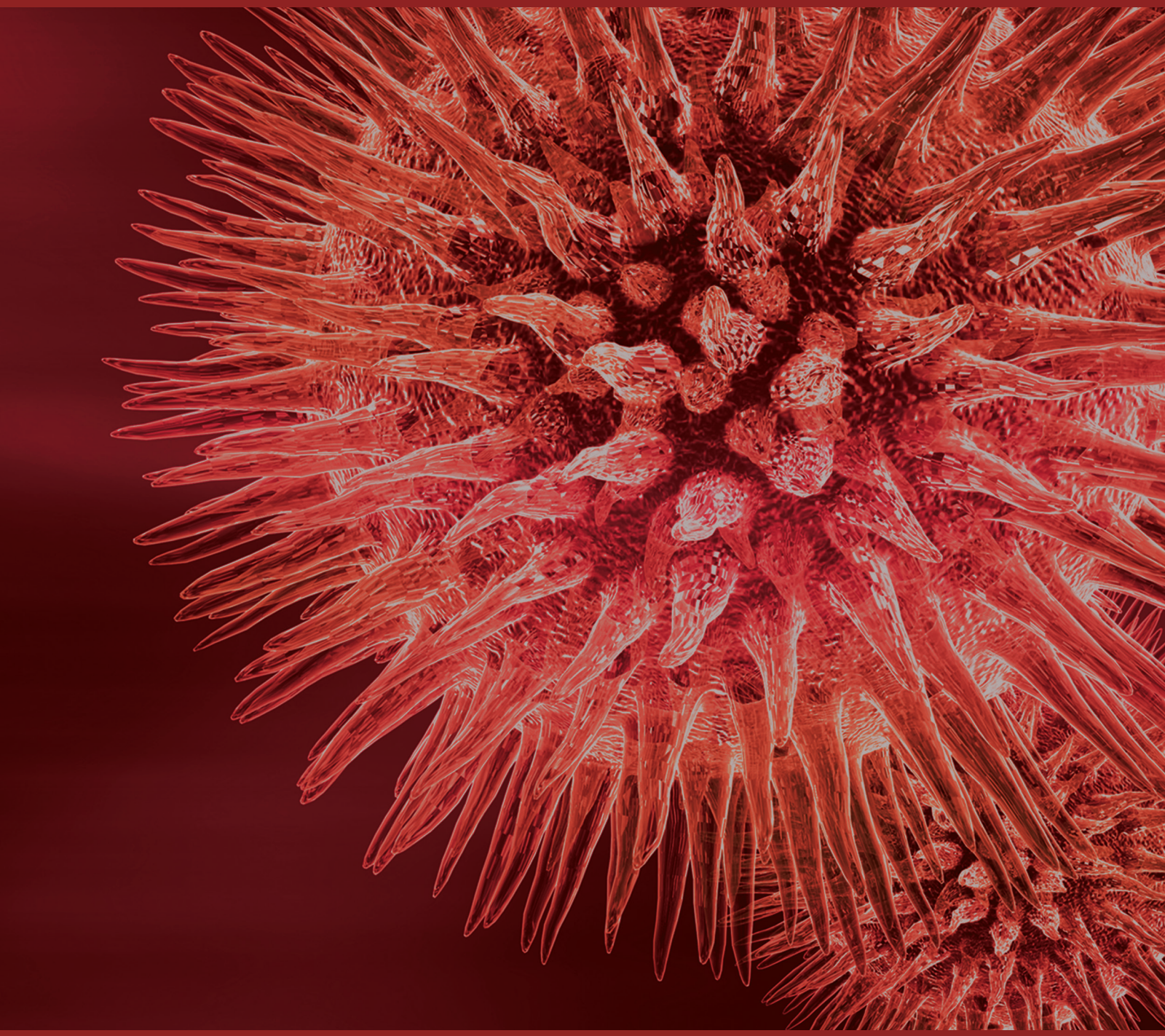
BioMed Research International

Normal and Pathological Placental Angiogenesis

Guest Editors: Nathalie Bardin, Padma Murthi, and Nadia Alfaidy



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Editorial

Normal and Pathological Placental Angiogenesis

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Placental angiogenesis is a pivotal process that establishes fetomaternal circulation, ensures efficient maternofetal exchanges, plays a key mechanistic role in the elaboration of the placental villous tree, and contributes to the overall development of the placenta throughout pregnancy. Failure in these processes is tightly linked to the development of placental pathologies such as preeclampsia (PE), early pregnancy loss, and intrauterine growth restriction (IUGR). It is now well established that a close relationship exists between embryonic development and the degree of placental angiogenesis. A key discovery in the investigation of placental pathologies was the understanding that major phenotypes of PE are associated with dysregulation in angiogenic factors. During the last decade multiple new key angiogenic proteins have been reported to play crucial roles in placental angiogenesis; these include netrins, EG-VEGF (endocrine gland derived endothelial growth factor), and angiogenin. Furthermore, placental angiogenesis appears to also be regulated by specific microRNAs (miRNAs), deregulations of which have also been shown to be associated with pregnancy pathologies. By affecting placental angiogenesis, numerous insults have been shown to influence pregnancy outcomes. These include (i) oxidative stress, (ii) hyperglycemia, and (iii) failure in immune system adaptations to pregnancy, such as the presence of antiphospholipid antibodies (aPL) and/or soluble Mic (sMIC).

In the present scope authors reported new findings in the field of placental angiogenesis, discussed the advancements made in the diagnosis of pathologies reported to be associated with placental angiogenesis, and brought new insights into the processes of vasculogenesis and angiogenesis occurring throughout pregnancy in the placenta. More importantly, regulators of the key protagonists of vascular and angiogenic processes have been reported and their roles discussed.

The reviews by M. Dakouane-Giudicelli et al., N. Pavlov et al., and N. Alfaidy et al. reported the role of three newly discovered angiogenic placental factors, netrin-1 and netrin-4, angiogenin, and EG-VEGF, respectively. M. Dakouane-Giudicelli et al. highlighted the opposite role that netrin-1 and netrin-4 might play in normal and pathological pregnancies. Netrin-1 and netrin-4 have been found to be either proangiogenic or antiangiogenic factors in the human placenta. These opposite effects appear to be related to the endothelial cell phenotype studied and seem also to depend on the type of receptor to which each netrin binds.

N. Pavlov et al. demonstrated that the proangiogenic protein, angiogenin, might play a key role in placental blood vessel formation as well as in the cross talk between trophoblasts and endothelial cells. The review by N. Alfaidy et al. recapitulates EG-VEGF mediated-angiogenesis within the placenta and at the fetomaternal interface and proposes that its deregulation might contribute to the pathogenesis of several placental pathologies including IUGR and PE. More importantly, this article argues for EG-VEGF clinical relevance as a potential biomarker of the onset of pregnancy pathologies and discusses its potential usefulness for future therapeutic directions.

In a second set of reviews of the scope Dr. R. D. Pereira et al. reported the effect of oxidative stress on the expression of a number of transcription factors that are important in mediating angiogenesis and stressed that the understanding of how oxidative stress affects redox-sensitive transcription

factors within the placenta may elucidate potential therapeutic targets to correct abnormal placental angiogenesis and function. In the same context, S. Cvitic et al. described the major regulators of placental angiogenesis and demonstrated how the diabetic environment *in utero* alters their expression.

In relation to immune placental pathologies that affect placental vascularization and angiogenesis, J. B. Haumonte et al. work suggests that detection of sMIC molecules in maternal plasma may constitute a hallmark of altered maternal immune functions associated with vascular disorders that contributes to poor pregnancy outcomes, notably by impairing NK-cell mediated production of IFN gamma, an essential cytokine favoring vascular modeling. In the same perspective, O. Paulmyer-Lacroix et al. highlighted the menace of antiphospholipid antibodies (aPL) positive patients on placental vascular development during implantation. These findings propose aPL assessment, in particular aβ2GPI IgA antibodies, as a novel therapeutic strategy to improve pregnancy outcome.

Finally, M. Tsochandaridis et al. highlighted a major topic of interest for clinicians and biologists that concern maternal and fetal monitoring and their importance in predicting pregnancy complications. Her work has addressed the potential role of circulating as well as placental miRNAs that are associated with abnormal angiogenesis.

Overall the present scope on this special issue summarizes key aspects of placental angiogenesis during normal and pathological pregnancies. The scope will expand our knowledge in this field and allow better understanding of the associated mechanisms to placental angiogenesis.

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Review Article

Circulating MicroRNAs as Clinical Biomarkers in the Predictions of Pregnancy Complications

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Predicting pregnancy complications is a major topic for clinicians and biologists for maternal and fetal monitoring. Noninvasive biomarkers in maternal blood such as circulating microRNAs (miRNAs) are promising molecules to predict pregnancy disorders. miRNAs are noncoding short RNAs that regulate mRNA expression by repressing the translation or cleaving the transcript. miRNAs are released to the extracellular systemic circulation via exosomes. The discovery of plasma- or serum-derived miRNAs and of free-circulating exosomes that contain miRNAs provides useful information about the physiological or pathophysiological roles of the miRNAs. Specific placental miRNAs are present in maternal plasma in different ways depending on whether the pregnancy is normal or pathological or if there is no pregnancy. This paper focuses on placental miRNAs and extracellular miRNAs to the placenta whose misregulation could lead to pregnancy complications.

1. Introduction

The discovery of fetal DNA in maternal blood has opened new perspectives on noninvasive prenatal diagnosis development [1]. Since then, other fetal specific nucleic acids related to placenta circulating throughout the plasma and the serum of pregnant women have been found: mRNA [2] and miRNA [3]. Specific placental miRNAs circulating through the plasma reflect the physiological state of pregnancy and are undetectable after delivery [4]. This suggests that their concentration and their profiles in the plasma make them possible candidates as biomarkers for the detection of pregnancy complications linked to placental pathologies. The identification of specific placental miRNAs in maternal blood as noninvasive biomarkers in pregnancy management is developing rapidly. Early noninvasive diagnosis techniques could facilitate medical monitoring for both the mother and the fetus. In this paper, we review the misregulated expression of circulating placental miRNAs related to specific pregnancy pathologies. These molecules could be promising biomarkers for noninvasive diagnosis or the prediction of preeclampsia, intrauterine growth restriction, and early pregnancy loss.

2. MicroRNAs

In the early 2000s the term microRNA (miRNA) was first introduced. MicroRNAs are small noncoding RNAs about 20–24 nucleotides long. These molecules have been shown to play critical regulatory roles in a wide range of biological and pathological processes [5]. miRNAs regulate cellular gene expression at the posttranscriptional level by suppressing the translation of protein coding genes. This process is made possible by the perfect pairing of miRNAs and mRNAs or their imperfect pairing leading to cleft mRNAs of protein coding genes. In both cases the pairing is performed at 3′UTR (untranslated region) or 5′UTR of mRNA [6]. The miRNA biogenesis is a multistep complex process (Figure 1): miRNA genes are transcribed as primary miRNA (pri-miRNA) in the nucleus by the enzyme RNA pol II. RNA pol II is bound to the promoter region of a specific DNA sequence and forms

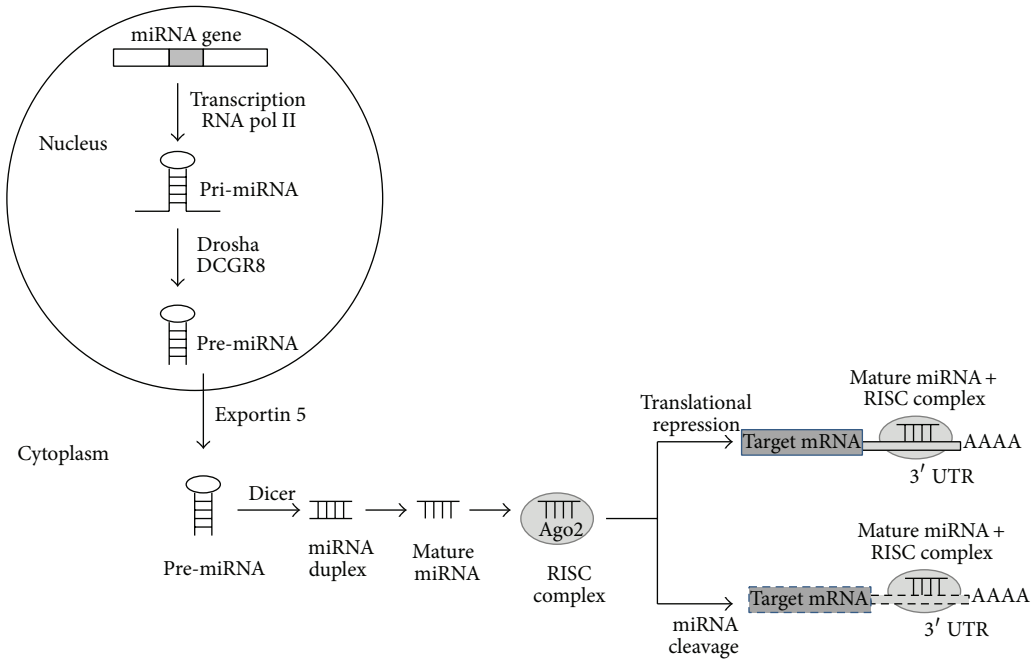

Figure 1: miRNA biogenesis. miRNA gene is transcribed by RNA pol II to form a hairpin loop primary transcript, pri-miRNA, which is processed by Drosha/DCGR8 to form pre-miRNA. Pre-miRNA is exported to the cytoplasm by exportin 5 where Dicer cleaves off the hairpin loop to form a duplex that contains the mature miRNA. The mature miRNA is then incorporated into the RNA-induced silencing complex RISC to target the 3′ untranslated region of the target mRNA to silence expression by repression or cleavage.

the pri-mRNA which presents a hair pin structure. This pri-miRNA transcript is processed by the Drosha endonuclease associated with the double-stranded RNA binding protein DGCR8 to form the precursor miRNA (pre-miRNA) by cleaving the nucleotides on both sides of the hair pin. Pre-miRNAs are exported by exportin 5 into the cytoplasm. The stem-loop miRNA is then processed by the Dicer RNase III endonuclease to produce the double-strand mature miRNA. The mature miRNA is associated with Ago2 to form the RNA-induced silencing complex (RISC), which prevents target mRNAs expression in a specific manner relative to the stability of the bound of the RISC complex at 3′UTR on the target mRNA [5]. When the sequences are perfectly matched, the RISC complex is bound tightly to the mRNA; and the mRNA is degraded by the enzyme Ago2. When the sequences are not perfectly matched, the RISC complex inhibits the translation of the mRNA without degradation. The two different pathways lead to the same final outcome: a decrease in the protein level of the target gene [7]. RISC complex can be bound efficiently not only on the 3′UTR but also on the 5′UTR of the target mRNA to inhibit the translation [6]. The binding with 3′UTR has been well studied; however the binding with 5′UTR needs further investigation to better understand this regulation. Interestingly, recent studies have shown that animal miRNAs are able not only to repress, but also to activate gene expression by acting on mRNA stability and translational regulation [8]. The binding of RISC complex on 5′UTR is a necessary condition for the translation activation. It has been shown that the association of miRNAs with 5′UTR generally induces translation activation rather than repression [9].

According to the miRNA database, today 2578 mature and 1872 precursor forms of miRNAs have been identified in humans [10]. One of the first identified characteristics of the miRNAs is the very well conserved sequences in all species and these are expressed in tissues, in a specific manner [11]. miRNAs are critical in cell development, proliferation, communication, and tissue differentiation. They have been involved in regulating pregnancies [5]. Aberrant miRNA expression patterns have been found in pathologies and physiological processes including pregnancy and angiogenesis [12].

3. MicroRNA Functions in Human Placental Development

Development of the human placenta is critical for embryonic development and successful pregnancy. Differentiation, migration, invasion, angiogenesis, proliferation, and apoptosis mechanisms play an important role in key processes of placental development [13, 14]. The first step begins with the implantation of the blastocyst followed by the formation of various trophoblast cell types from the outer epithelial layer of the blastocyst, the trophectoderm [13]. Primitive syncytium is generated from the invasive trophoblast cell type migrating into the maternal endometrium. After the formation of the lacuna system, which is the ancestor of the intervillous space, cytotrophoblasts emanating from the trophectodermal layer produce primary villi by proliferation and invasion through the primitive syncytium. These primary villi change into secondary and tertiary villi characterized by

the invasion of extraembryonic mesenchymal cells, villous branching, and vascularization. The cytotrophoblast fuses into multinucleated syncytiotrophoblasts which are eventually in close contact with placental vessels allowing efficient nutrient uptake by the fetus [14].

Studies have shown that miRNAs are produced in the human placenta and their expression is regulated by environmental factors such as hypoxia, signaling pathways, and epigenetic modification [15–17]. MicroRNAs are expressed in different ways during the various stages of placental development relative to their functions in regulating placental development and trophoblast cell activities [12]. miRNAs regulate placental development and functions through trophoblast cell proliferation, apoptosis, migration, invasion, and angiogenesis: miR-378a-5p [18], miR-376c [19], and miR-141 [20] enhance trophoblast cell proliferation whereas miRNA-155 [21] and miRNA-675 [22] inhibit this process. Apoptosis of trophoblast cells is induced by miR-29b [23] and inhibited by miR-182 [24]. It has been shown that miRNAs are involved in trophoblast cell migration and invasion: miR-376c [19] and miR-378a-5p [18] enhance trophoblast migration and invasion; miR-195 [25] and miR-21 [26, 27] promote trophoblast invasion. Conversely, miR-210 [28], miR-34a [29], and miR-29b [23] reduce trophoblast cell invasion; miR-155 [30] has an inhibiting effect on trophoblast cell migration. In placental angiogenesis, the abundant expression of Dicer in the perivascular villous stroma suggests that miRNAs play a role in placental vascularization and spiral artery remodeling [31]. It has been shown that miR-16 and miR-29b reduce placental angiogenesis [12].

More than 500 miRNAs are expressed in the human placenta which exhibits a specific miRNA expression pattern [11]. Numerous miRNAs, which are predominantly or exclusively expressed during pregnancy, are clustered in chromosomal regions and work synergistically [32]. The three most significant miRNA clusters are C19MC and miR-371-3 cluster (located on chromosome 19) and C14MC (located on chromosome 14) [33]. C19MC and C14MC are specifically expressed in the placenta [26, 34]. C19MC is the largest miRNA cluster identified with 46 pre-miRNAs transcribed only in the placenta [35] and expressed from the paternal allele [36]. C14MC contains 46 miRNAs and is encoded by maternally imprinted genes [17]. miR-371-3 cluster is adjacent to C19MC and contains 3 miRNAs. The expression of C19MC and miR-371-3 miRNAs increases from the first to the third trimester while C14MC miRNAs decrease during the same period [33]. These different expressions suggest that the placental miRNAs have specific functions at the various stages of the pregnancy.

Despite these interesting findings, the majority of placental miRNAs remain unknown. Only a small number of placental specific miRNAs have been studied for their role in placental development. Hence further research is required in this study.

4. Circulating Placental MicroRNA

In 1997, the discovery by Lo et al. of fetal cell free DNA in the plasma of pregnant women has led to the development of noninvasive diagnostic methods based on maternal blood for clinical applications such as fetal rhesus D genotyping, fetal sex determination, and recently fetal chromosomal aneuploidy detection [37]. With the discovery of miRNAs expression in placental tissue, new molecules with a strong diagnostic potential role as biomarkers for pregnancy-related diseases should be considered, inasmuch as circulating miRNAs have been detected in maternal plasma [3]. The absolute concentration of fetal DNA increases with the pregnancy as the fetus and the placenta grow [38]. Yet, it has been outlined that circulating miRNA concentration varies depending on the type of miRNA during the three trimesters of pregnancy [39, 40]. Therefore, the noninvasive circulating placental miRNA quantification for prenatal diagnosis should be performed in a specific time of the gestational age, preferably during the first trimester for early treatment or management of complicated pregnancies.

MicroRNAs are exported from placental syncytiotrophoblasts and released into the maternal blood flow via exosomal nanoparticles [41]. Exosomes are small 60–80 nm membrane vesicles that are secreted by a multitude of cell types as a consequence of fusion of multivesicular lysosomes/late endosomes with the plasma membrane. Depending on their original tissue, exosomes can come into play in different physiological processes [42]. Exosomes originating from the placenta have played a crucial role in setting up an immune privilege for the developing fetus. Compared to nonpregnant women, exosomes are found in larger quantities in pregnant women [43]. Exosomes can be isolated from blood sample, for *in vivo* or *in vitro* studies from specific tissues of the human cell line performed by ultracentrifugation, and then visualized by electron microscopy [44] (Figure 2). In addition to the exosomal pathway, extracellular miRNAs derived from human trophoblasts can be released from the trophoblast layers in other forms, like microvesicles, apoptotic bodies, and protein-bound miRNAs [45]. Circulating miRNAs have been found to be complexed with circulating ribonucleoprotein and high-density lipoproteins [45, 46]. The extracellular form of miRNAs gives them relative stability and protection from digestion by RNase in human blood [41] thus reinforcing these small molecules as potential biomarkers.

5. Identification of Specific Circulating MicroRNA Expression Linked to Placental Pathology

Placental miRNAs are instrumental in placentation mechanisms such as trophoblast proliferation, syncytialization, and invasion. This has impact upon the major human placental diseases such as preeclampsia [47], intrauterine growth restriction [48], and early pregnancy loss [49]. The misregulated expression of placental circulating miRNAs has been detected in maternal blood with pregnancy complications (Table 1).

To identify miRNA biomarkers for noninvasive diagnosis on maternal blood of specific placental diseases, the first step is to describe precisely the expression of miRNAs in both normal and pathological human placentae of a specific

Table 1: Altered expression of circulating placental miRNA in maternal blood with pregnancy complication for potential application in noninvasive diagnosis.

Pregnancy complication	miRNA upregulated	miRNA downregulated	References
Preeclampsia	miR-210		[50]
		miR-376c	[19]
	miR-24, miR-26a, miR-103, miR-130b, miR-181a, miR-342-3p, miR-574-5p		[65]
		miR-144	[47]
IUGR	miR-516-5p, miR-517, miR-518b, miR-520a, miR-520h, miR-525, miR-526a		[39]
	miR-27a-1, miR-30d, miR-93, miR-141, miR-200c, miR-205, miR-224, miR-335, mir-424, miR-451, miR-491		[48]
Early pregnancy loss		miR-17, miR-19a Found only on placental tissue To be validated on maternal blood	[49]

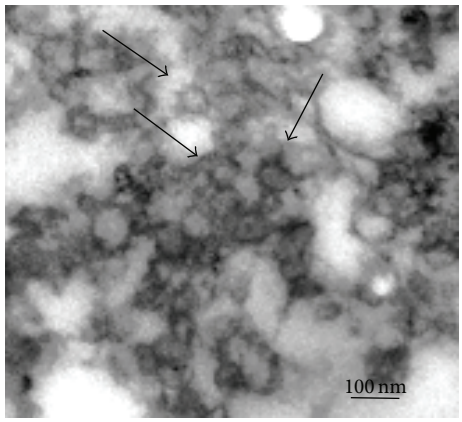

Figure 2: Isolated exosomes analyzed on electron microscopy.

disease using microarray or next generation sequencing methods to characterize the misregulated miRNAs in the pathology: the miRNAs are either under- or overregulated. Once the miRNAs have been determined as either under- or overregulated, they are studied by real-time PCR to establish the repeatability of the results both in the placenta and the maternal blood to identify biomarkers for clinical application [4]. A few expression studies have shown that the expression of miRNAs may not be the same in the placenta and in the maternal blood for certain pathologies. However other studies have shown that miRNAs in plasma or in the placenta increase or decrease at the same rate [47, 48].

The human placenta is a highly invasive and proliferative structure. Successful placentation is the one and only way to establish optimal blood and nutrient supply for normal growth. The invasive capacity is similar to cancer cell invasion with which it shares similar mechanisms. Therefore, miRNAs involved in human cancer and referred to as oncomiRs can be analyzed to suggest important miRNAs for placentation [49].

6. MicroRNA as Noninvasive Biomarkers in Preeclampsia

Preeclampsia (PE) is a major cause of maternal and fetal morbidity or mortality, preterm birth, and intrauterine growth restriction. This disease is characterized by maternal high blood pressure. If the systolic blood pressure is greater than 140 mmHg, the diastolic blood pressure is greater than 90 mmHg, and proteinuria occurs after 20 weeks' gestation, then one can diagnose the mother with preeclampsia. [47]. Risk factors for preeclampsia are maternal age, primiparity, previous preeclampsia, multiple fetuses during the same pregnancy, diabetes, high blood pressure, kidney disease, autoimmune diseases, the antiphospholipid syndrome, obesity, previous history, and ethnicity [47]. Preeclampsia is often diagnosed in the third trimester of the pregnancy; but the disorder is present long before the clinical symptoms appear [50]. Despite the lack of therapeutic treatment, predicting preeclampsia is a state-of-the-art method for managing pregnancy and improving the health of both the mother and the fetus. At present, there are no reliable biomarkers for early detection of preeclampsia because most of them are not sensitive or specific enough for clinical diagnosis [51]. To enhance the predictive value of the diagnosis, it is necessary that new biomarkers be found. Several studies have placed emphasis on proteins and metabolites in biofluids [51] but few have been concerned with miRNAs, which are yet becoming a promising class of biomarkers. Some studies have compared miRNAs expression on placenta between normal pregnancies and pregnancies with minor and severe preeclampsia; these studies have led to the identification of specific miRNAs involved in preeclampsia. Misregulated miRNAs were analyzed in maternal plasma for researching noninvasive biomarkers. MiR144 is significantly underexpressed both in severe and minor preeclampsia when compared with normal control [47]. Misregulation cases of

miRNAs circulating in the plasma are more frequent in minor than in severe preeclampsia as opposed to the placenta tissue. These dysregulated miRNAs circulating in the plasma may be associated with the early pathological preeclampsia related changes. Another miRNA, miR-210, has been found to significantly increase in the plasma of preeclampsia-affected women [50].

Preeclampsia is a consequence of inadequate placental cytotrophoblast invasion, trophoblast invasion, and maternal spiral artery remodeling. These lead to reduced uteroplacental perfusion pressure and oxygen availability which in turn results in placental ischemia and hypoxia [52]. miR-144 is an important regulator in ischemia and hypoxia [47] and miR-210 is induced by hypoxia and regulated by transcriptional factor HIF-1 (hypoxia inducible factor) and NF-κB [53]. Moreover, another research has shown that miR-210 inhibits trophoblast invasion [50]. These findings and the above-mentioned ones show that miR-144 and miR-210 play an important role in the pathogenesis of PE and that they might be useful biomarkers for the diagnosis of preeclampsia.

7. MicroRNAs as Noninvasive Biomarkers in Intrauterine Growth Restriction

Intrauterine growth restriction (IUGR), also known as fetal growth restriction (FGR), is characterized as birth weight less than the 10th percentile of the expected weight relative to the gestational age and the sex of the fetus. IUGR increases the risk of perinatal complications considerably [54]. IUGR has been traced to a malfunction of the placenta along with uteroplacental insufficiency at the interface between fetal and maternal circulation [55]. The uteroplacental insufficiency leads to deteriorated placenta transport of nutrients, mainly amino acids, lipids [56], and micronutrients such as iron and folate [57]. Preeclampsia, aneuploidies, or any genetic syndrome is major risk factors of IUGR. The mother's dietary deficiencies, her exposure to environmental factors, and placental epigenetic modifications are additional risk factors [58].

The role of miRNAs in IUGR has not yet been clearly understood compared to preeclampsia. However, we know that miR-141 contributes to IUGR by regulating pleomorphic adenoma gene 1 (*PLAG1*) expression [59]. The expression level of miR-141 in the placenta of pregnancies with IUGR is 3.4 times greater than that in normal placentae. Besides, the expression level of the miR-141 target gene, that is, *PLAG1*, decreases significantly in the IUGR placental tissue compared to controls. PLAG1 is mainly expressed in the placenta during pregnancy [59]. Another miRNA, miR-424, is a critical mediator of oxygen-dependent miRNAs; it is physiologically overregulated in placentae undergoing abnormal vascular development. Increased levels of miR-424 in placenta with IUGR have been found [60].

Local placental miRNAs such as mir-518b, mir-1323, mir-516b, mir-515-5p, mir-520h, mir-519d, and mir-526b are significantly lower in placentae in IUGR pregnancies compared to normal ones. However the corresponding circulating miRNA level does not change significantly [55]. This discrepancy has also been found for specific miRNAs regulated by hypoxia (miR-27a, miR-30d, miR-141, and miR-200c). The same observations are applicable to the following ubiquitous miRNA species (miR-205, miR-424, miR-451, and miR-491) and miRNAs from cluster C19MC known to be expressed mainly by the placenta (miR-517a, miR-518b, miR-518e, and miR-524) [48]. However, the overall level of these twelve specific miRNAs increases in the plasma of women with IUGR complicated pregnancies. The difference in misregulation level of the miRNAs between the plasma and the placenta may be indicative of placental injury in IUGR; placental injury lowers miRNA biogenesis while increasing the release of miRNAs onto the plasma [48].

Another high level of pregnancy-related extracellular miRNAs (miR-516-5p, miR-517, miR-518b, miR-520a, miR-520h, miR-525, and miR-526a) has been observed at the early stage of the pregnancy (between the 12th and the 16th weeks of gestation) in late-onset IUGR as opposed to normal pregnancies [39]. What has already been discovered needs to be completed to find reliable biomarkers for noninvasive diagnosis of IUGR on maternal plasma.

8. MicroRNAs as Noninvasive Biomarkers in Early Pregnancy Loss

The fetal loss before 20 weeks of pregnancy is called early pregnancy loss or miscarriage. This is a common event which complicates up to 15% of pregnancies [61]. Mechanisms leading to early pregnancy loss from noncytogenetic causes are related to defective trophoblast growth and impaired invasiveness by trophoblast cells leading to defective placentation [62].

Few studies have focused on miRNA implication and misregulation in early pregnancy loss. miR-17 and miR-19a are strikingly underregulated in the placenta when it comes to early spontaneous miscarriage [49]. This suggests that the above-mentioned miRNAs may play a critical role in early placentation. These results obtained from placenta samples have yet to be confirmed for maternal plasma to identify noninvasive miRNA biomarkers in early pregnancy loss.

9. Conclusion

miRNAs are relatively stable and expressed specifically depending on the various tissues. This makes them ideal candidates for diagnosis. The close link between altered expression of miRNAs in maternal plasma and pathological pregnancies has been well described.

Circulating miRNAs provide a new fetal genetic material easily collectable from maternal blood samples. They offer new potential for the development of noninvasive prenatal diagnostic tests.

Implementing noninvasive prenatal diagnosis using miRNAs involves the analysis of the misregulated miRNAs linked to the pathology. Under- or overexpressed miRNAs can be analyzed by relative quantification using real-time PCR. Important points need to be included when reporting investigation of cell-free microRNAs such as sample collection,

miRNA isolation and quantification, and the normalization methods [63]. As for the screening of Down syndrome in maternal serum [64], the design of noninvasive tests to anticipate pregnancy complications should include results with multiple of the median (MoM) for each miRNA analyzed for measuring the level of the misregulation. Additionally, a risk should be calculated from biological and clinical parameters. National or international studies should be performed to determine the cut-off risk to conclude to a low or high risk for developing the pathology.

Discoveries of potential miRNAs for noninvasive biomarkers should be improved by deeper analyses and appropriate normalization methods to confirm the results obtained prior to application for clinical care. The levels of circulating miRNAs change relative to the gestational age. Studies have to include results for each trimester of the pregnancy. Then, microRNAs circulating in the maternal plasma might have the potential to be noninvasive diagnostic and prognostic biomarkers for pregnancy monitoring. They might prevent the development of gestational disorders and form the basis of personalized therapeutic strategies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Angiogenesis in the Placenta: The Role of Reactive Oxygen Species Signaling

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Proper placental development and function are central to the health of both the mother and the fetus during pregnancy. A critical component of healthy placental function is the proper development of its vascular network. Poor vascularization of the placenta can lead to fetal growth restriction, preeclampsia, and in some cases fetal death. Therefore, understanding the mechanisms by which uterine stressors influence the development of the placental vasculature and contribute to placental dysfunction is of central importance to ensuring a healthy pregnancy. In this review we discuss how oxidative stress observed in maternal smoking, maternal obesity, and preeclampsia has been associated with aberrant angiogenesis and placental dysfunction resulting in adverse pregnancy outcomes. We also highlight that oxidative stress can influence the expression of a number of transcription factors important in mediating angiogenesis. Therefore, understanding how oxidative stress affects redox-sensitive transcription factors within the placenta may elucidate potential therapeutic targets for correcting abnormal placental angiogenesis and function.

1. Introduction

The placenta is located at the maternal-fetal interface and modulates the *in utero* environment to promote optimal fetal development. The dense networks of blood vessels within the placenta are responsible for exchanging respiratory gases, nutrients, and wastes between the mother and fetus throughout pregnancy, which is essential for proper fetal growth [1]. Throughout gestation the vasculature of the placenta is continually evolving to accommodate the mounting demands of the fetus and can be directly influenced by a number of exogenous factors such as maternal diet, smoking, and medication use [2–5]. Furthermore, conditions which subject the placenta to stress, such as increased dietary fats and exposure to the chemicals in cigarette smoke, can also result in altered levels of immune and growth factors which may impact the proper development of placental vasculature [6–8].

The establishment of proper placental function first requires successful implantation of the fertilized oocyte followed by the coordinated invasion of trophoblast cells, from the trophectoderm layer of the blastocyst into the maternal decidua. Following invasion, trophoblasts remodel the maternal spiral arteries to promote expansion of the placenta's vascular circuitry, which is central to improving uterine and umbilical blood flow. This facilitates efficient exchange of nutrients and thereby permits exponential fetal growth and development [9–11].

Abnormal development of the placental vasculature leads to placental insufficiency, which can result in a decrease in the exchange of nutrients and wastes between maternal and fetal circulations. Such changes can manifest in adverse uterine conditions leading to various pregnancy complications for both the mother and the fetus including gestational hypertension [12], intrauterine growth restriction [13, 14], preeclampsia [15, 16], stillbirth [17, 18], preterm delivery [19], or miscarriage [20]. Therefore, the proper establishment of blood vessels within the placenta is central to fetal growth and survival and may serve as a therapeutic target for mitigating

clinical conditions that are associated with altered placental vasculature.

Appropriate development of the placental vascular network requires vasculogenesis, angiogenesis, and trophoblast mediated arterial remodeling [21]. Vasculogenesis is the development of blood vessels *de novo* from pluripotent mesenchymal stem cells, occurring between 18 and 35 days after conception in humans. Angiogenesis is the creation of new blood vessel networks by branching and elongating previously existing vessels to make new connections. Angiogenesis occurs during most of the pregnancy, beginning 21 days after conception and continuing throughout human gestation [16, 22]. It is important to recognize that vascular development is mediated not only by the vascular endothelial cells, but also by the invading trophoblast cells. The extravillous trophoblasts contribute to the development of the placental vasculature by secreting angiogenic factors [23, 24] and invading the maternal decidua to remodel maternal spiral arteries creating a "low resistance, high capacitance vessel," increasing the exchange between maternal and fetal circulations [16]. All of these functional changes require the coordinated actions of various signaling molecules to regulate the expression of genes that govern placental vasculature development [25].

In order to elucidate the mechanistic signaling pathways involved in the development of the placental vasculature, researchers have sought to identify the maternal and fetal exposures that are linked to placental dysfunction. For instance, maternal smoking, obesity, and preeclampsia are three conditions that are linked to altered placental angiogenesis. Examining placental vascular development in these conditions may provide us with greater insights into factors that are important for proper placental blood vessel development.

2. ROS Signaling in Placenta Angiogenesis

Reactive oxygen species (ROS) are hyperreactive molecules resulting from the reduction of molecular oxygen. Some of the most commonly known species are superoxide ($O_2^{-\bullet}$), hydroxide ($OH^{-\bullet}$), and hydrogen peroxide (H_2O_2) [26]. These ROS are primarily formed from mitochondrial oxidative phosphorylation, where electrons are transferred across respiratory chain enzymes and leak onto molecular oxygen. At physiological levels, ROS are involved in cellular signaling pathways important for proper development and cellular function. However, excess ROS can cause cellular damage and impact tissue function as a result of lipid peroxidation, protein and amino acid modifications, and DNA oxidation. Antioxidants are molecules and enzymes capable of reducing the consequences of these ROS and/or mitigating their damaging effects [27, 28]. Typically, there is a dynamic balance between the generation of ROS and the actions of antioxidants. This balance is critical for maintaining ROS at optimal levels for signaling of various cellular processes, while avoiding a state of damaging oxidative stress [29].

The effects of altered placental oxygenation, with increasing gestational age, on placental development have been nicely reviewed by Burton [30]. Fluctuating oxygen conditions can contribute to increased ROS production where they can act as signaling molecules. This may be particularly pertinent to tissues which have a high-energy demand or those which contain large amounts of mitochondria, such as the placenta [31], brain, heart, and skeletal muscle. Moreover, ROS production changes over the course of pregnancy, underscoring the importance of oxidative signaling in the placenta. In the early stages of human pregnancy, the establishment of placental circulation is associated with a dramatic increase in the oxygen level within the placenta [32], resulting in increased ROS production and oxidative stress. As pregnancy progresses and the metabolic demands of the fetus rise, there is an increase in both placental mitochondria mass and mitochondrial electron chain enzyme activity. This contributes to elevated ROS production and increased oxidative stress [33]. Moreover, there is an increase in systemic oxidative damage during the course of human pregnancy which corresponds to extravillous trophoblast invasion and the development of the placental vasculature (Figure 1). Therefore, it is possible that ROS signaling during human pregnancy is important for central processes such as placental vasculature development.

There are a number of maternal conditions such as smoking, obesity, and preeclampsia that can disturb the profile of ROS production that occurs during healthy pregnancies. Moreover, because maternal smoking, maternal obesity, and preeclampsia can also cause placental dysfunction and pregnancy complications, it is important to elucidate the contribution of excessive ROS production and increased oxidative stress to proper placental function and pregnancy outcomes. Here we explore the existing associations between altered placental angiogenesis and oxidative stress in conditions of maternal smoking, maternal obesity, and preeclampsia.

3. Smoking and Angiogenesis

15–20% of women smoke during pregnancy despite maternal smoking being a significant risk factor for a number of adverse pregnancy outcomes [41]. Maternal smoking is associated with an increased risk of fetal growth restriction, low birth weight, and perinatal mortality [41–43]. Such changes are often linked to abnormal placental development and specifically to aberrant placental vascularization. Indeed, an increase in capillary/villous tree branching and vascular density within the placental terminal capillary convolutes has been observed in women who smoke. In such cases, poor development of the placental vasculature was inferred by an increased umbilical artery Doppler resistance index which is indicative of abnormal vascular tree formation [44]. Exposure of placental explants to cigarette smoke extracts has also been shown to shift the balance between proangiogenic (promoted by placental growth factor, PlGF) and antiangiogenic factors (fms-like tyrosine kinase-1, sFlt-1) [3]. Moreover, a number of animal studies have demonstrated that maternal chronic exposure to carbon monoxide, a major combustion product produced by cigarette smoke, increases uterine blood flow and uteroplacental vascular growth by shifting the placenta to a more proangiogenic state [45]. However, some controversy still exists with respect to the proangiogenic effects of smoking during pregnancy; several studies report a reduction in the number of placental

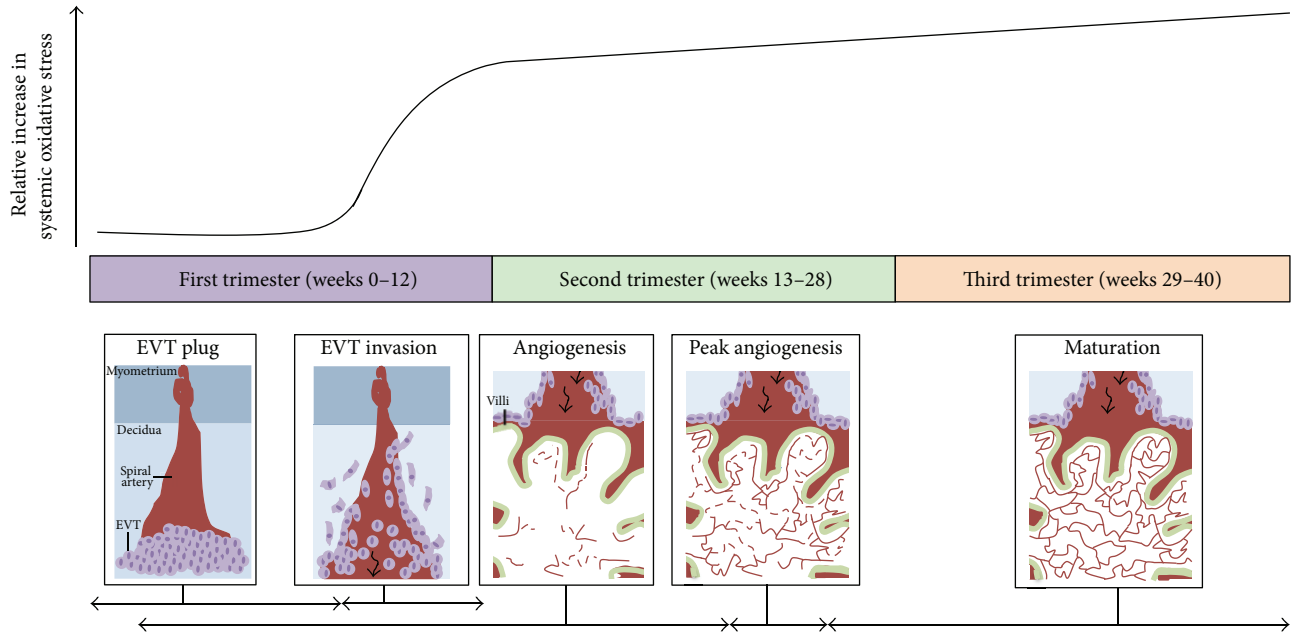

Figure 1: Oxidative stress throughout human pregnancy and its relation to placental angiogenesis. At the beginning of the first trimester of pregnancy, there are low levels of systemic oxidative stress and no blood flow into the placenta because extravillous trophoblasts (EVTs) (depicted as light purple circles) plug the maternal spiral arteries (depicted in red) in the decidua as shown in the first panel. Between 8 and 12 weeks of gestation, the EVT plug dissipates and the EVTs invade maternal spiral arteries to allow blood to enter the placenta (black arrow), as illustrated in the second panel. This coincides with a sharp increase in maternal oxidative stress. Furthermore, the state of oxidative stress increases with gestational age as depicted by the black curve. The first signs of placental angiogenesis occur at 3 weeks of gestation. However from about 12 weeks onwards, blood vessels (red lines) protrude towards the trophoblastic layers of the villi (outlined in green), where blood exchange between maternal and fetal circulation is optimal (shown in panel three). From about 9–23 weeks of gestation, there is an expansion of the fetal capillary bed by branching and nonbranching angiogenesis (dashed red lines in angiogenesis panel). From 23-24 weeks of gestation, the greatest changes in blood vessel development and villous composition are observed (peak angiogenesis panel) [22, 34]. Angiogenesis continues until term with the maturation of blood vessels and development of a more complex vascular network to facilitate exponential fetal growth (last panel). The horizontal black arrows indicate the approximate time each process depicted in the panels occurs. Note: this graph has been constructed by interpretations of multiple studies reporting findings of systemic oxidative stress markers present in women during normal pregnancy [35–40] as studies on placental/uterine oxidative stress are limited [32].

capillaries among smokers [46, 47]. In fact, the addictive component of cigarette smoke (nicotine) can inhibit trophoblast migration, a key process initiating trophoblast invasion and spiral artery remodeling, within human placental explants [48]. Recently it has been shown that nicotine inhibits trophoblast interstitial invasion, downregulates transcription factors required for trophoblast differentiation, and impairs placental vascularization [49]. Therefore, maternal smoking clearly influences both placental development and function; however there is still considerable uncertainty regarding the mechanism(s) underlying these effects.

Smoking is generally considered to be an oxidative insult and as a result it has been suggested that smoking causes abnormal placental vascular development via oxidative stress mediated pathways. Although this has not been shown unequivocally, recent studies support that maternal smoking results in changes in genes important for the management of oxidative stress [50], which may contribute to adaptive changes in the state of oxidative stress within the placenta. For example, smoking throughout pregnancy has been associated with an increased expression of antioxidants, such as heme-oxygenase within the basal plate of term placenta as well as in HTR8/SVneo trophoblast cells exposed to cigarette smoke extract [51]. Sidle and colleagues suggested that the increased expression of heme-oxygenase within the placenta may be facilitating trophoblast invasion of the spiral arteries and thus decreasing the placental oxidative damage incurred from smoking. However, increased oxidative damage to lipids and DNA is observed within term placenta of women who smoked during pregnancy [52]. Additionally, the total antioxidant capacity was significantly lower in the placentae of active smokers, while the total oxidant status and oxidative stress index was significantly increased in both the placentae from active and the passive smokers compared to placenta from nonsmokers during pregnancy [53]. Taken together, the current body of evidence suggests that oxidative stress dependent mechanisms play an important role mediating the effects of cigarette smoke on the placenta, potentially by influencing the development of the vascular network.

4. Maternal Obesity and Angiogenesis

Maternal obesity during pregnancy also results in adverse maternal and fetal outcomes including gestational diabetes, preeclampsia, macrosomia, preterm delivery, and stillbirth

[6, 54–56]. Many of these outcomes have also been associated with alterations in the placental vasculature. Indeed, maternal obesity has been clinically linked with placental abruption and infarction [57] along with abnormal placental spiral artery modification, which may result from inadequate trophoblast invasion [17, 58]. The importance of proper trophoblast invasion in dictating functional development of the placental vasculature is well supported [59, 60]. In fact, our group has demonstrated that the altered progression of trophoblast invasion is correlated to incomplete spiral artery remodeling in rodents exposed to a life-long high-fat diet [7]. Other animal models of obesity during pregnancy have also reported impaired placenta vascular development and increased capillary density [61] as well as significant reductions in uterine blood flow [62]. Additionally, a study done with obese ewes found that the fetal component of the placentomes displayed larger arteriole diameter in early to midgestation, with a decreased gene expression of angiogenic factors from mid to late gestation [63]. Therefore, maternal obesity causes adverse development of the placental architecture, which results in poor nutrient and waste exchange, in turn compromising fetal growth and survival [6, 62, 64]. Mechanistically, a higher body mass index during pregnancy is associated with altered levels of maternal serum angiogenic markers (low levels of sFlt-1 and altered levels of PlGF after first trimester and differential patterns of change near term) [65]. Abnormalities in the distribution of proangiogenic vascular endothelial growth factor (VEGF) and its receptors in the placentae of obese women have also been noted, with a particular mention of a predominance of nonbranching angiogenesis [66].

Maternal obesity has also been linked with placental oxidative stress. First trimester placenta from obese pregnancies has been shown to have a 31% increase in total oxidized protein content (a marker of oxidative damage) compared to placenta of nonobese pregnant women [67]. Term placentae from obese women also have altered redox balance as indicated by increased lipid peroxidation (malondialdehyde measurement) and activity of antioxidant enzymes such as the superoxide dismutases, catalase, and glutathione peroxidase, compared to control placenta [68]. However, the role of oxidative stress in the placentae of obese individuals is still not clear since some research suggests that there is a decrease in total antioxidant capacity and regulators of angiogenesis within term placentae of obese pregnancies compared to nonobese placentae [2]. In addition to oxidative stress, the work of Myatt and colleagues has also raised the possibility of nitrative stress being a route to vascular dysfunction in the placentae of obese women [69, 70]. Elucidation of the transcriptional and posttranscriptional processes that contribute to obesity-mediated changes in placentae will advance our understanding of the mechanisms linking oxidative stress to functional changes in the placenta, such as those involved in placental angiogenesis.

5. Preeclampsia and Angiogenesis

Preeclampsia can lead to intrauterine growth restriction, preterm delivery, and stillbirth [71]. There are a number of good reviews that discuss the connection between altered placental vascular development and preeclampsia [72–74], many of which highlight the role of elevated oxidative stress in causing reduced trophoblast invasion as a mechanism underlying the development of preeclampsia [33, 70, 75]. A recent proteomics evaluation of preeclamptic placentae demonstrated a reduction in the levels of several mRNAs associated with mitochondrial respiratory chain function [76]. Since mitochondria are intimately associated with oxidative stress and signaling [29, 77], understanding their role in dictating placental function/dysfunction may be important to elucidating disease pathogenesis [78].

While oxidative stress in the placenta is associated with adverse pregnancy outcomes, the mechanistic connection between the role of ROS and altered vascular development is not clearly defined (Figure 2). Therefore, the elucidation of how oxidative stress/signaling facilitates placental dysfunction as a consequence of maternal smoking, obesity, and preeclampsia will contribute to a better understanding of the specific cellular pathways linking these uterine stressors to placental dysfunction.

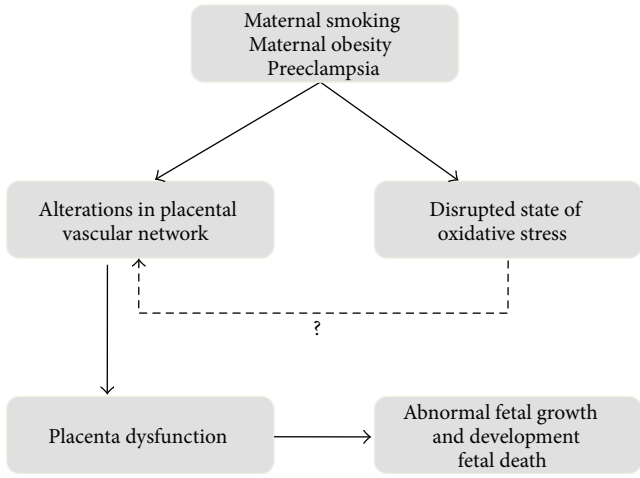

Figure 2: Summary of proposed mechanisms linking maternal smoking, maternal obesity, and preeclampsia with pregnancy complications and adverse fetal outcomes. It is well documented that both the placental vasculature and the state of oxidative stress are altered in pregnancies complicated by maternal smoking, maternal obesity, and preeclampsia (see text). These alterations in the placental vascular network are known to contribute to placental dysfunction and adverse pregnancy outcomes as well as abnormal fetal growth, development, and even death. We hypothesize that an increase in oxidative stress contributes to aberrant signaling in placenta, resulting in changes in processes essential for placental vascular development. This could be a potential mechanism leading to the adverse pregnancy outcomes observed in maternal smoking, maternal obesity, and preeclampsia. The solid arrows represent well documented findings, while the dashed arrow identifies the knowledge gap where more research needs to be done, to clarify the role of oxidative stress in placental angiogenesis.

6. Putative Role of ROS Activated Transcription Factors in the Placenta

While oxidative stress has been proposed as a contributory pathway for many instances of placental dysfunction, the association of specific signaling pathways with ROS still remains unclear. Therefore, in this section, we describe the roles of six transcription factors which may link oxidative stress with trophoblast invasion and vascular development in the placenta [79]. Specifically we address the roles of E26 transformation specific oncogene homolog 1 (Ets-1); Krüppel-like factor 8 (KLF8); nuclear factor kappa-light-chain-enhancer of activated B (NF-κB); NF-E2-related factor 2 (Nrf2); specificity protein 1 (Sp1) and specificity protein 3 (Sp3); and signal transducer and activator of transcription 3 (STAT-3).

6.1. Ets-1. Transcription factor Ets-1 is upregulated by hypoxia and ROS and regulates angiogenesis and invasion [80, 81], processes central to the normal development and function of the placenta. For instance, in bovine aortic endothelial cells it has been demonstrated that Ets-1 mRNA levels are increased by hypoxic conditions as well as elevated levels of cellular ROS [82]. More specifically, in endothelial cells and ovarian carcinoma cells, H_2O_2 has been shown to regulate Ets-1 through the hypoxia response element (HRE) and the antioxidant response element (ARE) [80]. In general, the Ets family has been linked with a number of other cellular processes such as apoptosis [83] and cellular differentiation [84]. Furthermore, other transcription factors, such as Nrf2, also play a role in the upregulation of Ets-1, by forming a complex with Ets-1 that associates with the ARE [80]. Under hypoxic conditions, other transcription factors such as hypoxia-inducible factor (HIF) can also trigger the increased expression of Ets-1 [81].

While conditions that increase free radical signaling can regulate the expression Ets-1, Ets-1 itself upregulates VEGF, a key protein in angiogenesis [85]. Additionally, Ets-1 can also interact with other transcription factors known to be important for cellular responses to oxidative stress and hypoxia, such as HIF-2α, to regulate the expression of VEGF receptor 2 (VEGFR-2) [86]. Furthermore, proangiogenic factors, such as VEGF, also induce Ets-1 expression in human umbilical vein endothelial cells which then goes on to bind to the promoter region of angiopoietin-2, upregulates the protein, and destabilizes vessels for angiogenesis [87]. Clearly Ets-1 has important roles in regulating the angiogenic response in a variety of cell types; however its role in the placenta is not clearly understood. We do know that Ets-1 expression in normal human placenta correlates with trophoblast invasion and has been observed to peak during the first trimester [88]. A number of investigators have suggested that, based on its correlation with increased invasion, Ets-1 may serve to increase the expression of matrix metalloproteinase 9 (MMP-9) and urokinase-type plasminogen activator (uPA), which are important players in trophoblast invasion [89].

Importantly, the regulation of Ets-1 dependent signaling pathways has been linked to the dysregulation of genes in smoking, obesity, and preeclampsia. While the role of Ets-1 in placenta is not well characterized, the observations outlined below argue for the importance of examining the role of Ets-1 dependent signaling pathways in placental vascular development and function. Firstly, the expression of MMP-1 is regulated in human epithelial cells as consequence of exposure to cigarette smoke [90] through an Ets-1 dependent pathway. Secondly, the addition of serum from obese humans increases MMP-3 and Ets-1 expression in human aortic endothelial cells [91]; this increase may be triggered by serum VEGF. The current evidence suggests that Ets-1 is specifically associated with proteins important to trophoblast invasion (such as the MMPs) and placental angiogenesis (such as VEGF), parameters that are frequently altered in smokers and obese individuals. Along with evidence that hypoxia and oxidative stress can also increase Ets-1 expression, it suggests that this transcription factor may play an important role in pregnancies complicated by oxidative stress.

6.2. KLF8. KLF8 is important in facilitating cellular differentiation [92, 93] as well as angiogenesis [94]. KLF8 has been extensively investigated within the context of different cancers, including hepatocellular carcinoma and breast cancer, where its activity has been found to induce invasion and metastasis [93, 95]. It has been suggested that KLF8 also plays a role in the activation of MMP-9 in breast cancer, and this may be an important signaling mechanism underlying invasion and metastasis [95]. Furthermore, the work of Yang et al. (2014) suggests that hypoxia-reoxygenation (H/R) also serves to reduce the expression and nuclear colocalization of KLF8 resulting in a downregulation of MMP-9, which ultimately inhibits trophoblast invasion [96]. Since H/R leads to the production of ROS which can modulate protein kinase C (PKC) [97] and KLF8 has a binding site for PKC, it is possible that the altered interaction between KLF8 and PKC may contribute to the reduction of nuclear KLF8. This hypothesis is further supported by research showing that matrix degradation by MMP-9 is PKC dependent; thus a decrease in PKC activity would also reduce the matrix degrading activity of trophoblasts [96]. While we do not know the exact role of KLF8 in the placenta, the existing evidence suggests that it may be regulated in conditions that affect trophoblast invasion, especially as a consequence of oxidative stress. In support of this hypothesis, decreased KLF8 expression has been associated with reduced MMP-9 mRNA and protein expression in trophoblasts from preeclamptic placentae. Furthermore, HTR8 human trophoblast cells subjected to H/R injury, which mimics changes in oxygen tension observed following the initiation of trophoblast invasion, also downregulate KLF8 and MMP-9 expression [96].

In addition to its effects in preeclampsia, KFL8 also plays a role in 3T3-L1 adipocyte differentiation by acting as an upstream regulator of peroxisome proliferator-activated receptor gamma (PPARγ) [92]. If KFL8 also regulates PPARγ in the placenta, a key factor in placental angiogenesis [98], it is plausible that under obesogenic conditions KLF8 may also have an important role in altering the development of the placenta vasculature. Therefore, KLF8 is another transcription factor of interest in linking oxidative stress with altered placental development and angiogenesis.

6.3. NF-κB. Perhaps the most recognized transcription factor associated with ROS signaling is NF-κB. Increases in cellular ROS production can result in an increase in the expression of NF-κB, leading to the upregulation of factors involved in angiogenesis [99, 100]. Furthermore, the addition of H_2O_2 to endothelial cells has been shown to result in the increased expression of VEGFR-2 mRNA via ROS and NF-κB dependent pathways. In addition to NF-κB inducing angiogenic factors and the expression of their receptors, it can also be activated by angiogenic factors such as VEGF [101]. Such a relationship can potentially amplify the angiogenic response triggered by cellular oxidative stress.

NF-κB can also influence angiogenesis by regulating the expression of cytokines such as interleukin-6 (IL-6) and interleukin-8 (IL-8), as shown in cancer cell models [102, 103]. The role of NF-κB may be to link the detection of ROS to regulation of cytokine expression. For example, H_2O_2 can stimulate the cellular levels of NF-κB and also increase DNA binding activity of NF-κB resulting in an increase in the production of IL-8 and the formation of tube-like structures [99]. Cytokines can regulate angiogenesis by directly acting on cell growth and differentiation and indirectly by inducing the release of secondary cytokines that influence the expression of angiogenic factors or receptors (reviewed in [104]).

Preeclamptic placentae express increased levels of NF-κB; in some cases the increase is reported to be as much as 10-fold [105]. More recent evidence suggests that other conditions leading to placental dysfunction, such as increased weight gain [106] and exposure to a high fat diet [2], may also result in increased expression of NF-κB. Such conditions are linked to increased oxidative stress and placental dysfunction [7, 69], providing justification for further examining the role of NF-κB in connecting placental oxidative stress to angiogenesis.

External stressors such as diet and cigarette smoke are known to affect changes in NF-κB expression in a variety of tissues. For instance, exposure of human airway epithelial cells to cigarette smoke increases the expression of NF-κB within 2 hrs [90]. It has also been suggested that aldehydes, present in cigarette smoke, may interfere with NF-κB binding of its target promoters [107]. Taken together these data suggest that NF-κB may be an important link between oxidative stress, smoking, and angiogenesis. There is also strong evidence that NF-κB is upregulated in the placenta of obese ewes [108] as well as obese women [2]. However, whether this increase is strictly the consequence of increased oxidative stress in these placentae is not clear.

In summary, ROS and oxidative stress can regulate the expression of NF-κB in a variety of tissues leading to changes in angiogenesis. Therefore, NF-κB may be having the same regulatory effect within the placenta and be a key transcription factor in regulating ROS-induced placental angiogenesis.

6.4. Nrf2. Nrf2 is a transcription factor that is involved in the regulation of nonmitochondrial antioxidant defense response [109], as well as mitochondrial proteins related to the management of ROS production [110]. Nrf-2 and its partner Kelch-Like ECH-Associated Protein 1 (Keap-1) have been linked to the oxidative stress response in tissues such as adipocytes [111], as a consequence of obesity, and in peripheral blood mononuclear cells, as a consequence of smoking [112]. Furthermore, increased expression of Nrf2 in the placenta is associated with preeclampsia and fetal growth restriction [113]. In such cases, trophoblast invasion is usually reduced and this is thought to be the result of increased cellular oxidative stress [114]. However, the link between Nrf2 and angiogenesis in the placenta is less clear.

Nrf2 is also associated with proangiogenic potential in endothelial cells [115] and may be triggered in response to reduced oxygen conditions. Nrf2 is thought to have its proangiogenic effects by modulating known regulators of angiogenesis, such as VEGF, in response to oxidative stress [116]. This has been demonstrated in extravillous trophoblasts where the placentae from women with early onset preeclampsia and intrauterine growth restriction exhibited increased levels of Nrf2 expression in association with decreased expression of VEGF and elevated levels of 4-hydroxynonenal (a marker of lipid oxidative damage) [113]. Since VEGF is also associated with the activation of Nrf2, perhaps the early decrease in VEGF may lead to insufficient activation of Nrf2 and this may play a part in modulating trophoblast invasion [117]. Clearly our understanding of the role of Nrf2 in linking trophoblast oxidative stress to placental angiogenesis is incomplete and requires further investigation.

6.5. Sp1 and Sp3. Sp1 and Sp3 are zinc finger proteins that are ubiquitously expressed in most mammalian cell types and are associated with enhanced gene promoter activity [118] by binding to GC boxes [119]. While there is little direct evidence for the role of Sp1 and Sp3 in placental angiogenesis, both of these transcription factors have been associated with the regulation of VEGFR-2 in pancreatic tumors [120]. Furthermore, Sp3 has been linked to the regulation of VEGF through a mechanism involving the posttranslational phosphorylation of a serine residue by extracellular related kinases [121]. In addition Sp1 has also been shown to modulate another transcription factor, PPAR-γ [122], which is known to be involved in placental angiogenesis [98]. Both Sp1 and Sp3 are also thought to be involved in the regulation of angiogenesis by cytokines such as IL-6 [123]. Such mechanistic pathways may be important in cases of altered placental angiogenesis due to obesity since cytokine balance is often affected as a consequence of overnutrition. Therefore, Sp1 and Sp3 may be important transcription factors in regulating placental angiogenesis through their regulation of VEGF, VEGFR-2, and PPAR-γ, as well as cytokines.

Oxidative stress, generated by the addition of exogenous H_2O_2, was also found to increase VEGF-A expression via Sp1 and Sp3 dependent pathways, by binding to two GC boxes in the VEGF-A promoter region [124]. In addition, Sp1 has been linked to the regulation of tissue inhibitors of metalloproteinase-2 (TIMP-2) [125]. TIMP proteins are important in regulating MMP activities that are central to trophoblast invasion and spiral artery remodeling. Furthermore, cigarette smoke has been shown to induce MMP-1 expression, an enzyme that is important in angiogenesis, through Sp1 dependent pathways [90]. Clearly, Sp1 and Sp3 are responsive to oxidative stress, modulate proteins important for vascular

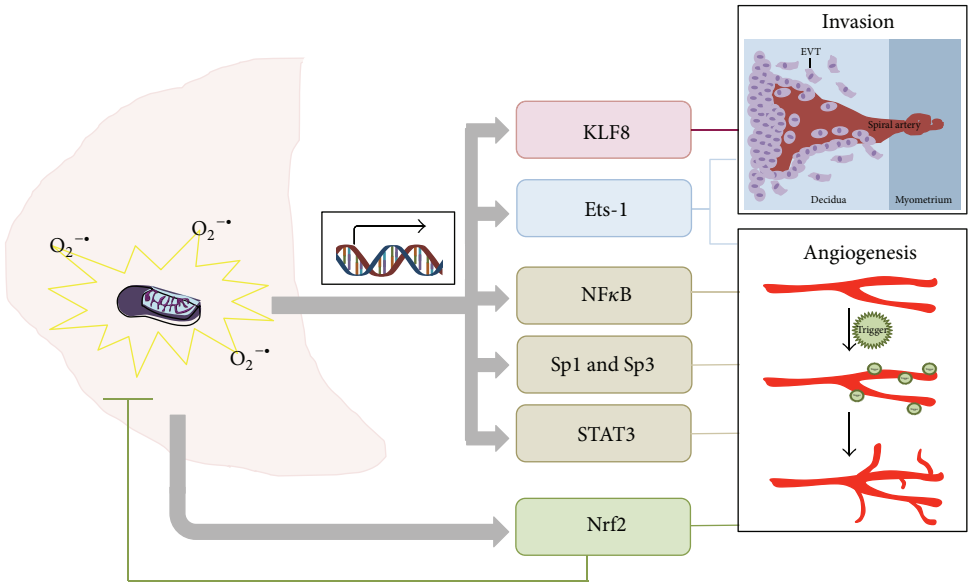

FIGURE 3: Placental oxidative stress triggers the expression of transcription factors to regulate angiogenesis and trophoblast invasion. Mitochondria within the placenta (depicted in pale pink) are a major producer of ROS, such as $O_2^{-\bullet}$ which can cause a state of oxidative stress (illustrated in yellow). Oxidative stress within the placenta can act as a signaling pathway to influence the expression of transcription factors, such as KLF8, Ets-1, NFκB, Sp1, Sp3, STAT-3, and Nrf2 (depicted by the grey arrows). These transcription factors regulate the expression and activity of proteins related to angiogenesis and trophoblast invasion (as shown by the lines linking to the invasion and angiogenesis panels).

development, and therefore may play a significant, as of yet undiscovered, role in placental angiogenesis.

6.6. STAT-3. STAT-3 is part of a group of transcription factors that provide cellular regulation in response to cytokines and growth factors. This transcription factor has been shown to respond to reactive nitrogen species [126] and plays a central role in regulating a range of signaling molecules involved in cellular differentiation and proliferation in a variety of cell types [127, 128]. STAT-3 has also been linked to the attenuation of oxidative damage in different cellular compartments such as the mitochondrion [129]. Additionally, STAT-3 can respond to cytokine or oxidative signals by affecting the expression of angiogenic factors such as VEGF [117, 130]. The proangiogenic role of STAT-3 is linked to its ability to regulate both the expression and the secretion of MMP-2, MMP-9, and uPA, proteins involved in the modulation of trophoblast invasion [131].

Importantly this transcription factor also enhances the promoter activity of MMP-1 in the lung, as a consequence of exposure to cigarette smoke [90]. The activation of STAT-3 in response to cigarette smoke appears to be a robust response demonstrated in a variety of tissues including human bladder cells lines [132], human bronchial epithelial cell lines [133], and mouse brain endothelial cells where it has been associated with the regulation of antioxidant defense response [134]. Not only is the expression of STAT-3 important but so is the activation of STAT-3 which is regulated by the extent of STAT-3 phosphorylation. The amount of activated STAT-3 in isolated trophoblasts has been found to increase with maternal body mass index [135]. Aye and colleagues also demonstrated that STAT-3 phosphorylation can be regulated by the cytokine TNF-α [135], which is known to be elevated in obese individuals [136]. Furthermore, STAT-3 expression and activity are reduced in preeclampsia, which is associated with decreased invasiveness of trophoblasts [137]. Our own data along with that of others has demonstrated that altered trophoblast invasion is linked to insufficient spiral artery remodeling and decreased oxygenation of the placenta [6, 7]. Therefore STAT-3 may be an important regulator for the adaptive responses to oxidative stress within the placenta.

7. Conclusion

We suggest that the transcription factors mentioned above warrant further investigation as to their roles in connecting increased oxidative stress with altered trophoblast invasion and placental angiogenesis (summarized in Figure 3). However, we do recognize that there remain many unexplored possibilities, which may provide further insights into mechanistic links between increased placental free radical production and changes to placental blood vessel development and function. One such example is cellular prion protein, thought to be involved in cellular copper regulation, which has been linked to altered placental oxidative stress and fetal growth and survival [138]. Elucidating the mechanisms that link adverse uterine conditions and adaptive placental development will lead to greater opportunities to develop therapeutic strategies for a number of obstetrical conditions which are associated with oxidative stress.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Glucose, Insulin, and Oxygen Interplay in Placental Hypervascularisation in Diabetes Mellitus

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The placental vasculature rapidly expands during the course of pregnancy in order to sustain the growing needs of the fetus. Angiogenesis and vascular growth are stimulated and regulated by a variety of growth factors expressed in the placenta or present in the fetal circulation. Like in tumors, hypoxia is a major regulator of angiogenesis because of its ability to stimulate expression of various proangiogenic factors. Chronic fetal hypoxia is often found in pregnancies complicated by maternal diabetes as a result of fetal hyperglycaemia and hyperinsulinemia. Both are associated with altered levels of hormones, growth factors, and proinflammatory cytokines, which may act in a proangiogenic manner and, hence, affect placental angiogenesis and vascular development. Indeed, the placenta in diabetes is characterized by hypervascularisation, demonstrating high placental plasticity in response to diabetic metabolic derangements. This review describes the major regulators of placental angiogenesis and how the diabetic environment *in utero* alters their expression. In the light of hypervascularized diabetic placenta, the focus was placed on proangiogenic factors.

1. Diabetes in Pregnancy

With the rise of maternal obesity in the Western world, diabetes in pregnancy has become more prevalent and affects a wide range of 3 to 20% of pregnancies. Diabetes in pregnancy comprises several metabolic diseases including gestational diabetes mellitus (GDM), a maternal glucose intolerance that clinically manifests in the 2nd gestational trimester, and type 1 and type 2 diabetes mellitus (T1D, T2D). These diseases cause increased short term fetal risks, foremost increased fetal fat accretion, but they also predispose the offspring to develop metabolic disorders or cardiovascular disease in later life [1, 2]. The placenta as the essential fetal organ nourishing fetal demands is characterized by various changes in morphology and function which may contribute to the fetal complications of diabetes. Particularly the fetoplacental vasculature and endothelium were shown to be susceptible to the diabetic intrauterine environment. Thus, diabetes associated derangements of factors regulating angiogenesis are a major cause of altered placental angiogenesis and hypervascularisation in diabetes [3–9].

2. Placental Angiogenesis in Normal Pregnancy

The central role of the placenta in pregnancy is highlighted by the fact that it is the first fetal organ to develop [10]. The placenta is highly vascularized to allow adequate oxygen and nutrient transfer from mother to fetus and back transfer of fetal waste products to the mother. Placental vascular development is tightly regulated by pro- and antiangiogenic factors and is divided into two stages. The first stage of vessel development, vasculogenesis, begins at day 21 after conception when a vascular plexus forms by differentiation of pluripotent mesenchymal progenitor cells into endothelial cells. In the second stage, these first vessels connect and further expand by angiogenesis that continues from day 32 after conception until delivery [11].

3. Regulation of Placental Angiogenesis

Various growth factors have been implicated in the regulation and stimulation of angiogenesis in the human placenta with

Table 1: *In vitro* expression of receptors of proangiogenic factors in the fetoplacental endothelium in normal third trimester human placenta.

Angiogenic factors	Receptors of angiogenic factors			
Gene symbol	Gene symbol	RefSeq ID	Mean intensity	Sd
VEGFA/PlGF	FLT1/VEGFR1	NM_002019	9.5	0.59
VEGFB	KDR/VEGFR2	NM_002253	10.5	1.43
FGF1/FGF2	FGFR1	NM_023110	9.5	0.25
	FGFR2	NM_000141	5.3	0.11
	FGFR3	NM_000142	6.8	0.13
	FGFR4	NM_213647	6.1	0.09
ANGPT1	TIE1	NM_005424	10.8	0.34
ANGPT2	TEK/TIE2	NM_000459	10.1	0.61
EPO	EPOR	NM_000121	7.6	0.15
TNFA	TNFR1	NM_001065	10.8	0.15
	TNFR2	NM_001066	8.6	0.57
IL6	IL6R	NM_000565	6.7	0.28
INS	INSR	NM_000208	6.4	0.70
IGF1	IGF1R	NM_000875	7.5	0.54
IGF2	IGF2R	NM_000876	9.3	0.48
LEP	LEPR	NM_002303	8.51	0.17
ADIPOQ	ADIPOR1	NM_015999	11.6	0.11
	ADIPOR2	NM_024551	9.6	0.18

Mean mRNA signal intensities from arterial ($n = 9$) and venous ($n = 9$) endothelial cells measured by Affymetrix GeneChip Human 1.0 ST arrays (Cvitic et al., unpublished data). Signal intensities range from 1 to 13. Sd: standard deviation.

distinct actions throughout gestation. Among these the most prominent are vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF2). Furthermore erythropoietin (EPA), leptin (LEP), adiponectin (ADIPOQ), placental growth factor (PlGF), angiopoietins (ANGPT), and the insulin/insulin-like growth factor (INS/IGF) system have also been demonstrated to promote placental angiogenesis. Their effects are mediated by specific cell surface receptors on the endothelium. Using gene expression analysis we compared expression levels of receptors for these hormones, growth factors, and cytokines in isolated human fetoplacental endothelial cells (Table 1). Indeed, receptors for all aforementioned proangiogenic factors were expressed with highest levels detected for VEGF, angiopoietin, and adiponectin receptors.

In the placenta, proangiogenic factors targeting the fetoplacental endothelium are often produced by neighboring cells such as trophoblasts, macrophages (Hofbauer cells), and smooth muscle cells. Moreover, proangiogenic factors of placental and fetal origin are also present in the fetal circulation. A subgroup of these factors is sensitive to reduced oxygen levels, and hypoxia is able to stimulate their expression.

3.1. Hypoxia. Hypoxia is the major regulator of angiogenesis. In general, it arises when vascular oxygen supply fails to meet metabolic demands. In early pregnancy, however, low oxygen is a physiological condition and drives developmental processes of placenta and embryo proper. During the establishment of the uteroplacental blood flow at the end of the first trimester, fetoplacental oxygen levels steeply rise [12]. Hypoxia affects the expression of multiple proangiogenic factors by regulating their transcription, mRNA stability, and translation. Thus, the low oxygen environment of the early placenta is paralleled by high levels of hypoxia sensitive proangiogenic factors. Mechanisms upregulating their expression under hypoxia are well studied. Whereas global protein synthesis is attenuated under low oxygen by masking the translation initiation sites for ribosomes, specific mRNAs contain alternative, internal ribosome binding sites (IRES) and are preferentially translated. Examples for mRNAs containing IRES include proangiogenic molecules, such as *VEGF*, and hypoxia inducible factors (HIFs), such as *HIF1A*. HIFs subsequently transactivate proangiogenic genes by binding to hypoxia response elements (HRE) in their promoters, introns, or enhancers [13].

3.2. VEGF System. The VEGF system constitutes a family of growth factors and their receptors, which are important regulators of blood vessel formation. With the founding factor and most important member, VEGFA (also known as VEGF), this family includes placental growth factor (PlGF), VEGFB, VEGFC, and VEGFD. VEGFs mediate their biological function by a family of protein tyrosine kinase receptors, VEGFR1 (Flt1), VEGFR2 (KDR/Flk1), and Flt4 [14].

In early pregnancy, VEGF is a key driver of placental vascular development and is held responsible for differentiation, growth, and aggregation of the endothelial precursors and formation of the haemangiogenic cords [15]. While villous trophoblasts initiate formation of first placental vessels by secretion of VEGF in the first trimester, it seems that placental macrophages and other stromal cells, for example, smooth muscle cells, take over angiogenic control at later stages of pregnancy [16–18]. VEGFR2 is expressed on the fetoplacental endothelium [19, 20] and mediates mitogenic cellular responses (reviewed in [21]), whereas VEGFR1 localizes to placental macrophages and trophoblast [19] and is an important regulator of VEGFR2 signalling (reviewed in [21]). As for VEGF, the expression of VEGFR2 is most intense in the early stages of pregnancy and declines steeply as pregnancy advances [20, 22].

In contrast to VEGF, the expression of PlGF and the soluble form of VEGFR1 (sFlt1) increase towards term [23–25]. sFlt1 captures free VEGF and prevents its binding to cell surface receptors, Flt1 and KDR, which further attenuates proangiogenic VEGF signaling in the third trimester, when VEGF levels are decreasing. PlGF is expressed in the syncytiotrophoblast [18] and in the smooth muscle cells around the fetoplacental vessels [26] and it stimulates the formation of highly branched capillary networks [27, 28]. *In vitro*, PlGF stimulates proliferation of microvascular endothelial cells from human term placenta [29].

Oxygen tension is the key physiological regulator of both VEGF expression and PlGF expression. Hypoxia regulates

VEGF transcription at the transcriptional level through HREs and posttranscriptionally via IRESs [18]. A similar posttranscriptional regulation was also demonstrated for PlGF [30].

3.3. FGF System. The fibroblast growth factor (FGF) system encompasses 22 members of the heparin-binding fibroblast growth factor family (FGF1 to 10 and FGF16 to 23) and four FGF receptors (FGFR1 to 4) (reviewed in [31]). Basic FGF (bFGF or FGF2) exerts proangiogenic functions on endothelial cells by binding to tyrosine kinase receptors FGFR1 and FGFR2. These functions include stimulation of proliferation, ECM degradation, migration, and modulation of cell-cell interactions (reviewed by [32]). In early placenta, FGF2 is thought to be involved in the recruitment of haemangiogenic progenitor cells, as its expression, similar to VEGF, reaches a maximum in this period of gestation. At term of gestation FGF2 is predominantly expressed in the syncytiotrophoblast, villous stroma, and fetal vessels [33–35]. FGFR1 parallels FGF2 expression [33], thus making the fetoplacental endothelium a target of FGF2 action. Similar to VEGF, FGF2 promoters harbor also both HREs and IRES, which mediate the regulation by hypoxia at the translational and posttranslational levels. The prevailing mechanism seems to be cell type specific [36].

3.4. Angiopoietin System. Whilst the VEGF system plays a key role in vessel sprouting and new vessel initiation, angiopoietins have a role in remodeling and maturation phases [37]. Establishment and maintenance of the outer layers of the vessel walls are thought to be controlled, at least in part, by the balance of angiopoietin 1 (ANGPT1) and angiopoietin 2 (ANGPT2), interacting with the angiopoietin receptors Tie1 and Tie2 [38]. Accordingly, ANGPT1 and ANGPT2 mRNA and protein have been detected in perivascular cells of immature intermediate villi [39], where larger arterioles/arteries and venules/veins develop. Both Tie1 and Tie2 mRNA localize to placental trophoblast and vascular endothelium [40, 41]. ANGPT2 belongs to the hypoxia sensitive proangiogenic factors and is regulated by HRE mediated transcriptional induction and by increasing mRNA stability [42, 43].

3.5. EG-VEGF/PROKR System. A novel family of angiogenic mitogens with high tissue specificity composed of EG-VEGF/PROK1 and PROK2 and the respective receptors, PROKR1 and PROKR2, has been characterized (reviewed in [44]). Whereas PROK2 mainly associates with the nervous system, EG-VEGF is associated with the reproductive tract including the placenta [45, 46]. EG-VEGF is mainly localized to syncytiotrophoblast with a mild expression in cytotrophoblast [47]. It has a strong vascular bed specificity and promotes proliferation, migration, tube-like formation, and permeability of placental microvascular endothelial cells without affecting angiogenesis in HUVEC [48]. EG-VEGF receptor PROKR1 is abundant in the cytotrophoblast, in the placental microvascular endothelial cells, and in the Hofbauer cells, whereas PROKR2 is expressed in the syncytiotrophoblast, Hofbauer cells, and extravillous trophoblast [47, 49, 50]. The oxygen tension regulates the expression of both EG-VEGF and the receptor PROKR1 at the transcriptional level [47].

3.6. Erythropoietin (EPO). Erythropoietin (EPO) is a hormone that regulates erythropoiesis in an oxygen-dependent manner. Generally, it is produced by perisinusoidal cells in the liver in the fetal and perinatal period and by peritubular interstitial fibroblasts in the kidney in adults [51]. The non-hematopoietic actions of EPO include regulation of angiogenesis during embryonic development [52], mitogenesis, stimulation of circulating endothelial progenitor cells, and cardioprotective effects in the ischemic heart by inhibiting apoptosis of cardiac myocytes [53, 54].

The placenta is also a source of EPO production, because EPO expression has been shown in various trophoblast subpopulations throughout gestation [55]. In addition, the EPO receptor (EPOR) was identified in villous and extravillous cytotrophoblast and syncytiotrophoblast at all gestational stages and also by cells within the villous core including fetoplacental endothelial cells [56].

EPO synthesis is primarily stimulated by hypoxia at the transcriptional level by HIF binding to HRE elements present in the *EPO* gene (reviewed in [57]). In addition, recent evidence suggests hypoxia regulation of EPO expression also at the translational level that does not involve IRES elements [58].

3.7. IL6 and TNFA. Interleukin 6 (IL6) and tumor necrosis factor alpha (TNFA) are multifunctional cytokines with main functions in the inflammatory response. In general, IL6 is produced by a variety of cell types, that is, macrophages, muscle cells, fibroblasts, and epithelial cells [59, 60], while TNFA is mainly produced by activated macrophages [61]. Both cytokines are also expressed by the human placenta.

In the first trimester placenta, IL6 expression is moderate and increases up to 4-fold in the third trimester placenta. In early pregnancy IL6 expression is observed in trophoblasts and fetal vessels [62, 63], with potential implication in angiogenesis and vascular remodeling [64]. IL6 acts by binding to an IL6-specific receptor, IL6R, which is expressed by placental trophoblasts and fetoplacental endothelial cells [65]. Hypoxia is reported to increase transcription, translation, and release of IL6 gene product from endothelial and smooth muscle cells [66].

TNFA mRNA and protein were also identified in first trimester villi, with strong prevalence in the syncytiotrophoblast and low to none in cytotrophoblasts and villous stromal cells. In term placentas, strong TNFA expression was observed in syncytiotrophoblast and villous stromal cells [67]. TNF signalling is mediated through two receptors, TNFR1 and TNFR2, which are expressed also in fetoplacental cells (reviewed in [68]). Regulation of TNFA transcription is complex and cell type specific [69]. Although *TNFA* mRNA does not contain HRE, chronic hypoxia is able to stimulate TNFA expression [70].

3.8. Insulin and IGFs. The insulin/insulin-like growth factor (INS/IGF) system comprises three ligands, insulin and the

insulin-like growth factors 1 and 2 (IGF1 and IGF2), three cell-surface receptors that mediate the biological effects of the INS and IGFs, insulin (IR) and the IGF1 and IGF2 receptors (IGF1R and IGF2R), and a family of IGF-binding proteins (IGFBPs) (reviewed in [71]).

Insulin and IGFs are implicated in the regulation of fetal and placental growth and development. IGF1 and IGF2 are synthesized in placental mesenchymal cells, such as macrophages and endothelial cells, with little change throughout gestation. However, while IGF1 is present in the trophoblast compartment at all gestational stages, IGF2 is not found in the syncytiotrophoblast and its expression in villous and extravillous cytotrophoblasts in the first trimester becomes undetectable at term of gestation [72–77].

In early pregnancy, the IGF1R is expressed on the villous cytotrophoblasts, the syncytiotrophoblast, and extravillous cytotrophoblast and additionally on placental macrophages and on fetoplacental endothelium in the third trimester [75]. The IR is localized predominantly at the syncytiotrophoblast with low occurrence in cytotrophoblasts in the first trimester. At term, however, IR is found mainly in the fetoplacental vessels [78]. Absent or low expression of insulin and IGF receptors on fetoplacental endothelium in the first trimester suggests that insulin and IGFs regulate fetoplacental angiogenesis only in later stages of pregnancy but will not contribute to placental vasculogenesis.

3.9. Leptin. The adipokine leptin (LEP) regulates food intake and satiety, but it also exerts growth factor and proangiogenic actions. In the placenta it is expressed in cytotrophoblasts, syncytiotrophoblast, amnion, and the fetoplacental endothelial cells [79, 80]. Most of the leptin produced by the placenta is secreted into the maternal circulation and may contribute to the increased maternal leptin levels during pregnancy [81]. Only 5% of leptin is secreted in the fetal circulation [82]. Leptin receptor (LEPR) is markedly expressed during the third trimester of pregnancy and is located primarily on the syncytiotrophoblast [83]. *In vitro*, its expression was also noted in fetoplacental endothelial cells [84].

Similar to *VEGF* and *FGF2*, leptin mRNA expression is also regulated by hypoxia, although solely at the transcriptional level by the hypoxia-inducible transcription factor HIF1A [85].

3.10. Adiponectin. Adiponectin (ADIPOQ) is an adipokine with insulin sensitizing properties and is proposed to exert angiogenic actions. ADIPOQ gene and protein expression were reported in the human term placenta, with expression primarily in the syncytiotrophoblast [86]. However a recent study contradicts this finding and shows absence of adiponectin in the term placenta [87]. The expression of the adiponectin receptor, ADIPOR2, but not of ADIPOR1, was observed in the cytoplasm of placental cytotrophoblasts and syncytiotrophoblast suggesting that adiponectin may have a physiological function in the placenta during pregnancy [88]. Whether adiponectin receptors are present in the fetoplacental endothelium has not been fully clarified, but high expression levels of ADIPOR1 and ADIPOR2 in isolated fetoplacental cells *in vitro* (Table 1) may suggest their expression also *in vivo*.

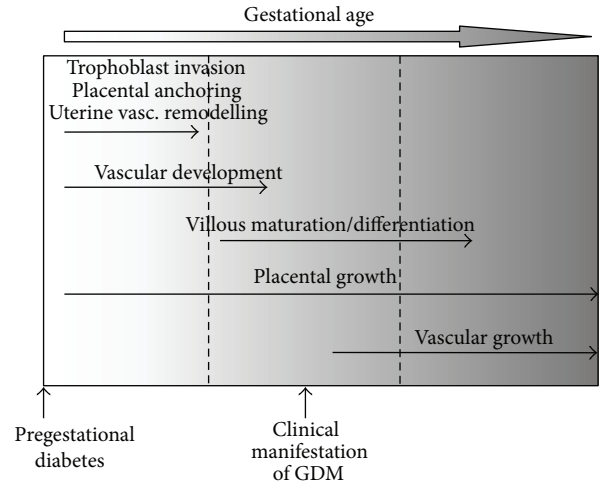

Figure 1: Graphical representation of specific windows of placental development susceptible to metabolic insults of pregestational and gestational diabetes, respectively.

4. Placental Angiogenesis in Diabetes Mellitus

It is well established that maternal diabetes mellitus affects placental vascular development. Depending on the type of diabetes, that is, pregestational (T1D and T2D) or gestational diabetes mellitus (GDM), different windows of placental vascular development are exposed to the diabetic derangements (Figure 1). While pregestational diabetes may affect the entire placental and fetal development, the hyperglycemia of GDM develops during pregnancy and clinically manifests only in the late second trimester. Thus, GDM may have an impact on placental processes occurring in later stages of pregnancy, such as angiogenesis and microvascular remodeling, and will not affect developmental events that occur in early pregnancy, such as vasculogenesis.

This difference in the onset and duration of pregestational diabetes and GDM suggests that the effect of diabetes on placental vascular development and, hence, on the morphology of the placental vascular tree will differ. Indeed, in T1D, three-dimensional power Doppler ultrasound of the fetoplacental vasculature revealed changes in vascular indices already in the first trimester. Thus, T1D affects the placental vasculature already in a period that cannot be affected by GDM. However, the results are conflicting, and both reduced [89] and increased [90] placental vascular indices were reported.

In the third trimester of gestation T1D and GDM lead to similar change of placental vascular structure despite different duration of the diabetic metabolic insult. In T1D the capillary surface area is increased by both longitudinal growth and enhanced branching of villous capillaries [3–7]. Although less investigated, similar observations were made for the placenta in GDM. There are increased capillary

branching [8] and capillary surface area [9]. The extent by which capillary branching is increased in T1D and GDM is similar, as reflected by a 2.0-fold versus a 1.8-fold increase in the number of redundant connections per villus, respectively [7, 8]. Further evidence for an impact of maternal diabetes on fetal vascular growth and angiogenesis is provided by findings on longer umbilical cord length in GDM [91] with higher risk for both hyper- and hypocoiled cords [92, 93].

Besides this general finding of placental hypervascularisation, evidence also suggests altered endothelial resistance. Measurements of surface expression of the adherens junctional molecules, vascular-endothelial cadherin (VE-cadherin) and beta-catenin (β-catenin), revealed a decreased expression in both T1D [94] and GDM placentas [95]. Moreover, in GDM, the tight junctional molecules, occludin and zonula occludens-1 (ZO1), were also reduced [95]. As these molecules play important roles in angiogenesis and barrier function these results implicate a disturbance of these processes in diabetes. Indeed, microscopic observations of cords after GDM pregnancies revealed rupture of the endothelium paralleled by extravasation of blood within Wharton's jelly [96].

These changes in the fetoplacental vasculature in response to maternal diabetes may imply potential differences also in the vasculature of the fetus proper. In fact, fetuses exposed to diabetes have vessel changes in the iris that resolve after birth [97] and an increased risk in developing cardiovascular defects. The prevalence of cardiovascular defects, however, is different between T1D and GDM. Pregestational diabetes associates with 50% of the investigated cardiac and noncardiac defects in the offspring, while the associations with GDM are weaker and limited to offspring of women with increased prepregnancy BMI [98]. Mechanisms underlying the association of diabetes mellitus with birth defects are not clear. Eriksson and colleagues report a positive correlation between exposure to hyperglycemia during *in utero* development and the risk of congenital malformations in the infants of diabetic mothers [99], suggesting indeed glucose as the driving force of the detrimental effects that diabetes has on the fetus.

5. Effect of Maternal Diabetes on Proangiogenic Factors in Placenta and Fetus

The maternal diabetic environment clearly differs between T1D and GDM. However, the emerging metabolic and hormonal changes in the fetus resemble, and it is likely that these changes in the fetus will likewise affect the placental and fetal levels of proangiogenic factors. Some of these changes are summarized in Table 2. The primary origin of fetal derangements in all types of diabetes mellitus is fetal hyperglycaemia resulting from maternal hyperglycaemia. This hyperglycaemia ensues metabolic and hormonal changes in the fetus. Once the fetal pancreas commences to produce and secrete insulin in the late first trimester [100, 101], fetal hyperglycaemia leads to fetal hyperinsulinemia and both stimulate fetal metabolism. Consequently, fetal oxygen demands rise often leading to chronic fetal hypoxia [102–104]. Fetal hypoxia was demonstrated directly by measuring cord blood oxygen [102] and indirectly by measuring cord blood EPO levels and by increased erythropoiesis [103–105].

Because of the strong proangiogenic potency of hypoxia [106] through regulating multiple steps of vascular growth, chronic fetal hypoxia as a consequence of maternal diabetes may thus stimulate placental vasculogenesis and angiogenesis by increasing the growth factors expression in the placenta and fetus. As mentioned before, various proangiogenic factors secreted by the placenta or present in the fetal circulation

Table 2: Expression and levels of proangiogenic factors in placenta and cord blood in pregnancies complicated by different types of diabetes.

Factor	Type of diabetes	Placenta	Cord blood
VEGF	GDM	↓protein [115]	↓[108]
	T1D	=mRNA [35]	↓[107]
PlGF	GDM	↓protein [115]	↓[108]
	T1D	=mRNA [35]	↓[107]
FGF2	GDM	↑mRNA and protein [112, 132]	↑[112, 142]
	T1D	↑mRNA [33, 111] ↑protein [34] =mRNA [35]	↑[142]
Angiopoietin	GDM	↑mRNA [113]	
	T1D	=mRNA [35]	
Erythropoietin	GDM		↑[103, 108]
	T1D		↑[103, 126]
IL6	GDM	↑mRNA [119]	↓[121]
	T1D		
TNFA	GDM	↑protein [120] ↑mRNA [113] =mRNA [119]	=[122] ↓[121]
	T1D		
Insulin	GDM		↑[124, 125, 127, 143]
	T1D		↑[5, 125, 126]
IGF1	GDM	=mRNA [112] ↓mRNA [113]	↑[112, 144, 145]
	T1D		=[5]
	IDDM		↑[146]
	T2D		=[144]
	D	=mRNA [147]	↑[147]
IGF2	GDM	↑mRNA [113]	↑[148] =[145]
	T1D		
	IDDM		↑[146, 148]
	D	=mRNA [147] ↓peptide [147]	↑[147]

↑ indicates elevated levels, ↓ indicates reduced levels, and = indicates unchanged levels in diabetes.
GDM: gestational diabetes mellitus; T1D: type 1 diabetes; T2D: type 2 diabetes; IDDM: insulin dependent diabetes mellitus without further classification; D: diabetes without further classification.

harbor hypoxia sensitive regulatory sites, that is, IRES or HREs, and may thus be elevated as a result of maternal diabetes. Potential factors regulated by fetal chronic hypoxia in maternal diabetes include VEGF, PlGF, FGF2, EPO, ANG2, and leptin. However, factors of the diabetic environment other than hypoxia may also contribute and modify its effect.

Parallel to its regulation by oxygen, placental VEGF levels are high in the first trimester when oxygen levels are low and decline thereafter towards term of gestation [16–18]. However, in the third trimester, fetal hypoxia as a result of maternal diabetes does not stimulate VEGF expression. In fact, placental expression and fetal cord levels in maternal T1D and GDM are unchanged or even lower than normal [107, 108]. Also PlGF levels are not altered in maternal diabetes [109]. Nevertheless, whole placental tissue from GDM pregnancies contains more VEGFR1 (Flt1) and VEGFR2 (Kdr) [110], which may compensate for the reduced VEGF levels and thus maintain VEGF activity.

Similar to VEGF, also FGF2 levels are higher in the low oxygen environment of the first trimester placenta than in the term placenta [111]. However, in contrast to VEGF, FGF2 is increased in placenta and fetal cord blood in diabetic pregnancies [33, 112]. The presence of an IRES and HRE sequence within its promoter represents a mechanism by which hypoxia increases *FGF2* expression and translation [36].

Hypoxia is also known to induce the expression of EG-VEGF and PROKR1, but their levels were never investigated in diabetes. Nevertheless, since the EG-VEGF and the receptors, PROKR1 and PROKR2, are predominantly expressed during the first trimester of pregnancy the derangement in the level of these factors would affect placental vasculogenesis [47].

Different from the VEGF and FGF2 system, both of which promote sprouting angiogenesis, the angiopoietin system is a major regulator of vessel maintenance and maturation [37]. ANG2 is transcriptionally regulated by hypoxia. This may explain why *ANG2* mRNA expression is increased in the placenta in maternal diabetes [113].

The hormone EPO is also induced by hypoxia and increased in the fetal circulation of diabetic pregnancies at the end of gestation [103]. The EPOR is expressed in fetoplacental vessels [56], making placental endothelial cells a target for EPO action. Experiments in mice revealed that proangiogenic effects of the EPOR are mediated by upregulation of the VEGF/VEGF receptor system [114]. While fetal VEGF levels are unchanged or even lower in maternal diabetes, the VEGFR is increased in the placenta [115]. This would suggest that in a situation of increased fetal oxygen demand fetal and perhaps also placental EPO can promote placental angiogenesis through the VEGF/VEGF receptor system. Although an attractive hypothesis, this awaits demonstration in human.

Collectively, the contribution of hypoxia in altering placental and fetal levels of proangiogenic factors is diverse. While *FGF2* and *EPO* are upregulated in maternal diabetes, the classical hypoxia regulated proangiogenic genes VEGF, PlGF,and ANG2 remain unchanged. This suggests that additional mediators present in the diabetic milieu may also contribute to the diabetes associated hypervascularization. Hyperglycaemia and hyperinsulinemia are such candidates. They may further modify the effect of hypoxia. The contribution of mediators in diabetes in addition to hypoxia is highlighted by differences in placental vascularization between diabetes and other conditions associated with fetal hypoxia, such as high altitude, anemia, or smoking [116]. While increased branching angiogenesis is a common observation, other vascular features, such as total vascular volume, surface area, and capillary length, are increased in maternal diabetes but remain unchanged in the other hypoxic conditions [116].

In addition to hypoxia, hyperglycaemia is one factor that may further impact placental vascular changes, since it is the main reason causing proinflammatory environment and cytokine derangements that will further act on the endothelium. Hyperglycaemia contributes to the generation of reactive oxygen species (ROS) and, thus, to oxidative stress. ROS are generated on one hand by stimulation of the glucose metabolism and the respiratory chain in the mitochondria and on the other hand by the production of advanced glycation end products (AGE). When binding to their receptor (RAGE), AGE induce the formation of ROS as second messengers, a process also termed ROS signaling (reviewed by [117]). As a speculation, this oxidative stress may then contribute to the proinflammatory environment of diabetes and may affect angiogenesis.

Expression of both IL6 and TNFA is sensitive to oxidative stress. Indeed, hyperglycemia stimulates expression of IL6 in trophoblasts [118] and placental expression of IL6 and TNFA is increased [113, 119, 120], but their levels in the fetal circulation are unchanged or even reduced [121, 122]. Thus, TNFA and IL6 may affect placental angiogenesis locally, that is, in a paracrine manner. TNFA and IL6 are not only regulated by the diabetic environment and they are regulators and initiators of the proinflammatory environment of diabetes. Both modulate the expression of adiponectin and leptin. Moreover, TNFA stimulates DNA binding of HIF1 [123] and thus may augment hypoxia induced transcription.

Besides hypoxia and hyperglycaemia, hyperinsulinemia is another hallmark of changes in the fetal circulation associated with maternal diabetes mellitus [5, 124–127]. The potent role of insulin in angiogenesis of fetoplacental endothelial cells has been shown by *in vivo* observations and *in vitro* studies. Fetal cord insulin is positively associated with capillary surface area of the placenta at the end of gestation [5, 128]. In isolated fetoplacental endothelial cells insulin binding to its receptors stimulates several pathways that promote angiogenesis, including the activation of the small GTPase Rac1, eNOS, and expression of the matrix metalloproteinase MT1-MMP [129, 130].

Leptin is regulated by multiple aspects of the fetal diabetic environment. In pregnancies complicated by maternal diabetes mellitus, placental leptin production and fetal plasma leptin is increased [5, 79, 113, 124, 125, 127, 131, 132]. Correlation of fetal insulin with fetal leptin levels suggests that fetal hyperinsulinemia, by stimulating white adipose tissue growth, the main leptin source, leads to higher leptin production in fetal adipose tissue [133]. Additionally, insulin was recently shown to stimulate leptin expression in human placental explants [134]. However, it is unclear whether this

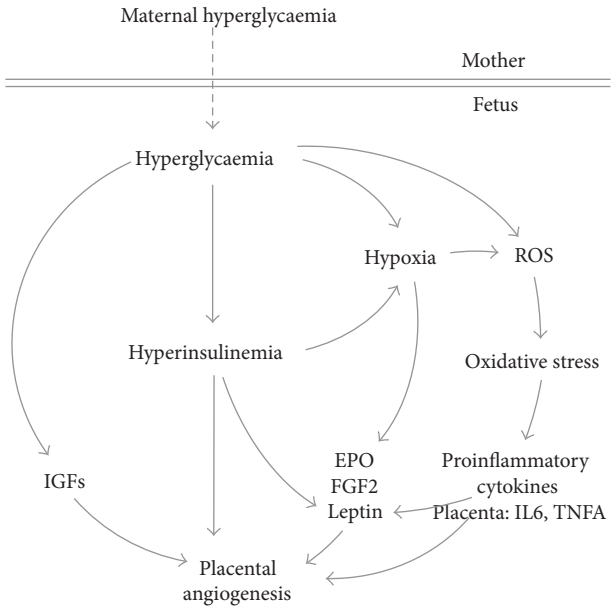

Figure 2: Hypothetic scheme indicating how fetal hyperglycaemia, hyperinsulinemia, and hypoxia may induce metabolic, hormonal, and inflammatory changes that may lead to placental hypervascularisation.

leptin will act in a paracrine manner or what proportion is secreted into the fetal circulation. A proinflammatory environment also stimulates leptin secretion. TNFA and IL6 upregulate leptin expression in trophoblasts *in vitro* [135, 136]. Furthermore, correlation of fetal leptin levels with fetal EPO levels in T1D [104] parallels the finding that leptin is regulated by hypoxia through transcriptional induction by HIF1 [85] and can thus represent another manifestation of fetal hypoxia in diabetes mellitus.

Similar to leptin, adiponectin is multifactorially regulated. Both, hyperinsulinemia and hypoxia suppress adiponectin concentrations in adipocytes [137, 138]. Thus, lower levels of adiponectin in the fetal circulation and in the placenta associated with maternal diabetes [85, 86, 139–141] may result from various alterations of the intrauterine environment of diabetes, but reduced levels of the proangiogenic adiponectin suggest that it will not contribute to placental hypervascularisation.

6. Conclusion

Diabetes mellitus in human pregnancy strongly affects fetoplacental vascular morphology. The fetal diabetic environment resulting from maternal and, hence, fetal hyperglycemia causes fetal hyperinsulinemia and hypoxia. Hypoxia is a key modulator of angiogenesis and works in concert with the diabetic environment to induce placental hypervascularization (Figure 2).

Whether vascular changes occur also in the fetus proper is still unknown. Altered cord blood levels of the majority of proangiogenic factors, as summarized above, however, may suggest potential consequences in the fetus. This speculation is supported by an increased risk for cardiovascular defects [98] and vessel changes in the iris [97] in offspring born to diabetic mothers.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Enhanced Prevalence of Plasmatic Soluble MHC Class I Chain-Related Molecule in Vascular Pregnancy Diseases

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The major histocompatibility complex class I related chain (MIC) is a stress-inducible protein modulating the function of immune natural killer (NK) cells, a major leukocyte subset involved in proper trophoblast invasion and spiral artery remodeling. Aim of the study was to evaluate whether upregulation of soluble MIC (sMIC) may reflect immune disorders associated to vascular pregnancy diseases (VPD). sMIC was more frequently detected in the plasma of women with a diagnostic of VPD (32%) than in normal term-matched pregnancies (1.6%, $P < 0.0001$), with highest prevalence in intrauterine fetal death (IUDF, 44%) and vascular intrauterine growth restriction (IUGR, 39%). sMIC levels were higher in preeclampsia (PE) than in IUFD ($P < 0.01$) and vascular IUGR ($P < 0.05$). sMIC detection was associated with bilateral early diastolic uterine notches ($P = 0.037$), thrombocytopenia ($P = 0.03$), and high proteinuria ($P = 0.03$) in PE and with the vascular etiology of IUGR ($P = 0.0038$). Incubation of sMIC-positive PE plasma resulted in downregulation of NKG2D expression and NK cell-mediated IFN-γ production in vitro. Our work thus suggests that detection of sMIC molecule in maternal plasma may constitute a hallmark of altered maternal immune functions that contributes to vascular disorders that complicate pregnancy, notably by impairing NK-cell mediated production of IFN-γ, an essential cytokine favoring vascular modeling.

1. Introduction

Pregnancy is a unique situation in which angiogenesis and the establishment of maternal-fetal tolerance are essential steps to ensure placental and fetal development.

Inadequate placentation leads to a large spectrum of vascular pregnancy diseases (VPD) that affects the mother and/or the fetus and include preeclampsia (PE) intrauterine fetal growth retardation (IUGR) and intrauterine fetal death (IUFD).

VPD represents a leading cause of fetomaternal morbidity and mortality. The etiology of VPD is often unknown and the search for molecules that may better characterize the physiopathological processes that lead to their adverse outcome is

the subject of extensive investigation. Identification of reliable indicators of adverse outcome is a clinically relevant issue to optimize prevention of severe pregnancy complications [1], and several reports highlight the importance of both angiogenic factors in insuring adequate placentation [2–4]. Indeed, circulating antiangiogenic proteins such as soluble fms-like tyrosine kinase 1 ([sFlt1]) and soluble endoglin have been associated with the pathogenesis of PE [5, 6]. Enhanced serum levels of PAPP-A, ADMA, homocysteine, and sFlt-1 have also been associated with the severity of this disease. Despite recent advances in understanding how inadequate placental vascularization and incomplete spiral artery remodeling may lead to vascular pregnancy diseases, the prognosis of VPD often remains severe, and placental removal remains the only treatment to manage severe preeclampsia and major intrauterine growth restriction. Understanding the complex and multifactorial mechanisms that favor these severe complications is thus essential to design new therapeutic approaches. Cross-talk between fetal and maternal immune cells is an essential requisite to achieve early pregnancy placental development, vasculogenesis, and immune tolerance of the fetus [7–9]. Natural killer immune cells (NK) are a major source of angiogenic growth factors and cytokines that insure transformation of the uterine spiral arteries, fetal implantation, and placentation [2, 7, 10–12]. Abnormal NK cell receptor and cytokine production profiles have been associated with pregnancy disorders such as recurrent pregnancy loss, implantation failure, and preeclampsia [13–15]. IFN-γ secretion by NK cells has been identified as an essential regulatory pathway favoring vascular modeling and first trimester extravillous cytotrophoblast migration. Lowered levels of IFN-γ were observed in decidual NK cells from women with hypertensive disorders complicating pregnancy. In contrast, enhanced peritoneal NK cell mediated IFN-γ was observed in women with severe endometriosis which may promote abnormal proliferation and angiogenesis of endometrial cells [16, 17]. NK cell-mediated cytotoxic activity and angiogenic factor/cytokine production are regulated by the integration of signals that target inhibitory and activating receptors expressed on NK cells [18–23].

Various molecules have been shown to regulate uterine NK (uNK) cell-mediated transformation of decidual arteries, thereby allowing increase of placental blood supply.

Apart from its role in protecting fetal cells from maternal NK cell cytotoxicity, engagement of the KIR2DL4 receptor by HLA-G has been shown to stimulate NK-cell mediated IFN-γ production, thus providing a favorable environment promoting vascularization in maternal decidua during early pregnancy [24, 25]. Accordingly, lowered HLA-G expression was associated with the occurrence of preeclampsia and intrauterine growth retardation [26–29]. Engagement of NKG2D receptor by stress-inducible membrane-bound MHC class I chain-related (MIC) has been shown to stimulate NK cell-mediated cytokine production [30–35]. The shedding of a soluble form of this molecule (sMIC) has been shown to induce internalization of the NKG2D receptor in NK cells and consequently modulate immune responses in various pathological settings [35–37]. Endothelial expression of MIC has also been shown to target antibody-mediated vascular rejection after solid organ transplantation [38, 39]. The release of sMIC was also suggested to modulate NK cell function during pregnancy [40]. We showed that elevated sMIC serum levels, detected in the serum of 38% of infertile women candidate to in vitro fertilization, were predictive of both embryo implantation failure and pregnancy success following IVF [41].

In the present study, we first investigated whether sMIC could be detected in the plasma from women at time of VPD diagnostic. We then tested whether incubation with plasma from women with VPD could affect NKG2D expression and cell mediated production of IFN-γ, a major cytokine implicated in vascular modeling during pregnancy.

2. Subjects and Methods

2.1. Patients. The study population comprised 169 women with singleton gestations recruited between June 2004 and May 2006 within women attending gynecology units of Assistance Publique Hopitaux de Marseille (AP-HM, hopital de la Conception and hopital Nord, Marseille, France). All these women gave their informed consent to collect plasma and participate in the study, which was approved by the local Hospital ethics committee of our institution (CPP-Marseille 1 n°05/33).

The study population included 81 patients with a diagnostic of vascular pregnancy diseases (VPD) that were further subdivided in 3 groups: 40 patients with PE, 23 patients with IUGR, 18 patients with IUFD, 25 patients with a diagnostic of non-vascular IUGR and 63 term-matched normal pregnancies (NP control group).

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PE was defined by persistent diastolic arterial blood pressure greater than 90 mm Hg and a systolic blood pressure greater than 140 mm Hg, associated with proteinuria (≥300 mg in a 24-hour urine collection or at least one dipstick measurement ≥2+). Severity of preeclampsia was defined according to ACOG guidelines [42], by high blood pressure ≥160 mm Hg systolic or to 110 mm Hg diastolic or presence of thrombocytopenia (platelet count less than 100.000/μL) or renal failure (serum creatinine concentration greater than 1.1 mg/dL or a doubling of the serum creatinine concentration in the absence of other renal diseases), impaired liver function indicated by abnormally elevated blood concentrations of liver enzymes (2-fold normal concentration). Patients included in this study did not present other signs of severe PE such as pulmonary edema or brain or visual disturbances. Vascular IUGR was defined as ultrasonographic measurement <2.5th percentile for gestational age and baby birth weight <5th percentile associated with at least one biological or sonographical marker of "placental insufficiency" as abnormal uterine, or umbilical artery Doppler, or elevated plasma fibronectin level. Exclusion criteria of vascular

Table 1: Characteristics of study population.

	Normal pregnancies	Vascular pregnancy diseases	Nonvascular IUGR	P value
Number of patients	63	81	25	
Age (yrs, mean ± sd)	29.6 ± 6.6	30.2 ± 6.3	29 ± 6.3	ns
Gestation (*n*)	3 [1–9]	2 [0–7]	2 [0–6]	ns
Parity (*n*)	1 [0–5]	0 [0–4]	0 [0–3]	ns
Body mass index (kg/cm^2)	23 [17–48]	23.1 [16.3–37.2]	23 [18–37]	ns
Systolic blood pressure (mmHg)	12 [10–13]	14 [10–22]	12 [10–13]	$P < 0.001^*$
Diastolic blood pressure (mmHg)	7 [5–8.3]	8.7 [6–12]	7 [5.5–8.5]	$P < 0.001^*$
Uterine height (cm)	32 [29–38]	26 [15–36]	26 [15–36]	$P < 0.001^§$
Term at sampling (weeks of gestation)	31.7 [15.3–41]	30 [17–41]	30 [17–41]	ns
Term at delivery (weeks of gestation)	40.3 [35–42]	32.3 [17–41.1]	32 [22–41]	$P < 0.001^§$
Baby weight at birth (g)	3300 [2640–4680]	1330 [80–3410]	1745 [400–2500]	$P < 0.001^§$

Comparison between groups was performed with nonparametric Kruskal-Wallis test followed by the Dunn posttest. Values indicate median [25–75 interquartile ranges].
$^*P < 0.001$ between vascular pregnancy diseases and other groups.
$^§P < 0.001$ between normal pregnancies and other groups.

IUGR group were the presence of congenital malformations or chromosomal abnormalities in the fetus, recent cytomegalovirus or toxoplasma infection, trauma, drugs or alcohol abuse during pregnancy.

The nonvascular IUGR group consisted of 25 women with isolated IUGR, related to chromosomal fetal aneuploidy in 4 cases, fetal polymalformative syndrome in 8 cases, toxic or infectious origin in 3 cases, and fetus small for gestational age in the 10 remaining cases.

Nonvascular IUGR was diagnosed as ultrasonographic measurement <2.5th percentile for gestational age and baby birth weight <5th percentile with a normal fibronectin level, uterine and umbilical artery Doppler velocity.

IUFD was defined by ultrasound examination as a visible fetus without cardiac activity after 12 weeks of gestation. Only IUFD occurring after severe vascular IUGR were included in the study. The term at sampling was the term of IUFD diagnosis (Table 1).

The control group consisted of 63 healthy pregnant women, seen for routine gynecologic examination and followed until delivery to confirm normal pregnancy (NP) outcome. Normal pregnancies were recruited between 17 and 41 weeks of gestation and matched on the pregnancy term of at least one patient of the distinct VPD groups analyzed (PE, IUGR, and IUDF).

2.2. Plasma Collection. Blood samples were collected at time of diagnosis of vascular pregnancies diseases or isolated IUGR and at time of obstetrical examination for the control group of term matched normal pregnancies. Samples were collected into 0.129 mol/L sodium citrate (3.8%) centrifuged and stored at −80° according to standard procedures.

2.3. Isolation of PBMCs. Peripheral blood from nonpregnant healthy volunteer blood donors was collected and PBMCs used for in vitro experiments were separated by Ficoll-Hypaque density gradient centrifugation.

2.4. Capture ELISA for sMIC/B. Soluble MIC concentrations were measured in the plasma using a sandwich enzyme-linked immunoabsorbent assay as previously described [37]. The detection threshold of recombinant soluble MICA protein, used as standard in each experiment, was 0.1 ng/mL and plasma levels higher than 0.3 ng/mL were considered as positive. Human recombinant MIC proteins standards were purified from baculovirus, as previously described [43].

2.5. Analysis of Cell Surface NKG2D Expression. Flow cytometry analysis of NKG2D expression was conducted after incubating control PBMCs from nonpregnant donors with 20% of human serum obtained from VPD patients or normal pregnancy controls. Intensity of PE-labeled anti-NKG2D mAb (IgG1, ON72, Beckman-Coulter) or isotype control mouse IgG1 antibody staining (Beckman-Coulter) was expressed as % of cells and mean fluorescence intensity (mfi) of NKG2D positive cells within the $CD3^-CD56^+$ peripheral blood NK cell subset. PBMCs were incubated for 48 h with 20% of sMIC-positive plasma from 8 VPD patients and analyzed in reference to sMIC-negative plasma from 8 women with normal term matched pregnancies.

3. Interferon-γ Assay Using Quantitative Real-Time PCR and ELISA

For the interferon-γ assay, 2×10^6 NK-92 cells (ATCC number: CRL-2407) were incubated for 72 h in the presence of 20% MIC-positive or MIC-negative plasma. Total RNA was isolated from NK culture using RNeasy mini kits (QIAGEN Inc., Valencia, CA, USA) followed by reverse transcription using SuperScript RTII (Invitrogen Life Technologies) according to the manufacturer's protocol. The resulting cDNA was amplified with primer pairs specific for interferon-γ and

Table 2: Frequency of sMIC detection and plasma levels in the study population.

	Frequency of sMIC positive plasma number (proportion %)	Comparison of sMIC frequency in reference to control normal pregnancy group	Median sMIC plasma levels in positive samples (ng/mL) [25–75 interquartile range]
Normal pregnancies $n = 63$	1 (1.6%)	—	0.5
Vascular pregnancy diseases $n = 81$	26 (32%)	$P < 0.0001$	2.2 [1.15–11.47]
Preeclampsia $n = 40$	9 (22.5%)	$P < 0.0008$	7.5 [1.37–32.69]
Vascular IUGR $n = 23$	9 (39%)	$P < 0.0001$	1.63 [0.86–5.2]
IUFD $n = 18$	8 (44%)	$P < 0.0001$	2.18 [0.86–7.58]
Nonvascular IUGR $n = 25$	1 (4%)	ns	1.63

GAPDH (forward and reverse sequences): INF-γ 5′-TGCAGAGCCAAATTGTCTCCTT-3′ and 5′-CATGTATTGCTTTGCGTTGGAC-3′; GAPDH 5′-GGTGGTCTCCTCTGACTTCAACA-3′ and 5′-GTTGCTGTAGCCAAATTCGTTGT-3′. Real-time PCR amplification was performed with the FastStart DNA Master^{PLUS} SYBR Green I Kit as recommended by the manufacturer, on a LightCycler Roche instrument. The cycling conditions were 10 min at 95°C (hot-start PCR), followed by 40 cycles of 5 s at 95°C (denaturation), 10 s at 62°C (annealing), and 20 s at 72°C (elongation). Melting curve analysis was then performed to check the specificity amplification. In relative quantification, reported values were expressed as the relative numbers of specific transcripts detected per 10^6 GAPDH transcripts ($2^{-\Delta Ct}$ method). All experiments were performed at least in duplicate. IFN-γ supernatant concentration of NK-cell line (Beckman Coulter, France) was measured using commercial ELISA according to the manufacturer's instructions. The limit of detection was 0.1 IU/mL.

3.1. Statistics. Analyses were performed using Prism software (GraphPad 4.0b, GraphPad, San Diego, CA) implementing the nonparametric Kruskal-Wallis test followed by the Dunn posttest to compare 3 or more continuous variables, the Mann-Whitney test to compare 2 unpaired groups, and the Wilcoxon matched pairs test for the NKG2D expression. Data were expressed as the median (25–75 percentile range) and mean (±sd), depending on the distribution. Association between categorical variables was tested after cross tabulation by the Pearson chi square (and by Fisher exact test if $n < 5$). Correlation was improved by spearman test. A 95% confidence interval ($P < 0.05$) was considered significant.

Multivariable logistic regression analysis was used to explore the relationship between sMIC and the following explanatory variables: parity, gestation, systolic and diastolic blood pressure, gestational age at sampling, thrombocytopenia, term of delivery, and baby weight at birth using SPSS v.12.0 (SPSS Inc., Chicago, IL, USA) statistical packages. A P value of <0.05 was considered significant.

4. Results

4.1. Increased Frequency of Plasma sMIC in Women with Vascular Pregnancy Diseases. Plasma levels of sMIC were evaluated in 3 groups of pregnant women: term-matched women undergoing normal pregnancies (NP), women with a diagnostic of VPD (PE or vascular IUGR or IUFD) or with a diagnostic of non-vascular IUGR. No significant differences were found in age, number of gestation, body mass index, and gestational age at sampling between normal pregnancies, VPD, and nonvascular IUGR. As expected the systolic and diastolic blood pressure were significantly higher in women with VPD. As expected the median of baby's birth weights and gestational age at delivery were significantly lower in the group with VPD and nonvascular IUGR compared to normal pregnancies ($P < 0.001$). The main clinical and biological characteristics of these patients are summarized in Table 1.

In the NP group, only one of the 63 women had detectable but low sMIC levels in plasma (0.5 ng/mL). However, it must be noted that this patient was followed for systemic lupus erythematosus, with normal pregnancy evolution. Of the 25 women with nonvascular IUGR, only one had detectable sMIC plasma levels (1.63 ng/mL). By contrast, sMIC molecules were detected in 26 of the 81 patients (32%) in the VPD group ($P < 0.0001$ in reference to NP). Highest prevalence of sMIC detection was associated with IUFD (44%) and vascular IUGR (39%) (Table 2). sMIC levels detected in the PE group (median, 25–75 interquartile ranges: 7.5 ng/mL, 1.37–32.69) were higher than those detected in the IUFD (2.18 ng/mL, 0.86–7.58, $P < 0.01$) and vascular IUGR (1.63 ng/mL, 0.86–5.2, $P < 0.05$) groups (Table 2).

4.2. The Presence of sMIC in PE Plasma Is Associated with Thrombocytopenia and High Proteinuria. We then determined whether the presence of sMIC in maternal plasma could be associated with parameters that depict preeclampsia associated disorders. sMIC was detected in 22.5% of PE patients while being only observed in 1.6% of term matched normal pregnancies ($P < 0.0008$, Table 2). Sonographical analysis of maternal uterine artery Doppler velocity waveform performed at time of PE showed that bilateral early

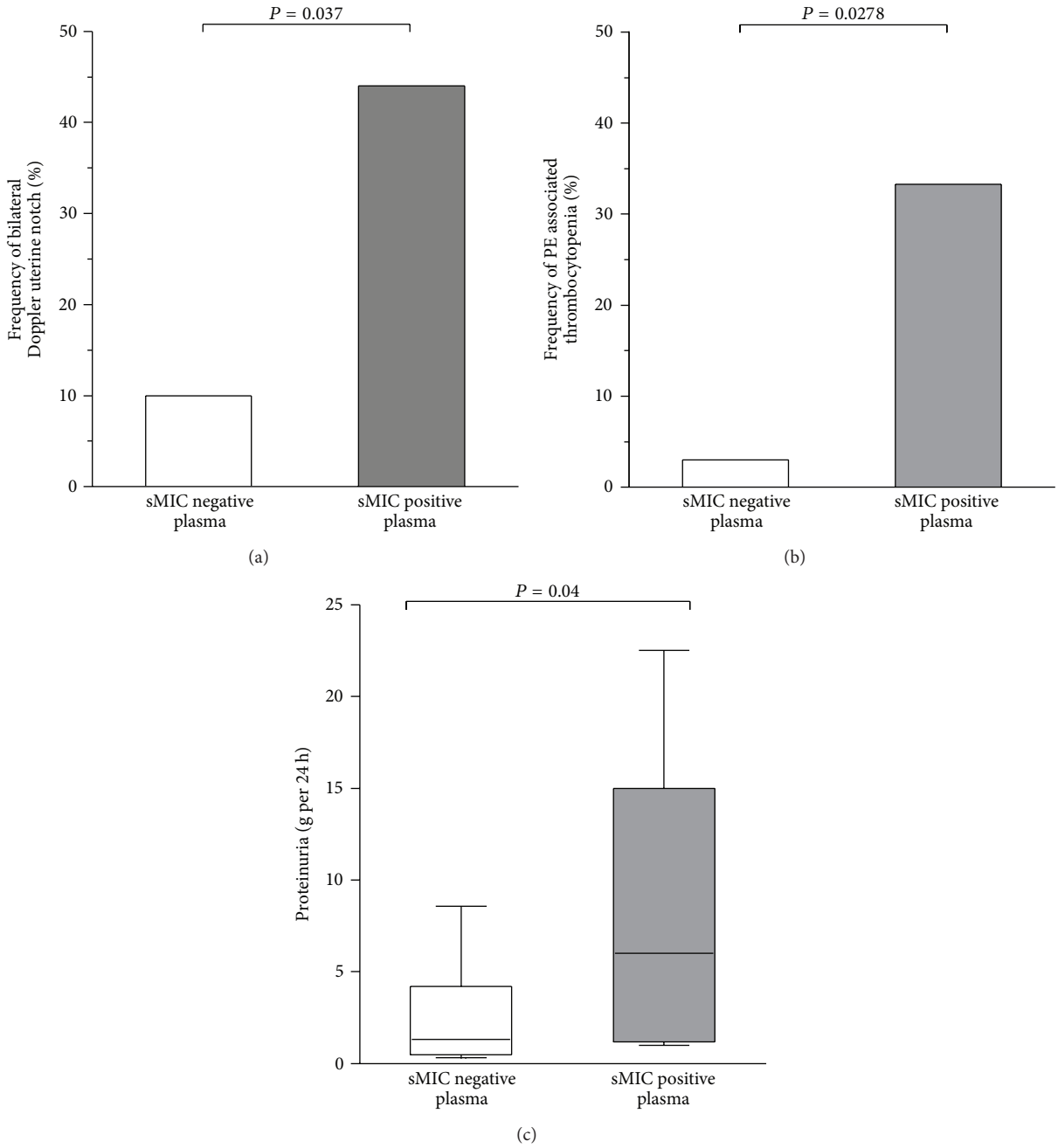

Figure 1: (a) Frequency of bilateral early diastolic uterine notch, in sMIC-positive and -negative subgroups of preeclamptic patients. (b) Frequency of thrombocytopenia in sMIC-positive and -negative subgroups of preeclamptic patients. (c) Median and interquartile range of proteinuria per day in sMIC-positive and -negative preeclampsia subgroups.

diastolic uterine notch, a reflection of placental insufficiency, was more frequent in the sMIC-positive group of PE ($P = 0.037$, Figure 1(a)). Severe PE diagnostic criteria such as high blood pressure (≥ to 160 mm Hg systolic), thrombocytopenia, or renal failure were observed in 75% of the PE cases evaluated. Among MIC^+ patients, 89% presented criteria of PE severity: 7 patients with high blood pressure (≥ to 160 mm Hg systolic) and one patient with renal failure. In the MIC-negative group, 64% of patients were defined as severe cases of PE on the basis of hypertension or renal failure criteria. Thrombocytopenia was significantly more frequent in the sMIC-positive patients group, compared with the sMIC-negative patients group (3 to 9 patients (33%) versus 1 to 31 patients (3.0%), resp., $P = 0.03$, Figure 1(b)).

We could also show that median proteinuria per day was significantly higher in PE women with sMIC-positive plasma than in PE women without detectable sMIC ($P = 0.04$, Figure 1(c)). Multivariate analysis of parity, gestation, systolic

and diastolic blood pressure, gestational age at sampling, thrombocytopenia, PAS ≥ to 160 mm Hg systolic, PAD ≥ to 110 mm Hg diastolic, term of delivery, and baby weight at birth further confirmed that proteinuria was an independent factor associated with detection of sMIC in plasma (P = 0.03).

4.3. The Presence of sMIC in Plasma Is Associated with Vascular IUGR. To further address whether the presence of plasma sMIC could be associated with the vascular origin of IUGR, we compared sMIC levels in women with vascular and nonvascular IUGR. Among the 23 patients with vascular IUGR, 9 (39%) had detectable plasma sMIC, whereas sMIC could be detected in one out of the 25 (4%) nonvascular IUGR cases. Thus while age, term at sampling, baby birth weight, and term of delivery were not different between the 2 groups, the presence of sMIC in maternal plasma was significantly associated with the vascular etiology of IUGR (P = 0.0038). Furthermore, analysis of the 48 pregnancies at IUGR diagnostic showed that detection of plasmatic sMIC levels in plasma is a specific factor associated with vascular etiology of IUGR, (96% specificity and 39% sensitivity).

4.4. Downmodulation of NKG2D Expression at the Surface of NK Cells by sMIC-Positive Plasma from VPD Patients. We then analyzed whether plasma from sMIC-positive VPD patients induced modification of NKG2D expression at the surface of NK cells within peripheral blood mononuclear cells (PBMCs) isolated from healthy donors (n = 8). PBMCs were incubated for 48 h with 20% of sMIC-positive plasma from 8 VPD patients and analyzed in reference to sMIC-negative plasma from 8 women with normal term matched pregnancies. Flow-cytometry analysis of NKG2D expression in $CD3^{-}CD56^{+}$ NK cells showed that incubation with sMIC-positive plasma from VPD resulted in a significant decrease in NKG2D expression in both % (Figure 2(a)) and mean fluorescence intensity (mfi, Figure 2(b)) (median, 25–75 interquartile ranges: % of NKG2D 72.25, 34.7–78.8; mfi 3.4, 2.7–4.2) when compared to sMIC-negative plasma of NP matched for term (% of NKG2D 87.2%, 67.1–90.6; MFI 5, 3.6–5.3, P = 0.0078). Moreover, the levels of plasma-induced downregulation of NKG2D at the surface of NK cells (Δ in % of NKG2D stained NK cells observed after incubation with MIC-positive plasma relative to MIC-negative plasma) were correlated to VPD sMIC plasma levels (Spearman correlation, r = 0.76, P = 0.04).

4.5. sMIC-Positive Plasma of VPD Patients Impairs NK Cell-Mediated IFN-γ Production. To gain insight into the mechanisms by which sMIC could affect NK-cell dependent vascular placental remodeling, we then investigated whether sMIC-positive VPD plasma could modulate NK cell-mediated INF-γ production. NK-92 cell lines were cultured for 72 hours in medium containing either 20% sMIC-positive plasma from VPD patients or 20% sMIC-negative plasma of term-matched women undergoing normal pregnancies. Impact of plasma was evaluated by ELISA assay of IFN-γ secretion levels in NK cell supernatants and real-time qRT-PCR analysis of INF-γ mRNA transcript levels in NK cells. We showed that INF-γ levels in culture supernatants were significantly reduced (15-fold decrease) after incubation of NK cells with sMIC-positive VPD plasma (median, 25–75 interquartile range: 2.5 ng/mL, 0.42–14.4), when compared to sMIC-negative NP plasma (41 ng/mL, 28.1–57 P = 0.002, Figure 3(a)). Furthermore, when analyzed by quantitative RT-PCR, relative levels of IFN-γ transcripts were also significantly decreased in the presence of sMIC-positive VPD plasma (median values relative to GAPDH, 25–75 interquartile range: 19170, 3140–25295), as compared to sMIC-negative NP plasma (123279, 112000–183011, P = 0.04, Figure 3(b)).

5. Discussion

Identification of specific biomarkers that characterize VPD, whose values may differ from observations occurring outside of the pregnancy context, is a challenging issue to anticipate adverse outcomes of these diseases for the mother and the child. Hypertensive disorders of pregnancy are a major factor associated with 5–10% of pregnancies and represent the second commonest cause of direct maternal death [44]. Gestational hypertension, renal failure, and congenital thrombophilic defects represent major criteria of PE diagnostic and have been associated with the occurrence and severity of PE [45, 46]. While standard diagnostic criteria have evolved, PE remains an extremely heterogeneous disease reflecting interplay of multiple underlying processes that can vary from one woman to another. It is thus important to unravel mechanisms and molecules that may reflect the heterogeneity of the clinical syndromes and enhance our understanding of how they relate to adverse pregnancy outcome in one woman and not in the other.

Deregulated expression profiles of a wide array of vascular and thrombotic markers have been identified to be associated with the onset and severity of vascular disorders that complicate pregnancies [5, 6, 47, 48]. Apart from angiogenic factors *per se*, the relevance of immunological markers has also been highlighted in vascular disorders of pregnancy and notably in the multisystem disorders associated with preeclampsia. The primary defect that leads to preeclampsia indeed involves alteration in the dialogue between invasive placental trophoblast cells and maternal immune cells in the uterine wall. Extravillous trophoblast cells express an unusual combination of polymorphic and nonpolymorphic HLA class I molecules that impact uterine NK-cell (uNK) angiogenic function during pregnancy. Genetic and functional studies suggested that maternal KIR/fetal HLA-C and G interactions regulate the delivery of an optimal blood supply to mother and fetus. The adverse impact of genetic KIR/HLA-C combinations on trophoblast-cell invasion during PE has been reported, as a support for the diversity of maternal immune responses during placentation [3].

Our study provides the first evidence that detection of soluble MIC-A, a stress inducible immunomodulatory molecule, is more frequent in plasma from women with vascular pregnancy complications than in gestational age-matched normal pregnancies or nonvascular IUGR.

Considering the result of this study, we expect that sMIC could be a stress marker reflecting disorders of the maternal

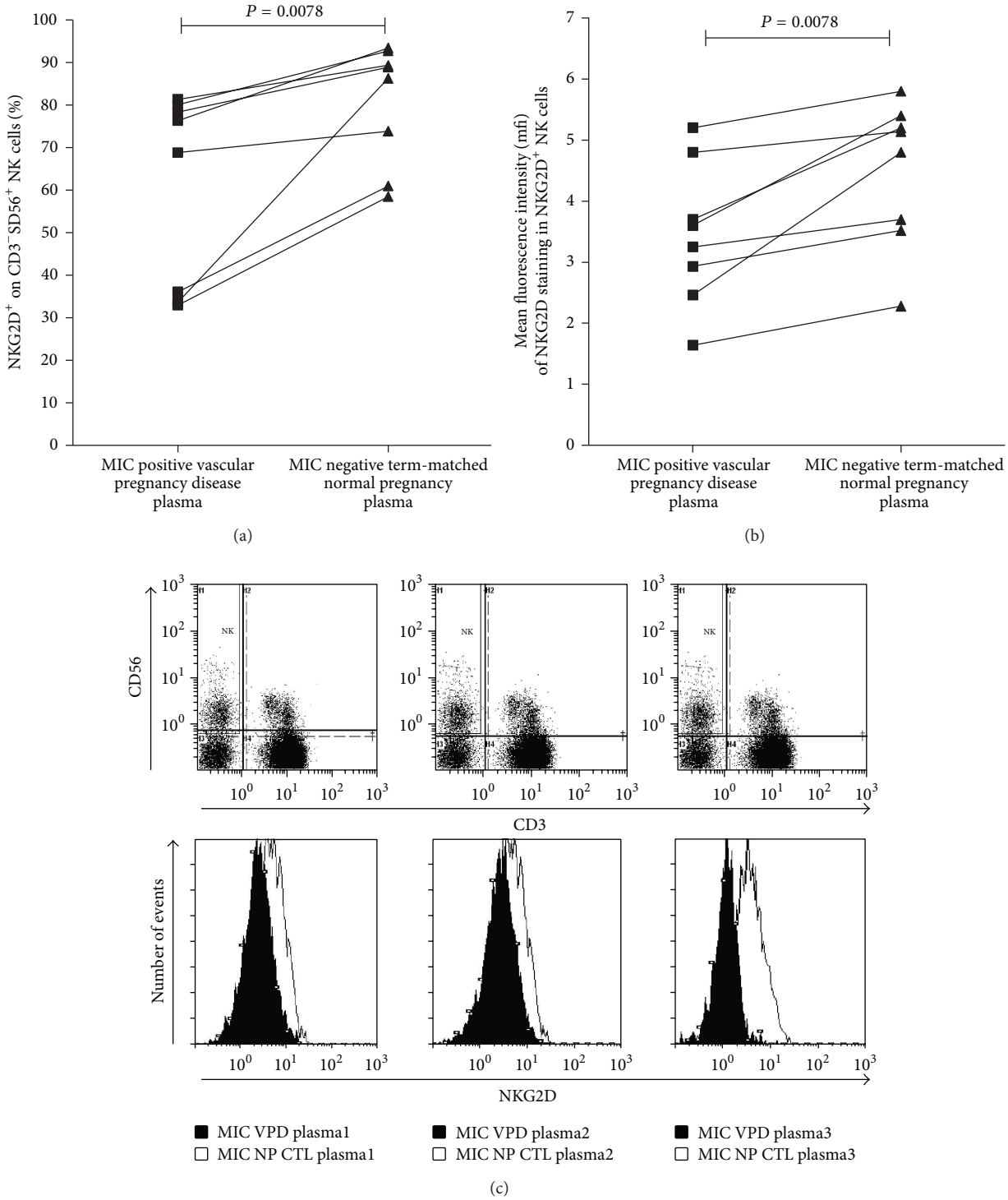

Figure 2: Differential effect of plasma isolated from sMIC-positive VPD patients or term-matched sMIC-negative plasma of women undergoing normal pregnancy (NP) on percentage (Figure 2(a)) and mean fluorescence intensity (mfi, Figure 2(b)) of NK cell surface NKG2D expression within PBMC. For each experiment, PBMC isolated from healthy nonpregnant control donors (NP) was cultured for 48 hours before flow cytometry analysis in media containing either 20% sMIC-positive plasma from women experiencing vascular pregnancy diseases (black) or 20% sMIC-negative plasma from normal pregnancies (NP, gray). Gestational age at plasma sampling of VPD cases was matched to that of NP plasma used as control in 8 independent experiments. Plasma-induced modifications of NKG2D cell surface expression are illustrated as variation in the % of NKG2D positive $CD3^{-}CD56^{+}$ NK cells found within PBMC (a) and mean fluorescence intensity of NKG2D staining in NKG2D positive $CD3^{-}CD56^{+}$ NK cells (b). (c) Flow cytometry plots illustrate detection of NK cells and gating of NKG2D expression within $CD3^{-}CD56^{+}$ NK cells in 3 representative experiments. Overlay of NKG2D downregulation resulting from incubation of NK cells with sMIC-positive VPD plasma (black) versus sMIC-negative plasma from control normal undergoing pregnancies (NP Figure 2(c), right panel). Wilcoxon matched pairs tests were used to compare the two groups.

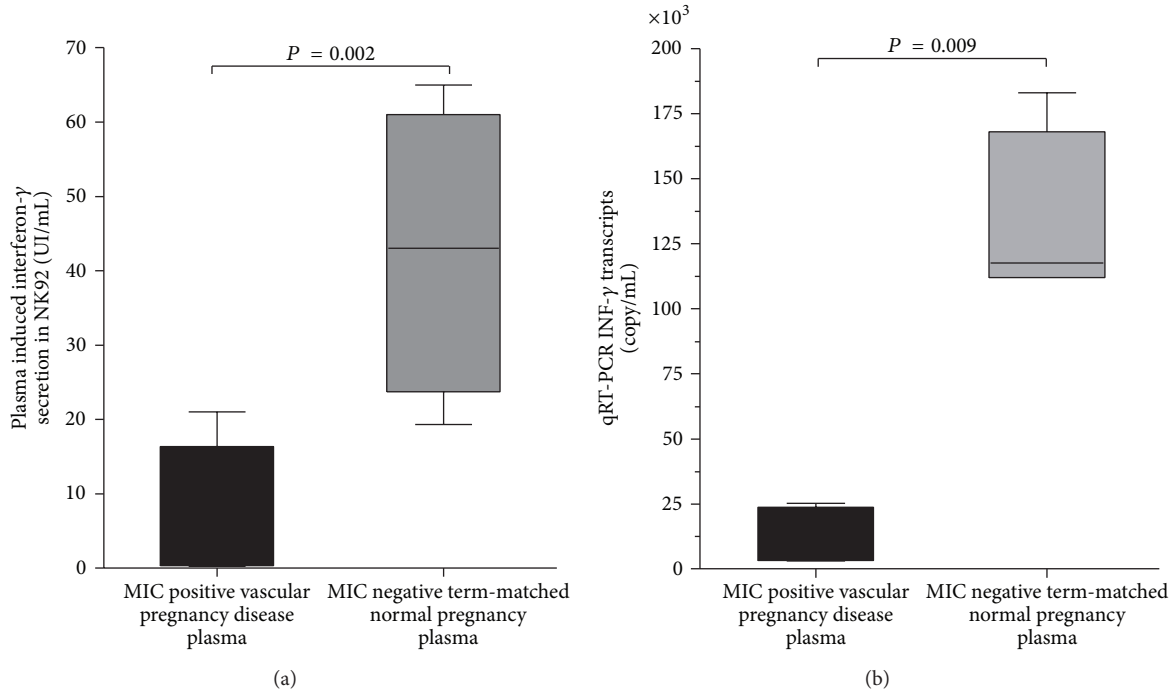

Figure 3: Quantification of INF-γ secretion by the NK-92 NK cell line after 72 hours incubation in media containing 20% plasma from representative sMIC-positive vascular pregnancy diseases (VPD, sMIC$^+$) and gestational age-matched sMIC-negative plasma normal pregnancies (NP, sMIC$^-$). (a) ELISA detection of INF-γ levels in supernatants of NK-92 cells cultured in 20% plasma. (b) Real-time PCR transcriptional analysis of INF-γ mRNA levels in NK-92 cells. mRNA transcript levels are represented as relative copy numbers per 10^6 GAPDH transcripts. Results are expressed as median and 25–75 interquartile ranges ($n = 4$).

immune system that impact NK cell angiogenic function at the fetomaternal interface in some women. As previously described for women that experience fetal implantation failure after IVF, plasmatic levels of maternal sMIC are only detected in about one third of PVG cases, with higher prevalence in women that experience intrauterine fetal death after IUGR or with a diagnostic of vascular intrauterine growth retardation or PE. This suggests that the level of sMIC upregulation in maternal serum may vary in response to stress signals provided by the pathological environment during complicated pregnancies. Although the nature of these signals is presently unknown, various viral, hormonal, and environmental stimuli have been reported to induce NKG2D ligand expression [49, 50] and may influence MIC-driven modulation angiogenic function in VPD. Although the number of PVG cases evaluated in this preliminary study may be too limited to address this point, no direct association was made between sMIC levels and diastolic or systolic blood pressure measurement in the PE subgroup. In PE patients, we suggest that high levels of sMIC could rather be associated with thrombocytopenia and high proteinuria, a former diagnostic criterion of severity of PE that is no longer retained in the American College of Obstetricians and Gynecologists' 2013 guidelines. Of interest, our results suggest that presence of sMIC in maternal plasma was also associated with the vascular nature of pregnancy-related disorders that lead to IUGR. IUGR is a pregnancy complication with many etiologies that associate to the highly variable prognosis of this disease. Placental insufficiency constitutes the major cause of vascular IUGR. Most vascular IUGR share the physiopathology signs of PE [51], and vascular IUGR is sometimes diagnosed in the presence of PE or alteration of uterine Doppler Velocimetry wave flow. By contrast, isolated IUGR may represent the only sonographical sign of severe fetal infection, aneuploidy, or genetic syndrome associated with abnormal neurologic outcomes. However, there is a lack of noninvasive and specific markers that allow differentiating vascular from nonvascular IUGR. Indices of uterine Doppler Velocimetry, known as the best marker in the prediction of placental hypoxic-ischemic lesions in IUGR, show 63% specificity for 97% sensibility [52]. In the absence of obvious IUGR etiology, invasive fetal explorations are required to determine fetal prognosis, with nonnegligible rate of premature delivery and late fetal loss [53]. Our observation that sMIC detection in plasma of women who developed IUGR may discriminate the vascular origin of IUGR with 96% specificity prompts further larger scale prospective studies that may confirm the value of sMIC to characterize or anticipate the vascular etiology of IUGR.

Although the mechanisms by which sMIC may participate to vascular pregnancy diseases remain unclear, we bring evidence that sMIC-containing plasma from patients with vascular pregnancy disorders can impact NKG2D expression and modulate NK-mediated IFN-γ production. Although factors that impair NK-mediated IFN-γ production have been suggested to impact vascular remodeling, their

alteration in association with vascular disorders of pregnancy is still poorly documented. Recent findings indicate that IFN-γ-secreting NK cells play a proangiogenic role and promote VEGF expression and corneal neovascularization [54, 55]. Several reports highlighted the role of dNK-derived INF-γ as a regulator of the size of uterine blood vessels that favors adequate decidualisation in a murine model [55–57]. In humans, various studies bring evidence for a direct role for dNK in modulating extravillous trophoblast cell differentiation and migration from anchoring villi, which is in part dependent from their potential to deliver IFN-γ [58]. Impaired NK cell mediated release of IFN-γ has been recently reported in women with hypertensive disorders that complicate pregnancies [15]. Integration of microenvironment signals delivered to NK cells at the fetomaternal interface is expected to affect this essential process of successful pregnancy. Fine tuning of NK-mediated cell cytokine production is dependent on engagement of cell surface receptors that sense HLA ligand expression on trophoblast cells. The high level of diversity of receptors and ligand that may regulate these interactions may in part condition the efficiency of placentation [59]. Genomic evidence suggests that deleterious combinations of maternal NK cell receptor repertoire and fetal HLA-C and HLA-G antigens can shape a strong inhibitory NK phenotype associated with impaired vascular placental remodeling [60]. Altered expression of the HLA-G NK cell ligand has also been reported to have a significant influence on IFN-γ production by NK cells [21] and development of preeclampsia [61, 62]. Consistent with this view, lowered levels of soluble HLA-G protein in maternal plasma were associated with occurrence of severe PE and IUGR during the third trimester [28, 63]. Similarly, we can speculate that stress-induced upregulation of the highly polymorphic MIC molecule may also differentially impact essential immune functions during placentation. Our results indeed suggest that presence of sMIC in plasma of women with vascular pregnancy diseases can deliver a strong inhibitory signal to NK cells through downmodulation of cognate NKG2D receptor expression at the surface of NK cells and NK-cell interferon-γ secretion. We have recently shown that detection of sMIC levels in the serum of some infertile women is predictive of implantation failure and successful term pregnancy following in vitro fertilization [41]. These data suggest that deregulated expression of this stress-inducible immunomodulatory protein may be associated with immune and vascular dysfunction during pregnancy, notably by impairing NK-mediated production of IFN-γ, an essential cytokine to allowing implantation and placentation.

Results from this exploratory study thus suggest that noninvasive evaluation of sMIC may be a hallmark of underlying mechanisms that complicate pregnancies in some women. It prompts larger prospective studies that may allow determining how a stress-inducible immunomodulatory molecule reflecting immune disorders may relate to other markers defining "angiogenic" and "nonangiogenic" profiles associated with pregnancy outcome.

In summary, we bring the first evidence that detection of high sMIC plasma levels may constitute a noninvasive indicator of underlying immune-mediated disorders of placentation in some women with severe VPD and may notably characterize the vascular etiology of IUGR. We further suggest that, in the pathogenic context of some pregnancies, sMIC release may constitute a factor that impairs immune cell's potential to establish the proper context of vascular remodeling steps, thus favoring the onset of severe vascular disorders that affect pregnancy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Netrins and Their Roles in Placental Angiogenesis

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Netrins, a family of laminin-related proteins, were originally identified as axonal guidance molecules. Subsequently, netrins were found to modulate various biological processes including morphogenesis, tumorogenesis, adhesion, and, recently, angiogenesis. In human placenta, the most vascularized organ, the presence of netrins has also been reported. Recent studies demonstrated the involvement of netrins in the regulation of placental angiogenesis. In this review we focused on the role of netrins in human placental angiogenesis. Among all netrins examined, netrin-4 and netrin-1 have been found to be either pro- or antiangiogenic factors. These opposite effects appear to be related to the endothelial cell phenotype studied and seem also to depend on the receptor type to which netrin binds, that is, the canonical receptor member of the DCC family, the members of the UNC5 family, or the noncanonical receptor members of the integrin family or DSCAM.

1. Vascular and Nervous Systems Similarities

The vascular and nervous systems share similarities at the anatomical and cellular levels: indeed both systems are a web of highly branched and complicated networks. The vessel system uses specialized tip cells which are located at the front of navigating blood vessels and which are morphologically and functionally similar to the axonal growth cone [1]. Moreover, both the vascular and nervous systems appear early during the embryonic development and evolve throughout the entire life. These observations have promoted investigations looking for the presence of guidance molecules outside the nervous cells; this led to the discovery of some axon guidance factors. Among these factors are netrins, which have since been shown to play a key role in the angiogenesis processes [2–4].

2. Netrins (Figure 1)

Netrins belong to a family of laminin-related proteins. All netrins comprise an N-terminal laminin-type domain which is followed by several epidermal growth factor-like (EGF) domains and a positively charged C-terminal domain. Five members of the netrin family have been identified in vertebrates. Two of them (netrin-G1 and netrin-G2) are bound to the cell membrane via glycosylphosphatidylinositol anchors [5]. Three are secreted proteins, including two netrins structurally related to the γ chain of laminin (netrin-1 and netrin-3) [6, 7] and one related to the laminin β chain (β netrin also known as netrin-4) [8, 9]. Netrins were initially described as regulators of axonal guidance during embryogenesis and are so called because netrin means one who guides in Sanskrit [6].

Besides the central nervous system, netrin expression was also reported in pancreas, lung, breast, and recently in the placenta [10, 11]. Netrins have been shown to play various important roles in central biological processes including cell guidance, adhesion, differentiation, and survival and recently in angiogenesis. Netrin-1 and netrin-4 are the most extensively studied members of the netrin family [12].

Netrin-1 is closely related to the laminin chain [13, 14]. The human netrin-1 gene is localized at 17p13-p12 [15] and encodes 604 amino acid protein of 70–84 kDa. It is a secreted protein that is involved in axonal outgrowth and migration orientation during the development of the central nervous

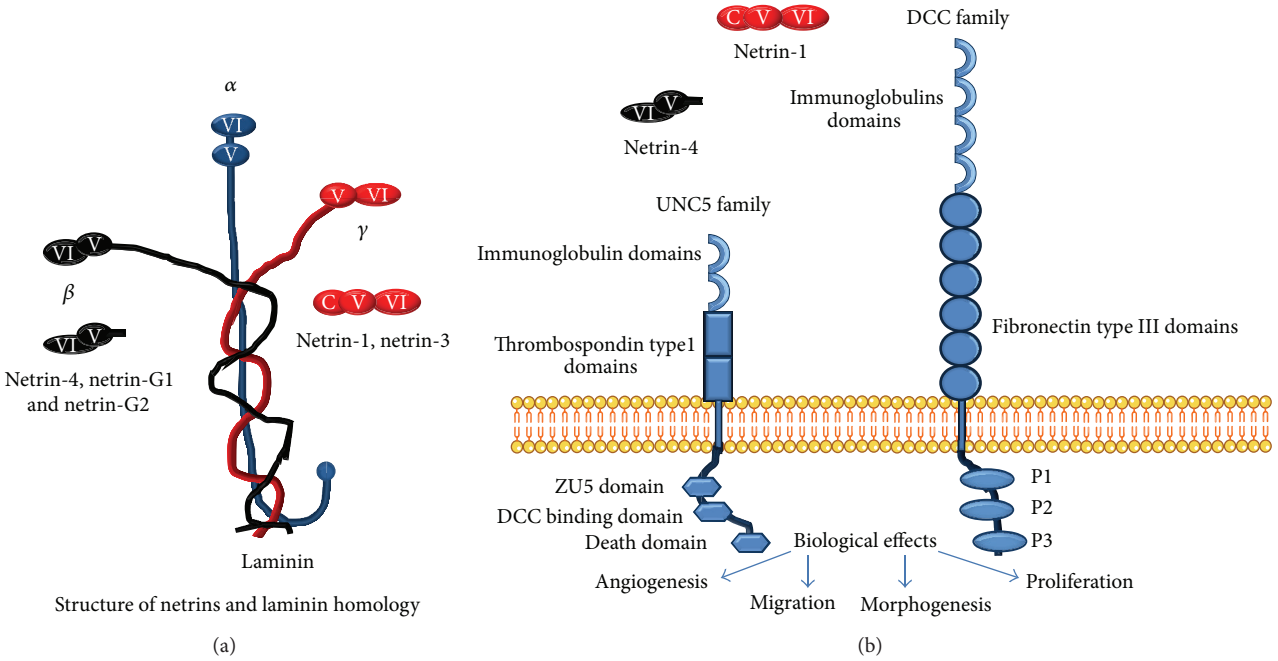

Figure 1: Netrins and their receptors. (a) Netrins show homology with laminin. Netrin-1 and netrin-3 are related to the γ chain of laminin, and β netrin, also known as netrin-4, is related to the laminin β chain. (b) Netrins bind to receptor members of the DCC family and to members of the UNC5 family. Their most important roles are given [12].

system [14]. Netrin-1 was also reported to control morphogenesis of endothelial cells and vascular smooth-muscle cells. Bouvrée et al. demonstrated that netrin-1 inhibits sprouting angiogenesis in cloned chick through UNC5B-binding [16]. Netrins are also involved in cytoskeleton reorganization and in epithelial cell adhesion and migration in lung, mammary gland, and pancreas [4, 17–20], as well as in tumor growth [21] and in the regulation of inflammation [22].

The human netrin-4 gene is localized in 12q22-q23 [8] and encodes a 629-amino acid long protein of approximately 70–84 kDa. Netrin-4 is a secreted protein involved in neurite growth [8] and migration orientation during the development of the central nervous system [12, 23]. Besides the central nervous system, netrin-4 has been shown to regulate epithelial branching and morphogenesis in the lung [24], pancreas [25], salivary gland branching [26], lymphangiogenesis, angiogenesis, and tumor growth [27]. It was recently shown that netrin-4 promotes mural cell adhesion and recruitment to endothelial cells [28].

3. Netrin's Receptors

As far as we know, the biological effects of netrin-1 and netrin-4 are mediated through two different classes of transmembrane receptors. The DCC (deleted in colorectal cancer) family includes the DCC and neogenin receptors. These receptors mediate the attraction response of axons to netrins. The second family is the UNC5 (uncoordinated-5 homolog) family receptors which are responsible for mediating axonal repulsion (as a response to UNC5B homodimer) or axonal attraction (as a response to UNC5B and DCC heterodimers) [13, 29, 30]. In addition, in lymphatic endothelial cells, netrin-4 was shown to bind with high affinity to two different noncanonical receptors, namely, $\alpha 6\beta 1$ integrin and laminin-1, both inducing local adhesion [23]. Whereas netrin-4 stimulates endothelial cell adhesion and migration, no effects were observed when UNC5B or neogenin was inactivated by siRNA [23]. These findings indicate that the initial step of netrin signaling pathway is more complex than the model of a single ligand signaling through a single receptor binding. Larivée et al. showed that UNC5B activation inhibits sprouting angiogenesis, and this data indicate that UNC5B is a potential antiangiogenic target [31].

Finally, disruption of one netrin-1 allele or of its UNC5B receptors was reported to be lethal during early embryogenesis in mice, suggesting a crucial role for netrin-1 and its UNC5B receptors in this process [32, 33].

4. Netrins and Angiogenesis

4.1. Netrin-1. In 2004, Anne Eichmann and colleagues reported the role of netrin-1 and its receptor UNC5B in controlling morphogenesis of the vascular system [4]. As a matter of fact, upon addition of netrin-1 to aortic ring in *in vitro* cultures, these authors observed inhibition of filopodia formation and sprouting, suggesting that netrin-1 is an antiangiogenic factor [1, 16, 31]. However, pro- and antiangiogenic activities were reported later on to be cell type dependent, presumably because of the existence of different patterns of angiogenesis-controlling genes expression. In this regard, other studies brought evidences for proangiogenic effects of netrin-1 in human umbilical vein endothelial cells

(HUVEC) and human umbilical and arterial endothelial cells (HUAVEC) by demonstrating an increase in the proliferation, migration, tubal formation, and capillary branching after exposure to netrin-1 [18]. In another report, netrin-1 was found to promote angiogenesis through the control of the endothelial cells survival due at least in part to the apoptosis blockade induced by its unbound UNC5B receptor [34].

4.2. Netrin-4. Another netrin family member, netrin-4, was first investigated by Plouet et al. in HUVEC and HUAVEC cells. These authors showed that netrin-4 gene is specifically overexpressed in VEGF-stimulated endothelial cells *in vitro* as well as *in vivo* [3]. Knockdown of netrin-4 expression in these endothelial cells increased their ability to form tubular structures on Matrigel [3]. In contrast to netrin-1, netrin-4 binds only to neogenin but not to UNC5B or UNC5C receptors. Neutralization of netrin-4 binding to neogenin using blocking antibodies abolished the chemotactic effect of netrin-4. Furthermore, the silencing of either neogenin or UNC5B abolished netrin-4 inhibitory effect on endothelial cell migration, suggesting that both receptors mediate this function *in vitro*. Finally, netrin-4 significantly reduced tube formation structure on Matrigel and laser-induced choroidal [3] These observations led the authors to conclude that netrin-4 acts as an antiangiogenic factor through binding to neogenin and recruitment of UNC5B. Other studies have provided further support to this conclusion. As a matter of fact, Nacht et al. demonstrated that netrin-4 markedly inhibits endothelial cells migration and tube formation [35]. Moreover netrin-4 was found to have only negligible effects on endothelial cell proliferation [35]. In contrast, Mehlen's group showed that netrin-4 significantly protected HUVECs and HUAECs from serum deprived-induced apoptosis, as measured by the caspase-3 activity assay [2]. Endothelial cell migration was also studied and revealed that netrin-4 stimulated HUVECs migration. Furthermore, netrin-4 significantly induced angiogenesis in a dose-dependent manner with the optimal effect being observed at 150 ng/mL of netrin-4. These results demonstrate that netrin-4 can promote endothelial cell survival, proliferation, migration, and angiogenesis using *in vitro* systems [2].

From this short literature review, no clear consensus arises concerning the precise roles played by netrin-1 and netrin-4 in placental angiogenesis. Some studies described these signals as promoters of angiogenesis, whereas others stated opposite conclusions. These discrepancies in the available data leave open the question on the potential role of netrins in the control of angiogenesis [4, 36]. A possible explanation for these discrepancies could be the heterogeneity of the endothelial cell population studied [37]. Most of the investigations on the role of netrins in angiogenesis were performed using HUVEC; these cells have been used as a model for endothelial cells in many studies that considered placental angiogenesis. However, nowadays, growing literature shows that the placental macrovascular endothelial cells differ in phenotype, gene expression, and physiology from the microvascular endothelial cells, such as those present within the placental villi (HPEC) [37]; another possible explanation for the above-mentioned discrepancies is the receptor type to which netrins bind in the placenta, which is still not clear. As all receptors seem to be present in the placenta, further analyses are needed to determine the netrin receptors that are involved in the angiogenic processes. A last explanation could be a variation in specific mRNA levels between endothelial cells of different lineage; in fact, in our laboratory we were astonished to note a difference in mRNA expression of netrin-1, netrin-4, UNC5B, and neogenin between HPEC and HUVEC cells (Figure 2). Other investigators reported variation in mRNA level between HUVEC harvested in their laboratory and HUVEC purchased from American Type Collection [38]; in fact ATCC proposes both HUVEC (CRL-1730) and primary umbilical vein endothelial cells, which have different characteristics.

5. Therapeutic Potential of Netrins

A study of Delloye-Bourgeois et al. in 2009 has shown that netrin-1 inactivation induced vessel loss and inhibited primary tumor growth and metastases in animal models [39, 40]. Recently, the same team found that combining conventional chemotherapies with netrin-1 interference could be a promising therapeutic approach [41]. On the other hand, netrin-4 overexpression decreased tumor recurrence and metastases after surgical resection in mouse models [40, 42, 43]. All these data suggest that inactivation of netrin-1 and overexpression of netrin-4 can be useful in tumor therapy. Other studies indicated that netrin-1 inhibits migration of monocytes, neutrophils, and lymphocytes via its receptors UNC5B. Another study by Zhang and Cai in 2010 demonstrates that netrin-1 potently protects the heart from I/R injury by stimulating NO production from cardiac ECs and myocytes [44]. Concerning the relationship between inflammation and netrin-1 van Gils et al. in 2010 established that netrin-1 inhibited macrophage migration via UNC5B, in case that the presence of an atherosclerotic plaque and that deletion of netrin-1 in myeloid cells severely reduced atherosclerosis lesion size [45, 46].

Contrary to these studies, others reported that netrin-1 reduced ischemia-reperfusion injury by decreasing apoptosis in endothelial cells and that netrin-1 enhanced focal neovascularization, reduced infarct size, and improved long-term functional recovery after transient focal cerebral ischemia [47]. Hence, netrin-1 can serve as an innovative agent for the treatment of strokes. Durrani et al. have shown that, by combining an increase of angiogenesis and a decrease of cardiomyocytes apoptosis, netrin-1 effectively reduced ischemia-reperfusion injury to preserve global heart function [48].

All these studies demonstrated dual roles for netrin-1. The question remains whether netrin-1 therapies using netrin-1 treatment or inactivation of netrin-1 give clinical benefit compared to acute side effects such as loss or unwanted angiogenesis or apoptosis.

6. Netrins in the Placenta (Figure 3)

We recently investigated and characterized the expression of netrin-1 and its receptors DCC and UNC5B in the human

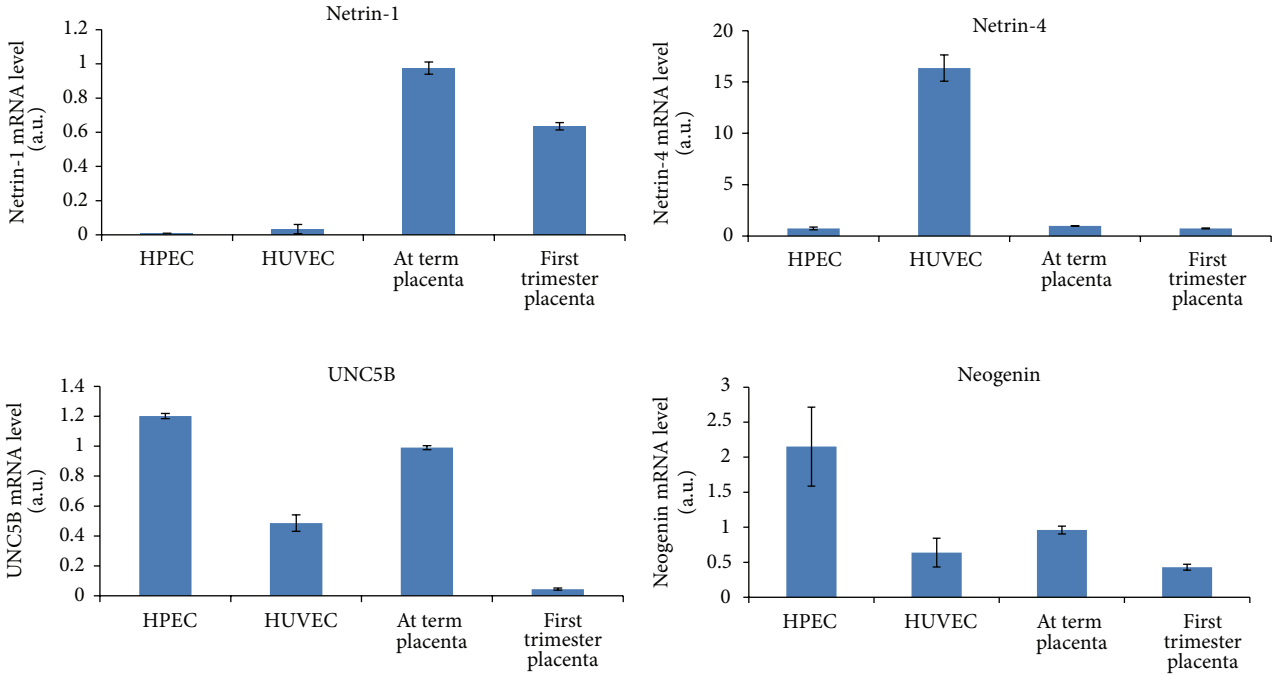

Figure 2: Netrin-1, netrin-4, UNC5B, and neogenin mRNA expression in first trimester and at term placenta and in endothelial cells HPEC and HUVEC normalized to term placenta (data not published).

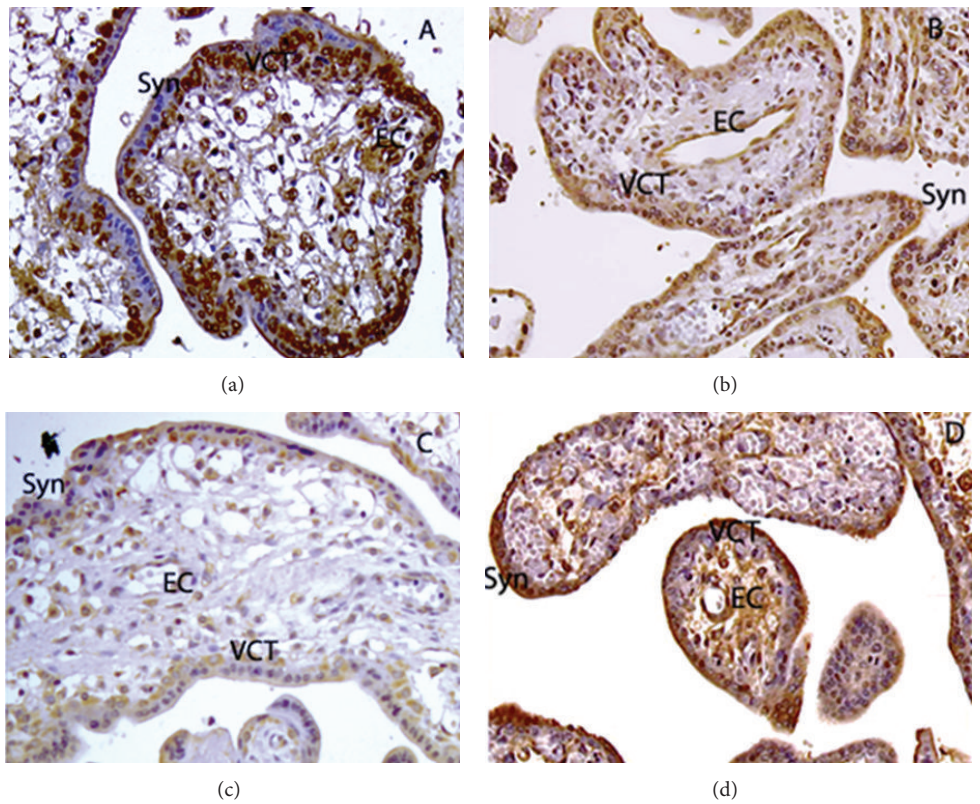

Figure 3: Immunohistochemical localization of netrin-1 (a), netrin-4 (b), UNC5B (c), and neogenin (d) in human first trimester placenta. EC: endothelial cells; Syn: syncytiotrophoblast; VCT: villous cytotrophoblasts.

placenta [10]. Coexpression of both netrin-1 and UNC5B in villous cytotrophoblasts and endothelial cells suggested an autocrine regulatory mechanism in these cells [10]. We have also demonstrated that netrin-1 plays a key role in cytotrophoblast proliferation and found that gene expression of UNC5B is upregulated by hypoxia [49] Ramkhelawon et al. also showed an upregulation of UNC5B by hypoxia in macrophage and also an upregulation of netrin-1 by hypoxia via HIF [50].

In a recent investigation, we demonstrated the expression and the cellular localization of netrin-4 and neogenin in human first trimester and term placenta [11]. Netrin-4 was found in the syncytiotrophoblast, together with neogenin. On the other hand, villous cytotrophoblast cells, which we had previously described as expressing the UNC5B receptor [10], also express netrin-4 [11]. Moreover, we observed that villous cytotrophoblast cells did not express neogenin. Other localizations of netrin-4 were observed in proximal extravillous and distal invasive cytotrophoblast cells. However, because neogenin staining was absent from these cells, it became clear that netrin-4 effects, if any, are mediated through receptors other than neogenin. Neogenin was however expressed by villous mesenchymal cells. We also showed a strong netrin-4 and neogenin expression in placental endothelial cells suggesting that netrin-4 might have both paracrine and autocrine signals in these cells [11].

7. Placental Angiogenesis

During placental development, angiogenesis follows vasculogenesis and leads to the remodeling of the vascular plexus into a branched vascular tree to ensure increased nutritional and gas exchanges and efficient elimination of fetal waste. This placental angiogenesis is dependent on various growth factors including the vascular endothelial growth factor (VEGF), the placental growth factor (PlGF), and the basic fibroblastic growth factor (bFGF) [51]. More recently, the axonal guidance molecules, netrins, have also been suggested to play a key role in the regulation of angiogenic processes. Importantly, the most threatening placental pathology, the preeclampsia, has been reported to be associated with alterations in the expression of these factors [52]. The present review focuses on the potential roles of netrins and their receptors in placental angiogenesis.

8. Netrins and Placental Angiogenesis

It was also observed that netrin-1 accelerated neovascularisation in the placenta of gravid rats [53]. More importantly, suppression of netrin-1 expression in the placenta resulted in reduced vascular sprouting *in vivo*. These findings suggest that netrin-1 is essential for the proper functioning of HUVECs and for angiogenesis in rat placenta and appears therefore to be necessary for placental and foetus development [53, 54]. Exposure of human placental microvascular endothelial cells (HPECs) to netrin-1 also resulted in enhanced cell viability, migration, and tube formation [54]. Taken together, these observations provide strong support for a key role of netrin-1 as a promoter of blood vessels formation in human placenta. This is an additional argument for netrin-1 to be a potential target for new therapeutic strategies in placental vasculature-related diseases.

Further lines of evidence for a proangiogenic effect of netrin-1 in HUVECs were reported by Xie et al. [53]. Involvement of netrin-1 in placental pathologies was first evoked by Yang et al. who reported decreased netrin-1 mRNA and protein expressions together with a reduced vascular density in term placenta from pregnant women with preeclampsia [52]. Moreover, Qian-Hua et al. demonstrated that netrin-1 expression was significantly reduced in placenta from women bearing foetuses with growth restriction when compared to pregnant control women [55]. Furthermore, as netrin-1 was shown to enhance viability of HPECs and to inhibit their apoptosis, it can be proposed that this protein controls vascular growth in the placenta and that failure in its expression might be associated with the development of placental pathologies such as fetal growth restriction [55].

9. Conclusions

In summary, netrins are present in the placenta and are important for placental development (proliferation of cytotrophoblast) and also for placental vascular development, but what about their role in maternal vascular adaptation to pregnancy?

Here, we highlighted the roles of netrins as angiogenic factors in the placenta. However, the molecular mechanisms underlying netrin effects remain largely unknown in human placenta. Therefore, additional research may provide new insights into the role of netrins in normal human placenta and associated diseases, such as preeclampsia and growth restriction, which are both characterized by failures in angiogenesis processes.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Angiogenin Expression during Early Human Placental Development; Association with Blood Vessel Formation

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The placenta is a transient organ essential for fetal development. During human placental development, chorionic villi grow in coordination with a large capillary network resulting from both vasculogenesis and angiogenesis. Angiogenin is one of the most potent inducers of neovascularisation in experimental models *in vivo*. We and others have previously mapped angiogenin expression in the human term placenta. Here, we explored angiogenin involvement in early human placental development. We studied, angiogenin expression by *in situ* hybridisation and/or by RT-PCR in tissues and primary cultured trophoblastic cells and angiogenin cellular distribution by coimmunolabelling with cell markers: CD31 (PECAM-1), vascular endothelial cadherin (VE-cadherin), vascular endothelial growth factor receptor-2 (VEGF-R2), Tie-2, von Willebrand factor, CD34, erythropoeitin receptor (Epo-R), alpha-smooth muscle actin, CD45, cytokeratin 7, and Ki-67. Extravillous and villous cytotrophoblasts, isolated and differentiated *in vitro*, expressed and secreted angiogenin. Angiogenin was detected in villous trophoblastic layers, and structured and nascent fetal vessels. In decidua, it was expressed by glandular epithelial cells, vascular cells and macrophages. The observed pattern of angiogenin expression is compatible with a role in blood vessel formation and in cross-talk between trophoblasts and endothelial cells. In view of angiogenin properties, we suggest that angiogenin may participate in placental vasculogenesis and organogenesis.

1. Introduction

The placenta is a transient organ essential for fetal development. It acts as an interface between the fetal and maternal blood circulation, ensuring (1) oxygen and nutrient transfer and waste removal, (2) immune protection and maternal tolerance of the semiallogeneic fetus, and (3) endocrine functions ([1, 2], for reviews). Chorionic villi are the essential structural and functional components of the human placenta (schematic representation in Figure 1). Their mesenchymal core is covered by a two-layered trophoblast epithelium that rests on a basement membrane in contact with the stromal core. This trophoblast layer is composed of villous cytotrophoblasts that proliferate and differentiate by fusion, forming a multinucleated syncytiotrophoblast that covers the entire surface of the villus, in direct contact with a maternal blood exudate during the first trimester of pregnancy and with maternal whole blood thereafter. The syncytiotrophoblast plays a major role in fetomaternal exchanges throughout pregnancy and in synthesis of steroid and peptide hormones required for fetal growth and development. A subset of chorionic villi anchors the placenta to the uterine wall.

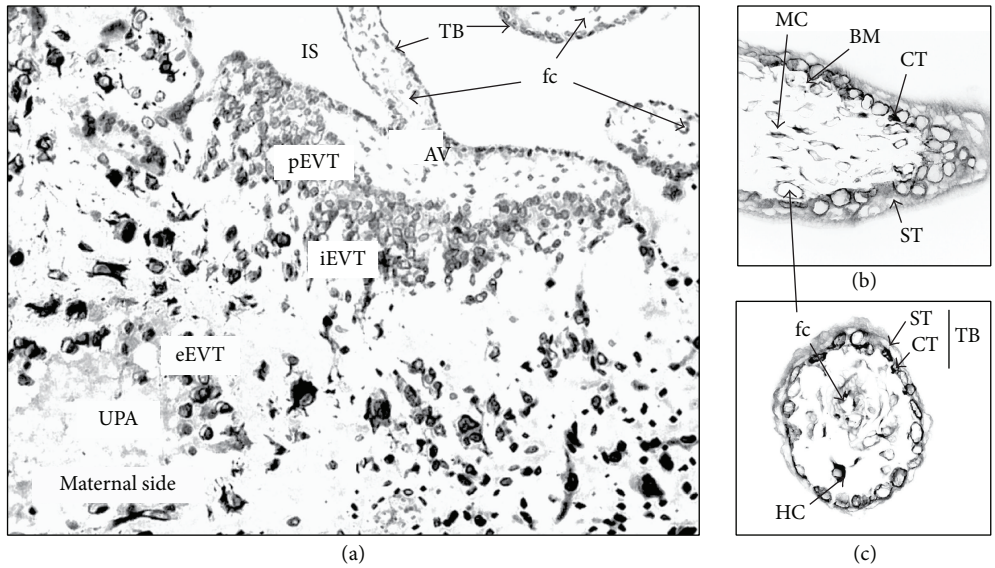

Figure 1: Schematic representation of an anchoring villus from a midgestational placental section, attached to the basal plate by a cell column (a). Schematic representation of a first-trimester mesenchymal villus: sagittal section (b) and cross-section (c). AV: anchoring villus; BM: basement membrane; CT: cytotrophoblast; eEVT, iEVT, and pEVT: endovascular-, interstitial-, and proliferating- extravillous trophoblast, respectively; fc: fetal capillary, HC: Hofbauer cell (macrophage); IS: intervillous space; MC: mesenchymal cell; ST, syncytiotrophoblast; TB: trophoblast layer; and UPA: uteroplacental artery.

The base of these villi contains proliferating extravillous cytotrophoblasts aggregated in columns (Figure 1(a)). During the first and second trimesters, highly invasive extravillous cytotrophoblasts stop proliferating and invade the uterine interstitium, while maternal spiral arteries are remodelled into uteroplacental arteries. The maternal arterial endothelium, unlike the venous endothelium, is replaced by extravillous cytotrophoblasts which adopt an endothelial phenotype [3, 4]. This endo- and perivascular trophoblast invasion and arterial remodelling ensures optimal exchanges between the maternal and fetal circulation.

Angiogenin [5] is one of the most potent inducers of neovascularization in experimental models *in vivo* ([6, 7], for reviews). Its expression is developmentally regulated in rats and humans [8, 9]. We and others have previously shown that the secreted 14 kDa protein is expressed by placental cells in the human term placenta, including vascular, trophoblastic, and amnionic cells [10, 11]. Here, we further probed the role of angiogenin in placental development. Indeed, early placental villi start to develop as mesenchymal villi at week 5 of gestation, with fetal capillary segments being formed by vasculogenesis. These villi develop into immature intermediate villi from week 7 to 8. The capillary segments then fuse and elongate to form a simple capillary network. Starting at week 9, the preexisting capillary network expands by vasculogenesis and branching angiogenesis [12–16]. Decidual sections provide access to the maternal environment, site of intense morphological, physiological and immunological reorganizations. In this work, we examined the distribution and cellular sources of angiogenin in early human placental tissues and maternal decidua. Angiogenin transcripts were detected by *in situ* hybridisation in tissues and by RT-PCR in both tissues and primary cultures of villous trophoblasts. The protein was localized by immunofluorescence from 7.5- to 9-week placental cryosections, and its cellular distribution was established by dual immunolabeling with markers for trophoblastic, epithelial, mesenchymal, and endothelial cells; vascular smooth muscle cells; endothelial, hematopoietic, and erythroid precursors; leukocytes and mature monocytes; and proliferating cells. Angiogenin expression was also studied in primary cultures of first-trimester extravillous and villous trophoblasts. We interpreted our findings in view of recent knowledge of the biological activities of angiogenin.

2. Materials and Methods

2.1. Reagents. Aprotinin, DNase I, ovalbumin, and Triton X-100 were from Sigma Chemical Co. (St. Louis, MO). Tween 20 was from Merck (Darmstadt, Germany). Percoll was from Amersham Pharmacia (Uppsala, Sweden). Culture media, Hanks buffered saline solution (HBSS), and Hepes were from Gibco Laboratories (Grand Island, NY). Trypsin was from Difco Laboratories (Detroit, MI), and penicillin and streptomycin were from Invitrogen (Illkirch, France). Fetal bovine serum (FBS) was from Biological Industries (Kibbutz Beit Haemek, Israel) or PAA Laboratories GmbH (Les Mureaux, France). Sera were heat-inactivated before use. Paraformaldehyde (PFA) was from Electron Microscopy Sciences (Washington, PA). Antibodies used in the study are listed in Table 1. Normal serum from donkey or goat, human IgG, and IgG- and protease-free bovine serum albumin (BSA) were from Jackson ImmunoResearch (West Grove, PA). All chemicals were of analytical grade.

Table 1: Antibodies used in this study.

Antibody specificity	Species, isotype	Concentration used	Cell specificity in placenta	Source
Angiogenin	Rabbit IgG	6 μg/mL	Aim of the study	[10]
	Mouse IgM Clone MANG-1	6 μg/mL		Bachem (San Carlos, CA)
Cytokeratin 7	Mouse IgG1 Clone OV-TL 12/30	2 μg/mL	Trophoblastic cells	Dako (Glostrup, Denmark)
Pan cytokeratin (5, 6, 8, 17, and probably 19)	Mouse IgG1 Clone MNF116	0.9 μg/mL	Epithelial glandular cells Trophoblastic cells	Dako
Vimentin	Mouse IgG1 Clone V9	5 μg/mL	Endothelial cells, mesenchymal cells, and vascular smooth muscle cells Monocytes Macrophages	Immunotech (Marseille, France)
Alpha smooth muscle actin	Mouse IgG2a Clone 1A4	Ascites fluid 1/300	Vascular smooth muscle cells	Sigma ImmunoChemicals (St. Louis, MO)
Ki-67	Mouse IgG1 Clone MIB-1	4 μg/mL	Proliferating cells	Immunotech
CD31, PECAM-1 (Platelet/endothelial cell adhesion molecule-1)	Mouse IgG1 Clone JC/70A	4 μg/mL	Endothelial cells	Dako
CD34	Mouse IgG1 Clone Qbend 10	4 μg/mL	Endothelial cells Hematopoietic precursors	Immunotech
Tie-2 (Tyrosine kinase with immunoglobulin and epidermal growth factor homology domains-2)	Rabbit IgG	6 μg/mL	Endothelial cells	Santa Cruz Biotechnology (Santa Cruz, CA)
VE-cadherin (Vascular endothelial cadherin), CD144, cadherin 5	Mouse IgG1 Clone TEA1/31	4 μg/mL	Endothelial cells	Immunotech
VEGF-R2 (Flk-1) (Vascular endothelial growth factor receptor-2)	Mouse IgG1 Clone A3	4 μg/mL	Endothelial cells	Santa Cruz Biotechnology
vWF (von Willebrand factor)	Mouse IgG1	4 μg/mL	Endothelial cells	Roche Diagnostics (Meylan, France)
Epo-R (Erythropoietin receptor)	Rabbit IgG	6 μg/mL	Endothelial cells Erythroid precursors	Santa Cruz Biotechnology
CD14	Mouse IgG2b Clone MY4	6 μg/mL	Mature monocytes Macrophages	Immunotech
CD45 (Leukocyte common antigen)	Mouse IgG1 Clone HI30	6 μg/mL	Leukocytes	BD Pharmingen (Le Pont-de-Claix, France)
Isotype control	Mouse IgG1		Nonspecific	Coulter Immunology (Hialeah, FL)
	Mouse IgG2a			Immunotech
	Mouse IgG2b			Coulter Immunology
	Mouse IgM			Coulter Immunology
	Rabbit IgG			Jackson ImmunoResearch Laboratories, Inc (West Grove, PA)

Table 1: Continued.

Antibody specificity	Species, isotype	Concentration used	Cell specificity in placenta	Source
FITC-conjugated donkey anti-mouse IgG	Affinity Pure donkey IgG	3.5 μg/mL		Jackson ImmunoResearch
Texas Red-conjugated goat anti-mouse IgG (subclasses 1 + 2a + 2b + 3)	Affinity Pure goat IgG, Fc fragment specific	3.5 μg/mL		Jackson ImmunoResearch
FITC-conjugated goat anti-mouse IgM	Affinity Pure goat IgG, μ chain specific	3.75 μg/mL		Jackson ImmunoResearch
FITC-conjugated goat anti-rabbit IgG	Affinity Pure goat IgG, Fc fragment specific	3.75 μg/mL		Jackson ImmunoResearch
Rhodamine (TRITC-) labelled goat anti-rabbit IgG	Affinity Pure goat IgG, absorbed against human IgG	4.4 μg/mL		Sigma BioSciences
Texas Red-conjugated donkey anti-rabbit IgG	Affinity Pure donkey IgG	3.5 μg/mL		Jackson ImmunoResearch

2.2. Tissue Collection. Human placental tissues from first-trimester pregnancies were collected after legal voluntary termination (7–14 weeks of gestation) at Broussais and Saint-Vincent de Paul hospitals (Paris, France). Second-trimester placentas were collected after medical termination for major fetal abnormalities [17]. Fetal karyotyping was normal. Term placentas (>37 weeks) were obtained after elective Caesarean section from healthy mothers with uncomplicated pregnancies delivered at Robert Debré, Saint-Vincent de Paul, and Tenon hospitals (AP-HP, Paris, France). The study was approved by our local ethics committee (CCPRB Paris Cochin no. 18-05) and the patients gave their informed consent. For immunofluorescence experiments and *in situ* hybridisation, pieces of placenta and decidua were embedded in Tissue-Tek O.C.T Compound (Sakura Finetek Europe, The Netherlands), frozen in isopentane, cooled with liquid nitrogen, and stored at −80°C until cryostat sectioning.

2.3. Villous and Extravillous Trophoblast Isolation and Primary Culture. Villous placental tissues were dissected free of membranes and vessels and then rinsed and minced in Ca^{++}- and Mg^{++}-free HBSS supplemented with 100 IU/mL penicillin and 100 μg/mL streptomycin. Extravillous cytotrophoblasts were isolated by trypsin-DNase digestion as previously described [18]. Cells were plated on 7 μg/cm^2 Matrigel-coated dishes in DMEM/10% FBS/2 mM L-glutamine/25 mM Hepes/100 IU/mL penicillin/100 μg/mL streptomycin (5-6 × 10^5 cells/2 mL/8 cm^2 culture dish) and maintained at 37°C in humidified 5% CO_2/95% air ($n = 8$). Villous cytotrophoblasts were isolated with the method of Kliman et al. [19], essentially as previously described [20]. Cells were seeded in HAM F12/DMEM (vol./vol.) containing 10% FBS, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin (3.3 million cells/3 mL/20 cm^2 culture dish) and maintained at 37°C in humidified 5% CO_2/95% air ($n = 11$). The medium was changed daily for three days. The collected medium was centrifuged, frozen in liquid nitrogen, and stored at −20°C until use. In parallel experiments, cells were collected for RNA extraction. RT-PCR and ELISA studies ($n = 3$) were performed in triplicate.

2.4. Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from frozen placental tissues with TRIzol reagent (Invitrogen SARL, Cergy Pontoise, France). Total RNA was isolated from cultured cells as described in the Atlas Pure Total RNA Labeling System user manual (Clontech Laboratories, Palo Alto, CA). The total RNA concentration was determined by absorbance at 260 nm and its integrity was checked by 1% agarose gel electrophoresis in the presence of ethidium bromide with UV visualisation.

First-strand complementary DNA was synthesised from 2 μg of total RNA using 0.2 μg oligo(dT)$_{15}$ as primer (Promega Co., France) and SuperScript II RNase H Reverse Transcriptase (Invitrogen SARL) in a 20 μL reaction volume as per the manufacturer's instructions. The synthesised cDNA was then subjected to PCR amplification as described in detail elsewhere [10]. Gene-specific primers (Table 2) were from Invitrogen SARL. Negative controls for RT-PCR lacked total RNA. Poly A+ RNA from human liver (Clontech laboratories, Inc.) was used as a positive control for angiogenin expression. The absence of contaminating DNA was confirmed by the obtention of a single RT-PCR product for β-actin, with the primers being located in different exons. In order to confirm that the PCR products are angiogenin amplicon, PCR products from angiogenin cDNA amplification were subjected to 2% agarose gel electrophoresis, extracted with JETsorb (GENOMED GmbH, Germany), cloned in the pCRII-TOPO plasmid vector (Invitrogen SARL), and sequenced using the ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer Applied Biosystems).

2.5. In Situ Hybridisation on Placental and Decidual Cryosections. Angiogenin cDNA (BBG28, R&D systems, Abingdon, UK) was labelled by incorporation of digoxigenin-labelled dUTP by random priming with the DIG High Prime

Table 2: Primer sequences.

Gene	GenBank accession number	Primer sequences	Amplicon length	Reference
Human angiogenin	M11567	5′-CAT CAT GAG GAG ACG GGG-3′ sense, bp 1964–1981 5′-TCC AAG TGG ACA GGT AAG CC-3′ antisense, bp 2227 > 2208	264 bp	[21]
β-actin	M10277	5′-ACA ATG AGC TGC GTG TGG CT-3′ sense, bp 1496–1515 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′ antisense, bp 2307 > 2284	371 bp	After [22]

Labelling and Detection Kit I according to the manufacturer's recommendations (Roche Diagnostics, Meylan, France).

Seven-micrometer-thick frozen sections of placenta or decidua were mounted on Polysine slides (Menzel-Gläser, Germany) and then dried and fixed with 4% PFA for 40 min at 4°C. Remaining free reactive groups were blocked with 0.2% glycine (w/v). After four washes in PBS, the sections were dehydrated with graded ethanol solutions (30%, 2 × 50%, 70%, and 2 × 100%), rapidly air-dried, and stored at −80°C. After rehydration with graded ethanol solutions (100%, 70%, and 2 × 50%), the sections were digested with 1 μg/mL Proteinase K (Sigma) in 20 mM Tris-HCl, 1 mM EDTA, and pH 7.6 for 15 min at 37°C. Postfixation was performed with 4% PFA for 20 min at 4°C, followed by blockade of remaining reactive groups with 0.2% glycine (w/v). The sections were prehybridised at 50°C for 4 hours in hybridisation buffer containing 5x standard saline citrate (SSC, Gibco), 0.1% N-lauroylsarcosine (Sigma), 20% blocking solution (Boehringer kit), 50% deionised formamide (Fluka Chemie, Buchs, Switzerland), and 0.02% SDS (Bioprobe, Montreuil, France). Hybridisation was performed overnight at 50°C in hybridisation buffer containing 0.25 ng/mL labelled angiogenin cDNA probe (BBG28). Sections were washed twice in 2x SSC for 5 min and twice in 1x SSC for 15 min, at 50°C. After saturating the sections with blocking buffer (Boehringer kit) for 45 min at room temperature, alkaline phosphatase-conjugated antidigoxigenin Fab (1 : 600) was added for 2 hours. Bound antibodies were revealed overnight by adding 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro-blue tetrazolium (NBT) in the presence of 2 mM levamisole to inhibit endogenous alkaline phosphatase activity. After counterstaining with Mayer's hematoxylin, the sections were mounted in Glycergel (Dako SA, Trappes, France). Negative controls were prepared either by pretreating the sections with 100 μg/mL RNase A for 1 h at 37°C or by using a nonspecific digoxigenin-labelled DNA probe (pBR 328).

2.6. Immunoassay. The angiogenin immunoassay was finalised in our laboratory by using a rabbit polyclonal anti-angiogenin antibody as previously described [10] with some modifications. Microtiter plates (Immulon 4 HBX; Dynatech, Chantilly, France) were coated with 1.8 μg/mL antiangiogenin IgG diluted in PBS (50 μL) overnight at 4°C. After three washes with PBS, 0.5‰ Tween 20 (PBS/Tween), the plates were blocked with 0.1% gelatine, PBS/Tween for 1.5 h at room temperature. Solutions (250 μL) containing either standard or samples buffered with 20 mM MOPS, pH 7.2, and diluted if necessary, were distributed and incubated overnight at 4°C in a moist chamber. The wells were then washed three times with PBS/Tween and blocked with 0.1% gelatine PBS/Tween for 1 h at room temperature. Then, 50 μL of peroxidase-conjugated affinity-purified antiangiogenin IgG (5 μg/mL in PBS/Tween containing 1% ovalbumin) was added for 2 h at 37°C. After three washes with PBS/Tween, bound antibody was revealed with O-phenylenediamine dihydrochloride (OPD) and H_2O_2; the reaction was stopped after 5 min by adding 4N H_2SO_4. Absorbance was determined at 490 nm in a microplate reader (Dynatech MR 5000). Normal human plasma was used as positive control. The detection limit was 7.8 pg/mL and the assay range was 7.8–500 pg/mL.

The human chorionic gonadotropin (hCG) concentration in conditioned media was determined by using an enzyme-linked fluorescence assay (Vidas System, Biomérieux, Marcy l'Etoile, France) with a detection limit of 2 mIU/mL.

2.7. Immunofluorescence Staining of Angiogenin in Cultured Trophoblasts. Cell cultures ($n = 5$) were washed twice with 60 mM Pipes/25 mM Hepes/10 mM EGTA/2 mM $MgCl_2$, pH 6.9 (PHEM) at 37°C. Cells were fixed with 4% PFA in PHEM for 20 min at room temperature. After two washes with PHEM, remaining reactive groups were blocked by adding 50 mM NH_4Cl in PBS for 10 min. Cells were washed twice in PBS and kept in PBS at 4°C until use. Immunofluorescence staining was performed on cells permeabilized with 0.5% Triton X-100 in PHEM for 2 min and then washed twice with PBS. Saturation was achieved with PBS, 10 mg/mL IgG-free BSA, 50 μg/mL human IgG. The monoclonal anti-angiogenin antibody (Table 1) was diluted in the same buffer at 6 μg/mL and incubated with the cells overnight at 4°C. After a sequence of five washes (two with PBS, one with PBS 0.1‰ Tween 20, and two with PBS) for 2 min, saturation was achieved with PBS, 5% goat serum, 5% donkey serum for 2 h at 4°C. The bound antibody was revealed by incubation for 2 h at room temperature with 3.5 μg/mL FITC-conjugated goat anti-mouse IgM in PBS, 10 mg/mL BSA. After a sequence of five washes for 2 min and a quick wash in distilled water, the cells were mounted in Vectashield mounting medium with 4′,6-diamidino-2-phenylindole dihydrochloride (Dapi, Vector Laboratories, Burlingame, CA). In negative controls, the primary antibody was replaced by nonspecific mouse IgM.

2.8. Immunolocalization of Angiogenin in Human First-Trimester Placenta and Decidua Sections. Ten-micrometer-thick cryosections were mounted on Superfrost Plus slides and air-dried. The sections were then either fixed and permeabilised (chorionic villi) or left untreated (decidua) before immunodetection. After two washes in PBS, the sections were fixed either with 100% acetone for 10 min at −20°C (Figures 4(a) and 4(b)) or with 4% PFA in PBS for 20 min at 4°C. In the case of PFA fixation, remaining free reactive groups were blocked by adding 50 mM NH_4Cl for 10 min. After two washes in PBS, chorionic villi cryosections were permeabilized by adding 0.5% Triton X-100 in PBS for 5 min. Following two washes with PBS, saturation was achieved with blocking buffer (PBS, 10% donkey or goat serum depending on the secondary antibody, 50 μg/mL human IgG) for 30 min at room temperature. Angiogenin was immunodetected with either angiogenin-specific rabbit IgG or mouse IgM in blocking buffer overnight at 4°C. After 6 washes in PBS for 2 min, sections were incubated with blocking buffer for 30 min and bound antibody was revealed by incubation for 1.5 h in blocking buffer with a TRSC-conjugated donkey antibody against rabbit IgG, FITC-conjugated goat antibody against mouse IgM, or a TRITC-labelled goat antibody against rabbit IgG, at room temperature. Finally, the sections were washed six times with PBS and once with distilled water. Cryosections of chorionic villi were mounted in Vectashield mounting medium with Dapi, while decidual sections were mounted in Mowiol. Negative controls were generated by omitting the primary antibody or by using the same concentration of isotypic control immunoglobulin. In order to characterise the structures exhibiting angiogenin staining, antibodies raised against cell-type-specific markers (Table 1) were added to the incubation medium. Bound antibodies were revealed with FITC- or TRSC-conjugated secondary antibodies, the choice of fluorochrome depending on the antibody used for angiogenin detection (Table 1). The slides were examined with an Olympus phase-contrast microscope with a fluorescence attachment (BX-60). Images were captured with a Hamamatsu C4742-95 CCD camera and VisionStage VA software (Graftek, France). Immunolocalization studies (n = 22) were performed on cryosections from 5 placentas.

3. Results

3.1. Angiogenin Is Expressed throughout Human Placental Development. Angiogenin transcripts were detected by RT-PCR in placental tissues from 7.5 weeks gestation to term (Figure 2).

3.2. Angiogenin Is Expressed by Isolated Trophoblasts In Vitro. Extravillous cytotrophoblasts were cultured from first-trimester placentas. Angiogenin was detected with a sandwich ELISA in conditioned media from six of eight cultures. The angiogenin concentration after 48 h of culture ranged from 10 to 255 pg/mL.

Villous cytotrophoblasts were isolated and cultured from first- and second-trimester placentas. They did not proliferate but aggregated, fused, and formed a functional endocrine syncytium *in vitro*, secreting hCG as shown in Figure 3(B)(b) [20]. Angiogenin transcripts were detected by RT-PCR in freshly isolated trophoblasts, as well as in functional syncytiotrophoblasts produced *in vitro* (Figure 3(A)). Angiogenin was detected by ELISA in conditioned media throughout the differentiation process (Figure 3(B)(a)). Angiogenin immunostaining intensity increased with cell differentiation (Figure 3(C)). Labelling was cytoplasmic, with marked heterogeneity across single, aggregated, and fused cells. Angiogenin staining was either diffuse (more pronounced around nuclei) or located in granules. Staining specificity was shown by the negativity of controls (Figure 3(C)(c)).

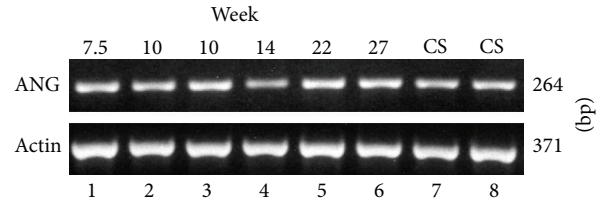

Figure 2: RT-PCR detection of angiogenin transcripts in human placenta. PCR products were obtained from first-trimester placenta RNA at weeks 7.5, 10, and 10: lanes 1, 2, and 3, respectively; from second-trimester placenta RNA at weeks 14, 22, and 27: lanes 4, 5, and 6, respectively; from term placenta RNA: lanes 7 and 8.

3.3. Angiogenin Is Immunodetected In Situ in First-Trimester Chorionic Villi. The location of angiogenin was studied by indirect immunolocalization on cryosections of human 8- to 9-week placentas, using two angiogenin-specific antibodies: a mouse monoclonal antibody (mANG) and a rabbit polyclonal antibody (pANG). Schematic villus sagittal sections and cross-sections are presented in Figures 1(b) and 1(c). A similar labelling pattern was obtained with the two antibodies: it covered the trophoblastic layer (identified by cytokeratin 7 (CK7) staining) and cells located within the villous stroma (Figure 4). Marked heterogeneity in the labelling intensity was observed from one villous to another and also within the villous itself (Figure 4(a)).

The trophoblastic layer was stained specifically for CK7 (Figures 4(e) and 4(f)) and was typically organised, with the syncytiotrophoblast lying on a continuous cytotrophoblastic layer (Figure 4(e)) in contact with the trophoblastic basement membrane (not visible here). In some villi, the trophoblastic layer was thinner and straightened (Figure 4(f)), as observed in term villi. In a few places, CK7-positive cells seemed to plunge from the trophoblastic layer into the core of the villi (Figures 4(e) and 4(f)). The villous trophoblastic layer was always immunolabelled for angiogenin, either uniformly (Figure 4(c)) or more strongly in some cytotrophoblasts (Figure 4(a)).

In the villous stroma, angiogenin was detected in some single cells close to the trophoblastic layer (Figures 4(a), 4(c), 4(e), and 4(f), arrowhead) and also in cell masses located deeper in the villous stroma (Figures 4(a) and 4(e)) (see Section 3.5). No signal was observed in negative controls (Figures 4(b) and 4(d)).

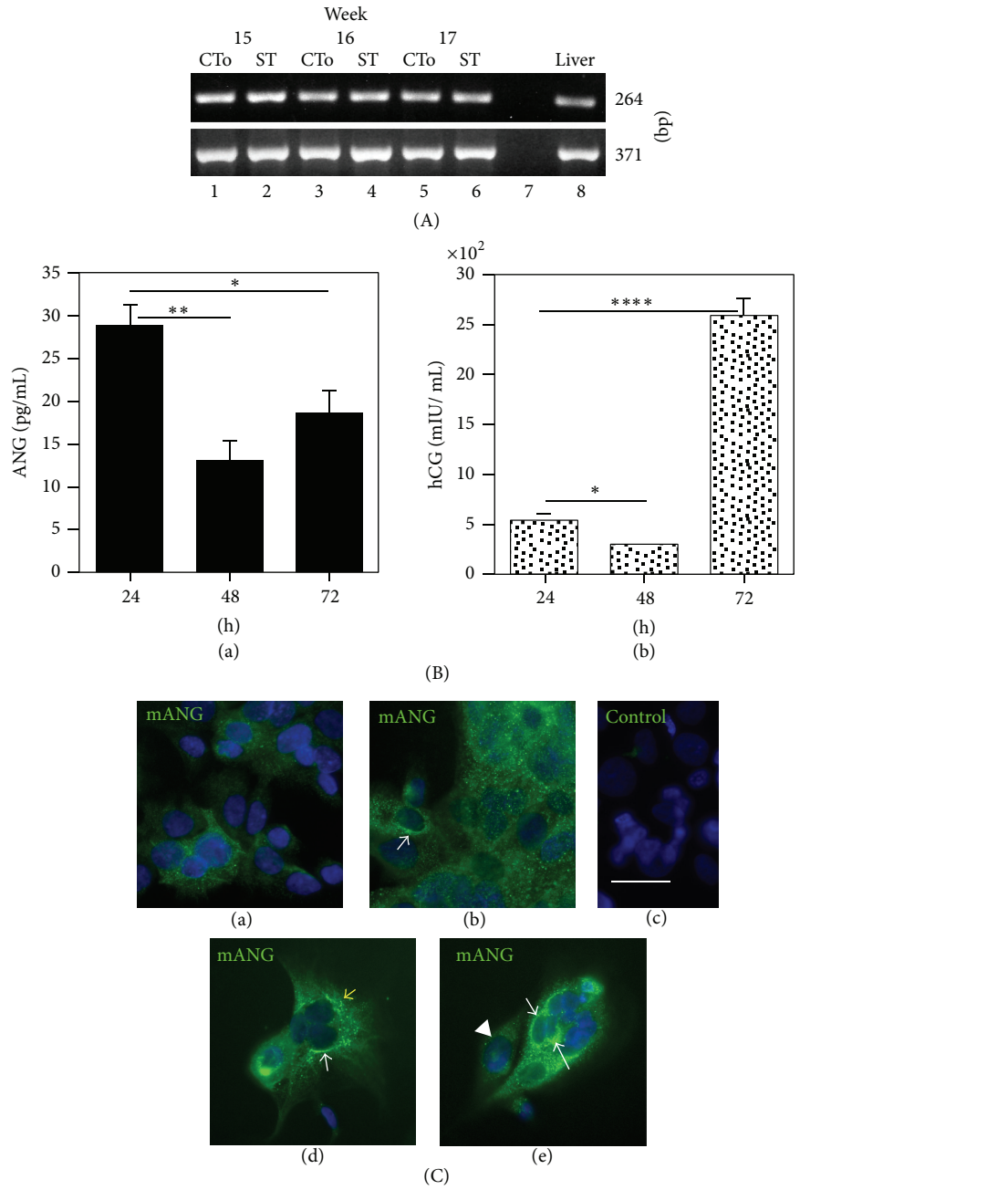

Figure 3: Angiogenin expression by trophoblastic cells. (A) Expression of angiogenin transcripts by trophoblasts isolated from second-trimester placenta. RT-PCR detection of a 264 bp fragment of the angiogenin transcript on 2% agarose gel stained with ethidium bromide. Lanes 1, 3, and 5: cytotrophoblasts from 15-, 16-, and 17-week placentas prior to culture (CT0) and 2, 4, and 6: RNA from 72 h cultured cytotrophoblasts, respectively, differentiated *in vitro* into a syncytiotrophoblast (ST); 7 is a negative control without RNA; 8 is a positive control using human liver RNA. β-actin PCR gave a product at 371 bp. (B) Angiogenin release by cultured villous cytotrophoblasts from 14-week placenta. Angiogenin (ANG) was released into the culture medium during *in vitro* differentiation of cytotrophoblasts into a syncytiotrophoblast (a). Human choriogonadotropin (hCG) was maximally expressed on day 3, indicating a functional syncytium (b). Results are means ± SD of triplicate determinations in a representative experiment ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$). (C) Angiogenin immunodetection in cultured cytotrophoblasts from first-trimester chorionic villi *in vitro*. The cells were fixed with paraformaldehyde and permeabilized and then reacted with monoclonal antiangiogenin. The bound antibody was revealed with FITC-conjugated goat anti-mouse IgM (mANG, in green). Nuclei were counterstained with Dapi (in blue). Angiogenin staining increased with cell differentiation: villous cytotrophoblasts at day 1 (a) compared to the cells at day 2 (b); control with nonspecific mouse IgM was negative (c). Angiogenin labelling was heterogeneous: diffuse in single cells (e, arrowhead), dense and more pronounced around nuclei in aggregating cells (b, e, and white arrow), punctuated and associated with granules here in the syncytium (d, yellow arrow). Cells were from 13-week (a, b, and c), 12.5-week (d), and 8.5-week (e) placenta, respectively. Bar, 20 μm.

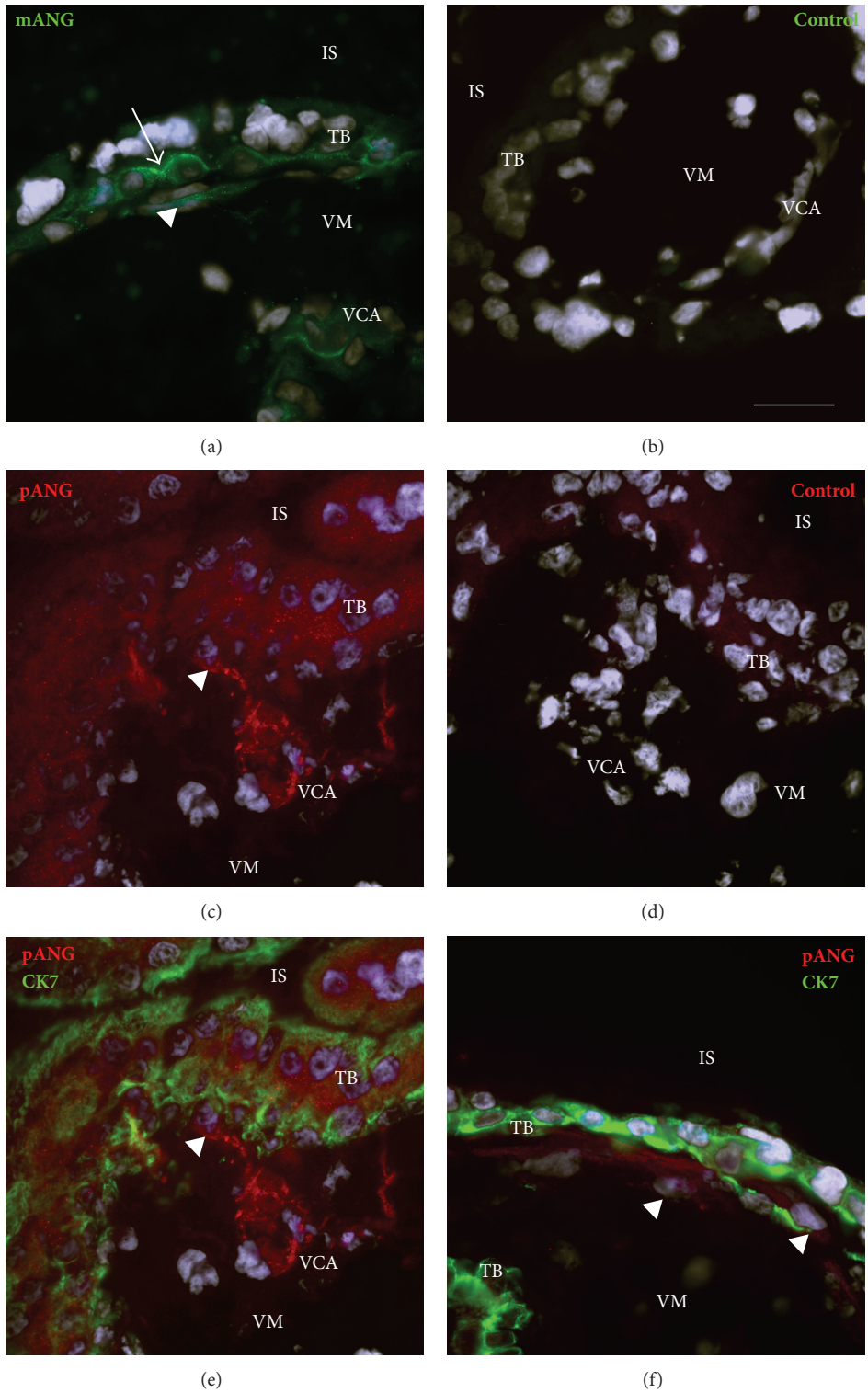

Figure 4: Angiogenin immunoreactivity in chorionic villi from human first-trimester placentas. Frozen sections of placentas at week 8 to 9 were reacted with either a monoclonal antibody against angiogenin (a, mANG in green) or a polyclonal antibody purified by affinity on immobilised angiogenin (c, e, f, and pANG in red). Bound antibodies were detected with fluorescent secondary antibodies. Angiogenin immunoreactivity (pANG, in red) was detected in the trophoblastic layer immunostained for cytokeratin 7 (CK7, in green) (e, f), in some cells in the close vicinity of the layer (arrowhead: a, in green; c, e, and f, in red) and in isolated cell masses (VCA), located deeper in the villous stroma (a, in green; c and e, in red). The signal was stronger in some cytotrophoblasts (arrow: a). No signal was observed on cryosections reacted with either nonspecific mouse IgM (control, b) or nonspecific rabbit IgG (control, d). Nuclei were counterstained with Dapi, in blue. Bar, 20 μm. IS: intervillous space; TB: trophoblast layer; VCA: vascular cell aggregate; and VM: villous mesenchyme.

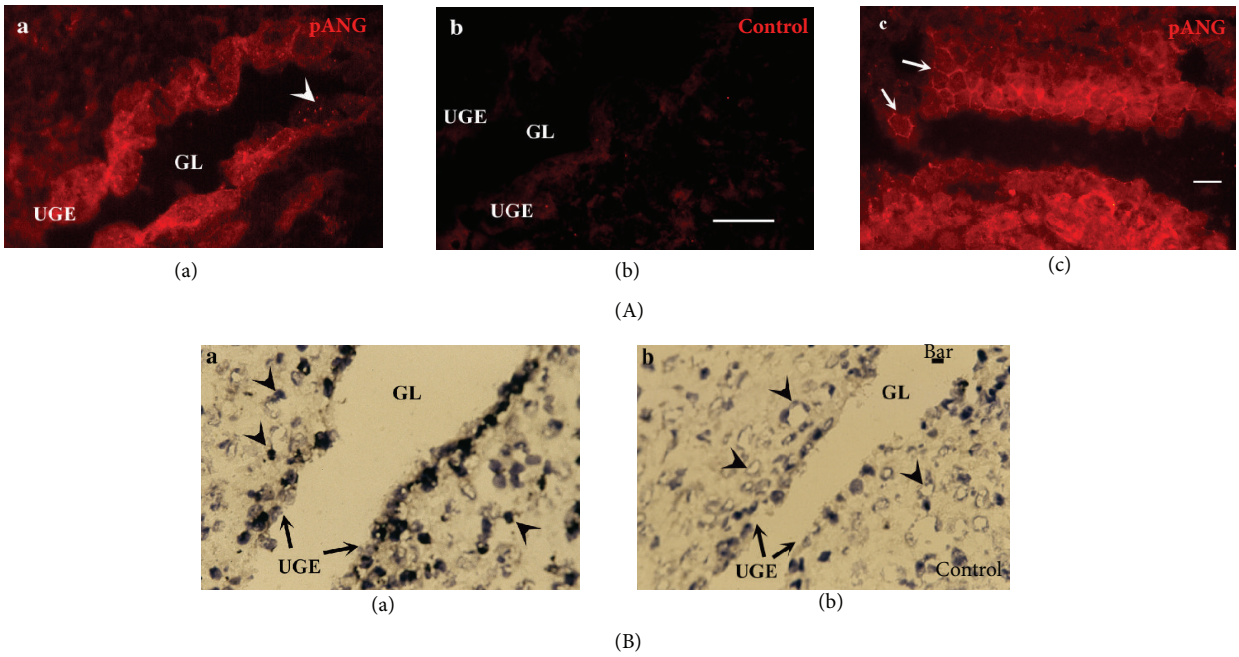

Figure 5: Angiogenin expression in parietal decidua: glandular epithelium and decidual cells. (A) Angiogenin immunoreactivity on frozen sections at week 7.5, using an angiogenin-specific polyclonal antibody; the glandular epithelium showed punctuate signal corresponding to secretory granules (a, arrowhead) and strongly delineated polyhedral decidual cells (c, arrows). No signal was observed on cryosections which reacted with nonspecific rabbit IgG (b). (B) Angiogenin transcripts were detected in the glandular epithelium (a, arrows) and in endothelial cells of small capillaries (a, arrowhead) on frozen sections hybridised with a digoxigenin-labelled angiogenin cDNA probe. Probe binding was detected with an alkaline phosphatase-coupled antibody to digoxigenin and visualised with a colorimetric substrate (NBT/BCIP) (a). No signal was observed in RNase pretreated frozen sections (b). Counterstaining with Mayer's haematoxylin. Bar, 20 μm. GL, glandular lumen and UGE, uterine glandular epithelium.

3.4. Angiogenin Is Expressed in the Decidua at Week 7.5 (Glandular Epithelium, Decidual Cells, Maternal Artery, and Macrophages). Tissue pieces were identified as parietal decidua based on the absence of invading cytotrophoblasts (CK7-positive cells) and remodelled spiral arteries. Uterine glands were present on the cryosections (Figure 5). Glandular epithelial cells, positive for cytokeratin (not shown), were immunolabelled for angiogenin. The intense, punctuate signal thus obtained likely corresponded to secretory vesicles (Figure 5A(a), arrowhead). No signal was observed in the control (Figure 5A(b)). Angiogenin was also detected on the outline of polyhedral decidual cells (Figure 5A(c), arrows). Angiogenin messengers were expressed by glandular epithelial cells and in endothelial cells of small maternal capillaries, as shown by *in situ* hybridisation (Figure 5B(a)). No signal was observed on negative controls either pretreated with RNase (Figure 5B(b)) or reacting with a nonspecific probe (not shown). The wall of spiral arteries was immunolabelled for angiogenin (Figure 6A(a)). Specific punctuate labelling was also observed in vimentin-positive cells (Figure 6A(a), arrowheads). The latter cells, in close contact with spiral arteries or sparser in the decidua, were shown to be macrophages, based on their CD14 reactivity (Figure 6A(b)). Thus, maternal macrophages were immunolabelled for angiogenin. Angiogenin transcripts were detected in spiral arteries by *in situ* hybridisation (Figure 6B(a)). Angiogenin transcripts were also strongly detected in unidentified small round cells. Negative controls demonstrated the specificity of the signal (Figure 6B(b)).

3.5. Angiogenin Expression Is Associated with First-Trimester Chorionic Villi and Blood Vessel Formation. The structure of the fetal vessels in the villus followed a gradient: the least organised vessels were close to the trophoblastic layer, while more organised ones were located deeper in the villus. In order to identify the cells that were immunopositive for angiogenin in developing chorionic villi, double immunolabelling with cell markers was performed on 8-week cryosections. Besides the trophoblastic layer (Figure 4), angiogenin staining was associated with three different structures: single cells, cell aggregates, and cell cords (Figures 7–9). Angiogenin labelling was associated with chorionic villi undergoing intense morphological changes (Figure 7). The labelled cells in close proximity of the trophoblastic layer expressed very early endothelial markers such as VE-cadherin (Figures 7(a) and 7(b)) and VEGF-R2 (Figures 7(c) and 7(d)). Figure 7(a) shows a typical view of a single cell double stained for angiogenin and VE-cadherin establishing a bridge between cytotrophoblasts and cell aggregates (double stained), corresponding to a nascent fetal blood vessel. Proliferative cells labelled for Ki-67 antigen were observed in aggregates (Figure 7(e)). In less organised aggregates, Ki-67 was present both in some trophoblastic nuclei and in nuclei of underlying nontrophoblastic cells that were positive for

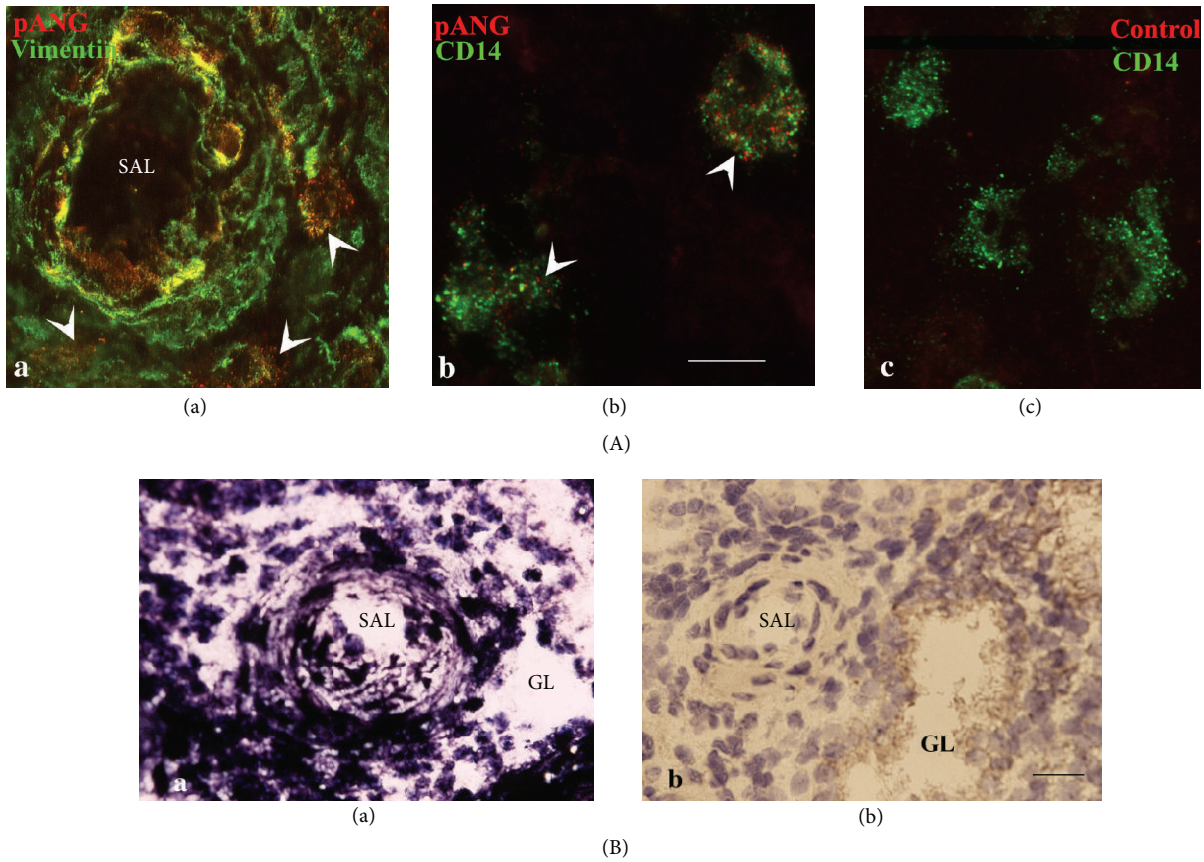

Figure 6: Angiogenin expression in decidua: maternal artery and macrophages. (A) Angiogenin immunoreactivity on frozen sections at week 7.5: (a) angiogenin specific polyclonal antibody (in red) highlighted vimentin-positive cells (in green): endothelial cells of the spiral artery (in yellow (red + green)) and sparse cells in the decidua (arrowhead). (b) Maternal macrophages (CD14 positive, in green) in a section of 11-week placenta were immunolabelled for angiogenin (in red), compared with the isotypic negative control (c). (B) *In situ* hybridisation on a frozen section of a 10-week placenta with a digoxigenin-labelled angiogenin cDNA probe revealing (in purple) angiogenin transcripts in spiral artery, glandular epithelium, and unidentified decidual cells (a). No signal was observed in RNase pretreated frozen sections (b). Counterstaining with Mayer's haematoxylin. Bar, 20 μm. GL: glandular lumen and SAL: spiral artery lumen.

angiogenin (Figure 7(f)). Tiny cytoplasmic processes, which are also labelled for angiogenin, were in intimate contact with the trophoblastic layer and connected with primitive established vessels in the core of the villi (Figures 8(a) and 8(b), arrowheads). Colabelling not only with CD31, an early endothelial marker, but also with *vWF*, a later endothelial marker, was shown in Figures 8(a) and 8(b), respectively. These observations suggest the existence of coordinated sites of proliferation composed of villous trophoblasts and facing nontrophoblastic cells. In cell aggregates, double staining for angiogenin and Tie-2, an endothelial marker, was observed (Figure 8(c)). As in term placenta, angiogenin and erythropoietin-receptor (EpoR) labelling were colocalized in the trophoblastic layer and in nascent or established fetal vessels (Figure 8(d)). However, EpoR labelling appeared stronger in cytotrophoblasts. In cell aggregates, colabelling with alpha-smooth muscle actin, a marker for vascular smooth muscle cells and pericytes, was detected (Figure 8(e)).

Angiogenin immunolabelling was observed in cords positive for the following early endothelial markers: VE-cadherin, CD34, and Tie-2 or for *vWF*, a later vascular marker (Figure 9). These nascent structures, corresponding to fetal vessels in formation and previously called angioblastic strands [23] or haemangioblastic cell cords [14], were located in a peripheral position in the villus. CD34-positive cells with rounded nuclei were present in these vessel segments (Figure 9(d)) and likely corresponded to haematopoietic precursors [24].

In the villous core, fetal macrophages (Hofbauer cells) were immunoreactive for CD45 (Figure 8(f)) but negative for CD14 (data not shown). The CD45-positive cells were angiogenin-negative but lay close to angiogenin-positive mesenchymal cells or in the close vicinity of the trophoblastic layer which was labelled strongly for angiogenin (Figure 8(f)).

In some villi, the trophoblastic layer was labelled for early endothelial cell markers such as VE-cadherin on its basal side (Figures 7(a) and 7(b) and Figure 9(a), arrows) and also for VEGF-R2 (Figures 7(c) and 7(d), arrows) but not for CD31 (Figure 8(a)), CD34 (Figure 9(d)), Tie-2 (Figure 8(c)), or *vWF* (Figure 8(b)). In areas labelled for both angiogenin and VE-cadherin, the trophoblastic layer was thickened

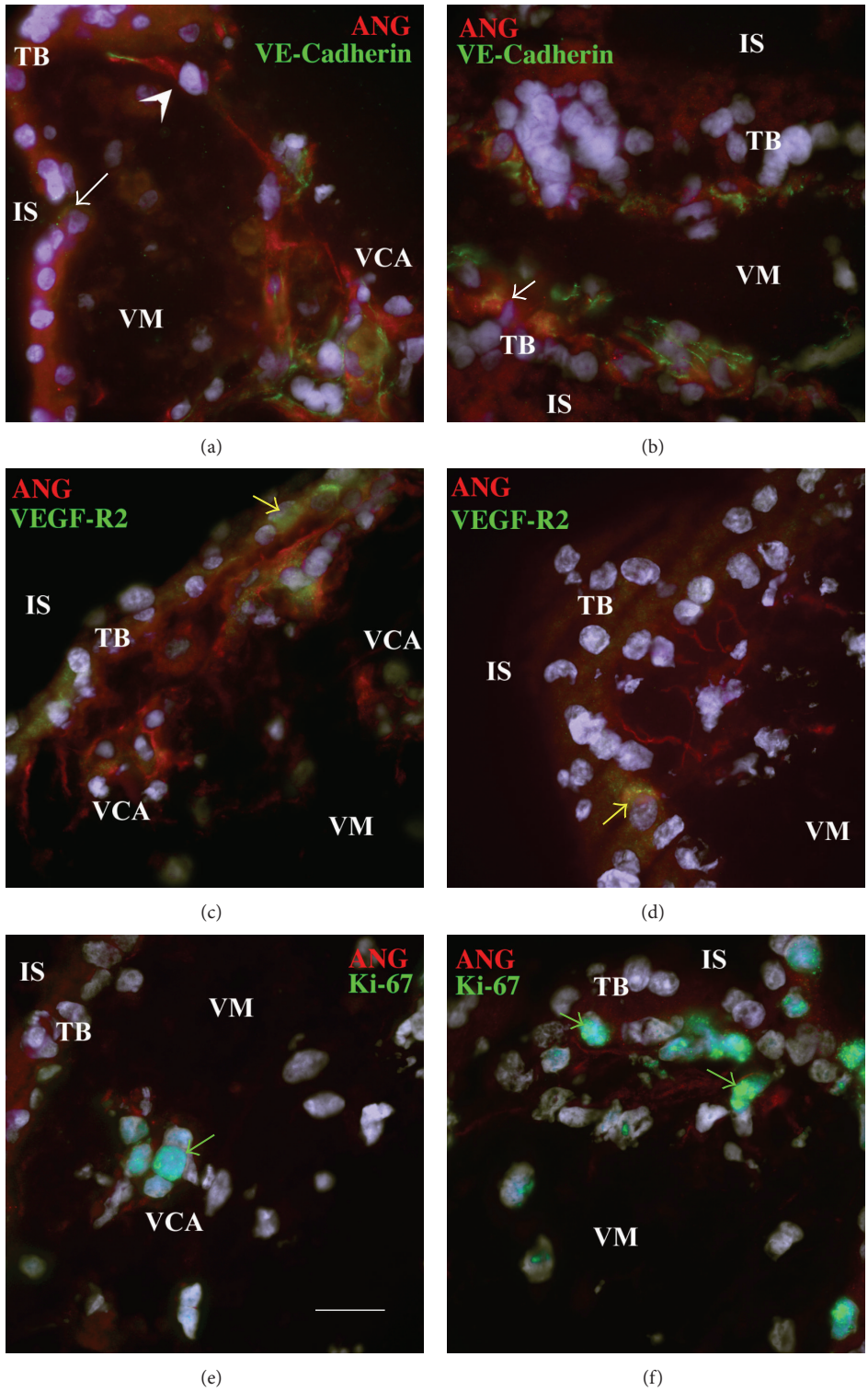

Figure 7: Characterisation by double labelling of chorionic villi undergoing intense morphological changes. Frozen cross-sections of human chorionic villi at week 8 were reacted with a mix of two primary antibodies. Angiogenin was detected with a specific polyclonal antibody (ANG, in red). The other antibody (in green) was directed against either the early endothelial markers: VE-cadherin (a, b) and VEGF-R2 (c, d), or the proliferation marker Ki-67 (e, f). Cells positive for the endothelial markers were stained for angiogenin. Note the angiogenin- and VE-cadherin-positive single cell (a, arrowhead) beneath the trophoblastic layer establishing a bridge between the layer and a vascular cell aggregate (VCA) stained for VE-cadherin; VEGF-R2-labelled VCA in close contact with the trophoblast layer (c); and angiogenin-labelled cytoplasmic processes (d). Proliferative cells labelled for Ki-67 and angiogenin were observed in aggregates (e, green arrow). Coordinated sites of proliferation (Ki-67+) composed of villous trophoblasts and facing nontrophoblastic cell were positive for angiogenin (f, green arrow). White and yellow arrows point to VE-cadherin and VEGF-R2 staining of trophoblast layer, respectively. Nuclei were counterstained with Dapi, in blue. Bar, 20 μm. IS: intervillous space; TB: trophoblast layer; VCA: vascular cell aggregate; and VM: villous mesenchyme.

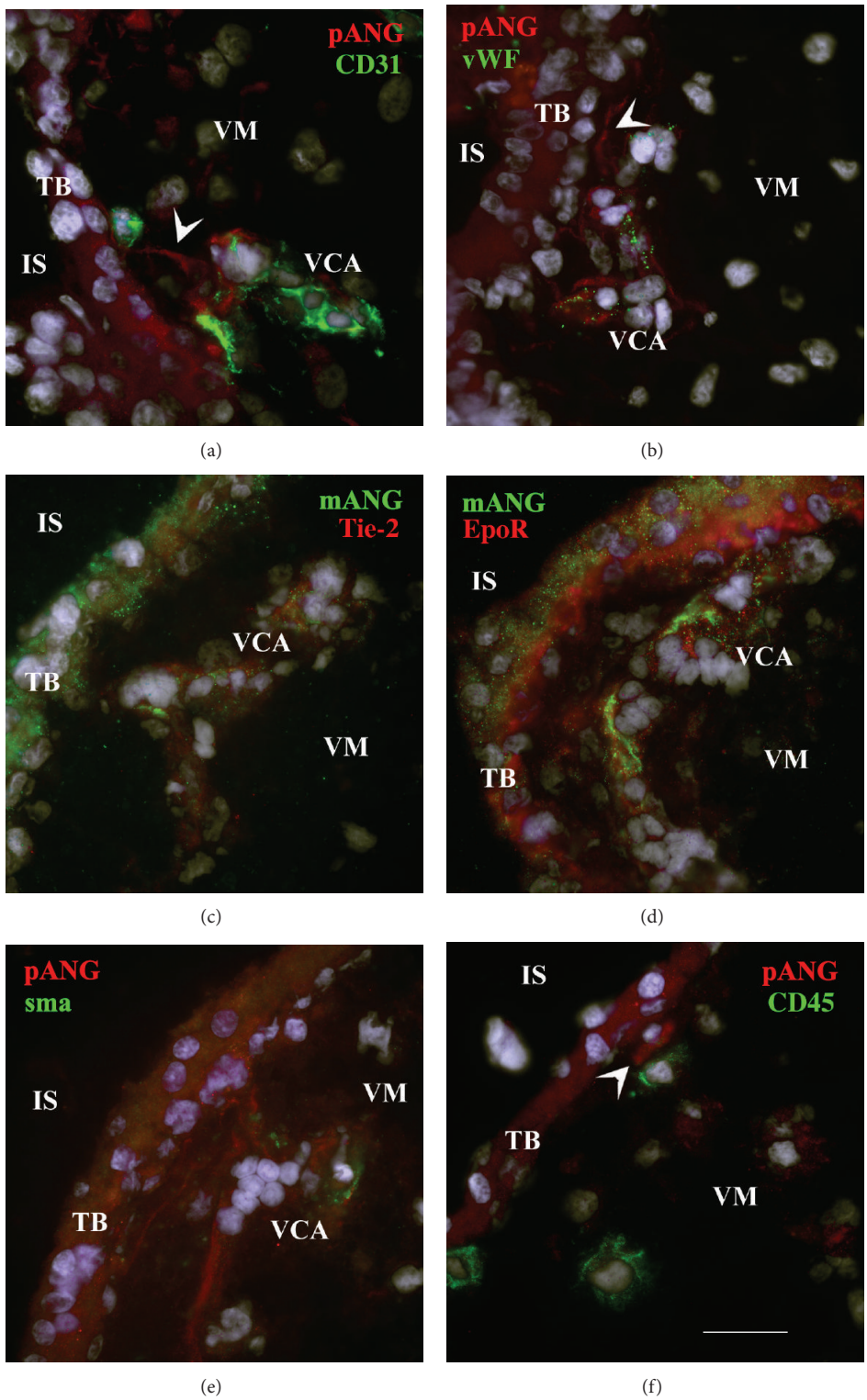

FIGURE 8: Immunolocalization of angiogenin in chorionic villi showing vascular cell aggregates. Frozen cross-sections of human chorionic villi at week 8 were reacted with a mix of two primary antibodies. The bound antibodies were detected with fluorescent secondary antibodies. The anti-angiogenin antibody was either polyclonal (a, b, e, f, and pANG in red) or monoclonal (c, d, and mANG in green). Angiogenin colabelling with CD31 (a, in green), vWF (b, in green), Tie2 (c, in red), Epo-R (d, in red), and α-smooth muscle actin (e, sma, in green), respectively, stained vascular cell aggregates. Arrowheads point to tiny cytoplasmic processes in close vicinity with the trophoblast layer connected with vascular cell aggregates (a, b). Angiogenin immunoreactivity was also detected in the trophoblastic layer, in cytotrophoblasts (also labelled for EpoR (d, in red)) and in single cells close to CD45-positive cells (f, in green, arrowhead). Nuclei were counterstained with Dapi, in blue. Bar, 20 μm. IS: intervillous space and TB: trophoblast.

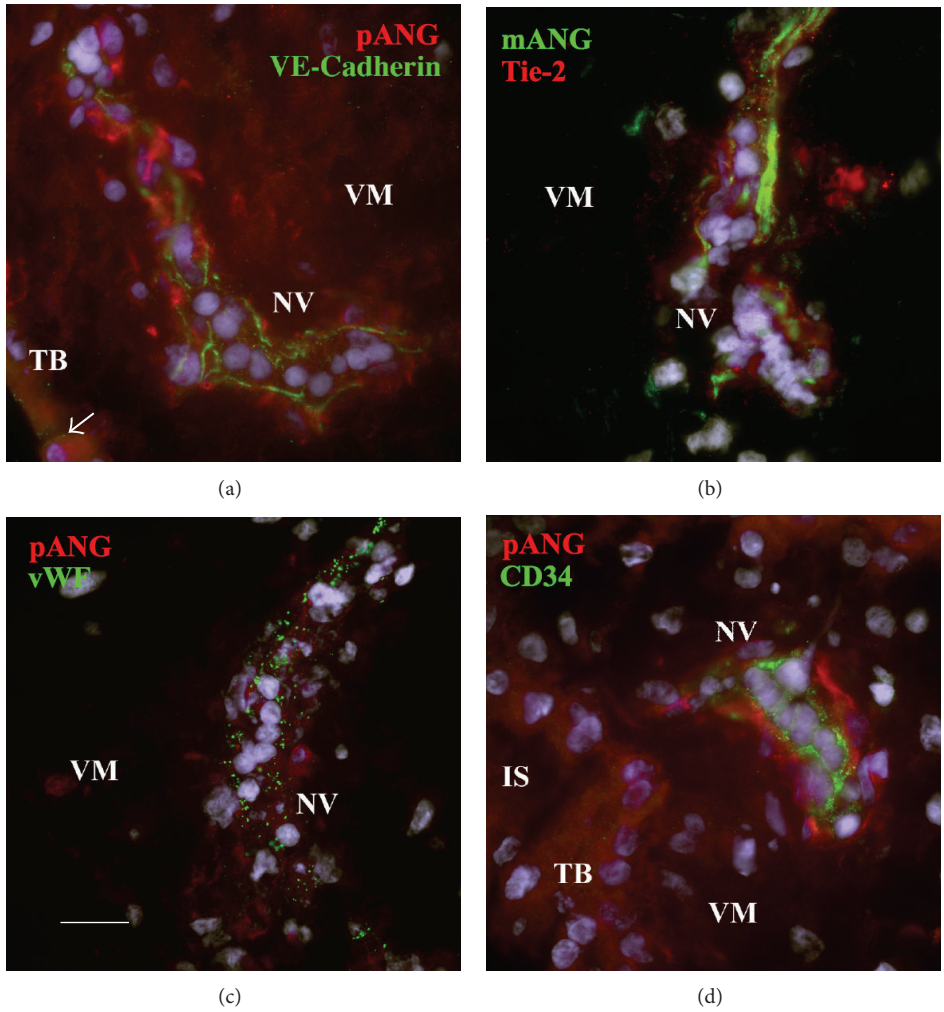

FIGURE 9: Angiogenin immunoreactivity in nascent vessels. Frozen sections prepared at week 8 were reacted with either a polyclonal antibody specific for angiogenin (a, c, d, and pANG in red) or a monoclonal (b, mANG in green). The other antibody, directed against VE-cadherin (a, in green), Tie2 (b, in red), vWF (c, in green), and CD34 (d, in green) colabelled nascent vessels. Nuclei were counterstained with Dapi, in blue. Bar, 20 μm. IS: intervillous space; TB: trophoblast layer; VCA: vascular cell aggregate; and VM: villous mesenchyme.

by superimposed nuclei (Figure 7(b)). Unlike in term villi, the trophoblastic basement membrane of first-trimester villi was not highlighted by angiogenin immunolabelling.

Taken together, these results show that angiogenin protein is present in the villous trophoblastic layer, structured fetal vessels, and sites of nascent fetal vessels.

4. Discussion

Vessel formation during human placental development occurs by means of both vasculogenesis (from *in situ* differentiating endothelial cells) and angiogenesis (sprouting of capillaries from existing vessels) [14, 25]. Fetoplacental vasculogenesis proceeds by formation of haemangioblastic cords which progressively linked up from day 22 to the 26th week [14, 26, 27]. Current knowledge of the cellular and molecular mechanisms involved in human placental blood vessel formation relies mainly on morphological and ultrastructural observations [14, 23, 28] and immunohistochemistry [27, 29, 30], but further insights are being obtained by the study of angiogenic growth factors [10, 30–33] and molecular methods [2].

Angiogenin has been first isolated from supernatants of colon carcinoma cells on its property to induce angiogenesis in chicken chorioallantoic membrane [5]. Angiogenin (RNase 5) belongs to the secreted RNase family, which is vertebrate-specific and displays weak ribonucleolytic activity. An intact catalytic site and cell-binding domain are both required to induce neovascularization [34]. Angiogenin is also a permissive factor for angiogenesis induced by other angiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF-1 and FGF-2) [35], stimulating rRNA transcription and proliferation [36]. Among its other properties, angiogenin promotes cell survival, a property related to its ability to cleave tRNA and release tiRNAs (stress-induced small RNAs), which inhibit protein translation [37–40]. Angiogenin-induced tiRNAs

promote stress granule assembly [37], an adaptive process that reprograms protein translation during stress [41, 42].

In this work, in agreement with previous observations of early placentas [30, 33, 43–45], we observed *in situ* immunostaining for vessel markers on single cells in not only close contact with the trophoblastic layer but also deeper in the villous stroma, on cell aggregates referred to as angioblasts by Florence Sabin in the chick [46]. Both sites were strongly labelled for angiogenin. The current consensus is that angioblastic cords arise from *in situ* differentiation of stem cells present in the embryonic-derived mesoderm that has invaded the chorionic villi [47]. These structures were found here to express endothelium-specific markers at 8 weeks of gestation, namely VE-cadherin, CD31 (PECAM-1, also present in leukocytes and platelets), VEGF-R2, Tie-2, vWF, CD34 (also present on haematopoietic stem cells), EpoR (also present on erythroid precursors), and angiogenin. Some of them also expressed the smooth muscle cell marker alpha-actin, suggesting the presence of pericytes.

Single cells in close contact with the trophoblastic layer were found to express VE-cadherin and PECAM-1 and also showed strong angiogenin expression. They were negative for the trophoblast marker CK7 and for the leukocyte differentiation antigens CD14 and CD45. Arthur Hertig suggested that these single cells might derive from trophoblast delamination and differentiation [23]. However, ultrastructural studies showing a continuous trophoblastic basal lamina throughout this early stage of development argue against this hypothesis [14, 28, 48]. It might be reconsidered [49] since epithelial-mesenchymal transitions are part of the developmental program [50] and the human cytotrophoblastic cell has been qualified as a "mononuclear chameleon" [51]. In certain areas, we observed trophoblastic expression of early endothelial markers such as VE-cadherin and VEGFR-2, as previously described [30, 33]. A switch in cadherin expression has been linked to cytotrophoblast differentiation [4]. However, this epithelial-/endothelial-like conversion has been only described for invasive cytotrophoblasts [52]. In any case, we observed that these cells in close contact with trophoblasts and the cellular extensions that bridge the cytotrophoblast layer to nascent vessels expressed endothelial markers. They were also strongly labelled for angiogenin as were the trophoblasts facing them. Together, these observations point to the existence of paracrine or juxtacrine interactions involving angiogenin. Paracrine interaction has been shown to mediate angiogenin neuroprotection. Skorupa et al. [53] have shown that angiogenin secreted by motoneurons is endocytosed by astroglia via syndecan 4. Interestingly, the paracrine RNA fragments in astrocytes differ from those previously described but their biological function is unknown. These observations from studies of the nervous system highlight the fundamental properties of angiogenin.

Angiogenin immunolabelling in early chorionic villi was associated with actively dividing endothelial and trophoblastic cells, possibly reflecting coordinated morphological development. In early placenta, vasculogenesis might be controlled by villous cytotrophoblast [30]. In this work, angiogenin was expressed by cytotrophoblasts. The fact that angiogenin binds to endothelial cells *via* high affinity binding sites [54] and stimulates proliferation [55, 56] and differentiation *in vitro* [57] suggests that cytotrophoblasts might release angiogenin as a paracrine signal to endothelial cells. Reciprocally endothelial cells might signal to trophoblastic cells in early villus development. It has been shown in developing organs such as liver, pancreas, or prostate that early endothelial cells and nascent vascular structures provide developmental signals to the growing organ throughout its development, even prior to blood vessel formation [58, 59]. In this work, angiogenin expression observed *in situ* corroborates its expression by endothelial cells *in vitro* [60]. However, evidence for angiogenin receptor on cytotrophoblasts is not yet documented. Taken together, these observations suggest cooperation between villous trophoblasts and nascent fetal blood vessels.

Unlike term placenta, the villous tree in first-trimester placenta (up to 10–12 weeks of gestation) does not bathe in maternal blood but in an exudate. Indeed, maternal spiral arteries are obstructed by intra-arterial cytotrophoblastic plugs, which block the entry of maternal blood into the intervillous space throughout the first trimester. The epithelium of endometrial glands is known to secrete material that is discharged into the lumen (uterine milk) [61, 62]. These secretions, a source of growth factors such as epidermal growth factor (EGF), VEGF, and leukaemia inhibitory factor, are delivered to the placental intervillous space. Fetal nutrition may thus be histiotrophic during this period [63, 64]. Angiogenin is a secreted protein present in amniotic fluid [65], follicular fluid [66], and plasma [67]. The punctuate angiogenin immunostaining of glandular epithelial cells from 7.5-week decidua points to the presence of secretory vesicles. At this time, the oxygen tension is <20 mm Hg [63]. Low but physiological oxygen tension may stimulate villous sprouting and capillary growth [15]. Angiogenin expression has been shown to be upregulated by hypoxic conditions in cultured cells, including granulosa cells [66], decidual cells [68], choriocarcinoma cells, and Simian virus 40 transformed placental cells [69]. Angiogenin might participate, along with other cytokines such as EGF, VEGF, and tumor necrosis factor-α, in regulating trophoblast proliferation and/or migration and might influence the remodelling of uteroplacental arteries.

Angiogenin was also expressed in decidual cells from first-trimester placenta, where it was strongly associated with the matrix which reminds that angiogenin is a heparin-binding protein [70] that binds to the extracellular matrix [54]. It has been shown that angiogenin is present in placental stromal and epithelial cells, with increased expression in the endometrium in the mid- and late secretory phases and early gestation [68]. In the decidua, we found that the uterine arteries and surrounding maternal macrophages also expressed angiogenin. The decidual environment is known to be immunosuppressive [71]. Angiogenin has been reported to function as an immunomodulator [72–74]. It is conceivable that angiogenin could thus participate in maternal immune tolerance towards the semiallogenic fetus.

Based on the other known biological activities of angiogenin and on its pattern of placental expression, our findings

suggest that angiogenin, in concert with other regulators, may play a fundamental role in placental organogenesis.

5. Conclusions

This work shows that angiogenin is expressed throughout human placental development. In the early placenta, angiogenin is expressed by extravillous and villous cytotrophoblasts, as well as by functional syncytiotrophoblasts differentiated *in vitro*. A compatible pattern of angiogenin expression was observed *in situ*. Angiogenin expression in extravillous cytotrophoblasts *in vitro* and in the maternal decidua *in situ* (uterine glands, decidual cells, maternal artery and small capillaries, and macrophages) suggests that angiogenin may play a role in the decidual environment. The cellular pattern of angiogenin distribution in early first-trimester mesenchymal villi suggests a role in blood vessel formation. Fine analysis with cell-type-specific markers suggests a role of angiogenin in cross-talk between trophoblasts and endothelial cells. In view of its known biological activities, our findings suggest a fundamental role of angiogenin in early placental development.

Abbreviations

EGF: Epidermal growth factor
FGF: Fibroblast growth factor
hCG: Human chorionic gonadotropin
IgG: Immunoglobulin G
RNase: Ribonuclease
VEGF: Vascular endothelial growth factor.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Nadine Pavlov set up and performed the immunoassays and the *in situ* hybridisation experiments, carried out immunodetection, and drafted the paper. Jean-Louis Frendo participated in RT-PCR studies. Danièle Evain-Brion directed the lab and participated in coordination. Jean Guibourdenche carried out hCG detection and contributed to first-trimester tissue collection. Séverine A. Degrelle built Figure 1 and carried out statistical analysis. Josette Badet led the angiogenin project, carried out the RT-PCR and immunocytochemistry experiments, and wrote the paper. All coauthors participated in helpful discussions and read the paper.

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Research Article

Antiphospholipid Antibodies in Women Undergoing In Vitro Fertilization Treatment: Clinical Value of IgA Anti-β2glycoprotein I Antibodies Determination

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Implantation failure could be related to antiphospholipid antibodies (aPL). We retrospectively analyzed the usefulness of aPL determination in women undergoing IVF. Conventional aPL of the antiphospholipid syndrome, lupus anticoagulant (LA), anticardiolipin antibodies (aCL), anti-β2glycoprotein I (aβ2GPI) antibodies, and IgG and IgM isotypes as well as IgA isotype were analyzed in women presenting with at least two implantation failures after in vitro fertilization (IVF). In a population of 40 IVF patients, a total prevalence of 20% (8/40) of aPL was found, significantly different from that of the control population (100 healthy blood donors, $P < 0.0005$). Among the panels of aPL tested, aβ2GPI IgA antibodies were the most prevalent (62.5% 5/8), significantly higher in IVF patients (12.5%, 5/40) than in controls (1%, 1/100) ($P = 0.01$). No difference according to the numbers of IVF attempts and success of embryo implantation was found between aPL positive and negative IVF patients. In contrast, no accomplished pregnancy with full-term live birth was observed in aPL positive IVF patients. Altogether our data led us to propose aPL assessment, in particular aβ2GPI IgA antibodies, in support of IVF treated women. In a perspective way, an early aPL detection could be the basis for defining novel therapeutic strategy.

1. Introduction

Infertility is defined as the inability to conceive after 12 months of unprotected sex and represents about 10% of the couples of childbearing age [1]. The most frequent factors of infertility are ovulation disorders, tubal obstruction, male infertility, and unexplained infertility also called idiopathic causes.

Autoimmune diseases are not considered as a major cause of infertility and their explorations are usually neglected although many of them are associated with infertility and fetal loss (defined as the loss of two by American Fertility Society or three pregnancies for others), as is the case of antiphospholipid syndrome (APS) [1, 2]. Obstetric complications are at the forefront of the symptoms of antiphospholipid syndrome. A nonimplantation could be related to unexplained infertility [3]. There are some similarities between unexplained infertility and recurrent miscarriages in APS such as defective embryonic implantation and the presence of antiphospholipid antibody (aPL) [3]. Therefore a cause of implantation failure could be related to the presence of an aPL or the existence of an APS.

In addition to the conventional markers for classification of APS, lupus anticoagulant (LA), anticardiolipin antibodies (aCL), and anti-β2glycoprotein I (aβ2GPI) antibodies with IgG and/or IgM isotypes and according to the last international consensus guidelines on antiphospholipid antibodies, testing for IgA isotype is recommended for both aCL and aβ2GPI when results of conventional markers are negative and APS is still suspected [4].

However the usefulness of aPL determination in women undergoing IVF treatment is not established. We conducted a retrospective study to assess the usefulness of aPL in laboratory testing of women undergoing IVF. To this aim, the conventional aPL of APS-LA, aCL, aβ2GPI, IgG, and IgM isotypes as well as IgA isotype were analyzed. Finally, the relationship between the presence of aPL and embryo implantation as well as pregnancy issue was studied.

2. Materials and Methods

2.1. Patients. We retrospectively studied aPL results in women undergoing IVF attempts from 2005 to 2011. The aPL assessment was offered in our center to women when no pregnancy occurred after at least two IVF attempts with good quality embryos available for the transfer (even cleavage, even cell sizes, <20% fragmentation).

IVF treatment was performed at the Reproductive Department of University Hospital La Conception and was proposed to couples in female (ovulation disorders, tubal obstruction, and endometriosis), male, mixed, or unexplained infertility. All samples were from a declared Biobank (DC 2012-1704) with respect to ethical directives.

The control population is composed of healthy blood donors ($n = 100$).

2.2. Methods

2.2.1. Lupus Anticoagulant Detection. For LA detection, venipuncture was performed on 0.129 M sodium citrate tubes (Vacutainer, Becton Dickinson) and platelet-poor plasma was prepared by Q1 centrifugation (twice at 3,000 g for 10 min). Whole blood and plasma fractions were stored at −80°C until use. Plasma samples were tested as recommended by the scientific and standardization subcommittee for LA of the ISTH.13 Screening for LA included activated partial thromboplastin time (aPTT, performed with LA sensitive reagent), dilute prothrombin time (1/500 dilution of thromboplastin in $CaCl_2$), and dilute Russell's viper venom time (dRVVT). In the case of prolongation of screening test(s), mixing studies were performed on 1 : 1 dilution of patient plasma with pooled normal plasma. Confirmation step consisted of a dRVVT-based test with and without addition of exogenous phospholipids. In some patients, associated coagulopathies were investigated by the measurement of coagulation factors on serial dilutions of patient plasma when necessary.

2.2.2. Determination of Antiphospholipid Antibodies. In-house enzyme-linked immunosorbent assays (ELISAs) were used for the determination of aCL (IgM, IgG, and IgA) and aβ2GPI IgA, as previously described [5, 6]. Determination of aβ2GPI (IgM and IgG) was obtained by using Orgentec ELISA kit.

In each ELISA, each sample was tested in three wells, two with antigen and one without. The latter corresponding to sample background was subtracted from the specific binding. One negative and two positive controls were included in each run. Positive controls were from patients with antiphospholipid syndrome and negative control from a healthy blood donor. For each aPL-ELISA, the cutoff level was determined by the analysis of the samples of 100 blood donors (control group) and was calculated at the 99th percentile. The results were expressed in GPLU and MPLU for IgG and IgM-aCL and aβ2GPI, in delta optical density, for IgA isotype of aCL and aβ2GPI. The cutoff values were the following: 1/aCL-ELISA : IgG = 20 GPLU; IgM = 8 MPLU; IgA = 0.25; 2/a2GPI-ELISA : IgG = 8 B2GU; IgM = 8 B2MU; IgA = 0.26 (GPLU, MPLU, and B2GU are arbitrary unit for, respectively, IgG IgM phospholipids and IgG β2glycoprotein I antibodies).

2.2.3. Statistical Analysis. The results are expressed as mean ± standard deviation or percentage prevalences. The comparison of quantitative data was performed by t-test. The χ^2 test was used for qualitative data. A $P < 0.05$ was considered significant.

3. Results

A total of 40 patients could be included in an IVF program when no pregnancy was obtained after at least two embryo transfers with good quality embryos available for transfers and were examined for the presence of antiphospholipid antibody. Consequently, the studied population represented a small part of the whole population attempting IVF in our center. Mean women's age was 35 ± 4.15 years at the time of aPL detection. IVF indications were distributed as follows: female infertility (8 patients), male infertility (21 patients), mixed infertility (10 patients), and unexplained infertility (1 patient). Before starting IVF treatment, 8 women already became pregnant spontaneously (14 early miscarriages) inside the actual couple or not. At the time of aPL detection, mean number of IVF attempts was 3.85 ± 1.5 (ranging between 2 and 6) and aPL assessment was proposed only in cases where we could exclude a poor embryo quality, which is a major factor of implantation failure. Embryo transfers were performed 48 to 72 h after oocyte retrieval and the mean number of transferred embryos/transfer was 1.96 ± 0.22 (ranging between 1 and 3). At the end of the IVF program, 21 pregnancies occurred (15 patients), with 10 early miscarriages, 2 ectopic pregnancies, 6 normal deliveries, 1 premature delivery in preeclampsia context, and 2 fetal deaths in utero. We can note that one fetal death in utero occurred after the fourth IVF attempt because of venous thrombosis of umbilical cord, and the second one occurred in severe preeclampsia context within the same patient who previously had a premature delivery also in preeclampsia context. For the 8 patients presenting with secondary infertility (mean age: 35 years ± 4 ans,

Table 1: IVF cycles and pregnancy outcome in aPL positive and negative IVF treated patients.

IVF treated patients ($n = 40$)	aPL positive patients ($n = 8$)	aPL negative patients ($n = 32$)	P value
Patients with secondary infertility (%) (before IVF attempts)	37.5% (3/8)	15.6% (5/32)	>0.05
Mean number of IVF attempts/patient	3.75 ± 0.45	3.85 ± 0.28	>0.05
Mean number of pregnancies after IVF/patient	0.5 ± 0.7 (4/8)	0.53 ± 0.75 (17/32)	>0.05
Outcome of IVF pregnancies			
Abnormal outcome			
Ectopic pregnancies/pregnancy	0%	11.7% (2/17)*	<0.05*
Early miscarriages/pregnancy	25% (1/4)	52.9% (9/17)*	
Fetal death in utero or premature delivery (preeclampsia)/pregnancy	75% (3/4)	0%*	
Normal outcome			
Full-term live birth/pregnancy	0%	35.3% (6/17)	

Table 2: Description of aPL positive patients ($n = 8$) in IVF treated population.

Women's age (years)	IVF indication	N IVF attempts	N pregnancies	Pregnancy outcome	aPL	aβ2GPI IgA	aCL IgG
30	Unexplained infertility	3	0		+	−	−
31	Mixed infertility	3	0		+	−	−
39	Unilateral tubal obstruction	4	3 (sp)	Early miscarriages	+	+	−
33	Female infertility	2	2 (sp)	Early miscarriages	+	+	−
30	Mixed infertility	2	1 (IVF)	Early miscarriage	+	+	−
36	Mixed infertility	6	2 (IVF)	1 premature delivery and 1 fetal death in utero (preeclampsia during these 2 pregnancies)	+	+	−
37	Male infertility	5	1 (IVF)	Fetal death in utero (venous thrombosis of umbilical cord)	+	+	−
33	Mixed infertility	4	1 (sp)	Early miscarriage	+	−	+

sp: spontaneous pregnancy, IVF: pregnancy after IVF cycle.

mean number of IVF attempts: 3.5 ± 1.2), no pregnancy was obtained after IVF.

Conventional aPL as well as aCL and aβ2GPI of IgA isotype was analyzed in this cohort of patients undergoing IVF. A total prevalence of 20% (8/40) of aPL was found, significantly different from that of the control population ($P < 0.0005$). Among the panels of aPL tested aβ2GPI IgA antibodies were the most prevalent (5/8, 62.5%), with a prevalence significantly higher in patients (12.5%, 5/40) than in controls (1%, 1/100) ($P = 0.01$). No significant difference was found for LA, aCL (IgG, IgM, and IgA), or aβ2GPI (IgG and IgM). No difference was found between aPL positive and negative patients, according to history of spontaneous pregnancies before IVF treatment, the numbers of IVF attempts, and success of embryo implantation after IVF (Table 1). In contrast no accomplished pregnancy was evidenced in the aPL positive patients (Table 1). Positivity of aPL was significantly associated with abnormal IVF pregnancy outcome such as premature embryo or fetal losses. Therefore, the percent of full-term live birth after embryo implantation was significantly lower in aPL positive compared to aPL negative patients. A detailed analysis of aPL + patients presented in Table 2 evidenced that pregnancy outcome was abnormal in every case of our series (early miscarriages, fetal death in

utero, or premature delivery in preeclampsia context). Within the subgroup of patients with secondary infertility, three patients were positive for aPL (Table 2). Importantly, 3 of the 5 positive women for aβ2GP1 IgA antibodies became pregnant after IVF but without any normal delivery since one early miscarriage was evidenced for the first patient, one premature delivery and one fetal death in preeclampsia context were evidenced for the second one, and one fetal death because of venous thrombosis of umbilical cord was evidenced for the third one.

4. Discussion

In this study, we showed a significant higher prevalence of aPL, in particular aβ2GPI IgA antibodies, in women undergoing in vitro fertilization treatment compared to controls. One should note that the studied population was not representative with the whole population, because patients were previously selected after at least two IVF attempts with good quality embryos available for transfers but not followed by pregnancy (implantation failure). Comparison between positive and negative aPL patients revealed no difference in success of embryo implantation, as shown by the outcome of IVF. This result was also obtained in the subgroup of patients with secondary infertility. In contrast, no accomplished pregnancy with full-term live birth was observed in aPL positive IVF patients. Altogether results led us to propose the assessment of aPL, in particular aβ2GPI IgA antibodies, in support of IVF treated women.

Antiphospholipid antibodies (aPL) are a family of autoantibodies that are associated with pregnancy complications including stillbirth and recurrent miscarriage [7, 8]. According to data from the literature, the prevalence of aPL in infertile women varies from 4% to 66% [9]. These differences can be explained by the lack of assay standardization, the panel of antibodies tested, and the definition of population of patients. In our study, as the sample size is perhaps not representative of all the patients having a treatment of IVF, we find a total prevalence of 20%, agreeing with the average of combining results of the literature [10]. Our study highlights the high prevalence of aβ2GPI IgA antibodies in the population of women undergoing IVF. Beta 2 glycoprotein 1, the major antigen in the antiphospholipid syndrome, is synthesized by hepatocytes, endothelial cells, and also trophoblast cells. It has been shown that this protein can adapt to different conformations, a circular, an S-shaped, and a J-shaped conformation [11]. Several studies have showed that aβ2GPI antibodies may represent the main pathogenic antibodies in obstetrical APS [12] that could cause thrombosis of placental blood vessels, dysfunctions of trophoblasts in the peri-implantation period, or an imbalance of maternal hormones [7, 13]. In particular, Chamley et al. have proposed the action of aβ2GPI on trophoblast proliferation as a mechanism of fetal death [14]. In addition, other studies underlined the necessity to search IgA antibodies for β2GPI in women undergoing IVF [6, 15]. Recently, we emphasize the determination of aβ2GPI IgA in patients with clinical manifestations of antiphospholipid syndrome [16]. This was corroborated by Murthy et al. that recommended testing aβ2GPI IgA when the other aPL are negative [17].

Although a larger cohort of patients should be tested to comfort our data, we did not find association between the presence of aPL and embryo implantation failure. In contrast we found a significant association between aPL positivity and abnormal pregnancy outcome as no accomplished pregnancy was obtained in aPL positive patients. These IVF treated patients could not be classified as APS patients because of their incomplete clinical expression and/or their positivity for "nonconventional aPL" (IgA isotype). Nevertheless, our results suggest that they should be considered as APS patients and thus cared for and treated accordingly. The presence of aPL and in particular aβ2GPI IgA antibodies does not explain implantation failure but should be regarded as a pejorative factor for successful ongoing pregnancy.

5. Conclusions

There are various causes of reproductive failure and more than 10% are still unexplained. Testing aPL and in particular aβ2GPI IgA should be important to exclude thrombotic state and autoimmune disease and to inform clinical practitioners. In a perspective way, it could be the basis for defining novel therapeutic strategy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

The Multiple Roles of EG-VEGF/PROK1 in Normal and Pathological Placental Angiogenesis

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Placentation is associated with several steps of vascular adaptations throughout pregnancy. These vascular changes occur both on the maternal and fetal sides, consisting of maternal uterine spiral arteries remodeling and placental vasculogenesis and angiogenesis, respectively. Placental angiogenesis is a pivotal process for efficient fetomaternal exchanges and placental development. This process is finely controlled throughout pregnancy, and it involves ubiquitous and pregnancy-specific angiogenic factors. In the last decade, endocrine gland derived vascular endothelial growth factor (EG-VEGF), also called prokineticin 1 (PROK1), has emerged as specific placental angiogenic factor that controls many aspects of normal and pathological placental angiogenesis such as recurrent pregnancy loss (RPL), gestational trophoblastic diseases (GTD), fetal growth restriction (FGR), and preeclampsia (PE). This review recapitulates EG-VEGF mediated-angiogenesis within the placenta and at the fetomaternal interface and proposes that its deregulation might contribute to the pathogenesis of several placental diseases including FGR and PE. More importantly this paper argues for EG-VEGF clinical relevance as a potential biomarker of the onset of pregnancy pathologies and discusses its potential usefulness for future therapeutic directions.

1. Introduction

The placenta is one of the most densely vascularized organs in the organism [1]. At term, it has developed a capillary network that is ≅550 km in length and 15 m^2 in surface area [1, 2]. During the course of 9 months, placental vascular network expansion is a dynamic process characterized by intravilli vasculogenesis followed by branching and nonbranching angiogenesis [3]. Vasculogenesis starts during the third week after conception and involves *de novo* formation of new vessels. This process is characterized by the formation of the first blood vessels from differentiation of pluripotent mesenchymal cells into haemangiogenic stem cells [4]. The subsequent step, angiogenesis, starts during the fifth week after conception and refers to the development of new vessels from preexisting vessels [4, 5]. From day 32 to week 25 after conception, haemangioblastic cords formed by vasculogenesis develop into a richly branched villous capillary bed by

two mechanisms: elongation of preexisting tubes and lateral ramification of these tubes (sprouting angiogenesis). Around week 25, this process switches from branching to nonbranching angiogenesis [4, 5]. Nonbranching angiogenesis occurs in mid and late gestation and it is mainly characterized by endothelial cell proliferation leading to an increase in the surface of the endothelial tissue. These processes ensure the increasing supply of gas and nutrient for the growing fetus [4, 5].

For many years, morphological and functional diversity among endothelia were thought to result from vascular bed-specific response to ubiquitous and tissue-restricted mediators. In this context, several ubiquitous growth factors (i.e., vascular endothelial growth factor (VEGF) and basic fibroblastic growth factor (bFGF)), as well as numerous pregnancy-specific angiogenic factors (i.e., placental growth factor (PlGF) and human chorionic gonadotropin hormone (hCG)), have been reported to regulate either the intravilli or the fetomaternal angiogenesis [4, 5].

The existence of tissue-specific angiogenic factors has been postulated for many years [6–9] but it only recently received confirmation when such a factor, named endocrine gland-derived vascular endothelial growth factor/prokineticin 1 (EG-VEGF/PROK1), was finally characterized [10].

2. EG-VEGF/PROK1 in the Placenta

2.1. EG-VEGF, a New Angiogenic Factor Highly Expressed in the Reproductive Organs. In 2001, a novel family of angiogenic mitogens, named the prokineticins, has been characterized with restricted expression profiles and selective endothelial cell activity [10]. This family is composed of two members, EG-VEGF/PROK1 and PROK2, with multiple roles in physiological and pathological conditions. Human EG-VEGF and PROK2 proteins exhibit 44% amino-acid identity and share the same G protein-coupled receptors (PROKR), termed PROKR1 and PROKR2 [11]. PROKR bind the peptide hormones EG-VEGF and PROK2, with PROK2 showing a moderately higher affinity than EG-VEGF for both receptors [11]. Although *prokr1* and *prokr2* genes are located on two different chromosomes (2q14 and 20p13, resp.), they encode proteins that share 85% amino acid identity and that exhibit the greatest differences in the N-terminal domains [11]. The exact functions of each receptor are not fully elucidated, but recent data reported specificities of actions in the heart and the placenta, where PROKR1 is preferentially involved in proliferation and angiogenic processes and PROKR2 is mainly implicated in endothelial permeability [12–14]. The prokineticins show different patterns of expression and preferential sites of actions. PROK2 is mainly associated with the nervous system, whereas EG-VEGF is predominantly associated with the reproductive tract and the endocrine organs, including the ovary and the placenta [10]. In the last decade, many studies have shed light on the angiogenic roles of EG-VEGF in the reproductive organs. In the gonads, EG-VEGF has been reported to impact physiological and pathological angiogenic processes [15–18]. In endothelial cells isolated from steroidogenic tissues, EG-VEGF has been shown to promote proliferation, differentiation, survival, chemotaxis, and fenestration of capillary endothelial cells [10, 19, 20]. Interestingly, the effect of EG-VEGF on endothelial cells seems to be tissue specific as it has no effect on endothelial cells derived from brain capillary, aorta, umbilical vein, or cornea [10, 21].

2.2. Placental Expression of EG-VEGF/PROKR Throughout Pregnancy. EG-VEGF is the major prokineticin in the female reproductive tract. In the placenta, EG-VEGF and its receptors are highly expressed [10, 22–26]. EG-VEGF is mainly localized to the syncytiotrophoblast layer (ST) with a mild expression in the cytotrophoblast layer (CT) [22]. EG-VEGF receptor PROKR1 is abundant in the CT, the placental microvascular endothelial cells (HPEC), and the Hofbauer cells (Ho), whereas PROKR2 is expressed by ST, HPEC, Ho, and extravillous trophoblasts (EVT) [22–25]. EG-VEGF and its receptors show a dynamic profile throughout pregnancy. In the placenta, EG-VEGF, PROKR1 and PROKR2 are predominantly expressed during the first trimester of pregnancy. During early pregnancy, EG-VEGF/PROKR1 peaks at 8–11 weeks of gestation (wg) and then gradually decreases by the end of the first trimester, whereas PROKR2 expression is maintained over the first trimester [22, 23, 26]. In nonpregnant women, circulating EG-VEGF levels are around 50 pg/mL [23]. During pregnancy, these levels significantly increase fivefold during the first trimester (≅250 pg/mL) and then gradually decrease to reach those observed in nonpregnant women by the end of the second trimester of pregnancy [23].

2.3. EG-VEGF/PROKR System and Placental Development. EG-VEGF is directly involved in the growth of the placental villi with multiple actions on various cell types. This factor is mainly produced by the ST [22, 23] and acts on the adjacent CT to increase their proliferation [27] at the expense of their differentiation to ST. This phenomenon contributes to the overall growth of the placental villi, an important aspect of placental development during the first trimester of human pregnancy. Moreover, EG-VEGF promotes the proliferation of anchoring trophoblasts and inhibits early EVT migration and invasion. In first trimester human placenta, anchoring trophoblastic plugs obstruct the spiral arteries and prevent maternal oxygenized blood from entering into the intervillous space. This physiological process creates a local hypoxic environment indispensable for normal placental and fetal development. At the end of the first trimester, anchoring trophoblast generates multilayered columns of EVT that invade the uterine blood vessels and remodel the maternal spiral arteries from minimal-flow/high-resistance vessels into larger diameter vessels with low resistance and high flow. The contribution of EG-VEGF in the formation and maintenance of the trophoblastic plugs throughout the first trimester protects the fetoplacental unit from early oxidative stress against which the first trimester placenta is not equipped [28, 29]. Altogether, these data demonstrate that EG-VEGF is a new placental growth factor that contributes to ensure

the maintenance of pregnancy during the first trimester of pregnancy.

3. EG-VEGF Control of Placental Angiogenesis

Beside its effects on the trophoblastic component of the placental villi, growing evidences established the involvement of EG-VEGF and its receptors in placental angiogenesis. The following paragraphs will discuss how EG-VEGF controls the two main types of placental angiogenesis, the intravilli and the fetomaternal interface one.

3.1. EG-VEGF Effects on Fetomaternal Angiogenesis. Trophoblastic invasion of spiral maternal arteries and decidua is the key process that establishes the fetomaternal circulation by the end of the first trimester of pregnancy. This process is known to be temporally and spatially controlled. Key studies from our group showed that EG-VEGF is a negative regulator of human EVT invasion. This statement was based on the demonstration that EG-VEGF inhibits EVT migration and invasion in HTR-8 cells (an extravillous trophoblastic cell line) and in first trimester villous explant culture systems and the demonstration that EG-VEGF inhibits HTR-8 cells organization into tube-like structures [23]. These data strongly suggest that EG-VEGF acts as an inhibitor of trophoblast differentiation towards an invasive phenotype and are consistent with a model of normal placentation in which downregulation of EG-VEGF expression at around 11 wg promotes differentiation of EVT. Therefore, the decrease in EG-VEGF circulating and placental levels at the end of the first trimester could contribute, with other factors, to extravillous trophoblast (EVT) invasion and to the establishment of fetomaternal circulation (Figure 1).

3.2. EG-VEGF Effects on Intravillous Angiogenesis. The placenta is composed of two types of endothelial cells: the microvascular endothelial cells (HPEC for human placental endothelial cells), cells that lie in the fetal capillaries of chorionic villi, and the umbilical vein macrovascular endothelial cells (HUVEC). It is well established that the endothelial cells that comprise the vascular beds of specific tissues are extremely diverse and display numerous tissue-specific characteristics in their phenotypes, growth properties, functions, and ultrastructure such as the intercellular junctions or the presence of fenestrae (for reviews see [30–32]). In accordance with these data, HPEC clearly differ from HUVEC in their phenotype and physiological functions [33–35]. HPEC show a spindle-shape that largely differs from the more polygonal phenotype of HUVEC [33–35]. HPEC grown *in vitro* secrete higher amounts of thromboxane and angiotensin II than HUVEC [34]. Furthermore, HPEC also show higher proliferative responses to tissue-restricted mediators (i.e., PlGF) in comparison to HUVEC [33–35]. Interestingly, ubiquitous angiogenic factors (i.e., FGF-2 and VEGF-A) exhibit similar effects on HPEC and HUVEC, suggesting that some tissue-restricted factors might contribute to endothelial singularity [6, 7, 33–35].

In 2010, the angiogenic effects of EG-VEGF have been investigated in HPEC and HUVEC. Interestingly, EG-VEGF displayed specificity towards distinct vascular beds with major effects on HPEC-mediated angiogenesis (Figure 2). EG-VEGF increased HPEC proliferation, migration, tube-like formation, and sprouting, without affecting HUVEC-mediated angiogenesis. Both EG-VEGF receptors are expressed *in vivo* by placental HPEC and HUVEC. Quantification of PROKR1 and PROKR2 protein levels in endothelial cell primary cultures revealed larger expression of both receptors in HPEC than in HUVEC. This difference suggests a higher sensitivity of HPEC for EG-VEGF. Altogether, these data confirm the two distinct endothelial identities of HPEC and HUVEC and stress the importance to investigate placental angiogenesis with appropriate microvascular endothelial models.

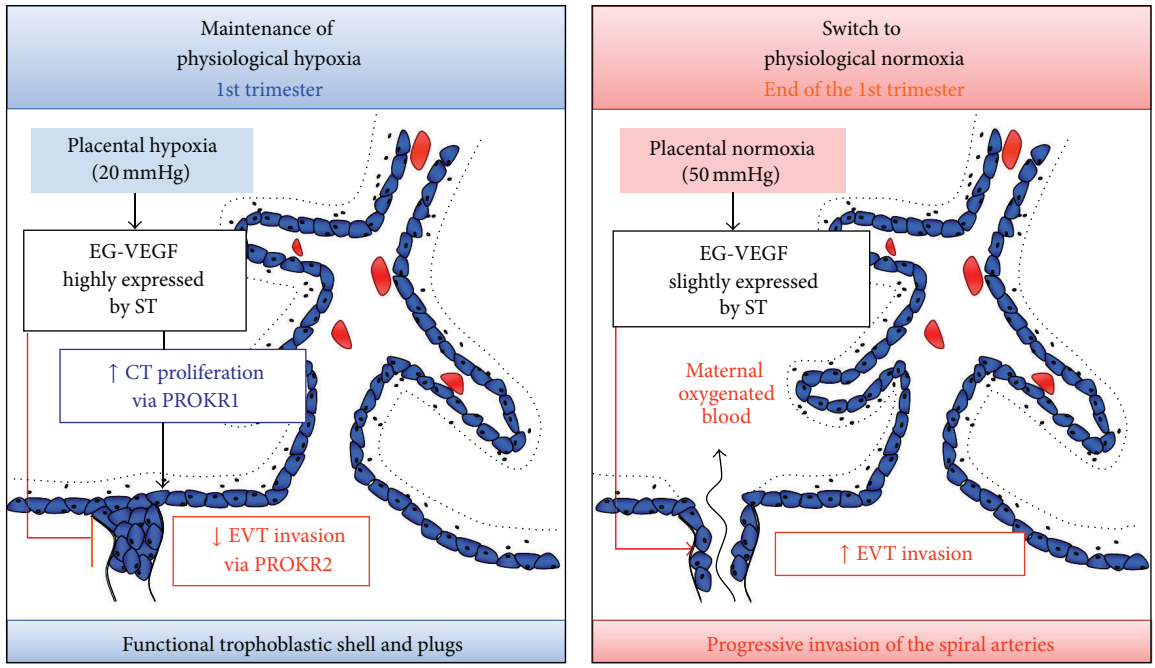
The understanding of the mechanisms underlying placental angiogenesis was significantly improved by the use of *in vitro* models using appropriate endothelial cell cultures. In the last decades, numerous two- and three-dimensional assays helped to bring new insight into the understanding of EG-VEGF-mediated placental angiogenesis.

3.2.1. Investigation of EG-VEGF Angiogenic Roles Using 2D-Primary Culture Models

(i) Placental Endothelial Cells Primary Culture. HPEC can be successfully isolated from the placental microvasculature by enzymatic perfusion of the placenta [21, 34] or from digestion of placental tissues [36–40]. Despite growing evidence demonstrating placental endothelial heterogeneity, HUVEC are still the most commonly used cell type for angiogenesis studies [8, 41]. Nevertheless, their above-mentioned differences in phenotype, gene expression, and physiology substantiate that microvascular endothelial cells are the unique model to use to investigate placental angiogenesis.

(ii) EG-VEGF Effect on HPEC Proliferation and Survival. Using complementary 2D-models, recent experiments have established the positive effect of EG-VEGF on HPEC proliferation and survival [21]. HPEC proliferation has been shown to be stimulated under EG-VEGF treatment, as assessed by [3H]-thymidine incorporation and Ki-67 staining [21]. EG-VEGF was also shown to promote endothelial survival as evidenced by decreased caspase-3 activity [21]. These results demonstrate that EG-VEGF is a new mitogenic and prosurvival factor for microvascular endothelial cells of the placenta [21].

(iii) EG-VEGF Effect on Placental Endothelial Cell Migration and Tubulogenesis. Using a quantifiable 2D-model of tubulogenesis, we established that EG-VEGF strongly promotes HPEC morphogenesis into tube-like structures [42–44]. Using a monolayer wound-healing assay, EG-VEGF effect on HPEC migration has been investigated. This assay is one of the earliest developed methods to study directional cell migration *in vitro* [45]. Our data demonstrates that EG-VEGF



(a) (b)

Figure 1: Proposed model of EG-VEGF-mediated effects on the fetomaternal angiogenesis during the first trimester of pregnancy. (a) and (b) represent cartoons of placental villi with EG-VEGF expression and actions on CT and EVT. (a) During the first trimester, EG-VEGF increases CT proliferation via PROKR1 activation and inhibits EVT invasion via PROKR2 activation. EG-VEGF/PROKR actions participate actively in trophoblastic shell and plugs constitution and contribute to the maintenance of physiological hypoxia during the first trimester of pregnancy. (b) At the end of the first trimester, EG-VEGF secretion declines. This contributes with other factors to EVT invasion and to the establishment of the fetomaternal circulation.

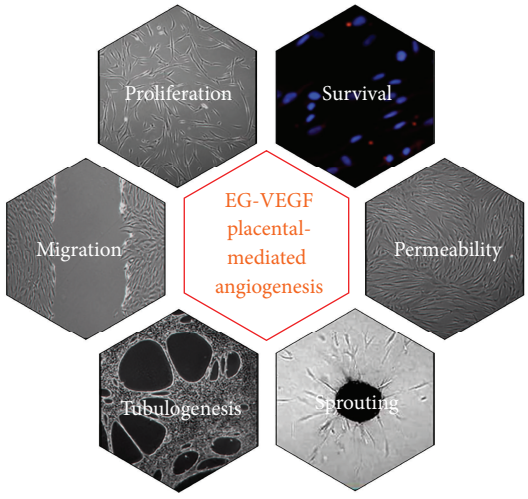


Figure 2: EG-VEGF is a new placental angiogenic factor. It controls placental growth via its multiple actions on endothelial cells within the chorionic villi.

significantly increased the migration of HPEC in this model [21].

(iv) EG-VEGF Effect on Placental Endothelial Permeability. In the placenta, the microvascular endothelium is known to form a selective permeable interface that participates in the fetomaternal transports of solutes and nutrients. Therefore, the maintenance of a semipermeable endothelium is critically important for the development of the fetus. Placental microvasculature is not static and can be modulated by exposure to specific stimuli that affect intracellular permeability and paracellular transport. Using the HPEC model, we demonstrated that EG-VEGF increased both transendothelial permeability and paracellular transport [21]. Measurement of the ion flux through the primary HPEC monolayer was evaluated by transendothelial electrical resistance (TEER) measurement [21]. The use of siRNAs and blocking antibodies demonstrated that PROKR2 was specifically mediating EG-VEGF effects on cell permeability. In addition, the effect of EG-VEGF on the paracellular transport was also investigated by measuring [3H]-mannitol transport through HPEC monolayer. We observed that EG-VEGF almost doubled the [3H]-mannitol transport capacities of HPEC [21]. These results imply that EG-VEGF controls not only placental angiogenesis but also some physiological properties of placental vasculature such as permeability and transport of solute molecules. Altogether, the results suggest that EG-VEGF acts as a vascular bed-specific angiogenic factor providing an optimal vascular supply during human pregnancy.

3.2.2. Investigation of EG-VEGF Angiogenic Roles Using 3D-Primary Culture Models. In addition to two-dimensional cell

culture systems, 3D-models have also been employed to investigate EG-VEGF effect on placental angiogenesis.

(i) EG-VEGF Effect on Intravilli Vascularization Using Placental Explant Model. Explants of human placenta are commonly used to study many tissue functions including cellular proliferation and differentiation [46]. In this system, EG-VEGF has been described to increase the number of differentiated endothelial cells (CD31+) within the villous tissue, suggesting an increase in the vascularisation within the placental villi [46]. This result is consistent with its proliferative and prosurvival effects observed in the 2D-primary microvascular cells model [21].

(ii) EG-VEGF Effect on Endothelial Cell Sprouting. The 3D endothelial spheroid model was used to study the role of EG-VEGF on placental endothelial cell sprouting. EG-VEGF significantly increased HPEC sprouting in a dose-dependent manner. Importantly, EG-VEGF treatment has a stronger effect than VEGF on HPEC sprouting [21]. The use of 3D-endothelial spheroid models confirmed the positive effect of EG-VEGF on placental angiogenesis previously reported using 2D-models. The use of siRNAs and blocking antibodies demonstrated that the effect of EG-VEGF on HPEC sprouting was specifically mediated by PROKR1. Such a selectivity of PROKR1 action in angiogenesis has also been found in other organs [12–14].

Using multifaceted strategies that included molecular, immunochemical, and functional approaches, several recent publications have shed lights on EG-VEGF key roles in placental angiogenesis via its specific effect on microvascular endothelial cells proliferation, migration, survival, tube organization, sprouting, permeability, and paracellular transport. Altogether, these findings imply that EG-VEGF might act in concert with other angiogenic factors to coordinate series of events that ensure the success of placental vascular development.

3.3. EG-VEGF, a Mediator of Placental Angiogenesis. During the last decade, many angiogenic actors have been described as regulators of the EG-VEGF/PROKR system, suggesting that EG-VEGF regulation of placental angiogenesis could be of direct or indirect form.

3.3.1. Hypoxia: A Key Actor of Placental Angiogenesis and a Regulator of EG-VEGF. The human placenta develops in a low oxygen environment from the beginning of implantation to the end of the first trimester of pregnancy, due to proliferative trophoblast plugs within the maternal arteries that restrict blood flow into the intervillous space. This physiological hypoxia plays a key role in the modulation of the expression of several angiogenic factors [47, 48], including EG-VEGF. Numerous studies demonstrated that EG-VEGF is upregulated by hypoxia suggesting that this cytokine might mediate some of its angiogenic effects.

3.3.2. Human Chorionic Gonadotropin (hCG): A Pivotal Hormone in Placental Angiogenesis That Increases EG-VEGF/PROKR System. Increasing evidence suggests that angiogenic effect of hCG on placental endothelial cells could be mediated by prior induction of EG-VEGF [49–51]. EG-VEGF and hCG are mainly secreted by the syncytiotrophoblast layer and exhibit similar patterns of expression with a peak around 8–10 wg. Recent findings demonstrate a new physiological regulation of EG-VEGF/PROKR system by hCG during the first trimester of pregnancy [51]. Using placental explants and primary trophoblast cultures, it has been established that hCG significantly increases EG-VEGF mRNA synthesis and protein secretion via the activation of the cAMP and protein kinase A signaling pathway [51]. HCG also induces mRNA and protein expression of PROKR1 and PROKR2 in first trimester human placenta [51]. These results reveal a new role for hCG in human placentation through its stimulation of the EG-VEGF/PROKR system and might explain the peak of expression of EG-VEGF and its receptors during the first trimester of pregnancy (8–11 wg). Moreover, EG-VEGF/PROKR regulation by hCG strongly suggests that some of the angiogenesis effects of hCG on placental villi might be mediated by EG-VEGF [49–51]. HCG is involved in many important functions in placental angiogenesis, including HPEC proliferation and sprouting [49, 50, 52].

3.3.3. MAPK and PI3K/AKT: Key Signaling Pathways of EG-VEGF Angiogenesis. The MAPK and PI3K/AKT signaling pathways are highly involved in angiogenesis [53–57]. They play an essential role in the formation of normal blood vessels during development via their direct effects on endothelial cell proliferation, survival, differentiation, migration, and angiogenesis and contribute indirectly to the induction of angiogenesis by increasing the expression of numerous angiogenic factors such as VEGF, nitric oxide, and angiopoietin [53–57]. In HPEC, EG-VEGF induces a strong phosphorylation of MAPK and AKT proteins [21]. These data confirm the involvement of EG-VEGF in HPEC migration and survival and highly suggest its contribution in the induction of others angiogenic factors. Further investigations are required to determine the veracity of this hypothesis.

3.3.4. IL-8: A Crucial Placental Angiogenic Factor Upregulated by EG-VEGF. Using third trimester placental explant model, a recent study has demonstrated IL-8 induction by EG-VEGF, potentially via activation of PROKR1 [24]. IL-8 is an important placental angiogenic factor that promotes endothelial cell chemotaxis and proliferation [58]. This cytokine is expressed in the human placenta throughout pregnancy and facilitates vascular permeability [59, 60]. Moreover, IL-8 is upregulated by HIF1α and is increased in conditions characterized by pathological angiogenesis such as placental vascular insufficiency [39, 61] and preeclampsia [62].

Altogether, these results demonstrate that EG-VEGF can directly and/or indirectly control placental angiogenesis. Hence, deregulations in EG-VEGF and/or its receptors could well be associated to vascular-associated pathologies during pregnancy.

4. Role of EG-VEGF in Pregnancy-Related Pathologies

It is well established that placental development depends on controlled growth, invasion, and differentiation of the trophoblast cells and on an adequate vascular development [63]. Hence, placental angiogenesis is highly linked to feto-placental growth and fetomaternal exchanges. Abnormal angiogenesis has been associated with different pregnancy-related pathologies such as ectopic pregnancy, recurrent pregnancy loss (RPL), gestational trophoblastic diseases (GTD), preeclampsia (PE), and fetal growth restriction (FGR). EG-VEGF/PROKR expressions vary across normal pregnancy and in complicated pregnancies [21–23, 27, 51, 64, 65]. Recent studies have established correlations between abnormal EG-VEGF expression and pregnancy-specific diseases, ranging from miscarriage to intrauterine growth restriction and preeclampsia. These results strongly suggest that EG-VEGF deregulation could be associated with adverse pregnancy outcomes.

4.1. EG-VEGF in Recurrent Pregnancy Loss (RPL). Recently, several publications demonstrated the involvement of EG-VEGF and its receptors in the etiology of RPL [66–68]. This pathology is widely attributed to chromosomal aneuploidy in the conceptus and/or to a deregulation in the expression of uterine factors. In the last decade, histological and ultrasound studies illustrated a link between recurrent miscarriage and abnormal vascularization in the placental bed, suggesting that early disturbance in placental vascular development might contribute to the pathogenesis of miscarriages [69–71]. In 2010, EG-VEGF receptor gene polymorphisms and haplotypes have been associated with RPL [67]. These data advocate that a deregulation in EG-VEGF-mediated signaling pathways could affect placental angiogenesis contributing to the pathogenesis of RPL. Further investigations are required to validate this hypothesis.

4.2. EG-VEGF in Gestational Trophoblastic Diseases (GTD). Recent data have shown that maternal circulating levels of EG-VEGF are increased in patients undergoing molar pregnancies, a severe form of the gestational trophoblastic disease (GTD) [72]. GTD includes a wide spectrum of pathologies ranging from partial/complete hydatidiform moles to gestational trophoblastic tumors. Increasing data report a poor placental vascularization during the first trimester of GTD [23, 72–74]. EG-VEGF controls numerous angiogenic processes during the first trimester of pregnancy [64] and it is significantly increased by hCG [51], a hormone that is also highly upregulated in GTD. Altogether, these results suggest that EG-VEGF increased circulating levels reported in GTD could be a consequence of hCG deregulation and propose that its angiogenic effects might contribute to the pathogenesis of GTD during the first trimester of pregnancy. Further investigations are ongoing to identify the participation of EG-VEGF in the development of gestational trophoblastic diseases and to investigate the potential use of this placental specific factor for early diagnosis and treatment of GTD.

4.3. Preeclampsia (PE). Recent publications report EG-VEGF and PROKR1 deregulations in PE and suggest their involvements in the development of this pregnancy-related pathology [23, 75]. PE is a systemic syndrome that is characterized by hypertension and proteinuria that appears around 22 weeks of gestation. PE affects approximately 5-6% of pregnancies worldwide accounting for nearly 18% of maternal deaths [76, 77]. The etiology of PE remains largely unknown but increasing evidences suggest that it originates from abnormal placentation. In the fetomaternal unit, PE is marked by insufficient trophoblast invasion and poor maternal spiral artery remodeling [78]. Further investigations pointed out the potential involvement of angiogenic factors and their receptors in PE development and stressed their potential importance in the prediction of its occurrence [79]. For instance, increased expression of soluble Flt1 and soluble endoglin in the maternal circulation weeks before the onset of PE has been reported and suggested as predisposing factors of the disease [79]. Recently, several findings strongly suggest that deregulation of EG-VEGF expression in the placenta might be associated with the development of PE [21–23, 27, 51, 64, 65]. In 2008, we reported a significant increase in EG-VEGF levels in the sera of third trimester PE patients as compared to age-matched controls [23]. More recently, a significant decrease in EG-VEGF mRNA expression has been reported in PE placentas [75], suggesting that local expression of EG-VEGF could be impaired at the transcriptional level in PE placentas. Determining the impact of these deregulations on placental development is difficult, as the relationship between maternal EG-VEGF circulating level and its local expression in the placenta remains unclear. EG-VEGF is also upregulated by hypoxia and hCG, two factors that are highly associated with the occurrence of PE [80–83]. The dynamic profile of EG-VEGF expression throughout pregnancy and its control of trophoblast invasion and placental angiogenesis strongly suggest that this cytokine contributes to the etiology of PE. Altogether, these results show that systemic and local EG-VEGF deregulation is associated with this pathology in the third trimester of pregnancy. However, we cannot conclude whether abnormal EG-VEGF levels are a cause or consequence of PE development. Further studies are needed to clarify whether EG-VEGF could be a predictive marker of PE.

4.4. Fetal Growth Restriction (FGR). Optimal growth of the fetus throughout pregnancy depends on an adequate vascular network in the fetomaternal unit [84]. Therefore, abnormalities in placental microvascular development toughly compromise the supply of nutrients and hormones, leading ultimately to fetal growth restriction [85]. Interestingly, recent findings reported that EG-VEGF circulating levels were significantly higher in FGR patients during the third trimester of pregnancy [27]. These results were confirmed at the placental level where significant increases in EG-VEGF, PROKR1, and PROKR2 mRNA and protein expression were also found [27]. The authors proposed two hypotheses that could explain the association between EG-VEGF/PROKR system upregulation

and the FGR condition. The first one proposed that EG-VEGF increased levels in FGR pregnancies could be a cause of the pathology, as sustained expression of EG-VEGF over the first trimester of pregnancy may compromise the spiral arteries remodeling and contribute to utero-placental hypoxia, a key parameter in the etiology of FGR [86, 87]. The second hypothesis proposed that FGR condition is caused by other predisposing factors that consequently increase EG-VEGF/PROKR system in the placenta and sera of the patients. This hypothesis is supported by recent *in vitro* experiments that demonstrate the strong upregulation of EG-VEGF and its receptors by hypoxia and hCG [22, 51], two parameters that are known to be increased in FGR [86–89]. Further studies are required to determine whether EG-VEGF/PROKR deregulation is a cause or consequence of FGR.

Altogether, these results clearly demonstrate that EG-VEGF and its receptors are closely associated to several pathologies marked by deregulated placental angiogenesis. These recent publications bring evidences for EG-VEGF association to key angiogenic processes and support the interest of new investigations on the predictive value of this factor in several pregnancy-associated pathologies including recurrent pregnancy loss, gestational trophoblastic disease, and placental pathologies associated with fetal growth restriction and/or preeclampsia.

5. Concluding Remarks

Disruption in the balance of placental angiogenesis controlling factors may lead to abnormal vascular development and compromises the success of pregnancy. Alterations in numerous specific angiogenic-signaling pathways have been already described in pregnancy-related diseases. The multiple roles of EG-VEGF in the development of the chorionic villi argue for its clinical relevance as a diagnostic and/or prognostic marker for several placental diseases. The current challenge in the field of reproduction is to discover early biomarkers of abnormal placental angiogenesis to develop successful screening tests for pregnancy disorders. These biomarkers also represent potential new therapeutic targets to "rescue" placental vascular development and thus fetal growth in compromised pregnancies. In the last decade, compelling advances highlighted the pivotal role of EG-VEGF and its receptors in regard to their expressions, multiple roles, and regulations in normal and pathological human pregnancies. These fundamental and clinical results highly suggest that EG-VEGF might be a potential early marker for several pathologies including recurrent pregnancy loss, gestational trophoblastic diseases, FGR, and PE. Further studies are required to evaluate its potential relevance as an early marker of these pregnancy-associated pathologies, probably in combination with other predictive parameters such as uterine arteries blood flow measurements by Doppler ultrasound imaging [90].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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