The SK3 channel promotes placental vascularization by enhancing secretion of angiogenic factors

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Rada CC, Murray G, England SK. The SK3 channel promotes placental vascularization by enhancing secretion of angiogenic factors. Am J Physiol Endocrinol Metab 307: E935–E943, 2014. First published September 23, 2014; doi:10.1152/ajpendo.00319.2014.—Proper placental perfusion is essential for fetal exchange of oxygen, nutrients, and waste with the maternal circulation. Impairment of uteroplacental vascular function can lead to pregnancy complications, including preeclampsia and intrauterine growth restriction (IUGR). Potassium channels have been recognized as regulators of vascular proliferation, angiogenesis, and secretion of vasoactive factors, and their dysfunction may underlie pregnancy-related vascular diseases. Overexpression of one channel in particular, the small-conductance calcium-activated potassium channel 3 (SK3), is known to increase vascularization in mice, and mice overexpressing the SK3 channel (SK3T/T mice) have a high rate of fetal demise and IUGR. Here, we show that overexpression of SK3 causes fetal loss through abnormal placental vascularization. We previously reported that, at pregnancy day 14, placentas isolated from SK3T/T mice are smaller than those obtained from wild-type mice. To identify the mechanism by which these vascularization defects occur, we measured levels of vascular endothelial growth factor (VEGF), placental growth factor, and the soluble form of VEGF receptor 1 (sFlt-1), which must be tightly regulated to ensure proper placental development. Our data reveal that overexpression of SK3 alters systemic and placent al ratios of the angiogenic factor VEGF to antiangiogenic factor sFlt-1 throughout pregnancy. Additionally, we observe increased expression of hypoxia-inducing factor 2α in SK3T/− placentas. We conclude that the SK3 channel modulates placental vascular development and fetal health by altering VEGF signaling.

SK3 channels; pregnancy; endothelium; angiogenesis; placenta

Two key promoters of blood vessel development in the placenta are vascular endothelial growth factor (VEGF) (12) and a related family member, placental growth factor (PIGF). VEGF is high at the beginning of pregnancy and promotes the de novo vessel formation essential for development of the fetal-placental vasculature (2, 6, 17). Low levels of VEGF correlate with preeclampsia, spontaneous preterm delivery, and recurrent pregnancy loss (3, 13). Later in pregnancy, PIGF promotes angiogenesis and vasculogenesis during embryonic development (6) and can regulate VEGF-mediated angiogenesis under pathological conditions (5, 17). A third essential factor is the soluble form of VEGF receptor 1, sFlt-1, which is antiangiogenic owing to its ability to competitively bind to PIGF and VEGF and prevent these pro-angiogenic factors from binding to their surface membrane Flt-1 receptors (11). In a healthy pregnancy, the level of sFlt-1 increases at the end of pregnancy (17). However, if sFlt-1 levels are too high, the blood vessel development needed for fetal development will be inhibited; this endothelial dysfunction can lead to preeclampsia (18).

Recent evidence has shown that potassium channels are key regulators of vascular smooth muscle dilation, proliferation, and angiogenesis and thus may play a role in pregnancy-induced vascularization (20). Specifically, several pieces of evidence indicate that the small-conductance calcium-activated potassium channel 3 (SK3), predominantly found in the vascular endothelium, is an important regulator of vascular tone during pregnancy. First, activation of the endothelial SK3 channel induces release of endothelin-derivered hyperpolarizing factor (EDHF), causing vasodilation (9, 30). Second, in rat mesenteric arteries, SK3 activity modulates PIGF levels, thereby activating the EDHF pathway (19). Third, in pregnant sheep uterine arteries, the SK3 channel is regulated by hypoxia (34), a known driving force of angiogenesis (2). Finally, we recently demonstrated that mice overexpressing the SK3 channel (SK3T/T mice) had an increased rate of fetal demise; the surviving SK3T/− pups showed both IUGR and decreased placental thickness at pregnancy day 14 (P14). These effects were likely linked to altered vascular properties, as SK3T/T mice had larger uterine artery diameters (25). These observations suggest that the SK3 channel has important roles in both the timing of vascular development and placental function. We hypothesized that the increase in channel expression in the SK3T/− placentas alters the secretion of angiogenic factors, thereby affecting vascular development and leading to increased IUGR and fetal demise.

Here, we show that overexpression of the SK3 channel affects the structure of uteroplacental blood vessels. In SK3T/− mice, the size and number of the placental blood vessels were increased at P14, the time at which we previously had observed

THE PLACENTA IS ESSENTIAL for maternal-fetal exchange of nutrients and wastes. Additionally, it modulates signals conveyed to the fetus and is sensitive to changes in the maternal environment. Proper regulation of uteroplacental perfusion is essential to maintain a healthy pregnancy, and impairments in this process can lead to pregnancy complications including preeclampsia, intrauterine growth restriction (IUGR), and fetal loss (22, 28). Thus, placental blood vessel development is tightly controlled by a delicate balance of angiogenic and angiostatic factors (16) that regulate blood vessel proliferation and remodeling at the maternal-fetal interface during pregnancy (1, 4, 6).

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IUGR. We also observed decreased placental thickness at this time. Additionally, we report that the changes in placental vasculature were associated with changes in systemic levels of VEGF, PI GF, and sFlt-1 and in the ratio of VEGF to sFlt-1. Finally, we show that overexpression of the SK3 channel alters placental secretion of VEGF and disrupts the ratio of VEGF to sFlt-1. We argue that this disruption of the balance of VEGF to placental secretion of VEGF and disrupts the ratio of VEGF to PlGF, and sFlt-1 and in the ratio of VEGF to sFlt-1.

RESULTS

Overexpression of SK3 alters placental vascular morphology. It was previously reported that homozygous SK3T/T mice express threefold more SK3 protein than WT mice (7). To confirm that SK3T/T placentas overexpressed the SK3 channel protein, we mated WT and SK3T/T females with WT males and compared SK3 levels in heterozygous SK3T/T-/- and WT placentas by Western blot (Fig. 1A). The SK3T/T-/- placentas expressed 1.5-fold more SK3 than WT placentas at P10, P14, and P18.

We previously used ultrasound measurements to demonstrate that SK3T/T-/- placentas from SK3T/T-/- mothers are significantly smaller than WT placentas at P14 (25). To determine the mechanism underlying this reduction in size of the SK3T/T-/- placentas, we used hematoxylin and eosin staining to examine gross histological morphology of the placentas from the surviving pups. At P10, the labyrinth layer of the implantation sites was significantly thinner at its widest point, from the chorionic plate to the giant cell layer, in SK3T/T-/- than in WT (Fig. 1, B and C, top). Placental abnormalities were also apparent at P14; at this stage, the decidua of SK3T/T-/- placentas exhibited abnormally dilated blood vessels (Fig. 1C, middle). Despite the extensive placental changes seen in SK3T/T-/- placentas at P14, we detected no differences in either the labyrinth size or vascular morphology at P18. This resolution of the defects supports the earlier finding (25) that, relative to WT placentas, SK3T/T-/- placentas have an accelerated growth trajectory from P14 onward.

SK3T/T-/- placentas have increased blood vessel volume. We next used MicroCT to quantify differences in the placental vascular bed morphology of SK3T/T-/- and WT placentas at P14 (Fig. 2). MicroCT provides a three-dimensional view of the placental vessels, thereby allowing determination of average

METHODS

Animals and breeding. All animal procedures complied with the guidelines for the care and use of animals set forth by the National Institutes of Health. All protocols were approved by the Animal Studies Committee at Washington University in St. Louis. Kcnm3^{tm1Ips/T} (SK3T/T) mice on a C57BL/6 background were used for this study (7), with C57BL/6 littermates serving as wild-type (WT) controls. Adult females between 2 and 6 mo of age were bred for 2 h with C57BL/6 males. Pregnancy was confirmed by the presence of a copulatory plug, and the day it appeared was designated day 0 of pregnancy (P0).

Placental perfusion and microcomputed tomography. Immediately before placental dissection, Microfil radiopaque silicone rubber (Flow Tech, Carver, MA) was perfused through the umbilical artery with a 33-gauge needle into placentas as previously described for fetoplacental vascular casting (27). Microfil perfused into placentas was allowed to polymerize in 10% formalin fixative for 24 h at 4°C. Placentas were then embedded in 2% agar and scanned on a MicroCT40 scanner (Scanco Medical, Brüttisellen, Switzerland) at X-ray tube settings of 70 kVp and 114 μA with a sample holder diameter of 16 mm. An integration time of 200 ms was used with a nominal voxel size of 8 μm.

MicroCT images were filtered by using a Gaussian blur with sigma of 2.2 and support of 4. The vasculature was then segmented with the following parameters: Carlsbad, CA) containing 50 μg/ml gentamicin. Placentas were placed individually into the wells of a 12-well dish and incubated in endothelial growth medium (Lonza, Basel, Switzerland) with the following additives according to the manufacturer’s instructions: 2% FBS, hEGF, hydrocortisone, GA-1,000, VEGF, hFGF-b, R3-IGF-1, ascorbic acid, and heparin for 1 h at 37°C. Placentas were washed three times with DPBS and incubated at 37°C in DPBS with or without 10 μM NS309 (Tocris Bioscience, Bristol, UK). DPBS was collected at 0-, 2-, and 6-h time points, with one placenta per time point, and stored at −80°C until quantified by enzyme-linked immunosorbent assay (ELISA).

ELISA. Blood was collected by cardiac puncture, allowed to coagulate at room temperature for 20 min, and then spun at 14,000 g for 20 min. Serum or placental explant supernatant levels of sFlt-1, VEGF, and PI GF were measured with the mouse Quantikine immunoassays (R&D Systems, Minneapolis, MN) ELISA kit according to the manufacturer’s instructions.

Histology. P10 implantation sites and P14 and P18 placentas were fixed in 10% formalin for 24 h, embedded in 2% agar, and then embedded in paraffin. Sections (5 μm) were adhered to slides, stained with hematoxylin and eosin, and mounted. Slides were imaged by light microscopy. The Washington University in St. Louis Department of Comparative Medicine Research Animal Diagnostics Laboratory performed histological analyses of sections of placentas and implantation sites.

Statistical analysis. All data are presented as means ± SE. Statistical significance was determined by ANOVA followed by Bonferroni post hoc test or Student’s t-test by GraphPad Prism (San Diego, CA); n refers to number of animals in all cases.

RESULTS

Table:<ref>

<table>
<thead>
<tr>
<th>Group</th>
<th>Vessel Volume (μm³)</th>
<th>Vascular Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK3T/T-/-</td>
<td>3.5 ± 0.5</td>
<td>Normal</td>
</tr>
<tr>
<td>WT</td>
<td>4.0 ± 0.6</td>
<td>Abnormal</td>
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vessel diameter, vessel volume, vessel density, and volume of first-order vessels. We found that average vessel diameter was slightly larger in the SK3T/− placentas than in WT placentas at P14, but this difference was not significant (Fig. 2B). The total volume of blood vessels in the placentas (Fig. 2C) was significantly larger in SK3T/− placentas than in WT placentas at P14, although vessel density was not. Finally, we assessed the volume of first-order vessels off the main umbilical artery (Fig.
We again observed significantly larger vessels in the SK3T+/H11002 placentas than in WT placentas at P14, similar to what was seen in the SK3T+/- uterine artery (25). We conclude that overexpression of the SK3 channel results in increased volume of placental blood vessels. SK3T/T mice have an imbalance of angiogenic factors in maternal serum during pregnancy. We hypothesized that an imbalance of soluble angiogenic factors caused the enhanced vascular network in the SK3T+/H11002 placentas. To test this, we first examined the systemic levels of the proangiogenic factor VEGF by measuring serum concentrations. We found no differences between SK3T/T and WT in the serum VEGF concentrations in nonpregnant (NP), P10, P14, or P18–19 mothers (Fig. 3A) despite the increased vessel volume in the SK3T+/H11002 placentas. By contrast, the serum concentration of the other hallmark proangiogenic factor, PlGF, was significantly lower in SK3T/T mothers than in WT mothers at P18–19 (Fig. 3B). We also measured the antiangiogenic factor sFlt-1 and found that its concentration was significantly lower in serum from SK3T/T mothers than from WT at both P10 and P18–19 (Fig. 3C). These findings, in addition to the morphological changes, led us to speculate that an imbalance in the pro/antiangiogenic factors in SK3T/T mice disrupted proper blood vessel formation at the maternal-fetal interface. To explore this, we calculated the VEGF/sFlt-1 (Fig. 3D) and PlGF/sFlt-1 ratios (Fig. 3E). Although we found no significant differences at any measured gestational stages in the PlGF/sFlt-1 ratio between SK3T/T and WT mice, we found that the ratio of VEGF to sFlt-1 was significantly higher in SK3T/T mice than in WT mice at P10 (Fig. 3D). These results suggest that decreased sFlt-1 levels allow increased VEGF signaling to enhance de novo growth of blood vessels during early placental vascular-
The imbalanced ratio of VEGF to sFlt-1, rather than either factor alone, likely causes the hypervascularization. SK3 channel expression alters placental secretion of angiogenic factors. To determine whether the observed alterations in systemic levels of angiogenic factors were representative of local placental secretion differences, we dissected placentas from WT and SK3T/T/H11002 mice at P10, P14, and P18 and measured the levels of factors secreted into the media for up to 6 h in vitro. No significant differences were found after 2 h of incubation, so the 6-h time point was assessed (Table 1). We found that P10 SK3T/T/H11002 placentas secreted significantly more VEGF than did P10 WT placentas (Fig. 4A). To determine whether acute activation of the SK3 channel could also alter secretion of VEGF, we measured secretion from WT placentas incubated with NS309, an SK3 channel opener. NS309 was also added to the SK3T/T/H11002 placentas, but we observed no differences in any parameters between treated and untreated SK3T/T/H11002 placentas (data not shown). WT P10 placentas in the presence of NS309 secreted more VEGF than untreated placentas, and there was no statistically significant difference in VEGF secretion between SK3T/T/H11002 and NS309-treated WT placentas. No differences in VEGF levels were observed at P14 or P18. Although NS309-treated WT P10 and P14 placentas secreted more PlGF than untreated placentas, the levels of PlGF secreted by SK3T/T/H11002 placentas were identical to those of WT placentas at all gestational stages (Fig. 4B). This suggests that additional pathways influence PlGF secretion. Levels of sFlt-1 did not differ among the three groups of placentas isolated from any gestational stage (Fig. 4C). Finally, we examined whether there was an imbalance in the ratio of pro-to-antiangiogenic factors secreted by the placentas. The ratio of VEGF to sFlt-1 secretion from WT placentas was significantly lower than that from SK3T/T/H11002 and NS309-treated WT placentas. However, no differences were seen in PlGF/sFlt-1 ratios at any gestational age (Fig. 4E). Together, these results indicate that placental secretion con-

Table 1. Levels of VEGF, PlGF, and sFlt-1 secreted by placentas for 0, 2, and 6 h

<table>
<thead>
<tr>
<th></th>
<th>VEGF (pg/ml)</th>
<th>PlGF (pg/ml)</th>
<th>sFlt-1 (nM)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>WT P10</td>
<td>0</td>
<td>43.4</td>
<td>57.5</td>
</tr>
<tr>
<td>SK3T/T/H11002 P10</td>
<td>0</td>
<td>43.4</td>
<td>57.5</td>
</tr>
<tr>
<td>WT P10 NS309</td>
<td>0</td>
<td>108.0</td>
<td>192.0*</td>
</tr>
<tr>
<td>WT P14</td>
<td>0</td>
<td>6.95</td>
<td>16.0</td>
</tr>
<tr>
<td>SK3T/T/H11002 P14</td>
<td>0</td>
<td>5.88</td>
<td>22.3</td>
</tr>
<tr>
<td>WT P14 NS309</td>
<td>0</td>
<td>13.50</td>
<td>27.2</td>
</tr>
<tr>
<td>WT P18</td>
<td>0</td>
<td>7.79</td>
<td>20.6</td>
</tr>
<tr>
<td>SK3T/T/H11002 P18</td>
<td>0</td>
<td>4.39</td>
<td>28.4</td>
</tr>
<tr>
<td>WT P18 NS309</td>
<td>0</td>
<td>8.87</td>
<td>29.2</td>
</tr>
</tbody>
</table>

Mean placental secretion rates, in pg/ml, of vascular endothelial growth factor (VEGF), placental growth factor (PlGF), and the soluble form of VEGF receptor 1 (sFlt-1) into PBS at times indicated; n = 3–5. WT, SK3T/T/H11002, and WT placentas treated with the SK3 channel opener NS309 were assayed. Pregnancy days (P)10, 14, and 18 were assessed. *P < 0.05 vs. WT at same day of pregnancy.
tributes to the altered ratio of VEGF to sFlt-1 observed systemically between SK3T-/- and WT mice.

**HIF-2α is increased in SK3T-/- placenta.** Improper placental vascularization often leads to insufficient oxygen delivery to the fetus. Furthermore, recent studies suggest that some potassium channels are oxygen sensitive (33). HIF-2α, an oxygen-sensitive molecule that increases in endothelial cells during hypoxia, induces blood vessel formation during pregnancy (16, 24), in part, by regulating sFlt-1 levels (26). As we observed changes in the VEGF/sFlt-1 ratio both systemically and locally, we measured HIF-2α protein expression in SK3T-/- and WT placentas at P10, P14, and P18 (Fig. 5, A and B). SK3T-/- P10 and P18 placentas expressed significantly more HIF-2α (normalized to GAPDH) than WT placentas at the same stages. This observation suggests that P10 and P18 placentas are in a hypoxic state, likely due to improper, possibly mistimed, placental vascularization caused by the SK3 channel overexpression.

**DISCUSSION**

Here, we provide evidence that the SK3 channel plays an important role in the development of the placental vasculature. Our structural studies showed that placentas from mice overexpressing the SK3 channel had increased blood vessel number and size at P14. Further investigation demonstrated that these effects were associated with changes in placental secretion of the proangiogenic factor VEGF. These changes occurred at the same time in development at which we previously reported IUGR and fetal demise in SK3-overexpressing mice (25).

This study suggests that SK3 dysregulation alters placental vascularization, potentially through changes in proangiogenic factors, leading to pathological outcomes. Understanding the complex effects of SK3 overexpression on placental vascular development requires an appreciation of the physiological impact of SK3 dysregulation on the placental vasculature in vivo.
SK3 INDUCES ANGIOGENIC-DEPENDENT PLACENTAL VASCULARIZATION

Fig. 6. Schematic model of enhanced vessel growth in SK3\textsuperscript{T/-} placentas. An increase in SK3 channel expression in SK3\textsuperscript{T/-} placentas leads to the release of endothelium-derived hyperpolarizing factor (EDHF) and nitric oxide (NO)/prostaglandins as well as changes in the secretion of the angiogenic factors VEGF, PlGF, and sFlt-1. SK3 overexpression decreases PlGF and sFlt-1 levels and increases VEGF secretion, thereby further increasing vessel growth. This vessel growth is exacerbated by an enhanced hypoxic response, as shown by increased HIF-2\alpha levels. The hypoxic response may also act directly on the channel itself making a multifactorial feedback loop. Ultimately, the excessive vessel growth leads to fetal distress.

![Diagram showing enhanced vessel growth in SK3\textsuperscript{T/-} placentas](image)

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Additional information about placental vessels, hypoxia, and fetal distress:

- Maternal-fetal communication occurs throughout pregnancy to ensure proper fetal development.
- The murine placenta is established by P10 and continues to adjust its structure and function to accommodate fetal needs throughout gestation.
- The placenta responds to early insults in a number of ways. One means was at the level of angiogenic signaling, specifically VEGF, which, along with vasodilation, might be driven by oxygen tension in small arteries.
- We observed decreased levels of the angiogenic factor sFlt-1 in serum of SK3\textsuperscript{T/-} mice at P10. The low sFlt-1 levels contributed to an increase in the VEGF/sFlt-1 ratio in SK3\textsuperscript{T/-} mice. With less available sFlt-1 to bind VEGF, free VEGF would have been able to bind to its insoluble tyrosine kinase receptor FLT to promote a proangiogenic state. Additionally, we observed an increase in the ratio of VEGF to sFlt-1 secreted by SK3\textsuperscript{T/-} placentas, and we suggest that the SK3\textsuperscript{T/-} placentas upregulated secretion of VEGF to compensate for the early defect and promote de novo blood vessel growth within the placenta.
- Moreover, the increase in HIF-2\alpha could promote angiogenesis, as hypoxia is known to be responsible for downregulation of sFlt-1 and PlGF, and upregulation of VEGF.
- Furthermore, HIF-2\alpha directly affects vascularization, as demonstrated in previous studies in which HIF-2\alpha knockout mice had insufficient placental vascularization, resulting in fetal death by P12.5.
- Further exacerbating the problems, hypoxia has been shown to cause an increase in intracellular calcium, which activates the SK3 channel. As a result, the membrane would become hyperpolarized, leading to the release of EDHF and angiogenic factors.
- Alternatively or additionally, hypoxia and HIF-2\alpha signaling could directly lead to the release of angiogenic factors. We argue that, together, these responses led to increased vascular development.
- This was manifested in our MicroCT observation that blood vessels were increased in volume in SK3\textsuperscript{T/-} placentas at P14. This hypervascularization in the placenta was likely detrimental to fetal health and contributed to the fetal demise and IUGR that we previously reported.
- Further highlighting the importance of balanced vascular regulation is the fact that hypovascularization is observed in preeclampsia, which also leads to IUGR.

In addition to the placental response to the early impairment of maternal-fetal exchange, the continued overexpression of SK3 may further worsen fetal health. Previous studies have shown that SK3 regulates endothelial-mediated pathways and...
is responsible for the release of endothelial-derived vasoactive factors, specifically EDHF (9, 30), which induces cell membrane hyperpolarization and vasodilatation of the vascular smooth muscle. This increased vasodilatation (larger diameter vessels) would lower arterial resistance, potentially altering the angiogenic signaling to remodel the blood vessels, as changes in hemodynamic forces are known to influence vascular branching (29). Vascular alterations would further impair fetal development. Strict spatial and temporal regulation of blood vessel development is essential for a healthy fetal environment; premature increases in oxygen can result in DNA, lipid, and protein damage in the fetus due to oxidative stress in the developing placenta (31, 32) and may explain the fetal loss and IUGR we detected in the SK3T/T mice (25). Moreover, excess production of ROS that result from premature changes in oxygen could alter potassium channel activity (33). Thus, the phenotypes of the SK3T−/− placentas are likely a result of actions of a multifactorial feedback loop.

By P18, the damage caused by SK3 overexpression was largely complete; we previously reported that roughly one-half of the pups were resorbed by this time (25). However, those placentas and fetuses that remained viable were able to “catch up” and achieve normal size (25). Nonetheless, we report here that some differences were still apparent. For example, the volume of blood vessels was higher in SK3T−/− placentas than in WT placentas at this stage. Additionally, we observed a decrease in systemic PlGF levels late in pregnancy in SK3T/T mice; this may reflect compensation for the increased VEGF measured in early pregnancy. Finally, those SK3T−/− placentas that remained appeared to be suffering from oxidative stress, as they expressed higher levels of HIF-2α than WT placentas.

Our experiments with the SK3 channel opener NS309 provided some insight about the effects of overactivity of this channel. Although NS309 treatment of placentas in vitro increased secretion of VEGF to a similar extent as overexpression of the channel, the opener did not mimic the effects of SK3 overexpression on secretion of PlGF. This likely reflects a difference between acute and chronic upregulation of the SK3 channel. A placenta in a chronic pathophysiological environment has external compensation mechanisms that are not measured when a placenta is being looked at acutely in culture. Alternatively, the increase in PlGF in response to the opener may reflect the fact that NS309 can also increase the opening of the intermediate conductance potassium channel (IK), which is also expressed in endothelial cells (8). The IK channel could have a larger impact on the release of PlGF in the placental vasculature than the SK3 channel. Such an effect would be tissue specific, however, as previous studies have shown that, whereas the SK3 channel modulates PlGF in mouse mesenteric arteries, the IK channel does not (19).

Our studies indicate that the SK3 channel is a key regulator of an angiogenic endothelium-mediated feedback pathway that must be properly regulated to achieve a successful pregnancy. Disruption of this pathway can have deleterious effects on the fetus by causing improper placental vascularization. Although studies have demonstrated that multiple pregnancy disorders, including preeclampsia, result in insufficient vascularization, our study indicates that overabundant placental vascularization is also detrimental. Our results indicate that strict regulation of oxygen tension by angiogenic signaling to create properly remodeled uteroplacental vessels is essential to maintain a healthy pregnancy.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: C.C.R. and S.K.E. conception and design of research; C.C.R. and G.M. performed experiments; C.C.R. and G.M. analyzed data; C.C.R. and S.K.E. interpreted results of experiments; C.C.R. prepared figures; C.C.R. drafted manuscript; C.C.R. and S.K.E. edited and revised manuscript; C.C.R. and S.K.E. approved final version of manuscript.

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