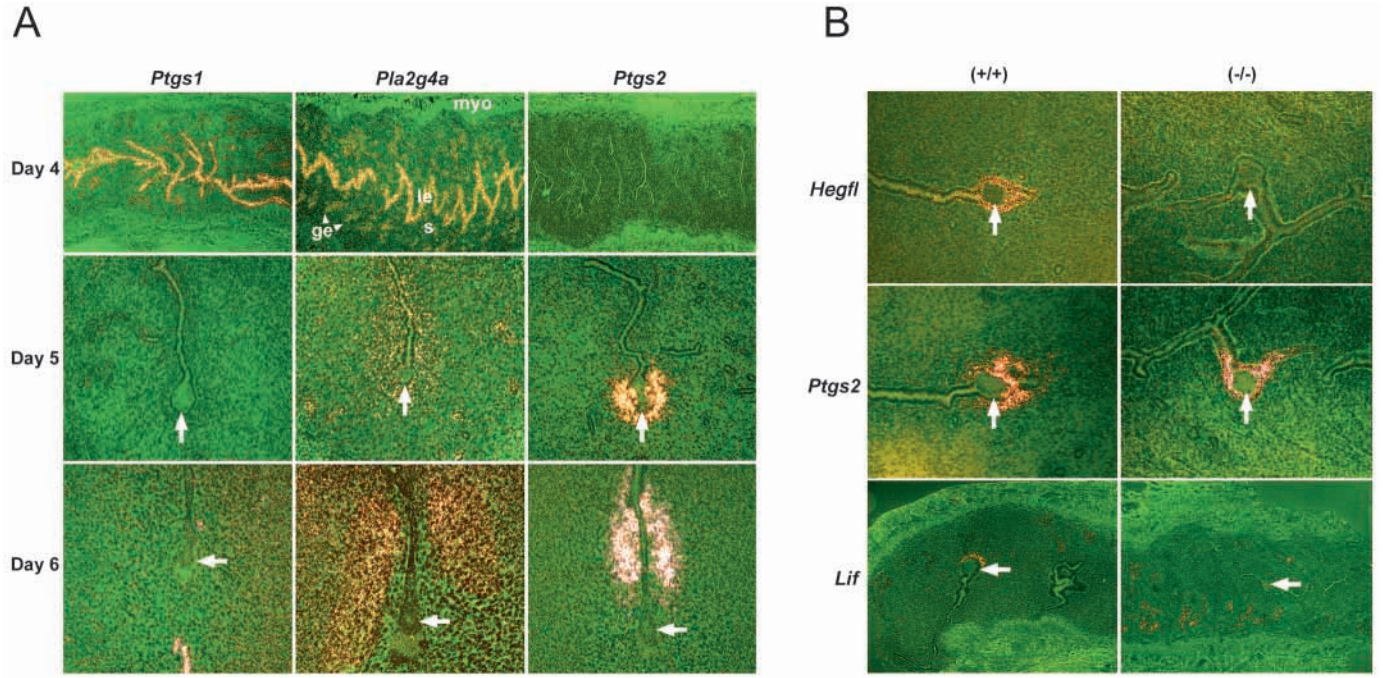

Fig. 3. Expression of *Pla2g4a* in wild-type uteri during implantation and expression of implantation-specific genes in *Pla2g4a*$^{-/-}$ uteri. (A) Comparison of expression of *Pla2g4a* with *Ptgs1* and *Ptgs2* in the mouse uterus during implantation. In situ hybridization of *Pla2g4a*, *Ptgs1* and *Ptgs2* on days 4-6 (09:00 hours) of pregnancy is shown. (B) In situ hybridization of *Hegfl*, *Ptgs2*, and *Lif* in uteri of wild-type and *Pla2g4a*$^{-/-}$ mice on day 5 of pregnancy (10:00 hours). Note aberrant expression of *Hegfl*, *Ptgs2* and *Lif* in the luminal epithelium and/or underlying stroma surrounding the blastocyst in *Pla2g4a*$^{-/-}$ mice. Arrows indicate the location of blastocysts. ge, glandular epithelium; le, luminal epithelium; s, stroma; myo, myometrium.

of pregnancy. On day 6-8, the expression was primarily restricted to the mesometrial pole of the implantation site. However, the expression of *Pla2g4a* was more widespread and apparently at lower levels than that of *Ptgs2*. These results suggest that cPLA$_2\alpha$ is available as an AA provider for uterine PG biosynthesis during implantation.

Expression of implantation-specific genes are dysregulated in *Pla2g4a*$^{-/-}$ mice

Deferred implantation in *Pla2g4a*$^{-/-}$ mice could be due to deficiency of uterine cPLA$_2\alpha$ and/or secondary to aberrant expression of genes considered important for implantation. Normal implantation requires preparation of the uterus to the receptive stage and embryo-uterine interactions for the attachment reaction followed by vascular permeability and stromal decidualization at the sites of blastocysts (Dey, 1996). Thus, we examined uterine genes involved in these events. Genes including *Areg* (amphiregulin), *Ptgs1*, *Hoxa10* and *Lif* are expressed on day 4 morning and implicated in uterine preparation (Benson et al., 1996; Chakraborty et al., 1996; Lim et al., 1997; Song et al., 2000). These genes were appropriately expressed in *Pla2g4a*$^{-/-}$ mice, suggesting that uterine preparation was not altered (data not shown). However, *Hegfl*, the gene encoding HB-EGF, the earliest known molecular marker of embryo-uterine interaction for implantation (Das et al., 1994), was not induced in the luminal epithelium surrounding the blastocyst prior to the attachment reaction on day 4 night (data not shown) or on day 5 morning in *Pla2g4a*$^{-/-}$ mice not showing implantation sites (blue bands) (Fig. 3B). Furthermore, *Ptgs2* and *Lif*, which are normally induced in the uterus surrounding the blastocyst during the attachment reaction (Chakraborty et al., 1996; Song et al., 2000), were either undetectable or aberrantly expressed at the sites of blastocysts in *Pla2g4a*$^{-/-}$ mice on day 5 in the absence of implantation (Fig. 3B). These results show that the expression of genes involved during early implantation is altered when on-time implantation does not occur in the absence of uterine cPLA$_2\alpha$, further confirming that the implantation process had been deferred.

Maternal cPLA$_2\alpha$ is crucial for implantation

Although *Pla2g4a* is expressed in the uterus during implantation and the attachment reaction is temporarily deferred in the absence of cPLA$_2\alpha$, it is possible that embryonic cPLA$_2\alpha$ is also a contributing factor in directing proper embryo-uterine interactions during implantation. Thus, we performed reciprocal embryo transfer experiments. Day 4 wild-type or *Pla2g4a*$^{-/-}$ blastocysts were transferred into wild-type or *Pla2g4a*$^{-/-}$ recipients on day 4 of pseudopregnancy and implantation rate was examined 24 hours later. As shown in Table 1, day 4 wild-type blastocysts transferred into wild-type recipient uteri showed normal complementation of implantation (42.4%, n=6). By contrast, wild-type blastocysts transferred into *Pla2g4a*$^{-/-}$ recipients showed considerably reduced number of implantation sites (21.2%, n=8). However, reduced implantation rate was not observed when *Pla2g4a*$^{-/-}$ blastocysts were transferred into wild-type uteri; over 40% of the blastocysts transferred showed implantation in all seven mice. Collectively, the results suggest that maternal cPLA$_2\alpha$, but not embryonic, is the primary contributor to on-time implantation.

Table 1. Implantation of blastocysts transferred into pseudopregnant wild-type or *Pla2g4a*$^{-/-}$ mice

Genotypes		Number of blastocysts transferred	Number of recipients	Number of mice with IS	Number of mice without IS	Number of IS (%)
Blastocysts	Recipients					
+/+	+/+	85	6	6	0	36 (42.4)*
+/+	–/–	113	8	7	1	24 (21.2)†
–/–	+/+	94	7	7	0	38 (40.5)*

Day 4 wild-type or *Pla2g4a*$^{-/-}$ blastocysts were transferred into uteri of wild-type or *Pla2g4a*$^{-/-}$ recipients on day 4 of pseudopregnancy. Recipients were killed on day 5 to examine implantation sites (IS) by the blue dye method. Uteri without IS were flushed with saline to recover any unimplanted blastocysts.
* are significantly different from † (χ^2-test; $P<0.01$).

Uterine levels of PGs are reduced in *Pla2g4a*$^{-/-}$ mice

The production of eicosanoids is reduced in *Pla2g4a*$^{-/-}$ macrophages (Bonventre et al., 1997; Uozumi et al., 1997). It is not known whether similar situation exists in *Pla2g4a*$^{-/-}$ uteri. Thus, we examined whether cPLA$_2$α deficiency leads to the reduced PG levels prior to and during implantation. Our results show that the levels of PGI_2 were significantly reduced in *Pla2g4a*$^{-/-}$ uteri on day 4, and both PGI_2 and PGE_2 were reduced on day 5 of pregnancy (Fig. 4A,B). PGI_2 is the major PG that is produced at the implantation sites in mice (Lim et al., 1999a). The less significant difference in uterine PGE_2 levels on day 4 could be due to the expression of specific sPLA$_2$ family members as providers of AA for COX 1. This is consistent with co-localization of *Pla2g4a* and *Pla2g10* (sPLA$_2$-X) with *Ptgs1* on day 4 prior to the attachment reaction, and primarily of *Pla2g4a* with *Ptgs2* from the time of attachment reaction and thereafter (see Fig. 3A). Furthermore, the reduced levels, but not complete abrogation, of PGs in *Pla2g4a*$^{-/-}$ mice suggest that other sPLA$_2$ family members contribute partially to maintain basal levels of uterine PGs. In this respect, our results show that *Pla2g1b* (sPLA$_2$-IB), *Pla2g2a* (sPLA$_2$-IIA), *Pla2g2c* (sPLA$_2$-IIC), and *Pla2g5* (sPLA$_2$-V) are undetectable in day 4 pregnant uteri by RT-PCR, although they are detected in other tissues used as controls. However, RT-PCR detected uterine expression of three other members, *Pla2g2d* (sPLA$_2$-IID), *Pla2g2e* (sPLA$_2$-

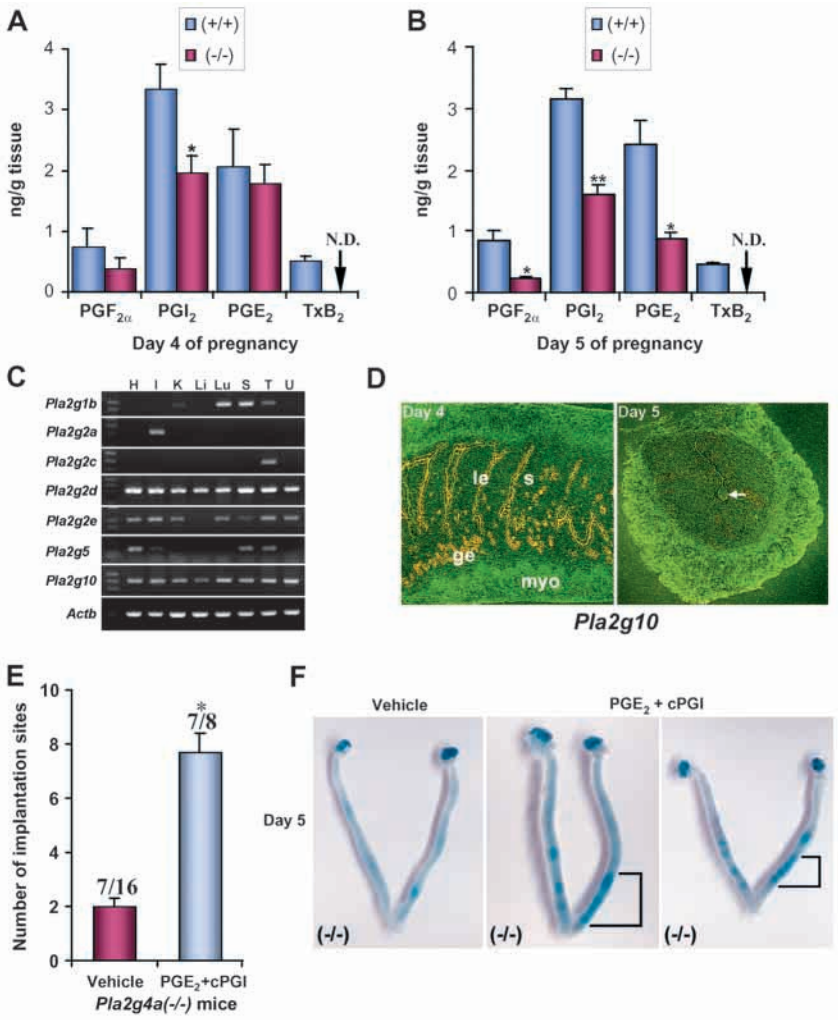

Fig. 4. Uterine status of PGs and expression of genes encoding sPLA$_2$s, and restoration of normal implantation in *Pla2g4a*$^{-/-}$ mice by PGs. (A,B) The levels of PGs in uteri of wild-type and *Pla2g4a*$^{-/-}$ mice on days 4 and 5 of pregnancy, respectively. PGI_2 was measured as 6-keto-$PGF_{1\alpha}$. N.D., not detectable (unpaired *t*-test, **P*<0.05; ***P*<0.01; *n*=4-5). (C) Expression of genes encoding sPLA$_2$ isoforms in various wild-type mouse tissues by RT-PCR. Heart (H), intestine (I), kidney (K), liver (Li), lung (Lu), spleen (S) and testis (T) tissue samples were used as controls along with uterine (U) samples obtained on day 4 of pregnancy. *Actb*, mouse β-actin. (D) In situ hybridization of *Pla2g10* (sPLA$_2$-X) in uteri of wild-type mice on days 4 and 5 of pregnancy. Note uterine expression of *Pla2g10* similar to that of *Pla2g4a* and *Ptgs1* on day 4 (compare with Fig. 3A). The arrow indicates the location of a blastocyst. ge, glandular epithelium; le, luminal epithelium; s, stroma; myo, myometrium. (E) Restoration of normal implantation in *Pla2g4a*$^{-/-}$ mice. *Pla2g4a*$^{-/-}$ mice were injected with saline or PGE_2 plus cPGI twice (10:00 and 18:00 hours) on day 4 and implantation sites were examined on day 5 (10:00 hours). The numbers above the bars indicate the number of *Pla2g4a*$^{-/-}$ mice with implantation sites/total number of *Pla2g4a*$^{-/-}$ mice used (unpaired *t*-test; **P*<0.001). The mice without implantation sites or blastocysts were excluded from the experiments. (F) Representative photographs of day 5 uteri of *Pla2g4a*$^{-/-}$ mice given the vehicle or PGs on day 4 of pregnancy. Note increased number of implantation sites with prominent blue reaction after PG treatment. Brackets indicate crowding of implantation sites.

IIE) and *Pla2g10* (sPLA$_2$-X), on day 4 (Fig. 4C). Further examination by in situ hybridization showed that while the uterine expression of *Pla2g2d* and *Pla2g2e* was insignificant (data not shown), *Pla2g10* was expressed in the uterine epithelium similarly to *Pla2g4a* on day 4, but the expression was very low on day 5 (Fig. 4D). These results suggest that sPLA$_2$-X could serve as an alternative source of AA in *Pla2g4a*$^{-/-}$ mice prior to implantation, but its role during implantation is questionable.

Exogenous administration of PGs restores normal 'window' of implantation in *Pla2g4a*$^{-/-}$ mice

The reduced levels of PGs in *Pla2g4a*$^{-/-}$ mice led us to restore normal implantation timing in *Pla2g4a*$^{-/-}$ mice by supplementing PGs (Fig. 4E,F). Administration of PGE$_2$ and carbaprostacyclin (cPGI, a more stable analogue of PGI$_2$) to *Pla2g4a*$^{-/-}$ mice twice on day 4 (10:00 and 18:00 hours, i.p.) restored implantation when examined on day 5. For example, seven out of eight mice injected with PGE$_2$ and cPGI showed an average of approx. eight implantation sites similar to normal day 5 implantation sites in wild-type mice (Fig. 4E,F). By contrast, parallel experiment without PG supplementation again showed poor implantation rate as described above; seven out of 16 *Pla2g4a*$^{-/-}$ mice injected with the vehicle had an average of two implantation sites, although blastocysts were recovered from all 16 mice examined. These results show that PG supplementation restores the normal 'window' of implantation in *Pla2g4a*$^{-/-}$ mice, further reinforcing a major role of cPLA$_2\alpha$ in PG biosynthesis. Although thromboxane B$_2$ (TxB$_2$) levels were drastically reduced in *Pla2g4a*$^{-/-}$ uteri (Fig. 4A,B), it is not likely to play any significant role in implantation, as mice deficient in thromboxane receptor do not show reproductive defects (Thomas et al., 1998).

Implantation beyond the normal 'window' of implantation in *Pla2g4a*$^{-/-}$ mice leads to defective postimplantation development

We demonstrate here that implantation occurs beyond the normal 'window' of uterine receptivity in *Pla2g4a*$^{-/-}$ mice (Fig. 2). This new finding of deferred implantation and small litter size led us to scrutinize postimplantation embryo development in *Pla2g4a*$^{-/-}$ mice. We examined the growth and development of the implantation sites on day 12 to assess the effects of deferred implantation on subsequent developmental processes. As shown in Fig. 5A, while most of the implantation sites in wild-type or *Pla2g4a*$^{+/-}$ mice were well spaced and developed normally, many implantation sites in *Pla2g4a*$^{-/-}$ mice were smaller and showed signs of resorption. The median weight of day 12 implantation sites or isolated embryos was reduced significantly in *Pla2g4a*$^{-/-}$ mice when compared with wild-type or *Pla2g4a*$^{+/-}$ mice (Fig. 5B). Although there was only a short delay in the timing of implantation, many of the isolated embryos from *Pla2g4a*$^{-/-}$ mice exhibited retarded growth at varying degrees (Fig. 5C). Furthermore, defective development of feto-placental unit with hemorrhagic placentas and preponderance of trophoblast giant cells was frequently noted, although decidual defect was not apparent (Fig. 5D). Similar dominance of trophoblast giant cells has previously been reported in mice with mid-gestational placental defects (Tremblay et al., 2001). Our results show that a transient delay in the attachment reaction produced heterogeneous feto-placental developmental phenotypes ranging from less severe to markedly retarded growth. Our observation of retarded postimplantation development and demise of embryos was reflected in high embryonic mortality (38% versus 2%) and the reduced number (4.8±0.8 versus 12.2±0.8) of pups delivered at birth by *Pla2g4a*$^{-/-}$ mice as compared to wild-type mice, confirming previous reports (Bonventre et al., 1997; Uozumi et al., 1997).

If deferred implantation is a cause for defective postimplantation embryonic development, administration of PGs prior to the attachment reaction in *Pla2g4a*$^{-/-}$ mice should improve later stages of embryo development. Thus, we examined day 12 embryos from *Pla2g4a*$^{-/-}$ mice injected with PGE$_2$ and cPGI at 10:00 and 18:00 hours on day 4 of pregnancy. We noted considerable improvement in embryonic development with concomitant decreases in the number of retarded embryos compared to vehicle-treated *Pla2g4a*$^{-/-}$ mice (Fig. 5E).

Embryo spacing is disturbed in *Pla2g4a*$^{-/-}$ mice

Limited information is available regarding cellular and molecular basis of embryo spacing in the uterus (McLaren and Michie, 1959). Previous reports using pharmacological inhibitors suggested that PGs are involved in embryo spacing in rats (Wellstead et al., 1989). This prompted us to examine more closely the spacing of embryos in *Pla2g4a*$^{-/-}$ mice. Although we observed increased implantation rates in *Pla2g4a*$^{-/-}$ mice on day 6, i.e. after a short delay, embryo spacing was aberrant (see sites within brackets in Fig. 2C). This abnormal spacing was more prominent when examined on day 12. Implantation sites were closely apposed or even fused together (see sites within brackets in Fig. 5A). Upon dissection, we often observed that two or more embryos were residing in the same decidual envelope or conjoined by a single placenta (Fig. 5F). This could be one reason for retarded embryonic development and resorption in *Pla2g4a*$^{-/-}$ mice, resulting from crowding of embryos. However, retarded growth of well-spaced embryos was also noted in *Pla2g4a*$^{-/-}$ mice.

Although PG treatment on day 4 of pregnancy restored normal implantation timing in *Pla2g4a*$^{-/-}$ mice (Fig. 4E), this treatment did not rescue altered embryo spacing (Fig. 4F), suggesting that other PGs or mediators, or AA itself are involved in normal embryo spacing prior to the attachment reaction. Alternatively, failure of exogenously delivered PGs to restore normal spacing could be due to inappropriate delivery of PGs at the right time at the target tissues responsible for embryo spacing. Our results provide genetic evidence for a role of cPLA$_2\alpha$ in this important event.

Deferred implantation in wild-type mice leads to late gestational defects

To reinforce that implantation timing affects postimplantation development, we used embryo transfers in wild-type mice. Wild-type day 4 blastocysts were transferred into day 4 or day 5 wild-type pseudopregnant mice. We observed that blastocysts transferred either on day 4 or day 5, when examined 48 hours later, showed similar implantation rates (Fig. 6A). However, severe developmental anomalies and resorption of implantation sites were noted later in pregnancy if blastocysts were transferred into day 5 recipients when compared with their transfer into day 4 recipients (Fig. 6B). Furthermore, the number of pups born at term was much lower for recipients

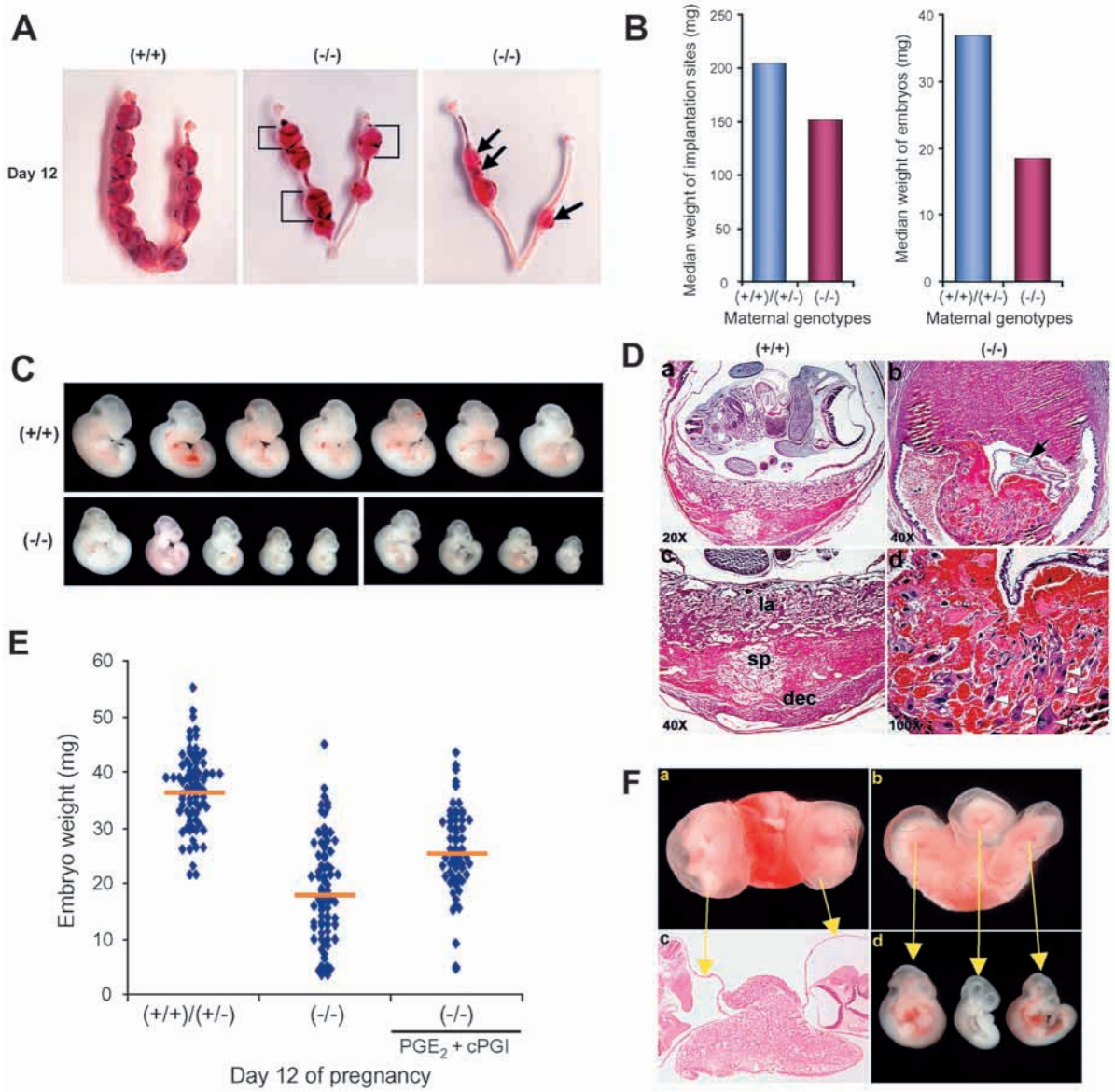

Fig. 5. Defective postimplantation developments in *Pla2g4a*$^{-/-}$ mice. (A) A composite photograph of uteri in wild-type and *Pla2g4a*$^{-/-}$ mice on day 12 of pregnancy. Resorption sites were often noted (arrows) and many implantation sites were closely apposed and even conjoined (brackets) to each other in *Pla2g4a*$^{-/-}$ mice. (B) Median weights of implantation sites and their embryos on day 12. The implantation sites without embryos (resorption sites) were excluded from this computation. (C) Photographs of embryos isolated from implantation sites of one representative wild-type and two *Pla2g4a*$^{-/-}$ mice on day 12. Note retarded and asynchronous development of embryos in *Pla2g4a*$^{-/-}$ mice. (D) Histological examination of day 12 implantation sites in *Pla2g4a*$^{-/-}$ mice. Feto-placental units from *Pla2g4a*$^{-/-}$ mice were examined on day 12. Embryos and placentas show defective development with a preponderance of trophoblast giant cells. Arrowheads and an arrow indicate trophoblast giant cells and degenerating embryo, respectively. (c,d) Higher magnifications of a,b, respectively. la, labyrinthine trophoblast; sp, spongiotrophoblast; dec, decidua. (E) Distribution of embryonic weights on day 12 (n=60-75). The horizontal orange lines represent median values of embryonic weights. *Pla2g4a*$^{-/-}$ mice were given the vehicle or PGs at 10:00 and 18:00 hours on day 4 of pregnancy and killed on day 12. Note a reduction in numbers of retarded embryos in the PG-treated group. (F) Representative photographs of conjoined embryos in a placenta (a,c) and three embryos in the same decidual envelope (b,d) from *Pla2g4a*$^{-/-}$ mice on day 12. (c) A histological section of (a) with two embryos; embryos shown in (d) are from (b). Yellow arrows indicate the source of the embryos from the decidual envelope.

receiving blastocyst transfers on day 5 (16/130, 12.5%) than those receiving transfers on day 4 (44/168, 26%) (Fig. 6C). By contrast, as in *Pla2g4a*$^{-/-}$ mice, there was no difference in the decidual response induced by intraluminal oil infusion either on day 4 or 5 of pseudopregnancy in wild-type mice (Fig. 6D). These results in wild-type mice, together with our findings in *Pla2g4a*$^{-/-}$ mice, clearly demonstrate that timing of implantation is a crucial determinant for normal feto-placental development and pregnancy outcome. It is surmised that an altered uterine environment resulting from the shifting of the normal 'window' of implantation cannot efficiently support normal pregnancy. This finding has a major clinical significance, as implantation in humans beyond the normal 'window' of uterine receptivity (8-10 days postovulation) is

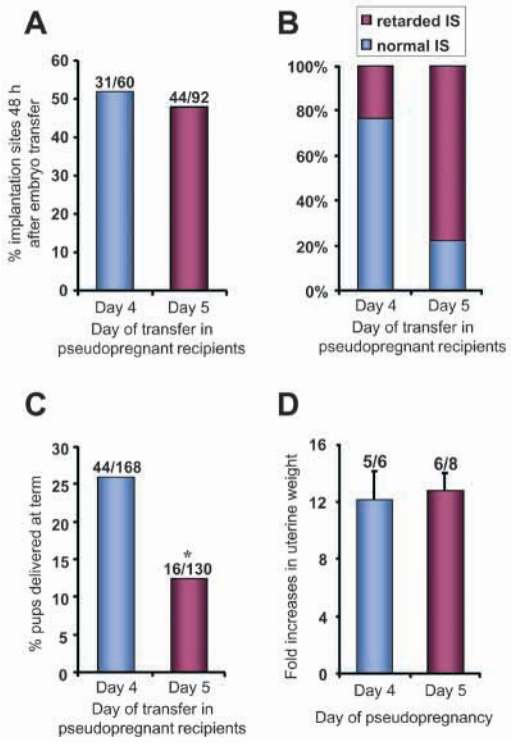

Fig. 6. Deferred implantation leading to retarded development and poor pregnancy outcome in wild-type mice. (A) Implantation of day 4 wild-type blastocysts transferred into wild-type recipients on day 4 (n=5) or 5 (n=7) of pseudopregnancy. The numbers above the bars indicate the number of implantation sites per total number of blastocysts transferred. Implantation sites were recorded 48 hours later by the blue dye method. Implantation rate was similar between the two groups. (B) Postimplantation developments of wild-type blastocysts transferred into wild-type recipients on day 4 (n=3) or 5 (n=4) of pseudopregnancy. Implantation sites were examined 8 days later after blastocyst transfer equivalent to day 12 of pregnancy. While resorption and retarded feto-placental growth were frequent in recipients that received blastocyst transfers on day 5, vastly normal development was noted in those receiving blastocyst transfer on day 4. Normal implantation sites (IS) represent sites with normally developing embryos, while retarded IS represents resorption sites and IS with retarded embryo development. On day 12, the number of normal IS was significantly higher in day 4 recipients than in day 5 recipients (18/23 versus 10/42; χ^2-test; P<0.001). (C) The pregnancy outcome of wild-type blastocysts transferred into wild-type recipients on day 4 (n=13) or 5 (n=11) of pseudopregnancy. The numbers above the bars indicate the number of pups delivered at term/total number of blastocysts transferred (χ^2-test; *P<0.01). The number of pups born was significantly lower for mothers receiving blastocyst transfers on day 5. (D) Decidualization in wild-type mice. Mice received intraluminal oil infusion on day 4 or 5 of pseudopregnancy. Uterine weights were recorded 4 days later. Fold increases denote comparison of weights between infused and non-infused uterine horns. The numbers above the bars indicate the number of responding mice/total number of mice. No significant difference in decidualization was noted between these two groups (unpaired t-test; P>0.05).

associated with higher risk of early pregnancy losses (Wilcox et al., 1999). There is evidence that the number of fetuses and their developmental stages determine the parturition process (Yoshinaga, 1983). Thus, retarded embryonic development and resorption observed in *Pla2g4a*$^{-/-}$ mice are most likely to be the causes of small litter size and parturition defects previously observed (Bonventre et al., 1997; Uozumi et al., 1997).

DISCUSSION

cPLA$_2\alpha$ is implicated in diverse biological functions (Clark et al., 1995; Bonventre et al., 1997; Uozumi et al., 1997; Fujishima et al., 1999; Gijon and Leslie, 1999). While cPLA$_2$-derived AA is a substrate for COX and lipoxygenase pathways for eicosanoid production, intracellular AA also has its own biological effects (Clark et al., 1995; Gijon and Leslie, 1999). Recent studies showed depressed PG synthesis, small litters and defective parturition in *Pla2g4a*$^{-/-}$ mice (Bonventre et al., 1997; Uozumi et al., 1997; Fujishima et al., 1999). However, the cause and underlying mechanism of these reproductive defects were not defined.

We here demonstrate that it is the initiation of implantation that is affected in *Pla2g4a*$^{-/-}$ mice, leading to the derangement of subsequent developmental processes. This is clearly evident from our results that while normal implantation is noted after 1-day delay i.e., on day 6 in *Pla2g4a*$^{-/-}$ mice, a gradual embryonic demise occurs during the remaining period of pregnancy. The postimplantation developmental defect is not due to a defect in the decidualization process, as decidualization occurs when embryos implant 1 day later or in response to an artificial stimulus in *Pla2g4a*$^{-/-}$ or wild-type mice (Fig. 2D, Fig. 6D). This raises a very intriguing proposition that initial attachment reaction is crucial to the fate of subsequent developmental processes. Our present work emphasizes the necessity for careful examination of mid or late gestational developmental anomalies resulting from specific gene mutation in mice.

The initiation of implantation and subsequent progression of pregnancy are the results of coordinated integration of various signaling pathways between the embryo and the uterus. The attachment reaction is followed by uterine decidualization, angiogenesis, embryonic growth and placentation. A defect in any of these events affects pregnancy outcome. The role of decidua and placentas in supporting pregnancy is well documented (Benson et al., 1996; Luo et al., 1997; Robb et al., 1998; Barak et al., 1999; Tremblay et al., 2001). For example, defective decidualization in mice deficient in Hoxa10 or IL11-Rα leads to pregnancy failures (Benson et al., 1996; Robb et al., 1998). Likewise, defective placentation attributed by embryos deficient in PPARγ or ERRβ also leads to midgestational embryonic lethality (Luo et al., 1997; Barak et al., 1999). Our results provide for the first time a novel concept that a short delay in the initial attachment reaction propagates detrimental effects during the later course of the pregnancy. This observation leads to the conclusion that the state of activity of the blastocyst and uterine environment conducive to support the initial stages of implantation must be precisely synchronized for normal pregnancy outcome.

The deferred implantation observed in *Pla2g4a*$^{-/-}$ mice is different from traditional lactational or experimentally induced delayed implantation that occurs in wild-type mice for an extended period in the absence of ovarian estrogen (Dey, 1996). In the latter, blastocysts undergo dormancy and uteri become non-responsive to implantation. The removal of the suckling stimulus or supplementation of estrogen terminates

blastocyst dormancy and resumption of implantation with normal pregnancy outcome.

A two-way interaction between the blastocyst and the uterus is essential for successful implantation and decidualization. Although growth factors, cytokines, transcription factors, and PGs are implicated in successful implantation and decidualization (Stewart et al., 1992; Das et al., 1994; Benson et al., 1996; Chakraborty et al., 1996; Lim et al., 1999a; Lim et al., 1999b; Song et al., 2000), the molecular interactions between these local mediators are not clearly understood. Our observation of aberrant expression of the genes encoding HB-EGF and LIF at the sites of blastocysts at the anticipated time of implantation without blue bands in *Pla2g4a*$^{-/-}$ mice suggests that cPLA$_2\alpha$ derived AA and/or eicosanoids coordinate these signaling pathways for implantation. It is possible that the absence of HB-EGF in the luminal epithelium surrounding the blastocyst before the attachment reaction in *Pla2g4a*$^{-/-}$ mice makes the blastocysts implantation incompetent. HB-EGF and LIF are effective in promoting blastocyst growth, zona-hatching and/or trophoblast outgrowth in vitro (Das et al., 1994; Dunglison et al., 1996). Deferred implantation could be due to either incompetence of blastocysts and/or uterine insufficiency for implantation. However, reduced implantation of normal wild-type blastocysts transferred to *Pla2g4a*$^{-/-}$ uteri suggests that uterine deficiency is the major cause of this temporary delay.

In rodents, embryos are evenly spaced along the uterus. However, little is known regarding the underlying mechanism regulating this process. Our observation of abnormal uterine spacing of embryos in *Pla2g4a*$^{-/-}$ mice provides genetic evidence for a role of PGs in this process and is consistent with reports of abnormal embryo spacing in rodents exposed to pharmacological inhibitors of PG synthesis (Wellstead et al., 1989). It is possible that embryo spacing is regulated by local factors associated with PG signaling. In other systems, bone morphogenetic proteins (BMPs) are implicated in the genesis of evenly spaced tissue structures during development (reviewed in Hogan, 1996). We have recently shown that local delivery of BMP-2 in the mouse uterus alters embryo spacing, suggesting that BMPs are involved in this process (Paria et al., 2001). As HB-EGF is an inducer of BMP-2 at the implantation site (Paria et al., 2001), as the EGF-like growth factors stimulate PG synthesis (DuBois et al., 1994) and as there is a relationship between BMP and PG signaling in other systems (Koide et al., 1999), it is conceivable that these regulatory pathways are operative during embryo spacing.

Genetic mutation of various components of the PG synthetic and signaling pathways in mice in recent years has provided important information on distinct and overlapping functions of various PGs in female reproduction with the conclusion that these lipid mediators are essential to successful pregnancy (Langenbach et al., 1995; Challis, 1997; Lim et al., 1997; Lim et al., 1999a). However, the reproductive deficiency in *Pla2g4a*$^{-/-}$ female mice reported here is different from that observed in *Ptgs1*$^{-/-}$ or *Ptgs2*$^{-/-}$ female mice. For example, while *Ptgs1*$^{-/-}$ mice are fertile with limited parturition defect (Langenbach et al., 1995), *Ptgs2*$^{-/-}$ female mice show profound defects in ovulation, fertilization, implantation and decidualization (Lim et al., 1997). By contrast, while *Pla2g4a*$^{-/-}$ mice show only modest effects on ovulation and fertilization, they clearly exhibit temporary postponement of the blastocyst attachment reaction leading to striking defects in subsequent postimplantation development. Distinct phenotypes between *Ptgs2*$^{-/-}$ and *Pla2g4a*$^{-/-}$ mice could be due to functional redundancy among the members of the PLA$_2$ superfamily. It is intriguing to see that although cPLA$_2\alpha$ is crucial to on-time implantation, implantation still can occur beyond the normal 'window' in both the wild-type and mutant females. Understanding the molecular basis of embryo-uterine interactions during the deferred 'window' of implantation will provide further insights regarding normal and abnormal implantation.

In conclusion, using *Pla2g4a*$^{-/-}$ mice and differential embryo transfers in wild-type mice, we show that a short deferral of implantation leads to late developmental defects. Our results provide a new concept that an early embryo-uterine interaction during implantation sets up the subsequent developmental programming.

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REFERENCES

Barak, Y., Nelson, M. C., Ong, E. S., Jones, Y. Z., Ruiz-Lozano, P., Chien, K. R., Koder, A. and Evans, R. M. (1999). PPARγ is required for placental, cardiac, and adipose tissue development. *Mol. Cell* **4**, 585-595.

Benson, G. V., Lim, H., Paria, B. C., Satokata, I., Dey, S. K. and Maas, R. L. (1996). Mechanisms of reduced fertility in Hoxa-10 mutant mice: uterine homeosis and loss of maternal Hoxa-10 expression. *Development* **122**, 2687-2696.

Bonventre, J. V., Huang, Z., Taheri, M. R., O'Leary, E., Li, E., Moskowitz, M. A. and Sapirstein, A. (1997). Reduced fertility and postischaemic brain injury in mice deficient in cytosolic phospholipase A$_2$. *Nature* **390**, 622-625.

Chakraborty, I., Das, S. K., Wang, J. and Dey, S. K. (1996). Developmental expression of the cyclo-oxygenase-1 and cyclo-oxygenase-2 genes in the peri-implantation mouse uterus and their differential regulation by the blastocyst and ovarian steroids. *J. Mol. Endocrinol.* **16**, 107-122.

Challis, J. R. (1997). Prostaglandins and reproduction–what do knockouts really tell us? *Nat. Med.* **3**, 1326-1327.

Clark, J. D., Schievella, A. R., Nalefski, E. A. and Lin, L. L. (1995). Cytosolic phospholipase A$_2$. *J. Lipid Mediat. Cell Signal.* **12**, 83-117.

Das, S. K., Wang, X. N., Paria, B. C., Damm, D., Abraham, J. A., Klagsbrun, M., Andrews, G. K. and Dey, S. K. (1994). Heparin-binding EGF-like growth factor gene is induced in the mouse uterus temporally by the blastocyst solely at the site of its apposition: a possible ligand for interaction with blastocyst EGF- receptor in implantation. *Development* **120**, 1071-1083.

Dey, S. K. (1996). Implantation. In *Reproductive Endocrinology, Surgery, and Technology* (ed. E.Y. Adashi, J.A. Rock and Z. Rosenwaks), pp. 421-434. Philadelphia: Lippincott-Raven.

DuBois, R. N., Awad, J., Morrow, J., Roberts, L. J. and Bishop, P. R. (1994). Regulation of eicosanoid production and mitogenesis in rat intestinal epithelial cells by transforming growth factor-α and phorbol ester. *J. Clin. Invest.* **93**, 493-498.

Dunglison, G. F., Barlow, D. H. and Sargent, I. L. (1996). Leukaemia inhibitory factor significantly enhances the blastocyst formation rates of human embryos cultured in serum-free medium. *Hum. Reprod.* **11**, 191-196.

Fujishima, H., Sanchez Mejia, R. O., Bingham, C. O., III, Lam, B. K., Sapirstein, A., Bonventre, J. V., Austen, K. F. and Arm, J. P. (1999). Cytosolic phospholipase A_2 is essential for both the immediate and the delayed phases of eicosanoid generation in mouse bone marrow-derived mast cells. *Proc. Natl. Acad. Sci. USA* **96**, 4803-4807.

Gijon, M. A. and Leslie, C. C. (1999). Regulation of arachidonic acid release and cytosolic phospholipase A_2 activation. *J. Leukoc. Biol.* **65**, 330-336.

Hogan, B., Beddington, R., Costantini, F. and Lacy, E. (1994). In *Manipulating the Mouse Embryo, A Laboratory Manual*, pp. 151-162. New York: Cold Spring Harbor.

Hogan, B. L. (1996). Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev.* **10**, 1580-1594.

Kennedy, B. P., Payette, P., Mudgett, J., Vadas, P., Pruzanski, W., Kwan, M., Tang, C., Rancourt, D. E. and Cromlish, W. A. (1995). A natural disruption of the secretory group II phospholipase A_2 gene in inbred mouse strains. *J. Biol. Chem.* **270**, 22378-22385.

Koide, M., Murase, Y., Yamato, K., Noguchi, T., Okahashi, N. and Nishihara, T. (1999). Bone morphogenetic protein-2 enhances osteoclast formation mediated by interleukin-1α through upregulation of osteoclast differentiation factor and cyclooxygenase-2. *Biochem. Biophys. Res. Commun.* **259**, 97-102.

Langenbach, R., Morham, S. G., Tiano, H. F., Loftin, C. D., Ghanayem, B. I., Chulada, P. C., Mahler, J. F., Lee, C. A., Goulding, E. H., Kluckman, K. D. et al. (1995). Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration. *Cell* **83**, 483-492.

Lim, H., Paria, B. C., Das, S. K., Dinchuk, J. E., Langenbach, R., Trzaskos, J. M. and Dey, S. K. (1997). Multiple female reproductive failures in cyclooxygenase 2-deficient mice. *Cell* **91**, 197-208.

Lim, H., Gupta, R. A., Ma, W. G., Paria, B. C., Moller, D. E., Morrow, J. D., DuBois, R. N., Trzaskos, J. M. and Dey, S. K. (1999a). Cyclo-oxygenase-2-derived prostacyclin mediates embryo implantation in the mouse via PPARδ. *Genes Dev.* **13**, 1561-1574.

Lim, H., Ma, L., Ma, W. G., Maas, R. L. and Dey, S. K. (1999b). Hoxa-10 regulates uterine stromal cell responsiveness to progesterone during implantation and decidualization in the mouse. *Mol. Endocrinol.* **13**, 1005-1017.

Luo, J., Sladek, R., Bader, J. A., Matthyssen, A., Rossant, J. and Giguere, V. (1997). Placental abnormalities in mouse embryos lacking the orphan nuclear receptor ERR-β. *Nature* **388**, 778-782.

MacPhee, M., Chepenik, K. P., Liddell, R. A., Nelson, K. K., Siracusa, L. D. and Buchberg, A. M. (1995). The secretory phospholipase A_2 gene is a candidate for the Mom1 locus, a major modifier of Apc^{Min}-induced intestinal neoplasia. *Cell* **81**, 957-966.

McLaren, A. and Michie, D. (1959). The spacing of implantations in the mouse uterus. *Mem. Soc. Endocrinol.* **6**, 65-74.

Matsumoto, H., Ma, W.-G., Smalley, W., Trzaskos, J., Breyer, R. M. and Dey, S. K. (2001). Diversification of cyclooxygenase-2-derived prostaglandins in ovulation and implantation. *Biol. Reprod.* **64**, 1557-1565.

Mechoulam, R., Fride, E. and di Marzo, V. (1998). Endocannabinoids. *Eur. J. Pharmacol.* **359**, 1-18.

Murakami, M., Nakatani, Y., Kuwata, H. and Kudo, I. (2000). Cellular components that functionally interact with signaling phospholipase A_2s. *Biochim. Biophys. Acta* **1488**, 159-166.

Paria, B. C., Das, S. K., Andrews, G. K. and Dey, S. K. (1993a). Expression of the epidermal growth factor receptor gene is regulated in mouse blastocysts during delayed implantation. *Proc. Natl. Acad. Sci. USA* **90**, 55-59.

Paria, B. C., Huet-Hudson, Y. M. and Dey, S. K. (1993b). Blastocyst's state of activity determines the 'window' of implantation in the receptive mouse uterus. *Proc. Natl. Acad. Sci. USA* **90**, 10159-10162.

Paria, B. C., Ma, W., Tan, J., Raja, S., Das, S. K., Dey, S. K. and Hogan, B. L. (2001). Cellular and molecular responses of the uterus to embryo implantation can be elicited by locally applied growth factors. *Proc. Natl. Acad. Sci. USA* **98**, 1047-1052.

Reddy, S. T. and Herschman, H. R. (1997). Prostaglandin synthase-1 and prostaglandin synthase-2 are coupled to distinct phospholipases for the generation of prostaglandin D_2 in activated mast cells. *J. Biol. Chem.* **272**, 3231-3237.

Robb, L., Li, R., Hartley, L., Nandurkar, H. H., Koentgen, F. and Begley, C. G. (1998). Infertility in female mice lacking the receptor for interleukin-11 is due to a defective uterine response to implantation. *Nat. Med.* **4**, 303-308.

Serhan, C. N. and Oliw, E. (2001). Unorthodox routes to prostanoid formation: new twists in cyclooxygenase-initiated pathways. *J. Clin. Invest.* **107**, 1481-1489.

Six, D. A. and Dennis, E. A. (2000). The expanding superfamily of phospholipase A_2 enzymes: classification and characterization. *Biochim. Biophys. Acta* **1488**, 1-19.

Smith, W. L. and DeWitt, D. L. (1996). Prostaglandin endoperoxide H synthases-1 and -2. *Adv. Immunol.* **62**, 167-215.

Song, C., Chang, X. J., Bean, K. M., Proia, M. S., Knopf, J. L. and Kriz, R. W. (1999). Molecular characterization of cytosolic phospholipase A_2-β. *J. Biol. Chem.* **274**, 17063-17067.

Song, H., Lim, H., Das, S. K., Paria, B. C. and Dey, S. K. (2000). Dysregulation of EGF family of growth factors and COX-2 in the uterus during the preattachment and attachment reactions of the blastocyst with the luminal epithelium correlates with implantation failure in LIF-deficient mice. *Mol. Endocrinol.* **14**, 1147-1161.

Stewart, C. L., Kaspar, P., Brunet, L. J., Bhatt, H., Gadi, I., Kontgen, F. and Abbondanzo, S. J. (1992). Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature* **359**, 76-79.

Takano, T., Panesar, M., Papillon, J. and Cybulsky, A. V. (2000). Cyclooxygenases-1 and 2 couple to cytosolic but not group IIA phospholipase A_2 in COS-1 cells. *Prostaglandins Lipid Mediat.* **60**, 15-26.

Thomas, D. W., Mannon, R. B., Mannon, P. J., Latour, A., Oliver, J. A., Hoffman, M., Smithies, O., Koller, B. H. and Coffman, T. M. (1998). Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxanes A_2. *J. Clin. Invest.* **102**, 1994-2001.

Threadgill, D. W., Dlugosz, A. A., Hansen, L. A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., Harris, R. C. et al. (1995). Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science* **269**, 230-234.

Tremblay, G. B., Kunath, T., Bergeron, D., Lapointe, L., Champigny, C., Bader, J. A., Rossant, J. and Giguere, V. (2001). Diethylstilbestrol regulates trophoblast stem cell differentiation as a ligand of orphan nuclear receptor ERRβ. *Genes Dev.* **15**, 833-838.

Underwood, K. W., Song, C., Kriz, R. W., Chang, X. J., Knopf, J. L. and Lin, L. L. (1998). A novel calcium-independent phospholipase A_2, cPLA$_2$-γ, that is prenylated and contains homology to cPLA$_2$. *J. Biol. Chem.* **273**, 21926-21932.

Uozumi, N., Kume, K., Nagase, T., Nakatani, N., Ishii, S., Tashiro, F., Komagata, Y., Maki, K., Ikuta, K., Ouchi, Y. et al. (1997). Role of cytosolic phospholipase A_2 in allergic response and parturition. *Nature* **390**, 618-622.

Valentin, E. and Lambeau, G. (2000). Increasing molecular diversity of secreted phospholipases A_2 and their receptors and binding proteins. *Biochim. Biophys. Acta* **1488**, 59-70.

Wellstead, J. R., Bruce, N. W. and Rahima, A. (1989). Effects of indomethacin on spacing of conceptuses within the uterine horn and on fetal and placental growth in the rat. *Anat. Rec.* **225**, 101-105.

Wilcox, A. J., Baird, D. D. and Weinberg, C. R. (1999). Time of implantation of the conceptus and loss of pregnancy. *New Engl. J. Med.* **340**, 1796-1799.

Yoshinaga, K. (1983). Superimplantation rats as a tool for study of onset of parturition. In *The Anterior Pituitary Gland* (ed. A. J. Bhatnagar), pp 367-375. New York: Raven.