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-17 β (*E*₂) + progesterone (*P*₄) in ovariectomized *CD31*^{-/-} mice, compared with *CD31*^{+/+} mice at 8 h after hormone treatment. *E*₂-induced protein synthesis was inhibited by *P*₄ in the *CD31*^{+/+} uterus, but not in the uterus of *CD31*^{-/-} mice. Also, STAT3, HAND2, LIF, and mTOR signals were enhanced in *CD31*^{-/-} mice. Stromal DNA replication was highly activated in the uterus of *CD31*^{-/-} mice, manifested by upregulated cyclin series signaling and PCNA expression after *E*₂ + *P*₄ treatment. Collectively, CD31 inhibits *E*₂-mediated epithelial proliferation via recruitment and phosphorylation of SHP-2 upon receiving *P*₄ 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-17 β (*E*₂) stimulates the proliferation of luminal and glandular epithelium at 1 day postcoitum (dpc) (Zhu *et al.* 2007). *E*₂, acting on stromal cells, initiates responses to mediate epithelial proliferation (Winuthayanon *et al.* 2010). *E*₂-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 *E*₂ treatment (Winuthayanon *et al.* 2010). *E*₂ 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 GSK3 β 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 (*P*₄) levels from the corpus luteum at 3 dpc inhibit the *E*₂-induced proliferation of uterine epithelial cells and initiate stromal cell proliferation. At the same time, *P*₄ 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 *E*₂-induced proliferation of the uterine epithelium and the induction of stroma proliferation by *P*₄ is essential for successful embryo implantation. In the first pathway of the anti-proliferative action of *P*₄ in the uterine epithelium, *P*₄ inhibits the activation of PI3K-AKT-GSK3 β 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 *E*₂ is mediated by PKC, which in turn induces ERK1/2 phosphorylation and thereby activates the mammalian target of rapamycin (mTOR). They also showed that *E*₂-stimulated protein synthesis was independent of the inhibition of epithelial proliferation by *P*₄.

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 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 2004) and embryo-endometrial interaction (Cullinan *et al.* 1996). 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 mice 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 *E*₂, and expression was evident in the luminal epithelium of the uterus at 1dpc and in the glandular epithelium at 4dpc (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 (Sunngam, 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 *E*₂; 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, (*E*₂ group) treatment with 50 ng *E*₂ on day 6, (*P*₄ group) on days 3–6, mice were treated with 1 mg *P*₄, and the other group (*P*₄ and *E*₂ group) was treated with 1 mg *P*₄ on days 3–6, and on day 6, they were treated with 50 ng *E*₂. 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 ovariectomized $CD31^{+/+}$ 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 Na_3VO_4 , and a protease inhibitor cocktail (Roche). Tissue lysates were centrifuged at 15,000g 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 signaling), p-RPS (Ser235/236) (Cell signaling), p-RPS6K1 (Thr421/Ser424) (Cell signaling), total RPS (Cell signaling), total mTOR (Cell signaling), p-mTOR (Cell signaling), 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 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](#)). E_2 and P_4 levels were measured to confirm whether difference of pregnancy rate and uterus weight were due to difference of E_2 and P_4 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

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. $n=13$ –26 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 $\pm$ 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 $E_2 + P_4$. 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 P_4 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 $E_2 + P_4$ 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

E_2 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 E_2 - or/and P_4 -induced signaling. Western blots of total uterine tissue at 4 h after E_2 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 E_2 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 E_2 in CD31^{+/+} and CD31^{-/-} mice (Fig. 3A and Supplementary Fig. 3). In contrast, the increases in the phosphorylation of mTOR, RPS, and RPS6K1 by E_2 were inhibited by P_4 4 h after

treatment in CD31^{+/+} mice, whereas the E_2 -induced phosphorylation of mTOR, RPS, and RPS6K1 in CD31^{-/-} mice were not blocked by treatment with P_4 (Fig. 3A and Supplementary Fig. 3). Consistent with the Western blot data, immunohistochemistry performed with anti-p-RPS (Ser235/236) Abs showed that E_2 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 E_2 -induced RPS phosphorylation was completely abolished by treatment with P_4 in CD31^{+/+} mice, whereas CD31^{-/-} mice showed persistent RPS phosphorylation at 4 h after P_4 treatment. These data suggest that E_2 -induced mTOR signaling in the uterus of CD31^{-/-} mice is not blocked by P_4 , as opposed to CD31^{+/+} mice.

CD31-deficient mice exhibit uterine hyperplasia

P_4 completely suppresses E_2 -induced epithelial proliferation, but sensitizes the stromal cells to E_2 for proliferation (Tong & Pollard 1999, Chen et al. 2005). E_2 -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 $E_2 + P_4$ treatment would affect DNA synthesis in CD31^{-/-} mice. Histological analysis showed similar overall uterine areas in CD31^{+/+} and CD31^{-/-} mice 15 h following E_2 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 $E_2 + P_4$, when compared with results from CD31^{+/+} mice (Fig. 4A and B). Unlike the results seen 4 h after P_4 treatment (Fig. 3B), E_2 -induced RPS phosphorylation was significantly abolished at 15 h following treatment with $E_2 + P_4$ in both groups (Fig. 4C and D and Supplementary Fig. 4). The E_2 -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-3 β , followed by the activation of cyclin E and cyclin A (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 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 E_2 alone (Fig. 4E, left), whereas treatment with $E_2 + P_4$ completely abolished the E_2 -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 $E_2 + P_4$ 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 $\pm$ s.d. Statistical analysis was performed using the *t*-test. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Figure 3 Activation of the mTOR pathway in *CD31*^{-/-} mice. Western blot analysis was performed with uterine lysates to determine the phosphorylation status of mTOR, RPS, and RPS6K1 in the uteri of *CD31*^{+/+} and *CD31*^{-/-} mice. GAPDH was used as a loading control. (A) Ovariectomized mice were sacrificed at 4 h after the following treatments: Con (vehicle), *P*₄, *E*₂, or *P*₄ + *E*₂. (B) Immunohistochemistry of the p-RPS protein in the uterine sections from ovariectomized mice treated with vehicle, *E*₂, or *P*₄ + *E*₂. Ovariectomized mice were killed at 4 h after hormone treatment and the uterine tissues were subjected to frozen sectioning and immunostained against p-RPS (Ser235/236).

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 increase 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) Abs, 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.

Figure 5 Upregulation of cyclin D, cyclin E, and cyclin A in uterus at 3.5 dpc in *CD31*^{-/-} mice. (A) Uterine lysate obtained from mice on 3.5 dpc of pregnancy were subjected to Western blotting using an antibody against cyclin D, cyclin E, and cyclin A. Quantitation of Western band intensities were normalized against GAPDH in each group. Data are means $\pm$ 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 FGs 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 ovariectomized 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-GSK3 β -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

Figure 6 A model of the action of CD31 on uterine development in early pregnancy. This figure summarizes data regarding the cooperative action of CD31 on P_4 signaling in uterine epithelial cells. The P_4 signal inhibits E_2 -mediated epithelial proliferation via CD31, which recruits and phosphorylates SHP-2. E_2 is coupled to cell cycle machinery and cell proliferation via the paracrine mechanisms of IGF-I and LIF, respectively, and also induces protein synthesis through the mTOR signaling pathway.

$CD31^{-/-}$ mice vs $CD31^{+/+}$ mice. Likewise, while E_2 -induced nuclear translocation of PCNA was equivalently detected in the uterine epithelium of both $CD31^{+/+}$ and $CD31^{-/-}$ mice, P_4 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 E_2 -induced proliferation (Wang *et al.* 2015). Taken together, these data suggest that the activation of mTOR signaling by E_2 and P_4 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 P_4 signal, CD31 regulates uterine receptivity by inhibiting E_2 -mediated uterine epithelial cell proliferation via SHP-2 during early pregnancy and may increase our understanding of hitherto unknown post- P_4 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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