Uterine activin receptor-like kinase 5 is crucial for blastocyst implantation and placental development

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Members of the transforming growth factor β (TGF-β) superfamily are key regulators in most developmental and physiological processes. However, the in vivo roles of TGF-β signaling in female reproduction remain uncertain. Activin receptor-like kinase 5 (ALK5) is the major type 1 receptor for the TGF-β subfamily. Absence of ALK5 leads to early embryonic lethality because of severe defects in vascular development. In this study, we conditionally ablated uterine ALK5 using progesterone receptor-cre mice to define the physiological roles of ALK5 in female reproduction. Despite normal ovarian functions and artificial decidualization in conditional knockout (cKO) mice, absence of uterine ALK5 resulted in substantially reduced female reproduction due to abnormalities observed at different stages of pregnancy, including implantation defects, disorganization of trophoblast cells, fewer uterine natural killer (uNK) cells, and impairment of spiral artery remodeling. In our microarray analysis, genes encoding proteins involved in cytokine–cytokine receptor interactions and NK cell-mediated cytotoxicity were down-regulated in cKO decidua compared with control decidua. Flow cytometry confirmed a 10-fold decrease in uNK cells in cKO versus control decidua. According to these data, we hypothesize that TGF-β acts on decidual cells via ALK5 to induce expression of other growth factors and cytokines, which are key regulators in luminal epithelium proliferation, trophoblast development, and uNK maturation during pregnancy. Our findings not only generate a mouse model to study TGF-β signaling in female reproduction but also shed light on the pathogenesis of many pregnancy complications in human, such as recurrent spontaneous abortion, preeclampsia, and intrauterine growth restriction.

Significance

Although many studies have yielded tremendous insights into the roles of TGF-β superfamily signaling pathways in physiologial and pathophysiological processes, the in vivo roles of TGF-β signaling pathways in many aspects of reproduction remain largely unknown. To address these functions in females, we conditionally deleted the TGF-β type 1 receptor (activin receptor-like kinase 5, ALK5) and demonstrated that absence of TGF-β signaling through ALK5 in the uterus leads to striking abnormalities at different stages of pregnancy, including delayed implantation, disorganization of the trophoblast cells, significantly fewer uterine natural killer cells, and defects in spiral artery remodeling. Our findings provide a mouse model to investigate TGF-β signaling in reproduction and pave the way toward a better understanding of the pathogenesis of pregnancy-related complications in women.

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subfamily, and Alk5 null mice are embryonic lethal because of severe vascular defects (19). In this paper, we conditionally ablated uterine ALK5 to study its important roles during pregnancy. Deletion of uterine ALK5 led to multiple abnormalities during pregnancy including early implantation defects, disorganization of trophoblast cells, a significant reduction in the number of uNK cells, and impairment of spiral artery remodeling.

**Results**

**Generation of Alk5 cKO Mice and Alk5 Expression in the Uterus.** Because complete loss of ALK5 results in embryonic lethality (19), we generated an Alk5 conditional knockout (cKO) mouse model using PR-Cre and an Alk5 floxed allele in which exon 3 is flanked by LoxP sites (Fig. 1A and B). Previous studies indicated that PR-Cre is expressed postnatally in the anterior lobe of pituitary glands, the corpus luteum, oviducts, and epithelial, stromal, and myometrial cellular compartments of uteri (18). To confirm PR-Cre mediated recombination in the female reproductive system, we performed PCR on DNA extracted from tails (as negative controls) and organs in the female reproductive tract of cKO mice (Fig. 1C). An Alk5 floxed allele was detected in the tail, ovary, oviduct, and uterus, whereas an Alk5 null allele was only detected in the female reproductive tract. Efficiency of Alk5 deletion was further examined by real-time quantitative PCR (qPCR) to compare the mRNA levels among different organs (Fig. 1D–G). qPCR primers were designed to detect the deleted exon 3 of Alk5. Significant reduction of the Alk5 mRNA level was only detected in the uterus of virgin females as well as the decidua of pregnant females (Fig. 1F and G), although PR-Cre was expressed in the entire female reproductive tract.

Previous studies have characterized the ALK5 expression pattern in the ovary, oviduct, and uterus by using an Alk5βgal knock-in allele (20). However, uterine ALK5 expression during pregnancy still remains unknown. Thus, we performed qPCR to measure decidual Alk5 mRNA levels measured by qPCR in wild-type decidua from 5.5 to 10.5 dpc. Data are presented as mean ± SEM (n = 3). **P < 0.01; ***P < 0.001 compared with controls. (H) Relative Alk5 mRNA levels measured by qPCR in wild-type decidua from 5.5 to 10.5 dpc. Data are presented as mean ± SEM (n = 3). (I–N) Expression and localization of uterine ALK5 were examined by immunofluorescence at sequential time points. Asterisk indicates an embryo. AM, antimesometrium; De, decidua; GE, glandular epithelium; LE, luminal epithelium; Lu, lumen; M, mesometrium; My, myometrium; S, stroma; TB, trophoblast. (Scale bar, 100 μm.)

Fig. 1. Generation of the Alk5 cKO female mice and expression of uterine ALK5. (A) Schematic representation of the breeding strategy to generate control and cKO females. (B) The Alk5 null allele was generated by PR-Cre–mediated recombination. (C) PCR analysis for Alk5 floxed and null alleles. OD, oviduct; OV, ovary; TL, tail; UT, uterus. (D–G) Relative Alk5 mRNA levels in the ovary, oviduct, uterus, and 8.5 dpc decidua of control and cKO females. Data are presented as mean ± SEM (n = 3). **P < 0.01; ***P < 0.001 compared with controls. (H) Relative Alk5 mRNA levels measured by qPCR in wild-type decidua from 5.5 to 10.5 dpc. Data are presented as mean ± SEM (n = 3). **P < 0.01; ***P < 0.001 compared with controls. (I–N) Expression and localization of uterine ALK5 were examined by immunofluorescence at sequential time points. Asterisk indicates an embryo. AM, antimesometrium; De, decidua; GE, glandular epithelium; LE, luminal epithelium; Lu, lumen; M, mesometrium; My, myometrium; S, stroma; TB, trophoblast. (Scale bar, 100 μm.)
Table 1. Six-month breeding trial with wild-type males

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Females</th>
<th>Pups</th>
<th>Litters</th>
<th>Pups/litter</th>
<th>Litters/female</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>365</td>
<td>43</td>
<td>8.49 ± 0.31</td>
<td>6.14 ± 0.26</td>
</tr>
<tr>
<td>cKO</td>
<td>12*</td>
<td>9</td>
<td>3</td>
<td>3.00 ± 1.00</td>
<td>0.25 ± 0.13</td>
</tr>
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*Five females died secondary to abnormal vaginal bleeding during pregnancy.

in stromal cells (Fig. 1J). When the embryo attaches to the uterine wall at 4.5 dpc, Alk5 started to be expressed in the primary decidual zone at the antimesometrial (AM) pole (Fig. 1J). Alk5 expression extended to the decidual cells at the mesometrial (M) pole at 5.5 dpc (Fig. 1K) and was subsequently restricted to the AM decidua at 6.5 dpc (Fig. 1L). Consistent with the previous qPCR results, Alk5 protein levels gradually declined after 6.5 dpc (Fig. 1 M and N).

**Alk5 cKO Female Mice Exhibited Severe Defects in Fertility but Displayed Normal Histology of the Female Reproductive System.** To evaluate the fertility of Alk5 cKO female mice, we performed a continuous breeding study for control and cKO female mice (Table 1). We mated 6-wk-old female mice (n = 7 for control and n = 12 for cKO) with known fertile wild-type male mice for 6 mo. The seven control females had normal breeding activity during the test period. In contrast, ablation of uterine Alk5 led to sterility in 4 of the 12 females tested; 3 of the 12 females produced a total of 9 pups during the 6-mo breeding period, and the other 5 females died secondary to abnormal vaginal bleeding during pregnancy. Vaginal plugs were observed at similar frequencies between the two genotypes, which eliminated the possibility of abnormal mating behavior.

We investigated ovarian function to find out the possible cause of the severe fertility defects in the Alk5 cKO females. In histological sections of the AM of cKO uterus from normal pregnant uterine, and nonpregnant uterine structures (Fig. S1 A–H), indicating that the severe fertility defects in Alk5 cKO female mice were likely caused by uterine defects during pregnancy. Serum P4 and E2 levels were measured at different time points during pregnancy (Fig. S1 I and J). No significant variation of P4 was detected between control and cKO female mice (Fig. S1J). The two groups exhibited comparable E2 levels in early pregnancy, whereas the increase of E2 with gestational age was slightly lower in the cKO female mice, suggesting lower E2 production during placental development (Fig. S1J).

**Alk5 cKO Females Exhibited Extra Epithelial Folds and Luminal Closure Defects During Peri-Implantation.** Because there were no apparent defects in the reproductive tract of nonpregnant female mice, we next investigated the potential role of Alk5 in early implantation. Embryo attachment leads to a local increase in capillary permeability, so implantation sites can be visualized at early stages of pregnancy. Vaginal plugs were observed at similar frequencies between the two genotypes, which eliminated the possibility of abnormal mating behavior.

By 6.5 dpc, the size of the cKO implantation sites appeared comparable to controls (Fig. 2C).

During blastocyst attachment, uterine luminal closure occurs, resulting in formation of the implantation chamber (2). In our histological analysis, luminal closure was observed in control uterus at 4.5 dpc, which led to the closure of the uterine lumen with an AM–M axis (Fig. 2D). Meanwhile, incomplete luminal closure and extra epithelial folds were found in cKO uteri (Fig. 2E). At 5.5 dpc, the extra epithelial folds almost disappeared in cKO females (Fig. 2 F and G), and no obvious defect was observed by 6.5 dpc (Fig. 2 H and I). To further study the implantation defects in Alk5 cKO mice, we examined prostaglandin-endoperoxide synthase 2 (PTGS2, an implantation marker) and cytokeratin 8 (CK8, an epithelial marker) protein expression and localization by immunofluorescence. PTGS2 has a dynamic localization correlated with different stages during implantation (22). At 4.5 dpc, PTGS2 was expressed in subepithelial stromal cells at the AM pole in controls (Fig. 2F). Besides the AM pole, PTGS2 was also localized around the extra epithelial fold in cKO implantation sites (Fig. 2K). At 5.5 dpc, PTGS2 shifted to decidual cells at the M pole in controls (Fig. 2L), but the shift of PTGS2 localization was delayed in cKO uteri, with PTGS2 remaining at the AM pole (Fig. 2M). At 6.5 dpc, PTGS2 was only visualized at the M pole in both control and cKO implantation sites (Fig. 2 N and O). Thus, the different expression pattern of PTGS2 in control and cKO suggested that lack of uterine ALK5 led to a delayed implantation.

Because the process of luminal closure during implantation is mainly controlled by P4 and E2 (2), we hypothesized that the lack of uterine lumen closure in Alk5 cKO females resulted from an abnormal P4 and/or E2 response. To test this hypothesis, we first examined the uterine expression of Esr1 (the gene encodes estrogen receptor α) and Pgr (the gene encodes PR) at 4.5 dpc. The mRNA levels of Esr1 were significantly elevated in Alk5 cKO females, whereas Pgr expression was comparable in the two genotypes (Fig. 2P). We tested another two E2-responsive genes, Mac1 and Lf (23), and found their expression was significantly increased in Alk5 cKO females (Fig. 2P). Several fibroblast growth factors (FGFs) have been previously reported to promote luminal epithelial proliferation via a paracrine manner (24), so we examined those FGF genes and found Fgfl8 was up-regulated in the Alk5 cKO uteri (Fig. 2P). To characterize possible defects of initial decidualization during implantation, we tested two decidual marker genes, Bmp2 and Wnt4 (16, 25). Bmp2 was significantly decreased in the cKO uteri, whereas Wnt4 expression was similar in the two genotypes (Fig. 2P). We also performed immunohistochemical examination to examine the expression of phosphorylated estrogen receptor α (pERα) and Kit67 (a proliferation marker) at 3.5 dpc. pERα was only detected in the stroma of control uterus (Fig. 2Q), but pERα-positive epithelial cells were also found in the cKO uterus (Fig. 2R). As we expected, Kit67 was only expressed in the stroma of control uterus (Fig. 2S), whereas Alk5 cKO females highly expressed Kit67 in uterine epithelial cells (Fig. 2T). Collectively, these data indicate that the defective luminal closure and increased epithelial folding are likely associated with enhanced epithelial proliferation caused by increased uterine E2 response during peri-implantation.

**Alk5 cKO Females Exhibited Fetal and Placental Abnormalities in Postimplantation.** To determine if there were defects later during pregnancy in Alk5 cKO mice, we determined the number and weight of implantation sites from 7.5 to 10.5 dpc (Fig. 3 A and B and Fig. S2 A–D). At 7.5 dpc, implantation sites from the two groups showed similar numbers and normal weight. However, cKO females began to show fewer and smaller implantation sites at 8.5 dpc, and the differences between the two groups became more substantial over time. By 10.5 dpc, cKO implantation sites dropped to 60% in number and 50% in weight compared with controls. Furthermore, fetuses dissected from Alk5 cKO females exhibited severe intrauterine growth restriction (IUGR) at 10.5 dpc (Fig. 3C). Because the fetuses are not mutants, conditional ablation of Alk5 in the maternal uterus led to the poor fetal growth.

To further characterize the uterine defects in postimplantation stages, we performed histological analysis of the implantation sites from 7.5 to 10.5 dpc (Fig. 3 D–K and Fig. S2 E–L). At 7.5 dpc, uterine decidualization, embryonic development, and extraembryonic structures appeared comparable between control and cKO females (Fig. 3 D and E and Fig. S2 E and F). Beginning at 8.5 dpc, hemorrhage and unlosed uterine lumens were observed in the cKO implantation sites (Fig. 3 F and G), concomitant with shallow
trophoblast invasion (Fig. S2 G and H). The hemorrhage was more extensive at 9.5 dpc, and we also observed a thicker AM uterine wall, which could restrict fetal growth (Fig. 3 H and I and Fig. S2 J and K). At 10.5 dpc, cKO females exhibited apparent abnormalities of the placenta, including expanded giant cells and an attenuated labyrinth layer (Fig. 3 J and K and Fig. S2 L and M).

Immunofluorescence analysis of PL-1 (a marker for trophoblast giant cells) and TPBPA (a marker for spongiotrophoblasts) was also performed to examine the critical role of ALK5 in postimplantation placentation (Fig. 3 L–S). No visible abnormalities were observed in the implantation sites at 7.5 dpc (Fig. 3 L and M), whereas Alk5 cKO started to show shallow trophoblast invasion at 8.5 dpc (Fig. 3 N and O). TPBPA+ spongiotrophoblasts began to appear at 9.5 dpc in the two genotypes (Fig. 3 P and Q). At 10.5 dpc, controls had mature placentas with extended TPBPA+ spongiotrophoblast cells and a very thin layer of PL-1+ trophoblast giant cells (Fig. 3 R). In addition to the labyrinth attenuation, fewer TPBPA+ spongiotrophoblasts were observed in the center of Alk5 cKO placenta (Fig. 3 S). The excessive trophoblast giant cells likely restricted the lateral extension of spongiotrophoblasts. Alternatively, it also could be secondary to more differentiated trophoblast giant cells at the expense of the TPBPA+ progenitor cells.

**Deletion of Uterine ALK5 Resulted in Defects in uNK Cell Differentiation and Spiral Artery Remodeling.** uNK cells are the most abundant types of immune cells at the maternal–fetal interface in early pregnancy (26). To investigate whether ALK5 is required for...
uNK recruitment and differentiation during decidualization, we used two well-established methods for detecting uNK cells, Dolichos biflorus agglutinin (DBA) and Periodic acid Schiff (PAS) staining (5, 27). Whereas a large number of DBA+ uNK cells were detected in the control implantation sites by 9.5 dpc (Fig. 4A and C), there were very few DBA+ uNK cells in the cKO decidua basalis (Fig. 4B and D). Similarly, many PAS+ uNK cells with granules were present in the control implantation sites (Fig. 4E); PAS+ uNK cells were almost absent in the decidua of Alk5 cKO females (Fig. 4F). Thus, our data suggest that ALK5 is likely to be essential for precursor NK cell recruitment and uNK cell differentiation in the implantation sites.

Loss of uNK cells has been shown to cause structural abnormalities in pregnancy-induced modification of decidual arteries including smooth muscle thickening and luminal narrowing (8). To further establish the roles of ALK5 in spiral artery remodeling during placentation, we performed immunohistochemical analysis to examine the vascular defects with different molecular markers (Fig. 4G–N). Upon staining for smooth muscle actin (SMA; a marker for smooth muscle cells within the vessel wall), we observed thinning and discontinuous smooth muscle cells of the arterial wall in controls (Fig. 4G and I), indicating increased vessel permeability to facilitate nutrient exchange between fetus and mother. By contrast, thicker smooth muscle and smaller vessel lumen of uterine arteries were found in the cKO decidua basalis (Fig. 4H and J). CD31 (a marker for endothelial cells) was used to confirm the lack of large vessels in Alk5 cKO (Fig. 4K and L). Furthermore, impaired formation of fetal blood vessels was also detected in cKO implantation sites by staining with laminin (a basement membrane marker) (Fig. 4O–R).

Normal Artificial Decidualization but an Absence of uNK Cells Were Found in Alk5 cKO Uteri. Uterine luminal closure defects were observed in Alk5 cKO females, indicating possible impairment in decidualization. We artificially induced a decidual reaction to investigate roles of ALK5 in decidual cell proliferation and differentiation as well as to eliminate possible effects from the fetus (Fig. 5A). During artificial decidualization, one uterine horn is traumatized by a needle to mimic embryo attachment, and the other uterine horn is intact and serves as a control. Four days later, the decidual reaction is evaluated. As shown, both of the genotypes responded well to the mechanical stimulation (Fig. 5B),

Fig. 3. Defects in decidualization and placentation were observed in the Alk5 cKO females. (A and B) The number and weight of implantation sites were recorded from 7.5 to 10.5 dpc. Data are presented as mean ± SEM (n = 5). *P < 0.05; **P < 0.001 compared with controls. (C) IUGR in the Alk5 cKO uterus was observed at 10.5 dpc. (Scale bar, 1.0 mm.) (D–K) Histological analysis of implantation sites from 7.5 to 10.5 dpc. Blue arrow indicates unclosed uterine lumen, red arrow indicates hemorrhage, and blue dashed area indicates placental labyrinth. (Scale bar, 1.0 mm.) (L–S) Immunofluorescence analysis of PL-1 and TPBPA. (Scale bar, 500 μm.) Asterisk indicates embryo or fetus. AM, antimesometrium; M, mesometrium.
and there was no significant difference in uterine weight (Fig. 3C) or histological analysis (Fig. 5 D–G). Similar levels of Ki67 and alkaline phosphatase (ALP; a differentiation marker) were detected in the two genotypes (Fig. 5 H–K). Notably, uNK cells were almost absent in cKO decidua after artificial induction, indicating that the critical roles of ALK5 in uNK recruitment and maturation are independent of the defects observed in trophoblasts and embryos (Fig. 5 L–G).

**uNK Deficiency in Alk5 cKO Was Confirmed by Gene Expression Profiling and Flow Cytometry.** To investigate the downstream pathways of TGF-β signaling via ALK5 during the decidualization process, we used microarrays to examine the gene expression profile in control and cKO decidua basalis collected at 8.5 dpc. As shown in the heat map, we found that the expression of 862 unique genes was significantly affected by the uterine deletion of Alk5 (Fig. 6A). To extract biological insight from the 862 genes, we performed Gene Set Enrichment Analysis (GSEA) based on Kyoto Encyclopedia of Genes and Genomes (KEGG) gene sets and identified five main pathways (Fig. 6B). Of note, our data showed significant reduction in cytokine–cytokine receptor and NK cell-mediated cytotoxicity, which confirmed the reduced number of uNK cells and vascular abnormalities previously observed in the Alk5 cKO mice. To better understand the biological importance of the five main pathways, we further grouped...
leading edge subsets to show gene sets as core members of each pathway (Fig. 5C). qPCR was performed to confirm the expression of genes involved in uNK maturation and spiral artery remodeling (Fig. 6D). mRNA levels of Acta2, the gene encoding SMA, were increased more than twofold in Alk5 cKO decidua, indicating defects in the attenuation of the smooth muscle layer during vascular remodeling. IL-15 is a critical cytokine for NK cell differentiation (28). In Alk5 cKO, Il15 mRNA was substantially decreased, which explains the reduction of uNK during decidualization. As we expected, other NK-specific genes, including Prf1 (29), Klrg1 (30), Nkg7 (31), and CtsG (32), and several granzyme genes (GzmA, GzmB, GzmD, GzmE, GzmF, and GzmG) (33) were significantly down-regulated in Alk5 cKO decidua. Previous studies reported that CORIN (an atrial natriuretic peptide-converting enzyme) is essential for physiological changes at the maternal–fetal interface, and Corin-deficient mice mimic the features found in preeclampsia patients (34). Advillin (AVIL), an actin binding protein involved in cell morphogenesis (35), is located in uNK cells during decidualization (36). Herein, we observed that both Corin and Avil mRNA levels were substantially decreased in Alk5 cKO females, suggesting the critical role of ALK5 in uNK maturation and spiral artery remodeling.

To further characterize and quantify the immune cell populations, we applied flow cytometry to compare the different leukocyte subsets including uNK cells, T cells, macrophages, and dendritic cells in both control and Alk5 cKO decidua at 8.5 dpc (Fig. 6E). By gating cells that are DBA+ cells but excluding CD3+ cells, we observed that the DBA+ CD3− uNK cells in Alk5 cKO decidua were reduced to about 10% of the control decidua (Fig. 6F and G). However, other types of leukocytes including T cells (CD3+), macrophages, and dendritic cells (CD11c+) were not significantly altered (Fig. 6E). Thus, uNK cells are the only leukocyte subset significantly decreased in Alk5 cKO decidua compared with control decidua.

**Discussion**

In our current studies, we conditionally deleted uterine ALK5 and demonstrated that absence of TGF-β signaling through ALK5 leads to many abnormalities at different stages of pregnancy. One of the striking phenotypes was the reduction of uNK cells at midgestation. A previous study reported that mouse uNK cells do not express the PR (37). Thus, it is possible that TGF-βs (secreted from uterine epithelium or stroma, or even from the embryo) act on decidual cells via ALK5 to induce expression of other cytokines, which are key regulators in uNK cell maturation after embryo implantation. Among those cytokines that were down-regulated in Alk5 cKO decidua, IL-15 has been previously reported as a key regulator in NK cell maturation (9). Il15 null mice revealed complete absence of uNK cells as well as severe defects in spiral artery remodeling, which mimicked features observed in other uNK-deficient mouse models (9). Notably, Il15
mutants and our Alk5 cKO shared very similar uterine gene expression profiles, particularly those genes specifically expressed in NK cells (e.g., Prf1, Klrg1, Nkg7, CtsG, and several granzyme genes) (36). However, no fetal loss was reported in either Il15 null mutants or other NK cell-deficient mouse models (8, 9, 38, 39), indicating that reduction of uNK cells is not likely the main reason for the severe fertility defects that we observed in Alk5 cKO females.

Several studies have reported that uNK cell-deficient mouse models exhibited no overgrowth of trophoblast or any consistent quantifiable impairment in fetus (6). However, no fetal loss was reported in either Il15 null mutants or other NK cell-deficient mouse models (8, 9, 38, 39), indicating that reduction of uNK cells is not likely the main reason for the severe fertility defects that we observed in Alk5 cKO females.

Although expression of TGF-β and their receptors was previously implicated to play key roles during implantation (41), the molecular mechanism of this signaling pathway during early pregnancy remains poorly defined. As we described in the current study, early defects were also identified in our Alk5 cKO females at 4.5 dpc, when blastocysts attached to the uterine wall, including extra epithelial folds and defective luminal closure. Interestingly, similar phenotypes have been recently reported in
multiple mouse models with excessive E2 response during im-
plantation (24, 42, 43). In mice, E2 stimulates the proliferation of 
uterine epithelium on the first 2 d after conception, whereas the 
production of P4 terminates the effects of E2 in epithelium 
growth from the third day. These hormone-regulated processes 
are required for preparing a receptive uterine environment for 
blastocyst attachment, which ensures successful reproduction (2, 
44). However, the abnormally high E2 response observed in our 
Alk5 cKO and other mouse models stimulates epithelial pro-
iferation, prevents luminal closure, and results in impaired im-
plantation (24, 42, 43). Although the early implantation defects 
found in Alk5 cKO mice did not lead to complete failure of 
blastocyst implantation, it was likely associated with the maternal 
and fetal defects during later placentation, including trophoblast 
expansion, IUGR, uNK deficiency, and vascular abnormalities.

A full-term pregnancy in humans lasts about 40 wk without 
experiencing major health problems. However, the total rate 
of pregnancy loss after implantation is about 30% in healthy 
women (45). Most cases of failed pregnancy develop many 
complications, including recurrent spontaneous abortion, pre-
clampsia, IUGR, and preterm birth. Unfortunately, the physi-
ological and pathological mechanisms of how those pregnancy 
complications develop in healthy mothers are still poorly un-
derstood. Because ethical issues are the main restrictions to 
the study of human pregnancy, animal models (and in particular 
mouse models) provide important insights into the molecular basis 
of human uterine physiology, pathology, and pregnancy compli-
cations. Herein, we successfully established a progressive mouse 
model mimicking multiple symptoms that appear at different 
stages of human pregnancy, such as implantation defects, tro-
phoblast disorganization, IUGR, and vascular abnormalities due 
to uNK deficiency. Thus, our studies not only provide a mouse 
model to better understand how TGF-β signaling regulates female 
reproduction but also shed light on the pathogenesis of 
important reproductive health issues in human pregnancy, which 
can lead to the development of treatments for female infertility.

Materials and Methods

Animals. All mice used in this study were maintained on a mixed C57BL/6/
129SvEv genetic background and handled under protocols approved 
by the Institutional Animal Care and Use Committee at Baylor College of Medicine. 
Sequences of primers used for genotyping are listed in Table S1.

Histological Analysis. Tissues were dissected and fixed in 10% (vol/vol) neutral 
buffered formalin for 24 h and embedded in paraffin. Paraffin sections were 
stained with hematoxylin and eosin (H&E), PAS, or PAS–hematoxylin using 
standard procedures.

Immunohistochemistry and Immunofluorescence. Paraffin sections were de-
paraffinized, rehydrated, and boiled for antigen retrieval using standard 
procedures. After blocking with 3% (wt/vol) BSA for 1 h, sections were incu-
bulated with the primary antibodies overnight at 4 °C. Antibody information
is listed in Table S2. Sections were incubated with biotinylated secondary antibodies and ABC reagent (Vector Laboratories), and immunoreactive sig-
als were developed using 3,3′-diaminobenzidine (DAB) substrate kit 1 (Vec-
tor Laboratories). Immunofluorescence used a similar protocol except that the 
secondary antibodies were Alexa Fluor 488 or 594 (Life Technologies).

Hormone Analysis. Blood was collected from 2-mo-old adult female mice by 
cardiac puncture. The serum was separated from the blood and stored at 
–80 °C before the test. Serum P4 and E2 levels were measured in the Ligand 
Assay and Analysis Core at University of Virginia.

Artificial Induction of Decidualization. As previously described (46), 2-mo-old 
female mice were ovariecotomized. After 2 wk, the mice were injected daily 
with 100 ng of E2 for 3 d, followed by 2 d of rest. The mice were injected 
daily with 1 mg P4 and 6.7 ng E2 for 3 d. One uterine horn was scratched with a 
nail to artificially induce decidualization. As a control, the other uterine 
horn was not traumatized. The mice were continuously injected with P4 and 
E2 and progesterone for another 5 d. After sacrifice, both uterine horns were 
dissected and fixed in 10% (vol/vol) neutral buffered formalin for 24 h and embedded in paraffin. Paraffin sections were 
stained with hematoxylin and eosin (H&E), PAS, or PAS–hematoxylin using 
standard procedures.

RNA Isolation, qPCR, and Microarray Analysis. Total RNA was isolated using the 
RNeasy Mini kit (Qiagen). Gene expression was analyzed by qPCR with the 
SYBR Green detection system (Life Technologies). Primer information is listed 
in Table S3. The relative fold change of transcript was calculated by the 
2−ΔΔCT method as described previously (47). For microarray analysis, the RNA 
samples were hybridized to Illumina Mouse WG-6 v.2.0 in the Microarray 
Core at University of Texas Health Science Center. Differentially expressed 
genes were defined by two-sided t test (P < 0.05) and fold change >2× and 
>2 were displayed in a heat map using GenePattern (48). GSEA was 
performed based on KEGG gene sets, and gene sets that met the false dis-
cover rate (FDR) < 0.25 criterion were chosen to further extract leading 
edge subsets (49).

Flow Cytometry. The decidual basals were dissected from implantation sites, 
mixed to small pieces, and then digested with 1 mg/mL collagenase IV 
and 0.2 mg/mL DNase I (Sigma-Aldrich) in HBSS for 45 min at 37 °C. Single cells 
were extracted by pipetting digested tissue and passed through a 40-μm 
cell strainer. Flow cytometry was performed with a BD LSR II (BD Biosciences) 
in the Flow Cytometry and Cell Sorting Core at Baylor College of Medicine. 
Data were analyzed with FlowJo software (Tree Star Inc.). Forward scatter/side 
scatter gate was used to identify leukocyte populations as well as to elimi-
nate debris. Antibodies (Table S2) were used to further identify different 
lymphocyte subsets.

Statistics. Comparison of means between two groups was conducted using 
the two-way ANOVA was used to determine the differences between 
groups with respect to time and genotype. Data are presented as mean ± SEM, 
and P < 0.05 was considered to be statistically significant.

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S.A.P.) and National Cancer Institute Grant R01-CA136828 (to S.A.P.).
Supporting Information

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Fig. S1. Control and Alk5 cKO virgins exhibited a normal female reproductive system. Control and cKO ovaries (A–D), oviducts (E and F), and nonpregnant uteri (G and H) were compared in histological analyses. [Scale bar, 1.0 mm (A and C) and 200 μm (B and D–H).] CL, corpus luteum; E, epithelium; GC, granulosa cell; Oo, oocyte; S, stroma. (I and J) The serum levels of progesterone (P_4) and estradiol (E_2) were measured in nonpregnant and pregnant females (from 7.5 to 10.5 dpc). Data are presented as mean ± SEM (n = 5). ***P < 0.001 compared with controls.
Fig. S2. Impaired implantation sites were observed in Alk5 cKO females. (A–D) We compared the gross morphology of control and cKO implantation sites from 7.5 to 10.5 dpc. (Scale bar, 10 mm.) (E–L) Histological analyses of control and cKO implantation sites from 7.5 to 10.5 dpc. [Scale bar, 200 μm (E and F) and 500 μm (G–L).] AL, allantois; CP, chorionic plate; E, embryo; EPC, ectoplacental cone; GC, trophoblast giant cells; LA, labyrinth; SP, spongiotrophoblasts.
### Table S1. Primer sequence information for genotyping

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence for genotyping</th>
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<td><em>Alk5</em> (floxed allele)</td>
<td>ACTCACATGTTGGCTCTCACCTGTC AGTCATAGCATGTGTTAGAGTC</td>
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### Table S2. Antibody and DBA information

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FC, flow cytometry; IF, immunofluorescence; IHC, immunohistochemistry.

### Table S3. Primer sequence information for qPCR

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