Title
Regulation of Placental Angiogenesis

Journal
Microcirculation, 21(1)

ISSN
1073-9688

Authors
Chen, Dong-bao
Zheng, Jing

Publication Date
2014

DOI
10.1111/micc.12093

Peer reviewed

Received Date : 22-Apr-2013
Revised Date : 20-Aug-2013
Accepted Date : 21-Aug-2013
Article type : Invited Reviews

Regulation of Placental Angiogenesis

Authors: Dong-bao Chen[1] & Jing Zheng[2]

Addresses:

- [1]Departments of Obstetrics & Gynecology, University of California, Irvine, CA 92697;
- [2]Department of Obstetrics and Gynecology, University of Wisconsin, Madison, WI 53715

Running title: Regulation of placental angiogenesis

Source of Funding: The present study was supported in part by the National Institutes of Health grants RO1 HL74947 & HL70562 and R21 HL98746 (to DB Chen), and R01 HL64703 (JZheng) and PO1 HD38843 (RR Magness/JZheng).

Disclosure: The author has no financial interest to disclose.

Correspondence: Dongbao Chen, PhD, Departments of Obstetrics & Gynecology, University of California, Irvine. Tel: 949-824-2409; dongbaoc@uci.edu.

Word Counts

- Abstract: **200**
- Main text: **4328**

Author profile

Dr. Dong-bao Chen is a Professor of Obstetrics & Gynecology and Pathology and the Director of Perinatal Research in the University of California Irvine. His research is accentuated on the cellular and molecular mechanisms underlying estrogen and growth factor regulation of vasodilation and angiogenesis at the maternal, fetal, and placental interface with a focus on reactive nitrogen and oxygen species as well as reactive sulfides.

Dr. Jing Zheng is an Associate Professor of Obstetrics & Gynecology at the University of Wisconsin-Madison. His major research interests focus on the cellular and molecular mechanisms governing placental angiogenesis and vasodilatation as well as ovarian cancer growth.

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 trophectoderm 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 trophoplastic 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 *neuropinin-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 hemagiogenic 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-β1 (TGF-β1) (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-β1 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 ioforms ($VEGF_{121}$, $VEGF_{145,}$ $VEGF_{148,}$ $VEGF_{165}$, $VEGF_{183,}$ $VEGF_{189}$, and $VEGF_{206,}$) 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 $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$, 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 neuropinin-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 $VEGF_{XXX}b$, such as $VEGF_{121}b$, $VEGF_{165}b$, 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, VEGFxxxb and the conventional sister VEGFxxx have different sequences but with the same size; however, they seem to possess opposite functions in angiogenesis. For example, $VEGF_{165}b$ inhibits $VEGF_{165}$-mediated endothelial cell proliferation and migration *in vitro* and $VEGF_{165}$-mediated vasodilation *ex vivo* (57) as well as angiogenesis *in vivo* (59). In tumors such as renal cell carcinoma $VEGF_{165}b$ is significantly decreased (57).

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

Apparently, the discovery of VEGFxxxb 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 VEGFxxx from the anti-angiogenic VEGFxxxb 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, VEGFxxx protein occupies the majority of the total VEGF protein expressed and VEGFxxxb occupies only less than 2% of the total VEGF protein; however, their concentrations are positively correlated ($r = 0.69$, $P<0.02$). In contrast, VEGFxxx isoforms are upregulated and VEGFxxxb isoforms are significantly downregulated in preeclamptic placentas, resulting in a significant negative correlation between VEGFxxxb and VEGFxxx 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 VEGFxxxb 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 VEGFxxx, the expression and function of VEGFxxxb 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, epherin, 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 (PPARγ) is another critical transcription factor that regulates placental vascular development. PPARγ 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). PPARγ-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 PPARγ-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 PPARγ activator rosiglitazone inhibits placental angiogenesis via regulating PRP and VEGF expression (89). To this end, it is speculating that the critical PPARγ dimerization partner retinoid X receptor (RXR) may also have a role in placental angiogenesis because RXR-null mice show a similar phenotype to PPARγ (90).

Mammalian embryogenesis and placental development are believed to take place under constant low-O_2 relative to ambient O_2 (91). For example, in a human placenta the intervillous

space O_2 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 O_2 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 O_2 level in the human fetus is even lower, ~ 2.2% O_2 (range 1.9-3.1%) and ~ 3.7% O_2 (range 2.3-5.1%) in the umbilical artery and vein, respectively (92). Low O_2 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 P38α in mouse placental development and angiogenesis has been confirmed by specific placental expression of *p38α* using lentiviral gene delivery technology. When *p38α* was specifically introduced into the *p38α*-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 phosphotidylinositol-3-kinase (PI3K) phosphorylates phosphatidylinositol 4,5-bisphosphate [Ptdlns(4,5)P_2] at the D-3 position of the inositol ring to produce PtdIns(3,4,5)P_3, which is then converted to PtdIns(3,4)P by the action of a 5'-phosphatase (105). Interaction with low micromolar concentrations of Ptdlns(3,4,5)P_3 or Ptdlns(3,4)P_2 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

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

Closing Remarks

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

Reference Cited

1. **Folkman J, Shing Y** 1992 Angiogenesis. J Biol Chem 267:10931-10934
2. **Carmeliet P** 2003 Angiogenesis in health and disease. Nat Med 9:653-660
3. **Reynolds LP, Redmer DA** 2001 Angiogenesis in the placenta. Biol Reprod 64:1033-1040
4. **Helmlinger G, Endo M, Ferrara N, Hlatky L, Jain RK** 2000 Formation of endothelial cell networks. Nature 405:139-141
5. **Carmeliet P** 2000 Mechanisms of angiogenesis and arteriogenesis. Nat Med 6:389-395
6. **Cross JC, Werb Z, Fisher SJ** 1994 Implantation and the placenta: key pieces of the development puzzle. Science 266:1508-1518
7. **Kaufmann P, Mayhew TM, Charnock-Jones DS** 2004 Aspects of human fetoplacental vasculogenesis and angiogenesis. II. Changes during normal pregnancy. Placenta 25:114-126
8. **Reynolds LP, Caton JS, Redmer DA, Grazul-Bilska AT, Vonnahme KA, Borowicz PP, Luther JS, Wallace JM, Wu G, Spencer TE** 2006 Evidence for altered placental blood flow and vascularity in compromised pregnancies. J Physiol 572:51-58
9. **Macara L, Kingdom JC, Kaufmann P, Kohnen G, Hair J, More IA, Lyall F, Greer IA** 1996 Structural analysis of placental terminal villi from growth-restricted pregnancies with abnormal umbilical artery Doppler waveforms. Placenta 17:37-48
10. **Mayhew TM, Charnock-Jones DS, Kaufmann P** 2004 Aspects of human fetoplacental vasculogenesis and angiogenesis. III. Changes in complicated pregnancies. Placenta 25:127-139
11. **Redman CW, Sargent IL** 2005 Latest advances in understanding preeclampsia. Science 308:1592-1594
12. **Cross JC** 2003 The genetics of pre-eclampsia: a feto-placental or maternal problem? Clin Genet 64:96-103
13. **Burton GJ, Charnock-Jones DS, Jauniaux E** 2009 Regulation of vascular growth and function in the human placenta. Reproduction 138:895-902
14. **Burton GJ, Jauniaux E** 1995 Sonographic, stereological and Doppler flow velocimetric assessments of placental maturity. Br J Obstet Gynaecol 102:818-825

15. **Kaufmann P** 1985 Basic morphology of the fetal and maternal circuits in the human placenta. Contrib Gynecol Obstet 13:5-17
16. **Li Y, Behringer RR** 1998 Esx1 is an X-chromosome-imprinted regulator of placental development and fetal growth. Nat Genet 20:309-311
17. **Li Y, Lemaire P, Behringer RR** 1997 Esx1, a novel X chromosome-linked homeobox gene expressed in mouse extraembryonic tissues and male germ cells. Dev Biol 188:85-95
18. **Watson ED, Cross JC** 2005 Development of structures and transport functions in the mouse placenta. Physiology (Bethesda) 20:180-193
19. **Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF** 1983 Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science (New York, NY 219:983-985
20. **Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N** 1989 Vascular endothelial growth factor is a secreted angiogenic mitogen. Science (New York, NY 246:1306-1309
21. **Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT** 1989 Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science (New York, NY 246:1309-1312
22. **Gospodarowicz D, Ferrara N, Schweigerer L, Neufeld G** 1987 Structural characterization and biological functions of fibroblast growth factor. Endocr Rev 8:95-114
23. **Maglione D, Guerriero V, Viglietto G, Delli-Bovi P, Persico MG** 1991 Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. Proc Natl Acad Sci U S A 88:9267-9271
24. **LeCouter J, Kowalski J, Foster J, Hass P, Zhang Z, Dillard-Telm L, Frantz G, Rangell L, DeGuzman L, Keller GA, Peale F, Gurney A, Hillan KJ, Ferrara N** 2001 Identification of an angiogenic mitogen selective for endocrine gland endothelium. Nature 412:877-884
25. **Derynck R, Jarrett JA, Chen EY, Eaton DH, Bell JR, Assoian RK, Roberts AB, Sporn MB, Goeddel DV** 1985 Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells. Nature 316:701-705

26. **Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM** 1994 Positional cloning of the mouse obese gene and its human homologue. Nature 372:425-432

27. **Sato TN, Tozawa Y, Deutsch U, Wolburg-Buchholz K, Fujiwara Y, Gendron-Maguire M, Gridley T, Wolburg H, Risau W, Qin Y** 1995 Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Nature 376:70-74

28. **Liao WX, Laurent LC, Agent S, Hodges J, Chen DB** 2012 Human placental expression of SLIT/ROBO signaling cues: effects of preeclampsia and hypoxia. Biol Reprod 86: 1-7

29. **Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, Schisterman EF, Thadhani R, Sachs BP, Epstein FH, Sibai BM, Sukhatme VP, Karumanchi SA** 2004 Circulating angiogenic factors and the risk of preeclampsia. N Engl J Med 350:672-683

30. **Poole TJ, Finkelstein EB, Cox CM** 2001 The role of FGF and VEGF in angioblast induction and migration during vascular development. Dev Dyn 220:1-17

31. **Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A** 1996 Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380:435-439

32. **Ferrara N** 2001 Role of vascular endothelial growth factor in regulation of physiological angiogenesis. Am J Physiol Cell Physiol 280:C1358-1366

33. **Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC** 1995 Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 376:62-66

34. **Fong GH, Rossant J, Gertsenstein M, Breitman ML** 1995 Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 376:66-70

35. **Tsoi SC, Wen Y, Chung JY, Chen D, Magness RR, Zheng J** 2002 Co-expression of vascular endothelial growth factor and neuropilin-1 in ovine feto-placental artery endothelial cells. Mol Cell Endocrinol 196:95-106

36. **Joukov V, Kaipainen A, Jeltsch M, Pajusola K, Olofsson B, Kumar V, Eriksson U, Alitalo K** 1997 Vascular endothelial growth factors VEGF-B and VEGF-C. J Cell Physiol 173:211-215

37. **Enholm B, Paavonen K, Ristimaki A, Kumar V, Gunji Y, Klefstrom J, Kivinen L, Laiho M, Olofsson B, Joukov V, Eriksson U, Alitalo K** 1997 Comparison of VEGF, VEGF-B, VEGF-C and Ang-1 mRNA regulation by serum, growth factors, oncoproteins and hypoxia. Oncogene 14:2475-2483

38. **Persico MG, Vincenti V, DiPalma T** 1999 Structure, expression and receptor-binding properties of placenta growth factor (PlGF). Curr Top Microbiol Immunol 237:31-40

39. **Robinson CJ, Stringer SE** 2001 The splice variants of vascular endothelial growth factor (VEGF) and their receptors. J Cell Sci 114:853-865

40. **Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, Abraham JA** 1991 The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. J Biol Chem 266:11947-11954

41. **Cheung CY, Singh M, Ebaugh MJ, Brace RA** 1995 Vascular endothelial growth factor gene expression in ovine placenta and fetal membranes. Am J Obstet Gynecol 173:753-759

42. **Zheng J, Vagnoni KE, Bird IM, Magness RR** 1997 Expression of basic fibroblast growth factor, endothelial mitogenic activity, and angiotensin II type-1 receptors in the ovine placenta during the third trimester of pregnancy. Biol Reprod 56:1189-1197

43. **Borowicz PP, Arnold DR, Johnson ML, Grazul-Bilska AT, Redmer DA, Reynolds LP** 2007 Placental growth throughout the last two thirds of pregnancy in sheep: vascular development and angiogenic factor expression. Biol Reprod 76:259-267

44. **Clark DE, Smith SK, Sharkey AM, Charnock-Jones DS** 1996 Localization of VEGF and expression of its receptors flt and KDR in human placenta throughout pregnancy. Hum Reprod 11:1090-1098

45. **Pepper MS, Ferrara N, Orci L, Montesano R** 1991 Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells. Biochem Biophys Res Commun 181:902-906

46. **Unemori EN, Ferrara N, Bauer EA, Amento EP** 1992 Vascular endothelial growth factor induces interstitial collagenase expression in human endothelial cells. J Cell Physiol 153:557-562

47. **Liao WX, Feng L, Zhang H, Zheng J, Moore TR, Chen DB** 2009 Compartmentalizing VEGF-induced ERK2/1 signaling in placental artery endothelial cell caveolae: a

paradoxical role of caveolin-1 in placental angiogenesis in vitro. Mol Endocrinol 23:1428-1444

48. **Liao WX, Feng L, Zheng J, Chen DB** 2010 Deciphering mechanisms controlling placental artery endothelial cell migration stimulated by vascular endothelial growth factor. Endocrinology 151:3432-3444

49. **Mata-Greenwood E, Liao WX, Wang W, Zheng J, Chen DB** 2010 Activation of AP-1 transcription factors differentiates FGF2 and vascular endothelial growth factor regulation of endothelial nitric-oxide synthase expression in placental artery endothelial cells. J Biol Chem 285:17348-17358

50. **Zheng J, Wen Y, Song Y, Wang K, Chen DB, Magness RR** 2008 Activation of multiple signaling pathways is critical for fibroblast growth factor 2- and vascular endothelial growth factor-stimulated ovine fetoplacental endothelial cell proliferation. Biol Reprod 78:143-150

51. **Zheng J, Wen Y, Austin JL, Chen DB** 2006 Exogenous nitric oxide stimulates cell proliferation via activation of a mitogen-activated protein kinase pathway in ovine fetoplacental artery endothelial cells. Biol Reprod 74:375-382

52. **Furchgott RF, Vanhoutte PM** 1989 Endothelium-derived relaxing and contracting factors. FASEB J 3:2007-2018

53. **Fukumura D, Gohongi T, Kadambi A, Izumi Y, Ang J, Yun CO, Buerk DG, Huang PL, Jain RK** 2001 Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability. Proc Natl Acad Sci U S A 98:2604-2609

54. **Armulik A, Abramsson A, Betsholtz C** 2005 Endothelial/pericyte interactions. Circ Res 97:512-523

55. **Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V, Ferrara N** 1998 Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J Biol Chem 273:30336-30343

56. **Jain RK** 2003 Molecular regulation of vessel maturation. Nat Med 9:685-693

57. **Bates DO, Cui TG, Doughty JM, Winkler M, Sugiono M, Shields JD, Peat D, Gillatt D, Harper SJ** 2002 VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma. Cancer Res 62:4123-4131

58. **Harper SJ, Bates DO** 2008 VEGF-A splicing: the key to anti-angiogenic therapeutics? Nat Rev Cancer 8:880-887

59. **Woolard J, Wang WY, Bevan HS, Qiu Y, Morbidelli L, Pritchard-Jones RO, Cui TG, Sugiono M, Waine E, Perrin R, Foster R, Digby-Bell J, Shields JD, Whittles CE, Mushens RE, Gillatt DA, Ziche M, Harper SJ, Bates DO** 2004 VEGF165b, an inhibitory vascular endothelial growth factor splice variant: mechanism of action, in vivo effect on angiogenesis and endogenous protein expression. Cancer Res 64:7822-7835

60. **Pritchard-Jones RO, Dunn DB, Qiu Y, Varey AH, Orlando A, Rigby H, Harper SJ, Bates DO** 2007 Expression of VEGF(xxx)b, the inhibitory isoforms of VEGF, in malignant melanoma. Br J Cancer 97:223-230

61. **Bates DO, MacMillan PP, Manjaly JG, Qiu Y, Hudson SJ, Bevan HS, Hunter AJ, Soothill PW, Read M, Donaldson LF, Harper SJ** 2006 The endogenous anti-angiogenic family of splice variants of VEGF, VEGFxxxb, are down-regulated in pre-eclamptic placentae at term. Clin Sci (Lond) 110:575-585

62. **Freitas C, Larrivee B, Eichmann A** 2008 Netrins and UNC5 receptors in angiogenesis. Angiogenesis 11:23-29

63. **Cheng N, Brantley DM, Chen J** 2002 The ephrins and Eph receptors in angiogenesis. Cytokine Growth Factor Rev 13:75-85

64. **Neufeld G, Kessler O** 2008 The semaphorins: versatile regulators of tumour progression and tumour angiogenesis. Nat Rev Cancer 8:632-645

65. **Brose K, Bland KS, Wang KH, Arnott D, Henzel W, Goodman CS, Tessier-Lavigne M, Kidd T** 1999 Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. Cell 96:795-806

66. **Itoh A, Miyabayashi T, Ohno M, Sakano S** 1998 Cloning and expressions of three mammalian homologues of Drosophila slit suggest possible roles for Slit in the formation and maintenance of the nervous system. Brain Res Mol Brain Res 62:175-186

67. **Kidd T, Russell C, Goodman CS, Tear G** 1998 Dosage-sensitive and complementary functions of roundabout and commissureless control axon crossing of the CNS midline. Neuron 20:25-33

68. **Kidd T, Brose K, Mitchell KJ, Fetter RD, Tessier-Lavigne M, Goodman CS, Tear G** 1998 Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. Cell 92:205-215

69. **Huminiecki L, Gorn M, Suchting S, Poulsom R, Bicknell R** 2002 Magic roundabout is a new member of the roundabout receptor family that is endothelial specific and expressed at sites of active angiogenesis. Genomics 79:547-552

70. **Park KW, Morrison CM, Sorensen LK, Jones CA, Rao Y, Chien CB, Wu JY, Urness LD, Li DY** 2003 Robo4 is a vascular-specific receptor that inhibits endothelial migration. Dev Biol 261:251-267

71. **Jones CA, London NR, Chen H, Park KW, Sauvaget D, Stockton RA, Wythe JD, Suh W, Larrieu-Lahargue F, Mukouyama YS, Lindblom P, Seth P, Frias A, Nishiya N, Ginsberg MH, Gerhardt H, Zhang K, Li DY** 2008 Robo4 stabilizes the vascular network by inhibiting pathologic angiogenesis and endothelial hyperpermeability. Nat Med 14:448-453

72. **Wang B, Xiao Y, Ding BB, Zhang N, Yuan X, Gui L, Qian KX, Duan S, Chen Z, Rao Y, Geng JG** 2003 Induction of tumor angiogenesis by Slit-Robo signaling and inhibition of cancer growth by blocking Robo activity. Cancer Cell 4:19-29

73. **Sheldon H, Andre M, Legg JA, Heal P, Herbert JM, Sainson R, Sharma AS, Kitajewski JK, Heath VL, Bicknell R** 2009 Active involvement of Robo1 and Robo4 in filopodia formation and endothelial cell motility mediated via WASP and other actin nucleation-promoting factors. Faseb J 23:513-522

74. **Suchting S, Heal P, Tahtis K, Stewart LM, Bicknell R** 2005 Soluble Robo4 receptor inhibits in vivo angiogenesis and endothelial cell migration. Faseb J 19:121-123

75. **Bedell VM, Yeo SY, Park KW, Chung J, Seth P, Shivalingappa V, Zhao J, Obara T, Sukhatme VP, Drummond IA, Li DY, Ramchandran R** 2005 roundabout4 is essential for angiogenesis in vivo. Proc Natl Acad Sci U S A 102:6373-6378

76. **Liao WX, Wing DA, Geng JG, Chen DB** 2010 Perspectives of SLIT/ROBO signaling in placental angiogenesis. Histol Histopathol 25:1181-1190

77. **Rossant J, Cross JC** 2001 Placental development: lessons from mouse mutants. Nat Rev Genet 2:538-548

78. **Mo FE, Muntean AG, Chen CC, Stolz DB, Watkins SC, Lau LF** 2002 CYR61 (CCN1) is essential for placental development and vascular integrity. Mol Cell Biol 22:8709-8720

79. **Duarte A, Hirashima M, Benedito R, Trindade A, Diniz P, Bekman E, Costa L, Henrique D, Rossant J** 2004 Dosage-sensitive requirement for mouse Dll4 in artery development. Genes Dev 18:2474-2478

80. **Krebs LT, Xue Y, Norton CR, Shutter JR, Maguire M, Sundberg JP, Gallahan D, Closson V, Kitajewski J, Callahan R, Smith GH, Stark KL, Gridley T** 2000 Notch signaling is essential for vascular morphogenesis in mice. Genes Dev 14:1343-1352

81. **Fischer A, Schumacher N, Maier M, Sendtner M, Gessler M** 2004 The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. Genes Dev 18:901-911

82. **Krebs LT, Shutter JR, Tanigaki K, Honjo T, Stark KL, Gridley T** 2004 Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. Genes Dev 18:2469-2473

83. **Karin M, Liu Z, Zandi E** 1997 AP-1 function and regulation. Curr Opin Cell Biol 9:240-246

84. **Schreiber M, Wang ZQ, Jochum W, Fetka I, Elliott C, Wagner EF** 2000 Placental vascularisation requires the AP-1 component fra1. Development 127:4937-4948

85. **Wahli W, Michalik L** 2012 PPARs at the crossroads of lipid signaling and inflammation. Trends Endocrinol Metab 23:351-363

86. **Fournier T, Tsatsaris V, Handschuh K, Evain-Brion D** 2007 PPARs and the placenta. Placenta 28:65-76

87. **Nadra K, Anghel SI, Joye E, Tan NS, Basu-Modak S, Trono D, Wahli W, Desvergne B** 2006 Differentiation of trophoblast giant cells and their metabolic functions are dependent on peroxisome proliferator-activated receptor beta/delta. Mol Cell Biol 26:3266-3281

88. **Barak Y, Nelson MC, Ong ES, Jones YZ, Ruiz-Lozano P, Chien KR, Koder A, Evans RM** 1999 PPAR gamma is required for placental, cardiac, and adipose tissue development. Mol Cell 4:585-595

89. **Nadra K, Quignodon L, Sardella C, Joye E, Mucciolo A, Chrast R, Desvergne B** 2010 PPARgamma in placental angiogenesis. Endocrinology 151:4969-4981

90. **Wendling O, Chambon P, Mark M** 1999 Retinoid X receptors are essential for early mouse development and placentogenesis. Proc Natl Acad Sci U S A 96:547-551

91. **Jauniaux E, Watson AL, Hempstock J, Bao YP, Skepper JN, Burton GJ** 2000 Onset of maternal arterial blood flow and placental oxidative stress. A possible factor in human early pregnancy failure. Am J Pathol 157:2111-2122

92. **Rodesch F, Simon P, Donner C, Jauniaux E** 1992 Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. Obstet Gynecol 80:283-285

93. **Meschia G** 2004 Placental respiratory gas and exchange and fetal oxygenation. In: Creasy RK, Resnik R,Iams JD, eds, Maternal-Fetal Medicine: Principles and Practice Else Elsevier Health Sciences:199-207.

94. **Ema M, Hirota K, Mimura J, Abe H, Yodoi J, Sogawa K, Poellinger L, Fujii-Kuriyama Y** 1999 Molecular mechanisms of transcription activation by HLF and HIF1alpha in response to hypoxia: their stabilization and redox signal-induced interaction with CBP/p300. EMBO J 18:1905-1914

95. **Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL** 1996 Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 16:4604-4613

96. **Adelman DM, Gertsenstein M, Nagy A, Simon MC, Maltepe E** 2000 Placental cell fates are regulated in vivo by HIF-mediated hypoxia responses. Genes Dev 14:3191-3203

97. **Kozak KR, Abbott B, Hankinson O** 1997 ARNT-deficient mice and placental differentiation. Dev Biol 191:297-305

98. **Cargnello M, Roux PP** 2011 Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol Rev 75:50-83

99. **Adams RH, Porras A, Alonso G, Jones M, Vintersten K, Panelli S, Valladares A, Perez L, Klein R, Nebreda AR** 2000 Essential role of p38alpha MAP kinase in placental but not embryonic cardiovascular development. Mol Cell 6:109-116

100. **Mudgett JS, Ding J, Guh-Siesel L, Chartrain NA, Yang L, Gopal S, Shen MM** 2000 Essential role for p38alpha mitogen-activated protein kinase in placental angiogenesis. Proc Natl Acad Sci U S A 97:10454-10459

101. **Okada Y, Ueshin Y, Isotani A, Saito-Fujita T, Nakashima H, Kimura K, Mizoguchi A, Oh-Hora M, Mori Y, Ogata M, Oshima RG, Okabe M, Ikawa M** 2007 Complementation of placental defects and embryonic lethality by trophoblast-specific lentiviral gene transfer. Nat Biotechnol 25:233-237

102. **Hatano N, Mori Y, Oh-hora M, Kosugi A, Fujikawa T, Nakai N, Niwa H, Miyazaki J, Hamaoka T, Ogata M** 2003 Essential role for ERK2 mitogen-activated protein kinase in placental development. Genes Cells 8:847-856

103. **Yamamoto H, Flannery ML, Kupriyanov S, Pearce J, McKercher SR, Henkel GW, Maki RA, Werb Z, Oshima RG** 1998 Defective trophoblast function in mice with a targeted mutation of Ets2. Genes Dev 12:1315-1326

104. **Fan X, Petitt M, Gamboa M, Huang M, Dhal S, Druzin ML, Wu JC, Chen-Tsai Y, Nayak NR** 2012 Transient, inducible, placenta-specific gene expression in mice. Endocrinology 153:5637-5644

105. **Vanhaesebroeck B, Leevers SJ, Panayotou G, Waterfield MD** 1997 Phosphoinositide 3-kinases: a conserved family of signal transducers. Trends Biochem Sci 22:267-272

106. **Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, Cohen P** 1997 Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr Biol 7:261-269

107. **Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA** 1995 Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378:785-789

108. **Deprez J, Vertommen D, Alessi DR, Hue L, Rider MH** 1997 Phosphorylation and activation of heart 6-phosphofructo-2-kinase by protein kinase B and other protein kinases of the insulin signaling cascades. J Biol Chem 272:17269-17275

109. **Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ** 1996 Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell 87:619-628

110. **Yang ZZ, Tschopp O, Hemmings-Mieszczak M, Feng J, Brodbeck D, Perentes E, Hemmings BA** 2003 Protein kinase B alpha/Akt1 regulates placental development and fetal growth. J Biol Chem 278:32124-32131

111. **Chen WS, Xu PZ, Gottlob K, Chen ML, Sokol K, Shiyanova T, Roninson I, Weng W, Suzuki R, Tobe K, Kadowaki T, Hay N** 2001 Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. Genes Dev 15:2203-2208

112. **Cho H, Thorvaldsen JL, Chu Q, Feng F, Birnbaum MJ** 2001 Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. J Biol Chem 276:38349-38352

113. **Woulfe D, Jiang H, Morgans A, Monks R, Birnbaum M, Brass LF** 2004 Defects in secretion, aggregation, and thrombus formation in platelets from mice lacking Akt2. J Clin Invest 113:441-450

114. **Easton RM, Cho H, Roovers K, Shineman DW, Mizrahi M, Forman MS, Lee VM, Szabolcs M, de Jong R, Oltersdorf T, Ludwig T, Efstratiadis A, Birnbaum MJ** 2005 Role for Akt3/protein kinase Bgamma in attainment of normal brain size. Mol Cell Biol 25:1869-1878

115. **Searles CD** 2006 Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression. Am J Physiol Cell Physiol 291:C803-816

116. **Zheng J, Li Y, Weiss AR, Bird IM, Magness RR** 2000 Expression of endothelial and inducible nitric oxide synthases and nitric oxide production in ovine placental and uterine tissues during late pregnancy. Placenta 21:516-524

117. **Myatt L, Eis AL, Brockman DE, Greer IA, Lyall F** 1997 Endothelial nitric oxide synthase in placental villous tissue from normal, pre-eclamptic and intrauterine growth restricted pregnancies. Hum Reprod 12:167-172

118. **Kwon H, Wu G, Meininger CJ, Bazer FW, Spencer TE** 2004 Developmental changes in nitric oxide synthesis in the ovine placenta. Biol Reprod 70:679-686

119. **Cooke JP** 2003 NO and angiogenesis. Atheroscler Suppl 4:53-60

120. **Myatt L, Brewer AS, Langdon G, Brockman DE** 1992 Attenuation of the vasoconstrictor effects of thromboxane and endothelin by nitric oxide in the human fetal-placental circulation. Am J Obstet Gynecol 166:224-230

121. **Chang JK, Roman C, Heymann MA** 1992 Effect of endothelium-derived relaxing factor inhibition on the umbilical-placental circulation in fetal lambs in utero. Am J Obstet Gynecol 166:727-734

122. **Buhimschi I, Yallampalli C, Chwalisz K, Garfield RE** 1995 Pre-eclampsia-like conditions produced by nitric oxide inhibition: effects of L-arginine, D-arginine and steroid hormones. Hum Reprod 10:2723-2730

123. **Kusinski LC, Stanley JL, Dilworth MR, Hirt CJ, Andersson IJ, Renshall LJ, Baker BC, Baker PN, Sibley CP, Wareing M, Glazier JD** 2012 eNOS knockout mouse as a model of fetal growth restriction with an impaired uterine artery function and placental transport phenotype. Am J Physiol Regul Integr Comp Physiol 303:R86-93

124. **Kulandavelu S, Whiteley KJ, Qu D, Mu J, Bainbridge SA, Adamson SL** 2012 Endothelial nitric oxide synthase deficiency reduces uterine blood flow, spiral artery elongation, and placental oxygenation in pregnant mice. Hypertension 60:231-238

125. **Kulandavelu S, Whiteley KJ, Bainbridge SA, Qu D, Adamson SL** 2012 Endothelial NO synthase augments fetoplacental blood flow, placental vascularization, and fetal growth in mice. Hypertension 61:259-266

126. **van der Heijden OW, Essers YP, Fazzi G, Peeters LL, De Mey JG, van Eys GJ** 2005 Uterine artery remodeling and reproductive performance are impaired in endothelial nitric oxide synthase-deficient mice. Biol Reprod 72:1161-1168

127. **Mata-Greenwood E, Liao WX, Zheng J, Chen DB** 2008 Differential activation of multiple signalling pathways dictates eNOS upregulation by FGF2 but not VEGF in placental artery endothelial cells. Placenta 29:708-717

128. **Feng L, Liao WX, Luo Q, Zhang HH, Wang W, Zheng J, Chen DB** 2012 Caveolin-1 orchestrates fibroblast growth factor 2 signaling control of angiogenesis in placental artery endothelial cell caveolae. J Cell Physiol 227:2480-2491

129. **Feng L, Zhang HH, Wang W, Zheng J, Chen DB** 2012 Compartmentalizing proximal FGFR1 signaling in ovine placental artery endothelial cell caveolae. Biol Reprod 87:1-9

130. **Zheng J, Bird IM, Melsaether AN, Magness RR** 1999 Activation of the mitogen-activated protein kinase cascade is necessary but not sufficient for basic fibroblast growth factor- and epidermal growth factor-stimulated expression of endothelial nitric oxide synthase in ovine fetoplacental artery endothelial cells. Endocrinology 140:1399-1407

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.

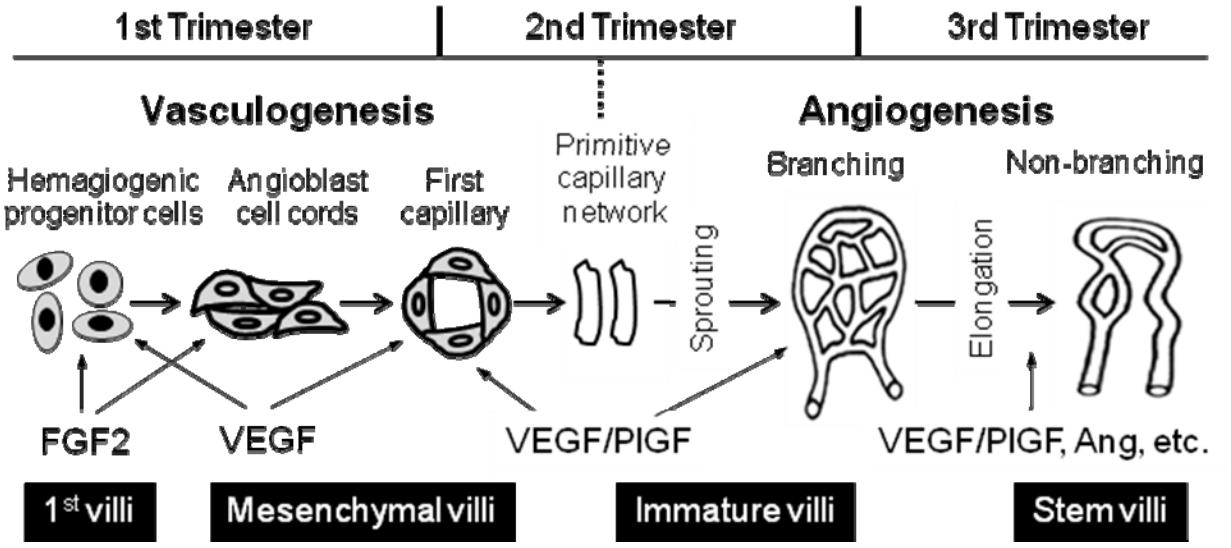

Fig. 2: Signaling control of vascular endothelial growth factor (VEGF)-induced placental endothelial angiogenesis. VEGF promotes placental endothelial proliferation, migration and tube formation via the activation of a complex signaling network involving the MAPK, PI3K/Akt1, and eNOS-NO pathways.

Fig.1