Transient $\beta_2$-Adrenoceptor Activation Confers Pregnancy Loss by Disrupting Embryo Spacing at Implantation*§

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Pregnancy loss is a serious social and medical issue, with one important cause associated with aberrant embryo implantation during early pregnancy. However, whether and how the process of embryo implantation is affected by environmental factors such as stress-induced sympathetic activation remained elusive. Here we report an unexpected, transient effect of $\beta_2$-adrenoreceptor ($\beta_2$-AR) activation (day 4 postcoitus) in disrupting embryo spacing at implantation, leading to substantially increased midterm pregnancy loss. The abnormal embryo spacing could be prevented by pretreatment of $\beta_2$-AR antagonist or genetic ablation of $\beta_2$-AR. Similar $\beta_2$-AR activation at day 5 postcoitus, when implantation sites have been established, did not affect embryo spacing or pregnancy outcome, indicating that the adverse effect of $\beta_2$-AR activation is limited to the preimplantation period before embryo attachment. In vitro and in vivo studies demonstrated that the transient $\beta_2$-AR activation abolished normal preimplantation uterine contractility without adversely affecting blastocyst quality. The contractility inhibition is mediated by activation of the cAMP-PKA pathway and accompanied by specific down-regulation of $lpa3$, a gene previously found to be critical for uterine contraction and embryo spacing. These results indicated that normal uterine contractile-mediated correct intrauterine embryo distribution is crucial for successful ongoing pregnancy. Abnormal $\beta_2$-AR activation at early pregnancy provided a molecular clue in explaining how maternal stress at early stages could adversely affect the pregnancy outcome.

EXPERIMENTAL PROCEDURES

Animals—Adult CD1 mice (7–8 weeks old) were purchased from Vital River Laboratories Co. Ltd., and the $\beta_2$-AR knock-out (Adrb1/Adrb2 double knock-out) mice were imported from The Jackson Laboratory (stock number: 003810). Adult female mice in estrus were mated with fertile males of CD1 at room temperature (25°C). The morning of finding a vaginal plug was designated as day 1 postcoitus (pc). The implantation sites on day 5 were identified by intravenous injection of 0.1 ml of 1% Chicago blue dye (Sigma) in saline. Implantation sites closely apposed or fused together were designated as “crowded sites.” To examine the midterm pregnancy status, implantation sites and embryos were isolated at day 12 of pregnancy and weighed individually after being fixed in Bouin’s solution overnight.

Drug Treatments—Different adrenergic drugs were administered twice (intraperitoneal injection) at 8:30 a.m. and 2:30 p.m., respectively on day 4 of pregnancy. These drugs include: prazosin hydrochloride (Sigma) (100 μg/mouse), $p$-lodoe-lonidine hydrochloride (Sigma) (50 μg/mouse), yohimbine

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The abbreviations used are: $\beta_2$-AR, $\beta_2$-adrenoreceptor; HB-EGF, heparin-binding EGF-like growth factor; EGFR, epidermal growth factor receptor; LPA, lysophosphatidic acid receptor; PG, prostaglandin; pc, postcoitus.
**β₂-AR Activation Disrupts Embryo Spacing and Pregnancy**

hydrochloride (Sigma) (200 μg/mouse), (S)-(−)-atenolol (Sigma) (100 μg/mouse), isoetharine mesylate salt (Sigma) (200 μg/mouse), terbutaline hemisulfate salt (Sigma) (2 mg/mouse), propranolol (Sigma) (1 mg/mouse), butoxamine (Sigma), and Salbutamol (Alfa Aesar) (2 mg/mouse). Drugs were prepared in saline. To reverse the effects induced by β₂-AR agonist (Salbutamol), mice were pretreated with β₂-AR antagonist (propranolol or butoxamine (1 mg/mouse)) 30 min before Salbutamol. The vehicle group mice received saline only. The dosage of each drug was referenced or modified as used previously (13–16). The optimal dosage and timing of β₂-AR agonist (Salbutamol) administration was defined by dose- and time-dependent assay (see supplemental Fig. S1, A and B).

Artificial Induction of Separated Deciduoma—CD1 virgin female mice were mated with vasectomized males to induce pseudopregnancy. On day 4 postcoitus, artificial decidualization with separated deciduoma was induced with a modified protocol by infusion of 1 μl of sesame oil (the previous standard protocol used 10 μl of oil (17)) into one uterine lumen, whereas the contralateral horn without any treatment served as control. The oil-induced deciduoma were detected on day 6 by the blue dye method as described above.

RNA Extraction and PCR—Total RNA was extracted from fresh tissues using the TRizol reagent (Invitrogen) according to the manufacturer’s protocol, and genomic DNA was removed using the RNase-free DNase (Promega, Madison, WI). RT-PCR and quantitative real-time PCR were conducted as described previously (18). Positive control consists of a mixture of cDNAs from brain, liver, and kidney, which covers the expression of all adrenergic receptor family members, and water served as the negative control. The primers used for amplifying mouse adrenergic receptors and implantation-related genes were listed in supplemental Table S1 and supplemental Table S2.

Immunostaining—Detections of β₂-AR on day 4 uteri were performed in a frozen section with standard protocols. Immunofluorescence detections of β₂-AR (Abcam), HB-EGF (R&D), EGF receptor (Santa Cruz Biotechnology), and ErbB-4 (Santa Cruz Biotechnology) on blastocysts recovered from day 4 uteri were incubated in an organ bath 10 min after adding 1-2 AR antagonist (propranolol), and forskolin were then added to the bath. For each sample, uterine contractility was recorded for at least 1 h.

**RESULTS**

Transient β₂-AR Activation at Preimplantation Causes Aberrant Intrauterine Embryo Distribution without Disturbing On-time Implantation—To examine the potential roles of adrenergic receptor signaling in embryo implantation, we first screened the expression profile of different adrenergic receptors in preimplantation uteri on day 4 pc. Among adrenergic receptor family, the β₂-AR showed dominant expression, with intense myometrial localization and relatively weak expression at luminal and glandular epithelium (Fig. 1A and B), suggesting a potential role in regulating preimplantation uterine function. We further performed systemic administration of various adrenergic drugs on preimplantation mice at day 4 pc, and the implantation status was examined on day 5 pc. We found that although none of the drugs caused significant changes on the numbers of implantation, the β₂-AR agonist, Salbutamol (similar results also obtained with terbutaline), severely disrupted intrauterine embryo distribution in 80% (24 of 30) of treated mice, with a substantial number (32.1%) of implantation sites severely crowded together (Fig. 1, C, D, and G).

Despite the abnormal embryo spacing, both vehicle-treated and β₂-AR agonist-treated groups did not adversely affect the timing of embryo implantation. As shown in Fig. 1, E and F, both groups showed distinguishable implantation sites at the predicted time (day 4 pc at 12:00 a.m.) of initial embryo attachment (Fig. 1E), and the implantation numbers of days 5
and 6 pc were also comparable (Fig. 1F). In the uterus with disrupted embryo spacing, the blue bands were sometimes so close together that it is difficult to distinguish how many implantation sites were present (Fig. 1G). Indeed, at the later stage of post-implantation, histological examination often revealed that two or more embryos shared one decidual or one placenta (Fig. 1G and Fig. 2A). The abnormal embryo spacing caused by Salbutamol could be substantially reversed by pretreatment of β2-AR antagonist (butoxamine or propranolol) (Fig. 1, G and H). Moreover, the adverse effect of Salbutamol is totally abolished in β-AR knock-out mice (Fig. 1, G and H), indicating that the observed disturbance on embryo spacing is a specific effect of β2-AR activation.

Aberrant Intrauterine Embryo Distribution Leads to Compromised Embryo Development and Pregnancy Loss—To examine the ongoing effects of abnormal embryo spacing at implantation, we next examined the pregnancy status at midterm gestation (day 12 pc) after transient β2-AR agonist treatment on day 4 pc. Although most vehicle-treated mice showed well developed implantation sites, mice after β2-AR activation showed increased embryo resorption rate, and the average implantation site weight and surviving embryo weight also significantly decreased (Fig. 2, A–D). The resorp-
**β₂-AR Activation Disrupts Embryo Spacing and Pregnancy**

**FIGURE 2. Transient β₂-AR activation before but not after embryo attachment caused compromised pregnancy.** (A) Representative pictures of day 12 mouse uteri after treatment of β₂-AR drugs on days 4 and 5. Red arrow shows resorption sites. Histological examination of crowded implantation sites reveals different degrees of retarded embryo growth. Illustration of a triplet shows three embryos with different developing stages in one placenta. agon, agonist; anta, antagonist. (B) Embryo resorption rate at day 12 (D12) of pregnancy (NS, p > 0.05, **, p = 0.0005). C, average implantation site weight at day 12 of pregnancy (NS, p > 0.05, *, p = 0.00287, **, p = 0.00003). D, average weight of surviving embryos (NS, p > 0.05, *, p = 0.0013). E, average litter size at birth (NS, p > 0.05, **, p = 0.0004, ***, p < 0.00001). *t* test, error bars represent S.E. Numbers within bars indicate the number of mice examined.

To rule out the possibility that the impaired pregnancy by β₂-AR agonist originated from abnormal post-implantation events rather than disrupted embryo distribution, we compared the effects of β₂-AR agonist treatment before (day 4 pc) and after initiation of embryo implantation (day 5 pc). As shown in Fig. 2, A–E, the same drug treatment on day 5 pc no longer adversely affected pregnancy outcome, indicating that the observed pregnancy loss specifically originated from pre-implantation events right before the initiation of embryo attachment.

**Transient β₂-AR Activation Does Not Adversely Affect Pre-implantation Embryo Quality**—To examine whether the transient β₂-AR activation at day 4 pc adversely influenced blastocyst quality, we checked blastocysts recovered from mice with or without β₂-AR agonist treatment. As shown in Fig. 3, the expression of β₂-AR itself also showed no apparent changes by immunofluorescence detection (Fig. 3A). We also found no significant changes in molecular markers (HB-EGF, EGFR, and ErbB4 (receptor tyrosine-protein kinase erbB-4)), which are important for embryo implantation (Fig. 3, B–D) (20–23). The embryo numbers and morphologies (Fig. 3, E and F) are comparable in both groups as well. More importantly, by carrying out embryo transfer experiments, we demonstrated that the transient β₂-AR activation did not adversely affect the quality and developmental potential of the embryos (Fig. 3G).

These experiments ruled out the possibility that the observed pregnancy loss originated from impaired embryo quality, suggesting an alternative cause by abnormal uterine function that fails to guide normal intrauterine embryo distribution.

**β₂-AR Activation Suppresses Preimplantation Uterine Contractility through Canonical cAMP-PKA Activation**—To further address that the observed aberrant intrauterine embryo distribution caused by β₂-AR activation is maternally originated independent of the living embryo, we next figured out a simple *in vivo* method to examine the uterine function concerning distribution of intrauterine contents. By modifying a well used technique to induce early implantation responses in pseudopregnancy uterus (24), we injected a small amount of oil (1 µl) into uterine lumen at day 4 pseudopregnancy to induce separated deciduoma (checked at day 6 pc) (Fig. 4A). In contrast, after β₂-AR agonist administration, the same oil injection no longer induces separated but diffused deciduoma (Fig. 4A), indicating that the injected oil failed to be separated into small droplets along the uterine horn. This experiment suggested that the normal day 4 uterus has the ability to separate intrauterine contents in a similar fashion as intrauterine embryos (25), which could be disrupted by abnormal β₂-AR activation. These results indicated that the observed embryo spacing defects were caused by maternally originated uterine dysfunction, possibly due to aberrant uterine peristaltic movement guided by myometrial contraction (26, 27).

To directly demonstrate that β₂-AR activation influences uterine contractility, we carried out a well established *in vitro* uterine contractility assay. Although the normal day 4 uterus showed a regular contraction pattern, a very low dose (1 ng/ml) of exogenous β₂-AR agonist significantly decreased the...
amplitude of uterine contractility, whereas a relatively higher dose (5 ng/ml) could completely abolish the uterine contraction (Fig. 4, C and D). Because cAMP-PKA elevation is the classical signaling induced by β2-AR activation, we next measured the cAMP levels and PKA activities from uterine extracts and showed that Salbutamol could induce uterine cAMP and PKA elevation to an extent similar to that by forskolin, a well used activator for cAMP-PKA signaling (Fig. 4, E and F). Indeed, forskolin could similarly inhibit uterine contraction (Fig. 4G). These results indicated that the inhibitory effect of myometrium contractility after β2-AR activation is mediated through cAMP-PKA elevation. As expected, pretreatment of β2-AR antagonist (propranolol) made the uterus resistant to the inhibitory effect of β2-AR agonist, similar to that of the β-AR knock-out uterus, while still sensitive to forskolin-induced contractility inhibition (Fig. 4, H and I).

β2-AR Activation Is Associated with Specific Uterine Down-regulation of Ipa3—To further explore the potential molecular mechanisms of β2-AR activation on preimplantation uterus, we examined gene expression profiles relating to uterine receptivity and embryo distribution. As shown in Fig. 5A, most genes critical for uterine receptivity (lif (leukemia inhibitory factor), hb-egf, ihh (Indian hedgehog homolog), and so forth) (4, 24, 28, 29) were unchanged after transient β2-AR activation, which was consistent with the unchanged timing
and number of embryo implantations (Fig. 1, C, E, and F). Because prostaglandin (PG) signal has been highlighted as a key pathway for normal embryo spacing (6, 7, 30), we further carefully examined PG signal-related enzymes (COX-1, COX-2, and cytosolic phospholipases A2α (cPLA2)) and membrane prostaglandin E receptors 1–4 (EP1–4) between β2-AR agonist- and vehicle-treated groups. However, no significant changes were found at the transcription level (Fig. 5A) of these genes as well as in uterine PGE2 contents (vehicle versus Salbutamol, 1606.60 ± 82.93 and 1604.66 ± 325.58 pg/g of tissue; p > 0.05, n = 3 for each group, mean ± S.E.), suggesting that the β2-AR activation-mediated embryo crowding is PG-independent or at the downstream of PG signaling in regulating uterine contractility. Surprisingly, after transient β2-AR activation, we detected a significant decrease in Lpa3 expression (Fig. 5A), whereas in the β2-AR knock-out mice, Lpa3 did not show significant down-regulation after β2-AR agonist treatment (Fig. 5B), demonstrating that the observed down-regulation of Lpa3 in wild-type uteri resulted from β2-AR activation.

Lpa3 is a gene encoding lysophosphatidic acid receptor 3, which has been previously shown to play critical roles in normal on-time and on-site implantation through COX-2-mediated PG productions (6). However, like the previously reported cPLA2-null mice, exogenous administration of PGs in Lpa3-null mice could only rescue aberrant implantation timing but not embryo spacing (6, 7, 31). Recent evidence has demonstrated that LPA3 deletion-caused defects in implantation timing and embryo spacing were two independent events, and the Lpa3-null uterus indeed showed defects in stimulated muscular contraction (32), possibly linking with alternative pathway(s) (adrenergic signaling) that directly related to myometrium function. Indeed, there have been reports showing the cross-talk between LPA signaling and β2-AR function in other systems (33), suggesting potential interactions between these two G-protein-coupled receptor family-mediated pathways. Because LPA3 expression was reported to be primarily localized on uterine epithelium of day 4 pregnant mouse (6), it is still unclear how this molecule is linked with normal embryo spacing through muscular regulation (31), which might be due to potential interactions with β2-AR at the uterine epithelium. Nonetheless, the observed down-regulation of Lpa3 after β2-AR activation provided functional clues for further insights into the regulation of uterine muscular function in the context of early pregnancy events.

DISCUSSION

Together, the present study revealed an unexpected pathophysiological role of β2-AR activation in disrupting normal embryo spacing and its profound link to ongoing pregnancy. Previous investigations on β2-AR have been focused on the periparturition period because the administration of β2-AR agonist results in uterine relaxation, which could be utilized as an anti-parturition drug (34). In addition, it has been reported that β2-AR is the predominant AR subtype in murine oviducts and that disturbed β2-AR signaling resulted in aberrant oviductal embryo transport (35). Here we have expanded the understanding of the pathophysiological roles of β2-AR for normal intrauterine embryo distribution at preimplantation, which is regulated by classic cAMP-PKA activation and cross-talk with LPA3-mediated signaling.

In murine and other polyovular species such as rabbit and pig, the intrauterine embryo distribution is characterized by an evenly spaced manner along the uterine horn (36), which seems to share evolutionarily conserved mechanisms. The phenomenon of regulated embryo spacing has long been the interest of reproductive scientists since its discovery (36). However, the past decades have only provided limited information regarding its underlying mechanisms, and the physiological significance of this phenomenon also remained undereexplored. Recently, renewed interest of this topic has been raised from the discovery of two knockout mice that showed disrupted embryo spacing at implantation. In the cPLA2- and Lpa3-null female, the timing of embryo implantation was slightly delayed beyond the normal implantation window, concomitantly with disrupted embryo spacing and reduced litter sizes (6, 7). The primary cause of compromised pregnancy in these knockout mice has been attributed to aberrant timing of implantation due to the reduced level of prostaglandins in the preimplantation uteri, whereas the relative significance of intrauterine embryo distribution for pregnancy outcome has remained unsettled. In this study, our results demonstrated that without disturbing on-time implantation, the disrupted embryo spacing was enough to adversely affect embryo development and pregnancy outcome. This would...
raise the notion that the process of on-site intrauterine embryo distribution mediated by concerted uterine contraction, as an early preimplantation event, is critical for successful embryo implantation and ongoing pregnancy.

Although the phenomenon of embryo distribution along uterine horns is unique to rodents with multiple implantation sites, the mechanism that controls embryo spacing in the rodents can also be responsible for proper embryo location within the uterus in single birth species including monkey (37) and humans. In human beings, the normal embryo implantation site is largely restricted to the uterine fundus (38). Recently developed high resolution ultrasound has revealed that the preimplantation uterine activity involves well regulated kinetics such as wave-like movement from cervix to fundus, which is likely responsible for correct transportation of the embryo to the favored uterine compartment (38). Aberrant uterine movement would result in embryo implantation at unfavorable sites, which are prone to miscarriage or other pregnancy complications such as placenta previa or cornual pregnancy. Indeed, a recent finding has demonstrated that abnormal uterine peristalsis is directly linked with decreased pregnancy rates in humans (39). In addition, with the increasing application of in vitro fertilization pre-embryo transfer in the clinic, the vanishing twin syndrome has become an important clinical issue due to its high incidence and increased risks for postnatal abnormalities (40). One cause of vanishing twin syndrome might be due to embryo crowding at implantation and subsequent resorption of one embryo as the result of competition for space and nutrient supply. There is growing epidemiological evidence that stress at early pregnancy serves as a risk factor for infertility and pregnancy complications, and stress can elevate concentrations of endogenous β2-AR ligands such as norepinephrine (41). It is an open but intriguing possibility that maternal stress at the time of embryo transfer and/or implantation may cause similar sympathetic activation of uterine β2-AR in humans, resulting in suboptimal embryo location and pregnancy complications.

In our view, the present study raises the concept that normal uterine contraction-mediated on-site intrauterine embryo location is crucial for successful ongoing pregnancy. Our data also provided the first molecular and physiological clue as to how sympathetic activation at early pregnancy could adversely affect pregnancy outcome. Because both pharmacological silencing and genetic silencing of β2-AR are similarly efficient in controlling uterine contractility, our study provided β2-AR as a potential target in manipulating related uterine complications during early pregnancy.

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REFERENCES

β2-AR Activation Disrupts Embryo Spacing and Pregnancy

Supplemental Fig S1. Dose and time dependent effect of transient β2-AR activation on embryo spacing. (A) Dose dependent administration (NS, $P = 0.44$, $*P = 0.0119$, $**P = 0.0009$, $***P < 0.00001$ (2mg), $****P = 0.00003$ (4mg). Rank Sum Test). (B) Time dependent administration of β2-AR agonist was compared between Morn group (Morning, 0830 AM), After group (Afternoon, 1430 PM) and Morn+After group (Morning and afternoon, 0830 AM and 1430 PM) (Rank Sum Test). Numbers above bars indicate number of mice with crowded implantation sites per total mice examined.
**Supplemental Table. S1** Primers used for amplifying mouse adrenergic receptors

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**Supplemental Table. S2** Primers used for quantitative Real-Time PCR detection of implantation related genes

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