The critical role of uterine CD31 as a post-progesterone signal in early pregnancy

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Abstract

CD31 has been shown to play a role in endothelial cell migration and angiogenesis, which are critical to the formation and function of the endometrium and myometrium in uterine development during early pregnancy. However, the role of CD31 in uterine receptivity during blastocyst implantation is poorly understood. The pregnancy rate in CD31⁻/⁻ female mice mated with CD31⁺/+ male mice was higher than that observed in CD31⁺/+ female mice mated with CD31⁻/⁻ male mice. During the receptive phase of implantation, uterine glands were more developed in CD31⁻/⁻ mice than in CD31⁺/+ mice, and the uterine weights of CD31⁻/⁻ mice were increased. Leukemia inhibitory factor (LIF) was highly expressed in the CD31⁻/⁻ mice during implantation and the expression of LIF was up-regulated by estradiol-17β (E₂) + progesterone (P₄) in ovariectomized CD31⁻/⁻ mice, compared with CD31⁺/+ mice at 8h after hormone treatment. E₂-induced protein synthesis was inhibited by P₄ in the CD31⁺/+ uterus, but not in the uterus of CD31⁻/⁻ mice. Also, STAT3, HAND2, LIF, and mTOR signals were enhanced in the uterus of CD31⁻/⁻ mice, manifested by upregulated cyclin series signaling and PCNA expression after E₂+P₄ treatment. Collectively, CD31 inhibits E₂-mediated epithelial proliferation via recruitment and phosphorylation of SHP-2 upon receiving P₄ signal in early pregnancy.

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Introduction

Pregnancy is a complex process that is comprised of several discrete events, including implantation, decidualization, placentation, and the birth of the offspring. The uterus changes into an altered state when implantation-competent blastocysts are ready to initiate the implantation (Winuthayanon et al. 2010). This process enhances uterine receptivity to implantation. In mice, estradiol-17β (E₂) stimulates the proliferation of luminal and glandular epithelium at 1 day postcoitum (dpc) (Zhu et al. 2007). E₂, acting on stromal cells, initiates responses to mediate epithelial proliferation (Winuthayanon et al. 2010). E₂-induced uterine epithelial proliferation was independent on the uterine epithelial estrogen receptor (ER) α. The basis for this can be seen in the ability of the uterine epithelium to synthesize DNA in uterus epithelium-specific ER knockout (KO) mice after E₂ treatment (Winuthayanon et al. 2010). E₃ regulates uterine epithelial cell proliferation through the paracrine mechanisms of insulin-like growth factor 1 (IGF1), which acts on the epithelial IGF1 receptor to activate phosphatidylinositol 3-kinase (PI3K), leading to an inhibitory phosphorylation of GSK3β by AKT (Chen et al. 2005, Zhu et al. 2007, Winuthayanon et al. 2010).

However, uterine epithelial ER is essential in the up-regulation of leukemia inhibitory factor (LIF), which is a critical factor in embryo implantation (Stewart et al. 1992, Lee et al. 2006), and the increase of uterine weight (Winuthayanon et al. 2010). Rising progesterone (P₄) levels from the corpus luteum at 3 dpc inhibit the E₂-induced proliferation of uterine epithelial cells and initiate stromal cell proliferation. At the same time, P₄ is known to increase the basic helix-loop-helix transcription factor HAND2, and increased HAND2 is known to increase LIF (Li et al. 2011). The attenuation of E₂-induced proliferation of the uterine epithelium and the induction ofstroma proliferation by P₄ is essential for successful embryo implantation. In the first pathway of the anti-proliferative action of P₄ in the uterine epithelium, P₄ inhibits the activation of PI3K-AKT-GSK3β pathways, resulting in the inhibition of DNA synthesis (Chen et al. 2005). Moreover, Wang et al. reported that the activation of protein synthesis in the uterine epithelium by E₂ is mediated by PKC, which in turn induces ERK1/2 phosphorylation and thereby activates the mammalian target of rapamycin (mTOR). They also showed that E₂-stimulated protein synthesis was independent of the inhibition of epithelial proliferation by P₄.
LIF, a pleiotropic cytokine of the IL-6 family, regulates various cellular functions, including proliferation and differentiation (Hilton 1992, Dani et al. 1998). LIF acts on cells by binding to the heterodimeric LIF receptor (LIFR), which recruits nonreceptor tyrosine kinases, resulting in the phosphorylation of the signal transducer and activator of transcription 3 (STAT3) (Sun et al. 2013). p-STAT3 is well-known to help the development of the uterus by translocating to the nucleus and increasing the genes involved in proliferation (Annerén et al. 2008, Hiraoka et al. 2016). LIF is essential toward embryo implantation, as well as the events that follow that initiate the processes of endometrial receptivity (Aghajanova et al. 2004) and embryo-endometrial interaction (Cullinan et al. 2003). Reciprocal embryo transfer experiments showed that LIF−/− blastocysts can attach to the uterus of pseudopregnant LIF+/+ mice, but the blastocysts in LIF+/− mice had difficulty implanting in the uterus of pseudopregnant LIF−/− mice, suggesting that maternal LIF is essential for implantation (Stewart et al. 1992). The intraperitoneal injection of LIF into a LIF−/− female mouse is sufficient to induce blastocyst implantation and the subsequent development of the embryo until birth (Chen et al. 2000). LIF expression in the uterus is up-regulated by E2, and expression was evident in the luminal epithelium of the uterus at 1 dpc and in the glandular epithelium at 4 dpc (Bhatt et al. 1991, Shen & Leder 1992, Chen et al. 2000).

CD31 is an inhibitory receptor that is comprised of 6 immunoglobulin-like homology domains, a transmembrane domain, and two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (Kim et al. 2015). SHP-2 is bound to the ITIMs, thereby to be phosphorylated (Wang & Sheibani 2006). The activated p-SHP-2 dephosphorylates and inhibits p-STAT3 (Xu & Qu 2008). CD31 has previously been shown to negatively regulate cellular functions in various cellular and diseased-animal models (Mamdouh et al. 2003). In addition to its inhibitory properties, CD31 is localized to cell-cell borders between endothelial cells in blood vessels and newly formed blood and lymphatic vessels (Mamdouh et al. 2003, Kim et al. 2013). In the uterus, CD31 is expressed in the endometrium and myometrium, and fine mesh-like CD31-positive vessels were arranged in the anti-mesometrial region, and the mesometrial region of CD31-positive vessels were enlarged and elongated over time during decidualization (DeLisser 1997, Kim et al. 2013). Although CD31 has been shown to play a role in endothelial cell migration and angiogenesis, the CD31−/− mice were viable and were born at the expected Mendelian frequency. From there, they remained healthy and showed no signs of vascular developmental defects (DeLisser 1997, Hirota et al. 2010). The question regarding the involvement of CD31 in the signaling pathway during pregnancy remains without a clear answer. To this end, we utilized CD31−/− female mice to examine the consequences of CD31 deficiency in pregnancy.

Materials and methods

Animals and experimental design

C57BL/6 mice were obtained from OrientBio (Sungnam, South Korea). CD31+/− mice were kindly provided by T W Mak (University of Toronto). All animal studies were performed according to protocol approved by the Institutional Animal Care and Use Committee at Chonbuk National University (CBNU) Medical School (CBNU 2017-0028). All mice were used at 10–12 weeks-of-age. To investigate the effects of CD31 on pregnancy, CD31+/+ or CD31−/− C57BL/6 female mice were caged with CD31+/+ C57BL/6 male mice. The day of vaginal plug formation was taken as Day 0.5 of coitus. Mice were sacrificed at 7.5 dpc to determine pregnancy rates. Mating females were sacrificed on 3.5 dpc to determine uterine weight and the uteri were fixed for histological analyses. Mice underwent ovariectomized via a dorsal incision under anesthesia with rumpun and ketamine. After resting for 3 weeks, mice were injected subcutaneously with hormones in peanut oil as described in previous reports (Hilton 1992). On Days 1 and 2, mice were given daily subcutaneous injections of 100 ng E2; on Days 3 and 4, there was no treatment, and the mice were randomly divided into four groups and treated as follows: (Control group) peanut oil treatment on day 6, (E2 group) treatment with 50 ng E2 on day 6, (P4 group) on days 3–6, mice were treated with 1 mg P4, and the other group (P4 and E2 group) was treated with 1 mg P4 on days 3–6, and on day 6, they were treated with 50 ng E2. Mice were sacrificed at 4, 8, and 15 h after hormone treatment, and uteri were harvested for real-time PCR and Western blotting.

Measurement of pregnancy rates

To induce pregnancy, female mice 10–12 weeks-of-age were mated with fertile male mice of same age obtained from OrientBio. Five cages were set up for each group, with two female mice and one male mouse placed in each cage. Plug formation was checked every 6 h and female mice with plugs were isolated and sacrificed at 7.5 dpc, and the uterus was extracted and the number of pups were counted.

Measurement of hormones

Female mice in the estrous cycle were selected according to the method as described previously (Caligioni 2009). Blood was collected and centrifuged at 300g for 10 min at 4°C. Serum was used for the measurement of hormones. Estrogen and progesterone levels were measured with an ELISA kit (Cayman, 582251; Enzo, ADI-900-011).

Histological processing

Tissues at 3.5 dpc were fixed in 4% paraformaldehyde/PBS overnight at 4°C and embedded in paraffin. 7 μm thick
sections were stained with hematoxylin-eosin (HE) for general histological analyses. Images of slides were acquired using a Scan Scope digital scanner (Aperio ePathology Solutions). To compare the intensity of immunostaining between ovarioctomized CD31+/− and CD31−/− female mice, which were treated with hormones, segments of fixed uterus were equilibrated in 30% sucrose and then frozen in tissue freezing medium (Tissue Tek), and sectioned. Samples were blocked with 5% goat serum for 2 h at room temperature (RT) and incubated overnight at 4°C with anti-p-RPS Abs (Cell signaling), anti-CD105 Abs (eBioscience), or anti-PCNA Abs (BioLegend). After several washes, the samples were incubated with FITC-conjugated anti-rabbit IgG, anti-hamster IgG, or anti-mouse IgG Abs, or Cy3-conjugated anti-rat IgG Abs (Jackson Immune Research) for 2 h at RT, and sections were counterstained with DAPI (Sigma-Aldrich). Microscopy was performed using a Zeiss Observer Z1 (Carl Zeiss) fitted with a motorized stage LEP Biopoint 2 (Ludl Electronic products) and Zylar (ANDOR) sCMOS digital camera. 20× images were automatically collected using Meta Morph software (Molecular Devices).

Real-time quantitative PCR

Total RNA was isolated from uterine tissue using Hybrid-R (GeneAll, Seoul, Korea), and cDNA was synthesized with random hexamers using the ImProm-II Reverse Transcription System (Promega). The PCR reaction was carried out in 384-well plates using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The primers that we used are listed in Supplementary Table 1 (see section on supplementary data given at the end of this article). The PCR conditions were as follows: 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, up to 40 cycles.

Western blot analysis

Mice were sacrificed at 3.5 dpc or at various times following hormone treatment. Uterine tissues were lysed in protein extraction buffer, containing 20 mM Tris–HCl pH 7.2, 1% Triton X-100, 100 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM NaVO₄, and a protease inhibitor cocktail (Roche). Tissue lysates were centrifuged at 15,000 g for 15 min at 4°C and supernatants were collected for Western blotting. Protein concentrations were determined using the BCA assay kit (Thermo Scientific Pierce). Equal protein amounts (10 µg) were separated by SDS-PAGE and blotted onto PVDF membranes (Bio-Rad). Membranes were subsequently blocked with 3% BSA in TTBS buffer and incubated sequentially with primary antibodies against p-SHP-2 (Cell signalling), total SHP-2 (Cell signalling), p-STAT3 (Cell signalling), total STAT3 (Cell signalling), LIF (Santa cruz), HAND2 (Abcam), p-RPS6 (Ser240/244) (Cell signalling), p-RPS (Ser235/236) (Cell signalling), p-RPS6K1 (Thr421/Ser424) (Cell signalling), total RPS (Cell signalling), total mTOR (Cell signalling), p-mTOR (Cell signalling), cyclin D1 (Santa Cruz), cyclin A (Santa Cruz), cyclin E (Santa Cruz), GAPDH (Santa Cruz), or PCNA (Bio Legned, San Diego, CA, USA) overnight, followed by treatment with horseradish peroxidase-conjugated secondary antibody (Santa Cruz) for 1 h at room temperature. Proteins were visualized by ECL Select Western blotting Detection Reagent (GE Lifesciences, Pittsburg, PA, USA) and Image Quant LAS 4000 (GE Lifesciences).

Determination of morphometric densities

For morphometric measurements of blood vessels in the uterus, the anti-CD105 immunostained slides were analyzed by using AngioTool as described previously (Zudaire et al. 2011), which computes several morphological parameters including the blood vessel density.

Statistical analysis

Data are represented as the mean±s.d. of at least three independent experiments. Statistical analysis was performed by using the t-test as appropriate. P<0.05 was considered significant.

Results

The pregnancy rate of CD31−/− female mice is increased with up-regulation of LIF

Our initial finding of higher higher pup numbers in C57BL/6 CD31−/− female mice prompted us to compare the fertility of CD31+/− and CD31−/− female mice. Although there was no significant difference in the number of pups/litter between the two groups of plug-positive mice at 7.5 dpc, the pregnancy rate of CD31−/− mice was ~140% of that of CD31+/− female mice (Fig. 1A and B). In addition, we found that CD31−/− mice had a greater uterine weight at 3.5 dpc (~160% of that of CD31+/− mice) (Fig. 1C). In agreement with this finding, histological analysis revealed noticeable uterine size and gland development in CD31−/− female mice (Fig. 1D).

CD31 has been shown to play a role in angiogenesis (DeLisser 1997, Hirota et al. 2010). To determine the effect of CD31 on angiogenesis, we compared CD105+ blood vessel density at the implantation sites of CD31+/− and CD31−/− mice at 7.5 dpc, and found that there was no significant difference (Supplementary Fig. 1A and B). E₂ and P₄ levels were measured to confirm whether difference of pregnancy rate and uterus weight were due to difference of E₂ and P₄ levels in CD31−/− female mice during the estrous cycle, but we found no significant difference between CD31+/− and CD31−/− female mice (Supplementary Fig. 1C). These data indicate that CD31 does not significantly influence uterine angiogenesis or sex hormone production. We then explored the idea that CD31 signaling may transcriptionally alter the expression of genes that are essential for uterine receptivity, implantation, and decidualization. We found that some of the genes encoding proteins that are important for uterine receptivity and implantation, such as LIF, were increased in CD31−/− mice upon quantitative PCR analysis at 3.5 dpc when compared to CD31+/− mice (Fig. 1E). Western blot analysis also demonstrated

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Figure 1 Ablation of the CD31 gene increases blastocyst implantation by altering uterine receptivity. (A) Female mice 10–12 weeks-of-age were mated with fertile male mice of same aged obtained from a commercial facility. Ten female mice were subjected to mating with 5 male mice per group and plug formation was checked every 6 h, and female mice with plugs were isolated and sacrificed at 7.5 dpc. This experiment was repeated 5 times and stochastic statistics were performed by counting the number of successful pregnancies in the plug-positive mice. (B) The number of pups/litter was counted at 7.5 dpc in plug-positive mice. (C) Uterine weights of CD31+/+ and CD31−/− pregnancies at 3.5 dpc. n = 5 mice. (D) Histological evaluation of uteri with longitudinal sections in CD31+/+ and CD31−/− pregnancies at 3.5 dpc. (E) Comparison of gene transcriptional profiling associated with implantation in the uterus of CD31+/+ or CD31−/− mice at 3.5 dpc using real-time PCR. n = 3 mice. (F) Ablation of CD31 upregulates LIF in the uterus at 3.5 dpc. Expression levels of LIF protein were compared between the uteri of CD31+/+ and CD31−/− mice by Western blot (upper panel). Quantitation of the band intensities of LIF were normalized against GAPDH (bottom panel). Data are means ± s.d. Statistical analysis was performed using the t-test. *P < 0.05, **P < 0.01, ***P < 0.001.
a significant increase in LIF levels at 3.5 dpc (Fig. 1F). These data suggest that CD31 may affect uterine receptivity in embryo implantation.

**Phosphorylation of STAT3 is increased phosphorylation of SHP-2 is decreased in CD31+/− mice**

In order to elucidate why the uterus was highly developed in CD31+/− mice during implantation, we examined the expression levels of LIF in ovariectomized CD31+/− mice by treatment with E2 + P4. Ovariectomized mice are generally used to prepare for implantation in the uterus with an artificial exposure of hormones without influence of endogenous hormones. First, Lif mRNA levels were measured at 2 h intervals following hormone injection and found increased levels, with a peak at 4 h that decreased to control level by 8 h (Supplementary Fig. 2). Therefore, the levels of all related parameters were compared at 4 h and 8 h after hormone injection. The difference in p-SHP-2 levels in CD31+/+ and CD31+/− mice were insignificant at 4 h, but at 8 h, levels were significantly decreased in CD31+/− mice when compared to CD31+/+ mice. On the other hand, while p-STAT3 levels in CD31+/+ and CD31+/− mice were similar at 4 h, p-STAT3 levels in CD31+/− mice were decreased at 8 h, whereas they were maintained in CD31+/− mice during the same timeframe (Fig. 2A and B). Consistent with previous findings that HAND2 is increased by P4 and that HAND2 increases LIF expression (Li et al. 2011), Lif and HAND2 were significantly increased in CD31+/− mice vs CD31+/+ mice 8 h after E2 + P4 treatment (Fig. 2A and B). It was confirmed that LIF mRNA levels were increased at 4 h in both mice, but decreased at 8 h in CD31+/− mice, whereas it was maintained in CD31+/− mice (Fig. 2C).

**Activation of mTOR signaling in the uterus of CD31+/− mice**

E2 induces protein synthesis through ERK1/2 phosphorylation and the activation of the mammalian target of rapamycin (mTOR) (Wang et al. 2015). Therefore, we examined the effects of CD31 deficiency on E2- or/and P4-induced signaling. Western blots of total uterine tissue at 4 h after E2 treatment were analyzed for phosphorylation in ERK1/2-mTOR pathway-related molecules in CD31+/+ and CD31+/− mice. Phosphorylation levels of mTOR and its downstream target, ribosomal protein S6 (RPS6) at Ser235/236 and 240/244, were significantly increased by E2 in both groups 4 h after treatment (Fig. 3A and Supplementary Fig. 3). Consistent with the increased RPS6 phosphorylation, the phosphorylation of RPS6K1 at Thr421/Ser424 was also elevated by E2 in CD31+/+ and CD31+/− mice (Fig. 3A and Supplementary Fig. 3). In contrast, the increases in the phosphorylation of mTOR, RPS, and RPS6K1 by E2 were inhibited by P4 4 h after treatment in CD31+/+ mice, whereas the E2-induced phosphorylation of mTOR, RPS, and RPS6K1 in CD31+/− mice were not blocked by treatment with P4 (Fig. 3A and Supplementary Fig. 3). Consistent with the Western blot data, immunohistochemistry performed with anti-p-RPS (Ser235/236) Abs showed that E2 dramatically increased the phosphorylation of RPS in luminal and glandular epithelial cells, as well as some stromal cells in CD31+/+ and CD31+/− mice (Fig. 3B). The E2-induced RPS phosphorylation was completely abolished by treatment with P4 in CD31+/+ mice, whereas CD31+/− mice showed persistent RPS phosphorylation at 4 h after P4 treatment. These data suggest that E2-induced mTOR signaling in the uterus of CD31+/− mice is not blocked by P4, as opposed to CD31+/+ mice.

**CD31-deficient mice exhibit uterine hyperplasia**

P4 completely suppresses E2-induced epithelial proliferation, but sensitizes the stromal cells to E2 for proliferation (Tong & Pollard 1999, Chen et al. 2005). E2-induced protein synthesis is essential for the proliferation of uterine luminal epithelial cells (Wang et al. 2015). Therefore, we hypothesized that the uterine hyperplasia from E2 + P4 treatment would affect DNA synthesis in CD31+/− mice. Histological analysis showed similar overall uterine areas in CD31+/+ and CD31+/− mice 15 h following E2 treatment (Fig. 4A). However, uterine hyperplasia, along with a 2.5-fold increase in weight, was observed in CD31+/− mice 15 h only after exposure to E2 + P4, when compared with results from CD31+/+ mice (Fig. 4A and B). Unlike the results seen 4 h after P4 treatment (Fig. 3B), E2-induced RPS phosphorylation was significantly abolished at 15 h following treatment with E2 + P4 in both groups (Fig. 4C and D and Supplementary Fig. 4). The E2-induced DNA synthesis was mediated by the paracrine pathway of IGF, resulting in the nuclear accumulation of cyclin D1 through the inhibitory phosphorylation of GSK-3β, followed by the activation of cyclin E and cyclin A (Chen et al. 2005). Our data showed that E2 increased the expression levels of cyclin D, cyclin E, and cyclin A in the uterus of both groups, in an equivalent manner (Fig. 4C and Supplementary Fig. 4). However, expression levels of cyclin D, cyclin E, and cyclin A were significantly higher in CD31+/− mice after exposure to both hormones (Fig. 4C and Supplementary Fig. 4). PCNA, a marker for cell proliferation, was stained in the nuclei of luminal and glandular epithelial cells in both groups after being treated with E2 alone (Fig. 4E, left), whereas treatment with E2 + P4 completely abolished the E2-induced nuclear localization of PCNA in the epithelial cells of the uterus, but not in the stromal cells, of both groups. However, we found that E2 + P4 treatment significantly increased the nuclear translocation of PCNA in the stromal cells of CD31+/− mice when compared to CD31+/+ mice (Fig. 4E, right).
Figure 2 Increase in STAT3 phosphorylation and decrease in SHP phosphorylation in CD31^{+/−} mice. Ovariectomized CD31^{+/+} and CD31^{−/−} mice were injected with vehicle or E_{2}+P_{4}. These mice were sacrificed at 4 and 8 h after E_{2}+P_{4} injection and STAT3 phosphorylation levels and LIF expression levels were measured. (A) Western blotting was performed with anti-p-SHP-2, total-SHP-2, p-STAT3, total-STAT3, LIF, HAND2, and GAPDH. (B) Quantitation of band intensities of p-SHP-2 was normalized against total-SHP-2; p-STAT3 was normalized against total-STAT3; LIF and HAND2 were normalized against GAPDH. (C) Real-time PCR was performed to measure LIF mRNA expression levels. n = 3 mice. Data are means ± s.d. Statistical analysis was performed using the t-test. *P<0.05, **P<0.01, ***P<0.001.

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![Western blot images](image_url)
These findings suggest that the $E_2 + P_4$-induced uterine hyperplasia induces DNA synthesis in the uterus of CD31 deficient mice.

**Loss of CD31 upregulates cyclin D, cyclin E, and cyclin A at 3.5 dpc in the uterus**

Next, to determine whether the uterine hyperplasia and DNA synthesis in response to $E_2$ and $P_4$ could also be found in early pregnancy of $CD31^{-/-}$ mice, we performed Western blot for cyclin D, cyclin E and cyclin A. Although expression levels of cyclin D did not significantly increased in $CD31^{-/-}$ mice, cyclin E and cyclin A were significantly increased in $CD31^{-/-}$ mice compared to $CD31^{+/+}$ mice (Fig. 5) at 3.5 dpc. These findings are consistent with precedent data, suggesting that CD31 deficiency increases uterus receptivity for implantation through uterine hyperplasia and cell proliferation.

**Discussion**

The steroid hormones $E_2$ and $P_4$ sequentially coordinate epithelial and stromal functions via multiple paracrine, juxtacrine, and autocrine factors in a spatiotemporal manner. The bidirectional communication between the stroma and epithelium is essential for successful implantation. One of the major mediators of $E_2$ action is LIF. LIF is essential for uterine receptivity, as $LIF^{-/-}$ females are infertile as well as impaired blastocyst implantation (Stewart et al. 1992). Our data indicated that the LIF levels of $CD31^{-/-}$ mice were increased vs $CD31^{+/+}$ mice at 3.5 dpc. Moreover, uterine LIF expression levels were significantly increased by $E_2$ treatment in ovariectomized $CD31^{-/-}$ mice, suggesting that CD31 plays a role in implantation by regulating LIF.

Although there are many studies which show that LIF increases with $E_2$ and $P_4$, the exact mechanism has not yet been discovered. HAND2, which is increased...
Figure 4  Uterine hyperplasia in CD31−/− mice. (A and B) Ovariectomized CD31+/+ and CD31−/− mice were sacrificed at 15 h after the following treatments: Con, E2, and P4+E2. (A) Transverse sections of the uterus were stained with hematoxylin-and-eosin. (B) Uterine weights were determined. n = 4–5 mice. (C) Western blot analysis was performed with uterine lysates at 15 h following treatment with anti-p-RPS (Ser235/236), cyclin D, cyclin E and cyclin A Abs. (D) Uterine sections were immuno-stained with anti-CD105 (red) and anti-p-RPS (green) Ab, and DAPI (blue). CD105 was used for blood vessel marker. (E) Uterine sections obtained from CD31+/+ and CD31−/− mice at 15 h after hormone treatment were subjected to IHC using an antibody against PCNA (green). Magnified images of uterine sections after being treated with E2, or P4+E2 (right panels). Data are means ± s.d. Statistical analysis was performed using the t-test. *P<0.05, **P<0.01, ***P<0.001.
by $P_4$ (Li et al. 2011), is known to inhibit FGFs in $E_2$ downstream signals. However, it has also been shown to increase LIF. We found that LIF, as well as HAND2, were significantly increased and maintained at 8 h after $E_2+P_4$ treatment in CD31$^{-/-}$ mice when compared to CD31$^{+/+}$ mice (Fig. 2A). However, we do not know why HAND2 was only increased in CD31$^{-/-}$ mice after $E_2+P_4$ treatment.

p-STAT3 is known to translocate to the nucleus and increase proliferation-associated gene expression (Annerén 2008, Hiraoka et al. 2016). Accordingly, reduced p-STAT3 can induce infertility (Sun et al. 2013, Robker et al. 2014). Interestingly, p-STAT3 was not decreased at 8 h after $E_2+P_4$ treatment in CD31$^{-/-}$ mice (Fig. 2A). Given that p-SHP2 is known to dephosphorylate p-STAT3, and SHP-2 is known to be phosphorylated by CD31 and LIFR, it can be assumed that p-STAT3 levels were maintained due to the decreased p-SHP, in the absence of CD31 (Fig. 2A).

$E_2$-induced DNA synthesis commences at about 6 h and peaks at 12–15 h (Martin et al. 1973). $E_2$ treatment induces the proliferation of both uterine epithelium and stroma in immature mice, but only stimulates the proliferation of epithelium in adult mice (Quarmby & Korach 1984). Our studies with ovarioctomized mice indicate that exposure to $E_2$ similarly increased the wet weight of the uterus 6 h after treatment in both CD31$^{+/+}$ and CD31$^{-/-}$ mice (Fig. 1D and E). However, the ablation of CD31 led to an increase in uterine weight 15 h after $E_2+P_4$ treatment (Fig. 4A), suggesting that uterine CD31 negatively impacts the uterine growth response.

Recent studies report that $E_2$ induces protein synthesis through the PKC-ERK1/2-mTOR signaling pathway (Wang et al. 2015). Our data showed that the phosphorylation of mTOR, and RPS6 were increased by $E_2$ in both CD31$^{+/+}$ and CD31$^{-/-}$ mice to the same extent (Fig. 3). Our data showed that the $E_2$-induced phosphorylation of mTOR and RPS6 was inhibited at 4 h and 15 h after $P_4$ treatment in CD31$^{+/+}$ mice (Figs 3A and B and 4B and C). However, the $E_2$-induced phosphorylation of mTOR and RPS6 was not blocked at 4 h after $P_4$ treatment in CD31$^{-/-}$ mice, although it was eventually inhibited at 15 h post-treatment. Therefore, these findings suggest that activation of mTOR signaling occurs in the uterus of CD31$^{-/-}$ mice, even after $P_4$ treatment, thereby resulting in the hyperplasia of the uterus. In other words, these findings indicate that CD31 plays its role via SHP-2 in the $P_4$-mediated regulation of uterine hyperplasia.

$E_2$-induced uterine epithelial cell proliferation is completely suppressed by $P_4$, and $P_4$ signaling is essential in order for stromal cell proliferation to respond to $E_2$ (Tong & Pollard 1999, Chen et al. 2005). $E_2$ couples to the cell cycle regulatory mechanisms via the PI3K-AKT-GSK3β-cyclin D1 pathway to the uterine epithelium (Chen et al. 2005). While $E_2$ up-regulates the expression levels of cyclin D1, E, and A, and also induces the nuclear accumulation of cyclin D1 and cyclin A (Tong & Pollard 1999), $P_4$ abolished the $E_2$-induced cyclin A up-regulation and completely inhibited the nuclear localization of cyclin D1 and A in the epithelium. Moreover, $E_2$ also induced the nuclear accumulation of cyclin D1 and cyclin A in stromal cells in the presence of $P_4$ (Chen et al. 2005). Our data showed that $E_2$ increased the expression levels of cyclin D, cyclin E, and cyclin A in the uteri of CD31$^{+/+}$ and CD31$^{-/-}$ mice in an equal manner (Fig. 4B). However, cyclin D1 and E, and A protein accumulation after $E_2+P_4$ treatment were increased significantly more in
CD31−/− mice vs CD31+/+ mice. Likewise, while E2-induced nuclear translocation of PCNA was equivalently detected in the uterine epithelium of both CD31+/+ and CD31−/− mice, P4 greatly increased this translocation in the stromal cells of CD31−/− mice when compared to CD31+/+ mice (Fig. 4D). Previous studies suggest that an increase in protein synthesis is essential for E2-induced proliferation (Wang et al. 2015). Taken together, these data suggest that the activation of mTOR signaling by E2 and P4 augments cell proliferation in the uterine stroma of CD31−/− mice, resulting in increased uterine weight.

In conclusion, CD31+/+ mice showed higher pregnancy rates, with enhanced STAT3, HAND2, LIF, mTOR, and cyclin series signals (Fig. 6), compared to CD31−/− female mice. Our results show that, upon receiving the P4 signal, CD31 regulates uterine receptivity by inhibiting E2-mediated uterine epithelial cell proliferation via SHP-2 during early pregnancy and may increase our understanding of hitherto unknown post-P4 signaling in early pregnancy.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-17-0419.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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