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Regulation of Placental Angiogenesis

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10

Abstract

Ample interest has been evoked in using placental angiogenesis as a target for the development of diagnosis tools and potential therapeutics for pregnancy complications based on the knowledge of placental angiogenesis in normal and aberrant pregnancies. Although these goals are still far from reach, one would expect that two complementary processes should be balanced for therapeutic angiogenesis to be successful in restoring a mature and functional vascular network in the placenta in any pregnancy complication: (i) pro-angiogenic stimulation of new vessel growth and (ii) anti-angiogenic inhibition of vessel overgrowth. As the best model of physiological angiogenesis, investigations of placental angiogenesis provide critical insights not only for better understanding of normal placental endothelial biology but also for the development of diagnosis tools for pregnancy complications. Such investigations will potentially identify novel pro-angiogenic factors for therapeutic intervention for tissue damage in various obstetric complications or heart failure or anti-angiogenic factors to target on cancer or vision loss in which circulation needs to be constrained. This review summarizes the genetic and molecular aspects of normal placental angiogenesis as well as the signaling mechanisms by which the dominant angiogenic factor vascular endothelial growth factor regulates placental angiogenesis with a focus on placental endothelial cells.

Introduction

Sprouting new blood vessels from existing ones is called angiogenesis (1). In a healthy adult body, angiogenesis occurs for healing wounds to restore blood flow to tissues after injury or insult and in various pathological conditions such as cancer and retinopathy (2). In female eutherians, it occurs normally during the menstrual or estrous cycle to transform the ovulated follicles into the corpus luteum for progesterone synthesis and to rebuild the uterine endometrium receptive for the implanting embryos (3). It requires endothelial proliferation, migration, and differentiation within the preexisting blood vessels as they send out capillary sprouts to initiate the formation of new tube-like structures, and secondary vasodilatation to enhance circulation and nutrient uptake (1). This multi-step process begins with a rise in local and/or systemic angiogenic factors, followed by breakdown of endothelial basement membrane to facilitate endothelial migration and proliferation. Endothelial differentiation leads to newly formed tube-like structures that stabilizes as mature vessels with the recruitment of pericytes or

smooth muscle cells (4, 5). Deranged angiogenesis has a major impact on human health and contributes to the pathogenesis of numerous vascular diseases that are caused by either excessive angiogenesis in tumors, retinopathy, and cavernous hemangioma or insufficient angiogenesis in atherosclerosis, hypertension, diabetes and restenosis (2).

In eutherians, shortly after the embryo is implanted, its trophoderm develops into the placenta. This ephemeral organ is unique to the pregnancy of these creatures, critically enough to evolutionally escape them from distinction. It supports the development, growth, and survival of the fetus in the womb. The formation, growth, and function of the placenta are precisely regulated and coordinated to operate the bi-directional maternal-fetal exchanges of nutrients and respiratory gases (oxygen and carbon dioxide) and to exhaust fetal metabolic wastes at the maximal efficiency, which is executed through the circulatory system at the maternal, fetal and placental unit such that all the supports needed for early life of a mammal in the womb can be met (3, 6). Angiogenesis in the placenta takes similar steps as it occurs in any other organs; it also requires proliferation, migration, and differentiation of endothelial cells within the preexisting trophoblastic microvessels (7). However, unlike pathological angiogenesis, placental angiogenesis is a normal physiological process that must be tightly regulated during pregnancy. Deranged placental vasculature is the most common placental pathology that has been identified in numerous pregnancy complications in animals and women (8-11), attesting the importance of placental angiogenesis during pregnancy.

Placental vascular formation and development

The process of *de novo* vascular formation during embryogenesis is called vasculogenesis, which begins with the formation of the endothelial progenitor cells called angioblasts in the extraembryonic mesoderm allantois (12). The placental vasculature further expands during pregnancy and elaborates with the morphogenesis of the placenta (13). Extensive angiogenesis occurs in both the maternal and fetal placental tissues. The placenta develops as a highly vascularized organ during late gestation. For example, the capillary network in a normal human placenta is estimated to be 550 km in length and 15 square meters in surface area (14). Both branching (the formation of new vessels by sprouting) and nonbranching (the formation of capillary loops through elongation) angiogenesis have been described in the placenta, with a major switch around the last third of gestation. Specifically, normal human placental

development is characterized by branching angiogenesis prior to 24 weeks post-conception, followed by nonbranching angiogenesis that occurs thereafter to term (15).

There is compelling evidence to suggest that vasculogenesis and angiogenesis are sequentially regulated by different growth factors. Vascular endothelial growth factor (VEGF) is critically required for all steps of placental vascular formation and development. Targeted inactivation of a single *VEGF* allele (31, 32) or disruption of genes encoding VEGF receptors such as *VEGFR1* (33) and *VEGFR2* (34) as well as *neuropilin-1* and *-2* (35) causes embryonic lethality due to abnormal blood vessel formation during embryogenesis, suggesting a pivotal role of VEGF/VEGFRs in vasculogenesis. Fibroblast growth factor (FGF2) has a particular role in the formation of hemangiogenic progenitor cells (angioblasts) early during embryonic development (30). Placental growth factor (PlGF) seems to play a synergistic role with VEGF for the formation of the vascular network with the development of the villous tree (29). During the third trimester of gestation, placental expressions of many other growth factors (see below) increase substantially to facilitate the coordinated development of the vascular system via sprouting and elongation in the placental villi (Fig. 1).

Extensive neovascularization in the placenta is accompanied with periodic increases in uterine and placental blood flows during gestation. Blood flows to the maternal, fetal, and placental units are established during implantation and placentation when the maternal-fetal circulations connect within the placenta, gradually increases until mid-gestation, then substantially increases at the last one third portion of gestation, essentially keeping pace with the rate of the growing fetus (3). Animal studies have clearly shown that angiogenesis and vasodilatation of the uterine and placental vessels are the two key mechanisms to increase placental (umbilical cord) blood flow during late gestation, which is imperative for normal fetal growth and survival and is also directly linked to the well-beings of the fetus, newborn, and the mother during pregnancy and postpartum (8).

Trophoblast regulation of placental angiogenesis

Endothelial cells are in close contact with the trophoblast cells in the placenta; trophoblast-derived factors are expected to have a significant role in the regulation of placental vascular formation and morphogenesis. For example, the *Esx1* gene encodes a homeobox transcription factor that is expressed solely in trophoblast cells of the labyrinth (16, 17). Placentas from *Esx1*

mutants seem to undergo normal chorioallantoic branching morphogenesis but the fetal blood vessel growth into the labyrinth villi is severely impaired (16). The placental phenotype of *Esx1* mutant mice indicates that trophoblast cells are critically involved in the vascularization of the labyrinth, suggesting a paracrine pathway for regulating placental vascular formation and morphogenesis possible by transcriptional signals of *Esx1* from the trophoblast cells (18), although the downstream targets of *Esx1* are currently unknown.

As a primary active site of angiogenesis, the placenta is one of the richest sources of both pro-angiogenic and anti-angiogenic factors. During the third trimester of both ovine and human pregnancy, at a time when maternal-fetal interface vascular growth, blood flow, and fetal weight increase exponentially, the fetal and maternal compartments of the placentas produce numerous angiogenic factors, including VEGF (19-21), FGF2 (22), PlGF (23), endocrine gland-derived-VEGF (24), transforming growth factor- β 1 (TGF- β 1) (25), leptin (26), angiopoietins (27), and Slit/Robo signaling cues (28). It is noteworthy that this list is still expanding. It is also becoming clear that the placenta also produces a large number of anti-angiogenic factors, i.e., soluble VEGFR1 (sFlt1) and soluble TGF- β 1 receptor endoglin (29), etc. These factors are important for the fine tuning of placental angiogenesis.

Vascular endothelial growth factor and placental angiogenesis

VEGF is the first angiogenic factor identified (19). Among many growth factors surveyed, VEGF is the only one that is expressed almost ubiquitously at sites of angiogenesis and its expression correlates most closely with the spatial and temporal events of vascular growth. Following the discovery of a family of structurally related growth factors, *e.g.*, VEGF-B, -C, -D and -E as well as placenta growth factor (PlGF) (36-38), the conventional form has been renamed as VEGFA or simply VEGF. VEGF consists of at least seven structurally homologous isoforms (VEGF₁₂₁, VEGF₁₄₅, VEGF₁₄₈, VEGF₁₆₅, VEGF₁₈₃, VEGF₁₈₉, and VEGF₂₀₆), with a potent mitogenic activity for endothelial cells (39). These isoforms are produced from different splicing variants of VEGF pre-mRNA, differing from each other with the presence or absence of sequences encoded by exons 6 and 7 (40). The majority of VEGF-producing cells preferentially express VEGF₁₂₁, VEGF₁₆₅ and VEGF₁₈₉, whereas the others are comparatively rare.

During normal pregnancy, human placental VEGF expression increases with gestational age. The fetal cotyledon and maternal caruncle as well as placenta amnion and chorion produce large

amounts of VEGF during the third trimester of ovine (41-43) and human (44) pregnancy. In addition, fetal placental endothelial cells also express VEGF (35). We have found that akin to most arterial endothelial cells, placental artery endothelial cells express the high affinity VEGF receptor VEGFR1 (also called *fms*-related tyrosine kinase 1/Flt1) and VEGFR2 (also called kinase insert domain receptor/KDR) as well as the VEGF co-receptors neuropilin-1 and -2 (35). These data suggest that VEGF plays a paracrine and autocrine role in the regulation of placental angiogenesis. Furthermore, in maternal caruncle and fetal cotyledonary tissues, expression of VEGF and Flt1 and KDR is highly correlated positively to placental vascularization and uteroplacental and fetoplacental blood flows in pregnant ewes (42, 43), suggesting that the VEGF-VEGFR system is critically involved in placental angiogenesis.

VEGF has been shown to regulate all steps of the angiogenesis process. It stimulates endothelial expression of proteases such as urokinase-type and tissue-type plasminogen activators and interstitial collagenase that break down extracellular matrix and release endothelial cells from anchorage, allowing them to migrate and proliferate (45, 46). *In vitro*, VEGF strongly stimulates placental endothelial cell proliferation and migration as well as the formation of tube-like structures on matrigel ((47, 48). VEGF can activate endothelial cells, generating various vascular active agents that themselves affect angiogenesis. For example, VEGF strongly stimulates placental artery endothelial production of nitric oxide (NO) (49, 50), which serves as a potent vasodilator and angiogenic factor in the placenta (51) as it does in other organs ((52, 53). VEGF can also recruit pericytes to the newly formed vessels (54) and participates in the continued survival (55) of nascent endothelial cells, both of which promotes the maturation and vessel stability of the newly formed vessels (56).

Interestingly, Bates *et. al.* (2002) described a novel group of VEGF splice variants that were named VEGF_{XXXb}, such as VEGF_{121b}, VEGF_{165b}, and so on (57, 58). They are also encoded by the *VEGF* gene but with alternative splicing at the distal site in the terminal exon (called exon 9) that differs from the terminal exon 8 for the conventional VEGF isoforms, which encode their last 6 amino acids (57). Thus, VEGF_{xxx}b and the conventional sister VEGF_{xxx} have different sequences but with the same size; however, they seem to possess opposite functions in angiogenesis. For example, VEGF_{165b} inhibits VEGF₁₆₅-mediated endothelial cell proliferation and migration *in vitro* and VEGF₁₆₅-mediated vasodilation *ex vivo* (57) as well as angiogenesis *in vivo* (59). In tumors such as renal cell carcinoma VEGF_{165b} is significantly decreased (57).

Downregulation of VEGF_{165b} leads to metastatic melanoma while overexpression of VEGF_{165b} prevents metastasis of malignant melanoma (60). These observations support an anti-angiogenic role of VEGF_{165b}.

Apparently, the discovery of VEGF_{xxx}b has raised a critical question as to whether the existing VEGF literature needs to be reevaluated with new reagents and methods that can differentiate the pro-angiogenic VEGF_{xxx} from the anti-angiogenic VEGF_{xxx}b isoforms. This is of particular importance not only because VEGF is a focal point of angiogenesis but also because some of the published work might have been misleading particularly for disease-related conditions and with total VEGF as an angiogenesis index. For example, in normal human placentas, VEGF_{xxx} protein occupies the majority of the total VEGF protein expressed and VEGF_{xxx}b occupies only less than 2% of the total VEGF protein; however, their concentrations are positively correlated ($r = 0.69$, $P < 0.02$). In contrast, VEGF_{xxx} isoforms are upregulated and VEGF_{xxx}b isoforms are significantly downregulated in preeclamptic placentas, resulting in a significant negative correlation between VEGF_{xxx}b and VEGF_{xxx} protein expression ($r = -0.8$, $P < 0.02$) (61). These data indicate that preeclampsia uncouples VEGF splicing in human placenta, which further adds to the soluble Flt1/VEGF complex in the deranged angiogenesis during preeclampsia (29). These data also implicate that the discovery of VEGF_{xxx}b has greatly devalued total VEGF as an index of angiogenic activity in preeclampsia and most likely under other disease-related conditions as well. Contrasting to the conventional VEGF_{xxx}, the expression and function of VEGF_{xxx}b in normal and abnormal placental development and angiogenesis awaits further investigation.

Slit/Robo signaling cues and placental angiogenesis

The Slit/Robo signaling system are members of a conserved neuronal guidance cue family that also includes netrin/DCC/Unc5 (62), ephrin/Eph (63) and semaphorin/plexin/neuropilin (64). In these systems, the former ones (i.e., Slit, netrin, ephrin, and semaphorin) are secreted proteins that function as ligands; whereas the latter ones (i.e., Robo, DCC/Unc5, Eph, and plexin/neuropilin) are their corresponding receptors. Mammals have at least three *slit* genes (*slit 1*, *slit 2* and *slit 3*) (65, 66) that encode three Slit proteins with ~1500 amino acids, and four Robo proteins, Robo1, 2, 3 and 4 (65, 67-70). Robo4 seems to be a vascular-specific Slit receptor (69, 70) that is important for the maintenance of vascular integrity by inhibiting abnormal

angiogenesis and endothelial hyperpermeability (71). Slit2, upon binding to Robo1, functions as an attractant to promote the directional migration and vascular network formation *in vitro*. Moreover, these cellular effects are inhibited by an anti-Robo1 antibody and are blocked by a soluble Robo1 extracellular fragment (RoboN) (72). Slit2 is also able to promote endothelial cell migration and tube-formation *in vitro*, possibly mediated by Robo1/Robo4 (73). Secreted soluble Robo4 is able to inhibit *in vivo* angiogenesis and the VEGF- and FGF2 - stimulated endothelial cell proliferation and migration (74). Knockdown or overexpression of Robo4 leads to either lack of or misdirected intersomitic vessels (75). In human placenta, Slit2 and Robo1 proteins are expressed in the syncytiotrophoblast, while Slit3 and Robo1 and Robo4 are detected in capillary endothelium of the placental villi (28, 76). Moreover, levels of Robo1 and Robo4 are significantly greater in preeclamptic placentas compared to normal controls; hypoxia significantly increased both mRNA and protein levels of Slit2 in the trophoblast cell line BeWo and Slit3 and Robo1/4 in human umbilical cord endothelial cells (28). Trophoblast and endothelial co-expression of Slit/Robo implies an autocrine/paracrine regulatory system for the regulation of placental trophoblast and endothelial cell function. It is likely that the other neuronal guidance systems may also have a role in placental angiogenesis although whether they are expressed in the placenta is not known.

Transcription regulation of placental angiogenesis

Global and placenta-specific gene “knock-out” animal studies have provided informative evidence as to the relative significance of a large number of genes [reviewed in (18, 77)] in placental development and function based on embryonic lethality owing to the severity of the placental defects in the homozygous mutant mice. Surprisingly, reduced vasculature in the labyrinth generally occurs in mouse mutants of only a few genes, including the extracellular matrix protein *Cyr61* (78) and the Notch-signaling components *Dll4* (79), *Notch1/4* (80), *Hey1/2* (81), and *Rbpsuh* (82). Of note, these genes are expressed in the vasculature itself and their mutations lead to a poorly vascularized allantois where the placental vasculature stems from during mouse embryogenesis (12). Nonetheless, these studies implicate that these genes, especially these encoding the Notch-signaling components, are of significant importance for placental vasculogenesis.

Genetic studies also have provided convincing data showing that disruption of several transcription factors results in impaired placental angiogenesis although the downstream target genes are incompletely understood. For example, targeted inactivation of *Fra1* [a member of the activator protein-1 (AP-1) transcription factors] (83) results in fetal death between E10.0 and E10.5 owing to defects in extra-embryonic tissues in mouse. The placental labyrinthine layer is reduced in size and largely avascular, owing to a marked decrease in the number of VEGFR1-positive vascular endothelial cells, without affecting the spongiotrophoblast layer. The mutant fetuses are severely growth restricted possibly due to yolk-sac defects. Importantly, when the placental defect is rescued by injection of *Fra1*^{-/-} embryonic stem cells into tetraploid wild-type blastocysts, the pups obtained are no longer growth retarded and survived up to 2 days after birth without apparent phenotypic defects. These results suggest that *Fra1* plays a crucial role in establishing normal vascularization of the placenta, which is crucial for fetal development and survival (84).

Peroxisome proliferator-activated receptor- γ (PPAR γ) is another critical transcription factor that regulates placental vascular development. PPAR γ belongs to a family of ligand-activated transcription factors of the nuclear hormone receptor superfamily, which mainly regulate the expression of genes involved in lipid and energy metabolism (85). It is highly expressed in the trophoblast cells of the rodent labyrinth and in the cytotrophoblasts and syncytiotrophoblasts in human placentas (86), which is increased at late gestation (87). PPAR γ -null mice are embryonic lethal between E9.5 to E11.5 due to defects in placental vascularization, highlighting its role in placental vascular development (88). Placentas of PPAR γ -null mice are with an unsettled balance of pro- and anti-angiogenic factors, i.e., increased proangiogenic factor proliferin (*Prl2c2*, PLF) and decreased anti-angiogenic factor proliferin-related protein (PRP). This has been confirmed with “gain of function” studies because the PPAR γ activator rosiglitazone inhibits placental angiogenesis via regulating PRP and VEGF expression (89). To this end, it is speculating that the critical PPAR γ dimerization partner retinoid X receptor (RXR) may also have a role in placental angiogenesis because RXR-null mice show a similar phenotype to PPAR γ (90).

Mammalian embryogenesis and placental development are believed to take place under constant low-O₂ relative to ambient O₂ (91). For example, in a human placenta the intervillous

space O₂ is as low as ~ 2% at ≤8–10 weeks of gestation at a time when placental vasculature forms; at the end of the first trimester this level rises 3-fold to ~ 8% when maternal blood is delivered into the placenta from the uterine spiral arteries; thereafter O₂ level gradually declines to ~ 6% at the end of the third trimester (92, 93), possibly due to the substantial increased demand of fetus. At the end of the third trimester, the O₂ level in the human fetus is even lower, ~ 2.2% O₂ (range 1.9-3.1%) and ~ 3.7% O₂ (range 2.3-5.1%) in the umbilical artery and vein, respectively (92). Low O₂ or hypoxia is known to stimulate the expression of numerous hypoxia-responsive genes via hypoxia-inducible factor-1β [HIF-1β, also known as arylhydrocarbon receptor nuclear translocator (Arnt)] heterodimerization with HIF-1α (94). HIF-1β mediates hypoxia-induced transcription of many angiogenic genes in the placenta, including VEGF (95). Thus, one would expect that HIF should play a critical role in placental angiogenesis. Surprisingly, vascular defect is likely to be secondary to the primary trophoblast defect in the *Arnt*-null mice (96). This is because placentas of *Arnt*-null mice display greatly reduced size in the spongiotrophoblast and labyrinth layers but with increased numbers of giant trophoblast cells, suggesting that HIF-1β is critical for determining the fate of the trophoblasts (97).

Signaling regulation of placental angiogenesis

The MAPK pathways: The mitogen-activated protein kinase (MAPK) pathways are evolutionarily conserved signal transduction cascades that are implicated in control of different and even opposite cellular responses including proliferation, differentiation and cell death. In vertebrates, multiple isoforms of MAPK have been identified and categorized into three subfamilies, i.e., the extracellular signal-regulated kinases (ERKs), p38MAPK, and the Jun N-terminal kinases (JNKs) or stress-activated protein kinases. The MAPK signaling is important for transmitting extracellular signals including growth factors, hormones, and chemokines, etc., into the intracellular targets for nearly all fundamental cellular processes. The p38MAPK comprises four members, including p38α/MAPK14, p38β/MAPK11, p38γ/MAPK12, and p38δ/MAPK13 (98). Targeted disruption of the *p38α* gene results in embryonic lethality in mice at mid-gestation due to severe placental defects (99, 100). Although chorioallantoic placentation is initiated appropriately in *p38α*-null mice, defects are manifested in the placenta around E10.5, which is evidenced by nearly complete loss of the labyrinth layer and significant reduction of the

spongiotrophoblast. Lack of vascularization and increased rates of apoptosis in the labyrinth layer of the mutant placentas are consistent with a defect in placental angiogenesis (100). An essential role of P38 α in mouse placental development and angiogenesis has been confirmed by specific placental expression of *p38 α* using lentiviral gene delivery technology. When *p38 α* was specifically introduced into the *p38 α* -null mouse placenta, the embryo of the mutant mice is largely rescued with a normal vascularized placenta (101). Application of this method also can substantially rescue the placental defect-caused embryonic lethality due to targeted disruption of other MAPK family members such as ERK2 (102) and their nuclear target Ets2 (103). Thus, the development of placenta-specific gene incorporation by lentiviral transduction of mouse zona-free blastocysts is of specific interest to placental biology, especially with the use of inducible lentiviral vectors (104) by which potentially a desired dose of any genetic materials of interest can be expressed in the placenta spatiotemporally for functional analysis.

The PI3K/Akt pathways: In mammals, the V-akt murine thymoma viral oncogene homolog 1 (Akt1) family of kinases comprises three isoforms (e.g., Akt1, 2, and 3), which are encoded by distinct genes. Upon stimulation with growth factors, hormones, and cytokines, etc., activation of phosphatidylinositol-3-kinase (PI3K) phosphorylates phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] at the D-3 position of the inositol ring to produce PtdIns(3,4,5)P₃, which is then converted to PtdIns(3,4)P by the action of a 5'-phosphatase (105). Interaction with low micromolar concentrations of PtdIns(3,4,5)P₃ or PtdIns(3,4)P₂ triggers the activation process of Akt by phosphorylation (106). Activated Akt can directly phosphorylate glycogen synthase kinase-3 (107) and 6-phosphofructo 2-kinase (108) that are important for protein synthase and insulin signaling; it also phosphorylates the B-cell CLL/lymphoma (Bcl-2)-associated death promoter (BAD) that interacts with the Bcl family member BclxL, thus preventing apoptosis of some cells (109). Akt1 has been found to be widely expressed in the mouse placenta, including all types of trophoblast and vascular endothelial cells (110). Disruption of *Akt1* results in significant neonatal mortality and growth retardation in mice (110-112). *Akt1*-null mouse placentas display significant hypotrophy, with marked reduction of the decidual basalis and nearly complete loss of glycogen-containing cells in the spongiotrophoblast. Furthermore, the placentas also exhibit significantly decreased vascularization, further causing placental insufficiency, fetal growth impairment, and neonatal mortality (110). In addition, placentas of the *Akt1*-null pregnant mice are associated with markedly reduced phosphorylation of Akt1 and

eNOS (110), strongly suggest that Akt1 and eNOS-NO signaling is important for placental angiogenesis. Akt2 and Akt3 seem not to play a major in placental angiogenesis because *Akt2*-null mice display a type-II diabetes-like syndrome and mild growth-retardation and age-dependent loss of adipose tissue (113) and Akt3 has been shown to be important in postnatal brain development (114).

The eNOS-NO pathway: The potent vasodilator NO is generated during the conversion of L-arginine to L-citrulline by a family of NO synthases (NOS), including eNOS, inducible NOS (iNOS) and neuronal NOS (nNOS) (115). Placental NO production increases during pregnancy, which is highly correlated to eNOS, but neither iNOS nor nNOS expression (116, 117), suggesting that eNOS is the major NOS isoform responsible for the increased NO in the placenta. During normal sheep pregnancy placental NO production increases (116, 118) in association with elevated local expression of VEGF and FGF2, vascular density, and blood flow to the placentas (42, 43), suggesting that eNOS-derived NO is important in placental angiogenesis. Indeed, the eNOS-derived NO is critical for the VEGF and FGF2- stimulated angiogenesis *in vitro* (48, 119) and *in vivo* (53). The eNOS-derived NO is also a potent vasodilator in the perfused human muscularized fetoplacental vessels (120), which might be critical for the maintenance of low vascular resistance in the fetoplacental circulation in pregnant sheep *in vivo* (121). Early studies have shown that pharmacological NOS inhibition by L-NG-nitroarginine methyl ester results in preeclampsia-like symptoms and reduced litter size in rats (122). This has been confirmed in *eNOS*-null mice whose dams develop proteinuria (123) and fetuses are growth restricted (123-125). In *eNOS*-null pregnant mice, uteroplacental remodeling is impaired and their vascular adaptations to pregnancy are dysregulated (125, 126), resulting in decreased uterine and placental blood flows and greatly reduced vascularization in the placenta (124, 125). These studies suggest that eNOS is critical for both vasodilation and angiogenesis, i.e., the two rate-limiting mechanisms for blood flow regulation at the maternal-fetal interface.

Numerous studies have shown that activation of the MAPK (ERK1/2, JNK1/2, and p38MAPK), PI3K/Akt1, and eNOS/NO pathways is critical for VEGF- and FGF2-stimulated angiogenesis in various endothelial cells. In placental endothelial cells, we have shown that activation of the MAPK pathways are important for the differential regulation of placental endothelial cell proliferation, migration and tube formation (i.e., *in vitro* angiogenesis) in response to VEGF and FGF2 stimulation *in vitro* (50, 127-129). Inhibition of the ERK1/2

1 pathway partially attenuates the FGF2-stimulated cell proliferation, whereas it completely blocks
2 the VEGF-stimulated cell proliferation as well as the VEGF- and FGF2-stimulated cell migration
3 (47, 48, 50, 128, 129). VEGF stimulation of cell migration also involves stress fiber formation
4 and focal adhesion via the tyrosine kinase Src-mediated phosphorylation of the small actin
5 binding protein cofilin-1 and FAK kinase (48). Inhibition of p38MAPK moderately suppresses
6 FGF2-stimulated cell proliferation and migration, whereas it does not alter VEGF-stimulated cell
7 proliferation and migration (48, 50). Inhibition of JNK1/2 also blocks cell migration stimulated
8 by VEGF (48). Activation of Akt1 is required for VEGF- and FGF2-stimulated eNOS activation
9 and NO production (50, 127, 130) and *in vitro* angiogenic responses including cell proliferation
10 and migration as well as tube formation (48, 50). However, only FGF2 stimulates eNOS mRNA
11 and protein expression via sustained ERK1/2 activation and AP-1 dependent transcription in
12 placental endothelial cells (49, 127). Thus, our data hence suggest that a complex signaling
13 network is involved in the signaling regulation of placental angiogenesis (Fig. 2).

14 **Closing Remarks**

15 Normal placental development and function have long been recognized to be critical not only
16 for the *in utero* development and survival of the fetus and its later life after birth but also for the
17 mother's wellbeing during pregnancy and postpartum. This is best exemplified by the facts that
18 nearly all human pregnancy complications have been linked to aberrant placental development
19 with a deranged vasculature. Although a wealth of knowledge has been generated to date as to
20 how normal placental vascular formation and development are regulated and how they are
21 deranged under various pregnancy complications, there is much more to be learned in this
22 important research topic. Further investigations for in-depth understanding of the genetic,
23 epigenetic, cellular, molecular, physiological and pathological regulation of placental
24 angiogenesis are warranted, which is critically important for reaching an ultimate goal of
25 research in placental angiogenesis - using placental angiogenesis as a target for the development
26 of diagnosis tools and potential therapeutics for pregnancy complications.

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Figure Legend

Fig. 1: Sequential regulation of placental vasculogenesis and angiogenesis during human placental development. Vascular endothelial growth factor (VEGF) is critically important for both placental vasculogenesis and angiogenesis throughout gestation, while fibroblast growth factor (FGF2) and VEGF are important for the formation of angioblasts along with the formation of the first mesenchymal villi. VEGF and placental growth factor (PlGF) are critically important for the formation of placental capillary network via sprouting and elongation with the development of the villous tree. Angiopoietins and many other growth factors are upregulated to facilitate the expansion of placental vascular network during the third trimester.

- 1 **Fig. 2: Signaling control of vascular endothelial growth factor (VEGF)-induced placental**
 2 **endothelial angiogenesis.** VEGF promotes placental endothelial proliferation, migration and
 3 tube formation via the activation of a complex signaling network involving the MAPK,
 4 PI3K/Akt1, and eNOS-NO pathways.

Fig.1