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Transient β_2-Adrenoceptor Activation Confers Pregnancy Loss by Disrupting Embryo Spacing at Implantation*[S]

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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 β_2-adrenoreceptor (β_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 β_2-AR antagonist or genetic ablation of β-AR. Similar β_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 β_2-AR activation is limited to the preimplantation period before embryo attachment. *In vitro* and *in vivo* studies demonstrated that the transient β_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 contraction-mediated correct intrauterine embryo distribution is crucial for successful ongoing pregnancy. Abnormal β_2-AR activation at early pregnancy provided a molecular clue in explaining how maternal stress at early stages could adversely affect the pregnancy outcome.

Pregnancy loss is a common and painful condition for gestational women, accounting for 25–40% of total pregnancies worldwide (1, 2). Although the exact etiology of miscarriage varies, it is believed that for many patients, the cause of midterm miscarriage/abnormal pregnancy has been seeded very early during the onset of embryo implantation (3–5). Recent animal studies and clinical investigations indicated that suboptimal embryo implantation at the wrong time and wrong place could cause adverse effects on pregnancy outcome (6–8). However, it remains unclear how and to what extent these implantation abnormalities are influenced by environmental factors such as stress-induced sympathetic activation. Epidemiological data have shown that maternal stress at early pregnancy is strongly associated with various complications during ongoing gestation (9–11). Considering that the uterus is an organ with extensive sympathetic innervations (12), it is our hypothesis that stress-induced adrenergic receptor activation may directly affect embryo-maternal interactions during implantation, resulting in pregnancy complications and miscarriage. In this study, we demonstrated that a transient β_2-AR[3] activation at preimplantation disrupted embryo spacing and caused subsequent embryo loss at midgestation due to disrupted uterine contraction before embryo attachment. Our data highlighted that the concerted uterine contraction is a crucial factor for successful implantation and ongoing pregnancy. Also, this study provided β_2-AR activation as a molecular link in explaining how maternal stress at early stages could adversely affect pregnancy outcome.

EXPERIMENTAL PROCEDURES

Animals—Adult CD1 mice (7–8 weeks old) were purchased from Vital River Laboratories Co. Ltd., and the β-AR knockout (*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*-lodoelonidine hydrochloride (Sigma) (50 μg/mouse), yohimbine

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1 and S2 and Fig. S1.
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[3] The abbreviations used are: β_2-AR, β_2-adrenoceptor; HB-EGF, heparin-binding EGF-like growth factor; EGFR, epidermal growth factor receptor; LPA, lysophosphatidic acid receptor; PG, prostaglandin; pc, postcoitus.

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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 β_2-AR agonist (Salbutamol), mice were pretreated with β_2-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 β_2-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 β_2-AR on day 4 uteri were performed in a frozen section with standard protocols. Immunofluorescence detections of β_2-AR (Abcam), HB-EGF (R&D), EGFR (Santa Cruz Biotechnology), and ErbB-4 (Santa Cruz Biotechnology) on blastocysts recovered from day 4 uteri (8:00 p.m.) were performed as described previously (19).

Blastocyst Recovery and Embryo Transfer—At day 4, at 4:00 p.m., embryos were flushed from the uteri of vehicle- and β_2-AR agonist (Salbutamol)-treated groups. Morphologically normal blastocysts were counted in both groups. Recovered blastocysts were used for embryo transfer into recipient females of day 4 pc pseudopregnancy. The recipient mice were kept until term pregnancy, and the number of pups sired was documented at term pregnancy.

Uterine Contraction Measurements—The uterine contractile activity assay was performed as described previously (13). In brief, 5 mm of uteri were removed from wild-type and β-AR null(*Adrb1/Adrb2*) mice at day 4 pc and mounted in a thermostat organ bath (Chengdu Equipment Factory, China) at 37 °C with carbogen (95% O_2 + 5% CO_2). Each sample was mounted vertically in the organ bath, and their contractility was detected by a tension (strength) transducer (JZJ01H, Chengdu Equipment Factory) coupled to a RM6240BD multichannel amplifier (Chengdu Equipment Factory). The uteri were allowed to equilibrate for at least 1 h in the Krebs solution (118 mM NaCl, 4.5 mM KCl, 1.0 mM $MgSO_4$, 1.0 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.8 mM $CaCl_2$, and 6.0 mM glucose). After equilibration, the β_2-AR agonist (Salbutamol), β_2-AR antagonist (propranolol), and forskolin were then added to the bath. For each sample, uterine contractility was recorded for at least 1 h.

cAMP and PKA Assay—Uteri from day 4 pregnant mice were incubated in an organ bath 10 min after adding β_2-AR agonist Salbutamol (5 ng/ml) or forskolin (3.5 μg/ml), and the samples were flash-frozen and stored in liquid nitrogen for cAMP or PKA detection. cAMP detection was performed by using the commercially available cAMP enzyme immunoassay kit (Sigma), and PKA detection was performed by using a SignaTECT® cAMP-dependent protein kinase (PKA) assay system (Promega) following the manufacturer's instructions.

PGE_2 Assay—Uteri from day 4 pregnant mice were injected with β_2-AR agonist Salbutamol (2 mg/mice) or vehicle (saline) twice as described under "Drug Treatments." At day 4, at 4:00 p.m., mice were sacrificed, and their uteri were flash-frozen and crushed in liquid nitrogen. Specimens were used for PGE_2 detection with the prostaglandin E_2 affinity purification kit (Cayman Chemical, Ann Arbor, MI) and prostaglandin E_2 EIA kit (Cayman Chemical).

Statistical Analysis—Statistical analyses were performed with SPSS 11.5. The significances of embryo crowding after treatment of adrenergic drugs were analyzed by the rank sum test. Other statistical analyses were using the unpaired two-tailed *t* test.

RESULTS

Transient β_2-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 β_2-AR showed dominant expression, with intense myometrial localization and relatively weak expression at luminal and glandular epithelium (Fig. 1*A* 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 β_2-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 β_2-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. 1*E*), and the implantation numbers of days 5

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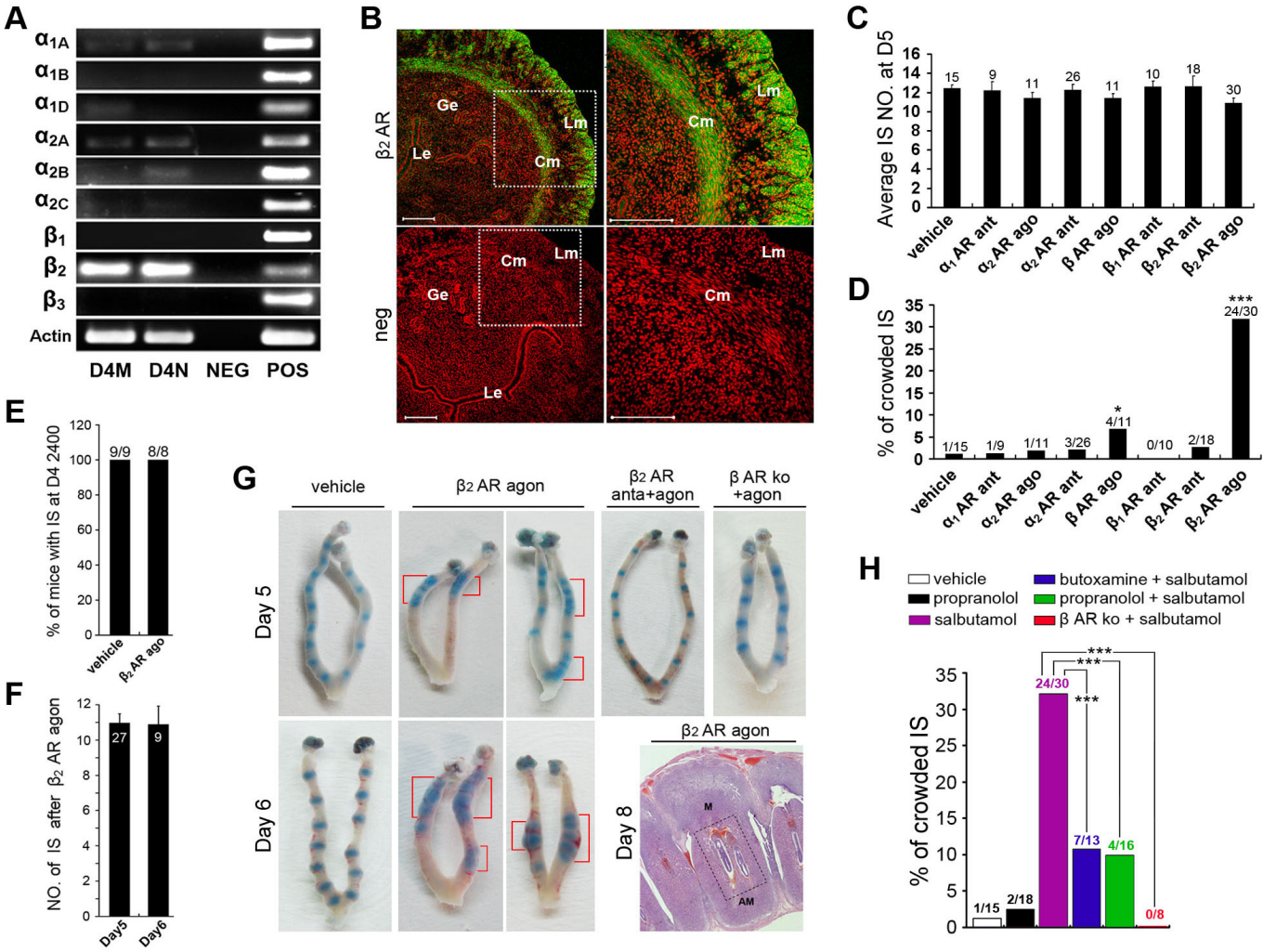

FIGURE 1. **Transient β_2-AR activation at implantation window caused aberrant embryo spacing.** *A*, dominant expression of β_2-AR mRNA during preimplantation uteri. *D4M*, day 4 morning; *D4N*, day 4 night; *NEG*, negative control; *POS*, positive control. *B*, β_2-AR localization in the day 4 mouse uterus showed intense expression at myometrium and weak expression at epithelium. FITC-labeled β_2-AR antibody is in *green*, and propidium iodide-labeled nuclei are in *red*. *Le*, luminal epithelium; *Ge*, glandular epithelium; *Lm*, longitudinal muscular layer; *Cm*, circular muscular layer. *Scale bars*, 200 μm. *C*, the day 5 (*D5*) implantation site (*IS*) number is comparable after different adrenergic agonist (*ago*)/antagonist (*ant*) treatment on day 4 (each treatment when compared with vehicle group, $p > 0.05$, *error bars* represent S.E., one-way analysis of variance). *D*, β-AR agonist and β_2-AR-selective agonist (when compared with vehicle, *, $p = 0.0044$ and ***, $p < 0.00001$. rank sum test) administration on day 4 (*D4*) caused aberrant embryo spacing on day 5. *E*, normal timing of implantation initiation on day 4 at 12:00 a.m. in both vehicle-treated and β_2-AR agonist-treated groups. *F*, the number of implantation sites was comparable on days 5 and 6 after β_2-AR agonist (*agon*) treatment on day 4 ($p = 0.9461$, *t* test). *G*, representative pictures of normal and crowded implantation sites by the blue dye method (days 5 and 6) and tissue section (day 8). Each *blue band* indicates an implantation site. The *bracket* shows crowded implantation sites. *anta*, antagonist; *M*, mesometrial pole; *AM*, anti-mesometrial pole. *H*, genetic ablation of β-AR or pharmacological blockade by β_2-AR antagonist (butoxamine or propranolol) reversed the abnormal embryo spacing by β_2-AR agonist (Salbutamol) (***, $p < 0.00001$, rank sum test). *Numbers* above/within bars indicate number of mice examined. For *D* and *H*, *numbers* above the bars indicate the number of mice with crowded implantation sites per total mice examined. All *error bars* represent S.E.

and 6 pc were also comparable (Fig. 1*F*). 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. 1*G*). Indeed, at the later stage of post-implantation, histological examination often revealed that two or more embryos shared one deciduum or one placenta (Fig. 1*G* and Fig. 2*A*). The abnormal embryo spacing caused by Salbutamol could be substantially reversed by pretreatment of β_2-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 β_2-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 β_2-AR agonist treatment on day 4 pc. Although most vehicle-treated mice showed well developed implantation sites, mice after β_2-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-

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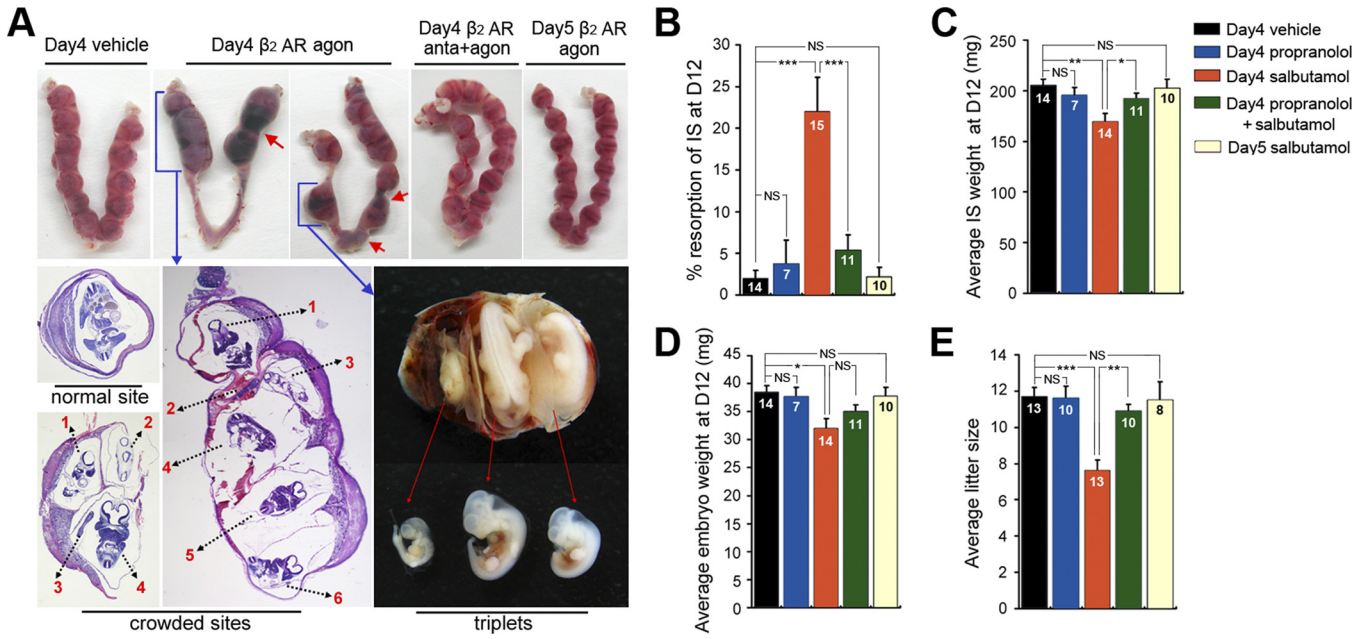

FIGURE 2. **Transient β_2-AR activation before but not after embryo attachment caused compromised pregnancy.** *A*, representative pictures of day 12 mouse uteri after treatment of β_2-AR drugs on days 4 and 5. *Red arrow* shows resorption sites. Histological examination of crowded implantation sites 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*, $p > 0.05$, ***, $p < 0.00005$). *C*, average implantation site weight at day 12 of pregnancy (*NS*, $p > 0.05$, *, $p = 0.0287$, **, $p = 0.0003$). *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.

tion sites and retarded embryo growth were usually found at regions with crowded embryos and were also found at the very end of the cervix pole (Fig. 2*A*). At the end of pregnancy, the litter size was significantly decreased in the β_2-AR agonist-treated group (Fig. 2*E*). Again, the adverse effects could be substantially rescued by pretreatment with β_2-AR antagonist (propranolol) at day 4 pc before β_2-AR agonist administration (Fig. 2, *A–E*).

To rule out the possibility that the impaired pregnancy by β_2-AR agonist originated from abnormal post-implantation events rather than disrupted embryo distribution, we compared the effects of β_2-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 β_2-AR Activation Does Not Adversely Affect Pre-implantation Embryo Quality—To exam whether the transient β_2-AR activation at day 4 pc adversely influenced blastocyst quality, we checked blastocysts recovered from mice with or without β_2-AR agonist treatment. As shown in Fig. 3, the expression of β_2-AR itself also showed no apparent changes by immunofluorescence detection (Fig. 3*A*). 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 β_2-AR activation did not adversely affect the quality and developmental potential of the embryos (Fig. 3*G*). 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.

β_2-AR Activation Suppresses Preimplantation Uterine Contractility through Canonical cAMP-PKA Activation—To further address that the observed aberrant intrauterine embryo distribution caused by β_2-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. 4*A*). In contrast, after β_2-AR agonist administration, the same oil injection no longer induces separated but diffused deciduoma (Fig. 4*A*), 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 β_2-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 β_2-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 β_2-AR agonist significantly decreased the

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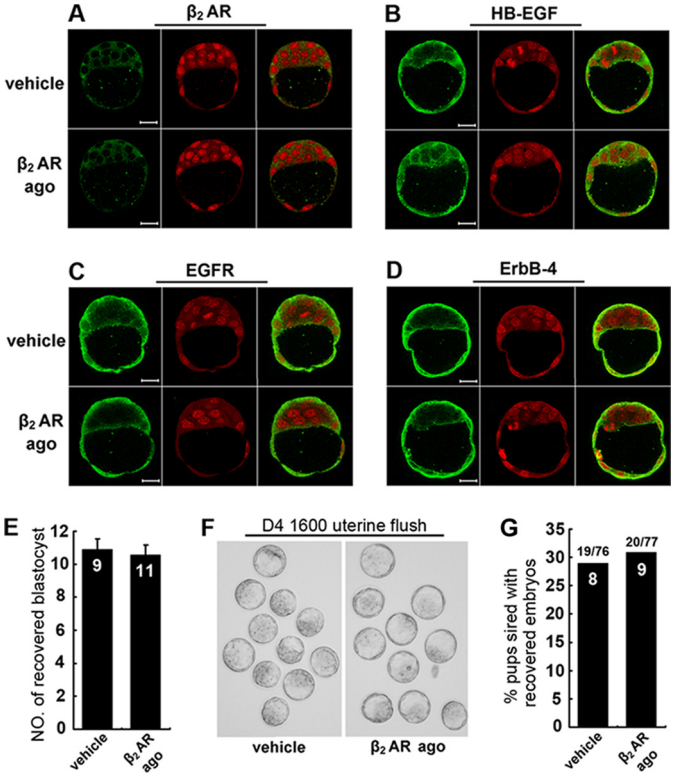

FIGURE 3. **Transient β_2-AR activation did not adversely affect preimplantation embryo quality.** *A–D*, immunofluorescence detection showing expression of β_2-AR (*A*), HB-EGF (*B*), EGFR (*C*), and ErbB-4 (*D*) on blastocysts (recovered at day 4 at 8:00 p.m.) from mice treated with vehicle or β_2-AR agonist (*ago*) on day 4 pc. FITC-labeled target antibody is in *green*, and propidium iodide-labeled nuclei are in *red*. *Scale bars*, 20 μm. *E*, the number of blastocysts recovered from day 4, at 4:00 p.m., after treatment of vehicle or β_2-AR agonist. *Numbers* within bars indicate the number of mice examined ($p > 0.05$, *error bars* represent S.E. *t* test). *F*, representative pictures of recovered blastocysts from vehicle- or β_2-AR agonist-treated groups (day 4, 4:00 p.m.) showed comparable morphology. *G*, the number of pups produced by transferring recovered blastocysts into uteri of day 4 pseudopregnant mice. *Numbers* within bars indicate the number of recipient mice used; *numbers* above the bars indicate pups sired per blastocysts transferred ($p > 0.05$, *t* test).

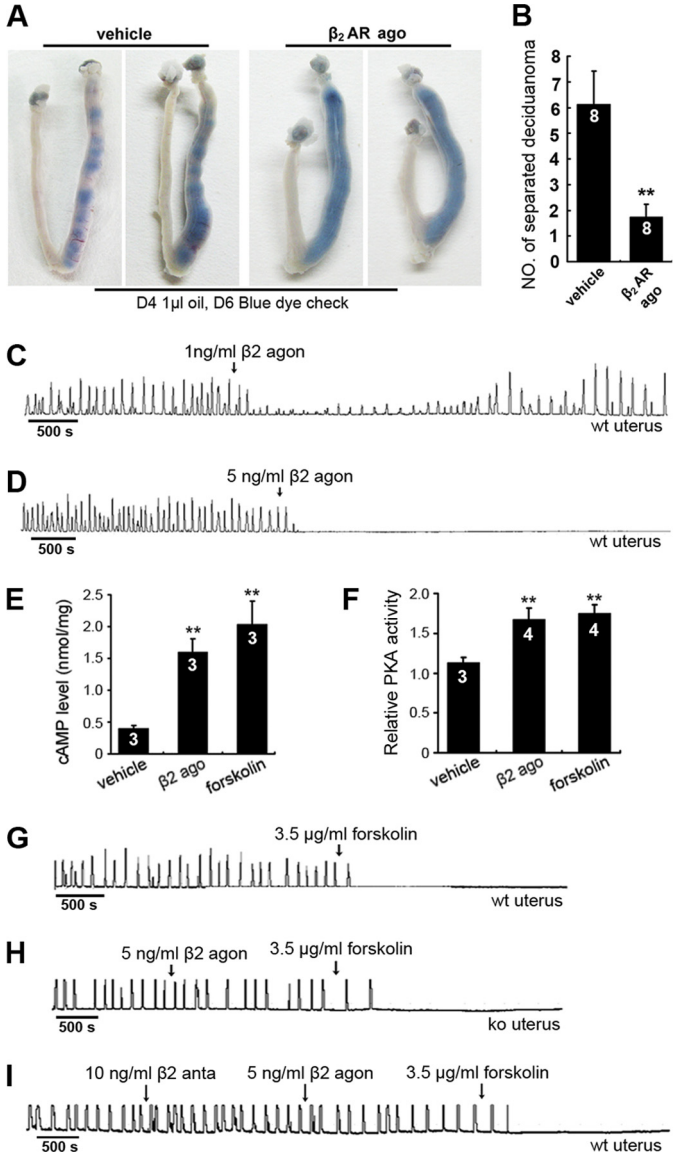

FIGURE 4. **β_2-AR activation suppressed preimplantation uterine contraction through cAMP-PKA elevation.** *A*, illustration of day 6 deciduoma (blue dye method) in pseudopregnant mice after day 4 intrauterine infusion of 1 μl of sesame oil. Note the separated blue bands in the vehicle-treated group and the diffused blue bands in the β_2-AR agonist (*ago*)-treated group. *B*, average numbers of separated deciduoma after vehicle or β_2-AR agonist treatment (**, $p = 0.0068$, *t* test). *C* and *D*, uterine contractile activities were recorded before and after administration of 1 ng/ml (*C*) or 5 ng/ml (*D*) β_2-AR agonist (*agon*) Salbutamol. *E* and *F*, uterine homogenates were used for cAMP level (*E*) and PKA activity (*F*) detection after vehicle or β_2-AR agonist treatment for 5 min (*versus* vehicle, cAMP, **, $p = 0.0063$; PKA, **, $p = 0.0047$). Forskolin treatment (*versus* vehicle, cAMP, **, $p = 0.0027$; PKA, **, $p = 0.0021$) was used as a positive control with *t* test. For PKA activities, data are presented as pmol of phosphate incorporated per minute per μg of total protein. *G*, forskolin (3.5 μg/ml) mimics the effect of Salbutamol in demobilizing uterine contractility. *H* and *I*, β-AR knock-out uterus (*H*) or pharmacological blockade of β_2-AR by its antagonist (*anta*) propranolol (*I*) made the uteri resistant to the inhibitory effect of Salbutamol but still sensitive to forskolin-induced muscular demobilization. For each muscular contraction assay, similar results were obtained from three mice. *Error bars* represent S.E. *Numbers* within the bars indicate the number of mice examined.

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 β_2-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. 4*G*). These results indicated that the inhibitory effect of myometrium contractility after β_2-AR activation is mediated through cAMP-PKA elevation. As expected, pretreatment of β_2-AR antagonist (propranolol) made the uterus resistant to the inhibitory effect of β_2-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*).

β_2-AR Activation Is Associated with Specific Uterine Down-regulation of lpa3—To further explore the potential molecular mechanisms of β_2-AR activation on preimplantation uterus, we examined gene expression profiles relating to uterine receptivity and embryo distribution. As shown in Fig. 5*A*, 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 β_2-AR activation, which was consistent with the unchanged timing

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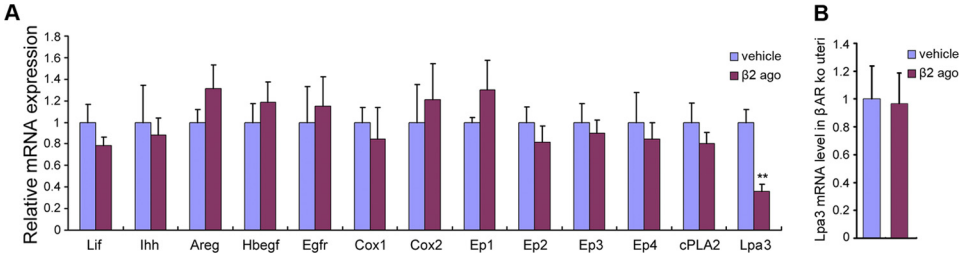

FIGURE 5. **Uterine gene expression after β_2-AR activation.** *A*, RNA was prepared from day 4 uteri (4:00 p.m.) after vehicle or β_2-AR agonist administration and processed for real-time RT-PCR analysis using gene-specific primers of *lif*, *ihh*, *hb-egf*, *areg* (amphiregulin), *egfr*, *cox1*, *cox2*, *cPLA2*, and *ep1–4* ($p > 0.05$, $n = 3$ for each gene) and *lpa3* (lysophosphatidic acid receptor 3) (**, $p = 0.0055$, $n = 10$). *Error bars* represent S.E., *t* test. *B*, in β-AR knock-out mice, the *lpa3* mRNA level did not show significant down-regulation after β_2-AR agonist treatments ($p = 0.925$, $n = 3$). *Error bars* represent S.E., *t* test.

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 β_2-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 PGE_2 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 β_2-AR activation-mediated embryo crowding is PG-independent or at the downstream of PG signaling in regulating uterine contractility. Surprisingly, after transient β_2-AR activation, we detected a significant decrease in *lpa3* expression (Fig. 5*A*), whereas in the β-AR knock-out mice, *lpa3* did not show significant down-regulation after β_2-AR agonist treatment (Fig. 5*B*), demonstrating that the observed down-regulation of *lpa3* in wild-type uteri resulted from β_2-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 β_2-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 β_2-AR at the uterine epithelium. Nonetheless, the observed down-regulation of *lpa3* after β_2-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 β_2-AR activation in disrupting normal embryo spacing and its profound link to ongoing pregnancy. Previous investigations on β_2-AR have been focused on the periparturition period because the administration of β_2-AR agonist results in uterine relaxation, which could be utilized as an anti-parturition drug (34). In addition, it has been reported that β_2-AR is the predominant AR subtype in murine oviducts and that disturbed β_2-AR signaling resulted in aberrant oviductal embryo transport (35). Here we have expanded the understanding of the pathophysiological roles of β_2-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 underexplored. Recently, renewed interest of this topic has been raised from the discovery of two knock-out mice that showed disrupted embryo spacing at implantation. In the *cPLA2*- and *Lpa3*-null female, the timing of embryo implantation was shortly 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 knock-out 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

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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 β_2-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 β_2-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 β_2-AR are similarly efficient in controlling uterine contractility, our study provided β_2-AR as a potential target in manipulating related uterine complications during early pregnancy.

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REFERENCES

1. Norwitz, E. R., Schust, D. J., and Fisher, S. J. (2001) *N. Engl. J. Med.* **345,** 1400–1408
2. Dey, S. K. (2010) *J. Clin. Invest.* **120,** 952–955
3. van Oppenraaij, R. H., Jauniaux, E., Christiansen, O. B., Horcajadas, J. A., Farquharson, R. G., and Exalto, N. (2009) *Hum Reprod. Update* **15,** 409–421
4. Wang, H., and Dey, S. K. (2006) *Nat. Rev. Genet.* **7,** 185–199
5. Lim, H. J., and Wang, H. (2010) *J. Clin. Invest.* **120,** 1004–1015
6. Ye, X., Hama, K., Contos, J. J., Anliker, B., Inoue, A., Skinner, M. K., Suzuki, H., Amano, T., Kennedy, G., Arai, H., Aoki, J., and Chun, J. (2005) *Nature* **435,** 104–108
7. Song, H., Lim, H., Paria, B. C., Matsumoto, H., Swift, L. L., Morrow, J., Bonventre, J. V., and Dey, S. K. (2002) *Development* **129,** 2879–2889
8. Wilcox, A. J., Baird, D. D., and Weinberg, C. R. (1999) *N. Engl. J. Med.* **340,** 1796–1799
9. Gold, K. J., Dalton, V. K., Schwenk, T. L., and Hayward, R. A. (2007) *Gen. Hosp. Psychiatry* **29,** 207–213
10. Neugebauer, R., Kline, J., Stein, Z., Shrout, P., Warburton, D., and Susser, M. (1996) *Am. J. Epidemiol.* **143,** 588–596
11. O'Hare, T., and Creed, F. (1995) *Br. J. Psychiatry* **167,** 799–805
12. Houdeau, E., Rousseau, A., Meusnier, C., Prud'Homme, M. J., and Rousseau, J. P. (1998) *J. Comp. Neurol.* **399,** 403–412
13. Allix, S., Reyes-Gomez, E., Aubin-Houzelstein, G., Noël, D., Tiret, L., Panthier, J. J., and Bernex, F. (2008) *Biol. Reprod.* **79,** 510–517
14. Irie, K., Fujii, E., Ishida, H., Wada, K., Suganuma, T., Nishikori, T., Yoshioka, T., and Muraki, T. (2001) *Br. J. Pharmacol.* **133,** 237–242
15. Song, D. K., Im, Y. B., Jung, J. S., Yan, J. J., Huh, S. O., Suh, H. W., and Kim, Y. H. (2000) *Br. J. Pharmacol.* **130,** 41–48
16. Im, Y. B., Won, J. S., Suh, H. W., Huh, S. O., Kim, Y. H., and Song, D. K. (1998) *J. Auton. Pharmacol.* **18,** 149–155
17. Brandon, J. M. (1990) *J. Reprod. Fertil.* **88,** 151–158
18. Zhao, P., De, A., Hu, Z., Li, J., Mulders, S. M., Sollewijn Gelpke, M. D., Duan, E. K., and Hsueh, A. J. (2008) *Endocrinology* **149,** 2782–2789
19. Kuang, H., Chen, Q., Fan, X., Zhang, Y., Zhang, L., Peng, H., Cao, Y., and Duan, E. (2009) *J. Cell. Physiol.* **221,** 448–457
20. Hamatani, T., Daikoku, T., Wang, H., Matsumoto, H., Carter, M. G., Ko, M. S., and Dey, S. K. (2004) *Proc. Natl. Acad. Sci. U.S.A.* **101,** 10326–10331
21. Wang, J., Mayernik, L., Schultz, J. F., and Armant, D. R. (2000) *Development* **127,** 33–44
22. Paria, B. C., Elenius, K., Klagsbrun, M., and Dey, S. K. (1999) *Development* **126,** 1997–2005
23. Paria, B. C., Das, S. K., Andrews, G. K., and Dey, S. K. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90,** 55–59
24. Dey, S. K., Lim, H., Das, S. K., Reese, J., Paria, B. C., Daikoku, T., and Wang, H. (2004) *Endocr. Rev.* **25,** 341–373
25. Restall, B. J., and Bindon, B. M. (1971) *J. Reprod. Fertil.* **24,** 423–426
26. Crane, L. H., and Martin, L. (1991) *Reprod. Fertil. Dev.* **3,** 233–244
27. Pusey, J., Kelly, W. A., Bradshaw, J. M., and Porter, D. G. (1980) *Biol. Reprod.* **23,** 394–397
28. Lee, K., Jeong, J., Kwak, I., Yu, C. T., Lanske, B., Soegiarto, D. W., Toftgard, R., Tsai, M. J., Tsai, S., Lydon, J. P., and DeMayo, F. J. (2006) *Nat. Genet.* **38,** 1204–1209
29. Xie, H., Wang, H., Tranguch, S., Iwamoto, R., Mekada, E., Demayo, F. J., Lydon, J. P., Das, S. K., and Dey, S. K. (2007) *Proc. Natl. Acad. Sci. U.S.A.* **104,** 18315–18320
30. Kennedy, T. G. (1977) *Biol. Reprod.* **16,** 286–291
31. Dey, S. K. (2005) *Nature* **435,** 34–35
32. Hama, K., Aoki, J., Inoue, A., Endo, T., Amano, T., Motoki, R., Kanai, M., Ye, X., Chun, J., Matsuki, N., Suzuki, H., Shibasaki, M., and Arai, H. (2007) *Biol. Reprod.* **77,** 954–959
33. Shumay, E., Tao, J., Wang, H. Y., and Malbon, C. C. (2007) *J. Biol. Chem.* **282,** 21529–21541
34. Berkman, N. D., Thorp, J. M., Jr., Lohr, K. N., Carey, T. S., Hartmann, K. E., Gavin, N. I., Hasselblad, V., and Idicula, A. E. (2003) *Am. J. Obstet. Gynecol.* **188,** 1648–1659

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35. Wang, H., Guo, Y., Wang, D., Kingsley, P. J., Marnett, L. J., Das, S. K., DuBois, R. N., and Dey, S. K. (2004) *Nat. Med.* **10,** 1074–1080
36. Wimsatt, W. A. (1975) *Biol. Reprod.* **12,** 1–40
37. Heuser, C., and Streeter, G. (1941) *Contrib. Embryo* **29,** 15–55
38. Bulletti, C., and de Ziegler, D. (2006) *Curr. Opin. Obstet. Gynecol.* **18,** 473–484
39. Yoshino, O., Hayashi, T., Osuga, Y., Orisaka, M., Asada, H., Okuda, S., Hori, M., Furuya, M., Onuki, H., Sadoshima, Y., Hiroi, H., Fujiwara, T., Kotsuji, F., Yoshimura, Y., Nishii, O., and Taketani, Y. (2010) *Hum. Reprod.* **25,** 2475–2479
40. Pinborg, A., Lidegaard, O., and Andersen, A. N. (2006) *Br. J. Hosp. Med.* (*Lond.*) **67,** 417–420
41. Ferry, B., Roozendaal, B., and McGaugh, J. L. (1999) *Biol. Psychiatry* **46,** 1140–1152

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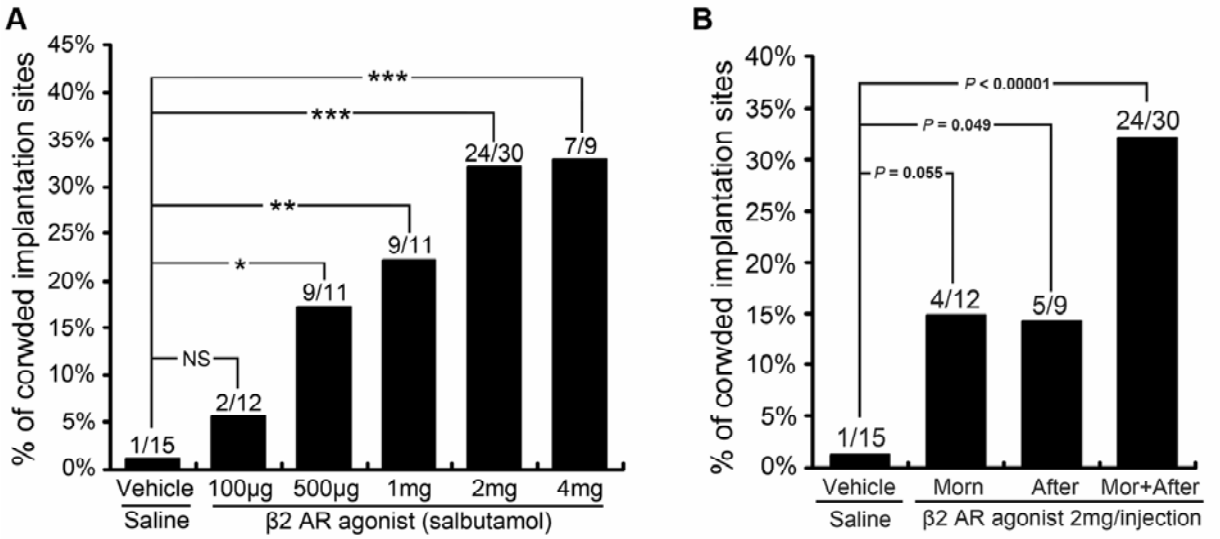

Supplemental Fig S1. Dose and time dependent effect of transient β_2-AR activation on embryo spacing. (**A**) Dose dependent administration (NS, $P = 0.44$, $*P = 0.0119$, $**P = 0.0009$, $***P < 0.00001$ (2mg), $***P = 0.00003$ (4mg). Rank Sum Test). (**B**) Time dependent administration of β_2-AR agonist 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.

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Supplemental Table. S1 Primers used for amplifying mouse adrenergic receptors

Transcript	Forward primer(5'-3')	Reverse primer(5'-3')	GenBank accession number
α1a AR	CCAGTGTCTTCGCAGAAGG	CAGCAGCAGACCTGCAAAAA	NM_013461
α1b AR	CGGACGCCAACCAACTACTT	AACACAGGACATCAACCGCTG	NM_007416
α1d AR	AGTGGGTGTCTTCCTAGCC	GCCTAGAACCTCCATAGTGGC	NM_013460
α2a AR	GTGACACTGACGCTGGTTTG	CCAGTAACCCATAACCTCGTTG	NM_007417
α2b AR	TCTTCACCATTTTCGGCAATGC	AGAGTAGCCACTAGGATGTCG	NM_009633
α2c AR	CTGTGGTGGGTTTCCTCATCG	ACTTGCCCGAAGTACCAGTAG	NM_007418
β1 AR	GAACCCTGCAACCTGTCGTC	CCACGAGTAGGCCCATACC	NM_007419
β2 AR	GGGAACGACAGCGACTTCTT	GCCAGGACGATAACCGACAT	NM_007420
β3 AR	AGAAACGGCTCTCTGGCTTTG	TGGTTATGGTCTGTAGTCTCGG	NM_013462
β-Actin	TGGAATCCTGTGGCATCCATGAAAC	TAAAACGCAGCTCAGTAACAGTCCG	NM_007393

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

Transcript	Forward primer(5'-3')	Reverse primer(5'-3')	GenBank accession number
β-Actin	TGGAATCCTGTGGCATCCATGAAAC	TAAAACGCAGCTCAGTAACAGTCCG	NM_007393
Hbegf	CGGGGAGTGCAGATACCTG	TTCTCCACTGGTAGAGTCAGC	NM_010415
Egfr	GCATCATGGGAGAGAACAACA	CTGCCATTGAACGTACCCAGA	NM_007912
Lif	ATTGTGCCCTTACTGCTGCTG	GCCAGTTGATTCTTGATCTGGT	NM_008501
Areg	CCCTGAAGTATCGTTTCCAAA	GCCATCATCCTCGCAGCTA	NM 009704
Ihh	CTGTGAACTGAGCTGACAAGCGTG	GGGAATCTAGCAGCATCGACTGAG	NM_010544
cPLA2	AGGCACAGCTACATTCCCTG	CATGCTGAACCGTAGGTCTGG	NM_008869
Lpar3	CAAGCGCATGGACTTTTTCTAC	GAAATCCGCAGCAGCTAAGTT	NM_022983
Cox1	CTGTTGTTACTATCCGTGCCAG	CTCAGGGATGGTACAGTTGGG	NM_008969
Cox2	TGAGCAACTATTCCAAACCAGC	GCACGTAGTCTTCGATCACTATC	NM_011198
EP1	GGGCTTAACCTGAGCCTAGC	GTGATGTGCCATTATCGCCTG	NM_013641
EP2	CCTCGACTTCCACTCCCTG	TGAGGGCACTAATGATGACAGAG	NM_133783
EP3	CAAGCGCAAGAAGTCTTTCCT	CCCCACTAAGTCGGTGAGC	NM_011196
EP4	ACCATTCCTAGATCGAACCGT	CACCACCCCGAAGATGAACAT	NM_008965

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