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NEW THERAPEUTIC TARGETS FOR HUMAN PLACENTAL ANGIOGENESIS DISEASES

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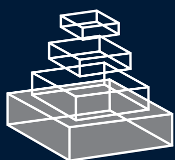

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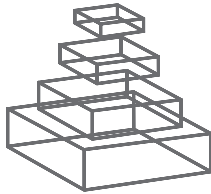

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NEW THERAPEUTIC TARGETS FOR HUMAN PLACENTAL ANGIOGENESIS DISEASES

Topic Editor:
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A large number of publications have described impaired angiogenesis and vasculogenesis present in the feto-placental circulation after pregnancy diseases such as pre-eclamptic pregnancies, gestational diabetes, and intrauterine growth restriction, among others. Results suggest impaired secretion and activity of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), interleukin 8 (IL-8), adenosine and nitric oxide, associates with compromised secretion and activity of anti-angiogenic factors such as soluble receptor of VEGF (sFlt-1), thrombospondin 2, endostatin among others. More recent evidences include the participation of endothelial progenitor cells (EPC), which is reduced in feto-placental circulation in pregnancies such as pre-eclampsia. Despite this knowledge, therapies for placental angiogenesis recovery during pathological pregnancies are far to be tested. However, from the cardiovascular field, it has been described the administration of EPC, alone or used as gene-transfer therapy; or it has been described the potential role of statins (HMGCoA inhibitors), or angiotensin-converter enzyme (ACE) inhibitors for enhancing angiogenesis. Finally, feto-placental tissue is an exceptional source of progenitor and stem cells, which could be used for treated other human diseases such as stroke, myocardial infarction, hypertension, or even cancer. In this research topic, authors highlight physiopatological and clinical importance of the impaired placental angiogenesis, and suggest potential targets for developing innovative therapies.

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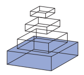

Editorial: New therapeutic targets for human placental angiogenesis disease

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Development of placental vascular tree is structurally and functionally required for both adequate placental growth and delivery of nutrients from mother to the fetus. Impaired placental angiogenesis has been implicated in the pathophysiology of pregnancy complications which have immediate and long-lasting effects on the mother and her child; such complications include fetal growth restriction and macrosomia as well as pre-eclampsia and gestational diabetes. The mechanisms underlying the deregulation of placental angiogenesis include a misbalance between the secretion and activity of pro-angiogenic vs. anti-angiogenic factors. Despite this, therapies for improving placental angiogenesis in pathological pregnancies have not been directly tested in humans and the aim of this Research Topic in Frontiers is to highlight potential therapeutic targets.

Physical activity during pregnancy might be effective for reducing the risk of developing pregnancy complications. In this regard, Rodriguez and Gonzalez (2014) explain how physical activity affects placental endothelial shear stress and vasodilation, via synthesis and release of nitric oxide (NO). Whilst these processes are relatively well-described in the adult circulation, they are not completely understood in the feto-placental circulation. Then, they analyze how training affects hemodynamics in the mother, which may favor blood supply to the placenta, favoring placental angiogenesis, glucose and oxygen delivery and thereby fetal growth and development. Considering this concept, the potential beneficial effects of moderate levels of maternal exercise may constitute a non-pharmacological intervention for improving maternal and fetal hemodynamic alterations observed during pathological pregnancies such as pre-eclampsia, gestational diabetes, or intrauterine growth restriction.

On the other hand, Herrera et al. (2014) characterizes the response of the umbilical-placental vasculature to hypoxia induced experimentally or through living at high-altitude. They later describe how hypoxia and oxidative stress may impact placental establishment and therefore have consequences for embryonic development from the early stages of gestation. The authors also highlight the implications of a prolonged hypoxic environment in inducing adaptive responses of the placenta in pregnancies at high altitude, or conversely in the development of placental vascular pathologies such as those observed during intrauterine growth restriction and pre-eclampsia. The clinical significance of these findings are discussed, emphasizing that balancing levels of oxidative stress may be a target for improving placental vascular alterations.

Impaired remodeling of maternal spiral arteries by invasive placental trophoblast is thought to be the primary cause of intrauterine hypoxia and the pathophysiology of pre-eclampsia and/or intrauterine growth restriction. In this regard, Salomon et al. (2014) presented novel data which improve our understanding in the interactions of trophoblast with vascular smooth muscle cells (VSMC) via exosomes. They assess exosomes release and content in two extravillous trophoblast cell lines (JEC-3 and HTR-8/SVneo), and relate their observations with the capacity of these exosomes for promoting migration of VSMC. They found high release, differential composition, and high promotion of VSMC migration in exosomes released from HTR-8/SVneo cells compared with JEC-3 cells. Interestingly, their findings indicate that modulation of VSMC migration depends on exosome cargo, exosomal structural integrity, and intracellular incorporation of these exosomes into VSMC. These promising results highlight the potential of exosomes as diagnostic biomarkers of normal or abnormal placentation, or perhaps may constitute an alternative method for introducing molecules with a therapeutic aim.

Bidwell and George (2014), also show that employing a carrier protein called elastin-like polypeptide (ELP) may provide a method for delivering therapeutic agents/drugs to the placenta during pathological pregnancies, including pre-eclampsia. These particular peptides may offer many advantages since researchers could manipulate their length, sequence, and therefore biochemical properties in order to selectively target a particular cell and/or tissue. More importantly, due to the high molecular weight of ELP, it cannot cross the placenta avoiding fetal exposure and potential developmental defects. Preclinical studies are currently underway exploring whether fusing ELP with proteins like vascular endothelial growth factor (ELP-VEGF), the p50 subunit of NF-kB or with the Nox2 docking sequence (Nox2ds) affects their half-life, or activity.

Cindrova-Davies (2014) provides an overview of the pathophysiology of pre-eclampsia. In particular, the contribution of

hypoxia, the equilibrium between oxidants-antioxidants, soluble vascular endothelial growth factor receptor 1 (s-Flt1), the bioavailability of NO and hydrogen sulfide (H_2S) and the levels of pro-inflammatory and endoplasmic reticulum (ER) stress in the development of this disease are discussed. Considering this biological background, She identifies potential pharmacological targets for improving placental function in pre-eclampsia. These pharmacological tools may include ER chaperones such as ursodeoxycholic acid, as well as vasoactive molecules including L-arginine, NO-donors, H_2S and statins; some of them are indeed being tested in clinical trials.

Also on the topic of pre-eclampsia, Escudero et al. (2014) propose a challenging hypothesis that the impaired adenosine-mediated placental angiogenesis observed in pre-eclampsia might also be present in the offspring at birth and lead to a reduction in microvascular formation and compromised hemodynamic regulation. According with this hypothesis, adenosine could constitute another avenue for recovering both impaired placental angiogenesis and future complication in the offspring.

On the other hand, Guzman-Gutierrez et al. (2014) raise the notion that the placenta controls the bioavailability of thyroxine (T_4) and tri-iodothyronine (T_3) in the fetal circulation. They propose that the placenta may respond in an adaptive fashion to low maternal T_4 although placental control of T_3/T_4 may be impaired during chronic maternal hypothyroxemia or hypothyroidism. They also present evidence suggesting that T_3/T_4 controls endothelial function, via effects on placental angiogenesis and the synthesis and release of vasodilators and vasoconstrictors. Additionally, they suggest that low levels of T_4 observed during gestational diabetes might contribute to impaired vascular function observed in this disease.

Nevertheless, Saez et al. (2014) review how ER stress could impair endothelium migration, one of the initial steps in the angiogenesis process. In addition they suggest that obesity, a well-described condition associated with ER stress, could also drive impaired placental angiogenesis. Their analysis includes characterizing potential intracellular signaling pathways that could link obesity mediated ER stress with alteration in the pro-migratory signals. Then, as described also by Cindrova-Davies in this issue, ER stress modulators might constitute a potential therapy for improving placental angiogenesis.

For understanding regulators of angiogenesis process, Murthi et al. (2014) describe how homeobox genes regulate the transcription of genes essential for angiogenesis in the human placenta. Their analysis includes a description of homeobox genes differentially expressed in the macrovascular and microvascular endothelium derived from the feto-placental vessels, which may improve our understanding of physiological and pathological placental angiogenesis. Therefore, manipulating the expression of homeobox genes and/or their targets in the placenta may serve as an alternate method to improve the outcome of pregnancies compromised by perturbed placental angiogenesis.

Another, cutting-edge analysis related with differential gene expression in placental endothelium and the vascular tree is reviewed by Casanello et al. (2014). The authors focus on how epigenetic mechanisms play a role in endothelial physiology, providing the example that the promoters of endothelial nitric oxide synthase (eNOS), and arginase 2 (Arg-2) are differentially methylated in the endothelium of arteries or veins, as well as in macro or microcirculation in the human feto-placental vasculature. Interestingly, by studying the patterns of DNA methylation, they suggest an "arterization" of human chorionic endothelium vein derived from pregnancies with intrauterine growth restriction. They also suggest that changes in the activity and/or expression DNA-methytransferases might comprise potential new targets for both understanding the control of gene expression in the fetal-placental unit, as well as for identifying new potential targets for therapy.

As presented in this Research Topic, the study of placental angiogenesis constitutes a niche of research not only for understanding pathophysiology of human pregnancy diseases such as pre-eclampsia, intrauterine growth restriction, or gestational diabetes; but also for the development of therapeutic tools which promote placental vascularization and function and thus improve fetal development with lasting effects into adult life. As described in each paper in this Research Topic so much is unknown in this field, therefore I would like to encourage researchers to continue contributing to our understanding of placental angiogenesis during normal and pathological conditions. Finally, but more importantly, I would like to thank all authors who have contributed papers, as well as, the reviewers and editorial board for helping us in underscore the importance of this Research Topic.

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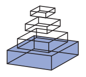

Maternally sequestered therapeutic polypeptides – a new approach for the management of preeclampsia

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The last several decades have seen intensive research into the molecular mechanisms underlying the symptoms of preeclampsia. While the underlying cause of preeclampsia is believed to be defective placental development and resulting placental ischemia, it is only recently that the links between the ischemic placenta and maternal symptomatic manifestation have been elucidated. Several different pathways have been implicated in the development of the disorder; most notably production of the anti-angiogenic protein sFlt-1, induction of auto-immunity and inflammation, and production of reactive oxygen species. While the molecular mechanisms are becoming clearer, translating that knowledge into effective therapeutics has proven elusive. Here we describe a number of peptide based therapies we have developed to target theses pathways, and which are currently being tested in preclinical models. These therapeutics are based on a synthetic polymeric carrier elastin-like polypeptide (ELP), which can be synthesized in various sequences and sizes to stabilize the therapeutic peptide and avoid crossing the placental interface. This prevents fetal exposure and potential developmental effects. The therapeutics designed will target known pathogenic pathways, and the ELP carrier could prove to be a versatile delivery system for administration of a variety of therapeutics during pregnancy.

Keywords: preeclampsia, elastin-like polypeptide, drug delivery, pregnancy, therapeutic peptide

INTRODUCTION

One of the most common complications encountered in obstetrical practice is preeclampsia, occurring in ~5% of all gestations. Preeclampsia was classically defined as new-onset hypertension and proteinuria, but recent diagnostic criteria released from the American Congress of Obstetricians and Gynecologists (ACOG) has recognized that proteinuria is one of many possible diagnostic criteria (thrombocytopenia, renal insufficiency, impaired liver function, pulmonary edema, or cerebral/visual symptoms) which, when manifested in combination with hypertension, indicate a preeclampsia diagnosis (American College of Obstetricians and Gynecologists and Task Force on Hypertension, 2013). Frustratingly, there is little in the way of pharmacological intervention at the disposal of the physician for the management of the preeclampsia patient, and the only definitive resolution of the disorder is parturition. Current management of these patients is limited to magnesium sulfate for seizure prophylaxis, bed rest, and administration of various anti-hypertensives which typically fail to fully control the progressing hypertension. Unchecked, preeclampsia can potentially develop into eclampsia, which leads to seizures and in some cases, death. Development of new therapeutics for the management of the preeclampsia patient remains an important area of research in obstetrics.

While a great deal of research has begun to elucidate the molecular and physiological mechanisms which are responsible for the maternal symptoms, the initiating causes remain unclear. What has become generally accepted is that the disorder is closely linked to defects at the maternal/fetal interface, particularly in the remodeling of the maternal spiral arteries which supply the blood flow to the placenta (Khong and Brosens, 2011). During gestation, the developing fetus requires copious amounts of blood flow to the placenta to allow for adequate exchange of nutrients and wastes between the maternal and fetal circulations. To ensure adequate delivery of blood, the maternal spiral arteries of the uterus undergo a dramatic remodeling. Fetally derived cytotrophoblasts invade the maternal vessels, displace the endothelium, and convert the normally small diameter, low capacitance vessels into dilated high capacitance vessels. Clues that the placenta was central to the etiology of preeclampsia came from case reports showing that delivery of the fetus alone was insufficient to remit the disease symptoms, and that delivery of the placenta was crucial for resolution (Shembrey and Noble, 1995). Early histological examination of placentas from preeclampsia patients suggested that the remodeling of these arteries in preeclampsia patients was deficient, with only very shallow trophoblast invasion and arterial remodeling. This led to the idea that in preeclampsia, the placenta-which even in normal pregnancy is relatively hypoxic-receives inadequate blood flow and in consequence experiences chronic hypoxia and ischemia. Indeed, a host of studies over the last 15 years have strongly implicated placental ischemia as a central factor in the manifestation of preeclampsia. Research into the molecular links between chronic placental ischemia and the symptomatic phase of the disorder continue, but several pathways have been intensively investigated and validated. This includes production of the anti-angiogenic

protein soluble fms-like tyrosine kinase-1 (sFlt-1), production of inflammatory cytokines such as TNFα, and increased production of oxidative stress in the placenta and maternal vasculature (**Figure 1**).

PATHWAYS DRIVING PREECLAMPSIA

ANGIOGENIC IMBALANCE

One of the pivotal breakthroughs in the understanding of preeclampsia was the recognition of increased circulating levels of the vascular endothelial growth factor (VEGF) antagonist sFlt-1 in the circulation of preeclampsia patients (Maynard et al., 2003). sFlt-1 is a soluble, alternatively transcribed isoform of the VEGF receptor Flt-1 and consists only of the receptor's recognition domain. This soluble protein is then secreted extracellularly, where it competes for VEGF binding, thus making VEGF unavailable to bind to its full length, active receptors (Wu et al., 2010). While the mechanisms which regulate sFlt-1 splicing are still under investigation, a number of preclinical studies have shown that production of sFlt-1 from placental tissue is increased by either *in vivo* chronic ischemia, or acutely by hypoxia *ex vivo* (Ahmad and Ahmed, 2004; Nagamatsu et al., 2004; Nevo et al., 2006; George et al., 2010).

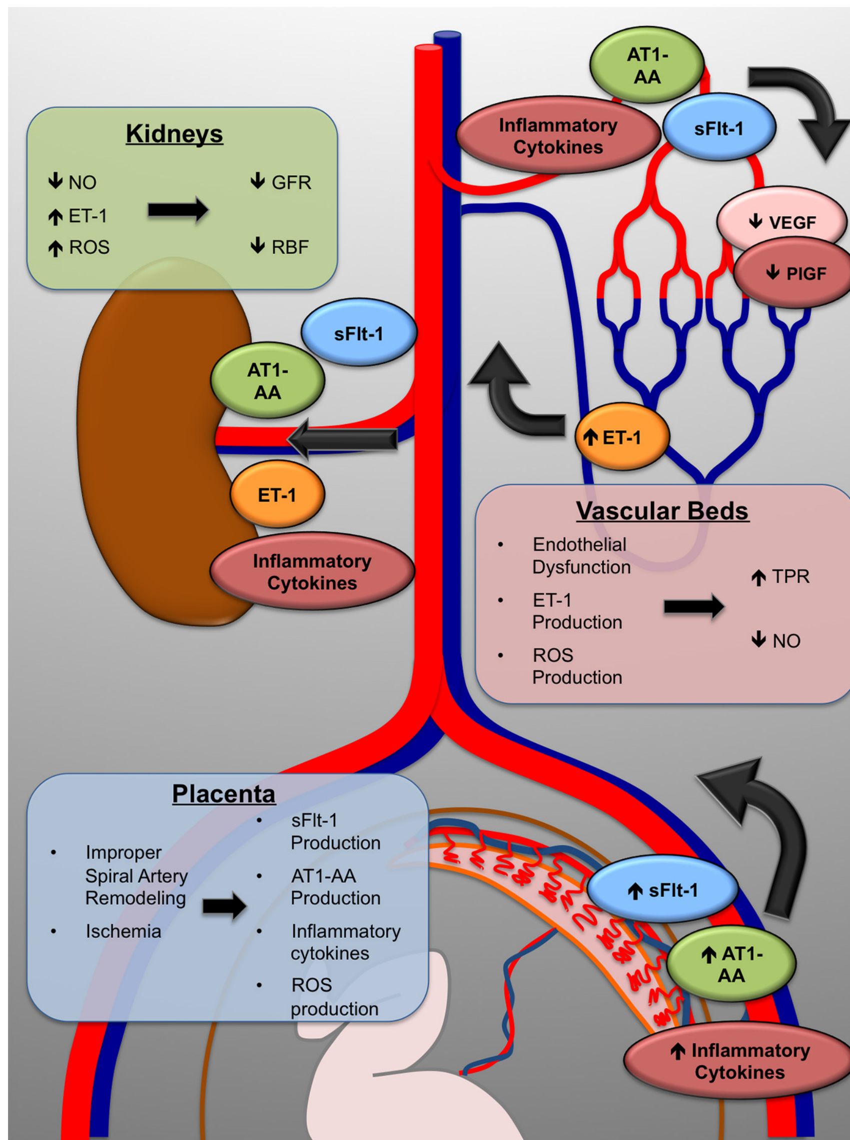

FIGURE 1 | The maternal symptoms of preeclampsia arise through multiple molecular mechanisms. Improper placentation leads to placental ischemia. As a direct result, the placenta produces the anti-angiogenic protein sFlt-1, inflammatory cytokines, and increased reactive oxygen species (ROS); as well as increased production of the agonistic AT-1 receptor autoantibody (AT1-AA). The maternal vasculature, including that in the kidneys, is exposed to decreased VEGF signaling and inflammatory mechanisms which cause endothelial dysfunction, marked by overproduction of the vasoconstrictor endothelin-1 (ET-1). In the kidneys, total peripheral resistance (TPR) increases, renal blood flow (RBF) and glomerular filtration rate (GFR) decrease, and maternal hypertension is the end result.

A variety of studies have supported a link between loss of VEGF activity and hypertension. Patients receiving the anti-VEGF antibody therapy bevacizumab experience hypertension and proteinuria – side effects which are remarkably similar to preeclampsia patients (Zhu et al., 2007). Likewise, inhibition of the VEGF receptors by small molecule tyrosine kinase inhibitors increases blood pressure, at least partially mediated by increased endothelin-1 expression – a known final effector of hypertension in preeclampsia patients (Kappers et al., 2010, 2011, 2012; George and Granger, 2011). Finally, a plethora of studies have demonstrated that increasing circulating sFlt-1 levels through direct administration or viral overexpression induces a hypertensive, preeclampsia-like phenotype in animal models (Maynard et al., 2003; Li et al., 2007; Bridges et al., 2009; Suzuki et al., 2009; Gilbert et al., 2010; Murphy et al., 2010). sFlt-1 has therefore become a major target of interest, and a recent study has shown beneficial effects of sFlt-1 removal by apheresis in a small cohort of preeclampsia patients (Thadhani et al., 2011). Therapeutics targeting sFlt-1 to restore angiogenic balance are a promising avenue for drug development.

THE MATERNAL INFLAMMATORY RESPONSE

Another well-characterized mechanism which has been extensively studied is the production of inflammatory cytokines in response to placental ischemia/hypoxia. Recent research has revealed that inflammatory processes play an important role in the etiology and progression of preeclampsia (Borzychowski et al., 2006; Ahn et al., 2011). The placenta is home to a variety of hematopoietic cells, including T cells, natural killer (NK) cells, and macrophages, and all have roles in production of cytokines including TNF-α and pro-inflammatory interleukins that exacerbate the immune response in preeclampsia (Azizieh et al., 2005). This highly inflammatory environment is a double-edged sword. High INF-γ and TNF-α levels inhibit trophoblast migration and are directly toxic to trophoblasts (Yui et al., 1994; Todt et al., 1996; Rasmussen et al., 1999), so they may contribute to the initial improper remodeling that leads to preeclampsia. Also, TNF-α and other inflammatory factors induce systemic endothelial dysfunction, including increased endothelin-1 release, induction of oxidative stress, and enhanced sensitivity to angiotensin II (AngII), which combine to exacerbate the maternal hypertension (Gilbert et al., 2008).

Of all the inflammatory cytokines examined, perhaps none have been as consistently described and characterized as TNF-α. Elevated TNF-α levels have been described in both the maternal circulation and amniotic fluid of preeclampsia patients (Kupferminc et al., 1994; Vince et al., 1995) as well as in the placenta and circulation of rodents undergoing placental ischemia (LaMarca et al., 2008). In rats, blockade of TNF-α signaling by etanercept partially attenuates the hypertension associated with placental ischemia, and infusion of TNF-α to levels seen in rodents with placental ischemia leads to a hypertensive phenotype associated with increased vascular production of endothelin-1 (LaMarca et al., 2005, 2008). Furthermore, one of the most recently elucidated pathways in preeclampsia is the production of agonistic auto-antibodies to the angiotensin type 1 receptor (AT1-AA) which are found in a large percentage of preeclampsia patients (Xia et al., 2003; Herse et al., 2009). Interestingly, the AT1-AA has been shown to induce the production of TNF-α in pregnant mice, suggesting that it might be one of the upstream regulators of TNF-α production in preeclampsia patients (Irani et al., 2010). These data and others suggest that TNF-α is an important regulator of the symptoms associated with preeclampsia and placental ischemia. Targeting of the inflammatory cascade set off by increased TNF-α levels could be an important target in the development of preeclampsia therapeutics.

OXIDATIVE STRESS

One other known player in the response to placental ischemia is the production of reactive oxygen species (ROS). The ischemic environment of the preeclamptic placenta has been shown to induce ROS production, (Staff et al., 1999a,b) either as a direct consequence of hypoxia or as a secondary response to the local inflammatory environment. Additionally, ROS production may also be induced in the systemic vasculature due to the highly inflammatory environment (Roggensack et al., 1999). Superoxide is the major ROS produced in the preeclamptic placenta, and its production might be a consequence of the action of the mitochondrial electron transport chain enzymes, xanthine oxidase, or NADPH oxidase (Nox) operating under low oxygen conditions (Myatt, 2010). Superoxide can act locally as a damaging oxidant, it can dismute to hydrogen peroxide, or it can react with nitric oxide to produce peroxynitrite (Myatt, 2010).

The increase in ROS in both the placenta and in the systemic vasculature might play a role in the development of PE symptoms. Within the placenta, superoxide and peroxynitrite act locally at the site of production in the vascular endothelium and surrounding stroma to induce damaging protein oxidation, lipid peroxidation, or protein nitration (Myatt, 2010). Systemically, ROS (produced by neutrophils (Lee et al., 2003a,b) or directly in vascular endothelial cells) can further exacerbate endothelial dysfunction, leading to endothelin-1 production and reduced NO bioavailability, which ultimately lead to hypertension via increased total peripheral resistance (TPR; George and Granger, 2011). In addition to the direct induction of ROS production in the placenta and the systemic vasculature, women with PE also have decreased superoxide dismutase (SOD), glutathione, and glutathione peroxidase levels and impaired SOD activity (Wang and Walsh, 2001). Therefore, they may have a reduced antioxidant capacity and thus a heightened response to the ROS production relative to a normal pregnant mother (Myatt and Cui, 2004). Supporting ROS as a target for intervention, rats with hypertension resulting from placental ischemia or sFlt-1 excess have significantly decreased blood pressure when administered anti-oxidant compounds (Sedeek et al., 2008; Tam Tam et al., 2011). These data suggest that ROS is a major contributor to the symptomatic manifestation of preeclampsia and that target modulation of ROS could be a potential therapeutic approach for the preeclampsia patient.

THERAPEUTIC STRATEGIES TARGETING THE MECHANISMS DRIVING HYPERTENSION IN PREECLAMPSIA

Above, we have outlined the evidence supporting ischemia-induced placental production of sFlt-1, activation of the innate

immune system, and induction of ROS production in the symptomatic manifestation of preeclampsia. Elucidation of these pathways that clearly drive the symptomatic phase of the disease lead us to hypothesize that interventions targeted to these pathways could be effective therapies for preeclampsia. Specifically, we hypothesize that supplementation with exogenous VEGF or placental growth factor (PlGF) to restore the depressed levels and sequester the overabundant sFlt-1 will have a positive effect on maternal hypertension, and as a result, fetal health both at birth and later in life. Similarly, we hypothesize that inhibition of the inflammatory pathway by inhibition of its master mediator NF-κB will serve to improve both maternal symptoms and fetal outcomes. Finally, we suggest that selective inhibition of enzymes responsible for ROS production could be beneficial for PE therapy. However, in order to achieve these outcomes, novel sFlt-1, NF-κB, and ROS inhibiting agents must be stabilized from degradation in the maternal circulation and ideally be prevented from crossing the placental interface and entering the fetal circulation, where they could be harmful to the developing fetus.

THE ELP DRUG DELIVERY SYSTEM

Our group has recently been developing a carrier protein called elastin-like polypeptide (ELP) for use as a drug delivery vector during pregnancy. ELP is a genetically engineered polypeptide consisting of repeated units of a five amino-acid motif (VPGxG, where x is any amino acid except P; Urry et al., 1991a). ELP has a unique property of reversibly forming aggregates in response to heat. Above a characteristic transition temperature, the polypeptide will form aggregates, and when the solution is lowered below the transition temperature, the aggregates re-dissolve (Urry et al., 1991a). There are several advantages of using ELP polypeptides for drug delivery. First, ELPs are genetically encoded rather than chemically synthesized. The coding sequence for ELP is built into a plasmid-based recombinant expression system. This means the researcher has absolute control over the ELP sequence and molecular weight (MW). Changes to the ELP sequence or modification of the number of ELP repeats are achieved by simple molecular biology techniques. This is important because the size of a polymer carrier influences the plasma pharmacokinetics and tissue distribution (Dreher et al., 2006), and because the size and sequence of ELP influences the transition temperature of its heat-induced aggregation (Urry et al., 1991a). Also, addition of targeting peptides and therapeutic proteins is easily achieved by modifying the DNA coding sequence. Second, ELP and ELP-fusion proteins can be expressed in *E. coli*, and large quantities of the molecules can be purified by simply taking advantage of the thermal responsiveness. Purification of ELP-fusion proteins is achieved by heating a bacterial lysate containing the recombinantly expressed ELP to a temperature above the polypeptides' transition temperature. This induces ELP aggregation, and it is collected by centrifugation (Meyer and Chilkoti, 1999; Bidwell and Raucher, 2005). Repeated centrifugation above and below the transition temperature leads to large quantities of very pure protein (Meyer and Chilkoti, 1999; Bidwell and Raucher, 2005). The third advantage for using ELP for drug delivery is that it is a large, biologically inert macromolecule. Therefore, ELP fusion can stabilize protein, peptide, or small molecule cargo in systemic circulation (Bidwell et al., 2012, 2013), and targeting agents can be used to direct the ELP-fused therapeutics' biodistribution (Bidwell et al., 2009). Previous work has used ELPs extensively for drug delivery in cancer models. These studies have carefully defined the polypeptide's pharmacokinetics and biodistribution (Liu et al., 2006; Bidwell et al., 2012, 2013; Moktan et al., 2012), confirming ELP as a long circulating, inert, biodegradable, and non-immunogenic (Urry et al., 1991b) drug carrier. In addition, our previous cancer work also serves as proof of principle for the ability to ELP to efficaciously deliver active therapeutic peptides (Bidwell et al., 2012, 2013). Recently, we have found that ELP has a long half-life after intravenous injection in a rat pregnancy model, but importantly, ELP does not cross the placenta (George et al., 2014). Our current goals are to leverage this powerful delivery system to (1) stabilize therapeutic proteins, peptides, or small molecules targeted to preeclampsia mechanisms in the maternal circulation and (2) prevent the penetration of these therapeutic agents into the developing fetus. We are currently developing ELP-fused therapeutics to target all three pathways described above using ELP-fused to VEGF or PlGF, ELP-fused peptide inhibitors of NF-κB, and peptide inhibitors of NOX (**Figure 2**).

The ELP delivery system can be utilized in either an untargeted manner, which is useful when attached to agents designed to circulate, or it can be targeted to enter cells or even to specific intracellular compartments using cell penetrating peptides (CPPs; Massodi et al., 2005; Bidwell et al., 2009). CPPs do not necessarily provide tissue specificity, but they do enhance deposition of ELP in many of the major organs, most notably the kidney (Bidwell et al., 2013). For delivery of VEGF or PlGF, we are utilizing a version of the polypeptide that is untargeted. This will allow the ELP-VEGF or ELP-PlGF to freely circulate, where it can bind and sequester the excess s-Flt-1. For delivery of the NF-κB inhibitory peptides or the NOX inhibitory peptides (which have intracellular targets), we are utilizing ELP vectors fused to CPPs to mediate cellular uptake of the polypeptide and direct them to the cytoplasm. We have previously shown that multiple CPPs are effective for intracellular delivery of ELP both *in vitro* (Massodi et al., 2005) and *in vivo* (Bidwell et al., 2012, 2013; Moktan et al., 2012).

In a recent study, we demonstrated that ELP and a CPP-tagged ELP are excluded from the fetus after systemic administration to the mother in a rat pregnancy model (George et al., 2014). Furthermore, this fetal exclusion held even after 5 days of continuous infusion of the polypeptides. Within the placenta, both ELP and CPP-tagged ELP were detectable in the cytoplasm of trophoblast cells, but were absent from the fetal portion of chorionic villi. We believe that the size of the ELPs prevents them from crossing the tight junctions between trophoblast cells in the chorionic villi.

NOVEL THERAPIES TARGETING THE sFlt-1/VEGF PATHWAY

As described above, one of the hallmarks of preeclampsia is the elevated maternal plasma levels of the VEGF antagonist sFlt-1. We hypothesize that VEGF or PlGF supplementation therapy could be a viable mechanism to sequester the excess sFlt-1 and thereby prevent or reverse the onset of hypertension. While we believe that VEGF supplementation therapy will be beneficial for treatment of preeclampsia, the therapeutic strategy is not as straightforward as

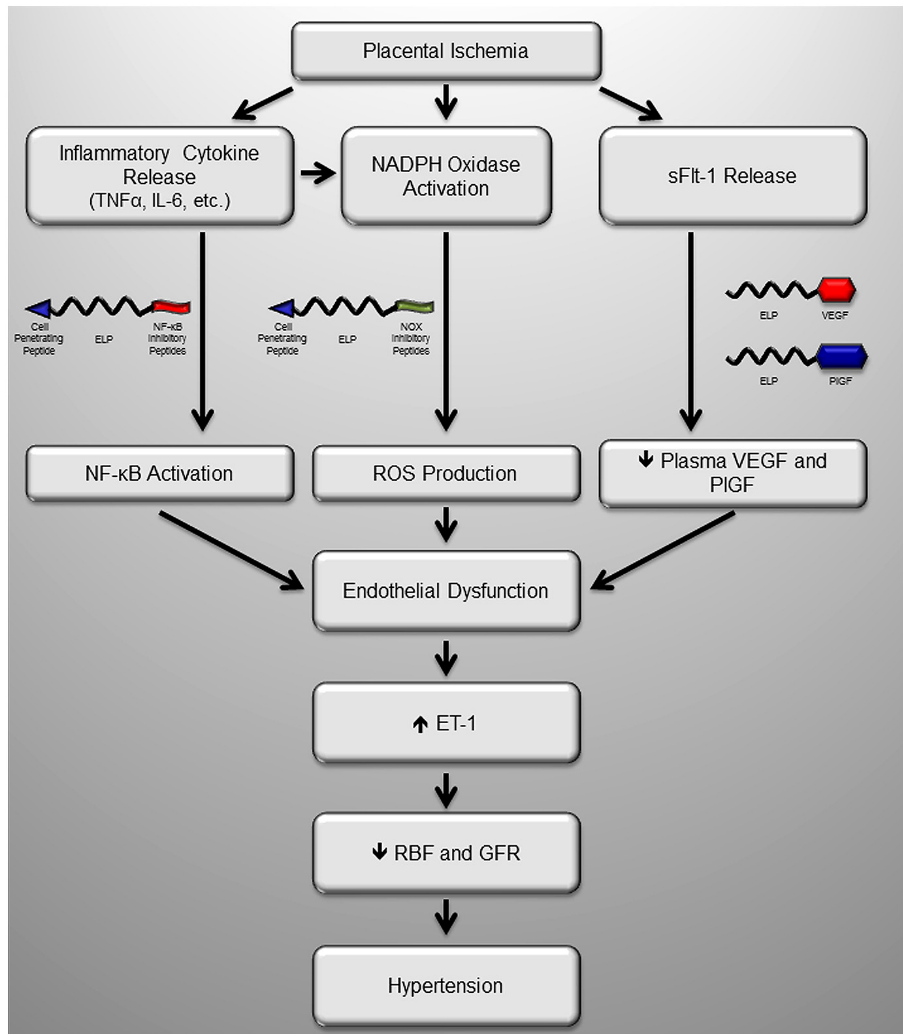

FIGURE 2 | Potential sites of intervention with maternally restricted therapeutic peptides during preeclampsia. We have recently produced several novel maternally restricted peptide therapeutics to target known pathologic molecules produced in response to placental ischemia in preeclampsia. Much of the activity of inflammatory cytokines is through the master regulator NF-κB, which could be blocked by the inhibitory ELP-p50 peptide which blocks nuclear translocation of NF-κB, a transcription factor. The major source of oxidative stress in the ischemic placenta is the NADPH oxidase (Nox) enzyme, which could be inhibited by the Nox inhibitory peptide, which blocks assembly of the complete complex. Finally, one of the most extensively characterized mechanisms is production of the VEGF inhibitor sFlt-1, which act as a decoy receptor. Introduction of ELP-stabilized VEGF or PlGF fusions would restore VEGF signaling by their direct activity, and/or by sequestering the excess sFlt-1 in the maternal circulation. Ultimately all of these proposed peptides help to restore endothelial function and renal function to block the maternal hypertension.

simply infusing VEGF. VEGF infusions have been tested in several disease models, and this approach has been hindered by many problems. First, free exogenous VEGF is very short-lived, with a plasma half-life in humans of about 34 min (as determined following a four hour intravenous infusion of recombinant human $VEGF_{165}$; Eppler et al., 2002). Exogenous VEGF therapy has shown potential in several disease models, including myocardial infarction (Banai et al., 1994; Pearlman et al., 1995), but due to the short half-life and poor stability of the protein, constant infusion via a pump-driven catheter placed directly at the diseased site was required. This type of treatment strategy is not a viable translational approach for preeclampsia therapy, where patients will need to be treated for several months.

The second limitation of free VEGF supplementation for preeclampsia therapy involves its potential for damage to the developing fetus. Several reports have demonstrated the severe potential consequences of overloading the fetus with VEGF. Overexpression of VEGF-A by two to threefold using a genetic strategy in mouse embryos resulted in embryonic lethality at day E12.5 (Miquerol et al., 2000). Death was due to cardiac failure resulting from malformation of the myocardium, improper ventricular septation, and abnormalities in the heart's outflow track. A separate study in which quail embryos were directly injected with exogenous VEGF showed similar results. The VEGF treated embryos had neovascularization in normally avascular regions and excessive, improper fusion of vessels (Drake and Little, 1995). Like the

mouse study, these VEGF treated embryos also had malformation of the hearts, including fusion of inflow and aortic outflow channels. These studies address the dire consequences of increasing VEGF levels directly in the developing fetus, but it has also been shown that administration of free VEGF to pregnant mice causes developmental problems in the embryos. Daily systemic injection of recombinant human VEGF from gestational day 9–day 17 resulted in an 18-fold increase in the fetal reabsorption rate and a significant decrease in fetal weight among the surviving fetuses (He et al., 1999). Given the limitations of short half-life and the potential for teratogenic effects of free VEGF, we have fused VEGF (or similarly PlGF) to the ELP carrier to both extend its plasma half-life and prevent its delivery across the placenta. The goal of this strategy is to supplement circulating VEGF or PlGF levels in order to achieve levels present in normal pregnancy. We have recently characterized the ELP-VEGF polypeptide and found that ELP-VEGF is equally potent at stimulating vascular endothelial cell proliferation, migration through a collagen matrix, and tube formation when compared to unbound VEGF (unpublished data). Given the favorable biodistribution profile of ELP-fused VEGF or PlGF and a careful dosing strategy to achieve proper VEGF or PlGF replacement, we believe that this approach represents a promising new method for preeclampsia therapy.

NOVEL THERAPIES TARGETING THE INFLAMMATORY PATHWAY

Many pro-inflammatory cytokines such as TNF-α, IL-1, and toll like receptors (TLRs, whose signaling has also been implicated in PE (Keelan and Mitchell, 2007; Tinsley et al., 2009; Chatterjee et al., 2012) exert their effects via receptor-mediated signaling pathways that are centrally routed through NF-κB. In fact, NF-κB is a master regulator of inflammation (Makarov, 2001). For this reason combined with the multitude of inflammatory factors at play, we chose to target NF-κB as a means to inhibit the inflammation associated with preeclampsia. Several previous studies have highlighted the importance of NF-κB activation in preeclampsia. Hypoxia and reoxygenation of villous explants leads to activation of the NF-κB pathway (Cindrova-Davies et al., 2007), and NF-κB activation plays an important role in the systemic endothelial dysfunction present in preeclampsia (Jiang et al., 2010). Furthermore, analysis of microarray data of placental tissue from preeclamptic women versus at-risk but non-preeclamptic controls reveals NF-κB as a major pathway that is upregulated in preeclampsia (Centlow et al., 2011). Within the placenta, NF-κB levels in preeclampsia are associated with increased trophoblast apoptosis (Aban et al., 2004), and systemic NF-κB activation is also present in the vasculature of preeclamptic mothers (Shah and Walsh, 2007). Systemic vascular NF-κB activation is associated with neutrophil infiltration, and these neutrophils release toxic substances such as TNF-α, ROS, and thromboxane, which promote vasoconstriction and vascular dysfunction (Shah and Walsh, 2007).

NF-κB activation is a complex pathway involving trimerization of NEMO, the regulatory domain of inhibitor of κ-B kinase (IKK), phosphorylation and deactivation of the NF-κB inhibitor I-κB by IKK, NF-κB phosphorylation on the p65 subunit, and active import of NF-κB into the nucleus (reviewed in Bidwell and Raucher, 2009). Extensive interest in developing NF-κB inhibitors exists in the cancer field, and several previous studies have described peptide inhibitors of all the activation steps mentioned above (Bidwell and Raucher, 2009). We have developed an ELP-fused peptide inhibitor of activated NF-κB. NF-κB activation upon extracellular signaling is mediated by phosphorylation and release of the natural inhibitor I-κB from the NF-κB p50/p65 heterodimer. I-κB release exposes a nuclear localization sequence (NLS) on the p50 subunit of NF-κB, and once exposed, this NLS mediates nuclear import of NF-κB. Once inside the nucleus, NF-κB binds to response elements on its target genes and regulates gene expression. A synthetic cell permeable peptide containing the p50 NLS is capable of blocking the nuclear import of NF-κB upon stimulation in a variety of cell lines (Lin et al., 1995). We have fused a copy of the p50 NLS to the CPP-ELP carrier and validated its activity *in vitro*. We are currently focusing on determining the pharmacokinetics and biodistribution of this NF-κB inhibitory polypeptide, and assessing its efficacy in our rat models of PE. Initial biodistribution data have shown that the CPP-ELP-delivered p50 NLS peptide accumulates highly in the kidneys and the placenta. This biodistribution is advantageous because these two organs are the most critical for modulating the drivers of hypertension and proteinuria in preeclampsia. We hypothesize that the polypeptide will function to inhibit NF-κB in these tissues (either in the tissue stromal cells directly or in invading lymphocytes) and reduce the local inflammatory environment.

NOVEL THERAPIES TARGETING REACTIVE OXYGEN SPECIES PRODUCTION

Of the possible sources listed above, the Nox family is thought to be the major contributor to ROS production in the placenta. Nox1 expression has been found in syncytiotrophoblasts and vascular endothelial cells in the placenta (Matsubara and Sato, 2001; Cui et al., 2006), and its levels are enhanced in tissue from PE patients (Cui et al., 2006). Elevated total Nox activity has also been seen in placentas from women with early onset PE, though no difference was seen when comparing all PE patients to normal pregnant controls (Raijmakers et al., 2004). It has also been demonstrated that ROS are produced via Nox2 in vascular smooth muscle cells and trophoblasts in response to the AT1-AA, and ROS production induced NF-κB activation (Dechend et al., 2003). The AT1-AA also induced Nox2 subunit production and ROS production in pregnant rats (Parrish et al., 2011). Furthermore, treatment of vascular endothelial cells with sera from preeclampsia patients induced production of the Nox2, an effect attenuated by an Ang II type 1 receptor antagonist (Matsubara et al., 2010).

We hypothesize that Nox inhibition could be a viable therapeutic strategy for PE, either alone or in combination with targeting other pathways. Patrick Pagano's lab has described a number of peptide inhibitors of the Nox family (reviewed in (Cifuentes-Pagano et al., 2012). The most specific and widely used agent, called Nox2 docking sequence (Nox2ds) or gp91ds, is an nine amino acid peptide that inhibits the interaction between Nox2 and its partner in the complex $p47^{phox}$ (Rey et al., 2001). The Nox2ds peptide, fused directly to the Tat CPP, has been shown to

inhibit superoxide production in a variety of cell and tissue types in response to multiple stimuli. The peptide has been used extensively in many disease models including AngII-induced hypertension, renovascular hypertension, and arterial balloon injury (reviewed in Cifuentes-Pagano et al., 2012). For application in PE, we fused the Nox2ds peptide to the CPP-ELP carrier (Bidwell and Raucher, 2009). The CPP-ELP carrier will increase the plasma half-life relative to the free peptide, and the use of the CPP will mediate uptake of the polypeptide into target cells in the placenta or systemic vasculature. We are currently testing the ability of this polypeptide to inhibit placental and/or vascular ROS production in our rat models of PE.

CONCLUSION

The explosion of research into the etiology of preeclampsia has provided a number of intriguing targets for therapeutic development. The ideal scenario would be to understand the pathways that lead to improper spiral artery remodeling and intervene very early in pregnancy to prevent the improper remodeling. However, in the absence of this knowledge and in the absence of a concrete biomarker to predict which patients will develop preeclampsia, this type of intervention is currently unrealistic. In contrast, we now know of many of the molecular pathways that lead to the precipitation of the symptomatic phase of preeclampsia. Our aim is to intervene during this symptomatic phase and modulate these pathways of interest with the goal of prolonging gestation and thereby improving fetal outcomes. While small molecule therapeutics are one potential method to affect these pathways, great care must be exercised in their development, as unwanted and potentially harmful effects to the fetus are possible. Peptide-based therapeutics, though an active area of research in cancer therapy, also have potential as therapeutics in cardiovascular disorders. However, free peptides suffer from very rapid plasma clearance and susceptibility to degradation *in vivo*. Our goal is to develop an appropriate polymeric carrier that can be fused to either small molecule drugs or to therapeutic peptides/proteins. By fusing these agents to the carrier, they can be stabilized in the maternal circulation and prevented from entering the fetal circulation. We feel that the proposed therapeutic agents, or agents like them that intervene in pathways of known importance in preeclampsia (angiogenic factors, maternal inflammatory/autoimmune response, and oxidative stress), have great promise as maternally sequestered therapeutics. Future studies will explore the therapeutic potential of these agents in the management of preeclampsia.

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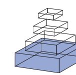

Extravillous trophoblast cells-derived exosomes promote vascular smooth muscle cell migration

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Background: Vascular smooth muscle cells (VSMCs) migration is a critical process during human uterine spiral artery (SpA) remodeling and a successful pregnancy. Extravillous trophoblast cells (EVT) interact with VSMC and enhance their migration, however, the mechanisms by which EVT remodel SpA remain to be fully elucidated. We hypothesize that exosomes released from EVT promote VSMC migration.

Methods: JEG-3 and HTR-8/SVneo cell lines were used as models for EVT. Cells were cultured at 37°C and humidified under an atmosphere of 5% CO_2-balanced N_2 to obtain 8% O_2. Cell-conditioned media were collected, and exosomes (exo-JEG-3 and exo- HTR-8/SVneo) isolated by differential and buoyant density centrifugation. The effects of exo-EVT on VSMC migration were established using a real-time, live-cell imaging system (Incucyte™). Exosomal proteins where identified by mass spectrometry and submitted to bioinformatic pathway analysis (Ingenuity software).

Results: HTR-8/SVneo cells were significantly more (~30%) invasive than JEG-3 cells. HTR-8/SVneo cells released 2.6-fold more exosomes ($6.39 \times 10^8 \pm 2.5 \times 10^8$ particles/10^6 cells) compared to JEG-3 ($2.86 \times 10^8 \pm 0.78 \times 10^8$ particles/10^6 cells). VSMC migration was significantly increased in the presence of exo-JEG-3 and exo-HTR-8/SVneo compared to control (−exosomes) (21.83 ± 0.49 h and 15.57 ± 0.32, respectively, vs. control 25.09 ± 0.58 h, $p < 0.05$). Sonication completely abolished the effect of exosomes on VSMC migration. Finally, mass spectrometry analysis identified unique exosomal proteins for each EVT cell line-derived exosomes.

Conclusion: The data obtained in this study are consistent with the hypothesis that the release, content, and bioactivity of exosomes derived from EVT-like cell lines is cell origin-dependent and differentially regulates VSMC migration. Thus, an EVT exosomal signaling pathway may contribute to SpA remodeling by promoting the migration of VSMC out of the vessel walls.

Keywords: exosomes, cell migration, placenta, pregnancy, proteomics

INTRODUCTION

Remodeling of the uterine spiral arteries (SpA) into low resistance, high capacity vessel begins as extravillous trophoblasts (EVT) invade the decidua during first trimester and is essential for successful pregnancy (Kam et al., 1999). When EVT "plugs" are lost during early second trimester, maternal blood flows through the modified vessels to deliver nutrients and oxygen to support fetal growth and development (Pijnenborg et al., 2006a). EVT continue to invade into the myometrium and remodel the SpA until mid-second trimester (Hamilton and Boyd, 1970; Pijnenborg et al., 1983; Blackburn et al., 2003; American Diabetes Association, 2012). The initial steps of uterine spiral artery remodeling consists of vessel dilatation, vascular smooth muscle cells (VSMC) separation, endothelial cell swelling, EVT infiltration, and fibrinoid deposition (Pijnenborg et al., 2006b). VSMC migrate or undergo apoptosis and are replaced by fibriniod material, in which EVT cells embed. Recently Bulmer et al. showed that during SpA remodeling, VSMC migrate outside of the artery, and this phenomenon is enhanced in the presence of EVT (Bulmer et al., 2012). While the mechanisms by which EVTs remodel SpA remain to be fully elucidated, available data are consistent with the hypothesis that EVT directly interact with VSMC of the uterine spiral arteries. We propose that the releases of nanoparticles (i.e., exosomes) that contain specific effector molecules (e.g., proteins and miRNAs) are released from EVT and affect the loss of VSMC.

Exosomes (30–100 nm) are nanovesicles released when late endosomes fuse with the cell membrane (Thery, 2011; Salomon et al., 2013a). Exosomes interact with target cells via multiple pathways including: by directly activating target cell membrane receptors; by modifying the extracellular *milieu* of the target cell; and by fusing with the cell membrane and releasing the molecular

cargo into the target cell (Pegtel et al., 2010). Their molecular cargo is: cell-specific (Kobayashi et al., 2014); regulated by tissue physiology and cellular function; and fundamental to their bioactivity.

Exosomes are identified in cell-conditioned media and body fluids indicate that they can be released from different types of cells (Vlassov et al., 2012). Recently, the role of exosomes isolated from placental cells (Salomon et al., 2013a,b) and other cell types (Chen et al., 2014; Lee et al., 2014; Yoon et al., 2014) on cell migration has been established. Exosomes released from first trimester placental mesenchymal stem cells (pMSC) increase endothelial cell migration and vascular tube formation *in vitro* (Salomon et al., 2013a). Similarly, cytotrophoblast-derived exosomes increase EVT migration *in vitro* (Salomon et al., 2013b).

Consistent with the proposal that exosomal signaling regulates cell migration and invasion, proteins associated with actin cytoskeleton, growth hormone, and VEGF signaling have been identified within exosomes. The effect of EVT-derived exosomes on VSMC migration, however, remains to be established. We, therefore, hypothesize that exosomes released by EVT act paracellularly to promote VSMC migration and thus contributing to SpA remodeling. The aims of this study were: (1) to compare the exosome release and exosomal protein composition derived from EVT cell lines from different origin (JEG-3 and HTR-8/SVneo); and (2) to establish the effect of exosomes from both JEG-3 and HTR-8/SVneo cells on human VSMC migration.

Numerous human trophoblastic cell lines have been established, which basically originated from normal tissues or from pathological tissues. JEG3 is a choriocarcinoma cell line cloned from primary choriocarcinoma (Kohler and Bridson, 1971), and HTR8/SVneo is a transformed extravillous trophoblast cell line established by immortalizing primary EVT cells via transfection with simian virus 40 large T antigen (SV40) (Graham et al., 1993); both cell lines are frequently used as models of physiologically invasive extravillous trophoblast. EVT invasion into the myometrium is a critical process for remodeling the uterine spiral artery (in this stage EVT interact with VSMC), however, the invasiveness capacity between these two cells lines are different. HTR-8/SVneo have significantly higher invasion capacity than JEG-3 (Suman and Gupta, 2012). Moreover, differences between these two cells lines are not just in the invasion capacity, but also in their miRNA profiles (Morales-Prieto et al., 2012) as well as their protease (e.g., metalloproteases-9) expressions (Suman and Gupta, 2012), however, functional differences between exosome vesicles derived from JEG-3 and HTR-8/SVneo remain to be established. Previously, these cells lines have been validated and used routinely as models of EVT function (Suman and Gupta, 2012; Weber et al., 2013).

The aim of this study was to test the hypotheses that: (1) exosomes from EVT act paracellularly to promote VSMC migration; and (2) The release, protein content and bioactivity of exosomes is cell origin-dependent (i.e., EVT cell lines from choriocarcinoma and chorionic villi). The effects of exosomes isolated from the EVT-like cell lines, JEG-3 and HTR-8/SVneo cells on VSMC migration were assessed.

The data obtained are consistent with the hypothesis that the function of EVT-derived exosomes is cell origin specific and showed differences in the release, content and effects on VSMC migration. EVT may communicate with VSMC during SpA remodeling, stimulating their migration through these specific nanovesicles (i.e., exosomes).

MATERIALS AND METHODS

MATERIALS

Medium RPMI 1640, Medium 231, Smooth Muscle Growth Supplement (SMGS), glutamine, antibiotics, HEPES, and phosphate buffered saline (PBS) were obtained from Life Technologies Corporation (Mulgrave, Victoria, Australia). CD63 ELISA kits were obtained from SBI (ExoELISA™, System Biosciences, Mountain View, CA).

CELL CULTURE

All experimental procedures were conducted within an ISO17025 accredited (National Association of Testing Authorities, Australia) research facility. All data were recorded within a 21 Code of Federal Regulation (CFR) part 11 compliant electronic laboratory notebook (Irisnote, Redwood City, CA, USA). JEG-3 human choriocarcinoma cell line was purchased from the European Collection of Cell Cultures (Porton Down, Salisbury, UK). The HTR-8/SVneo cell line was kindly donated by Dr. Charles H. Graham (Queen's University, Ontario, Canada). HTR-8/SVneo was established by the transfection of trophoblast cells isolated from first trimester villous explants, with a gene encoding simian virus 40 large T antigen to immortalize them (Graham et al., 1993). JEG-3 and HTR-8/SVneo cells were maintained in phenol red-free RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 1% non-essential amino acids, 1 mM sodium pyruvate and 100 U/mL penicillin, and 100 mg/mL streptomycin. Cultures were maintained at 37°C and humidified under an atmosphere of 5% CO_2-balanced N_2 to obtain 8% O_2 (pO_2 ∼54 mmHg) in an automated PROOX 110-scaled hypoxia chamber (BioSphericsacona, NY, USA). Cells were subcultured with dissociation media, TrypLE™ Express (Life technologies, USA) and cellular viability was determined by Trypan Blue exclusion and Countess® Automated cell counter (Life Technologies, USA).

Human Vascular Smooth Muscle cells (hVSMC) were purchased from LONZA (Lonza Group Ltd.). VSMC were cultured in 231 media (Life Technologies Corporation) supplemented with SMGS, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37°C and humidified under an atmosphere of 5% CO_2-balanced N_2 to obtain 8% O_2 (pO_2 ∼54 mmHg) in an automated PROOX 110-scaled hypoxia chamber (BioSpherics™, Lacona, NY, USA).

The *in vitro* cell migration rates of JEG-3 and HTR-8/SVneo we established using real-time cell imaging system (IncuCyte™ live-cell ESSEN BioScience Inc., Ann Arbor, MI, USA). Using a scratch assay format, cell were imaged every 3 h to monitor cell migration as previously described (Salomon et al., 2013b).

EXOSOME ISOLATION

Exosomes were isolated from cell-free JEG-3 and HTR-8/SVneo-conditioned media as previously described (Salomon et al., 2013a; Kobayashi et al., 2014). In brief, cell-conditioned media was

centrifuged at 300 × g for 15 min, 2000 × g for 30 min, and 12,000 × g for 45 min to remove whole cells and debris. The resultant supernatant were passed through a 0.22 μm filter sterilize Steritop™ (Millipore, Billerica, MA, USA) and then centrifuged at 120,000 × g for 70 min (Thermo Fisher Scientific Ins., Asheville, NC, USA, Sorvall, SureSpin™ 630/36, fixed angle rotor). The pellet was resuspended in PBS, washed and recentrifuged (120,000 × g, 75 min). The pellet was resuspended in PBS, layered on a cushion of 30% (w/v) sucrose and centrifuged at 110,000 g for 75 min. The fraction containing exosomes [~3.5 ml, 1.127 density using OPTi digital refractometer (Bellingham+Stanley Inc., Lawrenceville, GA, USA)] was recovered using a Pulse-Free Flow Peristaltic Pump with a flow rate range of 1 ml per min (GILSON Miniplus® model 3) and Fraction Collector (GILSON FC 203B model) and diluted in PBS, and then ultracentrifuged at 110,000 × g of 70 min. Recovered exosomes were resuspended in 50 μl PBS and their protein contents were determined using the Bradford assay (Bio-Rad DC). Exosome samples (5 μl) were prepared by adding RIPA buffer (50 mM Tris, 1% Triton × 100, 0.1% SDS, 0.5% DOC, 1 mM EDTA, 150 mM NaCl, protease inhibitor) directly to exosomes suspended in PBS and sonicated at 37°C for 15 s three times to disrupt exosomes and solubilise the proteins. Bovine serum albumin (BSA) diluted in RIPA buffer and PBS mixture (1:1) were prepared as protein standards (0, 200, 400, 600, 800, 1000, 1500 μg/mL). Standards and samples (exosomes) were transferred to 96-well plates and procedures outlined by the manufacture were followed. In brief, alkaline copper tartrate solution (Bio-Rab Laboratories, Hercules, CA, USA) and dilute Folin Reagent (Bio-Rab Laboratories,) were added to the samples and incubated for 15 min. The absorbance was read at 750 nm with Paradigm Detection Platform (Beckman Coulter, USA).

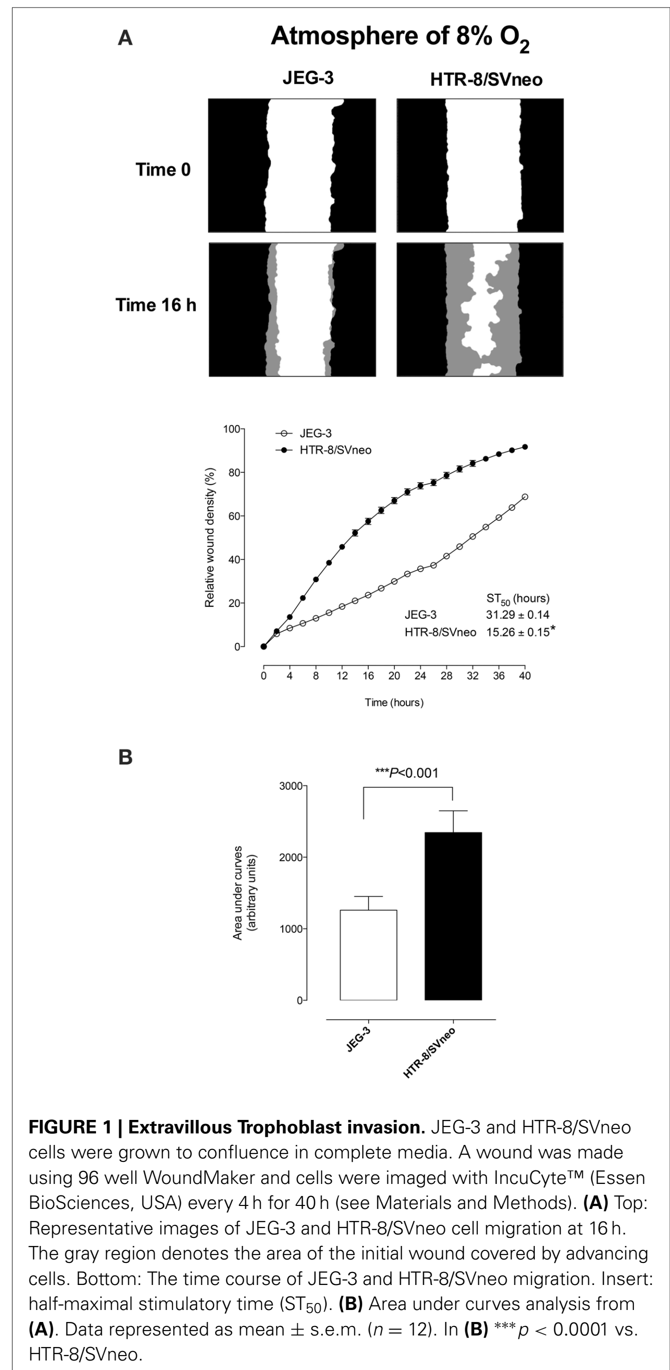

FIGURE 1 | Extravillous Trophoblast invasion. JEG-3 and HTR-8/SVneo cells were grown to confluence in complete media. A wound was made using 96 well WoundMaker and cells were imaged with IncuCyte™ (Essen BioSciences, USA) every 4 h for 40 h (see Materials and Methods). **(A)** Top: Representative images of JEG-3 and HTR-8/SVneo cell migration at 16 h. The gray region denotes the area of the initial wound covered by advancing cells. Bottom: The time course of JEG-3 and HTR-8/SVneo migration. Insert: half-maximal stimulatory time (ST_{50}). **(B)** Area under curves analysis from **(A)**. Data represented as mean ± s.e.m. ($n = 12$). In **(B)** ***$p < 0.0001$ vs. HTR-8/SVneo.

NANOPARTICLE TRACKING ANALYSIS (NTA)

NTA measurements were performed using a NanoSight NS500 instrument (NanoSight NTA 2.3 Nanoparticle Tracking and Analysis Release Version Build 0033) following the manufacturer's instructions. The NanoSight NS500 instrument measured the rate of Brownian motion of nanoparticles and consists in a light scattering system that provides a reproducible platform for specific and general nanoparticle characterization (NanoSight Ltd., Amesbury, UK). Samples were processed in duplicate and diluted with PBS over a range of concentration to obtain between 10 and 100 particles per image (optimal ~50 particles × image) before the analysis with the NTA system. The samples were mixed before introducting into the chamber (temperature: 25°C and viscosity: 0.89 cP) and the camera level set to obtain an image that had sufficient contrast to clearly identify particles while minimizing background noise with video recording (camera level: 10 and capture duration: 60 s). Afterwards, the capture videos (2 videos per sample) were processed and analyzed. A combination of high shutter speed (450) and gain (250) followed by manual focusing enables optimum visualization of the maximum number of vesicles. A minimum of 200 completed tracks per video were collected in duplicate for each sample analyzed. NTA post acquisition settings were optimized and kept constant between samples (Frames Processed: 1496 of 1496, Frames per Second: 30, camera shutter: 20 ms; Calibration: 139 nm/pixel, Blur: 3 × 3; Detection Threshold: 10; Min Track Length: Auto; Min Expected Size: Auto), and each video was then analyzed to give the mean, mode, and median particle size together with an estimated number of particles. An Excel spreadsheet (Microsoft Corp., Redmond, Washington) was also automatically generated, recording the concentration at each particle size.

TRANSMISSION ELECTRON MICROSCOPY

Exosome pellets (as described above, 30 μg protein) were fixed in 3% (w/v) glutaraldehyde and 2% paraformaldehyde in cacodylate

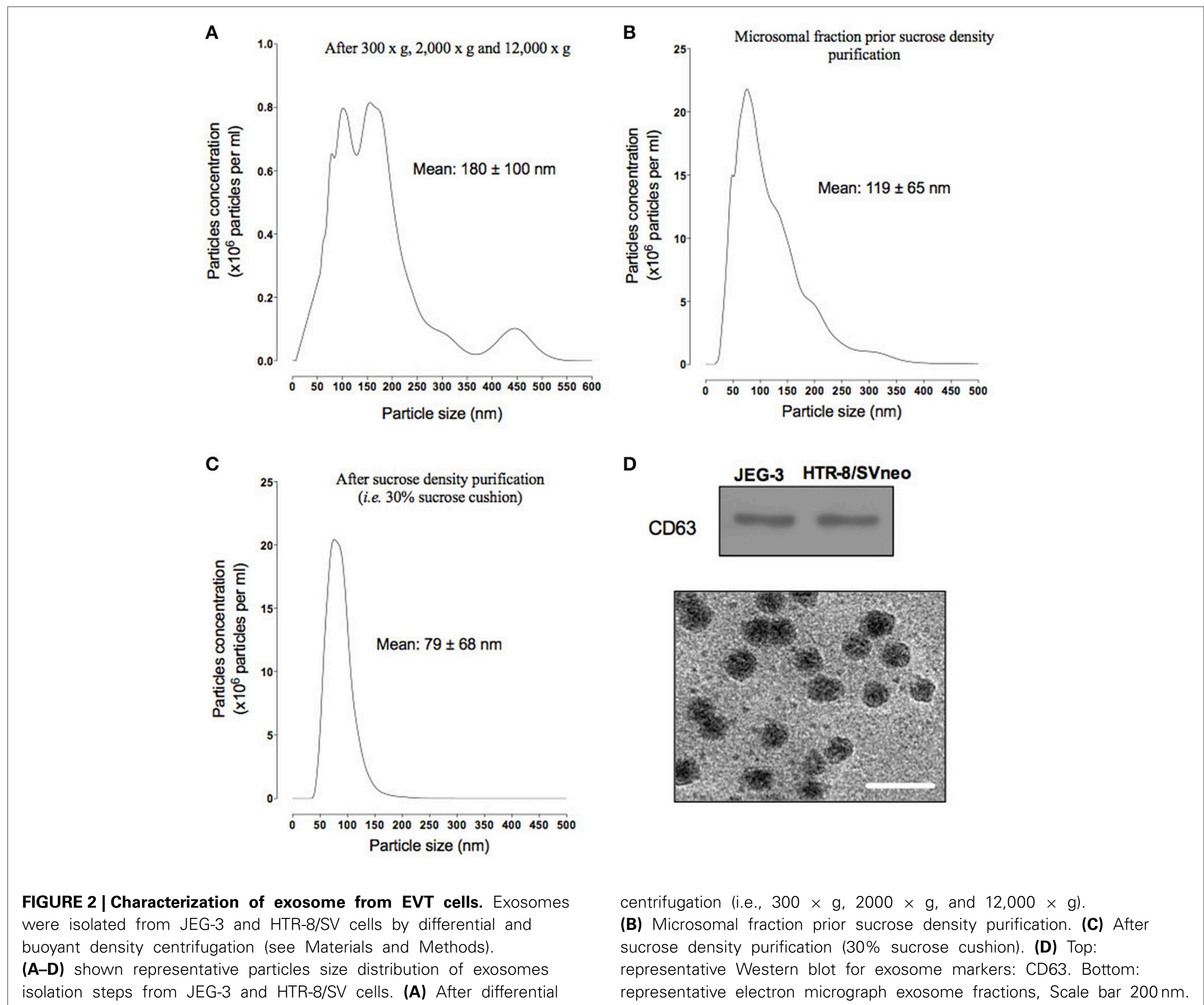

FIGURE 2 | Characterization of exosome from EVT cells. Exosomes were isolated from JEG-3 and HTR-8/SV cells by differential and buoyant density centrifugation (see Materials and Methods). **(A–D)** shown representative particles size distribution of exosomes isolation steps from JEG-3 and HTR-8/SV cells. **(A)** After differential centrifugation (i.e., 300 × g, 2000 × g, and 12,000 × g). **(B)** Microsomal fraction prior sucrose density purification. **(C)** After sucrose density purification (30% sucrose cushion). **(D)** Top: representative Western blot for exosome markers: CD63. Bottom: representative electron micrograph exosome fractions, Scale bar 200 nm.

buffer, pH 7.3. Exosome samples were then applied to a continuous carbon grid and negatively stained with 2% uranyl acetate. The samples were examined in an FEI Tecnai 12 transmission electron microscope (FEI™, Hillsboro, OR, USA).

QUANTIFICATION OF CELL-DERIVED EXOSOME

The concentration of exosomes from EVT cells lines (JEG-3 and HTR-8/SVneo) in maternal circulation was expressed as total immunoreactive exosomal CD63 (ExoELISA™, System Biosciences, Mountain View, CA). Briefly, 10 μg of exosomal protein was immobilized in micro-titer plate wells and incubated overnight (binding step). Plates were washed three times for 5 min using a wash buffer solution and then incubated with exosome specific primary antibody (CD63) at room temperature (RT) for 1 h on a shaker. Plates were washed and incubated with secondary antibody (1:5000) at RT 1 h on a shaker. Plates were washed and incubated with Super-sensitive TMB ELISA substrate at RT for 45 min while shaking. The reaction was terminated using Stop Buffer solution. Absorbance was measured at 450 nm. The number of exosomes/ml, (ExoELISA™ kit) was obtained using an exosomal CD63 standard curve calibrated against nanoparticle tracking data (i.e., number of exosomes, NanoSight™). The coefficients of intra- and inter-assay variations were less than 8%.

EFFECT OF EXOSOMES ON VASCULAR SMOOTH MUSCLE CELLS (VSMC) MIGRATION

VSMC were cultured in 231 media supplemented with 0.2% FBS-exosome free in 96-well culture plate (Corning Life Science, Tewksbury, MA, USA) according to the manufacturer's instructions for 18–24 h. Firstly, we analyzed the effect of cell proliferation on cell migration assay in our experimental conditions using an anti-proliferative drug Mitomycin C (SIGMA-ALDRICH). Cells were plated (1×10^5 cells per well) onto 24-well plate, and after 24 h Mitomycin C (50 and 100 ng/ml) was added to the cell for 48 h. Cell proliferation was quantified (time-curve) by measuring the cell confluence using a real time imagining

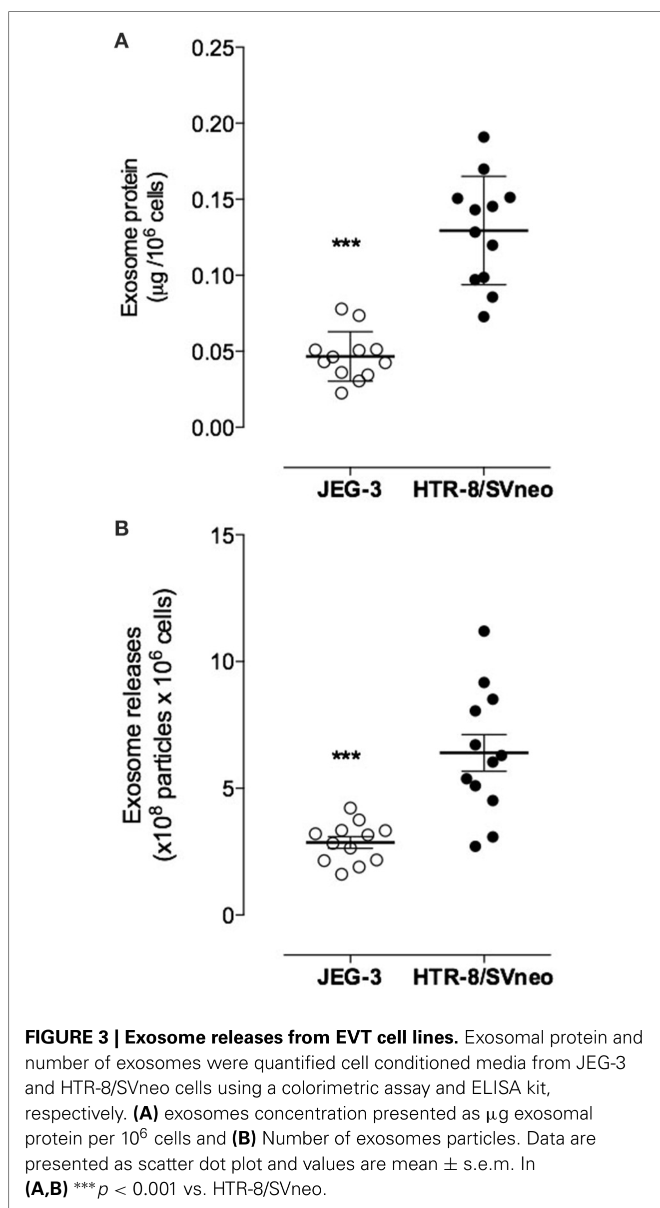

FIGURE 3 | Exosome releases from EVT cell lines. Exosomal protein and number of exosomes were quantified cell conditioned media from JEG-3 and HTR-8/SVneo cells using a colorimetric assay and ELISA kit, respectively. **(A)** exosomes concentration presented as µg exosomal protein per 10^6 cells and **(B)** Number of exosomes particles. Data are presented as scatter dot plot and values are mean ± s.e.m. In **(A,B)** $^{***}p < 0.001$ vs. HTR-8/SVneo.

system IncuCyte™. Simultaneously, VSMC migration assay in the presence of Mitomycin C (50 and 100 ng/ml) for 48 h was performed.

During experiments, VSMC cells were incubated in the presence (treatment: 100 µg exosomal protein/ml) or absence (control) of exosomes for up to 48 h under 8% O_2 ($n = 12$). The concentration used in this study was based upon exosome dose-response curves from our previously published studies (Salomon et al., 2013a,b, 2014). Exosomes were subjected to heat inactivation (30 min at 65°C) or sonication for 30 min (sonicator bath) before the incubation on VSMC. Cell migration was assessed using a scratch assay format. A scratch was made on confluent monolayers using a 96-pin WoundMaker™ (BioScience Inc, Ann Arbor, MI, USA). Wound images were automatically acquired and registered by the IncuCyte™ software system. CellPlayer™ 96-Well Invasion Assay software was use to fully automate data collection. Data were processed and analyzed using IncuCyte™ 96-Well Cell Invasion Software Application Module. Data are presented as the Relative Wound Density (RWD, Eizen, v1.0 algorithm). RWD is a representation of the spatial cell density in the wound area relative to the spatial cell density outside of the wound area at every time point (time-curve). Migration assays were performed in the presence of Mitomycin C (100 ng/ml) to minimize any confounding effects of cell proliferation. The rate of wound closure was compared using the half-maximal stimulatory time (ST_{50}) and area under the time course curve (AUC).

EXOSOME INTERNALIZATION

For exosome uptake analysis, the exosomes pellet (before purification using 30% sucrose cushion) isolated from JEG-3 and HTR-8/SVneo cells was resuspended in PBS and stained with PKH67 green fluorescent cell linker kit follow the manufacturer's instructions (Sigma-Aldrich). The staining reaction was stopped after 5 min with exosome-free FBS. Exosomes were then purified using 30% sucrose cushion as described above. Exosomes-PKH67 (100 µg/ml from JEG-3 and HTR-8/svneo cells) were incubated on VSMC for 24 h and exosome uptake was quantified using IncuCyte™ (fluorescent mode). Simultaneously, VSMC were grown in glass bottom chamber slides, and labeled exosomes (100 µg/ml) were added to subconfluent cells. After 24 h, VSMC were washed and fixed using 2% paraformaldehyde for 5 min at RT. Cells were immediately analyzed using a Zeiss fluorescence microscope (Axio Imager M1, Zeiss EC Plan-NEOFLUAR 40×/0.75, the Carl Zeiss Group, Germany) with FITC and DAPI absorbance setting.

PROTEOMIC ANALYSIS OF CYTOTROPHOBLAST DERIVED-EXOSOMES BY MASS SPECTROMETRY (MS)

Isolated exosomes from JEG-3 and HTR-8/SVneo were solubilized in 8 M urea in 50 mM ammonium bicarbonate, pH 8.5, and reduced with DTT for 1 h. Proteins were then alkylated in 10 mM iodoacetic acid (IAA) for 1 h in the dark. The sample was diluted to 1:10 with 50 mM ammonium bicarbonate and digested with trypsin (20 µg) at 37°C for 18 h. The samples were desalted by solid phase extraction using a STAGE tip protocol (Stop and go extraction tips for matrix-assisted laser desorption/ionization, nano-electrospray, and LC/MS sample pre-treatment in proteomics). The eluted peptides were dried by centrifugal evaporation to remove acetonitrile and redissolved in Solvent A. The resulting peptide mixture was analyzed by liquid chromatography (LC)/mass spectrometry (MS) LC-MS/MS on a 5600 Triple TOF mass spectrometer (AB Sciex, Framingham, USA) equipped with an Eksigent Nanoflow binary gradient HPLC system and a nanospray III ion source. Solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid in acetonitrile. MS/MS spectra were collected using Information Dependent Acquisition (IDA) using a survey scan (m/z 350–1500) followed by 25 data-dependent product ion scans of the 25 most intense precursor ions. All mass spectra were analyzed using the Mascot and Protein Pilot search engines against the Swissprot-database with the species set as human. Positive identifications were ascribed where Mascot scores were greater than 30. False discovery rate (FDR) was estimated using a reversed sequence

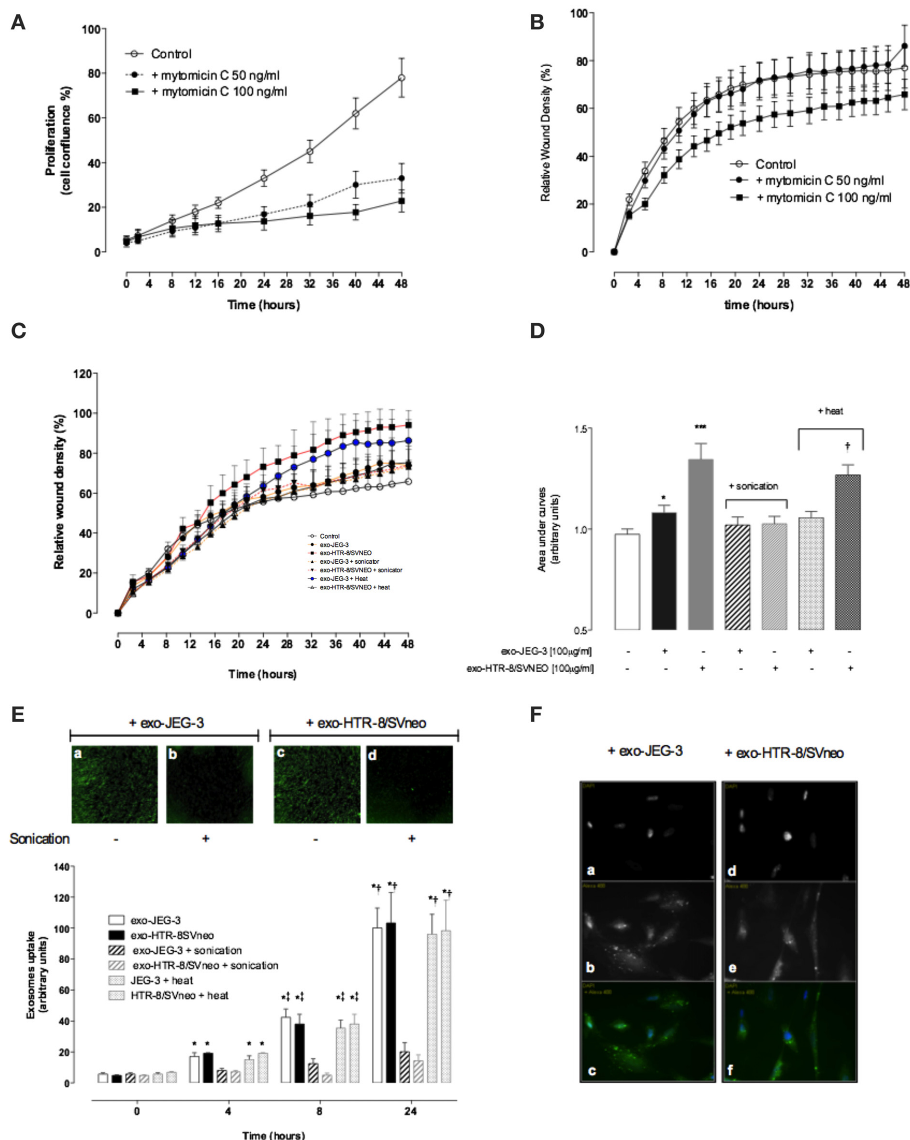

FIGURE 4 | EVT-derived exosomes effects on hVSMC migration. hVSMC were grown to confluence in 231 media and a wound was made using 96 well WoundMaker (see Materials and Methods). hVSMC Migration was measured in absence or presence of 100 ug/ml of exosomes from JEG-3 and HTR-8/SVneo cells and mitomycin C (100 ng/ml) for 48 h. Exosome particles were subjected to sonication (+sonication) or heat inactivation (+heat) before exposure to hVSMC cells **(A,B)** hVSMC proliferation and migration in the presence of Mitomycin C, respectively. **(C)** Time course of wound closure for hVSMC expressed as relative wound density (%). **(D)** Area under curves from data in **(C)**. **(E)** Time-dependent uptake of exosomes using a real time imaging system analysis. Top: images after 24 h; Bottom: Graphical representation of exosome uptake. **(F)** Fluorescent microscopy analysis of exosome uptake (40X). Data represent an $n = 12$ well each point with 3 different cells culture. Values are mean ± SD. In **(D)** $^{***}p < 0.001$ vs. all condition except exo-HTR-8/SVneo + heat; and $^{\dagger}p < 0.05$ vs. control. In **(E)** $^{*}p < 0.001$ vs. corresponding values at 0 h; $^{\ddagger}p < 0.05$ vs. corresponding values control at 4 h; $^{\dagger}p < 0.05$ vs. corresponding values control at 8 and 4 h.

database. Finally, proteins identified were submitted to bioinformatic pathway analysis (Ingenuity Pathway Analysis [IPA]; Ingenuity Systems, Mountain View, CA; www.ingenuity.com).

STATISTICAL ANALYSIS

Data are represented as mean ± s.e.m. Comparisons between two group means were performed by unpaired Student's *t*-tests. Multiple groups were compared using the analysis of variance (ANOVA). *Post-hoc* analyses were used for pairwise comparisons (Bonferroni correction test). Statistical significance was defined as at least $p < 0.05$. Statistical analyses were preformed using commercially available packages (Stata 11, StatCorp, College Station, TX, USA and Prism 6, GraphPad Inc, La Jolla, CA 92037 USA).

RESULTS

CHARACTERIZATION OF EVT CELL LINES

Figure 1A presents photomicrographs of wound closure over 40 h incubation for both cell lines and the percent change in relative

wound density over time. The migration rate of HTR-8/SVneo was significantly greater than that observed for JEG-3 cells. Half-maximal stimulatory time (ST_{50}) was 31.29 ± 0.14 h and 15.26 ± 0.15 h for JEG-3 and HTR-8SV/neo, respectively ($p <$ 0.05, **Figure 1A**, bottom). Area under curves analysis showed that the migration of HTR-8SV/neo was ~2.0-fold greater than that obtained for JEG-3 cells (**Figure 1B**).

CHARACTERIZATION OF EVT CELL LINE-DERIVED EXOSOMES

Defining vesicle size range is one of the standard metrics used to confirm that the vesicle preparation in not contaminated with non-exosome vesicles. After differential centrifugation (i.e., 300 × g, 2000 × g, and 12,000 × g), the supernatant was analyzed using a nanoparticles tracking analysis (NTA). Particle size distribution ranged from 30 to 500 nm, with an average of 180 ± 100 nm (**Figure 2A**). Supernatant was filtered and ultracentrifuged to obtain a particulate fraction (including exosomes particles) with a particle size ranged of 30–200 nm (with a mean diameter of 119 ± 65 nm, **Figure 2B**). The exosomes were purified by ultracentrifugation over a sucrose cushion and analyzed using a NTA. The exosome fractions displayed a particle size ranged from 30 to 150 nm in diameter (with mean of 79 ± 68 nm, **Figure 2C**). The presence of exosomal marker CD63 in exosomes isolated from both cell lines was confirmed by Western blot (**Figure 2D**, top). Finally, exosomes isolated from JEG-3 and HTR-8/SVneo were visualized by transmission electron microscopy (**Figure 2D**, bottom). Exosomes were identified as small vesicles between 40 and 100 nm. No significant differences in NTA data, CD63 expression and electron microscopy were identified between cell lines.

EXOSOME RELEASE FROM EVT CELL LINES

Exosomal protein release was expressed as total exosomal protein (i.e., particulate material with a buoyant density of 1.127 g/ml) per million cells/24 h. JEG-3 cells released 46 ± 16 ng exosomal protein per million cells per 24 h ($n = 4$; i.e., 4 different isolations from ~ 200×10^6 cells each) whereas HTR-8/SVneo cells released 120 ± 35 ng exosomal protein per million cells per 24 h ($n = 4$, **Figure 3A**). HTR-8/SVneo cells released significantly more exosomes (~2.6-folds) in 24 h compared to JEG-3 cells ($p < 0.0001$). These results were confirmed by quantifying immunoreactive exosomal CD63. The number of exosomes (NEP) released from HTR-8/SVneo cells ($6.39 \times 10^8 \pm 2.5 \times 10^8$/24 h) was ~2.3-fold greater ($p < 0.0001$) than that observed for JEG-3 cells ($2.86 \times 10^8 \pm 0.78 \times 10^8$/24 h, **Figure 3B**).

EFFECT OF EVT-DERIVED EXOSOMES ON CELL MIGRATION

A VSMC was used to establish the effects of EVT-derived exosomes on cell migration. An anti-proliferative drug Mitomycin C (50 and 100 ng/ml) reduces significantly ($p < 0.01$) VSMC proliferation (**Figure 4A**). A dose of 100 ng/ml decreased VSMC migration compared to 50 ng/ml of Mitomycin C and control (**Figure 4B**). The effects of exosomes (100 μg protein/ml) isolated from JEG-3 and HTR-8/SVneo cultured under 8% O_2 on VSMC migration under 8% O_2 are presented in **Figures 4C,D**. All experiments were done in the presence of Mitomycin C (100 ng/ml). The rate of wound closure was significantly increased in the presence of HTR-8/SVneo-derived exosome compared to control (-exosomes) as measured by ST_{50} (15.57 ± 0.32 vs. 25.09 ± 0.58, $p < 0.01$) (**Table 1**). Area under curves analysis showed that HTR-8/SVneo-derived exosome increased ~35 ± 0.2% VSMC migration compared to control. Similarly, exosomes from JEG-3 cells increased VSMC migration ~12 ± 0.1% compared to values in the absence of exosomes (control), however, the effect was smaller compared to exosomes from HTR-8/SVneo. Exosomes were exposed to heat inactivation before incubation on VSMC; however, heat inactivation did not affect the effect of exosomes on VSMC migration. In contrast, sonication completely abolished the HTR-8/SVneo and JEG-3-derived exosomes effect on VSMC migration.

The internalization of exosomes labeled with PKH67 (green) in VSMC was quantified and visualized using The IncuCyte and a fluorescence microscope, respectively (**Figures 4E,F**). Exosome uptake by VSMC was observed in a time-dependent manner with the maximum at 24 h (**Figure 4E**, top panel a and c). Sonication abolished the uptake of fluorescent exosomes (**Figure 4E**, top panel b and d) compared to exosomes without sonication. Exosome uptake is presented as fluorescent per cell confluence normalized to maximum uptake of 100%. Heat inactivation did not affect the exosomes uptake by VSMC (**Figure 4**, lower panel). Finally, fluorescence microscope analysis showed an intracellular fluorescent in VSMC cells exposed to intact vesicles from JEG-3 and HTR-8/SVneo (**Figure 4F**).

PROTEOMIC ANALYSIS OF EVT-DERIVED EXOSOME

Mass spectrometry analysis identified over 140 exosomal proteins, including cell-line specific exosomal proteins (**Table 2** and **Figure 5A**). Exosomal proteins isolated from JEG-3 and HTR-8/SVneo cells were associated with cellular movement and morphology, immune cell trafficking and cellular assembly and organization in accordance with Ingenuity Pathway Analysis (IPA) analysis. The canonical pathways associated with our exosomal proteins isolated from JEG-3 and HTR-8/SVneo cells and defined by IPA Core comparison analysis showed that the score (−log [p-value]) for proteins associated with cell movement and migration was significantly higher (~1.2-fold, $p < 0.05$) in HTR-8/SVneo-derived exosomes to compare to exosomes from JEG-3 cells (**Figure 5B**).

Table 1 | Kinetic characteristic of exosome effects on hVSMC migration.

Condition	ST_{50} (hours)
Control	25.09 ± 0.58
+exo-JEG-3	21.83 ± 0.49‡
+exo-HTR-8/SVneo	15.57 ± 0.32*
+exo-JEG-3 + sonication	25.06 ± 0.46
+exo-HTR-8/SVneo + sonication	23.27 ± 0.48
+exo-JEG-3 + heat	24.14 ± 0.35
+exo-HTR-8/SVneo + heat	19.43 ± 0.34†

*The effect of exosomes isolated from JEG-3 and HTR-8/SVneo-conditioned media on hVSMC in vitro migration. Data are expressed as half-maximal Stimulatory Time (ST_{50} in hours) and represent the mean ± SD. *$p < 0.05$ vs. control; †$p < 0.005$ vs. control and +exo-HTR-8/SVneo. ‡$p < 0.005$ vs. control.*

Table 2 | List of proteins identified in exosomes isolated from JEG-3 and HTR-8/SVneo cells.

Symbol	Entrez gene name	UniProt/Swiss-Prot accession	Location	Type(s)
COMMON PROTEINS IN EXOSOMES FROM JEG-3 and HTR-8/Svneo CELLS				
A2M	Alpha-2-macroglobulin	A2MG_HUMAN	Extracellular space	Transporter
ACTB	Actin, beta	ACTB_HUMAN	Cytoplasm	Other
AHSG	Alpha-2-HS-glycoprotein	FETUA_HUMAN	Extracellular space	Other
ALB	Albumin	ALBU_HUMAN	Extracellular space	Transporter
APOE	Apolipoprotein E	APOE_HUMAN	Extracellular space	Transporter
APOM	Apolipoprotein M	APOM_HUMAN	Plasma membrane	Transporter
C3	Complement component 3	CO3_HUMAN	Extracellular space	Peptidase
EEF2	Eukaryotic translation elongation factor 2	EF2_HUMAN	Cytoplasm	Translation regulator
ENO1	Enolase 1, (alpha)	ENOA_HUMAN	Cytoplasm	Enzyme
F5	Coagulation factor V (proaccelerin, labile factor)	FA5_HUMAN	Plasma membrane	Enzyme
FGB	Fibrinogen beta chain	FIBB_HUMAN	Extracellular space	Other
FLNA	Filamin A, alpha	FLNA_HUMAN	Cytoplasm	Other
FN1	Fibronectin 1	FINC_HUMAN	Extracellular space	Enzyme
HSP90AA1	Heat shock protein 90 kDa alpha (cytosolic), class A member 1	HS90A_HUMAN	Cytoplasm	Enzyme
HSPA8	Heat shock 70 kDa protein 8	HSP7C_HUMAN	Cytoplasm	Enzyme
KRT1	Keratin 1	K2C1_HUMAN	Cytoplasm	Other
KRT10	Keratin 10	K1C10_HUMAN	Cytoplasm	Other
LDHB	Lactate dehydrogenase B	LDHB_HUMAN	Cytoplasm	Enzyme
MSN	Moesin	MOES_HUMAN	Plasma membrane	Other
PGK1	Phosphoglycerate kinase 1	PGK1_HUMAN	Cytoplasm	Kinase
PKM	Pyruvate kinase, muscle	KPYM_HUMAN	Cytoplasm	Kinase
PZP	Pregnancy-zone protein	PZP_HUMAN	Extracellular space	Other
THBS1	Thrombospondin 1	TSP1_HUMAN	Extracellular space	Other
VEPH1	Ventricular zone expressed PH domain-containing 1	MELT_HUMAN	Nucleus	Other
YWHAB	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta	1433B_HUMAN	Cytoplasm	Transcription regulator
YWHAE	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon	1433E_HUMAN	Cytoplasm	Other
EXOSOMES FROM JEG-3 CELLS				
ACTN1	Actinin, alpha 1	ACTN1_HUMAN	Cytoplasm	Other
ADAMTS13	ADAM metallopeptidase with thrombospondin type 1 motif, 13	ATS13_HUMAN	Extracellular space	Peptidase
AFP	Alpha-fetoprotein	FETA_HUMAN	Extracellular space	Transporter
AHCY	Adenosylhomocysteinase	SAHH_HUMAN	Cytoplasm	Enzyme
AMY2A	Amylase, alpha 2A (pancreatic)	AMYP_HUMAN	Extracellular space	Enzyme
APOA1	Apolipoprotein A-I	APOA1_HUMAN	Extracellular space	Transporter
ARGLU1	Arginine and glutamate rich 1	ARGL1_HUMAN	Other	Other
ATP10A	ATPase, class V, type 10A	AT10A_HUMAN	Plasma membrane	Transporter
C5	Complement component 5	CO5_HUMAN	Extracellular space	Cytokine
C7	Complement component 7	CO7_HUMAN	Extracellular space	Other
C4A/C4B	Complement component 4B (Chido blood group)	CO4A_HUMAN	Extracellular space	Other
C8B	Complement component 8, beta polypeptide	CO8B_HUMAN	Extracellular space	Other
CLEC11A	C-type lectin domain family 11, member A	CLC11_HUMAN	Extracellular space	Growth factor
COL6A1	Collagen, type VI, alpha 1	CO6A1_HUMAN	Extracellular space	Other
COLGALT1	Collagen beta(1-O)galactosyltransferase 1	GT251_HUMAN	Cytoplasm	Other
COMP	Cartilage oligomeric matrix protein	COMP_HUMAN	Extracellular space	Other
CPN1	Carboxypeptidase N, polypeptide 1	CBPN_HUMAN	Extracellular space	Peptidase
DGKQ	Diacylglycerol kinase, theta 110 kDa	DGKQ_HUMAN	Cytoplasm	Kinase
EEF1A2	Eukaryotic translation elongation factor 1 alpha 2	EF1A2_HUMAN	Cytoplasm	Translation regulator
F2	Coagulation factor II (thrombin)	THRB_HUMAN	Extracellular space	Peptidase

(Continued)

Table 2 | Continued

Symbol	Entrez gene name	UniProt/Swiss-Prot accession	Location	Type(s)
F10	Coagulation factor X	FA10_HUMAN	Extracellular space	Peptidase
F13A1	Coagulation factor XIII, A1 polypeptide	F13A_HUMAN	Extracellular space	Enzyme
FBLN1	Fibulin 1	FBLN1_HUMAN	Extracellular space	Other
FGG	Fibrinogen gamma chain	FIBG_HUMAN	Extracellular space	Other
GC	Group-specific component (vitamin D binding protein)	VTDB_HUMAN	Extracellular space	Transporter
GSN	Gelsolin	GELS_HUMAN	Extracellular space	Other
H2AFV	H2A histone family, member V	H2AV_HUMAN	Nucleus	Other
HBB	Hemoglobin, beta	HBB_HUMAN	Cytoplasm	Transporter
HIST1H1T	Histone cluster 1, H1t	H1T_HUMAN	Nucleus	Other
HIST1H2BB	Histone cluster 1, H2bb	H2B1B_HUMAN	Nucleus	Other
HSPA1L	Heat shock 70kDa protein 1-like	HS71L_HUMAN	Other	Other
IGLL1/IGLL5	Immunoglobulin lambda-like polypeptide 1	IGLL5_HUMAN	Plasma membrane	Other
ITIH1	Inter-alpha-trypsin inhibitor heavy chain 1	ITIH1_HUMAN	Extracellular space	Other
ITIH2	Inter-alpha-trypsin inhibitor heavy chain 2	ITIH2_HUMAN	Extracellular space	Other
ITIH3	Inter-alpha-trypsin inhibitor heavy chain 3	ITIH3_HUMAN	Extracellular space	Other
LINC00083	Long intergenic non-protein coding RNA 83	YA021_HUMAN	Other	Other
LRP1	Low density lipoprotein receptor-related protein 1	LRP1_HUMAN	Plasma membrane	Transmembrane receptor
LUM	Lumican	LUM_HUMAN	Extracellular space	Other
MYH9	Myosin, heavy chain 9, non-muscle	MYH9_HUMAN	Cytoplasm	Transporter
MYL6	Myosin, light chain 6, alkali, smooth muscle and non-muscle	MYL6_HUMAN	Cytoplasm	Other
NHSL2	NHS-like 2	NHSL2_HUMAN	Other	Other
OR2W3	Olfactory receptor, family 2, subfamily W, member 3	OR2W3_HUMAN	Plasma membrane	G-protein coupled receptor
PIF1	PIF1 5′-to-3′ DNA helicase	PIF1_HUMAN	Nucleus	Enzyme
PITRM1	Pitrilysin metallopeptidase 1	PREP_HUMAN	Cytoplasm	Peptidase
PLG	Plasminogen	PLMN_HUMAN	Extracellular space	Peptidase
PPP6R3	Protein phosphatase 6, regulatory subunit 3	PP6R3_HUMAN	Cytoplasm	Other
RAN	RAN, member RAS oncogene family	RAN_HUMAN	Nucleus	Enzyme
RDH13	Retinol dehydrogenase 13 (all-trans/9-cis)	RDH13_HUMAN	Cytoplasm	Enzyme
SERPINA7	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7	THBG_HUMAN	Extracellular space	Transporter
SERPINF1	Serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	PEDF_HUMAN	Extracellular space	Other
SERPINF2	Serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 2	A2AP_HUMAN	Extracellular space	Other
THBS4	Thrombospondin 4	TSP4_HUMAN	Extracellular space	Other
TLN1	Talin 1	TLN1_HUMAN	Plasma membrane	Other
TUBA1B	Tubulin, alpha 1b	TBA1B_HUMAN	Cytoplasm	Other
TUBA4A	Tubulin, alpha 4a	TBA4A_HUMAN	Cytoplasm	Other
VTN	Vitronectin	VTNC_HUMAN	Extracellular space	Other
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	1433Z_HUMAN	Cytoplasm	Enzyme
ZNF480	Zinc finger protein 480	ZN480_HUMAN	Nucleus	Other
EXOSOMES FROM HTR-8/SVneo CELLS				
AASDH	Aminoadipate-semialdehyde dehydrogenase	ACSF4_HUMAN	Other	Enzyme
ACTN4	Actinin, alpha 4	ACTN4_HUMAN	Cytoplasm	Other
ALDOA	Aldolase A, fructose-bisphosphate	ALDOA_HUMAN	Cytoplasm	Enzyme
ANXA2	Annexin A2	ANXA2_HUMAN	Plasma membrane	Other
ANXA5	Annexin A5	ANXA5_HUMAN	Plasma membrane	Other
ANXA6	Annexin A6	ANXA6_HUMAN	Plasma membrane	Other
APOB	Apolipoprotein B	APOB_HUMAN	Extracellular space	Transporter

(Continued)

Table 2 | Continued

Symbol	Entrez gene name	UniProt/Swiss-Prot accession	Location	Type(s)
ARAP2	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 2	ARAP2_HUMAN	Cytoplasm	Other
ART4	ADP-ribosyltransferase 4 (Dombrock blood group)	NAR4_HUMAN	Nucleus	Enzyme
ATP1A1	ATPase, Na^{+}/K^{+} transporting, alpha 1 polypeptide	AT1A1_HUMAN	Plasma membrane	Transporter
B2M	Beta-2-microglobulin	B2MG_HUMAN	Plasma membrane	Transmembrane receptor
BASP1	Brain abundant, membrane attached signal protein 1	BASP1_HUMAN	Nucleus	Transcription regulator
BMS1	BMS1 ribosome biogenesis factor	BMS1_HUMAN	Nucleus	Other
C9	Complement component 9	CO9_HUMAN	Extracellular space	Other
C7orf61	Chromosome 7 open reading frame 61	CG061_HUMAN	Other	Other
CD59	CD59 molecule, complement regulatory protein	CD59_HUMAN	Plasma membrane	Other
CHM	Choroideremia (Rab escort protein 1)	RAE1_HUMAN	Cytoplasm	Enzyme
CLTC	Clathrin, heavy chain (Hc)	CLH1_HUMAN	Plasma membrane	Other
COL12A1	Collagen, type XII, alpha 1	COCA1_HUMAN	Extracellular space	Other
FLNB	Filamin B, beta	FLNB_HUMAN	Cytoplasm	Other
FSCN1	Fascin homolog 1, actin-bundling protein (Strongylocentrotus purpuratus)	FSCN1_HUMAN	Cytoplasm	Other
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	G3P_HUMAN	Cytoplasm	Enzyme
GLP2R	Glucagon-like peptide 2 receptor	GLP2R_HUMAN	Plasma membrane	G-protein coupled receptor
GNAI2	Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 2	GNAI2_HUMAN	Plasma membrane	Enzyme
GNB1	Guanine nucleotide binding protein (G protein), beta polypeptide 1	GBB1_HUMAN	Plasma membrane	Enzyme
GNG12	Guanine nucleotide binding protein (G protein), gamma 12	GBG12_HUMAN	Plasma membrane	Enzyme
HBD	Hemoglobin, delta	HBD_HUMAN	Other	Transporter
HIST1H1D	Histone cluster 1, H1d	H13_HUMAN	Nucleus	Other
HIST1H2BD	Histone cluster 1, H2bd	H2B1D_HUMAN	Nucleus	Other
HMCN1	Hemicentin 1	HMCN1_HUMAN	Extracellular space	Other
HSP90AB1	Heat shock protein 90 kDa alpha (cytosolic), class B member 1	HS90B_HUMAN	Cytoplasm	Enzyme
HSPB1	Heat shock 27 kDa protein 1	HSPB1_HUMAN	Cytoplasm	Other
HSPE1	Heat shock 10 kDa protein 1	CH10_HUMAN	Cytoplasm	Enzyme
ITGA3	integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	ITA3_HUMAN	Plasma membrane	Other
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	ITB1_HUMAN	Plasma membrane	Transmembrane receptor
KRT9	Keratin 9	K1C9_HUMAN	Cytoplasm	Other
LGALS1	Lectin, galactoside-binding, soluble, 1	LEG1_HUMAN	Extracellular space	Other
MFGE8	Milk fat globule-EGF factor 8 protein	MFGM_HUMAN	Extracellular space	Other
MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	MIF_HUMAN	Extracellular space	Cytokine
NCL	Nucleolin	NUCL_HUMAN	Nucleus	Other
NME2	NME/NM23 nucleoside diphosphate kinase 2	NDKB_HUMAN	Nucleus	Kinase
NRG1	Neuregulin 1	NRG1_HUMAN	Other	Growth factor
PARK7	Parkinson protein 7	PARK7_HUMAN	Nucleus	Enzyme
PGAM1	Phosphoglycerate mutase 1 (brain)	PGAM1_HUMAN	Cytoplasm	Phosphatase
PRDX1	Peroxiredoxin 1	PRDX1_HUMAN	Cytoplasm	Enzyme
PTGFRN	Prostaglandin F2 receptor inhibitor	FPRP_HUMAN	Plasma membrane	Other
RUVBL2	RuvB-like AAA ATPase 2	RUVB2_HUMAN	Nucleus	transcription regulator
S100A11	S100 calcium binding protein A11	S10AB_HUMAN	Cytoplasm	Other
SERPINA1	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	A1AT_HUMAN	Extracellular space	Other

(Continued)

Table 2 | Continued

Symbol	Entrez gene name	UniProt/Swiss-Prot accession	Location	Type(s)
SLC16A3	Solute carrier family 16 (monocarboxylate transporter), member 3	MOT4_HUMAN	Plasma membrane	Transporter
SLC2A1	Solute carrier family 2 (facilitated glucose transporter), member 1	GTR1_HUMAN	Plasma membrane	Transporter
STARD13	StAR-related lipid transfer (START) domain containing 13	STA13_HUMAN	Cytoplasm	Other
SYN1	Synapsin I	SYN1_HUMAN	Plasma membrane	Transporter
TMEM262	Transmembrane protein 262	YK025_HUMAN	Other	Other
TPI1	Triosephosphate isomerase 1	TPIS_HUMAN	Cytoplasm	Enzyme
TRRAP	Transformation/transcription domain-associated protein	TRRAP_HUMAN	Nucleus	Transcription regulator
TUBBP5	Tubulin, beta pseudogene 5	YI016_HUMAN	Other	Other
UBAC2	UBA domain containing 2	UBAC2_HUMAN	Cytoplasm	Enzyme
VIM	Vimentin	VIME_HUMAN	Cytoplasm	Other

All mass spectra were analyzed using the Mascot and Protein Pilot search engines against the Swissprot-swissprot database with the species set as human (score over 30). Exosomal proteins identified by mass-spectrometry were analyzed with the Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com). False discovery rate (FDR) was estimated using a reversed sequence database. List of total exosomal protein from cytotrophoblast cells exposed to different oxygen level are presented as Protein ID, Symbol, Entrez Gene Name, Location, and type.

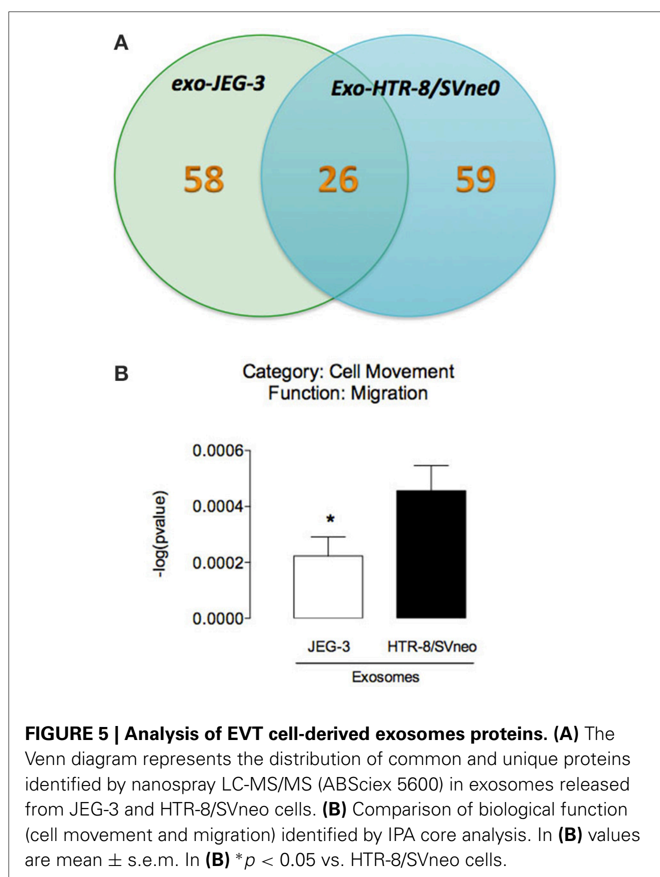

FIGURE 5 | Analysis of EVT cell-derived exosomes proteins. (A) The Venn diagram represents the distribution of common and unique proteins identified by nanospray LC-MS/MS (ABSciex 5600) in exosomes released from JEG-3 and HTR-8/SVneo cells. **(B)** Comparison of biological function (cell movement and migration) identified by IPA core analysis. In **(B)** values are mean ± s.e.m. In **(B)** $*p < 0.05$ vs. HTR-8/SVneo cells.

DISCUSSION

A tenet of contemporary obstetrics is that events that compromise placentation and the development of the materno-fetal exchange increase the risk of complications of pregnancy and contribute to poor pregnancy outcome. In particular, conditions that affect the differentiation and invasion of placental cells compromise placental perfusion and function (Jauniaux et al., 2006, 2010; Burton et al., 2009) and the subsequent growth and development of the fetus (Khong et al., 1987). EVT migrate into maternal decidua and myometrium and interact with VSMC in uterine spiral arteries. Conversion of these arteries is associated with the loss of VSMC from the vessel wall by migration out of the vessel. In this study we established that: (1) exosome isolated from EVT-like cell lines (JEG-3 and HTR-8/SVneo) with different cellular origins (choriocarcinoma and EVT) exhibit differences in their rate of release, protein content and bioactivity; and (2) both exosomes from JEG-3 and HTR-8/SVneo cells increase VSMC migration. However, the effect of exosomes from HTR-8/SVneo was significantly higher. Exosomes released from first trimester trophoblast cells (EVT), therefore, may regulate processes that play a key role in placentation and remodeling of the uterine spiral arteries.

The interactions between EVT and VSMC are not fully understood, in part due to difficulties of accessing first trimester samples and the lack of suitable animal models (Carter, 2007; Ackerman et al., 2013). We obtained nanovesicles with high purity. The data presented establish that the final preparation used display a size distribution and buoyant consistent with exosomes and the lack of significant contamination by microsomes. In this study, we quantified the release of exosomes from JEG-3 and HTR-8/SVneo cells (as indicated by immunoreactive exosomal CD63). The data obtained establish that exosome release is cell type specific and correlated with cell migration capacity. Consistent with these data, a correlation between exosome release and cell invasiveness has been described previously in ovarian cancer cells (Kobayashi et al., 2014).

The initial stages of uterine SpA remodeling involves a loss of the VSMC component by apoptosis, migration or a combination of both processes. In this study, we identify a novel exosomal signaling pathway by which EVT (HTR-8/SVneo cells) promote the

migration of VSMC. Consistent with these data, Bulmer et al. (2012) observed that VSMC migration plays a major role in remodeling of the artery by migration into the decidua and vessel lumen and this phenomenon is enhanced in the presence of EVT cells (Bulmer et al., 2012). Based upon the available data, we propose that perivascular EVT release exosomes that interaction with VSMC and promote their migration out of SpAs and alter the vasoreactive of these vessels. This effect of exosomes on VSMC migration was cell-type specific (i.e., only exosomes from HTR-8/SVneo cells promote cell migration) and may reflect the different cellular origin of the two cell lines compared in this study. HTR-8/SVneo cells are derived by transfection of first trimester trophoblast cells (Graham et al., 1993). JEG-3 cells are derived from a choriocarcinoma (Kohler and Bridson, 1971). Difference in the molecular cargo carried by exosomes released from HTR-8/SVneo and JEG-3 cell may account for their different effects on target cell migration. Consistent with this suggestion, proteins involved in cell migration were differentially represented in exosomes isolated from the two-cell lines.

The exosomal content is highly dependent on the cell origin and on pre-conditioning of the cell. Exosomes function as a carrier of specific molecules such as, proteins, lipids, mRNA, and miRNA and can interact with neighboring cells or travel long distances in the bloodstream to reprogram the phenotype and regulate their function (Denzer et al., 2000). In this study, we identified unique proteins (58 and 59 proteins in exosome from JEG-3 and HTR-8/SVneo cells) and common proteins (26) between these EVT cell lines. Ingenuity Pathway Analysis (IPA) of exosomal proteins identified cell-dependent changes in cell movement and migration signaling pathways. Exosomes have been reported to express a diverse range of cell surface receptors, proteins (including, heat shock proteins, cytoskeletal proteins, adhesion molecules, membrane transport, and fusion proteins), mRNA and miRNA with the potential to affect the acute and long-term function of the cells with which they interact (Ambros, 2004). Recent data demonstrate that trophoblast-derived exosomes induce proinflammatory cytokines such IL-1 β in human macrophages cells (Atay et al., 2011). Furthermore, *in vitro* exposure of PBMC and dendritic cells to exosomal proteins induce differentiation of stem cells; suppression of activation of natural killer cells and macrophages; and stimulation of cell migration (Mincheva-Nilsson et al., 2006; Knight, 2008; Soo et al., 2012). Interestingly, protein analysis revealed that exosome release from cytrophoblast cells increases with low oxygen tension and their exosome promotes cell migration in extravillous cytotrophoblast (HTR-8/SVneo) (Salomon et al., 2013b).

The observed effects of HTR-8/SVneo-derived exosomes on VSCM migration were also dependent upon exosome structural integrity. Disruption of exosomes by sonication (Delorme-Axford et al., 2013) completely abolished their effect on VSMC migration. While the precise mechanisms by which sonication abolishes the effects of exosomes remains to be established, preventing exosomal fusion with VSMC cell membrane and the intracellular delivery of signaling molecules and/or the loss of capacity to appropriately present exosomal surface moieties to target cell receptors represent possible pathways and warrant further investigation. We establish in this study that exosome integrity is critical to mediating their effects on VSMC migration. It is not possible from the data obtained in this study to differentiate between sonication–induced disruption of exosome: uptake; fusion; activation of VSMC cell surface receptors, or a combination of these (and other) mechanisms. The elucidation of contribution of these mechanisms will require additional extensive studies to establish.

In conclusion, using *in vitro* EVT-like cell lines, we have demonstrated difference in release, composition and bioactivity between exosomes from JEG-3 and HTR-8/SVneo cells. Exosomes released from EVT may play a role in remodeling SpA by promoting migration of VSMC. Exosomal-induced VSCM migration is associated with increased representation of proteins involved in cell migration processes within their molecular cargo and dependent upon exosomal structural integrity. The identification of this EVT-VSMC exosomal communication pathway not only affords opportunity for developing biomarkers of placentation but also the assessment of exosome targeted therapeutic innervation strategies.

AUTHOR CONTRIBUTIONS

Carlos Salomon, Sarah Yee, Katherin Scholz-Romero, Kanchan Vaswani, Miharu Kobayashi and David Kvaskoff contributed in generating experimental data. Carlos Salomon, Sebastian E. Illanes, Murray D. Mitchell and Gregory E. Rice contributed in discussion and reviewed/edited manuscript. Carlos Salomon and Gregory E. Rice wrote the manuscript and drew the figures.

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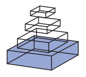

Physiological mechanisms of vascular response induced by shear stress and effect of exercise in systemic and placental circulation

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Physiological vascular function regulation is essential for cardiovascular health and depends on adequate control of molecular mechanisms triggered by endothelial cells in response to mechanical and chemical stimuli induced by blood flow. Endothelial dysfunction is one of the main risk factors of cardiovascular pathology, where the imbalance between the synthesis of vasodilator and vasoconstrictor molecules is common in the development of vascular disorders in systemic and placental circulation. In the placenta, an organ without autonomic innervations, the local control of vascular tone is critical for maintenance of fetal growth and mechanisms that underlie shear stress response induced by blood flow are essential during pregnancy. In this field, shear stress induced by moderate exercise is one of the most important mechanisms to improve vascular function through nitric oxide synthesis and stimulation of mechanical response of endothelial cells triggered by ion channels, caveolae, endothelial NO synthase, and vascular endothelial growth factor, among others. The demand for oxygen and nutrients by tissues and organs, especially in placentation and pregnancy, determines blood flow parameters, and physiological adaptations of vascular beds for covering metabolic requirements. In this regard, moderate exercise versus sedentarism shows potential benefits for improving vascular function associated with the enhancement of molecular mechanisms induced by shear stress. In this review, we collect evidence about molecular bases of physiological response to shear stress in order to highlight the relevance of moderate exercise-training for vascular health in adult and fetal life.

Keywords: endothelial dysfunction, shear stress, placental circulation, exercise, nitric oxide

INTRODUCTION

The endothelium is the main regulator of vascular physiology, controlling hemodynamics and angiogenesis in postnatal and fetal life. Dysfunction of endothelial cells have several clinical implications related with alteration of physiological regulation of capillary permeability, vascular homeostasis, leukocyte trafficking, vasomotor control, angiogenesis, acquired and innate immunity, among others. Furthermore, these cells exhibit morphological and functional heterogeneity, which give them high capacity for adaptation, according to environmental conditions to maintain homeostasis in different vascular beds (Aird, 2007). In human placenta, an organ without autonomic innervations, the control of vascular tone is dependent on local release of vasoconstrictors and vasodilators, released from endothelial cells in response to mechanical and chemical stimuli triggered by cardiac output and blood flow requirements (Fox and Khong, 1990; Myatt, 1992). In placental and systemic circulation, the main stimulus regarding control of vascular resistance and blood flow, is related to increments of shear stress by high placental perfusion throughout pregnancy. The vascular response of placental circulation to shear stress depends of a variety of factors: local release of vasoactive molecules, endocrine signaling, oxidative stress in vascular cells or vascular remodeling, among others. The maintenance of vascular tone and blood supply for placental circulation is a key factor for adequate placentation and fetal development.

Abbreviations: ACS, acute coronary syndrome; AMI, acute myocardial infarction; BAECs, bovine aortic endothelial cells; BH_4, tetrahydrobiopterin; Cav, caveolin; CVDs, cardiovascular diseases; DM2, diabetes mellitus type 2; eNOS, endothelial NO synthase; FGF-1, fibroblast growth factor 1; FMV, flow mediated vasodilatation; GDM, gestational diabetes mellitus; GPCRs, G-protein couple receptors; hCAT-1, human cationic amino acid transporter 1; HUVECs, human umbilical vein endothelial cells; IMPROVE, Improving Maternal and Progeny Obesity Via Exercise; IUGR, intrauterine growth restriction; K_{ATP}, ATP-sensitivity K^{++} channels; K_{Ca}, calcium-activated K^+ channels; K_{ir}, inwardly rectifying potassium channel; K_V, voltage-gated K^+ channels; L-NMMA, L-N^Gmonomethyl arginine; NADPH, nicotinamide adenine dinucleotide phosphate; NCDs, non-communicable diseases; NO, nitric oxide; oFPAEs, ovine fetoplacental artery endothelial cells; ORCCs, outwardly rectifying chloride channels; PlGF, placental growth factor; PP, pulse pressure; ROS, reactive oxygen species; sGC, soluble guanylate cyclase; SOD, superoxide dismutase; sVEGFR1, soluble VEGFR-1; TASK1, TWIK-related acid-sensitive K^+ channels 1; TS, tangential stress; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VSMCs, vascular smooth muscle cells; WTI, wall thickening increase.

Relevant pathologies in developing and middle-income countries are NCDs, like CVD, metabolic syndrome, obesity and diabetes mellitus. With changes in lifestyle and quality of nutrition in western civilization, there is a sustained increase in DM2, GDM, overweightness and obesity during pregnancy (Kopp, 2005; Zhang and Ning, 2011; Pan et al., 2012). There is a consensus that NCD are serious problems for public health, and several public policies in the western world are oriented toward prevention based on healthy nutrition and exercise. For this reason, the aim of this review is to contribute to the understanding about molecular mechanisms involved in vascular dysfunction and deterioration of shear stress response, considering the positive effect of physical exercise on systemic and placental circulation. In this field, it is well known the importance of intrauterine life conditions for the development of NCD in adults (Hanson and Gluckman, 2011), so our focus is to show evidence about the effects of physical exercise and moderate training to better vascular adaptations triggered by shear stress, and the potential application of exercise therapy for improve systemic and placental vasculature function affected by chronic or gestational diseases.

ENDOTHELIAL DYSFUNCTION

Endothelial dysfunction is established as one of the main risk factors for CVDs, where the imbalance between synthesis of vasodilators and vasoconstrictors is the most common factor (Qian and Chi, 2012). High synthesis and activity of vasoconstrictor molecules, such as endothelin-1 and thromboxane A2; as well as reduction in the availability of vasodilator molecules, like NO, associated with oxidative stress by high synthesis of ROS, are the cornerstone in poor capacity of endothelium to maintain vascular tone (Seals et al., 2011). Oxidative stress is a condition defined as an imbalance between systemic manifestation of ROS and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage (Valko et al., 2007). In pregnancy, a condition associated with high risk of oxidative stress, the equilibrium between vasodilators versus vasoconstrictors and oxidative stress versus antioxidant mechanisms is a key factor during gestation and fetal development (Sobrevia and González, 2009).

In HUVECs from GDM or incubated with high concentration of D-glucose, there is high synthesis of NO (Sobrevia et al., 1995), related with high expression of the main L-arginine transporter: hCAT-1 (González et al., 2004, 2011; Guzmán-Gutiérrez et al., 2014). L-Arginine is the substrate for NO synthesis by eNOS (Alderton et al., 2003) and the transport of L-arginine is essential for NO synthesis in the endothelium (Shin et al., 2011). In assays where cells are incubated with high concentrations of D-glucose, the activation of the L-arginine/NO pathway has been related with endothelial dysfunction induced by concomitant increased synthesis of ROS and lower bioavailability of NO (Sobrevia and González, 2009). In this regards, a hallmark for endothelial function is the availability of NO, which is regulated by a combination of synthesis and NO inactivation (Luiking et al., 2010). Reduction in synthesis or availability of NO is associated with lower expression or activity of eNOS as a result of endogenous and exogenous inhibitors or by reduced availability of L-arginine (Dhein et al., 2003; Endemann and Schiffrin, 2004; Kalinowski and Malinski, 2004). Another important mechanism for NO inactivation and endothelial dysfunction is oxidative stress induced by ROS synthesis mediated by NADPH oxidases; enzymes whose primary function is to generate ROS and which plays an important role in redox signaling (Lambeth, 2004). The over expression or high activity of NADPH oxidase induces the uncoupling of eNOS due to the oxidative degradation of BH_4, eNOS cofactor, leading to eNOS-dependent synthesis of superoxide anion (O_2^-) in detriment of NO synthesis (Antoniades et al., 2006; Dworakowski et al., 2008). Once synthesized, O_2^- is used by SOD to generate H_2O_2, which has greater stability and capacity to diffuse through biological membranes, acting as a modulator of signal transduction pathways (Li and Shah, 2004). In addition, the O_2^- reacts rapidly with NO to generate peroxynitrite ($ONOO^-$), a powerful oxidizing agent that induces DNA fragmentation and lipid oxidation (Carr et al., 2000). Currently, it is postulated that the mechanism by which oxygen "hijack" the NO is related with the $ONOO^-$ formation, which plays a central role in the development of endothelial dysfunction in diseases such as diabetes mellitus (Rolo and Palmeira, 2006; Hadi and Suwaidi, 2007; Rask-Madsen and King, 2008), preeclampsia (Gu et al., 2006; Escudero and Sobrevia, 2008), and hypertension (Harrison et al., 2003).

Also alterations related with endothelial dysfunction are associated with pro-thrombotic and pro-inflammatory states, and become the main etiologic factors for developing essential hypertension and atherosclerotic disease (Savoia et al., 2011). Determination of endothelial dysfunction in healthy and pathologic patients (especially during pregnancy) is a relevant challenge for physicians and researchers, regarding the obvious difficulties in extrapolating *in vitro* findings to the clinics. Therefore, non-invasive evaluation for endothelial dysfunction, such as FMV, are important tools to determine the association of endothelial dysfunction with wall thickness of conduit vessels, changes of pulse wave velocity and early cardiovascular risk predictors. In fact, these methods have been considered as complementary methods of the current evaluation guidelines for preventing CVD (Kozlov et al., 2012). Also the evaluation of endothelial dysfunction during pregnancy could be a potent tool in the prevention of CVD in early stages of development or in mothers that suffer pregnancy pathologies such as pregnant hypertension, preeclampsia, or GDM (Escudero and Sobrevia, 2008; Escudero et al., 2013).

SHEAR STRESS IN SYSTEMIC AND PLACENTAL CIRCULATION

Shear stress is defined as the force exerted by the blood flow on blood vessel walls. This stress generates a response in the vascular wall, characterized by release of endothelial mediators, which in turn stimulate structural remodeling through activation of gene expression and protein synthesis (Hudlicka and Brown, 2009). Hemodynamic forces exerted by the heart during the cardiac cycle, PP and TS, change the structure of vascular wall. PP (difference between systolic and diastolic pressure) induces distention of the vascular wall which increases the radial tension on the blood vessels. TS or shear stress depends on the inner diameter of the vessel, blood flow rate, viscosity of the blood, and pulsatility of blood

flow. It is estimated using Poiseuille's law, through the product of shear on the wall and blood viscosity:

$$\tau = \frac{4 * \eta * q}{\pi * r^3}$$

where η is fluid viscosity, q is flow, and r is radius. It is worth noting that this formula should be considered only for a blood vessel with circular cross section and in laminar flow regime. On the other hand, in clinical studies, shear stress is calculated through blood viscosity and shear rate (γ), which is estimated from the values of blood flow velocity (V) and internal arterial diameter (d) according to the following equation (Reneman et al., 2006):

$$\gamma = \frac{8 * V}{d}$$

Shear stress values calculated in this way might be held for *in vitro* assays, provided that the conditions meet Poiseuille's law. The latter statement cannot be applied to blood vessels *in vivo*, considering the presence of non-newtonian fluid, distensible vessels, pulsatile flow, and branching of the arterial tree. Moreover, blood flow velocity, and wall shear stress, is high in systole and relatively low in diastole. Thus, diastole comprises approximately two thirds of the cardiac cycle, and the level of wall shear stress during this phase of the cardiac cycle contributes substantially to the mean wall shear stress (Reneman et al., 2006).

In the case of placental shear stress, the same equations can be applied, considering that the placental flow is dependent on the umbilical blood flow, which is related with the umbilical vein diameter. In the placental vascular bed there are several hemodynamic adaptations in order to supply oxygen and nutrients to support the fetal growth. Endothelial cells are mainly responsible for these adaptations given that in the tunica intima where blood flow exerts longitudinal shearing forces (Sprague et al., 2010). Although there are obvious difficulties to determine changes in blood flow in fetuses during human pregnancy, some evidence obtained through non-invasive techniques like Doppler ultrasound has been used to determine the importance of placental vascular adaptation. To determine the umbilical blood flow in human pregnancy, Link et al. (2007) used this equation:

$$Q = V * d^2 * \pi * 0.15$$

where Q is the volume of umbilical blood flow (mL/min), V is the mean velocity (cm/s), and d is the diameter of umbilical vein (mm). In this study, the mean umbilical venous blood flow velocity was between 13 and 14 cm/s and was similar in preterm and full-term infants, whereas the diameter of the umbilical vein was greater in the full-term group. In preterm pregnancies, there was a decrease of umbilical blood flow in late pregnancy correlated with both gestational age and birth weight and the umbilical blood flow per unit body weight of the fetus or per placental weight was increased in preterm group. The authors argue that the increase of umbilical blood flow in the course of gestation is dependent of umbilical vein growth and there is a physiological decrease in the ratio between umbilical blood flow and fetal body weight that could be dangerous in post-term pregnancies (Link et al., 2007). These results show that the development and local regulation of umbilical vein diameter are determinants for an adequate blood flow to the fetus, considering that the endothelial cells respond to shear stress and there is no innervation in placental vasculature (Sprague et al., 2010). On the other hand, in isolated cotyledon from placenta, the increases of flow rate range from 1 to 10 ml/min increased the perfusion pressure, exhibiting a stronger effect when NO synthesis was inhibited (Wieczorek et al., 1995). Also, regulation of shear stress response in uterine vasculature is relevant for placental blood flow. For instance, in myometrial arteries from preeclamptic women there is no increase of flow rate by shear stress and lower capacity of induce NO-dependent relaxation. This might contribute to impaired utero-placental blood flow in this disease (Kublickiene et al., 2000).

Other important factors that regulate vascular response to shear stress are blood flow characteristics (magnitude and shape) and vascular tree anatomy (Friedman et al., 1987). For instance, it is well known that turbulence in zones of arterial branching, where oscillatory shear stress is generated, constitute areas of vascular remodeling associated with starting events leading to atherosclerosis (Giddens et al., 1993). It has been demonstrated that the flow patterns in ascending aorta contribute to pro-atherosclerotic environment, mainly that low and oscillator shear stress, specifically near of the aortic sinus. There is a correlation between low shear stress and increased incidence of vascular damage, especially near to the coronary arteries (Suo et al., 2008). Moreover, a study about structure and flow with 3D magnetic resonance in healthy subjects, established that the WTI is positively correlated with flow shear stress. Additionally, WTI is negatively correlated with atherosclerotic plaques wall stress, showing an increased progression of atherosclerotic plaques in zones of turbulent blood flow. This demonstrates that anatomic conformation of vascular beds and flow characteristics have important repercussions on endothelial damage development (Yang et al., 2010).

MOLECULAR MECHANISMS INDUCED BY SHEAR STRESS

Mechano-transduction induced by shear stress is widely studied, showing that there are multiple signaling pathways which are activated in response to stress in endothelial cells (Li et al., 2005; Gautam et al., 2006; Yu et al., 2006; Jacob et al., 2007; Kumagai et al., 2009; Herranz et al., 2012). These pathways are triggered by mechanical stimuli sensed by endothelial cells, and generate intracellular signaling through second messengers, which in turn lead to the establishment of an adaptative response in short or long term according to stimulus (Johnson et al., 2011). For instance, the adaptive response of endothelial cells to the acute increase of shear stress is characterized by high endothelial cell permeability and high expression of anti-inflammatory and antioxidant proteins. This process is generated in three phases: induction, early adaptive response and late remodeling response, showing a different phenotype according the phase in which it is found (Zhang and Friedman, 2012).

eNOS AND CAVEOLAE

Recently, a systematic review and 3-stage meta-analysis of studies that measured FMV under local infusion of saline or (L-NMMA; NOS inhibitor) solutions demonstrated that FMV of conduit

arteries in humans is, at least in part, mediated by NO (Green et al., 2014). Furthermore, one of the enzymes that increases its expression in response to shear stress is NOS (Yee et al., 2008), specifically eNOS (Luiking et al., 2010). The use of NOS inhibitors, like L-NMMA or L-NAME, showed that the inhibition of NO synthesis suppresses the effect of shear stress on angiogenesis associated with muscular stimulation (Hudlicka et al., 2006) or placental microcirculation (Wieczorek et al., 1995). Still there is little evidence about the relevancy of L-arginine transporters in the response to shear stress. However, considering that NO synthesis depends on hCAT-1 activity (Shin et al., 2011), and has been demonstrated the colocalization of hCAT-1 with eNOS in caveolae (McDonald et al., 1997), it is highly probable that hCAT-1 is part of this physiological response. Importantly, the structure and function of caveolae is relevant for endothelial physiology: several studies have revealed that this subset of lipid structures, highly enriched in cholesterol and sphingolipids, play an important role in regulation of cell signaling (Das and Das, 2012; Sowa, 2012). Proteins such as cav 1, 2, 3 are part of their structure and organization, being cav-1 the more important in vascular endothelium (Hansen and Nichols, 2010). Together with cav-1, other proteins found in the caveolae are tyrosine-kinase receptors (TKRs), GPCRs, VEGFR, Ca^{2+} channels, among others. These expression profiles show the relevance of this plasma membrane structure for endothelial cells metabolism and vascular health (Sowa, 2012). It has been demonstrated in BAECs, that 1–3 days of exposition to laminar shear stress, increased the total amount of caveolae in 45–48%; as well as the expression of cav-1, compared with the same conditions without flow (Boyd et al., 2003). In cav-1 knockout animals (cav-$1^{-/-}$) the decrease of shear stress for 14 days did not reduce the diameter of arterial lumen and exhibit high vascular wall thickness associated with reduction in the FMV and eNOS phosphorylation in serine 1176 (i.e., eNOS activation; Yu et al., 2006). Importantly, it has been observed that the association between cav-1 and eNOS is necessary for angiogenic response induced by shear stress, because cav-1 gene suppression decreases the response to VEGF stimulation, NO production and endothelial tube formation (Sonveaux et al., 2004). Like other endothelial cells, endothelial cells from the placenta and umbilical cord express cav-1. In oFPAEs, the effects of FGF-1 on proliferation and tube formation were abolished when stable cav-1 knockdown oFPAE was used (Feng et al., 2012). Also in HUVEC, the decrease of cav-1 suppressed the NO synthesis and tube formation induced by VEGF (Pan et al., 2006). Interestingly, in human and murine placenta there is a high expression of cav-1 and cav-2 in endothelium and VSMCs but there is a lack of expression in syncytiotrophoblast layer or in cytotrophoblast (Lyden et al., 2002; Mohanty et al., 2010). Although there is evidence that supports the role of cav-1 in placental vasculature, findings are lacking about specific effects of shear stress on co-localization of cav-1 or cav-2 with eNOS or hCAT-1 in human endothelium.

ION CHANNELS

Another mechanism that is involved with the response to shear stress is related with the activity of ion channels in vasculature. Some ion channels activated by mechanical stress suffer conformational changes which modifies the cell membrane potential through changes of ions conductance (Sukharev and Sachs, 2012). Vascular endothelium expresses a great variety of sensitive channels for calcium (Ca^{2+}), potassium (K^+) and chloride (Cl^-) ions, which elicit a rapid response of endothelial cells to shear stress (Nilius and Broogmans, 2001). In this context, $K_{ir}2.1$ has shown to be a sensor of laminar flow, responding according to shear stress intensity in order to induce cell membrane hyperpolarization (Hoger et al., 2002). Together with $K_{ir}2.1$, ORCCs are also activated simultaneously in presence of shear stress, whose stimulation induces endothelial cell membrane depolarization (Nilius and Broogmans, 2001). It has been demonstrated that chloride currents are saturated at 3.5 dyn/cm^2, meanwhile K^+ currents are saturated between 10 and 15 dyn/cm^2. This shows that ORCC and $K_{ir}2.1$ channels work in cooperation in order to provide sensibility to the endothelium for a wider range of shear stress. The Cl^- channel is responsible for sensing low levels of shear stress, and K^+ channel is responsible for sensing high levels of laminar shear stress (Gautam et al., 2006). In this context, as membranes hyperpolarize during high shear stress, exercise-induced shear stress would be an important hyperpolarizing stimulus which would induce vascular relaxation of smooth muscle cells (SMCs; Gautam et al., 2006; Gurovic and Braith, 2012).

In placental tissues expression has been shown of K_V, K_{Ca}, K_{ir}, and TASK1. Regarding function, NO-mediated relaxation of human umbilical arteries occurs via activation of K_V and K_{Ca} channels; $K_{IR}6.1$ play an important role by reverse constriction in disease states, such as IUGR (Wareing et al., 2006). In the last decade it has been determined that insulin induces relaxation in umbilical and placental veins in a mechanism that could be dependent on activity of potassium channels (González et al., 2004, 2011). Specifically in HUVEC, the L-arginine transport and hyperpolarization induced by insulin is blocked by pre-incubation with glibenclamide, an inhibitor of K_{ATP} (González et al., 2004). Despite the importance of K^+ channels in vascular response to shear stress and recent evidence about K^+ channel expression and activity in human placenta, the role of K^+ channels in placental shear stress and/or in complicated pregnancies is poorly understood (Wareing, 2014).

VEGF AND ANGIOGENESIS

Modifications of blood flow induce changes in growth patterns of vascular beds, where an increase of the capillary/fiber ratio (C:F) in response to prolonged stimulation to shear stress and ischemic remodeling, decreases the diameter of capillaries and angiogenesis of low blood flow areas (Hudlicka and Brown, 2009) associated with VEGFR-2 (De la Paz et al., 2012). There are three receptors of VEGF (VEGFR 1, 2, and 3), being VEGFR-2 a strong tyrosine-kinase protein with high expression in vascular cells but reduced affinity to VEGF compared to VEGFR-1. Both receptors have soluble splicing isoforms, which contribute to negative regulation of angiogenesis. In this context, membrane-linked VEGFR-2 is pro-angiogenic, whereas sVEGFR1 or sFlt-1 is anti-angiogenic (Shibuya, 2013). Angiogenesis induced by shear stress is associated with NO bioavailability because the increase of collateral blood flow induced by VEGF and FGF is dependent on NOS

activity (Yang et al., 2001). Also, L-arginine supplementation contributes to the increase in VEGF expression and angiogenesis in skeletal muscle and left ventricle of middle-aged rats, showing the importance of the L-arginine/NO pathway in VEGF expression in response to shear stress (Suzuki, 2006).

In placental circulation, it has been determined that the VEGF/angiogenesis pathway is relevant for early placental vascularization and deficiencies in this signaling pathway could be related with placental pathologies like IUGR or preeclampsia. Is well known that plasma levels of sFlt-1 is higher in mothers with preeclampsia (Shibuya, 2014) which is associated with lower NO synthesis in HUVEC obtained from mild or severe preeclampsia (Veas et al., 2011). Regarding placental responses to shear stress, these are similar to those reported in systemic circulation have been observed in oFPAEs, which shows high eNOS expression and rapid phosphorylation of eNOS on serine 1177 (Ser1177) through a PI3K-dependent pathway after applications of shear stress (Li et al., 2005).

EFFECTS OF PHYSICAL EXERCISE ON SYSTEMIC SHEAR STRESS

Clinical evidence shows that physical exercise applied in cardiovascular rehabilitation is effective in decreasing both hospitalization rate and mortality associated with CVDs (Heran et al., 2011). In this regard, a study including 18.809 patients from 41 countries, showed that physical exercise decreases the risk of AMI in people who have a history of ACS (Chow et al., 2010). On the other hand, there are recent advances shows that controlled exercise in pregnant women with risk factors for CVD, like obesity or overweight, improve cardiovascular parameters (Seneviratne et al., 2014).

IMPACT OF EXERCISE ON ENDOTHELIAL FUNCTION

Previously we mentioned that increases in shear stress causes the release of vasodilator substances from the endothelium and, consequently, FMV. FMV has been used as a parameter of endothelial function in clinical protocols and is the support of therapies for improving cardiovascular performance through shear stress induced by exercise (Inoue et al., 2008; Santos-García et al., 2011). When referring to the discussion about the effect of exercise on shear stress and vascular health, it is important to establish that there is a large variability of flow patterns in response to different types of exercise. For instance, in incremental exercise of the lower limbs, significant increases of blood flow peaks have been observed, associated with a biphasic increase of blood flow in the brachial artery due to anterograde and retrograde flow which is correlated positively with the intensity of workloads (Birk et al., 2012; Gurovic and Braith, 2012). This retrograde flow observed in the radial artery (and perhaps in other vessels) may be due to the redistribution or the influence of retrograde diastolic flow, which is associated with lower limb exercise in the upright position (Green et al., 2002a,b). Meanwhile exercise of upper limbs induces anterograde flow proportionally to the workload (Green et al., 2005). In the same way, Tinken et al. (2009) in compared the effects of blood flow modification and shear stress on FMV, reporting that when the anterograde flow was increased by 30 min, the FMV increased. Also, they observed that when the anterograde flow was decreased (through a brachial cuff), the elevation in FMV is blocked, suggesting that FMV is modulated by differences in the magnitude of anterograde flow and shear stress (Tinken et al., 2009). Furthermore, it has been observed that low retrograde flow predisposes to NO dependent endothelial dysfunction, because it generates an altered FMV response, which is a hallmark of endothelial dysfunction (Thijssen et al., 2009). Regarding the impact of exercise intensity on endothelial function, it has been shown that aerobic exercise of moderate intensity (50% VO_2 max) increases the endothelium-dependent vasodilatation through stimulation of NO synthesis. Nonetheless, high intensity exercise could be an oxidative stress signal (Goto et al., 2003). Thus, these authors evaluated the response of brachial blood flow to different exercise intensities (25% VO_2 max, 50% VO_2 max, and 75% VO_2 max) in healthy subjects and they demonstrated that exercise at 50% VO_2 max induces vasodilatation through high bioavailability of NO, whereas high intensity exercise was associated with an increase in the production of ROS (Goto et al., 2007).

IMPACT OF PHYSICAL TRAINING ON ENDOTHELIAL FUNCTION

Physiological bases concerning of physical training on endothelial function is related with the facts that increases of blood flow and shear stress affect the synthesis of NO (Naylor et al., 2011). In this context, it has been demonstrated in both animals and humans, that exposure to repetitive exercise carried out during a prolong period of time increases the bioavailability of endothelial NO, as well as vascular collateralization (Hambrecht et al., 2003; Heaps and Parker, 2011; Lee et al., 2011). Hambrecht et al. (2003) in a study using samples from patients with stable coronary disease and surgery of CABG scheduled, showed that a daily training program of 30-min with a cycle ergometer and 30-min with treadmill for 4 weeks before surgery, significantly increases the endothelium-dependent vasodilatation, the flow rate in response to acetylcholine (Ach) and the FMV in LIMA (Hambrecht et al., 2003). Furthermore, they also found that endothelial cells isolated from the LIMA of patients who were trained, exhibited a higher expression and activation of eNOS and PKB/Akt compared with untrained patients, showing that repetitive increases of shear stress, triggered by physical training, enhance the NO bioavailability (Hambrecht et al., 2003). Moreover, in relation to the time course of these vascular changes, it has been described that functional adaptations precede structural adaptations. Tinken et al. (2008) observed that 2–6 weeks of exercise-training triggered an increase in NO bioavailability due to an increase in exercise-induced shear stress. After 6 weeks, NO levels returned to baseline due the normalization of shear stress on the blood vessel wall associated by vascular remodeling and angiogenesis (Tinken et al., 2008, 2010). These results show that physical training with moderate intensity schemes would be useful tools to improve the outcomes of therapies and/or cardiovascular surgeries.

EFFECTS OF PHYSICAL EXERCISE ON PLACENTAL VASCULAR FUNCTION

Even though research shows a high increase of overweight and obesity in world population, especially in pregnant women, there are few reports published regarding the effect of moderate physical exercise in pregnancy. However, effects have been demonstrated

of exercise-training on pro-angiogenic molecules in pregnant animal models, showing evidences that exercise-based interventions would be effective in preventing the onset of preeclampsia. In this context, Gilbert et al. (2012b) showed that 6 weeks of training increases the levels of cytoprotective molecules such as heat shock proteins (HSPs) 27, 60, and 90 in placentas from trained rats compared with sedentary controls. The authors pointed out that small HSP are involved in cellular protection against oxidative stress and apoptosis, meanwhile larger HSP facilitate eNOS-mediated NO synthesis. In addition, it has been observed that exercise increases free VEGF, decreases sFlt-1 and increases endothelial cell tube formation *in vitro*. In addition, exercise augments endothelium-dependent vascular relaxation compared with non-exercise control rats (Gilbert et al., 2012a). These findings suggest that physical activity before and during pregnancy stimulate molecular pathways that may yield benefits with respect to placental and/or vascular function, since the HSP is associated with the VEGF overexpression which leads to development of angiogenesis (Gilbert et al., 2012a,b).

Moreover, exercise can mitigate hypertension-associated physiological consequences in pregnant rat models. It has been observed, in spontaneously hypertensive pregnant rats, that the exercise recovers the fetal weight decreased by hypertension. After an exercise-training program, the placentas of hypertensive rats had a higher number of blood vessels in relation to the sedentary control group (Abate et al., 2012). In addition, it has been observed in female Sprague Dawley rats exposed to 6 weeks of voluntary exercise, that the placental ischemia-induced hypertension was attenuated by exercise as well as the restoration of angiogenesis. These changes were associated with high sensitivity of Ach-induced vasodilation in mesenteric vessels of exercise-trained pregnant rats (Gilbert et al., 2012b). Apparently, these physiological effects induced by exercise could be transferred to the fetus, since there are evidences that physical training attenuates the detrimental effect of low-protein diet on fetal growth and development, glucose homeostasis, serum leptin concentration, and oxygen consumption in the offspring from exercised-trained mothers (Fidalgo et al., 2013). The underlying mechanism of these effects could be related to metabolic changes associated with long-term effects of perinatal physical training such as exercise-induced blood flow redistribution as well as increase of insulin-like growth factors, growth hormone, and leptin after training (Turgut et al., 2006; Amorim et al., 2009; de Mélo Montenegro et al., 2012; Fidalgo et al., 2013). However, more direct evidence is necessary to verify these hypotheses.

Regarding the effect of exercise-training in preeclampsia, it has been demonstrated in a mouse model of the disease (transgenic female mice over expressing human angiotensinogen, which develop preeclampsia when mated with males overexpressing human renin) that the exercise-training decreases the proteinuria, cardiac hypertrophy, and vascular reactivity of placental vessels. Also, it was observed that PlGF was normalized in trained transgenic mice (Falcao et al., 2010). Studies done on humans have shown that aerobic exercise is an effective tool in maternal weight gain and cardiovascular risk control during pregnancy (Clapp, 2008; Lamina and Agbanusi, 2013) and that physiological basis of preeclampsia are both vascular dysfunction and oxidative stress, which improve with exercise-training (Goto et al., 2007; Brown and Garovic, 2011). However, evidence related with the beneficial effect of exercise-training on vascular function and preeclampsia prevention in pregnant women is scarce (Yeo et al., 2000). In this context, Ramírez-Vélez et al. (2013) demonstrated that exercise-training during pregnancy led to a 2-fold increase in eNOS expression and 4-fold increase in NO production in placental cytosol, as well as, 6% decrease in O_2^- level and 26% in H_2O_2 production rate in human placental mitochondria. The training program consisted in 32 sessions, each session included 30-min of aerobic circuit training accompanied by an audio music recording and instructions which guided the participants to exercise at each station for approximately 1 min per station in a circuit of 10 stations. In a similar way of previously reported data by Gilbert in rats, there is an increase in placental efficiency (fetal weight/placental weight) in exercised-trained pregnant women. These responses are triggered, presumably, by exercise-induced shear stress (Ramírez-Vélez et al., 2013). Additionally, it has been observed that 20 min of moderate-intensity cycle ergometry is effective for improving angiogenic markers: higher serum PlGF and lower sFlt-1 and sEng concentrations in late gestation (Weissgerber et al., 2010). Regarding the effect of exercise and changes of lifestyle for control or prevention of GDM, a recent publication concludes that there is no strong evidence to support the benefits of exercise on insulin resistance or glucose tolerance in these patients, although they are known the effects on vascular function, oxidative stress and insulin tolerance in humans (Weissgerber et al., 2010; Halperin and Feig, 2014; Rynders et al., 2014). Additionally, it has been described that insulin influences beneficial changes in insulin signaling and NO/ROS synthesis in GDM placenta (Sobrevia and González, 2009; Westermeier et al., 2011). Interestingly, there is a new protocol being applied in New Zealand called IMPROVE. In this study, pregnant women (aged 18–40 years) with a BMI 525 $\geq$ 25 kg/m2 and will be subjected to an exercise regime that consists of home-based stationary cycling. Researchers will collect anthropometric data of mothers and children together with different metabolic and cardiovascular parameters (Seneviratne et al., 2014). In the future, it will be very important to follow these and others trials outcomes, aiming to determine if exercise, its type and intensity, improves cardiovascular parameters with special interest in the mechanism induced by shear stress in placental circulation. In a recent report, the effect was determined of 20 min/day of treadmill exercise in pregnant sow, on vascular function of the femoral artery from porcine offspring at 3, 5, or 9 months of age. Although it was not possible to determine changes in endothelial function, overall, this report demonstrates, for the first time, that maternal exercise during pregnancy can induce long-term vascular programming in adult offspring. The authors determined a decreased of relaxation in response to sodium nitroprusside (NO donor) and high levels of the regulatory subunit of myosin phosphatase MYPT1 in femoral artery of porcine (3 months age) from exercised pregnant sows (Bahls et al., 2014). The main limitation of this study is the use of only the femoral artery for the determination of vascular reactivity and protein expression, in detriment of resistance vessels, or directly determine the changes in placental or uterine vascular beds from exercised mothers. Also for the determination of molecular changes in endothelial function,

isolation of endothelial cells is necessary for *in vitro* determination of protein expression to avoid the overlap with protein profiles of SMC. Even so, this work opens new possibilities in the exploration of the effects of exercise during pregnancy, however, more evidence is necessary in order to clarify the subjacent mechanism that induce the cited beneficial effects. In this context, a previous study shows that exercise in late gestation increases the umbilical blood flow without changes in fetal weight, showing a potential effect of exercise on umbilical vein function that could be related with molecular mechanisms influenced by shear stress (Harris et al., 2013). These data suggest that the potential role of physical activity or exercise-training could be different in healthy and pathological pregnancy, where the exercise on the last (i.e., GDM, preeclampsia, hypertension, and obesity) has potential to improve fetoplacental function. In this regard, in a mouse model of preeclampsia superimposed on chronic hypertension induced by overexpression of renin–angiotensin system, exercise before and during pregnancy decreased mean arterial pressure and induced normalization of VEGF and sFlt-1 placental and plasma levels at the end of gestation. These findings show that preeclampsia is associated with an anti-angiogenic shift that is initiated by the placenta, and exercise-training restores angiogenic balance (Genest et al., 2013). This last idea supports the notion that exercise-training improves several parameters associated with the development of preeclampsia such as the risk of CVD, DM2, and improves the maternal health in patients at risk for preeclampsia (Genest et al., 2012).

Thus, mechanisms associated with benefits of exercise include: (1) VEGF-induced angiogenesis in early placentation due to short hypoxic events induced by redistribution of blood during exercise; (2) High levels of PlGF and reduction of circulating sFlt-1 and sEng in late gestation of exercised women; (3) High resistance against oxidative stress and lipid peroxidation, (4) Lower levels of endothelin-1 (vasoconstrictor) and high expression of eNOS and antioxidant enzymes, increasing the bioavailability of NO (Genest et al., 2012).

Even though studies are still scarce and controversial, new findings show the potential role of exercise-training on placental endothelial function. It is necessary to increase and improve experimental evidence, with carefully designed protocols on time and type of interventions, exploring molecular mechanisms in isolated endothelial cells and SMC from uterine (mother), placenta, umbilical cord (placental tissue), and systemic resistance vessels in offspring. The combination of animal models and human trials will give new insights about the benefits of exercise to avoid deleterious effects of pre-gestational and gestational diseases that are growing in the human population.

PERSPECTIVES

Evidence shows that hemodynamic stimulus plays a crucial role in modulating synthesis and bioavailability of endothelial NO both *in vitro* and *in vivo*. This allows us to provide the experimental evidence that supports the positive effects of physiological shear stress and exercise-induced shear stress in systemic and placental circulation.

The role of eNOS and VEGF in the regulation of physiological responses to shear stress is well known, and there is increasing evidence about the benefits of physical exercise (acute or

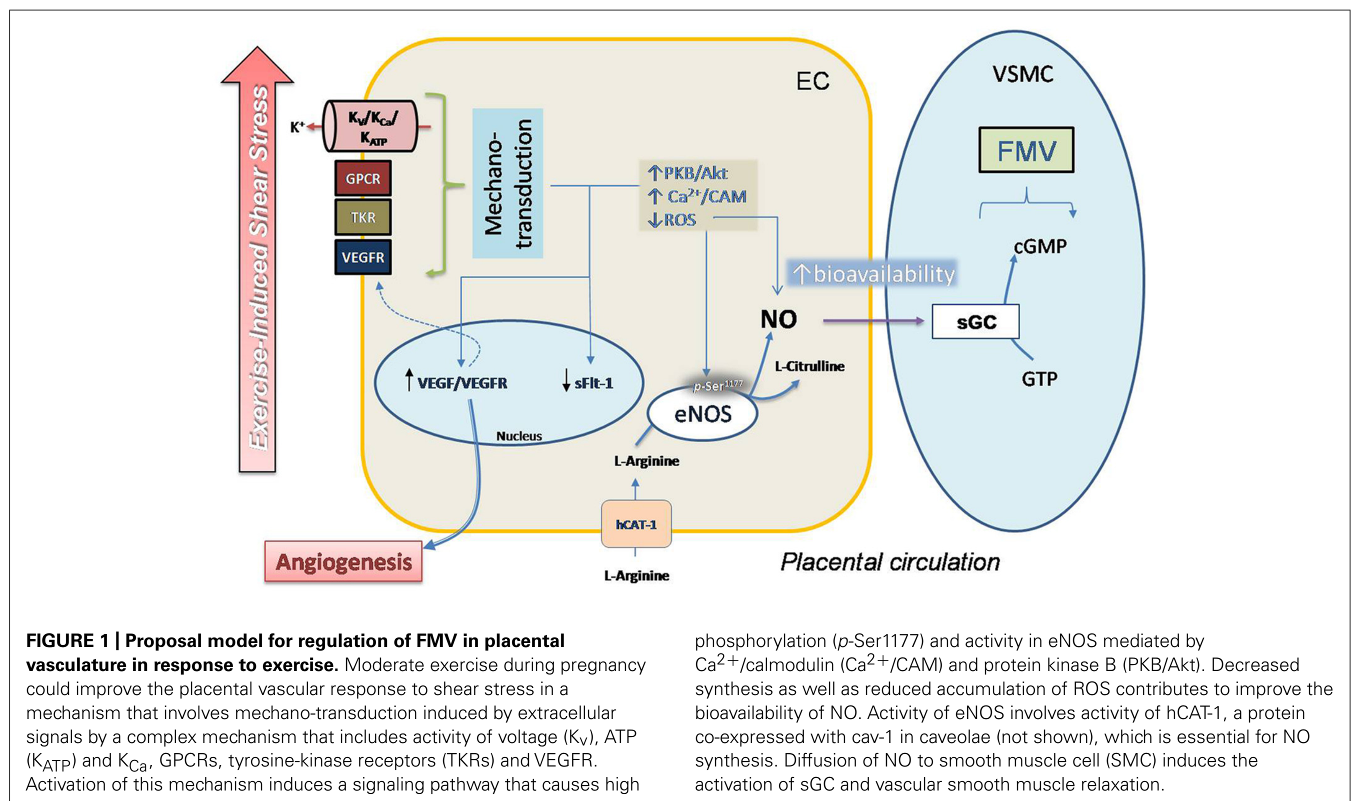

FIGURE 1 | Proposal model for regulation of FMV in placental vasculature in response to exercise. Moderate exercise during pregnancy could improve the placental vascular response to shear stress in a mechanism that involves mechano-transduction induced by extracellular signals by a complex mechanism that includes activity of voltage (K_V), ATP (K_{ATP}) and K_{Ca}, GPCRs, tyrosine-kinase receptors (TKRs) and VEGFR. Activation of this mechanism induces a signaling pathway that causes high phosphorylation (*p*-Ser1177) and activity in eNOS mediated by Ca^{2+}/calmodulin (Ca^{2+}/CAM) and protein kinase B (PKB/Akt). Decreased synthesis as well as reduced accumulation of ROS contributes to improve the bioavailability of NO. Activity of eNOS involves activity of hCAT-1, a protein co-expressed with cav-1 in caveolae (not shown), which is essential for NO synthesis. Diffusion of NO to smooth muscle cell (SMC) induces the activation of sGC and vascular smooth muscle relaxation.

training-exercise programs) for cardiovascular health. However, evidence about the effects of physical exercise on fetal and placental circulation remains obscure, mainly because of little information based on controlled trials in human pregnant women. In **Figure 1** we postulate a potential mechanism for response to exercise of placental vasculature, considering direct evidence and results obtained from systemic circulation and *in vitro* umbilical vasculature.

The endothelium is an inexhaustible source of cardioprotective substances which can be induced by exercise. For that reason, exercise-training can be considered an effective, economical and natural protector. Furthermore, light and moderate exercise does not have adverse effects, which cannot be said for the best medication synthesized in the pharmaceutical industry. These features, and the strong evidence about the high proportion of obesity and metabolic syndrome in pregnant women, demonstrate that the relevance of obtaining better knowledge about the effect of exercise-induced shear stress in fetoplacental vasculature.

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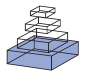

Modulation of endothelial cell migration by ER stress and insulin resistance: a role during maternal obesity?

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Adverse microenvironmental stimuli can trigger the endoplasmic reticulum (ER) stress pathway, which initiates the unfolded protein response (UPR), to restore protein-folding homeostasis. Several studies show induction of ER stress during obesity. Chronic UPR has been linked to different mechanisms of disease in obese and diabetic individuals, including insulin resistance (IR) and impaired angiogenesis. Endothelial cell (EC) migration is an initial step for angiogenesis, which is associated with remodeling of existing blood vessels. EC migration occurs according to the leader–follower model, involving coordinated processes of chemotaxis, haptotaxis, and mechanotaxis. Thus, a fine-tuning of EC migration is necessary to provide the right timing to form the required vessels during angiogenesis. ER stress modulates EC migration at different levels, usually impairing migration and angiogenesis, although different effects may be observed depending on the tissue and/or microenvironment. In the context of pregnancy, maternal obesity (MO) induces IR in the offspring. Interestingly, several proteins associated with obesity-induced IR are also involved in EC migration, providing a potential link with the ER stress-dependent alterations observed in obese individuals. Different signaling cascades that converge on cytoskeleton regulation directly impact EC migration, including the Akt and/or RhoA pathways. In addition, ER is the main intracellular reservoir for Ca^{2+}, which plays a pivotal role during EC migration. Therefore, ER stress-related alterations in Ca^{2+} signaling or Ca^{2+} levels might also produce distorted EC migration. However, the above findings have been studied in the context of adult obesity, and no information has been reported regarding the effect of MO on fetal EC migration. Here we summarize the state of knowledge about the possible mechanisms by which ER stress and IR might impact EC migration and angiogenesis in fetal endothelium exposed to MO during pregnancy.

Keywords: mesenchymal migration, unfolded protein response, RhoA, Akt, Scrib, polarization, cytoskeleton

INTRODUCTION

Endoplasmic reticulum is the major subcellular membrane organelle, playing a pivotal role in synthesis, folding and maturation of proteins, and providing the main Ca^{2+} reservoir inside the cell (Berridge et al., 2003; Cnop et al., 2012; Hetz et al., 2013). Under certain conditions, the environment induces ER stress and further activation of the UPR, which triggers a cascade of signaling events to restore protein-folding homeostasis (Kozutsumi et al., 1988). This cellular condition, known as ER stress, is induced by different types of stimuli, such as accumulation of unfolded proteins, fatty acids, cytokines, redox state dysregulation, and increased intracellular Ca^{2+} levels (Kozutsumi et al., 1988; Hotamisligil, 2010; Cnop et al., 2012; Fu et al., 2012; Garg et al., 2012; Hetz et al., 2013). Moreover, ER stress is linked to different diseases, including cancer, type II diabetes, and obesity (Hotamisligil, 2010; Cnop et al., 2012; Hetz et al., 2013). Importantly, most if not all of these pathologies are associated with vascular pathologies such as distorted angiogenesis or endothelial dysfunction (Minamino and Kitakaze, 2010; Basha et al., 2012). By affecting EC physiology, ER stress contributes to the vascular dysfunction observed in diabetic retinopathy, cancer, obesity, atherosclerosis, and ischemia (Amin et al., 2012; Hetz et al., 2013;

Abbreviations: 2-DG, 2-deoxy-D-glucose; ATF6, activating transcription factor 6; BiP/GRP78, immunoglobulin binding protein; BMI, body mass index; CHOP-10, C/EBP homologous protein-10; EC, endothelial cell; eIF2α, eukaryotic translational initiation factor 2α; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; GSK, Akt/glycogen synthase kinase; HUVECs, human umbilical vein endothelial cells; IFN-γ, interferon γ; IL, interleukin; IR, insulin resistance; IRE1, inositol-requiring enzyme 1α; MCPIP, monocyte chemotactic protein-induced protein; MO, maternal obesity; PERK, PKR-like eukaryotic initiation factor 2α kinase; PIP_3, phosphatidylinositol (3,4,5)-trisphosphate; RhoA, Ras homolog family member A; Scrib, scribbled planar cell polarity protein; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase pump; sFlt-1, soluble fms-like tyrosine kinase-1; TNF-α, tumor necrosis factor α; UPR, unfolded protein response; VEGF, vascular endothelial growth factor; XBP1, X-box binding protein 1.

Zeng et al., 2013; Paridaens et al., 2014). One of the most relevant functions of EC is angiogenesis, which is the capacity to form new capillary vessels (Lamalice et al., 2007). Interestingly, ER stress affects two of the basic mechanisms that contribute to angiogenesis (Lamalice et al., 2007): VEGF signaling, and EC migration (Iwawaki et al., 2009; Ghosh et al., 2010; Pereira et al., 2010; Banerjee et al., 2011; Zeng et al., 2013; Paridaens et al., 2014). However, obesity might impact EC migration directly through ER stress and induced IR; Westermeier et al., 2014), because several of the involved proteins, such as RhoA and Akt (also called protein kinase B), also modulate EC migration (Lamalice et al., 2007).

As expected, the development of obesity in adults produces altered angiogenic responses in adipose tissue (Christiaens and Lijnen, 2010). However, in the context of pregnancy, MO not only affects the mother but also can permanently damage fetal tissues [American College of Obstetricians and Gynecologists (ACOG), 2005]. Thus, the adverse intrauterine environment in MO pregnancies could modulate offspring physiology (Bruyndonckx et al., 2013), leading to *in utero* development of IR (Catalano et al., 2009), which ultimately might affect EC migration and angiogenesis. In support of this notion, MO is associated with alterations in serum levels of angiogenic markers (Zera et al., 2014) and changes in VEGF receptor expression patterns in the placenta (Dubova et al., 2011; Saben et al., 2014). Interestingly, very recent studies show that MO induces ER stress in offspring in murine models (Melo et al., 2014; Wu et al., 2014), suggesting that distortions in EC migration and angiogenesis might occur. Since cell migration commands angiogenesis, our goal is to give an integrative overview of how MO-induced ER stress and IR might affect the migratory potential of EC and hence angiogenesis in the offspring, with deleterious consequences for the offspring's development.

ER STRESS AND THE UNFOLDED PROTEIN RESPONSE

Multiple environmental stimuli are capable of triggering ER stress (Schroder and Kaufman, 2005). There are three major sensors of ER stress, all of which are ER membrane-associated proteins: ATF6 (α and β isoforms), PERK, and IRE1 (Hetz et al., 2013). While activation of both PERK and IRE1 involves dimerization and phosphorylation, ATF6 activation requires its cleavage and translocation to the nucleus (Hetz et al., 2013). These three pathways interact and produce ER-to-nucleus signaling that reduces protein translation and increases folding capacity (Hotamisligil, 2010). However, differential activation of ER sensors may occur depending on the type and timing of the ER stressor signal (Wu et al., 2007; Fu et al., 2012). The latter will produce different UPR profiles, associated with the specific stimuli triggering the ER stress, the affected cell type(s), and the microenvironment background. In addition, acute versus chronic ER stress may lead to different cellular responses (Wu et al., 2007; Fu et al., 2012).

ROLE OF ER STRESS IN CELL MIGRATION

The role of ER stress on cell migration and angiogenesis has been studied mainly in cancer cells (Hetz et al., 2013). Several ER stress-related proteins contribute to cell migration and/or angiogenesis in tumors (Paridaens et al., 2014). The three abovementioned ER stress branches usually contribute to angiogenesis in the tumoral context (Hetz et al., 2013). Interestingly, the ER chaperone immunoglobulin binding protein (BiP/GRP78), which is an early signal of ER stress activation, is required to give angiogenic potential to tumors (Dong et al., 2008), suggesting that ER stress might impact angiogenesis from the beginning of the response. However, in non-tumoral contexts, ER stress has been shown to impair cell migration and angiogenesis.

Tunicamycin is an antibiotic that inhibits synthesis of asparagine-linked glycoproteins (Takatsuki et al., 1971; Duksin and Bornstein, 1977) and is commonly used to induce ER stress in different *in vitro* and *in vivo* models. Promoting the accumulation of misfolded un-glycosylated proteins at the ER level, tunicamycin produces activation of all of the ER branches (Schroder and Kaufman, 2005). In support of the notion that ER stress impacts cell migration, early studies performed by Gipson et al. (1984) showed impaired epithelial sheet migration in the presence of tunicamycin. This study observed delayed wound healing in tunicamycin-exposed organotypic cultures of corneas (Gipson et al., 1984). Several years later, similar results were obtained *in vitro* with human epithelial airway cells (Dorscheid et al., 2001). In addition, recent observations in vascular smooth muscle cells show that tunicamycin activates the IRE1 and ATF6 pathways, imparing platelet-derived growth factor-induced *in vitro* migration (Yi et al., 2012). Similarly, neferine, an alkaloid used in cancer treatment, induces ER stress activation in an epithelial cell line, which produces concomitant inhibition of cell migration (Yoon et al., 2013).

Therefore, ER stress activation under resting or non-tumoral physiopathological conditions seems to impair collective cell migration, conversely to the tumoral context in which it seems to promote angiogenesis (Paridaens et al., 2014). This finding suggests that ER stress might play different roles in EC migration depending on the tissue environment.

ROLE OF ER STRESS IN EC MIGRATION AND ANGIOGENESIS

Collective EC migration is required as an initial event during angiogenesis. The EC migration process combines three different mechanisms: (1) chemotaxis, which is induced by soluble chemoattractants, (2) haptotaxis, which is mediated by chemoattractants bound to the substrate, and (3) mechanotaxis, which provides the mechanical forces to provide directionality (Lamalice et al., 2007). EC migrate according to the leader–follower model, in which a leader (or pioneer) cell with more protrusive and motile activity at the leading edge affects the signaling of the follower cells. Thus, the leader cell exerts mechanical pulling over the follower cells, providing the directionality of the sheet growth (Vitorino and Meyer, 2008; Rorth, 2009). At the cellular level, this process involves both actin and microtubule cytoskeleton rearrangements and changes in cell polarity (**Figure 1**) towards the edge of the monolayer (Etienne-Manneville, 2013).

While wound healing is commonly used to evaluate collective EC migration, tube formation in Matrigel has been used extensively to evaluate the angiogenic potential of these cells. These and other *in vitro* and *in vivo* models are used to evaluate different pro- or anti-angiogenic compounds (Lamalice et al., 2007; Aranda and Owen, 2009). As expected, VEGF is a potent chemoattractant for EC and contributes to angiogenesis (Lamalice et al., 2007). Interestingly, signaling of VEGF and other proteins involved in

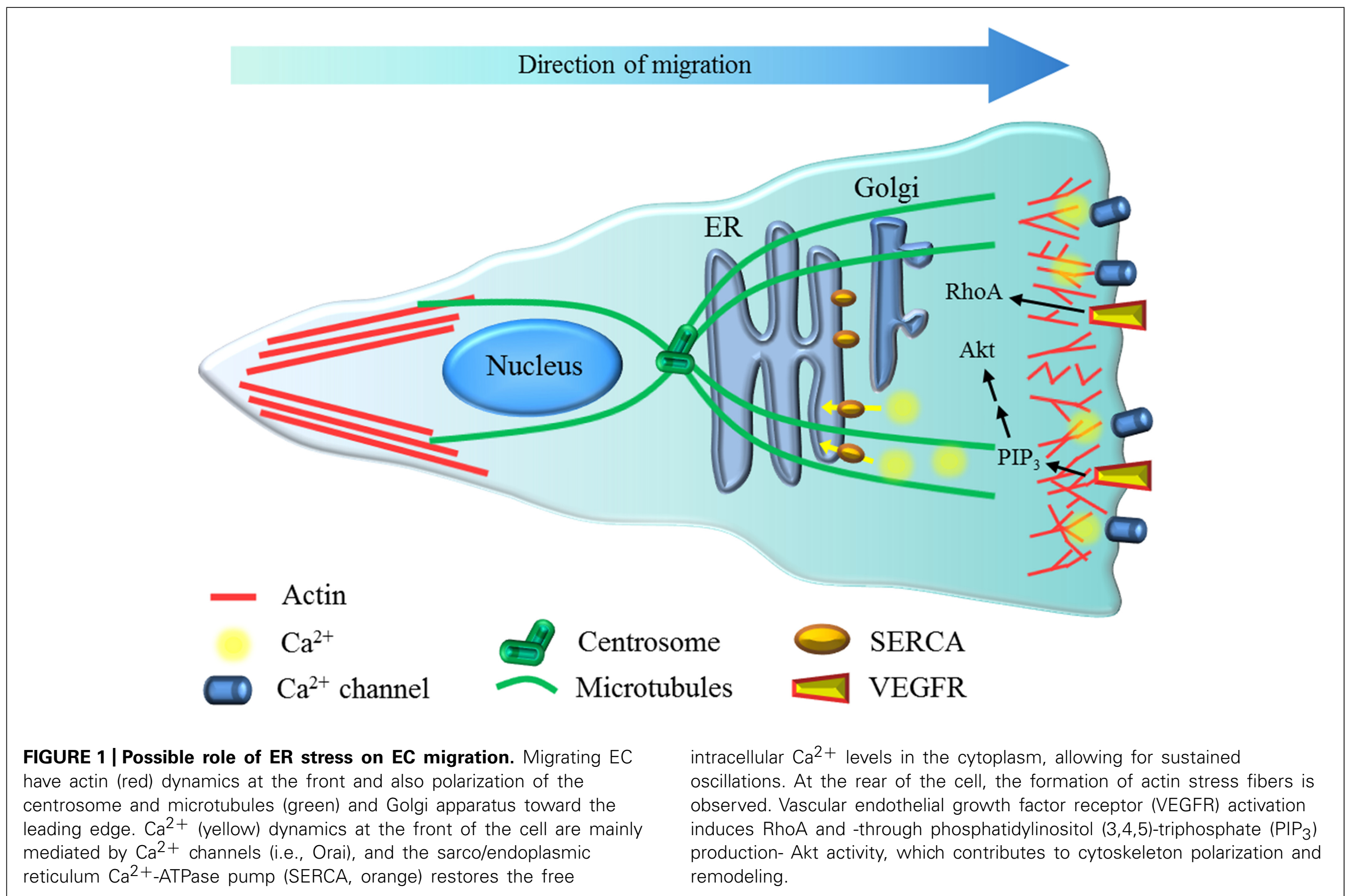

FIGURE 1 | Possible role of ER stress on EC migration. Migrating EC have actin (red) dynamics at the front and also polarization of the centrosome and microtubules (green) and Golgi apparatus toward the leading edge. Ca^{2+} (yellow) dynamics at the front of the cell are mainly mediated by Ca^{2+} channels (i.e., Orai), and the sarco/endoplasmic reticulum Ca^{2+}-ATPase pump (SERCA, orange) restores the free intracellular Ca^{2+} levels in the cytoplasm, allowing for sustained oscillations. At the rear of the cell, the formation of actin stress fibers is observed. Vascular endothelial growth factor receptor (VEGFR) activation induces RhoA and -through phosphatidylinositol (3,4,5)-triphosphate (PIP_3) production- Akt activity, which contributes to cytoskeleton polarization and remodeling.

EC migration and angiogenesis is affected by ER stress (Paridaens et al., 2014).

Tunicamycin exposure is associated with impairment of both spontaneous and VEGF-induced migration of capillary EC, involving inhibition of VEGF signaling (Banerjee et al., 2011). Similarly, 2-DG reduces spontaneous collective migration, showing an anti-angiogenic effect on cultures of HUVEC through activation of ER stress (Merchan et al., 2010). Moreover, this study also shows that 2-DG has an anti-angiogenic effect *in vivo* (Merchan et al., 2010). In addition, treatment with neferine, another ER stress inducer, inhibits *in vitro* angiogenesis in HUVEC (Yoon et al., 2013). Similarly, acrolein, which is an unsaturated aldehyde known as an environmental pollutant and also found in some foods, induces ER stress in EC (Haberzettl et al., 2009), affecting both migration and angiogenesis (O'Toole et al., 2014). This study shows that acrolein inhibits wound healing and tube formation in HUVEC (O'Toole et al., 2014). Importantly, the same study evaluated Akt signaling after insulin exposure, showing that acrolein impairs insulin signaling (O'Toole et al., 2014), supporting the hypothesis that IR might be linked to distorted EC migration.

In contrast, MCPIP mediates cytokine-induced angiogenesis in HUVEC by up-regulation of ER stress markers (Roy and Kolattukudy, 2012). In addition, a recent study shows that VEGF might induce PERK and ATF6 signaling, which contribute to survival and migration of EC (Karali et al., 2014). These data suggest that in EC cells, the induction of ER stress might affect both migration and angiogenesis in a stimulus-dependent manner. Moreover, in a murine model it has been shown that IRE1 activity, which contributes to proper placental development, is required for development of ER stress during pregnancy (Iwawaki et al., 2009). This study shows that lack of IRE1 reduces VEGF receptor expression and is lethal (Iwawaki et al., 2009). These data suggest that in EC, induction of ER stress might affect both migration and angiogenesis in a stimulus-dependent manner.

One additional explanation of the different effects of ER stress on angiogenesis capacity could be related to the involvement of microenvironmental factors such as inflammatory mediators. For example, diabetic and other models of retinopathy are associated with distorted retinal angiogenesis, which has been related to ER stress (Salminen et al., 2010; Wang et al., 2012). In a murine model of type I diabetes, increased levels of VEGF and TNF-α were observed in the retina, which were correlated with increased ER stress markers of the PERK and IRE1 branches (Li et al., 2009). In addition, oxygen-induced retinopathy was associated with development of ER stress, in a similar way to that induced by tunicamycin (Li et al., 2009). Importantly, resveratrol and some of its derivatives (Tabata et al., 2007), which have anti-inflammatory effects and inhibit ER stress development (Zhang and Kaufman, 2008), prevent retinal vascular degeneration induced by tunicamycin or ischemia/reperfusion (Li et al., 2012). Taken together, these data suggest that ER stress contributes to

angiogenesis and neovascularization *in vivo*. However, the target cells are not fully elucidated, and it is possible that the effect of ER stress inhibition might first impact immune cells, which through the release of inflammatory mediators might indirectly impact EC. Interestingly, several of these inflammatory mediators show altered levels during obesity (Snyder-Cappione and Nikolajczyk, 2013).

EFFECTS OF MATERNAL OBESITY AND INSULIN RESISTANCE ON CELL MIGRATION

Altered nutritional state is becoming a relevant and growing public health issue globally [World Health Organ (WHO), 2003]. The relationship between obesity-induced chronic ER stress and IR has been well established in murine and human adipose tissues (Cnop et al., 2012; Flamment et al., 2012; Jung et al., 2013; Boden et al., 2014). Interestingly, it has been shown that the obesity-dependent induction of ER stress markers is reduced in human adipose tissue after weight loss, suggesting that body weight change constitutes an important factor that modulates the ER stress response (Gregor et al., 2009). During pregnancy, excessive gestational weight gain and MO have been associated with increased risk of maternal pathologies and detrimental long-term effects on fetal tissues, through a process known as intrauterine programming (McMillen and Robinson, 2005). Since HUVEC provides a useful model to study neonatal evidence of fetal EC programming under multiple pregnancy conditions, in this section we focus on different IR- and migration-associated proteins that might be distorted by MO.

MO-RELATED FETAL PROGRAMMING

Obesity and overweight during pregnancy are well-recognized independent risk factors that contribute to the development of metabolic syndrome and several diet-related anomalies not only in the mother, but also in the fetus through fetal programming [American College of Obstetricians and Gynecologists (ACOG), 2005; Flenady et al., 2011; Triunfo and Lanzone, 2014]. This intrauterine programming can be observed as altered responses to physiological stimuli in HUVEC isolated from pathological pregnancies (Cheng et al., 2013; Krause et al., 2013). Indeed, it has been described that MO induces IR in fetuses *in utero* (Catalano et al., 2009), showing the relevance of metabolic fetal programming. Recently, it has been found that EC from obese adult subjects show ER stress (Kaplon et al., 2013), but it has not been determined whether MO induces these changes in fetal tissue. However, interesting recent evidence suggests that ER stress might be induced through fetal programming in animal models (Melo et al., 2014; Wu et al., 2014).

Using a murine model of MO, feeding dams a high-fat diet resulted in increased inflammation, ER stress markers, and IR in hypothalamic tissue of the MO offspring (post-natal day 28) compared to the control group (Melo et al., 2014). This study shows that lactation plays a major role in the development of ER stress (Melo et al., 2014). However, it was also noted that there was a significant increase in phosphorylation of eIF2α, downstream of PERK, in hypothalamic tissue at birth (day 0) of MO offspring (Melo et al., 2014), suggesting that at least the PERK ER stress branch is already activated during MO pregnancy.

In another study, using a similar model of diet-induced obesity, it was shown that MO offspring have increased ER stress and inflammatory markers compared to the control group (Wu et al., 2014). This study shows increased PERK and IRE1 activation in liver and adipose tissue of MO offspring at post-natal day 100 (Wu et al., 2014). Interestingly, treating dams during pregnancy and lactation with quercetin, which is an anti-inflammatory flavonoid (Indra et al., 2013) that inhibits ER stress (Suganya et al., 2014), prevented the development of ER stress in the offspring of MO pregnancies (Wu et al., 2014), suggesting that the development of ER in the offspring begins during pregnancy.

Altogether these data show that MO induces ER stress through fetal programming in murine models. Therefore, it is conceivable to suggest that MO in human pregnancies might produce a similar phenomenon.

HOW MIGHT MO IMPACT HUVEC MIGRATION AND ANGIOGENESIS?

Fetal programming is known to occur during MO pregnancies; however one remaining question is how MO might mediate fetal EC migration and angiogenesis. First, human chorionic gonadotropin has been shown to increase the proliferation of HUVEC in the presence of various adipokines, such as IL 6, leptin, adiponectin, and TNF-α (Polec et al., 2014). Moreover, there is interesting evidence that placental tissue from women with MO shows altered expression of VEGF receptors (Saben et al., 2014). In fact, a very recent study showed that increased body mass index (BMI) was associated with the presence of angiogenic markers in placental tissue (Zera et al., 2014). This work demonstrates an inverse correlation between BMI and serum levels of sFlt-1, which is associated with a pro-angiogenic profile (Zera et al., 2014). The authors propose that this might be due to excessive fetal growth, which requires a bigger placental vascular bed (Zera et al., 2014). This distorted angiogenic profile during MO is also supported by evidence showing the predominance of non-branching angiogenesis observed in placental tissue of obese women (Dubova et al., 2011).

Considering that: (1) MO results in distorted angiogenesis; (2) obesity has been associated with ER stress and IR; and (3) IR-related proteins also play a role in cell migration (RhoA, Akt), we hypothesize the possible contribution of different IR-related and others proteins to the modulation of EC migration capacity in the context of MO-dependent ER stress (**Table 1**).

RhoA signaling

RhoA and its downstream signaling has been linked to IR because they have targets such as Akt and eNOS (Kanda et al., 2006; Nunes et al., 2010) but also play a relevant role in cell migration (Jaffe and Hall, 2005). As mentioned above, in 2D cultures, EC migrate according to the leader–follower model (Rorth, 2009). In HUVEC, fibroblastic growth factor-induced collective cell migration is commanded by proteins that regulate cell–cell interactions, cell density, individual cell migration, and directed cell migration (Vitorino and Meyer, 2008). In the same study, RhoA was found to contribute to collective cell migration of HUVEC, a finding corroborated later by other groups (Vitorino and Meyer, 2008; Povero et al., 2013). Moreover, the role of RhoA is also relevant in other cell types where

Table 1 | Putative migration-related targets of ER stress signaling.

Target protein	Cell type	ER stress trigger	ER stress effect on target	Reference
RhoA	U87, HUVEC, HUVEC	↑IRE1, VEGF, ND	↓M ↑A, ↓M?	Dejeans et al. (2012), van Nieuw Amerongen et al. (2003), Song et al. (2012)
Spark	U87	↓IRE1	↑M ↑RhoA activity	Dejeans et al. (2012)
PI_3K/Akt/GSK3β/β-Catenin/E2F2 via	HUVEC	VEGF	↑P ↑A	Zeng et al. (2013)
eNOS	HUVEC	CHOP-10	↓M?	Loinard et al. (2012)
HO-1	VSMC	Tunicamycin	↓M	Yi et al. (2012)
Tsp-1	Athymic Balb/c (nu/nu), CEC	Tunicamycin	↓A	Banerjee et al. (2011)
MCPIP	HUVEC	TNF-α, IL-1β, IL-8	↓A	Roy and Kolattukudy (2012)
Scrib	HUVEC	ND	↓A?	Michaelis et al. (2013)

A, angiogenesis; M, migration; ND, not determined; P, proliferation.

it seems to be a typical feature of leader cells (Omelchenko et al., 2003; Jaffe and Hall, 2005; Rorth, 2009), because it contributes importantly to the mechanotaxis process (Reffay et al., 2014).

The contribution of RhoA to EC migration has been observed using a dominant-negative model and by its inhibition using ADP-ribosylation after bacterial toxin exposure. Both experimental conditions were associated with reduction of HUVEC migration (Aepfelbacher et al., 1997; Song et al., 2012) and angiogenesis (Povero et al., 2013). Previous studies suggest that RhoA mediates migration and VEGF-induced chemotaxis (van Nieuw Amerongen et al., 2003). However, interesting observations using a microfluidic device showed that RhoA contributes to HUVEC shear stress-induced mechanotaxis, although it does not affect VEGF-induced filopodia formation (Song et al., 2012). The explanation for these differential effects may also rely on the EC culture type studied; for example, RhoA contributes to VEGF-induced migration and angiogenesis of human foreskin microvascular EC (van Nieuw Amerongen et al., 2003). Thus, it is possible to suggest that RhoA contributes to migration in a stimulus- and cell type-dependent manner.

As mentioned above, ER stress is linked to cell migration. Supporting this notion, ER stress is associated with IRE1, which acts as an upstream protein of RhoA signaling (Dejeans et al., 2012). In a RhoA-dependent manner, cancer cells expressing a dominant-negative IRE1 protein show increased adhesion, impaired migration, and a reduced proliferation rate, but no change in invasive properties (Dejeans et al., 2012). As expected, RhoA inhibition restores the phenotype in IRE1 dominant-negative expressing cells (Dejeans et al., 2012). Therefore, as IRE1-lacking cells show over-activation of RhoA, it is possible to hypothesize that MO-induced ER stress, which increases IRE1 activity, might reduce RhoA activity, impairing EC migration (**Figure 2**).

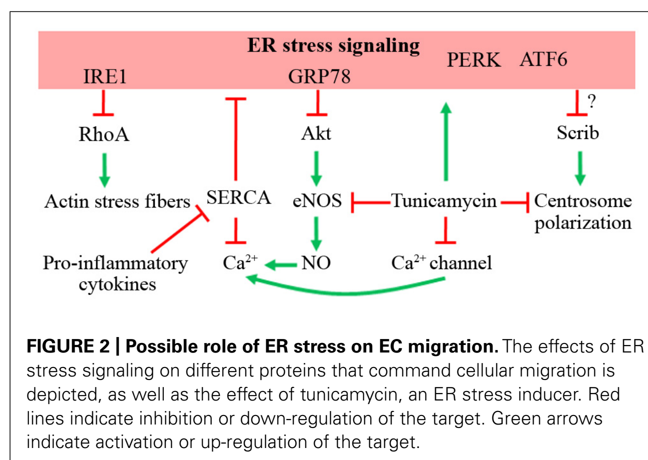

FIGURE 2 | Possible role of ER stress on EC migration. The effects of ER stress signaling on different proteins that command cellular migration is depicted, as well as the effect of tunicamycin, an ER stress inducer. Red lines indicate inhibition or down-regulation of the target. Green arrows indicate activation or up-regulation of the target.

Akt signaling

The Akt gene encodes three isoforms (Akt1-3). In EC, Akt activation is related to several signaling cascades, such as the insulin pathway, eNOS activation, cell survival, and migration (Shiojima and Walsh, 2002). In EC, it has been suggested that Akt3 contributes to cell migration (Vitorino and Meyer, 2008). However, Akt involvement in cellular migration and angiogenesis depends on the tissue context and simultaneously activated signaling (Somanath et al., 2006). For instance, Akt3 has defective signaling in muscle cells from obese insulin-resistant subjects (Brozinick et al., 2003), potentially affecting EC migration response downstream of the insulin receptors (Guo, 2014). In addition, extensive evidence has shown a potential link between Akt and ER stress signaling (Appenzeller-Herzog and Hall, 2012). Interestingly, BiP/GRP78 suppresses the Ser473 phosphorylation of Akt by direct interaction, which might prevent accessibility for activating kinases (Yung et al., 2011). Moreover, this study shows that

ER stress activates or inhibits Akt signaling depending on the magnitude or severity of this response (Yung et al., 2011). On the other hand, VEGF has been related to induction of the IRE1 branch of ER stress in HUVEC (Zeng et al., 2013). VEGF exposure was associated with IRE1-dependent splicing of XBP1 and activation of Akt/GSK signaling, which is required for the proliferation and angiogenesis induced by this growth factor (Zeng et al., 2013).

Downstream to Akt activation, the production of NO by eNOS has been linked in EC migration, because pharmacological inhibition of eNOS reduces *in vitro* migration capacity (Murohara et al., 1999; Lamalice et al., 2007). Moreover, aortic EC from eNOS-lacking mice have impaired *in vitro* and *in vivo* angiogenesis (Lee et al., 1999; Lamalice et al., 2007). Tunicamycin-induced ER stress reduces eNOS levels in mouse coronary artery EC (Galan et al., 2014). Accordingly, homocysteine-induced ER stress through C/EBP CHOP-10 signaling inhibits eNOS signaling in HUVEC (Loinard et al., 2012). As mentioned above, fetuses from pregnancies with MO develop IR (Catalano et al., 2009), which is maintained through childhood (Bruyndonckx et al., 2013). Since eNOS activation is regulated by insulin stimulation in EC, eNOS signaling could be altered in HUVEC from MO pregnancies as an outcome of fetal programming, as occurs in other maternal pathologies (Farías et al., 2006, 2010; Leiva et al., 2011; Westermeier et al., 2011). If the latter effectively occurs, HUVEC derived from MO pregnancies might have impaired Akt/eNOS signaling and migration and/or angiogenic capacity (**Figure 2**).

Soluble mediators and Ca^{2+} signaling

Pro-inflammatory mediators such as cytokines and adipokines are increased during MO (Catalano et al., 2009) and have detrimental effects on EC migration. Leptin is an adipokine with pro-angiogenic effects that induces HUVEC migration after activation of Akt and eNOS (Goetze et al., 2002). However, leptin exposure is not associated with significant effects on tube formation *in vitro* (Dubois et al., 2013). In contrast, adiponectin inhibits HUVEC migration in a wound-healing assay and also inhibits tube formation *in vitro* (Dubois et al., 2013), showing an opposite effect to that of leptin. In fact, leptin signaling is impaired by ER stress, which also contributes to leptin resistance (Hosoi et al., 2008; Ozcan et al., 2009). Conversely, adiponectin has been linked to inhibition of ER stress (Boddu et al., 2014).

Pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-8, have a pro-angiogenic effect (Dinarello, 2007). These cytokines exert this effect by up-regulation of MCPIP, which is required to induce angiogenesis *in vitro* by increasing ER stress (Roy and Kolattukudy, 2012), suggesting an association between cytokines and ER stress. Interestingly, most if not all cytokines regulate free intracellular Ca^{2+} levels in cells, providing another possible link between these soluble mediators and the development of ER stress, as we discuss next.

Ca^{2+} signaling is one of the most important players during cell migration (Wei et al., 2012). Ca^{2+} channel-dependent calcium dynamics are observed at the leading edge in migrating cells (Wei et al., 2009). Consequently, during HUVEC migration, a polarized generation of PIP_3 is found at the front of the migrating cells, which further increases the Ca^{2+} influx, allowing cytoskeleton rearrangements required for motility (Tsai and Meyer, 2012; Tsai et al., 2014). Simultaneously, an increase in the extrusion of Ca^{2+} towards the extracellular milieu is observed, hence maintaining the Ca^{2+} dynamics at the front (Tsai et al., 2014), showing that a fine-tuning of Ca^{2+} signaling is required for HUVEC migration. In this context, tunicamycin links ER stress with Ca^{2+} signaling because this antibiotic induces distorted function of Ca^{2+}-channels (Czyz et al., 2009). In addition, NO produced by eNOS contributes to Ca^{2+} dynamics (Huang et al., 2013), suggesting that deficient eNOS signaling induced by ER stress might affect Ca^{2+} signaling and hence cell migration. Interestingly, pro-inflammatory cytokines IL-1β and IFN-γ down-regulate the SERCA and increase ER stress markers in pancreatic β-cells (Cardozo et al., 2005). The possibility of a similar mechanism occurring in EC is interesting, because it would unveil the mechanisms by which cytokines might affect EC migration (**Figure 2**).

Cell polarity

During cell migration, a reorientation of several cellular structures occurs in a process called polarization (Rorth, 2009). One of the intracellular features exhibited by migrating EC is the polarization of the centrosome toward the direction of movement of the endothelial sheet (**Figure 1**; Gotlieb et al., 1981; Rorth, 2009; Etienne-Manneville, 2013). Moreover, microtubule-binding drugs that inhibit HUVEC migration exert this blockade effect by avoiding centrosome repositioning (Hotchkiss et al., 2002; Kamath et al., 2014). Therefore, ER stress might affect cellular polarization and hence impair cell migration.

One of the candidates potentially affected by ER stress is Scrib, which mediates chemotaxis-dependent, but not spontaneous, cell migration (**Figure 2**) and *in vitro* and *in vivo* angiogenesis (Michaelis et al., 2013). This protein contributes to cytoskeletal rearrangements and Golgi apparatus polarization toward the leading edge in wound-healing assays (Michaelis et al., 2013). Whether a similar distortion occurs with nuclei and/or mitochondrial and/or lysosomal reorientation (Rorth, 2009; Friedl et al., 2011; Etienne-Manneville, 2013; da Silva et al., 2014) has not yet studied. Thus, cell polarity-related proteins might be affected by ER stress and thus impair proper organelle and centrosome polarization.

CONCLUDING REMARKS AND PERSPECTIVES

Endothelial cell migration relies on tightly regulated signaling cascades that are activated by various stimuli. Adequate signaling events are required for proper remodeling of vessels during angiogenesis, and distorted intracellular cross-talk among the involved pathways would result in vascular dysfunction. We focus on the potential involvement of two main mechanisms of disease observed in obesity, ER stress, and IR. Interestingly, ER stress might impact EC migration and hence angiogenesis in different ways. Here, we summarize the current knowledge about how ER stress might provoke alterations in EC migration capacity and propose new targets (**Figure 1**). Specifically, both ER stress and IR might affect the coordination of endothelial chemotaxis, haptotaxis, mechanotaxis, Ca^{2+} signaling, and

cell polarity modulation, which are key steps associated with EC migration. Better understanding of these processes regarding the physiopathological mechanism underlying ER stress might provide new perspectives in the design of therapeutic targets.

Different ER stress stimuli and micro-environmental contexts play major roles in regulation of EC migration, as well as the timing of stimulation signals and the magnitude of ER stress activation. For example, a physiological role of ER stress has been shown during pregnancy, where it is required for placenta development (Iwawaki et al., 2009), but it still is unknown whether its overactivation under pathological conditions remains favorable or becomes detrimental. On the other hand, intracellular cascades associated with IR development may be also associated with impaired EC migration capacity. To further address these research topics, new models of *in vivo* and *in vitro* analysis are required. An interesting approach recently validated the use of rat mesenteric EC to evaluate angiogenesis, because these cells exhibit the same behavior as HUVEC during migration and angiogenesis (Mansouri et al., 2013). Another approach proposes 3D culture of adipocytes and HUVEC in microspheres, in an attempt to mimic adipose tissue (Yao et al., 2013). The zebrafish, a well-established model to evaluate migration and angiogenesis, has been recently used to evaluate the role of Akt and ER stress pathways (Lu et al., 2014). Furthermore, the chick embryo chorioallantonic membrane assay might be used to evaluate the impact of ER stress on EC migration and angiogenesis, in a way similar to its current use in evaluating the anti-angiogenic potential of different compounds (Lange et al., 2014). Taken together, these models might provide new tools for studying EC migration and angiogenesis during obesity-induced ER stress and IR.

Considering the state of knowledge, we propose that acute and chronic ER stress might induce different effects on EC migration. In addition, as observed in tumoral versus non-tumoral environments, ER stress might promote or impair EC migration and angiogenesis, respectively. Finally, based on the hypothesis of intrauterine programming during pregnancies affected by adverse conditions and the induction of ER stress and IR in the presence of obesity, we suggest that MO might induce fetal ER stress and IR, two intracellular mechanisms associated with altered EC migration and hence distorted angiogenesis in offspring endothelium.

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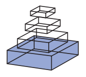

The placental pursuit for an adequate oxidant balance between the mother and the fetus

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The placenta is the exchange organ that regulates metabolic processes between the mother and her developing fetus. The adequate function of this organ is clearly vital for a physiologic gestational process and a healthy baby as final outcome. The umbilico-placental vasculature has the capacity to respond to variations in the materno-fetal milieu. Depending on the intensity and the extensity of the insult, these responses may be immediate-, mediate-, and long-lasting, deriving in potential morphostructural and functional changes later in life. These adjustments usually compensate the initial insults, but occasionally may switch to long-lasting remodeling and dysfunctional processes, arising maladaptation. One of the most challenging conditions in modern perinatology is hypoxia and oxidative stress during development, both disorders occurring in high-altitude and in low-altitude placental insufficiency. Hypoxia and oxidative stress may induce endothelial dysfunction and thus, reduction in the perfusion of the placenta and restriction in the fetal growth and development. This Review will focus on placental responses to hypoxic conditions, usually related with high-altitude and placental insufficiency, deriving in oxidative stress and vascular disorders, altering fetal and maternal health. Although day-to-day clinical practice, basic and clinical research are clearly providing evidence of the severe impact of oxygen deficiency and oxidative stress establishment during pregnancy, further research on umbilical and placental vascular function under these conditions is badly needed to clarify the myriad of questions still unsettled.

Keywords: hypoxia, oxidative stress, placenta, vascular dysfunction, high-altitude

INTRODUCTION

The placenta is the exchange organ between the pregnant woman and her developing fetus. The adequate function of this organ is clearly essential for a proper progress of gestation and a healthy baby as final outcome. As every developing organ, the placenta has an adapting capacity to variations in materno-fetal conditions, with short and long-lasting responses deriving in potential morphostructural and functional changes. These adjustments sometimes compensate the initial triggering insults and occasionally may switch to long-lasting remodeling processes arising maladaptation, affecting its own function and the materno-fetal health. Appropriate levels of oxygen and reactive oxygen species are determinants in placental development and function. This review will focus on placental responses to hypoxic environments, usually associated with oxidative stress and vascular functional impairments.

HYPOXIC CONDITIONS DURING DEVELOPMENT

One of the most challenging conditions in modern perinatology is hypoxia during development. This is considered harmful to the fetus due to the short- and long-term devastating effects, particularly in the central nervous and cardiovascular systems, resulting in functional alterations. Furthermore, *in utero* adverse conditions can increase the risk of developing chronic diseases later in life, phenomena known as fetal programming or developmental origins of health and disease-DOHaD (Fowden and Forhead, 2009; Gunn and Bennet, 2009; Li et al., 2012). However, less focus has been put on the placenta and its protective role during development.

Abbreviations: 4HNE, 4-Hydroxynonenal; ADMA, asymmetric dimethylarginine; ANGPT, angiopoietins; BH4, tetrahydrobiopterin; CO, carbon monoxide; DOHaD, developmental origins of health and disease; eNOS, endothelial nitric oxide synthase; EPO, erythropoietin; ET-1, endothelin-1; GPx, glutathione peroxidase; H_2O_2, hydrogen peroxide; HO, heme oxygenase; HIF, hypoxia-inducible factor; HSP70, heat-shock protein 70; IGF, insulin-like growth factor; IUGR, intrauterine growth restriction; K(V), voltage-gated potassium channel; K+, potassium; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor kappaB; NO, nitric oxide; O_2, oxygen; $^{\bullet}O2-$, superoxide anion; $^{\bullet}OH-$, hydroxyl radical; PDGFB, platelet-derived growth factor subunit B; PE, preeclampsia; ROS, reactive oxygen species; SGA, small for gestational age fetus; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor; XD, xanthine dehydrogenase; XO, xanthine oxidase.

Hypoxia is defined as a deficient oxygen (O_2) supply for the physiological demands of a tissue. This is a restrictive condition frequently faced during fetal life, either by maternal or umbilico-placental circumstances, or by environmental hypoxia as seen in highlands.

Nowadays, it is estimated that worldwide there are 150 million newborns per year and 5–10% of them will have low birth weight standardized for gestational age (Alberry and Soothill, 2007; Nardozza et al., 2012). Placental insufficiency leads to fetal growth restriction (IUGR) due to a decreased feto-placental perfusion and restricted oxygen delivery. This situation affects simultaneously O_2 and nutrient supply to the fetus (Baschat, 2011), overlapping conditions that difficult the isolation of the specific effects of O_2 deficiency in the determination of vascular impairment. Conversely, altitude decreases environmental pressure and consequently, alveolar PO_2, deriving in high altitude hypoxia (Niermeyer et al., 2009). There is no precise data about the number and complications in high altitude pregnancies, but hypoxia-related problems during gestation are dramatically increased above 2500 m (Keyes et al., 2003; Moore et al., 2004; Zamudio, 2007; Julian, 2011). Currently, it is estimated that more than 150 million people live in highlands worldwide (Niermeyer et al., 2009). Of those, 35 million live in the Andean Mountains, in important cities, such as La Paz, capital of Bolivia (Keyes et al., 2003). This has led to an increased clinical and scientific interest in dissecting the different noxas that induce fetal programming (Luo et al., 2006; Franco et al., 2007; Nuyt, 2008; Schleithoff et al., 2012), but few have focus on the lack of oxygen as a main cause.

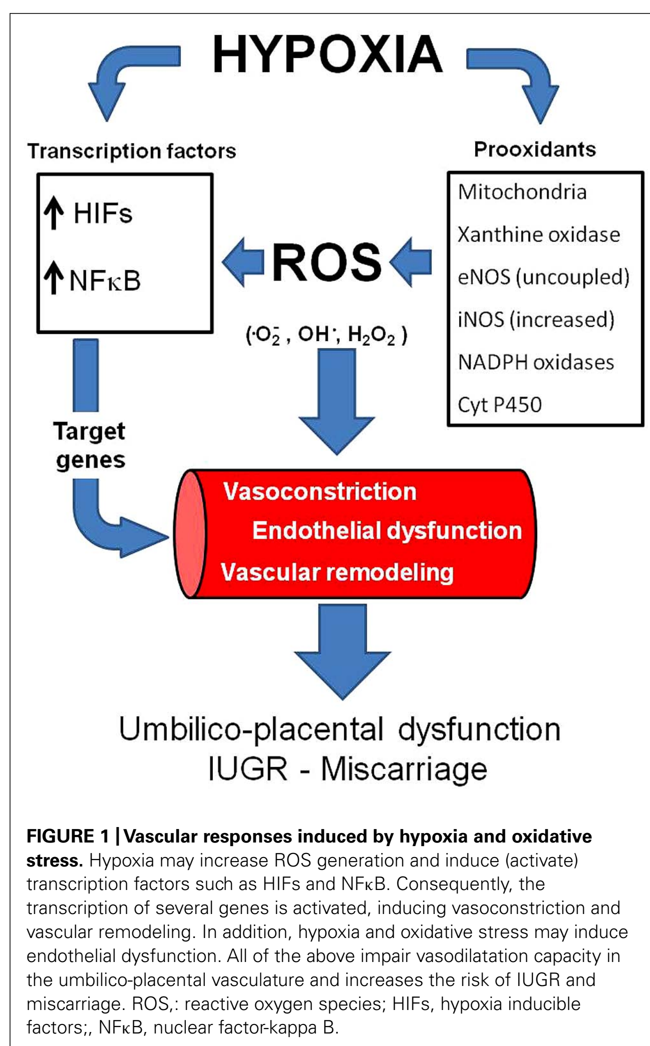

FIGURE 1 | Vascular responses induced by hypoxia and oxidative stress. Hypoxia may increase ROS generation and induce (activate) transcription factors such as HIFs and NFκB. Consequently, the transcription of several genes is activated, inducing vasoconstriction and vascular remodeling. In addition, hypoxia and oxidative stress may induce endothelial dysfunction. All of the above impair vasodilatation capacity in the umbilico-placental vasculature and increases the risk of IUGR and miscarriage. ROS,: reactive oxygen species; HIFs, hypoxia inducible factors;, NFκB, nuclear factor-kappa B.

HYPOXIA AND OXIDATIVE STRESS

One of the mechanisms by which hypoxia induces damage is as a result of the increased generation of reactive oxygen species (ROS) by an incomplete reduction of oxygen (Abramov et al., 2007; Giussani et al., 2012; Pialoux and Mounier, 2012). ROS are free radicals produced as by-products of oxidation–reduction reactions. There are various intracellular enzymatic pathways that produce ROS in mammals, such as mitochondrial electron transport, NADPH oxidase, the cytochrome P450 monoxygenase system, xanthine oxidase, nitric oxide synthases, cyclooxygenase, and lipoxygenase (Apel and Hirt, 2004; Dowling and Simmons, 2009). Particularly in the vasculature, the main sources of superoxide anion (•O2-) are NADPH oxidase, xanthine oxidase, uncoupled endothelial nitric oxide synthase (eNOS) and mitochondria (Katusic, 1996; Förstermann and Sessa, 2012). Relatively low concentrations of ROS are necessary to operate as signaling molecules in the normal regulation of cell differentiation and function. However, the increased ROS generation may overwhelm the antioxidant endogenous capacity and determine oxidative stress (**Figure 1**). For instance, it has been established in several experimental models that ischemia/reperfusion, hypoxia/reoxygenation and even hypoxia itself can promote oxidative stress (Comhair and Erzurum, 2002; Guzy and Schumacker, 2006; Waypa et al., 2006; Behn et al., 2007; Clanton, 2007; Ward, 2007; Loor et al., 2011; Sylvester et al., 2012).

An important source of ROS is the mitochondrial electrons chain. At physiological oxygen levels, it has been suggested that about 1–4% of the O2 reduced by mitochondria may form •O2- (Li and Shah, 2004). However, under conditions of metabolic perturbation such as hypoxia/reoxygenation (Pearlstein et al., 2002), the percentage of oxygen incompletely reduced can increase, promoting enhanced production of ROS by the mitochondria (Sanjuan-Pla et al., 2005; Guzy and Schumacker, 2006; Loor et al., 2011). Thus, studies have shown that a functional respiratory chain is required and that loss of cytochrome c (complex III), abolishes this ROS signal (Guzy et al., 2005; Guzy and Schumacker, 2006). Outstandingly, the direct addition of ROS such as H_2O_2 overcomes this blockade (Bell et al., 2007). These studies have led to the proposal that mitochondrial complex III is one of the main sources of ROS (Guzy and Schumacker, 2006).

In addition to ROS generated as a "byproduct" of cellular respiration, endogenous production of •O2- also arises from NADPH oxidases (NOX 1, 2, 4, and 5) normally at low levels in smooth muscle and vascular endothelium (van der Vliet, 2008; Rivera et al., 2010). Another source of ROS is cytochrome P450, an enzyme involved in the metabolism of arachidonic acid in the vascular endothelium (Fleming et al., 2001). Superoxide anion and hydroxyl radical (•OH-) are produced during

the cytochrome P450 reaction cycle when the electrons for the reduction of the central heme iron are transferred to the activated bound O_2 molecule in an NADPH-dependent reaction (Fleming, 2001). Another important source of ROS is xanthine oxidase (XO), a metalloflavoprotein which is generated by the post-translational modification of xanthine dehydrogenase (XD) (Hewinson et al., 2004). Functionally, both XD and XO catalyze oxidation of hypoxanthine to xanthine and xanthine to uric acid, with the generation of •O2- (Zhang et al., 1998; Meneshian and Bulkley, 2002). Endothelial nitric oxide synthase (eNOS) may also act as a generator of ROS and is dependent on NOS substrates and cofactors. Endothelial nitric oxide synthase is a calcium-dependent flavoenzyme that generates NO in a process that involves oxidation of the amino acid L-arginine via the reduction of O_2 (Cai and Harrison, 2000). Intracellular calcium levels control the eNOS-calmodulin association which is an important stage in the activation of eNOS (Stuehr, 1999). Another protein which can control eNOS activity is heat shock protein 90 (HSP90), a molecular chaperone that increases eNOS activity, possibly by increasing the rate of electron transfer between the two domains of eNOS (Stuehr, 1999). The process of NO production also requires the essential NOS cofactor tetrahydrobiopterin (BH4) as it plays a crucial role in coupling the reduction of O_2 to the oxidation of L-arginine as well as maintaining the stability of the NOS dimers (Vasquez-Vivar et al., 2003). If the production or efficiency of BH4 decreases, or if there is a deficiency in the NOS substrate L-arginine, then NOS can become uncoupled resulting in the production of •O2- instead of NO (Lei et al., 2013; **Figure 1**).

VASCULAR EFFECTS

Most studies on umbilico-placental vascular function are Doppler-based, therefore non-invasive and mostly present descriptive analysis. Nonetheless, *ex vivo* experimental approaches, such as vascular myography and placenta perfusion, have offered important clues of mechanisms modulating umbilico-placental vascular function in diverse perinatal conditions.

Vascular tone regulation

Vascular tone is dependent on the balance between vasodilator and vasoconstrictive agents. The endothelial-independent NO-induced vascular relaxation has been assessed in normal term placentas (Myatt, 1992; Wareing et al., 2005), and correlates inversely to Doppler pulsatility and resistance indexes (Wareing et al., 2005). Otherwise, in complicated pregnancies with fetal growth restriction umbilical artery pulsatility index inversely correlates with placental eNOS mRNA levels (Giannubilo et al., 2008). In addition, arginase, an endogenous negative regulator of eNOS which counteracts the NOS-dependent relaxation in umbilico-placental vessels (Krause et al., 2012), is increased by hypoxia in umbilical vein endothelium (Prieto et al., 2011) and in plasma from pre-eclamptic women (Sankaralingam et al., 2010). Furthermore, arginase inhibition restores the impaired NOS-induced relaxation in IUGR umbilical and chorionic arteries (Krause et al., 2013). Another vasodilator suggested to be involved in placental vasodilation is carbon monoxide (CO), which is decreased in preeclampsia and IUGR (Barber et al., 2001). In compensating CO and NO dysfunction in complicated pregnancies, an up-regulation of H2S has been shown to vasodilate the placental vasculature via potassium channels (Cindrova-Davies et al., 2013). Similar to other oxygen-sensitive vasculature, potassium channels are main determinants of vascular resistance (Wareing and Greenwood, 2011) and vasculogenesis (Brereton et al., 2013) in chorionic plate vessels. Previous studies have not been conclusive for the role of these channels in complicated pregnancies, but some authors suggests that K(V) may be involved in IUGR (Corcoran et al., 2008) and ROS might be regulating their function in the placenta (Mills et al., 2009), elevating vascular resistance. Furthermore, it has been shown that ROS enhanced basal tension and vasoconstriction in response to a thromboxane mimetic (Mills et al., 2009). Although the exact mechanism is unknown, K+ channels may be involved in maintaining a lower membrane potential, and therefore depolarization processes are easy to be activated and induce contraction. However, ROS may as well increase relaxation in response to NO (Mills et al., 2009).

The responses in placental vascular tone to different levels of oxygenation are still under debate and extensively reviewed by Wareing (2014). Some argue similar effects as those observed in the pulmonary bed, where oxygenation promotes vasodilation and others suggest a vasoconstriction response in umbilico-placental veins (Wareing, 2014). Even more, some authors sustain that vascular reactivity in chorionic arteries is independent of oxygen levels (Cooper et al., 2005; Wareing et al., 2006). The differences found might be dependent on the experimental conditions and the oxygenation levels, where much of these are made under relatively hyperoxic conditions when compared to the ranges of ~15–30 mmHg *in vivo* PO_2 (Wareing, 2014). Furthermore, there is a differential response in the *ex vivo* experiments depending on the mode of delivery (Mills et al., 2007). Clearly the debate is open, and the responses in hypoxic pathological conditions cannot be predicted. Despite their fundamental importance for normal perfusion of the placenta, still the umbilico-placental vascular reactivity has been poorly studied, particularly in pathological conditions.

Vascular protein expression

The vascular effects on gene expression and cellular responses to hypoxia and oxidative stress are alike, sharing similar effector pathways. For instance, both conditions activate the hypoxia-inducible factor (HIF-1) and therefore induce several proteins associated with remodeling processes. It has been proposed that mitochondria are O_2 sensors and signal HIF-1α stabilization by releasing ROS to the cytoplasm (Simon, 2006). HIF-1 is a transcription factor that is activated under hypoxia (Semenza and Wang, 1992) and regulates over 100 physiologically important genes that may affect the vascular function (Semenza, 2006). HIF-1α is in fact regulating the transcription of many vascular remodeling related genes (Yu et al., 1999; Schofield and Ratcliffe, 2004; Semenza, 2006), leading to increased tone, thickening and stiffening of the vasculature. Increases in HIF-1 and HIF-2 activity induce production of angiogenic growth factors and cytokines in hypoxic cells, such as VEGF, EPO, ANGPT, and PDGFB (Prabhakar and Semenza, 2012). All of these will induce vascular smooth muscle

cell proliferation and a balanced tone towards increased resistance under chronic hypoxia (Prabhakar and Semenza, 2012). In addition, HIFs also play a more general role in the response to a variety of cellular activators and stressors, many of which use ROS as signal transducers (Cash et al., 2007; Görlach and Kietzmann, 2007). Although HIF-1 and HIF-2 may upregulate the expression of pro-angiogenic factors, the first tend to diminish the inflammatory response, whereas the second increases the response (Loboda et al., 2012). Therefore, in endothelial cells, both factors may have opposite effects, probably a fine tuning depending on the oxygen or ROS levels. HIF-3 is a third isoform, which has been less studied but postulated to act as a negative regulator of HIF-mediated transcription (Loboda et al., 2012). Currently, the most studied and with more ascribable roles in vascular expression responses if HIF-1. HIF-1α is increased in high-altitude placentas (Soleymanlou et al., 2005; Zamudio et al., 2007) and has an important role in lowland preeclampsia (Tal, 2012; Akhilesh et al., 2013), such as it has been proposed as a predictive biomarker of late pre-eclampsia (Akhilesh et al., 2013). A recent discussion has proposed that preeclampsia does not lead to hypoxia as has been presumed (Huppertz et al., 2014). In fact, Huppertz et al. (2014) cited three papers where the calculated PO_2 seems to be higher in patients with preeclampsia and/or IUGR. However, no *in vivo* measurements have been ever done in uterine or placental tissue in complicated pregnancies, so the debate is still open.

Another transcription factor that is activated by hypoxia and oxidative stress is the nuclear factor kappaB (NF-κB) (Haddad, 2004). Although this factor is more related to innate immunity, inflammation, and apoptosis, it regulates genes that induce remodeling processes (Ungvari et al., 2006; Taylor and Cummins, 2009). Thus, NF-κB is involved in angiogenesis of the placenta (Min et al., 2003; Chen et al., 2008). The immune and inflammatory responses induced by NF-κB activation initiates and accelerates vascular remodeling, vascular inflammation, endothelium apoptosis, vascular oxidative stress and impaired NO bioavailability (Gao et al., 2008), which contribute to the blunted vascular function (Foncea et al., 2000; Csiszar et al., 2008). Furthermore, NF-κB may induce tumor necrosis factor-α (TNF-α), which plays a pivotal role in endothelial dysfunction (Gao et al., 2008; Zhang, 2008). It seems that NF-κB-induced local inflammatory reaction may have important placental dysfunctional features, at least in pre-eclampsia (Guedes-Martins et al., 2013). Recent studies have demonstrated that similar oxygen-sensing mechanisms, such as hydroxylases, are determinants on oxygen sensitivity for both HIF and NF-κB-dependent gene expression. Furthermore, there is an extensive degree of cross-talk occurring between NF-κB and HIF (Taylor and Cummins, 2009; Scholz and Taylor, 2013). Therefore, hypoxia and oxidative stress are activating transcription signaling pathways that may end in placental vascular dysfunctions (**Figure 1**).

HYPOXIA, UMBILICO-PLACENTAL DEFICIENCIES, AND IUGR

Hypoxia and oxidative stress levels in the placenta change along gestation. At initial stages of development, there is a reduced utero-placental vasculature and therefore, a low perfused and oxygenated environment. This hypoxic condition seems to be protective for the placenta and fetus as it is exposed to less oxygen radicals (Jauniaux et al., 2006). However, after trophoblastic invasion and placental development, the fetus and placenta demands for oxygen increases, and are covered by maternal blood supply in the intervillous space (Jauniaux et al., 2006; Schneider, 2011; Murray, 2012). In contrast, placental growth reaches its limits at term and terminal villi become over-crowded with diminished intervillous pore size. This physiologic condition will decrease the intervillous perfusion generating local hypoxia and oxidative stress (Schneider, 2011; Redman et al., 2014). Therefore, in the placental function, there is more chance of hypoxia and oxidative stress near delivery (**Figure 2**). Environmental hypoxic conditions or maternal problems that decrease PO_2 may anticipate this physiological response to hypoxia in the placental unit. This condition is particularly important in the last trimester of gestation, when overlapping situations as placental and fetal energy demands are dramatically increased and the feto-placental unit respond in order to optimize the allocation of oxygen between competing demands (Schneider, 2011; Murray, 2012). As oxygen is a vital regulator of placental and fetal development (Genbacev et al., 1997; Zamudio et al., 2007), these responses are adaptive for fetal survival with evident restrictions in their demands, as seen in IUGR fetuses (Burton and Jauniaux, 2004; Soleymanlou et al., 2005; Soria et al., 2013).

Reduced fetal growth is well documented under the conditions of chronic hypoxia at high altitude (Keyes et al., 2003; Moore et al., 2004; Zamudio, 2007; Julian, 2011; Soria et al., 2013). In human high-altitude placentas, there are increased nitrotyrosine residues in the syncytiotrophoblast, nitrative stress and lower concentrations of anti-oxidants (Zamudio et al., 2007). These results show that hypoxia is inducing oxidative stress in placentas (Bosco et al., 2012). This is also observed in placentae from lowland women with pre-eclampsia and IUGR (Myatt et al., 2000). Further, maternal exposure to an equivalent level of chronic hypoxia in a rat model, significantly elevate maternal and placental molecular indices of oxidative stress, such as HSP70 and 4HNE (Richter et al., 2012). However, placentas at 3100 m do not increase total oxidative stress at delivery (Zamudio et al., 2007; Tissot van Patot et al., 2010). This was suggested to be a hypoxic preconditioning response that might occur in placentas that develop at high altitude (Tissot van Patot et al., 2010). The differences found in the placental level of oxidative stress might depend on the species, the period of exposure to hypoxia, the severity of the insult and the genetic background of the individual (Jauniaux et al., 2006; Richter et al., 2009, 2012; Tissot van Patot et al., 2010).

Another condition where placenta dysfunction and oxidative stress are directly involved is pre-eclampsia. Pre-eclampsia is a multi-system disorder characterized by high blood pressure and proteinuria in pregnant woman (Sibai, 2005). Although, no clear cause is known to induce this pathology, several proposals point toward an inadequate placentation and placental dysfunction (Jauniaux et al., 2006; Redman et al., 2014). Interestingly, high-altitude populations have increased incidence of pre-eclampsia (Escudero and Calle, 2006; Zamudio, 2007; Julian, 2011; Gonzales, 2012). In fact, this has been replicated in gestational hypoxia inducing preeclamptic-like symptoms in pregnant rats (Zhou et al., 2013).

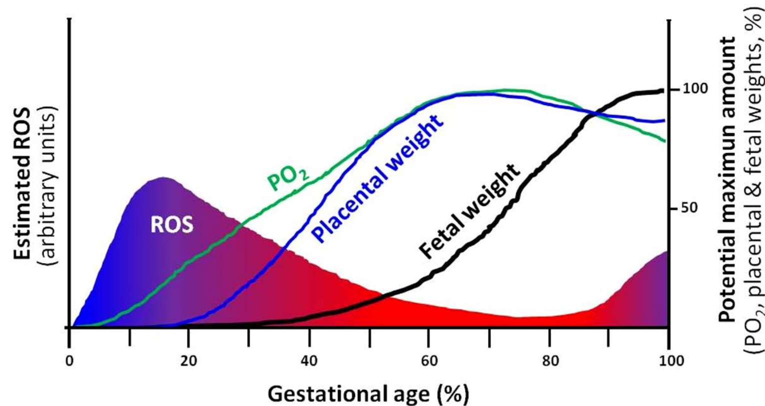

FIGURE 2 | Theoretical depiction proposing the correlation of ROS, PO_2 and development of the placenta and the fetus. The intrauterine (utero-placental) ROS formation is a function of PO_2 in which, hypoxia supports elevations in ROS. Furthermore, the placental and fetal weights are closely related and dependent on increases in PO_2 and limitation of ROS. Changes in any of the four variables, may directly affect the potential maximum amount of the rest of the variables. For instance, a decrease in PO_2 may diminish fetal growth and term weight (IUGR). ROS, reactive oxygen species.

Furthermore, hypoxic stabilization of HIF-1α seems to have an important role in pre-eclampsia pathogenesis (Tal, 2012). Similar as in hypoxic conditions, pre-eclamptic placentas show increased oxidative stress (Vaughan and Walsh, 2012; Redman et al., 2014), potentially contributing to the impairment of placental perfusion by affecting vascular function (Myatt et al., 2000; Richter et al., 2009; Bosco et al., 2012). In pre-eclampsia there is a failure in trophoblast invasion and insufficient modification of spiral arteries, which has been described to lead, although not necessarily, to placental hypoxia (Huppertz et al., 2014). In addition to the functional impairment, the placental vascular bed is greatly reduced in chronic fetal hypoxia (Kuzmina et al., 2005; Sankar et al., 2013). The mechanisms involved will not only impact fetal life, but will have mediate- and long-lasting effects in the newborn (Gheorghe et al., 2007; Fowden and Forhead, 2009; Gunn and Bennet, 2009; Li et al., 2012; Giussani et al., 2012). The programming effects on the feto-placental unit due to intrauterine stress such as hypoxia and the potential mechanisms are currently intensively under research. The proposed main modulating mechanisms are epigenenetic, such as DNA methylation and histone deacetylation, reviewed in this issue by Casanello et al. (2014).

Clearly, the placental and fetal developments are dependent on oxygen availability and ROS restraint. Moreover, the levels of oxygen and ROS are as well dependent on placental function, creating interdependence among these variables and fetal development and growth (**Figure 2**).

CLINICAL EVIDENCE

The combination of fetal biometry and maternal and fetal Doppler provides the best clinical approach, which are complementary to each other to identify small for gestational age fetuses (SGA) as well as those with intrauterine growth restriction (IUGR) at risk of adverse outcomes due to placental insufficiency (Figueras and Gardosi, 2011; Parra-Saavedra et al., 2014). Although the precise mechanisms by which placental function is affected remain unknown, there may possibly be a primary defect in placental development as the underlying abnormality. For the vast majority of the IUGR cases, the underlying defect is due to poor trophoblastic invasion of maternal spiral arteries and reduced uteroplacental flow (Brosens et al., 2011; Everett and Lees, 2012). When oxygen delivery to the fetus falls below a critical value, fetal oxygen uptake and glucose transfer are reduced and fetal hypoglycemia leads to gluconeogenesis from hepatic glycogen stores (Economides and Nicolaides, 1989; Nicolini et al., 1989). There is a down-regulation of active placental transport, independent of the presence of hypoxia or the severity of the IUGR and the fetus needs to mobilize other energy sources, resulting in more widespread metabolic changes such as alteration of amino acid metabolism and transport across the placenta (Cetin and Alvino, 2009; Avagliano et al., 2012). Maternal metabolic signals (leptin, insulin, and adiponectin plasma levels) lead to placental regulation of nutrient transport to the fetus (Lager and Powell, 2012) An increasing degrees of fetal metabolic compromise has been documented by cordocentesis in human fetuses showing that some SGA fetuses are hypoxemic, hypercapnic, hyperlacticemic, acidemic, and hypoaminoacidemic (Cetin and Alvino, 2009; Avagliano et al., 2012). In umbilical venous blood, mild hypoxemia may be present in the absence of hypercapnia or acidemia. However, in severe utero-placental insufficiency the fetus cannot compensate hemodynamically and thus, hypercapnia and acidemia increase exponentially. The immediate effect of decreased fetal glucose and amino acids levels is the down-regulation of the principal endocrine growth axis involving insulin, IGF I, IGF II and transforming growth factor beta adding negative impact on fetal growth (Randhawa, 2008; Lager and Powell, 2012).

The endothelial regulation of umbilico-placental vascular tone may be abnormal in pregnancies complicated by IUGR. Although the mechanisms may be diverse in the vascular dysfunction, hypoxia and oxidative stress play critical roles. Prostacyclin release during cordocentesis is decreased in pregnancies complicated by IUGR (Rizzo et al., 1996). Elevated levels of ET-1 were found in

samples of umbilical venous blood obtained from IUGR pregnancies with abnormal Doppler waveforms (Erdem et al., 2003) and the villous core receptors for ET-1 appear to be functional. On the other hand, nitric oxide is the main vasodilator of the uteroplacental blood flow and it has also been suggested that inhibition of its synthesis reduces basal perfusion and increases flow resistance (Myatt et al., 1997; Parra et al., 2001). However, it has been postulated that the marked hypoxic vasoconstrictive response in pathological pregnancies is mediated by inhibition of potassium channels in small intraplacental vessels (Hampl et al., 2002). Hypoxemic growth-restricted fetuses also show a whole range of haematological abnormalities, including erythroblastemia and thrombocytopenia. Hypoxia is a trigger for erythropoietin release and stimulation of red blood cell production, through both medullary and extramedullary sites, resulting in polycythemia (Pallotto and Kilbride, 2006). Furthermore, there is a reduction in platelet count which inversely correlates with umbilical artery Doppler, and can be explained by placental consumption or dysfunctional erythropoiesis and thrombopoiesis (Baschat et al., 2013).

In IUGR pregnancies, histological studies have shown that the process of spiral artery vascular transformation is incomplete. In a normal pregnancy, the luminal diameter of the spiral arteries is greatly enlarged, and the walls are remodeled such that they contain very little smooth muscle. These changes extend into the vessels to the inner third of the myometrium to provide a low-resistance circuit for perfusion of the intervillous space. These modifications are associated with endovascular invasion of the fetal trophoblast into these maternal vessels. In women with IUGR, endovascular invasion and spiral artery remodeling occur either very superficially or they do not occur, being associated with high resistance to flow in the maternal uterine arteries and relative placental hypoperfusion (Brosens et al., 2011; Everett and Lees, 2012). Increased impedance in the fetal umbilical arteries becomes evident only when at least 60% of the placental vascular bed is obliterated (Chaddha et al., 2004). In pregnancies with IUGR, those with absent end-diastolic frequencies, compared to those with normal Doppler, have more fetal stem vessels with medial hyperplasia and luminal obliteration, and those with reversed end-diastolic flow have more poorly vascularized terminal villi, villous stromal haemorrhage, "haemorrhagic endovasculitis," and abnormally thin-walled fetal stem vessels (Salafia et al., 1997). Absent/reversed end diastolic flow in umbilical artery represents the extreme end of the spectrum and this finding is associated with a high perinatal mortality, particularly after 30 weeks of pregnancy (Parra-Saavedra et al., 2014).

In fetal hypoxemia there is an increase in the blood supply to the brain and reduction in the perfusion of the kidneys, gastrointestinal tract and the lower extremities (the so-called brain sparing effect; Baschat, 2004). Although knowledge of the factors governing circulatory readjustments and their mechanism of action is incomplete, it appears that partial pressures of oxygen and carbon dioxide play a role, presumably through their action on chemoreceptors. This mechanism allows preferential delivery of nutrients and oxygen to vital organs, thereby compensating for diminished placental resources (Vyas et al., 1990). On the other hand, the hemodynamic changes occurring in fetal arterial vessels during hypoxaemia and acidaemia induced by uteroplacental insufficiency are vasoconstriction, expressing as increased impedance to flow in descending aorta and renal artery (Griffin et al., 1984). Therefore, as a consequence of the brain sparing condition, there are selective modifications in cardiac afterload with a decreased left ventricle afterload due to cerebral vasodilatation, and an increased right ventricle afterload due to the systemic and pulmonary vasoconstriction (Groenenberg et al., 1989). Furthermore, hypoxemia may impair myocardial contractility while the polycythemia, which is usually present, may alter blood viscosity and therefore preload (Soothill et al., 1987). Alterations in fetal venous circulation will manifest an increase of reverse flow in the inferior vena cava during atrial contraction, and progressive fetal deterioration, suggesting a higher pressure gradient in the right atrium (Hecher and Hackelöer, 1997). The next stage of this disease is the extension of these abnormal blood velocities to the ductus venosus, rising systolic pressure and reducing diastolic filling as an expression of myocardiac dysfunction (Baschat, 2004). Finally the high venous pressure induces a reduction of velocity at the end of diastole in the umbilical vein, causing typical end diastolic pulsations (Rizzo et al., 1995). The epilogue of these clinical circumstances is cardiovascular impairment and umbilico-placental dysfunction.

CONCLUSION

The full understanding of the umbilico-placental vascular functions will have important implications in developing therapies for oxidative-stress and hypoxia complicated pregnancies. Oxidative stress in chronic hypoxic conditions during gestation arises in multiple organ systems and subcellular compartments. This occurs due to an imbalance between cellular pro-oxidant and/or anti-oxidant detoxifying mechanisms. The umbilico-placental vasculature during pregnancy seems to be particularly affected as highly sensitive territories to changes in levels of oxygen and reactive oxygen species. Although these aspects have suggested the rationale for antioxidant therapy during pregnancy, still there are no effective treatments. Several randomized controlled trials have been performed to determine whether antioxidants supplementation in complicated pregnancies are beneficial, showing no evidence that these supplements may prevent preeclampsia (Poston et al., 2006, 2011; Basaran et al., 2010; Roberts et al., 2010; Xu et al., 2010; Conde-Agudelo et al., 2011; Kalpdev et al., 2011; Rumbold et al., 2011; Polyzos et al., 2012). In marked contrast, animal models and few human experiences have shown effectiveness in the use of different antioxidants preventing pregnancy complications and short- and long-term vascular dysfunction during pathologic pregnancies (Richter et al., 2009; Tara et al., 2010; Parraguez et al., 2011; Giussani et al., 2012; Wibowo et al., 2012; Jones et al., 2013; Kane et al., 2013). Nowadays, it is not recommended the use of antioxidants in pregnancy but the debate is still open and it merits new therapeutical approaches as it is clear that oxidative stress is partly determining complications. Further research on umbilical and placental vascular function under stressed conditions is still required.

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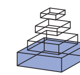

Analysis of homeobox gene action may reveal novel angiogenic pathways in normal placental vasculature and in clinical pregnancy disorders associated with abnormal placental angiogenesis

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Homeobox genes are essential for both the development of the blood and lymphatic vascular systems, as well as for their maintenance in the adult. Homeobox genes comprise an important family of transcription factors, which are characterized by a well conserved DNA binding motif; the homeodomain. The specificity of the homeodomain allows the transcription factor to bind to the promoter regions of batteries of target genes and thereby regulates their expression. Target genes identified for homeodomain proteins have been shown to control fundamental cell processes such as proliferation, differentiation, and apoptosis. We and others have reported that homeobox genes are expressed in the placental vasculature, but our knowledge of their downstream target genes is limited. This review highlights the importance of studying the cellular and molecular mechanisms by which homeobox genes and their downstream targets may regulate important vascular cellular processes such as proliferation, migration, and endothelial tube formation, which are essential for placental vasculogenesis and angiogenesis. A better understanding of the molecular targets of homeobox genes may lead to new therapies for aberrant angiogenesis associated with clinically important pregnancy pathologies, including fetal growth restriction and preeclampsia.

Keywords: homeobox gene, transcription factors, placenta, angiogenesis, endothelial cells, macrovasculature, microvasculature

INTRODUCTION

Placental angiogenesis has become a focus for the development of diagnostic tools and potential therapeutics for pregnancy complications. Strategies for pro-angiogenic therapies are grounded on our knowledge of normal placental angiogenesis and our understanding of the angiogenic pathways that are disrupted in pregnancy pathologies. However, it is clear that our comprehension of normal angiogenesis in the placenta is lacking in comparison with other tissues and organs, such as the cardiovascular system. Furthermore, unique aspects of placental angiogenesis offer the potential for identifying novel angiogenic pathways from which new pro-angiogenic factors could be identified as potential therapeutics for various obstetric complications associated with aberrant angiogenesis. This review summarizes the genetic and molecular aspects of normal placental angiogenesis with a focus on placental endothelial cells. Our laboratory has major interest in understanding the transcriptional control of placental angiogenesis, with a specific focus on a family of transcription factors called "homeobox genes" and their expression in placental endothelial cells.

Homeobox genes play an essential role in regulating the function of vascular systems (Douville and Wigle, 2007). They coordinate the processes required for proper vascular formation during development, as well as the maintenance and repair of the vasculature systems throughout life. Often, homeodomain proteins work in concert within the vascular cells to achieve proper vessel function. Homeobox genes regulate the transcription of genes necessary for many vascular cell processes such as cell migration, invasion, proliferation, and tube formation. Several new downstream targets of specific homeobox genes have been identified in vascular systems in recent years. However, there are many homeobox genes that regulate angiogenesis where we have little or no knowledge of the biological pathways they regulate and their target genes of action. This review focuses on the expression of homeobox genes in placental vascular systems and their potential role in regulating placental angiogenesis.

THE PLACENTA AND ITS VASCULATURE

An efficient and high capacity materno-fetal exchange system is crucial for the growth and development of the fetus and the

outcome of a healthy baby (Boyd and Hamilton, 1970). The placenta acts as a conduit between the maternal and fetal circulations and facilitates all gaseous and nutritive transfer between mother and fetus (Page, 1993; Moore and Persaud, 1998). This is achieved through a structural interface consisting of fetoplacental capillaries encased within terminal branches of the placental villous tree (i.e., the terminal villi), which are bathed in maternal blood perfusing into the intervillous spaces (see **Figure 1**; Gude et al., 2004). The metabolic needs of the fetus increase throughout pregnancy and the placenta adjusts to these demands through the continual development and adaptation of the placental villous vasculature thus ensuring sustained fetal growth and well-being (Chaddha et al., 2004).

In early placental development, villous vascularisation is preceded by trophoblast-mediated invasion of maternal uterine spiral arterioles, which establishes a maternal blood supply (Jaffe et al., 1997; Kingdom et al., 2000). The maternal blood filled lacunae then coalesce to create intervillous spaces, interposing pillars of trophoblasts, which gradually collapse and allow entry of blood from the uterine circulation (Lyall, 2005). The placental villous tree begins to form around day 13 post-conception, when remnants of the trophoblastic pillars proliferate into the intervillous spaces (Kingdom et al., 2000). A week later, vascularisation occurs by the *de novo* process of vasculogenesis (Risau, 1997; Kingdom et al., 2000). The villi are invaded by extraembryonic mesenchyme, which differentiates into endothelial and stromal support cells (Charnock-Jones et al., 2004). From these cells, a primitive placental vascular network is assembled and eventually connects with the embryonic circulatory system around day 32 post-conception (Kaufmann et al., 2004).

To perform the exchange functions required of it, the highly immature placental vasculature subsequently undergoes a phase of branching angiogenesis, which dramatically increases the number of villous blood vessels (Kaufmann et al., 2004). During this period, there is a corresponding rise in end-diastolic blood flow velocity, most likely reflective of a rise in fetal blood pressure (Hendricks et al., 1989). The increased villous capillary density improves fetoplacental blood flow to accommodate progressively increased fetal requirements (Ahmed and Perkins, 2000).

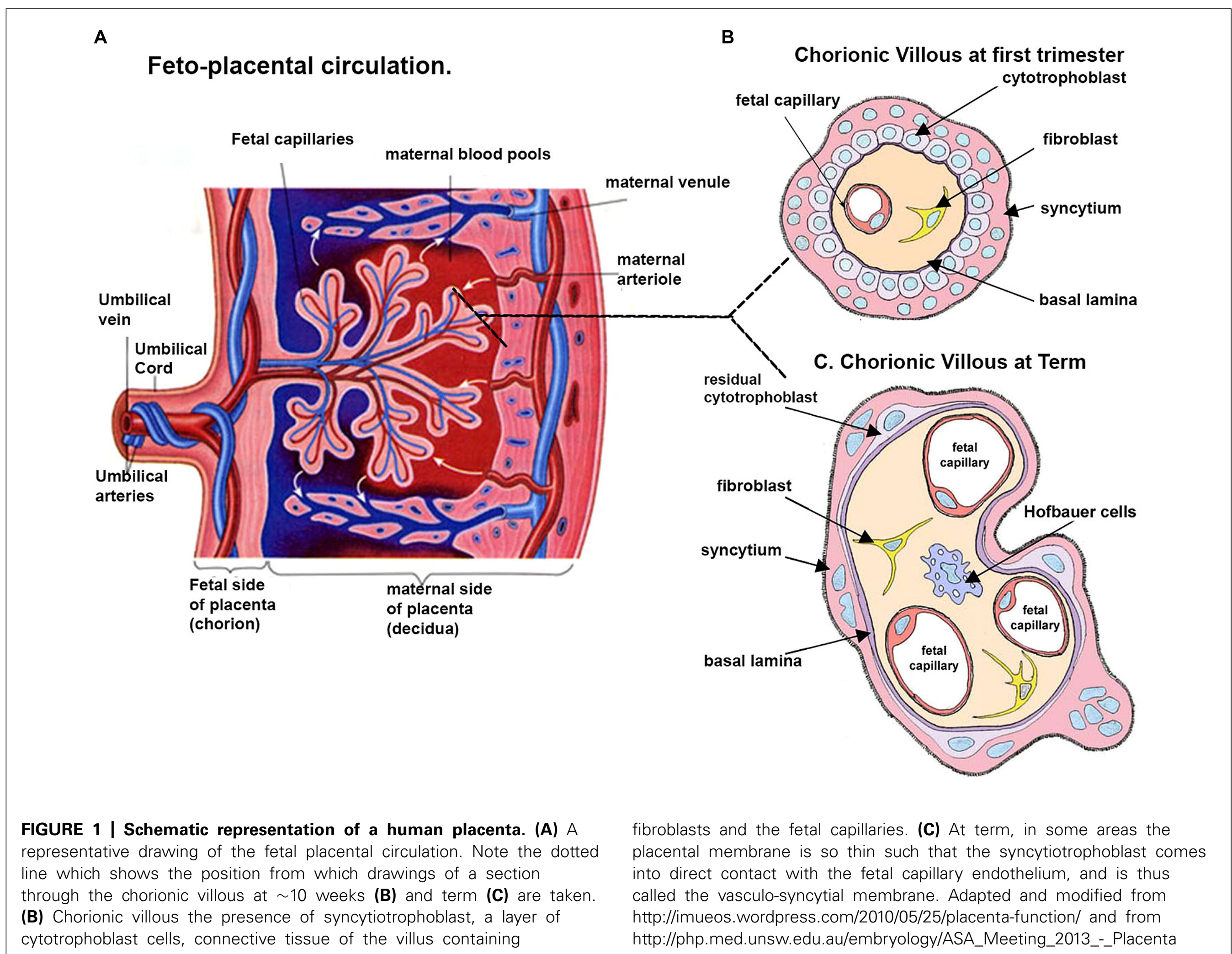

FIGURE 1 | Schematic representation of a human placenta. (A) A representative drawing of the fetal placental circulation. Note the dotted line which shows the position from which drawings of a section through the chorionic villous at ~10 weeks **(B)** and term **(C)** are taken. **(B)** Chorionic villous the presence of syncytiotrophoblast, a layer of cytotrophoblast cells, connective tissue of the villus containing fibroblasts and the fetal capillaries. **(C)** At term, in some areas the placental membrane is so thin such that the syncytiotrophoblast comes into direct contact with the fetal capillary endothelium, and is thus called the vasculo-syncytial membrane. Adapted and modified from http://imueos.wordpress.com/2010/05/25/placenta-function/ and from http://php.med.unsw.edu.au/embryology/ASA_Meeting_2013_-_Placenta

Around 26 weeks' gestation, villous vascular development enters the final phase of non-branching angiogenesis, characterized by longitudinal growth of capillaries exceeding that of the villi themselves. The capillary loops bulge into the overlying villous trophoblasts, forming structures called terminal villi (Kingdom et al., 2000). Focal sinusoids, which are unique to the placenta because they possess a continuous endothelium and complete basal lamina, may also form in the fetoplacental capillaries, causing the outer vessel wall to be separated from maternal blood only by a very thin layer of syncytiotrophoblast called the vasculo-syncytial membrane (Burton and Tham, 1992). Terminal villus formation occurs exponentially during the third trimester (Chaddha et al., 2004). The end result of terminal villus formation is a dramatic increase in the surface area to volume ratio (Charnock-Jones, 2002; Chaddha et al., 2004) and the terminal villi form the major sites for diffusional exchange between the maternal and fetal circulations (Kingdom et al., 2000; Charnock-Jones et al., 2004; Kaufmann et al., 2004).

Therefore, the adaptation of the placental vasculature to increasing fetal demands follows two main strategies. Firstly, blood flow *per se* increases by lowering vascular impedance (Kaufmann et al., 2004). Branching angiogenesis initially creates parallel vessels of reduced mean length, and hence reduced impedance (Kaufmann et al., 2004). As capillaries lengthen due to non-branching angiogenesis, the sinusoids formed in them counterbalance the effect on total fetoplacental vascular impedance (Charnock-Jones, 2002). Secondly, the rate of diffusion across the placenta is improved by an increase in available surface area, and a reduction in villous membrane thickness; the vasculo-syncytial membrane separating maternal blood from fetal blood can be as thin as 1–2 μm (Charnock-Jones, 2002). Angiogenesis and the formation of terminal villi are the main processes that culminate in remodeling the placental vascular bed (Mayhew, 2003).

IMPAIRED ANGIOGENESIS AND PREGNANCY-ASSOCIATED DISORDERS

Villous vascularisation is an important process in organogenesis and is essential for the placenta to function efficiently (Zygmunt et al., 2003). The spectrum of vascular defects associated with clinically significant pregnancy disorders attests to the close relationship between the placental vasculature and embryonic development. Compared with villi obtained from elective terminations, villi from placentae where intrauterine embryonic death and blighted ova was the outcome exhibit aberrant vascular characteristics manifest in significantly lower vascular density, fibrosis, and hydropic degeneration (Meegdes et al., 1988). Placentae from women with diabetes mellitus and gestational diabetes also show villous vascular maldevelopment and studies using light microscopy, electron microscopy and histochemical techniques have shown the length, diameter and surface area of fetoplacental capillaries to be increased (Jacomo et al., 1976; Jones and Fox, 1976; Mayhew et al., 1994). As well, some of the capillaries appear unduly immature (Kami and Mitsui, 1984).

Perhaps the most dramatic, best-characterized changes in the villous vasculature are seen in fetal growth restriction (FGR), which is a common and clinically significant disorder of pregnancy. FGR is defined as failure of the fetus to achieve genetically determined potential size to an extent where its health is adversely affected (Lin and Santolaya-Forgas, 1998). FGR affects 4–7% of live births in developed countries and contributes significantly to prematurity, perinatal morbidity, and mortality (Wang et al., 2007). Investigations using random block sampling and stereological studies reported reductions in the number, surface area, and volume of terminal villi in FGR-affected placentae, compared with placentae from uncomplicated pregnancies (Biagiotti et al., 1999; Egbor et al., 2006; Biswas et al., 2008; Vedmedovska et al., 2011; Almasry et al., 2012; Almasry and Elfayomy, 2012). Additionally, villous vessels exhibited fewer branches, and a majority of the vessels were slender and uncoiled (Teasdale, 1984; Teasdale and Jean-Jacques, 1988; Jackson et al., 1995; Chen et al., 2002; Mayhew, 2003; Tomas et al., 2010). A failure, or reduced capability, of branching angiogenesis in FGR is strongly associated (Kingdom et al., 2000) with a reduced supply of oxygen and nutrients to the fetus, and subsequent growth delay (Sanchez-Vera et al., 2005; Salafia et al., 2006).

Despite extensive research, it is unknown whether vascular changes cause FGR or whether these changes are a consequence of aberrant biological mechanisms in the FGR-affected placenta (Maulik, 2006; Maulik et al., 2006). Clearly, further research into the molecular regulation of angiogenesis in the placenta is vital.

MOLECULAR REGULATION OF ANGIOGENESIS

Angiogenesis involves distinct changes in the phenotype of endothelial cells, the central cellular organizational units of vascular structures. **Figure 2** shows the two distinct processes of vasculogenesis and angiogenesis involved in fetoplacental vascular development in human pregnancy.

In a multi-step event, quiescent endothelial cells are first activated to re-enter the cell cycle (Myers et al., 2002). As a consequence of increased cellular proliferation, proteolytic enzyme production is up-regulated in order to degrade the basement membrane. The endothelial cells then migrate into the surrounding stroma and gradually assemble into a tube-like capillary structure with a patent lumen. After a new basement membrane is synthesized, pericytes are recruited to the outside of the new capillary to complete the formation of a stable, quiescent vessel (Sato, 2000).

The stimuli for these complex, temporally coordinated changes are communicated from the microenvironment surrounding the endothelial cell surface to the nucleus through multiple signaling pathways (Patel et al., 2005). At the molecular level, the growth factors and receptors that activate these pathways have been extensively studied *in vitro and in vivo* (Arderiu et al., 2007; Winnik et al., 2009). Vascular endothelial growth factor (VEGF), placental growth factor (PlGF), and the angiopoietins are considered the most influential factors (Patel et al., 2005). The primary receptors for VEGF are VEGF receptor-1 (VEGFR-1) and VEGF receptor-2 (VEGFR-2), while PlGF only binds to VEGFR-1 (Patel et al., 2005). VEGF has been demonstrated to be a potent stimulator of endothelial cell proliferation, migration, and production of plasminogen activators required for basement membrane digestion (Regnault

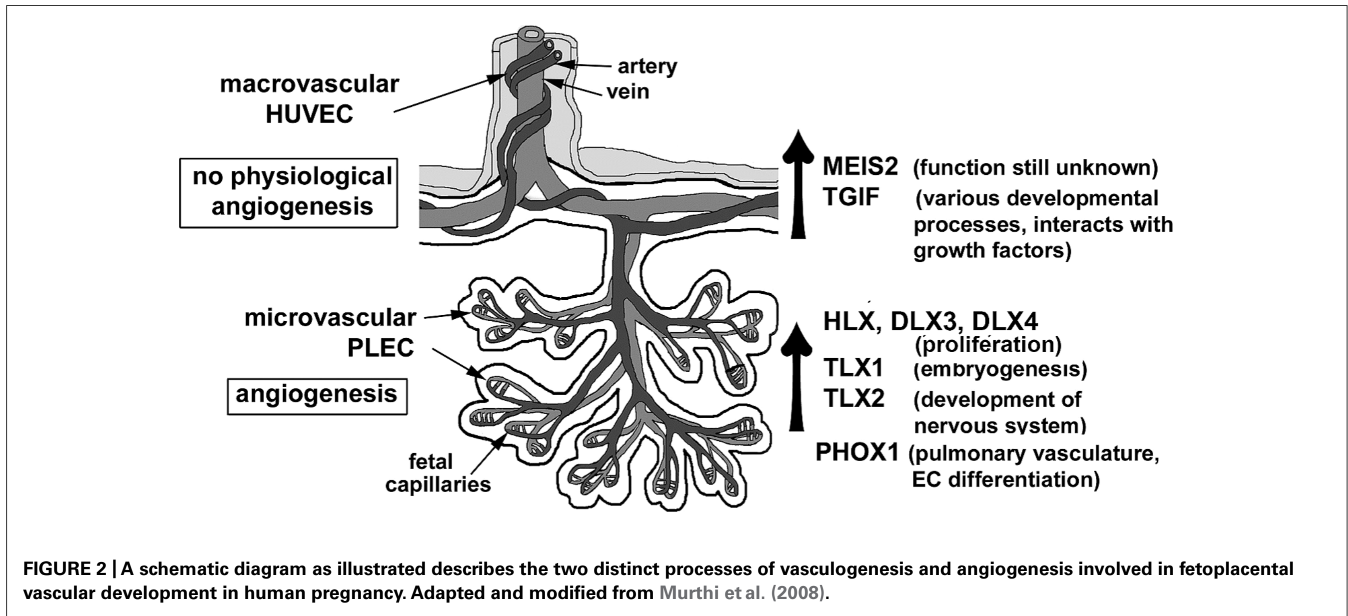

FIGURE 2 | A schematic diagram as illustrated describes the two distinct processes of vasculogenesis and angiogenesis involved in fetoplacental vascular development in human pregnancy. Adapted and modified from Murthi et al. (2008).

et al., 2003; Escudero et al., 2014). Studies of chicken chorioallantoic membranes have shown that VEGF binding to both VEGFR-1 and VEGFR-2 results in branching angiogenesis, while PlGF binding to VEGFR-1 alone mediates non-branching angiogenesis (Wilting et al., 1996). The angiopoietin family comprises two main factors, angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) and are both antagonistic ligands of a common receptor, the tyrosine-kinase with immunoglobulin-like and epidermal growth factor-like domains-2 (TIE-2). While Ang-1 binding to TIE-2 promotes vascular stabilization, Ang-2 allows vessels to remain in a more plastic state (Kurz et al., 1998).

In normal pregnancies, placental expression of important growth factors correlates with their established roles. For example, expression of VEGF and VEGFR-2 is highest during early gestation, which coincides with vasculogenesis and branching angiogenesis, but expression declines with advancing pregnancy (Jackson et al., 1994). Conversely, PlGF and VEGFR-1 expression is highest toward term, coinciding with non-branching angiogenesis (Clark et al., 1996). A concurrent decrease in Ang-2 expression, and increase in Ang-1 expression at the end of the second trimester, is believed to mediate the transition from branching to non-branching angiogenesis (Geva et al., 2002).

Our knowledge of the molecular regulation of angiogenesis in the placenta is incomplete. Nuclear transcription factors integrate upstream signals generated by the binding of growth factors to their receptors. Transcription factor binding to specific DNA sequences within the promoter regions stimulates or represses expression of batteries of downstream target genes (Hamik et al., 2006). Transcription factors are considered to be the master regulators that determine gene expression profiles that culminate in the activated, angiogenic phenotype. Loss of function studies clearly demonstrate that transcription factors including *TBX4, CDX2, CDX4, HAND1, FOXF1, CITED2* are required for placental development (Mahlapuu et al., 2001; Cross et al., 2002; Naiche and Papaioannou, 2003; Cross, 2006; Preis et al., 2006; van Nes et al., 2006). However, the target genes regulated by these transcription factors are largely undefined. Recent studies have provided evidence for transcriptional control of VEGF signaling by Notch ligand as well as hypoxia-inducible factor (*HIF1a*) in placental angiogenesis (Fang et al., 2013). Morphological and phenotypic analyses of the human placenta using whole mount immunofluorescence technique were employed to demonstrate that early human placental blood vessels express high levels of the pro-angiogenic receptors VEGFR1, VEGFR3 and the activated signal transduction and activator of transcription 3 (pSTAT3) suggesting that these molecules play a role in regulation of placental vascular development (Bushway et al., 2014). Thus, an understanding of transcriptional mechanisms would afford a valuable insight into the downstream angiogenic signaling cascades in the placenta, which as yet, remain largely unexplored in the human placenta.

HOMEOBOX GENES

A particular large family of transcription factors that provides a fertile area for studying placental angiogenesis is the homeobox gene family. Characterized by a common 60-amino-acid DNA-binding motif known as the homeodomain, and homeobox genes were first identified in *Drosophila* through investigations of mutations that gave rise to homeotic transformations (McGinnis and Krumlauf, 1992). Subsequently, it was discovered that three-dimensional patterning and body plan formation during embryogenesis are largely attributable to action of homeobox genes, due to their capacity to spatiotemporally regulate the basic processes of differentiation, proliferation, and migration (Manley and Levine, 1985; Han et al., 1989). Homeobox genes can regulate genes responsible for cell adhesion, migration, proliferation, growth arrest, and the expression of cytokines needed for extracellular matrix interactions (Graba et al., 1997; Svingen and Tonissen, 2006; Hueber et al., 2007) all of which are functions characteristic of the angiogenic phenotype.

HOMEOBOX GENES IN ANGIOGENESIS

Evidence in the literature increasingly supports a substantial role for homeobox genes in general vascular development, and particularly in endothelial cell function (Gorski and Walsh, 2000, 2003; Gorski and Leal, 2003; Douville and Wigle, 2007). A well-known example is *Gax*, a homeobox gene originally isolated from a rat aortic cDNA library, which is widely expressed in embryonic muscle precursors (Gorski et al., 1993). Initial investigations of the human homolog, *Gax,* were conducted primarily on vascular smooth muscle cells, where *Gax* was shown to induce G_1 cell cycle arrest and reduce cell migration (Witzenbichler et al., 1999). Gorski and Leal (2003) subsequently confirmed *GAX* expression in endothelial cells using immunohistochemical methods on sections of highly capillarised human kidney. Succeeding *in vitro* investigations revealed that *GAX* prevented VEGF-induced endothelial cell migration and tube formation through the repression of multiple genes involved in the pro-angiogenic nuclear factor kappa-beta (NFκ-B) signaling pathway (Patel et al., 2005). Hence, *GAX* emerged as an important inhibitor of the angiogenic phenotype. Other less well studied homeobox genes are implicated as positive regulators of angiogenesis. For example, human *HOXA9* promotes endothelial cell migration, in part by activating the expression of EphB4; a receptor tyrosine-kinase that shows increased expression in tumor-induced vascularisation (Bruhl et al., 2004).

HoxA9$^{-/-}$ mouse embryos display a poorer angiogenic response to hypoxia and have decreased numbers of endothelial cell precursors (Rossig et al., 2005). Complementary pro-angiogenic functions have also been described for the paralogous homeobox genes *HoxD3* and *HoxB3*. *HOXD3* not only promotes endothelial cell invasion of the extracellular matrix early in angiogenesis, but also regulates the subsequent capillary morphogenesis of these new vascular sprouts (Douville and Wigle, 2007). Although these findings generally emphasize the multi-faceted importance of homeobox genes in angiogenesis, the studies were conducted within the context of embryonic development and/or tumor-induced adult neovascularisation.

Studies by Shaut et al. (2008) have reported that *HoxA13* is essential for placental vascular patterning and labyrinth endothelial specification. In the absence of *HoxA13* function, placental endothelial morphology is altered causing a loss in vessel wall integrity, edema of the embryonic blood vessels and mid-gestational lethality. The authors have also reported on the novel transcriptional program by which *HoxA13* directly regulates *Tie2* and *Foxf1* in the placental labyrinth endothelia, providing a functional explanation for the mid-gestational lethality exhibited by *HoxA13* mutant embryos. However, homeobox gene contribution(s) to extraembryonic angiogenesis, particularly in the human placenta, remains largely unexplored.

HOMEOBOX GENES IN THE PLACENTA

Currently, information about the role of homeobox genes in placental tissues is mainly derived from studying mouse gene knockouts (Rossant and Cross, 2001). For example, targeted deletion of *Esx1* (Fohn and Behringer, 2001) and *Dlx3* (Morasso et al., 1999) resulted in disruption of the vascular network in the placental labyrinthine layer, which in mice is thought to be functional equivalent of the human placental villi (Cross et al., 2003a,b). Not only were embryos in both cases growth-restricted, but failure to establish an adequate placental circulation in *Dlx3*$^{-/-}$ mutants resulted in embryonic lethality (Morasso et al., 1999). Together, these studies provide genetic proof that homeobox genes are not only regulators of placental organogenesis but they are also specific regulators of placental vascular development. Furthermore, homeobox genes can directly or indirectly influence fetal viability. We carried out the first screening of a 32-week placental cDNA library for homeobox genes, which led to the isolation of *DLX4, MSX2, GAX,* and *HLX* (Quinn et al., 1997; Rajaraman et al., 2008). Immunohistochemical analyses identified the localisation of these homeobox genes in both trophoblasts and endothelial cells of the human placenta (Murthi et al., 2006a; Rajaraman et al., 2008; Chui et al., 2010).

HOMEOBOX GENE EXPRESSION IS ALTERED IN HUMAN FETAL GROWTH RESTRICTION CHARACTERIZED BY IMPAIRED PLACENTAL ANGIOGENESIS

Using a clinically well-defined cohort of idiopathic FGR ($n = 25$) and gestation-matched control ($n = 25$) pregnancies, we reported an overall decrease in homeobox gene *HLX* and *ESX1L* expression in all cell types, including endothelial cells, in FGR-affected placentae compared with GMC (Murthi et al., 2006a,b). Subsequently, we also reported that homeobox genes *DLX4* and *DLX3* showed increased expression in FGR-affected placentae (Murthi et al., 2006c; Chui et al., 2012), whereas *GAX* and *MSX2* showed no significant difference. Our studies represented the most comprehensive and extensive analyses of homeobox genes in placental pathologies undertaken. *In situ* mRNA hybridisation and immunohistochemical studies on placental sections localized the expression of these genes not only to placental trophoblasts but also to endothelial cells that comprise the fetal capillaries (Quinn et al., 1998a,b, 2000).

HOMEOBOX GENE EXPRESSION IN PLACENTAL ENDOTHELIAL CELLS

At least two functionally distinct endothelial cell types, macrovascular and microvascular exist within the human placenta (Lang et al., 1993; Ugele and Lange, 2001). Macrovascular endothelial cells [human umbilical vein endothelial cell (HUVEC)] line the large conduit vessels of the umbilical cord and isolated cells from the vein have been used extensively to model vasculogenic and angiogenic processes occurring in tissues such as the placenta (Demir et al., 1989; Wang et al., 2004). Microvascular endothelial cells vascularise the cotyledons of the placenta. It is important to study the microvascular environment of the placenta because in placental disorders such as FGR and PE, structural and vascular changes occur within the microvasculature of the terminal villi that impact on maternal-fetal gas and nutrient exchange (Demir et al., 1989; Kingdom et al., 2000; Dye et al., 2004; Wang et al., 2004). Lang et al. (2003) have reported that distinct morphogenetic, antigenic, and functional characteristics exist between microvascular and macrovascular endothelial cells of the human placenta and demonstrated differences in the secretion of vasoactive substances

and the proliferative response to cytokines between microvascular and macrovascular endothelial cells of the human placenta. The different reactions of microvascular and macrovascular endothelial cells to various stimuli (Lang et al., 2003) are likely to reflect differences in the activation of transcription factors that mediate signal transduction mechanisms in the two cell types.

In addition, several studies have found various morphological, antigenic, growth, and functional differences between the two endothelial cell types in association with pathological conditions (Thorin and Shreeve, 1998; Charnock-Jones et al., 2004; Pollheimer and Knofler, 2005). Therefore, it was concluded that isolated microvascular endothelial cells from the chorionic villi have advantages as a model to study placental vascular development over macrovascular HUVEC.

To isolate and enrich placental microvascular endothelial cells (PLEC), we used a modified methodology based on the perfusion-based technique described by Lang et al. (2003). After cannulation of the chorionic vessels and removal of fetal blood, Lang et al. (2003) introduced proteolytic enzymes into the perfused cotyledon in a specific volume of buffer that was perfused into the placenta at a variable flow rate by employing a gravity feed system. In contrast, our modified methodology involved pumping the enzymes into the vasculature at a constant flow rate for a variable length of time and until no further venous outflow was obtained. This modified technique achieved a controlled delivery of the enzymes and an enriched population of PLEC (Murthi et al., 2007).

Freshly isolated PLEC were used to identify the homeobox genes expressed in the placental microvasculature, and expression of homeobox genes was compared with that of macrovascular HUVEC. Conventional reverse transcriptase polymerase chain reaction (PCR) was used to detect mRNA levels of homeobox genes *DLX3, DLX4, MSX2, GAX,* and *HLX* (formerly known as *HLX1* or *HB24*) in both PLEC and HUVEC. Our study was the first to show *DLX3, DLX4,* and *MSX2* are expressed in macrovascular HUVEC. We also reported that the mRNA levels of *HLX* mRNA in HUVEC were significantly lower compared with PLEC (Murthi et al., 2007). These data provided further evidence of heterogeneity in homeobox gene expression between microvascular PLEC and macrovascular HUVEC, which most likely reflects significant differences in endothelial cell function in the two different cellular environments.

HLX is important in the proliferation and lineage commitment of haematopoietic cells (Deguchi et al., 1992). In the human placenta, *HLX* mRNA expression is restricted to proliferating cell types such as villous cytotrophoblast and extravillous cytotrophoblast cells in the proximal regions of the invading cell columns (Rajaraman et al., 2008). In our study, we showed *HLX* mRNA expression in placental endothelial cells (Murthi et al., 2007), which are also proliferative cell types. Microvascular endothelial cells of the placenta, particularly within the terminal and intermediate villi of term placentae, have a higher level of proliferative activity in comparison with their macrovascular counterparts (Murthi et al., 2007). Moreover, in response to PlGF, PLEC have a significantly greater proliferative activity compared with HUVEC (Lang et al., 2003). *HLX* levels in PLEC are relatively higher than in HUVEC, and PLEC have greater proliferative potential. Taken together, these data suggests a possible role for *HLX* in the proliferative capacity of microvascular endothelial cells. The transcriptional regulation of proliferation, migration, and invasion of PLEC by homeobox genes *DLX3, DLX4, MSX2, GAX,* and *HLX* is yet to be explored.

The reported the co-expression of *HLX, MSX2, GAX,* and *DLX4* in PLEC (Murthi et al., 2007) and this specific combination of homeobox genes may be important in mammals. For example, *Hlx* is co-expressed with members of the *Msx, Gax (also known as Mox)* and *Dlx* families in the mouse embryo. *HLX* is expressed with *MSX2, GAX* (also called *MOX2*) and *DLX4* in the trophoblast cell layers of the human placenta (Quinn et al., 1998b). Quinn et al. (2000) predicted that the combination of homeobox genes could play a significant role in the regulation of epithelial–mesenchymal cell interactions in the extraembryonic tissues. Therefore, co-expression of the homeobox genes in both trophoblast and endothelium may be important in the coordination of villous outgrowth and angiogenesis that is seen in the terminal villi and is essential for the efficient functioning of the placenta as it grows. Thus, our study on the homeobox gene expression profiling in placental endothelial cells (Murthi et al., 2007) further emphasized the importance of studying microvascular endothelial cells (i.e., PLEC) as a model for the placental microvascular bed, or other microcirculation systems.

IDENTIFICATION OF NOVEL HOMEOBOX GENES IN PLACENTAL ENDOTHELIAL CELLS

To further expand our knowledge of the repertoire of homeobox genes expressed in placental endothelial cells, in a subsequent study by Murthi et al. (2008) we carried out microarray expression profiling on endothelial cells and analyzed public microarray expression profile databases. We have employed PCR and real-time PCR methods to corroborate the microarray data and to compare relative expression levels of homeobox genes in PLECs and HUVEC.

Microarray expression data as reported in Murthi et al. (2008) suggested that novel homeobox genes are expressed in microvascular placental endothelial cells. These homeobox genes, *HEX, PHOX1, LIM6, HOX*B7 and *TGIF*, have not been previously detected in the placenta and were selected because they exhibited the greatest relative expression in the microarray data (Murthi et al., 2008). Novel homeobox genes *TLX1* and *TLX2* homeobox gene expression data was obtained from the GNF Microarray Analysis Data for the Human U95A microarray, Version 2 dataset (http://expression.gnf.org; Su et al., 2002). Homeobox genes *LIM6, HOXB7, TGIF, PHOX1,* and *HEX* were expressed in the endothelial cells of the placenta.

Expression of these homeobox genes in the placenta or in placental endothelial cells has not been previously reported. *HEX* (Nakagawa et al., 2003), *PHOX1/Prx1* (Ihida-Stansbury et al., 2004), and *HOXB7* (Care et al., 2001) homeobox genes have been previously described in endothelial cells from various sources but have not been described in any endothelial cell type and may represent novel endothelial regulatory genes. Validation of high-throughput gene microarray screening data of potentially novel homeobox gene expression in endothelial cells is essential. In our study (Murthi et al., 2008), the

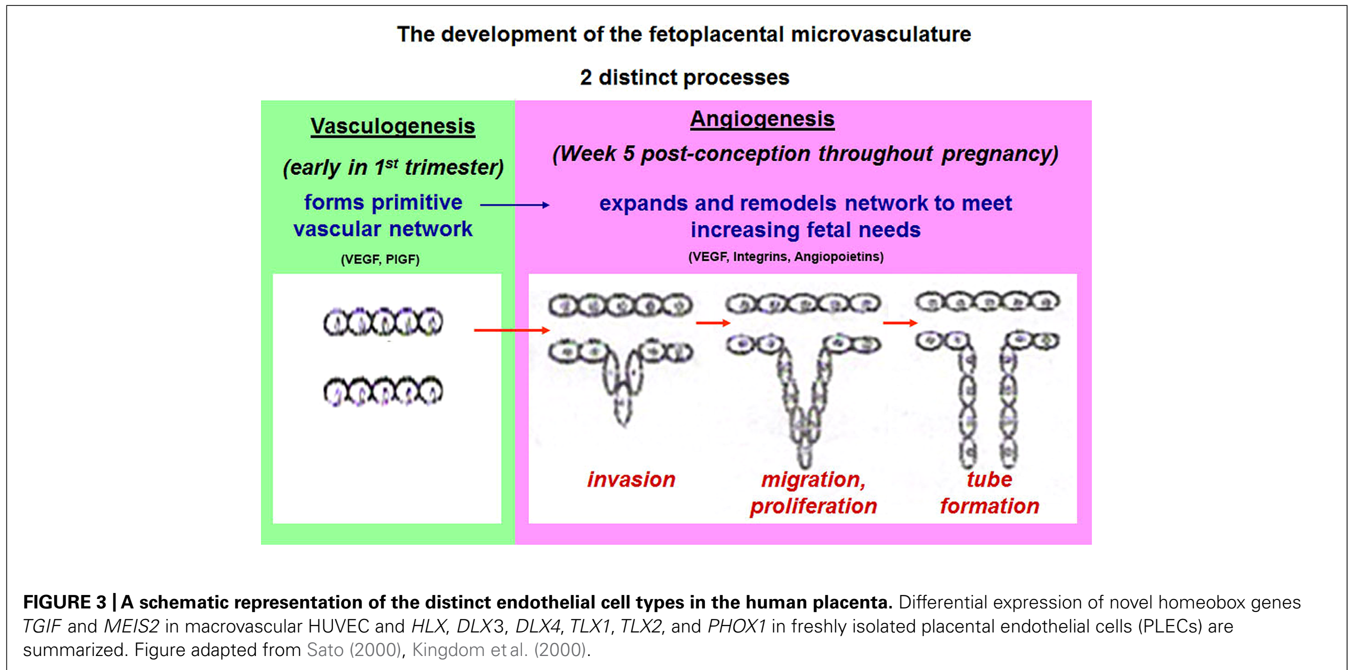

FIGURE 3 | A schematic representation of the distinct endothelial cell types in the human placenta. Differential expression of novel homeobox genes *TGIF* and *MEIS2* in macrovascular HUVEC and *HLX*, *DLX*3, *DLX4*, *TLX1*, *TLX2*, and *PHOX1* in freshly isolated placental endothelial cells (PLECs) are summarized. Figure adapted from Sato (2000), Kingdom et al. (2000).

microarray data were further corroborated by independent methods such as RT-PCR and real-time PCR. Thus, our study was the first to demonstrate that the novel homeobox genes *TLX1, TLX2, PHOX1, MEIS2,* and *TGIF* are expressed in PLEC. In addition, in the same study, we have also reported a differential expression of *TLX1, TLX2, PHOX1, MEIS2,* and *TGIF* mRNA levels in macrovascular and microvascular endothelial cells.

Thus, we have identified novel homeobox genes in microvascular endothelial cells, and consistent with our previous studies reported in Murthi et al. (2007), we have shown that homeobox genes are differentially expressed between micro- and

Table 1 | Examples of target genes downstream of homeobox genes required for the regulation of endothelial functions.

Homeobox genes	Target genes	Regulation	EC function	Reference
Pro-ang0iogenic				
HOXA3	uPAR	+	Migration	Mace et al. (2005)
HOXA9	MMP-14	+	MigrationProliferationActivation	Bruhl et al. (2004)
	EphB4	+		Rossig et al. (2005)
	eNOS	+		
	VEGFR2	+		
HOXB5	VEGFR2	+	Activation	Wu et al. (2003)
HOXB3	Ephrin A1	+	Vessel formation	Myers et al. (2000)
HOXD3	Collagen A1	+	Adhesion and migration	Boudreau and Varner (2004)
MEOX2	MLLT7	–	Apoptosis	Wu et al. (2005)
PROX1	Cyclin E1	+	Proliferation	Petrova et al. (2002)
Anti-angiogenic				
HHEX	VEGFR2	–	Activation	Nakagawa et al. (2003)
HOXD10	FGF2	+	Recruitment	Chen et al. (2009)
HOXA5	VEGFR2	+	Adhesion	Arderiu et al. (2007)
	Ephrin A1			
	HIF1a			
MEOX2	P21	+	Cell cycle arrest	Gorski and Leal (2003)

macrovascular endothelial cells. Our studies also provided further evidence of heterogeneity in homeobox gene expression between PLEC and HUVEC, which reflects the significant differences in endothelial cell function in the two different cellular environments.

In summary, our studies have reported homeobox genes that are novel not only in placental microvascular endothelial cells but also in the macrovascular endothelial cells of the placenta (Murthi et al., 2008). **Figure 3** summarizes the association between the detection of homeobox gene expression and the regions of angiogenic potential in the human placenta. In the microvasculature, where angiogenesis is predominant, we have identified increased expression of homeobox genes *TLX1*, *TLX2*, and *PHOX1*. In the macrovasculature, where there is limited angiogenesis, the level of *TGIF* and *MEIS2* are significantly increased suggesting that the heterogeneity in homeobox gene expression between PLEC and HUVEC that could reflect differences in the angiogenic potential in the two different endothelial environments. Functional studies in cultured endothelial cells are underway in our laboratory to determine the role of novel homeobox genes.

DOWNSTREAM TARGETS OF HOMEOBOX GENES

Homeobox genes control transcription by binding to regulatory elements in the promoter regions of target genes. Miano et al. (1996) first reported the expression of several homeobox genes in the cardiovascular and lymphatic vasculature. More recently, several homeobox genes were shown to affect processes in embryonic and adult tissues, including angiogenesis and wound healing (Kachgal et al., 2012). Homeobox genes activate either growth or migration of vascular cells to promote angiogenesis or wound healing or restore and maintain quiescent differentiated tissue function by modulating the expression of pro-angiogenic or anti-angiogenic factors. **Table 1** provides examples of downstream target genes of homeobox genes that are required for the regulation of endothelial function in general.

Studies using umbilical or uterine artery Doppler for identifying FGR, in the absence of maternal hypertensive disease, show that maternal serum sFLT-1 is increased in these pregnancies compared with pregnancies of normotensive women delivering average for gestational age infants (Crispi et al., 2006; Stepan et al., 2007; Wallner et al., 2007; Chaiworapongsa et al., 2008, 2013). More recent studies by Borras et al. (2014) have reported that maternal plasma free VEGF (f-VEGF) and s-Flt-1 were significantly higher in FGR compared with controls and the f-VEGF/sFlt-1 quotient was significantly lower in the FGR group compared with controls. Although the VEGF family has important roles in normal and complicated pregnancies, the current predictive value of the VEGF family as biomarkers appears to be limited to early onset preeclampsia (Andraweera et al., 2012).

Studies from our laboratory, using a real-time PCR-based gene profiling, recently identified candidate target genes of homeobox gene *DLX3* as regulators of trophoblast differentiation; *GATA2* and *PPARγ* (Chui et al., 2013). The expression of *GATA2* and *PPARγ* were further assessed in placental tissues and showed increased expression in FGR-affected tissues compared with gestation-matched controls. Our studies showed that *DLX3*

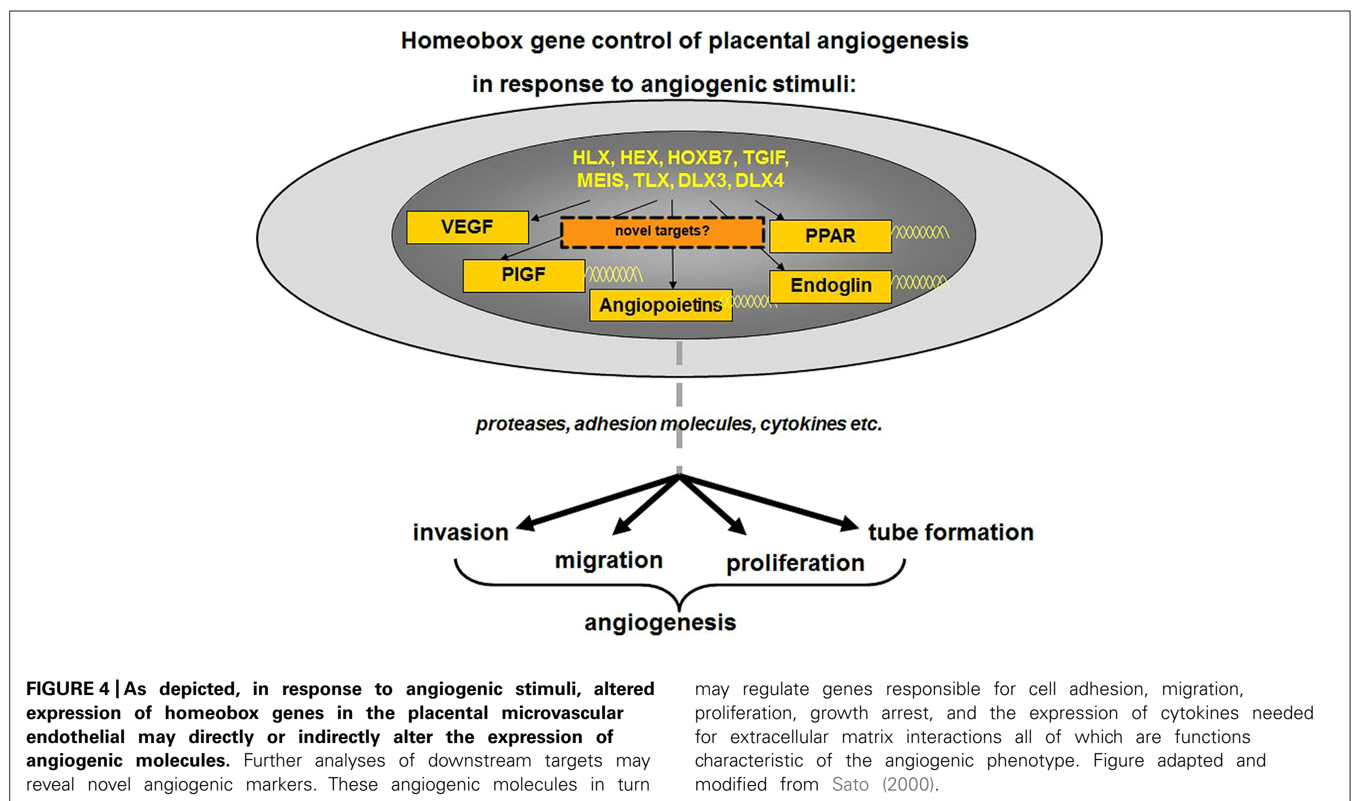

FIGURE 4 | As depicted, in response to angiogenic stimuli, altered expression of homeobox genes in the placental microvascular endothelial may directly or indirectly alter the expression of angiogenic molecules. Further analyses of downstream targets may reveal novel angiogenic markers. These angiogenic molecules in turn may regulate genes responsible for cell adhesion, migration, proliferation, growth arrest, and the expression of cytokines needed for extracellular matrix interactions all of which are functions characteristic of the angiogenic phenotype. Figure adapted and modified from Sato (2000).

orchestrates the expression of multiple regulators of trophoblast differentiation and that expression of these regulatory genes is abnormal in FGR.

Xin et al. (1999) have shown that *PPARγ* ligands suppress *VEGFR1* and *VEGFR2* expression in HUVECs. Anti-angiogenic actions of 15-lipoxygenase on angiogenesis is regulated by *PPARγ* and *VEGF* by inhibiting the expression of *VEGFR2* in endothelial cells (Mochizuki and Kwon, 2008; Viita et al., 2008). Because the chemical *PPARγ* ligands thiazolidinediones have been used widely for the treatment of type 2 diabetic patients, many of whom experience vascular diseases, clarifying the precise role of *PPARγ* in defective placental angiogenesis may be of clinical significance.

Current studies in our laboratory are also focused on identifying target genes of homeobox genes *TGIF, MEIS, HOXB7,* and *HHEX* in human placental endothelial cells, which may reveal molecular pathways responsible for fundamental cellular functions such as endothelial cell migration, invasion, proliferation, and tube formation that are important for placental angiogenesis. As depicted in **Figure 4**, in response to angiogenic stimuli or insult, as in the case of FGR or PE, altered expression of homeobox genes in the placental microvascular endothelial may directly or indirectly alter the expression of angiogenic molecules. These angiogenic molecules in turn may regulate genes responsible for cell adhesion, migration, proliferation, growth arrest, and the expression of cytokines needed for extracellular matrix interactions all of which are functions characteristic of the angiogenic phenotype.

CONCLUSION

Clearly, identifying target genes regulated by homeobox genes in placental microvascular endothelial cells will reveal the biological pathways regulated by homeobox genes. These pathways will provide important information on the function of homeobox genes in placental angiogenesis. Although homeobox gene nuclear transcription factors are unlikely to be ideal disease biomarkers or therapeutic targets, their target genes, if secreted, may provide viable biomarkers or diagnostic markers. A better understanding of cellular and molecular mechanisms that regulate homeobox genes in placental endothelial cells may lead to new approaches for correcting aberrant angiogenesis observed in pregnancy pathologies, including FGR and preeclampsia.

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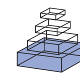

The therapeutic potential of antioxidants, ER chaperones, NO and H_2S donors, and statins for treatment of preeclampsia

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Preeclampsia is a complex multifactorial disease. Placental oxidative stress, a result of deficient spiral artery remodeling, plays an important role in the pathophysiology of preeclampsia. Antiangiogenic factors secreted from malperfused placenta are instrumental in mediating maternal endothelial dysfunction and consequent symptoms of preeclampsia; the mechanism is likely to involve increased ET-1 secretion and reduced NO bioavailability. Therapeutic interventions so far remain only experimental and there is no established remedy for the treatment of preeclampsia. This review concentrates on the evidence for the therapeutic potential of antioxidants, ER chaperones, NO and H_2S donors, and statins. These compounds display pleitropic antioxidant, anti-inflammatory, and pro-angiogenic effects in animal and *in vitro* studies. Although clinical trials on the use of antioxidant vitamins in pregnancy proved largely unsuccessful, the scope for their use still exists given the beneficial cardioprotective effects of antioxidant-rich Mediterranean diet, periconceptual vitamin use and the synergistic effect of vitamin C and L-arginine. Encouraging clinical evidence exists for the use of NO donors, and a clinical trial is underway testing the effect of statins in treatment of preeclampsia. H_2S recently emerged as a novel therapeutic agent for cardiovascular disease, and its beneficial effects were also tested in animal models of preeclampsia. It is risky to prescribe any medication to pregnant women on a large scale, and any future therapeutic intervention has to be well tested and safe. Many of the compounds discussed could be potential candidates.

Keywords: preeclampsia, angiogenesis, antioxidants, ER chaperones, NO, H_2S, statins

THE SYNDROME OF PREECLAMPSIA

Preeclampsia affects 3–5% of all pregnancies. The traditional definition of preeclampsia as *de novo* onset of hypertension and proteinuria after 20 weeks of gestation has been recently modified by the American College of Obstetricians and Gynecologists in recognition of the syndromic nature of preeclampsia. In the absence of proteinuria, preeclampsia is diagnosed as hypertension in association with thrombocytopenia, impaired liver function, new development of renal insufficiency, pulmonary edema, or new-onset cerebral or visual disturbances (American College of Obstetricians Gynecologists [ACOG], 2013). Hypertension, proteinuria, edema, and other systemic manifestations of the syndrome of preeclampsia are the direct consequences of maternal endothelial dysfunction. The syndrome is a major cause of maternal and fetal morbidity and mortality, and results in mild to severe microangiopathy of target organs, including the brain, liver, kidney, and placenta (Sibai et al., 2005). Severe maternal complications include seizures (eclampsia), stroke, renal failure, liver failure, and/or rupture, HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets), and death (Young et al., 2010). Fetal complications include prematurity due to preterm delivery, fetal growth restriction, fetal/neonatal hypoxic neurological injury, perinatal death, and long-term cardiovascular morbidity associated with low birth weight (Sibai et al., 2005).

Preeclampsia (PE) is a complex multifactorial disease; many factors, including genetic predisposition, immunological interactions, maternal endothelial function and environmental factors interact and culminate in the disease manifestation. In recent

Abbreviations: ADMA, asymmetric dimethylarginine; AKT, serine/threonine-specific protein kinase; ALK1 and ALK5, TGF-β receptors type I; ARE, antioxidant responsive element; ATF6, activating transcription factor 6; CBS, cystathionine β-synthase; CO, carbon monoxide; CSE, cystathionine γ-lyase; CTT, cholesterol treatment trialists; CV, cardiovascular; DAMPs, damage-associated molecular pattern; EDF, end diastolic flow; eIF2α, eukaryotic initiation factor 2α; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; ET-1, endothelin-1; GPx, glutathione peroxidase; GRP78, 78 kDa glucose-regulated protein, also known as binding immunoglobulin protein (BiP) or heat shock 70 kDa protein 5 (HSPA5); GSS, glutathione S-transferase; H_2S, hydrogen sulfide; HELLP syndrome, hemolysis, elevated liver enzymes and low platelets; HO-1, heme oxygenase; HIF-1α, hypoxia-induced factor; HR, hypoxia-reoxygenation; ICAM, intercellular adhesion molecule; I/R, ischemia-reperfusion; IRE1, inositol-requiring enzyme 1; IUGR, intrauterine growth restriction; LDL, low density lipoprotein; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; Nrf2, nuclear factor E2-related factor-2; p38 MAPK, p38 mitogen-activated protein kinase; PE, preeclampsia; PERK, PRKR-like ER kinase; PlGF, placental growth factor; ROS, reactive oxygen species; RUPP, reduced uterine perfusion pressure; sEng, soluble TGF-β co-receptor endoglin; sFlt1, soluble VEGF receptor 1, also referred to as soluble fms-like tyrosine kinase; SGA, small for gestational age; SOD, superoxide dismutase; StAmP trial, Statins to ameliorate early onset preeclampsia; TLR, Toll-like receptors; TUDCA, Tauroursodeoxycholic acid, bile salt; UDCA, ursodeoxycholic acid, bile salt; UPR, Unfolded Protein Response; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor; XBP-1, X-box binding protein-1.

years, PE is commonly subdivided into early and late-onset, as the two conditions seem to have different underlying etiologies. Both early and late onset diseases present with abnormal placental perfusion and oxidative stress, however, poor placentation and deficient conversion of maternal spiral arteries only underlie the pathology of the early onset disease, whilst the late onset condition is associated with normal uterine spiral artery conversion and normally grown baby (Huppertz, 2008). Redman recently proposed a novel hypothesis for the pathology of late-onset PE, suggesting an intrinsic cause due to microvillous overcrowding, as placental growth reaches its functional limit. In both conditions, oxidatively stressed syncytiotrophoblast over-secrets proteins that perturb maternal angiogenic balance (Redman et al., 2014). Abnormal placentation is recognized as a main prerequisite for the pathogenesis of early onset preeclampsia. The disease seems to progress in two stages; in stage I (preclinical), poor development of the early placenta leads to deficient remodeling of maternal spiral arteries and consequent ischemia-reperfusion type injury of the placenta and oxidative stress. In stage II (clinical), the ischemic/malperfused placenta triggers the secretion of a mixture of placental factors, including antiangiogenic factors, proinflammatory cytokines and apoptotic debris that culminate in an enhanced maternal inflammatory response and in turn induce systemic endothelial dysfunction and the maternal syndrome of preeclampsia (Roberts and Cooper, 2001; Redman and Sargent, 2005).

In conditions of PE and IUGR which result in fetal hypoxia, there is a tendency to refer to fetoplacental hypoxia as the underlying cause. This concept has been challenged by several groups who advocate caution when interpreting results from pregnancies complicated by PE and IUGR (Kingdom and Kaufmann, 1997; Mayhew et al., 2004; Huppertz et al., 2014). These studies suggest that late-onset PE and late onset IUGR with present end diastolic flow (EDF) are associated with ischemic uteroplacental hypoxia in which the delivery of blood to the intervillous space is compromised due to deficient trophoblast invasion. This placental malperfusion results in ischemia-reperfusion injury affecting trophoblast and vascular endothelium, however fetal extraction of O_2 is not compromised and there is evidence of increased placental branching angiogenesis (Mayhew et al., 2004). In contrast, pregnancies complicated by early onset PE, which is usually accompanied with early onset IUGR with absent/reversed EDF are often referred to as 'placental hyperoxia' due to compromised fetoplacental blood flow which fails to extract oxygen from the intervillous space, leading to poor fetal oxygenation and higher than normal intervillous oxygen levels (postplacental hypoxia; Kingdom and Kaufmann, 1997; Mayhew et al., 2004).

Medical intervention in preeclampsia remains limited, centering on management of maternal hypertension and systemic complications, seizure prevention with magnesium sulfate, and delivering the fetus before term as a measure of treating maternal effects and preventing further growth restriction. However, any benefits this confers are offset by the complications of prematurity. Hence, identifying interventions that would modulate the pathological processes involved in the disease pathogenesis are key to intervening effectively in preeclampsia to improve maternal and fetal prognosis. This review will focus on the role of angiogenic and antiangiogenic factors in mediating maternal endothelial dysfunction and explore some therapeutic strategies aimed at targeting these factors in order to reverse/ameliorate the disease pathology.

EVIDENCE FOR THE ROLE OF ANGIOGENIC FACTORS IN PREECLAMPSIA

Placental vasculature is plastic and changes constantly throughout pregnancy to facilitate the rapid growth, and supply the increasing metabolic needs of the growing fetus (Charnock-Jones et al., 2004). Normal placental development is regulated by proangiogenic and antiangiogenic factors. The processes of vasculogenesis, *de novo* formation of blood vessels from precursor cells, and angiogenesis, creation of vessels from pre-existing vessels, are critical in the fetal and placental development for effective transport of oxygen, nutrients and fetal growth and development (Charnock-Jones et al., 2004). In contrast, angiogenesis is a rare event in adult vascular networks under physiological conditions, being limited to organs of the female reproductive tract (endometrium, ovary, placenta). Appearance of new blood vessel growth in other organs is regarded as pathological, and can be involved in conditions such as wound healing, tumor growth, and retinopathy.

Placental abnormalities lead to secretion of a mixture of proteins that stimulate maternal endothelium and result in the disease presentation. Recently, circulating antiangiogenic proteins, soluble vascular endothelial growth factor receptor 1 [VEGF-R1, also referred to as soluble fms-like tyrosine kinase (sFlt1)] and soluble TGF-β co-receptor endoglin (sEng), have been implicated in the pathogenesis of many of the maternal features of preeclampsia. We will first consider the role of proangiogenic factors, VEGF and transforming growth factor (TGF-β), and their receptors in vascular homeostasis (**Table 1**). VEGFs are a family of secreted dimeric glycoproteins involved in vasculogenesis and angiogenesis. In humans and other mammals, VEGF-A (referred here as VEGF) and its placental homologue placental growth factor (PlGF) are the most important members with proangiogenic activity (Keck et al., 1989; Leung et al., 1989; Maglione et al., 1991). VEGF promotes survival and proliferation of endothelial cells and induces vascular permeability (Keck et al., 1989; Leung et al., 1989). Two VEGF receptors, VEGF-R1 (Flt-1) and VEGF-R2 (Flk-1/KDR) are present on endothelial cells. VEGF binds to both receptors whilst PlGF homodimers bind exclusively to VEGF-R1. VEGF-R2 it thought to primarily mediate the actions of VEGF on endothelial cells. The function of VEGF-R1 is less well defined, although it is thought to modulate VEGF-R2 signaling. Another function of VEGF-R1 is to act as a decoy receptor (sFlt-1), sequestering VEGF from VEGF-R2 binding (**Table 1**; Park et al., 1994; Waltenberger et al., 1994; Keyt et al., 1996). TGF-β is another important molecule involved in angiogenesis, it has been implicated in the development of the vasculature (Oshima et al., 1996). TGF-β signals via binding to both type I (ALK1 and ALK5) and type II receptors. In addition, TGF-β signaling in the vasculature involves coreceptors that act to modulate its effects. Endoglin (CD105) is a TGF-β coreceptor expressed by endothelial cells and placental syncytiotrophoblast. TGF-β effects

Table 1 | A brief description of function of angiogenic and antiangiogenic growth factors and their receptors in pregnancy.

Molecules	Function and changes in pathological conditions
Angiogenic	**Factors**
VEGF-A	Potent angiogenic protein and trophic cytokine essential for endothelial integrity Free VEGF concentration decreased in preeclamptic women at disease presentation and even before the onset of disease (Maynard et al., 2003; Taylor et al., 2003; Levine et al., 2004)
PlGF	Key molecule in angiogenesis and vasculogenesis, in particular during embryogenesis. The main source of PlGF during pregnancy is the placental trophoblast Reduced in maternal plasma in preeclampsia (Maynard et al., 2003; Taylor et al., 2003; Levine et al., 2004)
TGF-β	Important molecule involved in angiogenesis, it has been implicated in the development of the vasculature (Oshima et al., 1996). *In vitro* effects are concentration-dependent, inducing proliferation and migration of endothelial cells at low doses (ALK1 mediated), and inhibiting proliferation and migration of endothelial cells at high doses (via ALK5; Goumans et al., 2002). Important modulator of VEGF signaling, further linking TGF-β to angiogenesis (Shao et al., 2009)
Receptors	
VEGF-R1 (Flt-1)	All members of the VEGF family stimulate cellular responses by binding to tyrosine kinase receptors (the VEGF-Rs) on the cell surface, causing them to dimerize and become activated through transphosphorylation
VEGF-R2 (Flk-1)	VEGF-A binds to VEGF-R1 (Flt-1) and VEGF-R2 (KDR/Flk-1), whilst PlGF only binds to VEGF-R1. VEGF-R2 appears to mediate almost all of the known cellular responses to VEGF. The function of VEGF-R1 is less well defined, although it is thought to modulate VEGF-R2 signaling. Another function of VEGFR-1 is to act as a decoy receptor (sFlt-1), sequestering VEGF from VEGF-R2 binding (Park et al., 1994; Waltenberger et al., 1994; Keyt et al., 1996)
TGF-βRI (ALK1,5) TGF-βRII Endoglin (CD105)	TGF-β signals via binding to both type I (ALK1 and ALK5) and type II receptors. In addition, TGF-β signaling in the vasculature involves coreceptors, e.g., endoglin (CD105), that act to modulate its effects. Endoglin is a membrane glycoprotein expressed by endothelial cells and placental syncytiotrophoblast. It has a crucial role in angiogenesis, therefore, making it an important protein for tumor growth, survival and metastasis of cancer cells to other locations in the body (Goumans et al., 2002; Shao et al., 2009).
Antiangiogenic	**Factors**
sFlt-1	Formed by alternative splicing of the pre-mRNA encoding VEGF-R1. It can bind circulating VEGF and PlGF as it lacks the cytoplasmic and transmembrane domain, thus preventing their interactions with endogenous receptors. Increased in maternal plasma in preeclampsia (Taylor et al., 2003; Levine et al., 2004, 2006; Chaiworapongsa et al., 2005; Steinberg et al., 2009)
sEng	Reduces endothelial tube formation in vitro and leads to increased capillary permeability. It blocks TGF-β1-induced vasodilation through interference of TGF-β1 binding to its receptor (Venkatesha et al., 2006). sEng is elevated in the sera of preeclamptic women, its circulating levels increase markedly beginning 2–3 months before the onset of preeclampsia, concentrations correlating with disease severity (Levine et al., 2006)

in vitro seem concentration-dependent, inducing proliferation and migration of endothelial cells at low doses (ALK1 mediated), and inhibiting proliferation and migration of endothelial cells at high doses (via ALK5; Goumans et al., 2002). In addition, TGF-β is an important modulator of VEGF signaling, further linking TGF-β to angiogenesis (**Table 1**; Shao et al., 2009).

The vascular endothelium relies on proangiogenic factors. Release of placentally derived antiangiogenic factors into maternal circulation is thus likely to cause angiogenic factor imbalance, leading to endothelial dysfunction that manifests as the symptoms of preeclampsia. Two such factors, sFlt-1 and sEng, have been implicated in the pathophysiology of preeclampsia (**Table 1**). Soluble Flt-1 is formed by alternative splicing of the pre-mRNA encoding VEGF-R1. It can bind circulating VEGF and PlGF as it lacks the cytoplasmic and transmembrane domain, thus preventing their interactions with endogenous receptors. Soluble endoglin receptor sEng reduces endothelial tube formation *in vitro* and leads to increased capillary permeability. It blocks TGF-β1-induced vasodilation through interference of TGF-β1 binding to its receptor, this effect is likely to involve NO (Venkatesha et al., 2006).

Let us now consider the clinical and experimental evidence for the role of angiogenic factor imbalance in the pathophysiology of preeclampsia. The circulating levels of two placental-derived antiangiogenic factors, sFlt-1 and sEng, are elevated in the circulation of women with preeclampsia, and may begin to rise even

before the onset of clinical symptoms, whereas the circulating concentrations of VEGF and PlGF are reduced in preeclamptic women at disease presentation and even before the onset of clinical symptoms (Taylor et al., 2003; Levine et al., 2004, 2006; Chaiworapongsa et al., 2005; Steinberg et al., 2009). The levels of circulating sFlt-1 increase and PlGF decrease during the last two months of pregnancy in normotensive women, however, these changes are significantly more pronounced in women who later develop preeclampsia and occur on average about 5 weeks before the onset of the disease (Levine et al., 2004). sEng is elevated in the sera of preeclamptic women, its circulating levels increase markedly beginning 2–3 months before the onset of preeclampsia, concentrations correlating with disease severity (Levine et al., 2006). However, it should be noted that despite the strong evidence linking angiogenic factor imbalance with PE, not all preeclamptic patients present with increased sFlt-1 and decreased VEGF/PlGF levels, and other mechanisms such as inflammation, oxidative stress, immunological interactions, maternal endothelial function, etc. also play an important role in the pathophysiology of this multifactorial disease.

Animal studies provide strong evidence linking sFlt-1 to the pathogenesis of preeclampsia. Although animal models do not develop preeclampsia, exogenous administration of sFlt-1 to pregnant rats induces some of the symptoms similar to the preeclamptic phenotype, including hypertension, proteinuria, and glomerular endotheliosis (Maynard et al., 2003). Similarly, glomerular endotheliosis and proteinuria can be induced in non-pregnant mice using antibodies against VEGF (Sugimoto et al., 2003), or by reducing glomerular VEGF levels by 50% (Eremina et al., 2003). In addition, administration of sFlt-1 can block VEGF and PlGF-induced microvascular relaxation of rat renal arterioles *in vitro* (Maynard et al., 2003). These studies all demonstrate that abnormal inhibition of endogenous VEGF activity can induce maternal preeclampsia-like symptoms. Similarly, administration of a therapeutic VEGF neutralizing antibody leads to renal pathology which can be replicated in adult animals by conditional gene targeting (Eremina et al., 2008). sFlt-treated animals do not develop additional symptoms of PE such as hemolysis and thrombocytopenia, seen in the HELLP syndrome. However, co-administration of sFlt-1 with sEng amplifies the symptoms, resulting in severe PE and/or HELLP syndrome, suggesting that these two placenta-derived factors could act in concert to induce severe preeclampsia (Venkatesha et al., 2006). We have shown similar synergistic effects between sFlt-1 and TNF-α. sFlt-1 sensitized endothelial cells to pro-inflammatory factors, increasing endothelial cell activation, as measured by increases in endothelial intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1, endothelin 1 (ET-1), von Willebrand factor and leukocyte adhesion, and led to reduction of AKT Ser^{473} and endothelial nitric oxide synthase (eNOS) Ser^{1177} phosphorylation (Cindrova-Davies et al., 2011). The survival of endothelial cells *in vivo* under non-pathological conditions is critically dependent on autocrine VEGF signaling. Endothelial specific ablation of VEGF results in progressive endothelial degeneration and sudden death of mutant animals (Lee et al., 2007), whilst autocrine VEGF is necessary for the survival of hematopoietic stem cells (Gerber et al., 2002), demonstrating that paracrine actions of VEGF are not sufficient to maintain the target cells. Thus, in preeclampsia the elevated circulating sFlt-1 is able to act in a dominant-negative fashion at the endothelial cell surface, blocking these autocrine signals (Cindrova-Davies et al., 2011).

LINKING ANTIANGIOGENIC FACTORS WITH ENDOTHELIAL DYSFUNCTION

Maternal symptoms of preeclampsia present as a consequence of maternal endothelial cell dysfunction, not due to dysregulation of maternal angiogenesis. Secretion of antiangiogenic factors from the placenta can adversely affect maternal endothelial function. The higher relative concentrations of the antiangiogenic factors are thought to potentiate vascular endothelial cell injury in the liver, kidney, brain, and the placenta itself, and thus trigger the symptoms of preeclampsia. Placental oxidative stress due to malperfusion is now commonly considered as the underlying source for antiangiogenic factors which are at the root of the symptomatic phase of preeclampsia. However, the mechanism linking the angiogenic imbalance with endothelial dysfunction is still under investigation. Endothelin-1 (ET-1), a potent vasoconstrictor and pressor agent involved in the regulation of blood pressure, has been shown to play a crucial role in the development of hypertension in experimental animal models of placental hypoxia/ischemia (George and Granger, 2011). Long-term infusion of ET-1 into sheep also induced preeclampsia-like symptoms, i.e., hypertension, proteinuria, and decreased uteroplacental blood flow (Greenberg et al., 1997). In addition, several studies have reported elevated levels of plasma ET-1 in preeclamptic pregnant women, compared to controls (Taylor et al., 1990; Nova et al., 1991; Baksu et al., 2005).

The expression of VEGF and sFlt-1 can be regulated by the hypoxia-inducible factor-1 (HIF-1), a key component of a widely operative transcriptional response activated by hypoxia. Hypoxia increases HIF-1α stability, but it can also be up-regulated under non-hypoxic conditions by inflammatory cytokines or microtubule-depolymerising agents involving the NF-κB pathway (Jung et al., 2003). HIF-1-mediated sFlt-1 secretion can by induced in placental explants by hypoxia or hypoxia-reoxygenation (HR) *in vitro* (Achmad et al., 1997; Nagamatsu et al., 2004; Cindrova-Davies et al., 2007; Cudmore et al., 2007; Cindrova-Davies, 2009). These *in vitro* effects can be blocked by anti-oxidant vitamins, inhibitors of the p38 MAPK and NF-κB pathways, and by over-expression of the heme oxygenase (HO-1)/CO, suggesting the involvement of oxidative stress, NF-κB, p38 signaling and HO-1 in sFlt-1 and HIF-1α regulation (Cindrova-Davies, 2009). sEng inhibits TGF-β signaling in the vasculature, which includes effects on activation of eNOS and vasodilation. It has been postulated that increased circulating levels of sFlt-1 lead to reduction of VEGF and NOS, thus reducing NO production. Administration of sFlt-1 to pregnant rats induced hypertension and significantly increased the production of prepro-ET message levels in the renal cortex. The hypertensive response could be abolished with coadministration of an ET_A antagonist (Murphy et al., 2010). Administration of sFlt-1 to pregnant animals thus impairs VEGF-R2-mediated NO production, reducing NO

bioavailability, thereby increasing systemic peripheral resistance and inducing hypertension. NO bioavailability may be further reduced by increased oxidative stress, and leads to enhanced ET-1 production and increased blood pressure (George and Granger, 2011).

Differences have been reported in the oxygen sensing ability of early vs. late onset PE. Rolfo et al. (2010) reported a disruption of oxygen sensing in early onset disease but not in the late-onset, and they speculate this contributes to decreased HIF-1α hydroxylation and breakdown, leading to its accumulation in early onset PE (Rolfo et al., 2010). As mentioned previously, hypoxia is disputed by many as the leading cause of placental pathologies. Instead, ischemia-reperfusion (Hung et al., 2002; Burton et al., 2009) or hyperoxia (Kingdom and Kaufmann, 1997; Huppertz et al., 2014) have been proposed as the underlying etiological factors in PE. Nevertheless, all these conditions lead to generation of reactive oxygen species (ROS), which can increase HIF-1α levels and stability. In addition, HIF-1α can be stabilized under normoxic conditions by other mechanisms such as components of the immune system, especially inflammatory cytokines, and hormones (Zhou and Brüne, 2006). Preeclampsia is a condition of excessive inflammatory responses mediated by syncytiotrophoblast microparticles, pathogens and DAMPs (damage-associated molecular pattern), which activate toll-like receptors (TLR) and through binding to immune cells promote persistent inflammatory conditions in this syndrome (Laresgoiti-Servitje, 2010). TNF-α can promote the release of sFlt-1 (Parrish et al., 2010), and similarly, AT1 autoantibodies can promote the secretion of sEng and sFlt-1 through TNF-α-mediated mechanisms (Zhou et al., 2008; Irani et al., 2010).

OXIDATIVE STRESS AND ANTIOXIDANTS

Strong evidence exists that generation of placental oxidative stress is a key intermediary event in the pathology of preeclampsia (Hubel, 1999; Redman and Sargent, 2000; Burton and Jauniaux, 2004). It is thought that deficient conversion of the uterine spiral arteries and subsequent impaired perfusion of the placenta provides the initiating insult (Brosens et al., 2002). In early gestation of normal pregnancy, the spiral arteries undergo substantial remodeling by invasive extravillous trophoblasts, which penetrate into the myometrium and convert muscular spiral arteries into flaccid tubes with no muscularis or elastic lamina, capable of supplying the hugely expanded blood flow of the third trimester placenta. In contrast, the remodeling is minimal in PE, only affecting the decidual segments of the spiral arteries, which retain their high-resistance (Brosens et al., 2002). Maternal blood thus enters the intervillous space at a higher pressure and a faster rate, in a pulsatile jet-like manner, exposing placental villi to fluctuating oxygen concentrations, which contributes to the ischemia-reperfusion (I/R) type injury of the placenta (Jauniaux et al., 1994, 1995; Burton and Hung, 2003). The resulting oxidative stress is thought to induce the placenta to release a mixture of factors, including inflammatory cytokines, antiangiogenic factors, and apoptotic debris, which culminates in an enhanced maternal inflammatory response and endothelial dysfunction (Roberts, 1998; Redman and Sargent, 2005).

The role of oxidative stress in mediating PE pathophysiology is supported by reports of significantly decreased levels of antioxidant vitamins C, A, E, β-carotene, glutathione levels, and iron-binding capacity in the maternal circulation of women with preeclampsia. There is also evidence of diminished superoxide dismutase (SOD) levels and activity in the maternal and placental compartments of preeclamptic women, indicative of decreased total antioxidant protective capacity in preeclampsia (Walsh, 1998). In the experimental model of reduced uterine perfusion pressure (RUPP) of placental ischemia-induced hypertension, treatment with vitamins C and E did not decrease blood pressure, while the SOD mimetic Tempol attenuated RUPP hypertension. Interestingly, treatment with an NADP(H) oxidase inhibitor attenuated but did not normalize hypertension, suggesting other ROS-generating pathways (Gilbert et al., 2008; Sedeek et al., 2008). Challenging placental explants with hypoxia-reoxygenation (HR) or hypoxia *in vitro* induced sFlt-1 expression via HIF-1α upregulation (Ahmad and Ahmed, 2004; Nagamatsu et al., 2004; Cindrova-Davies et al., 2007). We showed that administration of vitamins C and E can block HR-mediated sFlt-1 secretion, via inhibition of p38 and NF-κB signaling pathways (Cindrova-Davies et al., 2007; Cindrova-Davies, 2009). Similarly, the *in vivo* challenge of labor results in increased oxidative stress, and increased expression of HIF-1 α, sFlt-1 and VEGF (Cindrova-Davies et al., 2007).

The regulatory role of oxidative stress in the I/R-type injury in preeclampsia and cardiovascular disease, together with encouraging *in vitro* and *ex vivo* data introduced the hypothesis that antioxidant vitamins might play an important role in the treatment of the ischemic disease pathology. However, despite encouraging *in vitro* data and cohort trials, clinical trials aimed to treat women at risk of preeclampsia with vitamins C and E (Poston et al., 2006; Rumbold et al., 2006) showed no reduction in the incidence of the disease. These results are disappointing. Periconceptional use of vitamins in women is associated with lower rates of severe preterm births and extreme SGA, which seems to suggest that vitamin use might be effective during conception and early pregnancy (Catov et al., 2007), and that the clinical trial administration of vitamins occurred too late in pregnancy to reverse already established disease pathology. The outcome could also be affected by the composition of natural vs. synthetic vitamins, as evidence exists that healthy Mediterranean diet lowers the incidence of preterm birth (Haugen et al., 2008) and coronary heart disease (Aravanis et al., 1970). Synergy between different mediators may also be important in disease treatment. Recent clinical trial found that whilst antioxidant vitamins alone did not have a protective effect for prevention of PE, there was a synergistic effect between L-arginine and vitamin C, reducing the incidence of PE in these patients (Vadillo-Ortega et al., 2011). Endogenous NADPH oxidase and manganese SOD are required to maintain VEGF signaling and vascular homeostasis (Abid et al., 2001, 2007). Interestingly, despite no effect of the vitamin trials on lowering the incidence of preeclampsia, antioxidant supplementation led to a significant decrease in the plasma concentration of sFlt-1 and an increase in the plasma concentration of PlGF (Poston et al., 2011), matching our *in vitro* findings and confirming the beneficial effect of antioxidants on VEGF

signaling. Antioxidant vitamins could interfere with the normal physiological roles of VEGF and there seems to be a scope for potential use of antioxidants in preventing or ameliorating preeclampsia.

NO SUPPLEMENTATION

Endothelium plays a crucial role in regulating vascular tone. In preeclamptic patients, endothelial activation is manifested by increased expression of markers of endothelial activation, including VCAM, endothelin, von Willebrand Factor, and thrombomodulin (Mutter and Karumanchi, 2008). Nitric oxide (NO), hydrogen sulfide (H_2S), and carbon monoxide (CO) are three gaseous vasodilators that maintain the vascular tone. NO release from endothelial cells counter-balances the vasoconstriction produced by the sympathetic nervous system and the renin–angiotensin system. In addition, NO exerts many vasoprotective and anti-atherosclerotic properties, including protection from thrombosis, reduction of adhesion molecule expression and leukocyte adhesion. NO is produced by nitric oxide synthase (NOS), using L-arginine as a substrate. In addition, endogenously produced methylated amino acids such as asymmetric dimethylarginine (ADMA) can act as competitive inhibitors, and their secretion is increased in patients with cardiovascular disease and renal failure (Schnabel et al., 2005), as well as in women with high resistance placental circulation at risk of preeclampsia, IUGR, or both (Savvidou et al., 2003).

NO formation was found to be impaired in women with preeclampsia and gestational hypertension, compared to healthy pregnant controls. A negative correlation exists between plasma nitrite levels and sFlt-1 and sEng, suggesting an inhibitory effect of the angiogenic factors on NO formation (Sandrim et al., 2008). Given the important role of NO in the pathophysiology of preeclampsia, much research has concentrated on targeting the NO pathway as a potential therapy for preeclampsia. These include organic nitrates and *S*-nitrosothiols (e.g., *S*-nitrosoglutathione), L-arginine, inhibitors of cGMP breakdown (e.g., sildenafil), and other novel inhibitors of NO donor metabolism. This topic has been reviewed extensively by Johal et al. (2013). *S*-nitroglutathione (GSNO), endogenous *S*-nitrosothiol found ubiquitously in tissue, has been infused in women with preeclampsia and it has been shown to target not only the endothelial dysfunction, but also reduce platelet aggregation and activation, reduce sEng, and improve utero-placental perfusion with no adverse side effects, making it a good potential candidate for PE therapy (de Belder et al., 1995; Lees et al., 1996; Johal et al., 2013). The effects of supplementation with NO precursor, L-arginine, have been studied in several clinical trials. A recent randomized control trial of high risk pregnant women showed that dietary supplementation with a combination of L-arginine and antioxidants was associated with a significant reduction in the incidence of preeclampsia, compared to antioxidants alone or placebo prevention of preeclampsia (Vadillo-Ortega et al., 2011). These are encouraging results although they have to be interpreted with caution, given that the effects of L-arginine alone were not studied, and as such it is difficult to dissect out the relative contributions of L-arginine and antioxidants in reducing the incidence of the disease. These encouraging results were echoed in a recent systematic review of randomized trials focused on the role of L-arginine in the prevention of preeclampsia (Dorniak-Wall et al., 2014). The authors analyzed 7 randomized controlled trials testing the effects of L-arginine in pregnant women, and they reported L-arginine supplementation was associated with a significant reduction in the risk of preeclampsia in pregnant women with either established hypertension or who were considered at risk of preeclampsia (Dorniak-Wall et al., 2014).

The mechanism of the beneficial effect of L-arginine supplementation has been studied in pregnant rats in whom preeclampsia-like phenotype was induced with sFlt-1 administration (Murphy et al., 2011). sFlt-1 infusion into pregnant rats induced hypertension associated with reductions in circulating levels of VEGF, significant proteinuria, and endothelial dysfunction, as marked by a 3.5% increase in renal cortical pro-ET mRNA expression (Maynard et al., 2005; Gilbert et al., 2007; Murphy et al., 2010, 2011). Administration of L-arginine decreased sFlt-1 hypertension but had no effect on the blood pressure response in non-pregnant rats. In addition, L-arginine abolished the sFlt-mediated expression of renal cortical prepro-ET, suggesting that a reduction in NO synthesis may play an important role in the enhanced ET-1 production in response to sFlt-1 hypertension in pregnant rats (Murphy et al., 2011). These data are supported by previous studies, which showed a link between reduced NO production and enhanced ET-1 production (Kourembanas et al., 1993; Edwards et al., 1996). It has been postulated that increased circulating levels of sFlt-1 lead to reduction of VEGF and NOS, thus reducing NO production (George and Granger, 2011).

ER STRESS AND ER STRESS INHIBITORS

Endoplasmic reticulum (ER) processes all secreted proteins, facilitating folding and post-translational modifications. Accumulation of misfolded proteins leads to activation of ER stress response pathways, collectively known as the Unfolded Protein Response (UPR). The aim of the UPR is to restore homeostasis by inhibiting translation of non-essential proteins, increasing the capacity of the chaperone and folding machinery, and stimulating degradation of misfolded proteins. Failure of the mechanism results in activation of apoptotic pathways. ER stress is a major regulator of cell homeostasis (Xu et al., 2005; Cullinan and Diehl, 2006; Yoshida, 2007). The UPR is initiated by three transmembrane sensors normally held inactive by binding of the principal ER chaperone, GRP78. These sensors are PERK (PRKR-like ER kinase) that phosphorylates eukaryotic initiation factor 2α (eIF2α) to inhibit translation, ATF6 (activating transcription factor 6) and IRE1 (inositol-requiring enzyme 1). ATF6 is cleaved to produce an active transcription factor, while IRE1 splices the mRNA encoding XBP-1 (X-box binding protein-1) to produce a second transcription factor. These factors have overlapping functions, and both upregulate ER chaperone proteins, including GRP78, and degradation pathways (Xu et al., 2005; Cullinan and Diehl, 2006; Yoshida, 2007). In addition, ER stress can generate and accumulate ROS, thus promoting oxidative stress. Activation of the NF-κB pathway links increased oxidative stress, ER stress and inflammation. NF-κB can be activated by an increase in

ER stress via PERK-eIF2α activation or IRE1α autophosphorylation. This effect can be blocked by both calcium chelators and antioxidants, suggesting NF-κB activation could be a result of the oxidative stress arising from excessive protein folding and/or ER-stress mediated Ca^{2+} leakage (Zhang and Kaufman, 2008).

Disorders of ER function are recognized to underlie many diverse pathologies, including diabetes and neurodegenerative diseases, and therapeutic interventions targeting it are being devised (Hetz et al., 2013). ER stress also seems instrumental in the pathophysiology of preeclampsia and IUGR (Yung et al., 2008, 2012). Use of ER chaperones proved a beneficial therapy for treatment of type 2 diabetes as it restored systemic insulin sensitivity, normalized hyperglycemia, resolved fatty liver disease, and enhanced insulin action in various tissues (Ozcan et al., 2006). Similarities exist between preeclampsia and metabolic syndrome, including dyslipidemia, inflammation, insulin resistance, oxidative stress and ER stress. We have demonstrated an increased expression of ET-1 and sFlt-1 in HR-treated placental explants (Cindrova-Davies et al., 2007; Cindrova-Davies, 2009). Conditioned medium from HR explants induced ER stress in JEG-3 cells, which was abolished by an ET-1 neutralizing antibody (Jain et al., 2012). Administration of ER chaperone TUDCA to placental explants challenged with HR reduced the levels of endothelin-1 (ET-1) in these explants, and it reduced the protein levels of sFlt-1 (unpublished observations). These data suggest that restoring ER function could also offer a therapeutic benefit for the treatment of preeclampsia. The ER chaperones are endogenously produced bile salts ursodeoxycholic acid (UDCA, TUDCA). The chaperones have excellent *in vivo* safety profiles and their use has been approved in clinical trials for the treatment of urea-cycle disorders, thalassemia and cystic fibrosis (Ozcan et al., 2006), and they are currently used to treat intrahepatic cholestasis of pregnancy (Geenes and Williamson, 2009).

STATINS

Preeclampsia has been associated with dyslipidemia, and there is evidence of increased antibodies for oxidized form of LDL in patients with preeclampsia, which is consistent with oxidative stress, and it is analogous to changes described in atherosclerosis (Branch et al., 1994). Although preeclampsia is unique to pregnancy, there are many biological and pathological similarities, as well as risk factors (e.g., hypertension, obesity, dyslipidemia, diabetes, etc.) with adult cardiovascular disease. The mechanism underlying both atherosclerosis (Hansson, 2005) and preeclampsia (Roberts and Cooper, 2001; Redman and Sargent, 2005) is initiated by underlying endothelial dysfunction and inflammation, which lead to disease progression and manifestation. Pregnancy is often viewed as a stress test, and preeclampsia an early manifestation of CV disease. In fact, women who develop preeclampsia have a two- to threefold risk of hypertension, ischemic stroke and heart disease in later life (van Pampus and Aarnoudse, 2005; McDonald et al., 2008), and the risk is further increased with the severity of the disease, and early manifestation before 34 weeks gestation (Mongraw-Chaffin et al., 2010). Given the analogies between the two diseases, some remedies and treatments used to prevent and treat cardiovascular disease have been tested in preeclampsia.

Natural and synthetic statins (or 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors) are the most commonly prescribed classes of medication worldwide, used in primary and secondary prevention of cardiovascular mortality and other cardiovascular events through lipid-lowering remedy, as well as many pleiotropic effects, including endothelial protection, antioxidant properties, anti-inflammatory, antithrombotic and proangiogenic effects (Costantine and Cleary, 2013; Girardi, 2014). The beneficial effects of statins in the treatment of cardiovascular disease have been confirmed by recent meta-analysis of individual data from 90,000 individuals in 14 randomized trials of statin therapy vs. placebo, called the Cholesterol Treatment Trialists' (CTT) Collaboration (Baigent et al., 2005). The CTT concluded that statin therapy can safely reduce the 5-year incidence of major coronary events, coronary revascularization, and stroke by about one fifth per mmol/L reduction in LDL cholesterol, largely irrespective of the initial lipid profile or other presenting characteristics (Baigent et al., 2005).

The effect of pravastatin administration in pregnancy has been tested in many rodent models of preeclampsia. Treatment with pravastatin significantly reduced maternal sFlt-1 levels, lowered blood pressure, improved the vascular profile, and prevented kidney injury (Ahmed et al., 2010; Costantine et al., 2010; Fox et al., 2011; Kumasawa et al., 2011; Saad et al., 2014). In addition, pravastatin also prevented the incidence of intrauterine growth restriction, without any adverse effects on pregnancy. In a mouse model of preeclampsia using placenta-specific lentiviral vector expression of sFlt-1, Kumasawa et al. elegantly demonstrated that pravastatin administration decreased sFlt-1 but importantly increased PlGF, thus restoring the angiogenic balance (Kumasawa et al., 2011). Pravastatin effects could be reproduced by administration of PlGF, which reduced sFlt-1 levels, ameliorated hypertension, glomerular endotheliosis, and proteinuria in the mice, suggesting that the beneficial effect of pravastatin on improving preeclampsia-like symptoms is mediated by increasing PlGF levels, which counteracts sFlt-1-mediated effects (Kumasawa et al., 2011). In addition, statins also exert protective effects on the endothelium and ameliorate preeclampsia symptoms by increasing the release of vasodilators NO (Redecha et al., 2009; Fox et al., 2011) and CO (Cudmore et al., 2007; Muchova et al., 2007). Pravastatin treatment of mice destined to develop preeclampsia using sFlt-1 overexpression increased eNOS protein expression in the vasculature (Fox et al., 2011), whilst pravastatin improved pregnancy outcome by increasing plasma NO levels in CBAxDBA/2 mice destined to develop recurrent miscarriage and IUGR (Redecha et al., 2009). Treatment of mice with statins resulted in increased HO-1 activity and increased CO release from tissues, as well as increased levels of plasma antioxidants (Muchova et al., 2007). Statins also induced the expression of HO-1 and inhibited sFlt-1 secretion in placental explants (Cudmore et al., 2007). These studies provide further evidence of a pleiotropic role of pravastatin in preventing the vascular dysfunction.

Given the encouraging evidence of the beneficial effects of statins on the prevention of cardiovascular disease in humans

and of preeclampsia in animal models, the ability of pravastatin to restore angiogenic balance is currently being tested in the first randomized placebo controlled StAmP trial (Statins to ameliorate early onset preeclampsia) in the UK. The aim of the trial is to establish if pravastatin can lead to a significant reduction in circulating antiangiogenic factors and alleviate the severity of early onset preeclampsia. The pleiotropic anti-inflammatory, anti-thrombotic, antioxidant and vascular protective effects suggest that statins might be a good therapeutic option to prevent preeclampsia.

FUTURE DIRECTIONS – H_2S DONORS

Endogenous H_2S plays an important role in regulating physiological processes such as blood flow, vasodilation, arterial diameter, and leukocyte adhesion (Wang, 2009). Endogenous production is catalyzed by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). H_2S is produced in the cardiovascular system by CSE, using cysteine as a substrate. It acts as a vasodilator, being able to hyperpolarize and relax SMCs by opening K_{ATP} channels (Zhao et al., 2001). Unlike NO and CO, which mediate vasorelaxation largely by cGMP pathway activation, the vasorelaxant effect of H_2S is independent of cGMP pathway (Zhao et al., 2001). In addition, NO effects depend largely on the endothelial function integrity, whereas H_2S is an endothelium-independent vasodilator and exerts its direct effect on SMCs. CSE knockout mice have markedly reduced serum H_2S levels, which results in pronounced hypertension and diminished vasodilation, providing direct evidence that H_2S is a potent vasodilator that can influence blood pressure (Yang et al., 2008). Similarly, genetic deficiency of CBS leads to homocysteinemia, which is associated with endothelial dysfunction and hypertension (Miles and Kraus, 2004). We reported reduced CSE expression in IUGR cases, as well as PE cases that were accompanied by abnormal UA Doppler profiles, and this could be recapitulated by subjecting placental explants to HR. In addition, we demonstrated a potent vasodilatory effect of an H_2S donor in perfused placentas, capable of K_{ATP} dependent-reduction of placental vascular resistance. Reduced bioavailability of H_2S may thus be implicated in placental vasoconstriction (Cindrova-Davies et al., 2013). In addition to being a potent vasodilator, many animal studies also showed a protective role of H_2S donors against ischemia-reperfusion injury and inflammation (Bos et al., 2009, 2013; Sodha et al., 2009). The pro-survival pathways AKT, PKC, and ERK1/2 have been identified as H_2S targets conferring its anti-apoptotic effects during reperfusion injury (Hu et al., 2008; Yong et al., 2008). Additionally, H_2S can promote direct anti-apoptotic signaling during ischemia-reperfusion by activating eNOS and thus inducing NO (Yong et al., 2008), and H_2S promotes DNA binding and transcriptional activation of anti-apoptotic genes regulated by NF-κB (Sen et al., 2012). The antioxidant properties of H_2S can be mediated via two distinct mechanisms; by acting as a direct scavenger of ROS and by up-regulating antioxidant defenses. H_2S may up-regulate endogenous antioxidants through a nuclear factor E2-related factor-2 (Nrf2; Calvert et al., 2009). Nrf2 binds the antioxidant responsive element (ARE) found in the promoter region of antioxidant genes, including heme oxygenase-1 (HO-1), thioredoxin, thioredoxin reductase, glutathione reductase, glutathione peroxidase (GPx), glutathione *S*-transferase (GSS), and catalase and is thus an important factor in controlling cardiac cellular susceptibility to reactive oxygen and nitrogen species-induced cytotoxicity (Zhu et al., 2005). Garlic oil can also promote Nrf-2 activation, further supporting the role for Nrf2 as the mediator of H_2S-induced antioxidant effects (Fisher et al., 2007). Additionally, CSE expression appears to be a critical component of the cytoprotective ATF4 transcriptional response; CSE deficiency increases sensitivity to apoptosis induced by ER stressors and homocysteine, indicating the importance of GSH up-regulation through the transsulfuration pathway to promote survival (Dickhout et al., 2012). H_2S also exerts important proangiogenic effects (Cai et al., 2007; Wang et al., 2010; Bir et al., 2012; Holwerda et al., 2014). H_2S promotes proliferation, adhesion, migration and tube-like structure formation of endothelial cells *in vitro* via AKT pathway phosphorylation. It also stimulates angiogenesis *in vivo* at physiologically relevant doses (Cai et al., 2007). NO-H_2S crosstalk seems critical in mediating these effects (Bir et al., 2012; Coletta et al., 2012). H_2S therapy restores blood flow to ischemic tissues in a NO-dependent manner, by stimulating NOS expression and HIF-1α and VEGF expression and activity (Bir et al., 2012).

Consistent with these roles of H_2S, the consumption of garlic is negatively correlated with the progression of CV disease, and causes a lower incidence of hypertension, enhances antioxidants and inhibits platelet aggregation (Banerjee and Maulik, 2002). It has been shown that the biological production of H_2S from garlic-derived organic polysulfides mediates the major beneficial effects of garlic-rich diets, specifically on CV disease and more broadly on overall health (Benavides et al., 2007). In animal models of hypoxic pulmonary hypertension, H_2S could reverse structural remodeling changes in pulmonary vessels of rats exposed to chronic HR (Hongfang et al., 2006). H_2S therapy also protected against acute myocardial ischemia/reperfusion (Calvert et al., 2009), and attenuated cardiac dysfunction following heart failure (Kondo et al., 2013; Polhemus et al., 2013). Recent animal studies have focused on the use of “medical food,” i.e., drugs derived from natural sources such as garlic, as a cardioprotective therapy for heart failure. These include diallyl trisulfide (DATS; Polhemus et al., 2013), a long acting H_2S donor derived from garlic, and sulfur-releasing “medical food” SG-1002 (Kondo et al., 2013), which is currently in clinical trials. Similar results have been reported in the placental field recently. Holwerda et al. (2014) evaluated the therapeutic effect of an H_2S donor in an animal model of sFlt-induced hypertension. They reported a significant reduction in blood pressure, and proteinuria, as well as concomitant reduction in sFlt-1 levels and increase in free maternal VEGF concentrations in the treated group of animals (Holwerda et al., 2014). Wang et al. (2013) demonstrated that CSE enzyme inhibition in pregnant mice induced hypertension, increased sFlt-1 and sEng levels, caused placental abnormalities and compromised fetal growth. H_2S donor therapy reduced the levels of the antiangiogenic factors and restored fetal growth, adding further weight to the evidence that a dysfunctional CSE/H_2S pathway may contribute to the pathogenesis of preeclampsia (Wang et al., 2013), and that H_2S donor therapy could have beneficial effects on prevention of preeclampsia.

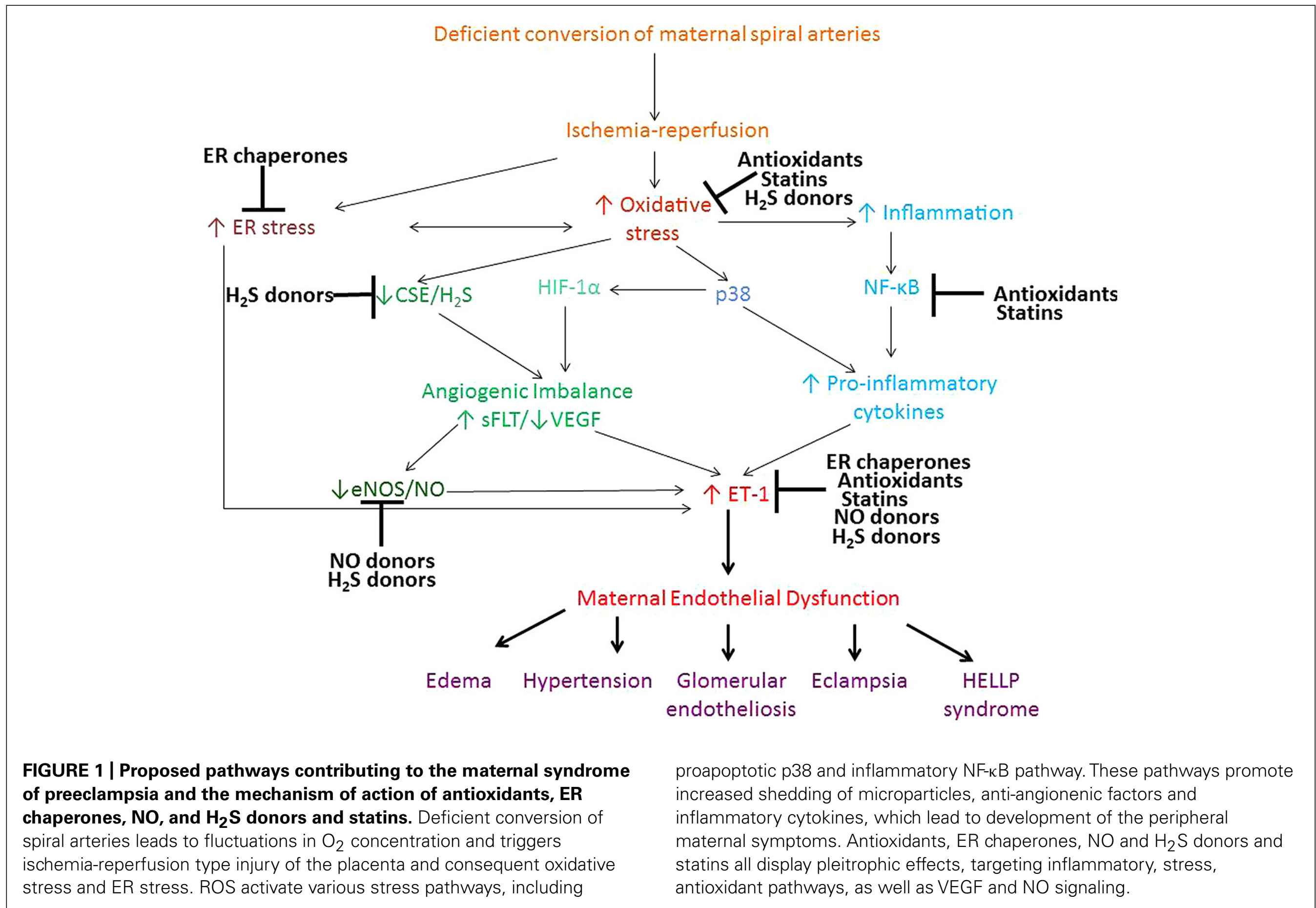

FIGURE 1 | Proposed pathways contributing to the maternal syndrome of preeclampsia and the mechanism of action of antioxidants, ER chaperones, NO, and H_2S donors and statins. Deficient conversion of spiral arteries leads to fluctuations in O_2 concentration and triggers ischemia-reperfusion type injury of the placenta and consequent oxidative stress and ER stress. ROS activate various stress pathways, including proapoptotic p38 and inflammatory NF-κB pathway. These pathways promote increased shedding of microparticles, anti-angionenic factors and inflammatory cytokines, which lead to development of the peripheral maternal symptoms. Antioxidants, ER chaperones, NO and H_2S donors and statins all display pleitrophic effects, targeting inflammatory, stress, antioxidant pathways, as well as VEGF and NO signaling.

CONCLUDING REMARKS

Therapeutic interventions to treat preeclampsia remain largely experimental and there is no established remedy for the treatment of this multifactorial disease. This review concentrated on the evidence for the therapeutic potential of antioxidants, ER chaperones, NO and H_2S donors, and statins. Mechanistic effects of these compounds on the signaling pathways involved in the pathophysiology of preeclampsia, are summarized in **Figure 1**. Antioxidants, ER chaperones, NO donors, statins and H_2S donors display pleitropic antioxidant, anti-inflammatory, and pro-angiogenic effects in animal and *in vitro* studies. Encouraging clinical evidence exists for the use of NO donors, and a clinical trial is currently underway testing the effect of statins in treatment of preeclampsia. Although clinical trials on the use of antioxidant vitamins in pregnancy proved largely unsuccessful, the scope for their use still exists given the beneficial cardioprotective effects of antioxidant-rich Mediterranean diet, periconceptual vitamin use and the synergistic effect of vitamin C and L-arginine. H_2S recently emerged as a novel therapeutic agent for cardiovascular disease and its effects are being tested.

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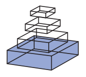

Is a low level of free thyroxine in the maternal circulation associated with altered endothelial function in gestational diabetes?

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Synthesis of thyroid hormones, thyroxine (T_4) and tri-iodothyronine (T_3), in the human fetus starts from 17 to 19th weeks of gestation. Despite the majority of normal pregnant women reaching adequate levels of circulating thyroid hormones, in some cases, women with normal pregnancies have low level of free T_4 during first trimester of pregnancy, suggesting that T_4 action may be compromised in those women and their fetuses. In addition, pathological low levels of thyroid hormones are detected in isolated maternal hypothyroxemia (IMH) and clinical hypothyroidism. Nevertheless, human placenta regulates T_3/T_4 concentration in the fetal circulation by modulating the expression and activity of both thyroid hormone transporters (THT) and deiodinases. Then, placenta can control the availability of T_3/T_4 in the feto-placental circulation, and therefore may generate an adaptive response in cases where the mother courses with low levels of T_4. In addition, T_3/T_4 might control vascular response in the placenta, in particularly endothelial cells may induce the synthesis and release of vasodilators such as nitric oxide (NO) or vasoconstrictors such as endothelin-1 mediated by these hormones. On the other hand, low levels of T_4 have been associated with increase in gestational diabetes (GD) markers. Since GD is associated with impaired placental vascular function characterized by increased NO synthesis in placental arteries and veins, as well as elevated placental angiogenesis, it is unknown whether reduced T_4 level at the maternal circulation could result in an altered placental endothelial function during GD. In this review, we analyze available information regarding thyroid hormones and endothelial dysfunction in GD; and propose that low maternal levels of T_4 observed in GD may be compensated by increased placental availability of T_3/T_4 via elevation in the activity of THT and/or reduction in deiodinases in the feto-placental circulation.

Keywords: thyroxine, gestational diabetes, endothelial dysfunction, pregnancy

INTRODUCTION

Thyroid gland produces tetra-iodothyronine (T_4 or thyroxine) and tri-iodothyronine (T_3). In the human fetus, the synthesis of these hormones starts from 17 to 19th weeks of gestation (wg), therefore it is well accepted that before this period, the circulating T_3/T_4 in the fetus depends on the maternal levels of these hormones (Pérez-López, 2007). Thus, an altered function of the thyroid gland at the maternal side could prejudice physiological levels of T_3/T_4 at the fetal circulation, and impair fetal growth and development. Worldwide studies indicate that ~10% of women may have hypothyroidism in their childbearing age (Mosso et al., 2012; Khalid et al., 2013; Ohashi et al., 2013). It has been also described that ~35 or ~3% of women with an apparent normal pregnancy have clinical hypothyroidism or exhibit maternal hypothyroxemia (low level of free T_4), respectively (Mosso et al., 2012), both maternal conditions associated with several alterations in the fetus development (Parkes et al., 2012). Nevertheless, isolated maternal hypothyroxemia (IMH), a pathological condition manifested during pregnancy (Sahay and Nagesh, 2012), have been associated with occurrence of gestational diabetes (GD; Olivieri et al., 2000), pre-eclampsia (Sardana et al., 2009), or intrauterine growth restriction, IUGR; Chan et al., 2006). Moreover, pregnant women with IMH have higher risk (fourfold) to develop insulin resistance and GD (Karakosta et al., 2012; Tudela et al., 2012). In fact, reduced T_4 level in the maternal circulation is associated with an increase in the incidence of GD pregnancies (Olivieri et al., 2000; Tudela et al., 2012) and with altered development of the central nervous system in children from pregnancies affected by these diseases (Smallridge and Ladenson, 2001; Casey et al., 2005). In addition, an incidence as high as ~70% of women coursing with pregnancies affected with GD exhibit IMH (Olivieri et al., 2000).

Gestational diabetes (GD) is associated with higher synthesis and release of vasodilators such as nitric oxide (NO) in the human fetal endothelium from GD (described as altered endothelial function) (De Vriese et al., 2000; Guzmán-Gutiérrez et al., 2011; Westermeier et al., 2011; Salomón et al., 2012). In addition, thyroid hormones are also involved in NO synthesis and release (Napoli et al., 2001; Fazio et al., 2004), but the potential contribution of reduced circulating T_4 on deregulation of fetal endothelial function seen in GD pregnancies is unclear. We here analyze the available information regarding the potential relationship between maternal and fetal thyroid hormones with the occurrence of endothelial dysfunction in GD. We propose that the low maternal levels of T_4 seen in GD may be compensated by higher placental availability of thyroid hormones via elevation in the activity of placental thyroid hormone transport and metabolism.

OVERVIEW OF SYNTHESIS AND RELEASE OF THYROID HORMONES

The thyroid hormones 3,5,3′,5′-tetraiodothyronine (T_4 or thyroxine) and 3,5,3′-triiodothyronine (T_3) are synthesized in the thyroid gland and is regulated by hypothalamus/pituitary/thyroid axis by a negative feedback. In this regulatory axis, hypothalamus releases thyrotropin releasing hormone (TRH), which interacts with TRH receptors in thyrotropin cells in the pituitary gland to release thyroid stimulating hormone (TSH). In turn, TSH is the main regulator of the release of thyroid hormones leading to TSH receptor (TSHr) activation and increased iodo (iodide) uptake in the thyroid gland (Szkudlinski et al., 2002).

Iodide intracellular uptake is mediated by cotransport with sodium (Na^+/I^-) in the basal membrane of follicular cells in the thyroid gland. In these cells, iodides are oxidized by thyroid peroxidase (TPO) in the presence of hydrogen peroxide. Iodine (oxidized iodine) binds to thyroxine residues belong to the tiroglobulins (Tg), then tyroxine residues can be mono (MIT), di (DIT), tri (T_3), or tetra-iodinated (T_4) (Sugenoya et al., 1984; Rousset, 1991). The release of thyroid hormones through the basolateral membrane in thyroid gland follicular cells requires endocytosis of iodinated Tg at the apical side of these cells. The Tg is then incorporated into phagolysosomes and digested by proteolytic proteins, with MIT and DIT being re-uptaked into Tg; however, T3 and T4 are released toward circulation (Rousset, 1991). T_4 is the main thyroid hormone released by thyroid gland follicular cells ($\sim$40-fold compared with T_3) and is almost all (99.97%) bound to thyroxine binding globulin (TBG), albumin and pre-albumin in the circulation. In addition, free T_3 accounts for $\sim$0.3% and the rest is bound to TBG and albumin.

Free T_3 is the hormone with biological activity and is the active form of thyroid hormones. It is derived from 5′-deiodination of free T_4 via iodothyronine deiodinases located in the target tissues (Sugenoya et al., 1984; Rousset, 1991; Schussler, 2000; Bianco and Kim, 2006). Deiodinases are grouped in three subtypes: I, II, and III (or D1, D2, and D3, respectively), all of which are involved in the regulation of T_3 activity (Bianco and Kim, 2006; Darras and Van Herck, 2012). For instance, D2 is specific to generate T_3 from T_4; however, D3 generates DIT from T_3, and reverse T_3 (rT_3, inactive form of T_3) from T_4 (Kilby et al., 2005). D1 has been reported as an enzyme that is much less active compared with the other forms (Bianco and Kim, 2006; Dentice and Salvatore, 2011; Darras and Van Herck, 2012).

VASCULAR EFFECTS OF THYROID HORMONES

Thyroid hormones reduce peripheral vascular resistance by promoting relaxation in human and murine vascular smooth-muscle cells (Klemperer et al., 1995; Ojamaa et al., 1996; Park et al., 1997), and improve vascular reactivity by endothelium-dependent and -independent mechanisms (Napoli et al., 2001; Fazio et al., 2004). In rat, T_4 released from the mesenteric arteries increases vasorelaxation when administrated at supraphysiological concentrations (Zwaveling et al., 1997). Moreover, T_3 and T_4 improve fibroblast growth factor-2 (FGF-2) expression, a recognized proangiogenic factor, in cultures of ECV304 cells (Davis et al., 2004). Interestingly, human umbilical vein endothelial cells (HUVEC) exposed to high T_3 levels exhibit high expression of endothelin-1 (vasoconstrictor) and fibronectin (profibrotic molecule), suggesting that pathological conditions such as hyperthyroidism could be associated with vasoconstriction (Baumgartner-Parzer et al., 1997; Diekman et al., 2001).

In addition, there is evidence that vasoconstriction associated with high levels of thyroid hormones may result from a non-genomic action, which seems mediated by αvβ3 integrin as reported in HUVEC (Luidens et al., 2010). In this regard, αvβ3 integrin is a membrane protein that should active the phosphatidylinositol 3 kinase and protein kinase B/Akt (PI3K/Akt) pathway in this cell type (Hiroi et al., 2006; Luidens et al., 2010). Nevertheless, in another study HUVEC and bovine aortic endothelial cells (BAEC) seems to respond to T_3 by increasing the phosphorylation of serine^{1177} (Ser^{1177}; Ser^{1177}-eNOS) at the endothelial nitric oxide synthase isotype (eNOS) in a time- and concentration-dependent manner (Hiroi et al., 2006). Similar results were seen in vascular smooth-muscle cells from rat thoracic aortae where T_3 increases Ser^{1177}-eNOS via PI3K/Akt pathway inducing eNOS, inducible (iNOS) and neuronal (nNOS) NOS expression. Thus, it is likely that T_3 increases NOS expression via a genomic and a non-genomic (i.e., via αvβ3 integrin) action (Carrillo-Sepúlveda et al., 2010). Therefore, the thyroid hormone concentration is a determinant factor involved in the modulation of vascular function (**Figure 1**). However, there is not information addressing thyroid hormone effects on the human feto-placental vasculature.

HUMAN THYROID HORMONES IN PREGNANCY

Human fetal thyroid hormones are secreted from the 17 to 19 wg, indicating that the fetus requires thyroid hormones delivery from the mother during the first and beginning of the second trimester of pregnancy (Pérez-López, 2007). Pregnant women have TSH and free T_4 levels that are normal and comparable to those in non-pregnant women; however, in the first trimester of pregnancy, there is an increase in the maternal free T_4 level most likely in response to chorionic gonadotropin hormone (hCG; Fantz et al., 1999; Pérez-López,

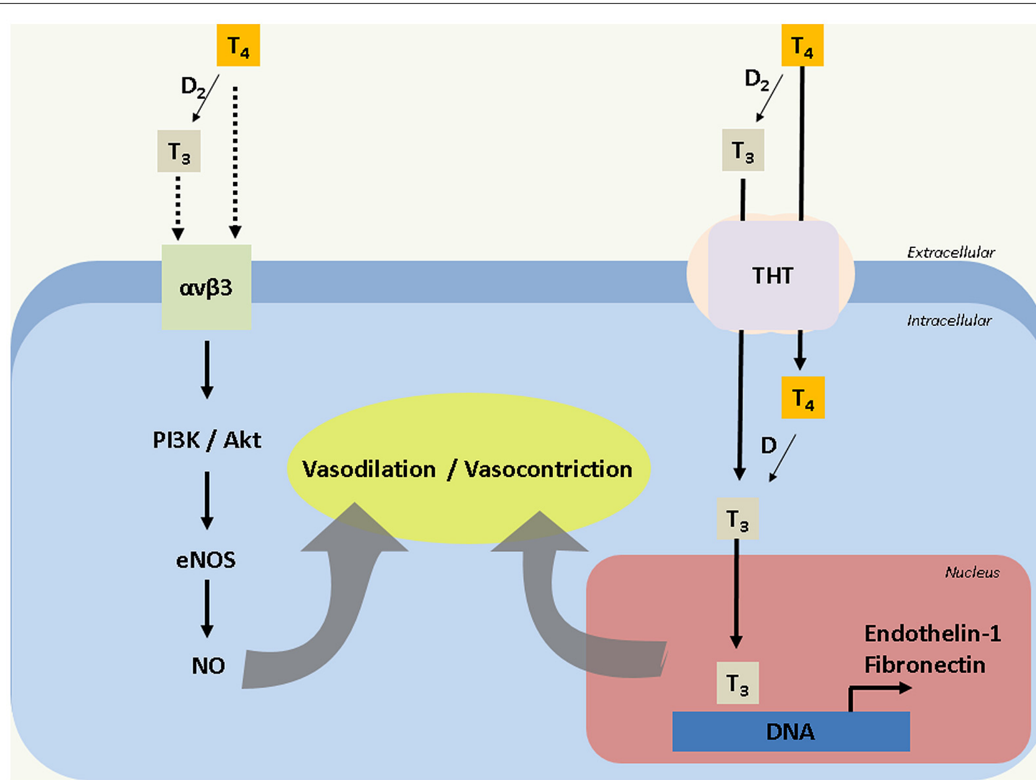

FIGURE 1 | Thyroids hormones effects on endothelial cells. T_4 (Tyrosine) is metabolized by deiodinase 2 (D_2) to T_3 (triiodothyronine) of manner extra and intracellular. T_3 and T_4 (Tyrosine) could activates αvβ3 integrin receptor which initiate a signaling that involve PI3K, Akt and eNOS, generating nitric oxide (NO) as vasodilator. But, also T3 and T4 are uptake by thyroid hormones transporters (THT). T3 bind to DNA and activate transcriptional activity for endothelin-1 and fibronectin which are associated with vasoconstriction.

2007). It has been shown that increased hCG level leads to reduced hypothalamus/pituitary/thyroid axis activity, but improved thyroid hormones delivery to the fetus in the first trimester of pregnancy (Fantz et al., 1999; Pérez-López, 2007; Chan et al., 2009). In this period, the concentration of free T_4 in the fetal circulation corresponds to a third of the level found in the maternal circulation (Chan et al., 2009). This phenomenon results from a reduced concentration of the TBG binding protein in the fetal circulation, which leads to a free T_4 concentration enough to exert its biological effects in embryonic tissues (Kilby et al., 2005; Chan et al., 2009). In the second trimester of pregnancy, free T_4 levels in the fetal circulation corresponds to about half of the concentration detected in the maternal circulation (Kilby et al., 2005; Chan et al., 2009). Therefore, it seems clear that thyroid hormone levels are regulated by the placenta tissue (Burrow et al., 1994). Thus, a role of this organ is crucial in the delivery of T_4 to the fetus.

THYROID HORMONES METABOLISM IN THE HUMAN PLACENTA

In addition to the paracrine effect of hCG on the hypothalamus/pituitary/thyroid axis, the human placenta regulates directly the thyroid hormone concentration in the fetal circulation by modulation of thyroid hormone transporters (THT), and by thyroid hormones metabolism mediated by deiodinases (Burrow et al., 1994). THT are located at the apical and basolateral membranes of the cytotrophoblasts, syncytiotrophoblast and microvascular endothelial cells (James et al., 2007). There are several THT, including monocarboxylate transporters (MCT), where MCT8 and MCT10 are the main forms. Moreover, a role has been reported for L-amino acid transporters (LAT) and organic anion transporter polypeptides system (OATPs), which operates with less selectivity for T_4 (James et al., 2007). Also in the human placenta MCT8 (Park and Chatterjee, 2005), MCT10, LAT1, LAT2 (Friesema et al., 2003), OATP1A2 and OATP4A1 (Hagenbuch and Meier, 2003; Hagenbuch, 2007) have been identified, but no studies addressing the role of these membrane transporters in any pathology of pregnancy have been documented. Moreover, D2 (located at the endoplasmatic reticulum) and D3 (located at the plasma membrane with a cytoplasmic active site; Koopdonk-Kool et al., 1996; Stulp et al., 1998; Chan et al., 2003) have been identified. D2 and D3 are referred as major factors controlling transplacental transport of T_4 to the fetus (Mortimer et al., 1996). Interestingly, D2 and D3 expression is up regulated by T_3 (Chan et al., 2003). In addition, since changes in the level of T_3/T_4 cause altered THT expression (Mortimer et al., 1996), modulation of D2/D3 and THT expression by T_3/T_4 could be a phenomenon serving as a defense mechanism for the fetus in pregnancies where the mother courses with hypothyroxemia.

ISOLATED MATERNAL HYPOTHYROXEMIA AND CLINICAL HYPOTHYROIDISM

The pathologies associated with low levels of free T_4 correspond to IMH and clinical hypothyroidism. IMH (1–2% of normal pregnancies) is characterized by low free T_4 (<10th percentile in normal range), but normal TSH level. Instead, clinical hypothyroidism characterizes by high levels of TSH, but low levels of free T_4 (Casey et al., 2005). In another pathological condition referred as "low T_3 syndrome," an increase in the expression of the membrane transporters MCT8 is reported, which could be a compensatory response to low levels of thyroid hormones (Mebis et al., 2009). The latter seems paralleled by an increase in the D1 and D2 levels as reported in human skeletal muscle and liver (Peeters et al., 2003; Weetman et al., 2003). While, using a *knock-out* mice model for MCT8 (*Mct8*$^{-/-}$) an increase in the plasma free T_4 and T_3 levels, and D1 and D2 expression and activity in the liver was shown (Dumitrescu et al., 2006). Therefore, these results support the fact that low levels of T_4 lead to changes in the THT and deiodinase expression and activity in target organs. However, there is no information addressing this possibility in the human placenta from pregnancies coursing with maternal hypothyroxemia.

On the other hand, minor changes such as D2 gene polymorphism (Thr92Ala) are associated with human type 2 diabetes mellitus and insulin resistance (Mentuccia et al., 2002). In this regard, despite there is not information regarding D2 gene polymorphism in women coursing with pregnancies without a diagnosis of thyroid gland pathology, a negative correlation between free T_4 level and metabolic markers of GD and insulin resistance (i.e., degree of glycosylated HbA_{1c}, fasting insulin, and HOMA-IR) has been shown (Bassols et al., 2011). Then, it is proposed that a potential relationship between low maternal free T_4 levels and occurrence of GD and perhaps its complications including endothelial dysfunction exists.

MATERNAL T_4 LEVEL AND GD

GD is a disease coursing with glucose intolerance first recognized or manifested during pregnancy [Metzger et al., 2007; Reece et al., 2009; American Diabetes Association [ADA], 2012]. This pathology accounts for ~5% of pregnant women worldwide and it is associated with high risk of fetal perinatal alterations (e.g., macrosomia, insulin resistance) and higher incidence of diseases in the adulthood (e.g., GD, obesity, dyslipidemia, hypertension, metabolic syndrome) (Poston, 2010; Negrato et al., 2012). GD is associated with reduced maternal circulating T_4 levels in the first trimester of pregnancy. To date, 5% of women coursing with GD pregnancies have been shown to correlate with IMH (Krcma et al., 2010). In addition, free T_4 levels are lower in women with GD pregnancy compared with women with normal pregnancies (Velkoska-Nakova et al., 2010) and a reduced free T_4 level is shown in 70% of patients with GD pregnancies (Olivieri et al., 2000). Therefore, maternal hypothyroxemia could be associated with GD.

Other pathologies associated with low levels of free T_4, such as clinical hypothyroidism (i.e., low free T_4 and high TSH levels), also have been associated with GD. Indeed, 6–15% of GD pregnancies are associated with hypothyroidism (Velkoska-Nakova et al., 2010; Tudela et al., 2012; Stohl et al., 2013). Moreover, if pregnant women have hypothyroidism, they have 4.3-fold higher risk for developing GD (Karakosta et al., 2012). There are no publications addressing the T_4 plasma levels at the fetal circulation in a pregnancy coursing with GD. However, since GD courses with endothelial dysfunction (De Vriese et al., 2000; Westermeier et al., 2011; Guzmán-Gutiérrez et al., 2011, 2014; Salomón et al., 2012) and thyroid hormones modulate endothelial function (Napoli et al., 2001; Fazio et al., 2004), it is likely that a low free T_4 level at the maternal circulation eventually could result in altered endothelial function in GD pregnancies.

GD AND ENDOTHELIAL DYSFUNCTION

One of the main alterations detected in GD pregnancies is the associated endothelial dysfunction of the fetoplacental circulation (De Vriese et al., 2000; Guzmán-Gutiérrez et al., 2011, 2014; Westermeier et al., 2011; Salomón et al., 2012). Since the vasculature in the human placenta lacks innervation (Marzioni et al., 2004), several local metabolic mechanisms, such as synthesis and release of vasoactive molecules (e.g., NO, adenosine) (Vásquez et al., 2004; Guzmán-Gutiérrez et al., 2011, 2014; Sobrevia et al., 2011) or release of nanovesicles (e.g., exosomes), most likely mediating autocrine and/or paracrine modulation of vasculature (Salomon et al., 2013), could lead to acute and rapid modulation of vascular tone in this vascular bed (Guzmán-Gutiérrez et al., 2011, 2014; Burnstock and Novak, 2013).

Arteries and veins in the human placenta from pregnancies with GD exhibit increased NO synthesis (Figueroa et al., 2000). Furthermore, similar results were early reported in primary cultures of HUVEC from pregnant women diagnosed with GD (Sobrevia et al., 1995). Therefore, it has been proposed that vascular dysfunction in GD could result from a functional dissociation between NO synthesis and its bioavailability in the human placental circulation (Guzmán-Gutiérrez et al., 2011, 2014; Sobrevia et al., 2011). Even when endothelial dysfunction, referred to as an alteration of NO synthesis and the uptake of cationic amino acid L-arginine (i.e., L-arginine/NO pathway), is associated with GD, a clear mechanism behind these effects of GD is still unavailable (Guzmán-Gutiérrez et al., 2014).

HUMAN PLACENTA ENDOTHELIAL FUNCTION AND T4 IN GD: A HYPOTHESIS TO BE TESTED

In normal pregnancies maternal free T_4 is taken up by THT by the syncytiotrophoblast, where it is metabolized by D2 and D3 to be converted into T_3 or rT_3, respectively. T_4 is then released via THT at the basolateral membrane of the syncytiotrophoblast into the intervillous space from where it is taken up by the microvascular endothelial cells via THT. In these cells a fraction of T_4 is metabolized again to produce T_3 and rT_3 via D2 and D3, respectively (**Figure 2**). As a result of this process, T_4 and T_3 are released into the fetal blood. However, currently there is no information regarding transport of thyroid hormones across the human placenta in GD pregnancies. We propose that low levels of T_4, lead to an increase in the number and activity of

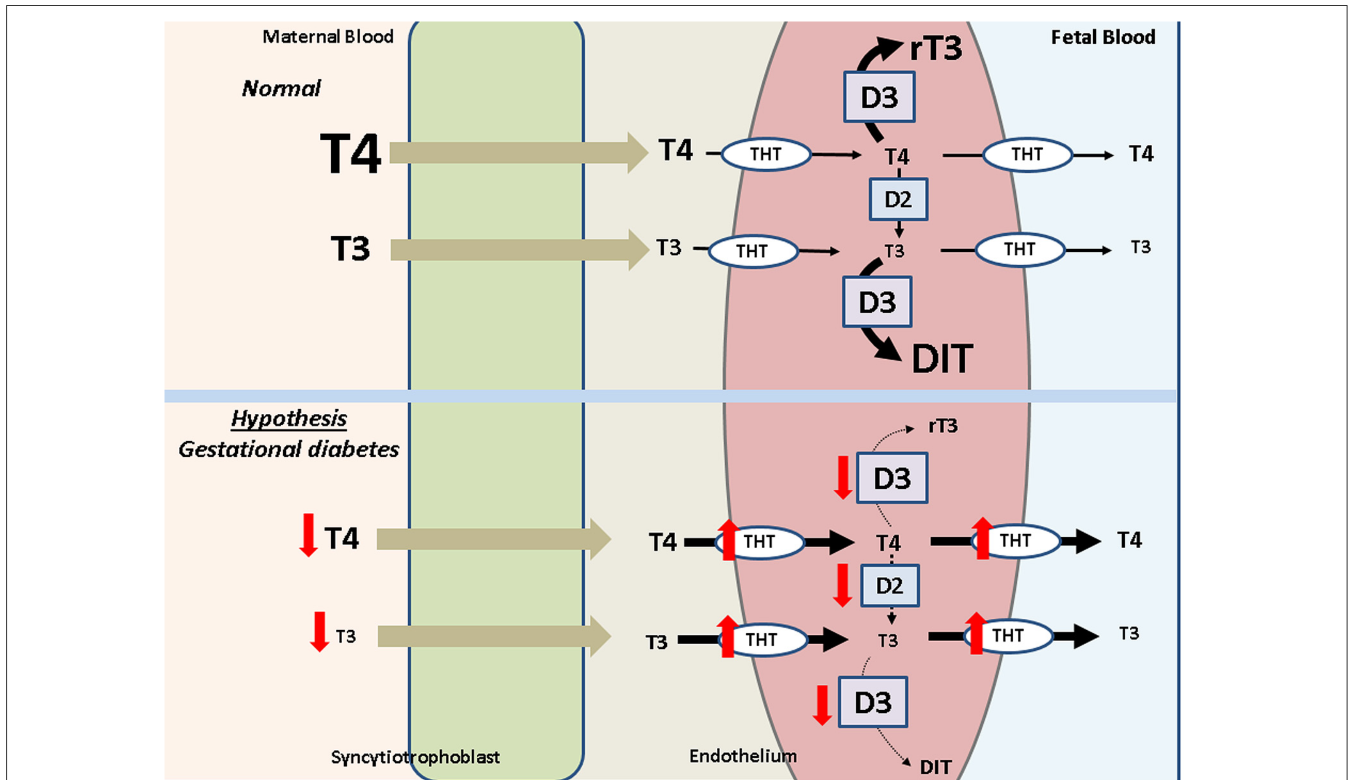

FIGURE 2 | Potential thyroid hormones transplacental transport in gestational diabetes. In cells from normal pregnancies (*Normal*) T3 and T4 in the maternal blood are transported to the fetal blood by thyroid hormone transporters (THT) through the microvascular endothelium (*Endothelium*). T3 and T4 are metabolized by deiodinases 2 (D2, forming T3) and 3 (D3, forming reverse T3 9 [T3 from T4 and diiodothyronine (DIT) from T3)], thus leading to modulation of these hormones delivery to the fetal blood. As hypothesis, in *Gestational diabetes* the mother courses with reduced T3 and T4 levels (red arrows). There is an increase THT and reduced D2/D3 activity, thus compensating T4 level in the fetal blood.

THT membrane transporters available at the plasma membrane of the human placental endothelial cells, and reduced deiodinase expression and activity, in order to supply T_4 necessities associated with fetal development in GD. These changes could constitute a mechanism by which the endothelium from the human placenta intends to maintain normal intracellular and circulating levels of T_4 in the fetus. The latter would be potentially reached by a greater delivery of maternal T_4 to the fetal blood.

FINAL COMMENTS AND CONCLUSION

Based on what was described in this review, our central research questions are: (1) is a low level of free T_4 in the maternal circulation associated with GD? (2) is GD a disease associated with increased placental THT, but reduced deiodinase expression and activity? and (3) would the potential changes caused by reduced free T_4 level in the maternal circulation and altered THT and deiodinases in the placenta in GD lead to placental endothelial dysfunction? Furthermore, nothing is known regarding the feto-placental vascular function/dysfunction in pregnancies where the mother courses with hypothyroxemia. Despite many benefits for using human placental tissue after birth, we acknowledge that information behind cellular mechanisms and adaptative response occurred at the beginning of pregnancy is difficult to extrapolate; however, it offers a good approximation for studying consequences of human pathologies. Potential more complex models, might include analysis of placentas collected from animal deficient in leptin receptor (*db*/+), since they develop GD during pregnancy (Bobadilla et al., 2010), offering a model that may to understand molecular mechanisms of THT and deiodinases in first trimester of pregnancy. Moreover, a therapeutical approach of pregnant women coursing with hypothyroxemia targeted to improve free T_4 circulating levels will likely reduce the risk of developing GD and the deleterious consequences of this disease in the feto-placental endothelial function. We also speculate that a normalization of free T_4 levels in the first trimester of pregnancy could reduce the risk to face GD-associated complication.

AUTHOR CONTRIBUTIONS

Enrique Guzmán-Gutiérrez and Luis Sobrevia generated the text and figures, Carlos Veas, Andrea Leiva, and Carlos Escudero contributed for design of text.

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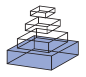

Impaired adenosine-mediated angiogenesis in preeclampsia: potential implications for fetal programming

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Preeclampsia is a pregnancy-specific syndrome, defined by such clinical hallmarks as the onset of maternal hypertension and proteinuria after 20 weeks of gestation. The syndrome is also characterized by impaired blood flow through the utero-placental circulation and relative placental ischemia, which in turn, may generate feto-placental endothelial dysfunction. Endothelial dysfunction in offspring born from preeclamptic pregnancies has been associated with an increased risk of cardiovascular disease, including hypertension, later in life. Interestingly, diminished endothelial function, manifested by low angiogenic capacity, leads to hypertension in animal studies. Recently, we have shown that the adenosine receptor A_{2A}/nitric oxide/vascular endothelial growth factor axis is reduced in human umbilical vein endothelial cells derived from preeclamptic pregnancies, an effect correlated with gestational age at onset of preeclampsia. We and others suggested that impaired vascular function might be associated with high cardiovascular risk in offspring exposed to pregnancy diseases. However, we are not aware of any studies that examine impaired adenosine-mediated angiogenesis as a possible link to hypertension in offspring born from preeclamptic pregnancies. In this review, we present evidence supporting the hypothesis that reduced adenosine-mediated angiogenesis during preeclamptic pregnancies might be associated with development of hypertension in the offspring.

Keywords: adenosine receptors, angiogenesis, placenta, preeclampsia, programming

INTRODUCTION

Preeclampsia is a major cause of maternal and infant morbidity and mortality worldwide (Xiong et al., 2002; Duley, 2009). Stillbirth is more common in preeclamptic pregnancies, and one third of infants of preeclamptic women exhibit growth restriction. Furthermore, the appropriate management of preeclampsia (i.e., delivery of women with increasingly severe disease) is responsible for 8% of preterm births, with attendant increased morbidity and mortality (Sibai et al., 2005; Villar et al., 2006). In the last 30 years, it has become evident that the fetal intrauterine environment has long-lasting consequences for the infant. Low or high birth weight, prematurity, gestational diabetes, and hypertension not only have an impact on perinatal outcomes; they also have long-term consequences, increasing the risk of neurological disability, obesity, and cardiovascular disease in adult life (Gluckman and Hanson, 2004; Hanson and Gluckman, 2008; Krause et al., 2009). The multi-organ dysfunction syndrome associated with preeclampsia could, directly or indirectly, affect the intrauterine environment.

The underlying pathophysiology of preeclampsia includes dysregulation of endothelial function in both the maternal and the feto-placental circulation (Roberts and Escudero, 2012). Several groups have suggested that long-term complications in offspring from preeclamptic pregnancies might be associated with loss of the endothelium's ability to regulate vascular tone by synthesizing vasoactive molecules. However, the endothelium is also responsible for the generation of new vessels through the process of angiogenesis. Imbalance of angiogenic factors (i.e., reduction in the activity of pro-angiogenic factors in association with high activity/expression of the anti-angiogenic factors) is a well-characterized feature of preeclampsia. The implication of this imbalance for the occurrence of long-term complications in offspring from preeclamptic pregnancies is not yet clear. Since adequate formation of blood vessels is required for controlling blood pressure and for tissue repair, it is likely

Abbreviations: NECA, 5′-*N*-ethylcarboxamido-adenosine; AP-1, Activator protein 1; AR, adenosine receptor; A_{2A}AR, adenosine receptor A_{2A}; A_{2B}AR, adenosine receptor A_{2B}; bFGF, basic fibroblast growth factor; EOPE, early-onset preeclampsia; eNOS, endothelial nitric oxide synthase; EPC, endothelial progenitor cells; hENT1, equilibrative nucleotide transporter type 1; hENT2, equilibrative nucleotide transporter type 2; Epac, exchange protein activated by cAMP ; ERK, extracellular signal-regulated kinase; hPMEC, human placental microvascular endothelial cell; HUVEC, human umbilical vein endothelial cell; HIF-1α, hypoxia inducible factor type 1 α; IL-8, interleukin 8; LOPE, late-onset preeclampsia; MAPK, mitogen-activated protein kinase; LLC, mouse Lewis lung carcinoma; NO, nitric oxide; PKA, protein kinase A; sFlt-1, soluble vascular endothelial growth factor receptor type 1 or soluble fms-like tyrosine kinase-1; sEnd, soluble endoglin; VEGF, vascular endothelial growth factor.

that impaired angiogenesis may contribute to future cardiovascular risk in the newborn "exposed" to preeclampsia *in utero.*

Adenosine is a naturally occurring nucleoside, which is increased in the feto-placental circulation with preeclampsia (Yoneyama et al., 1996; Escudero et al., 2009; Espinoza et al., 2011). It plays an important role in controlling the production and action of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), as well as anti-angiogenic factors including soluble fms-like tyrosine kinase-1 (sFlt-1; George et al., 2010) and, through this mechanism, may control placental angiogenesis (Escudero et al., 2012). Therefore, it is plausible that dysfunctional adenosine-mediated angiogenesis *in utero* and after birth may contribute to long-term complications in offspring from preeclamptic pregnancies.

PATHOPHYSIOLOGY OF PREECLAMPSIA: CURRENT HYPOTHESES

Preeclampsia has been defined by the onset of hypertension and proteinuria after 20 weeks of gestation and is additionally characterized by maternal endothelial dysfunction (Roberts et al., 1989). However, recently The American College of Obstetricians and Gynecologists has stated that proteinuria is no longer absolutely required for diagnosis of preeclampsia (ACOG, Task Force of Hypertension in Pregnancy, 2013). Alternatively, diagnosis may be established by the presence of hypertension associated with thrombocytopenia (platelet count less than 100.000/μL), impaired liver functions (elevated blood concentrations of liver transaminases up to twice the normal concentration), development of renal insufficiency (serum creatinine concentration greater than 1.1 mg/dL or a doubling of the serum creatinine concentration in the absence of other renal diseases), pulmonary edema, or new-onset cerebral or visual disturbances.

Current thinking on the pathophysiology of preeclampsia suggests that impaired invasion of trophoblastic cells into the maternal vascular bed leads to aberrant transformation of uterine resistance vessels to large diameter capacitance vessels (Burton et al., 2009a,b). This reduces maternal blood flow to the placenta and generates relative ischemia. Failure to increase the terminal caliber of the spiral arteries results in increased velocity of blood entering the intervillus space, with consequent shear stress on the fetal villus trophoblast (Burton et al., 2009b), leading to cell damage, detachment, and the release of cell fragments into the maternal circulation (Tannetta et al., 2013). These fragments contain elements that may impair maternal endothelial function and generate a vicious cycle that will chronically affect maternal and feto-placental endothelial function.

Among molecules released from the placenta, sFlt-1 has become a focus of study in preeclampsia. It is increased in the blood of women prior to and during clinical preeclampsia and has the potential to blunt angiogenic responses. Importantly, reduction of the plasma level of sFlt-1 in women with preterm preeclampsia using dextran sulfate apheresis has been reported to reduce proteinuria and blood pressure and prolong pregnancy by 23 days without apparent adverse events for mother or fetus (Thadhani et al., 2011). Moreover, the injection of mice with adenovirus expressing sFlt-1 results in pathophysiological findings resembling preeclampsia (Maynard et al., 2003; Bytautiene et al., 2011; Murphy et al., 2012). This animal model has been employed to study not only the pathophysiology of preeclampsia but also vascular alterations in the offspring (Lu et al., 2007a; Byers et al., 2009; Bytautiene et al., 2011).

DEVELOPMENTAL ORIGINS OF ADULT DISEASE AFTER PREECLAMPSIA

The fetal programming hypothesis proposes that chronic diseases may originate through adaptations of the fetus to an adverse intrauterine environment. These adaptations may include changes in the vascular, metabolic, or endocrine systems. Those changes permanently affect function in adult life.

Numerous epidemiological studies suggest an important role for the adverse intrauterine environment in the development of schizophrenia, depression, cardiovascular diseases, stroke, diabetes, cancer, pulmonary hypertension, osteoporosis, polycystic ovarian syndrome, and other conditions in adult life. These observational relationships are supported by animal experiments in which effects on fetal growth via manipulation of maternal nutrition or reduction of blood flow to the placenta (by various approaches) result in obesity, increased blood pressure, and other cardiovascular abnormalities in the offspring later in life (Hanson and Gluckman, 2008; Glover, 2011; Davis et al., 2012b). Applying this concept to preeclampsia brings in a number of other insults which may trigger programming. These include increased oxidative stress and elevated concentration of anti-angiogenic factors, which can also result in growth restriction or premature deliveries.

PREECLAMPSIA AND LONG-TERM ADVERSE OUTCOMES IN THE OFFSPRING

Many epidemiological studies (Kajantie et al., 2009; Wu et al., 2009, 2011; Davis et al., 2012a,b; Lawlor et al., 2012) indicate that preeclampsia is associated with long-term adverse outcomes in the offspring. The majority of studies (Palti and Rothschild, 1989; Seidman et al., 1991; Tenhola et al., 2003, 2006; Vatten et al., 2003; Swarup et al., 2005; Hiller et al., 2007; Oglaend et al., 2009; Kvehaugen et al., 2010; Lazdam et al., 2010; Palmsten et al., 2010; Lawlor et al., 2012), but not all (Ounsted et al., 1983; Jayet et al., 2010; Belfort et al., 2012; Lawlor et al., 2012) report that children and adolescents who were exposed to preeclampsia or hypertension in pregnancy exhibit higher systolic and diastolic blood pressure compared with non-exposed children or adolescents. These studies were reviewed in a recent meta-analysis (Davis et al., 2012a), which included individuals aged 4–30 years, born at term from preeclamptic pregnancies. This meta-analysis concluded that offspring born from preeclamptic women had ∼2 mm Hg greater systolic and ∼1.3 mm Hg greater diastolic blood pressure than individuals born from normotensive pregnancies. Interestingly, according to their prediction, "if the 2.4 mmHg difference in systolic blood pressure tracks into adulthood (Chen and Wang, 2008), this difference would be associated with an ∼8% increased risk of mortality from ischemic heart disease and 12% increased risk from stroke" (Davis et al., 2012a). Based on a study in a large population of preeclamptic pregnancies, Kajantie et al. (2009) reported that

the risk for stroke in subjects born from preeclamptic pregnancies was twice that of controls born from normotensive pregnancies. Other studies have described an increased risk for pulmonary hypertension (Jayet et al., 2010), metabolic and endocrine disease (Wu et al., 2009, 2011), depression (Tuovinen et al., 2010), cerebral palsy (Szymonowicz and Yu, 1987), poor cognitive outcome (Cheng et al., 2004), or intellectual disabilities (Griffith et al., 2011) in children born of preeclamptic pregnancies compared to non-exposed children.

These clinical and epidemiological observations are supported by a recent review of animal models of preeclampsia (Davis et al., 2012b), including those generated by systemic hypoxia, by mechanical reduction of maternal uterine artery blood flow, in genetically modified animals lacking endothelial nitric oxide synthase (eNOS), or by overexpression of sFlt-1 by infection with adenovirus carrying this protein. Notwithstanding differences in design and outcome of these models, the conclusion was that "animal studies support the potential relevance of these insults to programming of offspring blood pressure."

Although fetal programming by preeclampsia is suggested by human and animal studies, it is not easy to determine whether preeclampsia *per se* leads to high cardiovascular risk in the offspring or whether related factors, such as intrauterine growth restriction or preterm delivery, contribute. To allay these concerns, most studies have excluded individuals exposed to preterm delivery or intrauterine growth restriction associated with preeclampsia. Interestingly, the risks for hypertension, impaired neurological function, and stroke in offspring from preeclamptic pregnancies remain significant (Kajantie et al., 2009; Tuovinen et al., 2010, 2012). Moreover, a study performed in brothers exposed, or not, to preeclampsia suggested that impaired vessel function was associated with preeclampsia *per se* rather than genetic predisposition (Jayet et al., 2010). It is plausible, then, that exposure to preeclampsia *in utero* can predispose to adverse outcomes later in life.

Understanding the underlying mechanisms might suggest interventions to prevent the occurrence of future chronic disease in offspring exposed to preeclampsia. Several groups (Jayet et al., 2010; Lazdam et al., 2010; Kvehaugen et al., 2011; Davis et al., 2012a,b; Lawlor et al., 2012), including ours (Escudero and Sobrevia, 2008; Escudero et al., 2012), have presented evidence of endothelial dysfunction in the feto-placental circulation in preeclampsia, which may be a precursor to the long-term complications observed in offspring born of preeclamptic pregnancies.

ENDOTHELIAL DYSFUNCTION AND IMPAIRED ANGIOGENESIS IN OFFSPRING BORN FROM PREECLAMPTIC PREGNANCIES

Endothelial dysfunction is a pathological state characterized by an imbalance between vasodilators and vasoconstrictors produced by (or acting on) the endothelium (Brunner et al., 2005). Infants born of preeclamptic pregnancies have evidence of endothelial dysfunction shortly after delivery and months to years later (Davis et al., 2012a,b; Sobrevia et al., 2012; Wadsack et al., 2012). For instance, reduced flow-mediated vasodilatation (a hallmark of endothelial dysfunction) has been reported in children born from preeclamptic pregnancies as compared to children born from normotensive pregnancies (Jayet et al., 2010; Lazdam et al., 2010; Kvehaugen et al., 2011; Davis et al., 2012b). In animal studies using mice, male offspring born from mothers with preeclamptic-like syndrome, generated by administration of adenovirus carrying sFlt-1, exhibited high blood pressure (Lu et al., 2007a,b) and increased vascular reactivity (Byers et al., 2009; Bytautiene et al., 2011) compared to controls.

It is also clear that endothelial cells are main contributors to angiogenesis (Shibuya, 2006; Escudero et al., 2009), which leads to the growth of new blood vessels from pre-existing ones. The endothelium participates in angiogenesis through several processes, which include cell proliferation/migration, tube formation, as well as synthesis and release of pro-angiogenic factors including VEGF (Shibuya, 2013). In addition, neovasculogenesis, a process of blood vessel formation occurring by *de novo* production of endothelial cells, can occur not only at the embryonic stage but also in adult life (Risau, 1997). Endothelial progenitor cells (EPCs) play a critical role in postnatal blood vessel formation and vascular homeostasis. In preeclampsia, impaired feto-placental angiogenesis (Escudero et al., 2009, 2012, 2014) and neovasculogenesis (due to the reduced number of EPCs found in umbilical cord blood (Kwon et al., 2007; Xia et al., 2007; Monga et al., 2012) may be a result of endothelial dysfunction.

Reduced placental levels of several pro-angiogenic factors have been reported in the feto-placental circulation in early-onset preeclampsia (EOPE) when these are compared to late-onset preeclampsia (LOPE) or to age-matched controls (Gellhaus et al., 2006; Junus et al., 2012). Microarray analysis reveals lower expression of at least two angiogenesis-associated transcripts (Egfl7 and Acvrl1) in EOPE compared to LOPE or age-matched controls (Junus et al., 2012). Recently, we have reported that the proliferation/migration of human umbilical vein endothelial cells (HUVEC) is reduced in EOPE compared to LOPE or controls, whereas cells from LOPE exhibit elevated proliferation/migration compared to controls (Escudero et al., 2012). These reports suggest that angiogenesis could be modified in the feto-placental circulation in preeclampsia.

The mechanisms underlying impaired neovascularization in feto-placental circulation during preeclampsia are under investigation and may be associated with the reduced numbers of EPCs observed in umbilical blood (Kwon et al., 2007; Xia et al., 2007; Monga et al., 2012), an increase in circulating anti-angiogenic factors such as sFlt-1 (Staff et al., 2005; Tsao et al., 2005) and soluble endoglin (sEnd; Staff et al., 2007), or reduced expression and activity of pro-angiogenic signals such as VEGF (Lyall et al., 1997; Andraweera et al., 2012; Kim et al., 2012) or adenosine (Escudero et al., 2012). As presented in **Figure 1**, this imbalance is manifested mainly by elevation of sFlt-1and sEnd, associated with reduced numbers of EPCs, which may nullify pro-angiogenic signals from VEGF and placental growth factor (PlGF).

Several prior studies have examined feto-placental tissue; however, few studies (see for instance Staff et al., 2005; Tsao et al., 2005; Kvehaugen et al., 2010, 2011) have assessed endothelial dysfunction postnatally in newborn infants or children exposed to

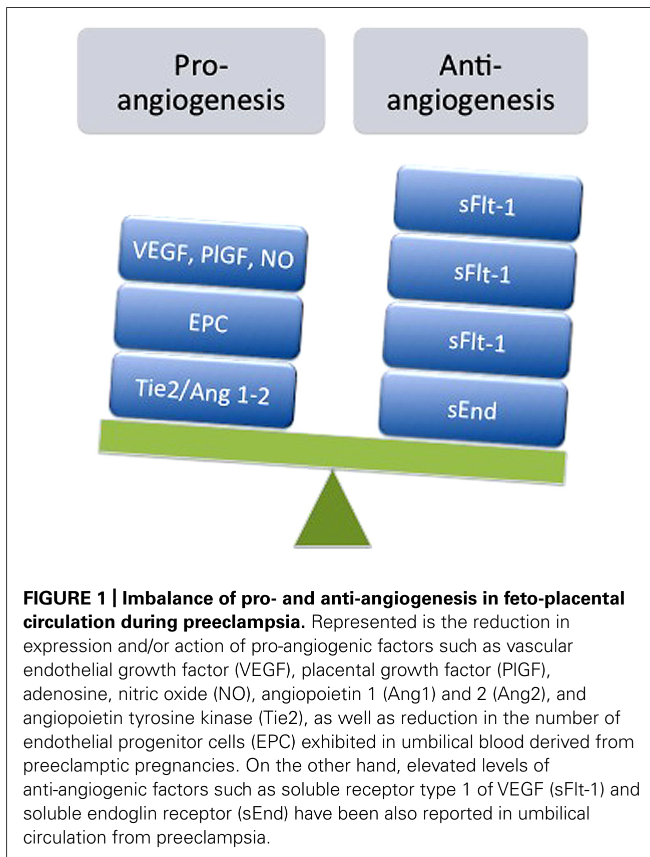

FIGURE 1 | Imbalance of pro- and anti-angiogenesis in feto-placental circulation during preeclampsia. Represented is the reduction in expression and/or action of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), placental growth factor (PlGF), adenosine, nitric oxide (NO), angiopoietin 1 (Ang1) and 2 (Ang2), and angiopoietin tyrosine kinase (Tie2), as well as reduction in the number of endothelial progenitor cells (EPC) exhibited in umbilical blood derived from preeclamptic pregnancies. On the other hand, elevated levels of anti-angiogenic factors such as soluble receptor type 1 of VEGF (sFlt-1) and soluble endoglin receptor (sEnd) have been also reported in umbilical circulation from preeclampsia.

preeclampsia. Considering that placental blood vessels on the fetal side form a continuous network with fetal systemic circulation, it is pertinent to ask whether offspring exposed to preeclampsia exhibit endothelial dysfunction and impaired angiogenesis after birth.

To the best of our knowledge, there is no direct answer to this question. However, indirect evidence includes increased concentrations of sFlt-1 in the fetus from preeclamptic pregnancies as measured in umbilical cord blood (Staff et al., 2005; Tsao et al., 2005). Offspring with high blood pressure whose parents also had high blood pressure showed fewer capillaries in the dorsum of the finger compared with either offspring with low blood pressure whose parents were either normotensive or hypertensive or hypertensive offspring whose parents were normotensive (Noon et al., 1997). More recently, Yu et al. (2012) reported that children born from preeclamptic pregnancies exhibited a 45% reduction in the risk of retinopathy of prematurity, a well-described example of pathological angiogenesis in premature infants, compared to preterm babies born from normotensive pregnancies. Moreover, Stark et al. (2009), studying blood flow immediately after birth in the microcirculation of children born from preeclamptic pregnancies, found altered fetal microvascular structure and function, particularly in male newborns.

In animal studies, at postnatal day 7, rat pups from spontaneously hypertensive mothers, exposed to hypoxic-ischemic brain injury, exhibited less brain damage than pups from normotensive mothers (Letourneur et al., 2012). Interestingly, this apparently protective phenomenon was associated with deficits in motor coordination and spatial learning in pups from hypertensive, compared to normotensive mothers. These results could be interpreted as a consequence of impaired angiogenesis. This could reduce the area of the lesion but also impair tissue recovery after ischemic insult in the brain. Therefore, it is plausible that offspring born from preeclamptic pregnancies may exhibit reduced angiogenic processes after birth, which may lead to cardiovascular complications later in life.

The latter concept is supported by the following findings: (1) VEGFR2 gene expression decreases with development (Greene et al., 2011). Also, vessel branching in the brain increases until 10 days postpartum and stabilizes to adult levels between days 10 and 25 in mice (Harb et al., 2013); (2) mature (4–5 month old) mouse brains lose their ability to undergo angiogenesis in response to hypoxia (Harb et al., 2013), suggesting that the process of angiogenesis, even in stressful conditions, is limited after birth; (3) VEGF production and activity are both impaired in the feto-placental circulation during preeclampsia (Lyall et al., 1997; Andraweera et al., 2012; Kim et al., 2012); (4) Inhibition of angiogenesis with humanized antibodies targeting VEGF or orally active small tyrosine kinase inhibitors targeting VEGF receptors is commonly associated with severe hypertension (Lankhorst et al., 2013); (5) Loss of microvessel growth has been reported to precede elevations in blood pressure (Murfee and Schmid-Schonbein, 2008); (6) Programming of elevated blood pressure in the offspring has been associated with a reduced angiogenic capacity of vessels cultured *in vitro* (Pladys et al., 2005). Taking all these data into account, we believe that abnormal angiogenic processes present after birth in offspring born from preeclamptic pregnancies may contribute to elevation in blood pressure later in life.

OVERVIEW OF ADENOSINE RECEPTORS

ADENOSINE RECEPTORS AND ANGIOGENESIS

Adenosine is a purinergic nucleoside which controls several physiological processes, including angiogenesis and vasculogenesis. Adenosine activates a family of G-coupled adenosine receptors, A_1AR, A_{2A}AR, A_{2B}AR, and A_3AR (Olah and Stiles, 2000; Jacobson and Gao, 2006). All of the adenosine receptors have been implicated in the modulation of angiogenesis (see **Table 1**). Briefly, stimulation of A_1AR on embryonic EPCs promotes their adherence to the vascular endothelium, suggesting an important role for this receptor subtype in vasculogenesis (Ryzhov et al., 2008). A_1AR have also been reported to upregulate VEGF production from monocytes, thus promoting angiogenesis (Clark et al., 2007).

Depending on the tissue or cell studied, A_{2A}AR and A_{2B}AR can play a dominant role in the regulation of angiogenic factors. For example, A_{2B}AR upregulates the pro-angiogenic factors VEGF, basic fibroblast growth factor (bFGF), insulin-like factor-1, and interleukin 8 (IL-8) in human microvascular endothelial cells (Grant et al., 1999; Feoktistov et al., 2002). Conversely, A_{2A}AR is reported to upregulate VEGF in macrophages (Leibovich et al., 2002; Pinhal-Enfield et al., 2003). Stimulation of A_3AR in mast cells and some tumors can result in upregulation of pro-angiogenic factors, complementing the actions of adenosine mediated via A_{2B}AR (Feoktistov et al., 2003). Of interest,

stimulation of $A_{2A}AR$ in HMEC-1 inhibits the release of the anti-angiogenic factor thrombospondin 1, providing yet another means by which adenosine may regulate angiogenesis (Desai et al., 2005; see **Table 1**).

While $A_{2A}AR$ and $A_{2B}AR$ have been shown to mediate the proliferative actions of adenosine in human retinal microvascular endothelial cells (Grant et al., 1999, 2001), HUVEC (Feoktistov et al., 2004; Escudero et al., 2012), or porcine coronary artery and rat aortic endothelial cells (Dubey et al., 2002), it remains unclear whether A_1AR and A_3AR are functionally expressed and what role, if any, they play in endothelial cells (Wyatt et al., 2002; Schaddelee et al., 2003).

ADENOSINE RECEPTORS AND INTRACELLULAR PATHWAYS DURING ANGIOGENESIS

Although some data suggest that cAMP may play a role in the pro-angiogenic effects of adenosine in certain cells (Takagi et al., 1996), other studies show that upregulation of angiogenic factors is mediated via coupling to Gq, possibly involving mitogen-activated protein kinase (MAPK) pathways (Grant et al., 1999, 2001; Feoktistov et al., 2002; Ryzhov et al., 2014). Further studies, using HMEC-1 demonstrated that adenosine receptor-dependent upregulation of VEGF production was associated with an increase in VEGF transcription, activator protein 1 (AP-1) activity and transcription factor JunB (JunB) accumulation (Ryzhov et al., 2014).

Mechanistically, $A_{2B}AR$ which are coupled to both Gs and Gq proteins (Feoktistov and Biaggioni, 1995) increase JunB protein levels and VEGF production via stimulation of protein lipase C and extracellular signal-regulated kinase (ERK), which are possibly linked by the calcium diacylglycerol guanine nucleotide exchange factor (CalDAG-GEF)–Ras-proximate-1 (Rap1) pathway (Ryzhov et al., 2014). These effects were protein kinase A (PKA)-independent because the PKA inhibitors had no effect on the $A_{2B}AR$-dependent increase in JunB protein levels and VEGF production. Because VEGF secretion and reporter promoter activity induced by the adenosine analog 5′-*N*-ethylcarboxamido-adenosine (NECA) were inhibited by the expression of a dominant, negative JunB or by JunB knockdown, these data suggest an important role for the A_{2B}

Table 1 | Summary of participation of adenosine receptor in angiogenesis using human cells.

AR	K_d (nM)	Angiogenic process	Cell type	Reference
A_1	3–30	⇧ Migration	EPC	Ryzhov et al. (2008)
A_{2A}	1–20	⇧ VEGF expression	Macrophages	Pinhal-Enfield et al. (2003), Ernens et al. (2010)
		⬇ Thrombospondin 1 expression	HMVEC	Desai et al. (2005)
		⬇ sFlt-1 release	Macrophages	Leonard et al. (2011)
		⇧ mFlt-1 expression	Macrophages	Leonard et al. (2011)
		⇧ Proliferation/migration and VEGF expression	HUVEC	Escudero et al. (2012)
A_{2B}	5.000–20.000	⇧ Permeability	HUVEC-PMN	Lennon et al. (1998)
		⇧ VEGF expression	HMVEC	Ryzhov et al. (2014)
		⇧ Migration	HREC	Afzal et al. (2003)
		⇧ VEGF, IL-8 and bFGF expression	HMEC-1	Feoktistov et al. (2002)
		⇧ Migration	EPC	Rolland-Turner et al. (2013)
		⇧ VEGF and IL-8 expression	Foam cell	Gessi et al. (2010)
		⇧ IL-8 secretion	Melanoma cells, HT29	Merighi et al. (2007, 2009)
		⇧ VEGF expression	HUVEC under hypoxia	Feoktistov et al. (2004)
		⇧ VEGF and IL-8 expression	HMEC-1	Feoktistov et al. (2003)
		⇧ Proliferation/migration and tube formation and VEGF expression	HREC	Grant et al. (1999, 2001)
A_3	> 1.000	⬇ Migration and tube formation	HUVEC	Kim et al. (2013)
		⇧ VEGF and IL-8 expression	Foam cell	Gessi et al. (2010)
		⇧ VEGF and IL-8 expression	Melanoma cells	Merighi et al. (2009)
		⇧ VEGF expression	HT29	Merighi et al. (2007)
		⇧ Angiopoietin-2 expression	HMEC-1	Feoktistov et al. (2003)

K_d (nM) for adenosine. VEGF, vascular endothelial growth factor; sFlt-1, soluble; mFlt-1, membrane-linked receptor type 1 for VEGF; IL-8, Interleukin 8; bFGF, basic fibroblast growth factor; HMVEC, human microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells; PMN, polymorphonuclear leukocyte; HREC, human retinal endothelial cells; HMEC-1, human microvascular endothelial cell line 1; EPC, endothelial progenitor cells; HT-29, human colon adenocarcinoma. Increase (⇧) and decrease (⬇) of pro or anti-angiogenic processes; AR, adenosine receptor.

receptor-dependent upregulation of JunB in VEGF production in various cell types, including endothelial cells (Ryzhov et al., 2014).

Another study, in HUVEC, reported that adenosine-mediated activation of ERK may involve an exchange protein activated by cAMP (Epac), a component of a family of cAMP-activated guanine nucleotide exchange factors for Rap GTPases (Fang and Olah, 2007). Thus, A_{2B}AR, coupled to $G\alpha_s$ promotes activation of adenylyl cyclase and an increase in intracellular cAMP. In turn, cAMP activates Epac 1, which may then activate a cascade of RapGTPase, B-Raf, and finally, ERK (Fang and Olah, 2007), demonstrating an alternative pathway for ERK activation involved in upregulation of pro-angiogenic proteins.

In addition to JunB, the mediators downstream of ERK and p38 MAPK activation may include molecules such as hypoxia inducible factor type 1 α (HIF-1α) and/or nitric oxide (NO). Using foam cells generated *in vitro*, Gessi et al. (2010) found that activation of A_3AR, A_{2B}AR, and to a lesser extent, the A_{2A} subtypes were associated with the production of VEGF induced by adenosine and hypoxia. This last effect was dependent on activation of ERK, p38 MAPK, Akt, and HIF-1α. Furthermore, adenosine has been reported to increase the synthesis of the angiogenic modulator NO in some, but not all, cultured endothelial cells (Sobrevia et al., 1996; Li et al., 1998; Wyatt et al., 2002). Whether, adenosine receptor-mediated activation of ERK-MAPK-HIF-1α increases NO is unknown. But, it has been reported that NO promotes a regulatory loop with ERK activation/deactivation (Schieke et al., 1999) and stabilization of HIF-1α and promotes HIF-1α binding to DNA (Kimura et al., 2000).

EXPRESSION OF ADENOSINE RECEPTORS IN ENDOTHELIAL CELLS DURING HYPOXIA

The expression of adenosine receptor subtypes and their function are subject to dynamic regulation by hypoxia (Bshesh et al., 2002; Eltzschig et al., 2003; Feoktistov et al., 2004). Because the A_{2B}AR promoter contains a functional binding site for HIF-1α (Kong et al., 2006), the onset of hypoxia strongly induces A_{2B}AR expression in different cell types including human dermal microvascular endothelial cells (Eltzschig et al., 2003), and HUVEC (Feoktistov et al., 2004). In addition, elevated expression of A_{2A}AR has also been reported after exposure to hypoxia in human placental homogenate (Von Versen-Hoynck et al., 2009), fetal chromaffin-derived cell line (Brown et al., 2011), and human lung endothelial cells, while no evidence of A_{2A}AR upregulation was seen in mouse endothelial cells (Ahmad et al., 2009).

Interestingly, despite the fact that all adenosine receptors contain a hypoxia response element in their promoters (St. Hilaire et al., 2009), regulation via HIF is differentially modulated. Whereas A_{2B}AR is regulated by HIF-1α, A_{2A}AR is regulated by HIF-2α, suggesting that transcriptional regulation might be part of the switch of A_{2A}AR toward A_{2B}AR expression observed in HUVEC exposed to hypoxia (Feoktistov et al., 2004). This switch may have important functional implications for regulation of angiogenesis. For example, in HUVEC, adenosine does not stimulate VEGF secretion under normoxic conditions, but hypoxia increases the expression of A_{2B}AR, which are then able to stimulate VEGF release (Feoktistov et al., 2004). Therefore, we could speculate that switching the expression of adenosine receptor toward A_{2B}AR rather than A_{2A}AR during hypoxia in the endothelium may offer some advantages in the angiogenic process, since high levels of adenosine may downregulate activation and/or expression of A_{2A}AR as mechanisms of desensitization. But at the same time, upregulation of A_{2B}AR will enhance or maintain the pro-angiogenic capacity of adenosine in conditions were high levels of this autocoid are expected. It is likely that this phenomenon may occur in preeclampsia.

ADENOSINE, ANGIOGENESIS, AND PREECLAMPSIA

ADENOSINE LEVELS DURING PREECLAMPSIA

The plasma level of adenosine is finely regulated by a series of enzymes responsible for synthesis and catabolism (see details in Escudero and Sobrevia, 2009). Compared with non-pregnant women, normal, pregnant women exhibit increased synthesis, but reduced catabolism, of adenosine (Yoneyama et al., 2000; Lee et al., 2007). Several studies have described high adenosine levels in both maternal (Yoneyama et al., 2001, 2002a,b,c) and fetal blood (Yoneyama et al., 1996; Espinoza et al., 2011; Escudero and Sobrevia, 2012) during preeclampsia, particularly in severe preeclampsia, compared with normal pregnancies (Yoneyama et al., 1996; Espinoza et al., 2011; Escudero and Sobrevia, 2012). Unexpectedly, these high levels are associated with high adenosine catabolism via adenosine deaminase 2 (ADA2; Yoneyama et al., 2002a; Kafkasli et al., 2006) as well as elevated adenosine uptake (Escudero et al., 2008). The causes and consequences of a high extracellular adenosine level in both maternal and fetal circulation during preeclampsia are unclear; however, it may be explained by an adaptive mechanism (Casanello et al., 2007; Escudero and Sobrevia, 2008, 2009, 2012; Escudero et al., 2009) associated with vasodilation or angiogenesis in preeclamptic placenta as occurs in other tissues, such as heart, muscle, or brain, in unfavorable conditions such as hypoxia (Eckle et al., 2007; Loffler et al., 2007).

The levels of adenosine in umbilical vein blood in preeclampsia (1.7 vs. 0.5 μmol/L, preeclampsia vs. normal pregnancy; Yoneyama et al., 1996; Espinoza et al., 2011) and in the culture medium of human placental microvascular endothelial cells (hPMEC) from preeclamptic pregnancies (2.7 vs. 0.6 μmol/L) are at least three times higher than in normal pregnancy (Escudero et al., 2008), making it likely that, in preeclampsia, all adenosine receptors are likely to be stimulated (Jacobson and Gao, 2006). However, only a few reports have described the effect of preeclampsia on the expression and function of adenosine receptors (Escudero et al., 2008, 2012; Kim et al., 2008; Von Versen-Hoynck et al., 2009). Thus, reduced expression of A_{2A}AR, without changes in A_{2B}AR (Escudero et al., 2008), was found in hPMEC isolated from preeclamptic placentas, whereas reduced A_{2A}AR (Escudero et al., 2012) but higher A_{2B}AR (Acurio et al., 2014) expression levels were found in HUVEC from preeclampsia. Yet, increased levels of all adenosine receptors have been reported in placental homogenate from preeclamptic placentas

compared with normal pregnancy (Von Versen-Hoynck et al., 2009).

ADENOSINE RECEPTOR ACTIVATION IN PREECLAMPSIA

It has been shown that activation of $A_{2A}AR$ leads to reduction in adenosine uptake by the equilibrative nucleotide transporter type 1 (hENT1) and hENT2, whereas $A_{2B}AR$ activation increases hENT2-mediated adenosine transport in cells from preeclamptic placentas (Escudero et al., 2008). Therefore, during preeclampsia, activation of adenosine receptors may control adenosine transport and, hence, extracellular adenosine levels. However, because adenosine levels are increased despite the elevation of total adenosine uptake, it is expected that the production of adenosine from sources such ATP or cell debris is higher in preeclampsia than in normal pregnancy (Spaans et al., 2014).

Recently, we observed reduced protein abundance of $A_{2A}AR$ in HUVEC derived from EOPE, but non-significant changes in LOPE, compared with cells from normal pregnancy. These findings were associated with a basal (i.e., without any treatment) reduction in cell migration/proliferation of HUVEC in EOPE compared with normal pregnancy or LOPE (Escudero et al., 2012). In addition, CGS-21680 (an $A_{2A}AR$ agonist) and NECA significantly increased HUVEC migration/proliferation in normal pregnancy, LOPE, and EOPE. However, considering that cells from EOPE exhibited the lowest migration/proliferation in the basal conditions, the magnitude of response to both adenosine receptor agonists in migration and proliferation tends to be higher in cells from EOPE than those from others groups. In agreement with these results, VEGF expression was significantly lower in HUVEC from EOPE, but higher in LOPE, compared to normal pregnancy. Also, CGS-21680 increases the protein abundance of VEGF in normal and EOPE-derived cells, an effect blocked by the $A_{2A}AR$ antagonist ZM-241385. Nevertheless, CGS-21680 did not affect VEGF expression in HUVEC from LOPE, but ZM-241385 led to a reduction ($41 \pm 6\%$, $p < 0.05$) in the level of this protein compared to corresponding levels at basal condition, suggesting that $A_{2A}AR$ is activated at basal condition in LOPE. Thus, $A_{2A}AR$-mediated HUVEC proliferation and migration was associated with VEGF synthesis in normal pregnancy, LOPE, and EOPE.

To elucidate potential intracellular pathways related to $A_{2A}AR$ activation, we determined that CGS-21680 increased the synthesis of NO as evidenced by activation of eNOS (i.e., the p-eNOS/eNOS ratio) and nitrite and nitrotyrosine levels in HUVEC from normal pregnancies and EOPE, but not in LOPE. The stimulatory effect observed in normal and EOPE cells was blocked by ZM-241385 co-incubation. In contrast, ZM-241385 reduced NO synthesis in LOPE cells compared to non-treated controls. Furthermore, using the non-selective nitric oxide synthase inhibitor, L-NAME, we found a significant reduction in the HUVEC migration/proliferation responses and VEGF protein levels in cells from normal pregnancies and LOPE, but not in EOPE cells stimulated with CGS-21680.

Thus, our study demonstrated that activation of $A_{2A}AR$ is associated with the following cascade: eNOS activation (i.e., ser^{1177} phosphorylation), NO synthesis, nitrotyrosine formation, VEGF expression, and cell proliferation/migration in normal pregnancy. However, cells derived from EOPE and LOPE were different in several aspects. Whereas EOPE cells exhibited low $A_{2A}AR$ expression and reduction of NO/VEGF synthesis and cell proliferation/migration; LOPE cells demonstrated increased cell proliferation/migration, mediated in part through the same pathway (see **Figure 2**). Existence of this pathway was recently confirmed using selective shRNA for $A_{2A}AR$ in HUVEC. Knockdown of $A_{2A}AR$ was associated with reduced formation of intracellular cAMP, NO metabolites, VEGF protein level, and the capacity for tube formation compared with controls (unpublished results).

As stated before, adenosine-dependent angiogenesis can be regulated by all four adenosine receptors. There is little information on the role of $A_{2B}AR$ in the angiogenic process during preeclampsia, whereas the participation of A_1AR and A_3AR in this process is unknown. In primary cultures of hPMEC, a cell type with high pro-angiogenic capacity compared to HUVEC (Dye et al., 2004), we found that $A_{2B}AR$ may be constitutively activated in cells from preeclamptic placentas, since the use of $A_{2A}/A_{2B}AR$ inhibitors in non-stimulated cells decreases adenosine uptake (Escudero et al., 2008). More recently, we found that activation of $A_{2B}AR$ in HUVEC accounts for at least 30% of the pro-proliferative response mediated by adenosine or NECA (Acurio et al., 2014). These data agree with prior reports (Feoktistov et al., 2002, 2004) that exposure of HUVEC to hypoxia increases the expression of $A_{2B}AR$, which is then able to stimulate VEGF release.

HYPOTHESIS FOR A ROLE FOR ADENOSINE IN PREECLAMPSIA

In view of available information, we can speculate that during preeclampsia, a condition associated with reduction in the expression and activity of $A_{2A}AR$, a compensatory increase in the expression and/or activity of $A_{2B}AR$ occurs that tends to compensate the impaired adenosine-mediated pro-angiogenic process. Moreover, since adenosine is pro-angiogenic, the reduction in $A_{2A}AR$ expression and down activation of $A_{2A}AR$-dependent intracellular pathway might be part of the apparent "adenosine paradox," in which increased adenosine levels do not stimulate angiogenesis in preeclampsia. The mechanism underlying this phenomenon is unclear, but may be associated with the capacity of adenosine to regulate the expression of its receptors, as exhibited in cells such as cardiomyocytes (Headrick et al., 2013) or PC12 cells (Saitoh et al., 1994). In particular, PC12 exposure to $A_{2A}AR$ agonists reduces ADORA2 gene expression (Saitoh et al., 1994), suggesting a transcriptional regulation of $A_{2A}AR$ by adenosine. Whether similar regulation is present in endothelium is unknown, but this is a possible mechanism for the pathophysiological deregulation observed in preeclampsia.

Although the intracellular signaling pathway related to adenosine receptor activation is an area of active research, only our recent study suggested a potential adenosine receptor-dependent mechanism in preeclampsia. On the basis of this study, we propose a model (**Figure 2**), in which low expression of $A_{2A}AR$ in EOPE leads to reduction in NO and VEGF expression (Escudero et al., 2012). The implication of these alterations for feto-placental angiogenesis is poorly understood, but might involve changes in the activation of HIF (Kimura et al., 2002) and changes in the promoter activity of several proteins, including anti-angiogenic factors such as thrombospondin 2 (MacLauchlan et al., 2011) or

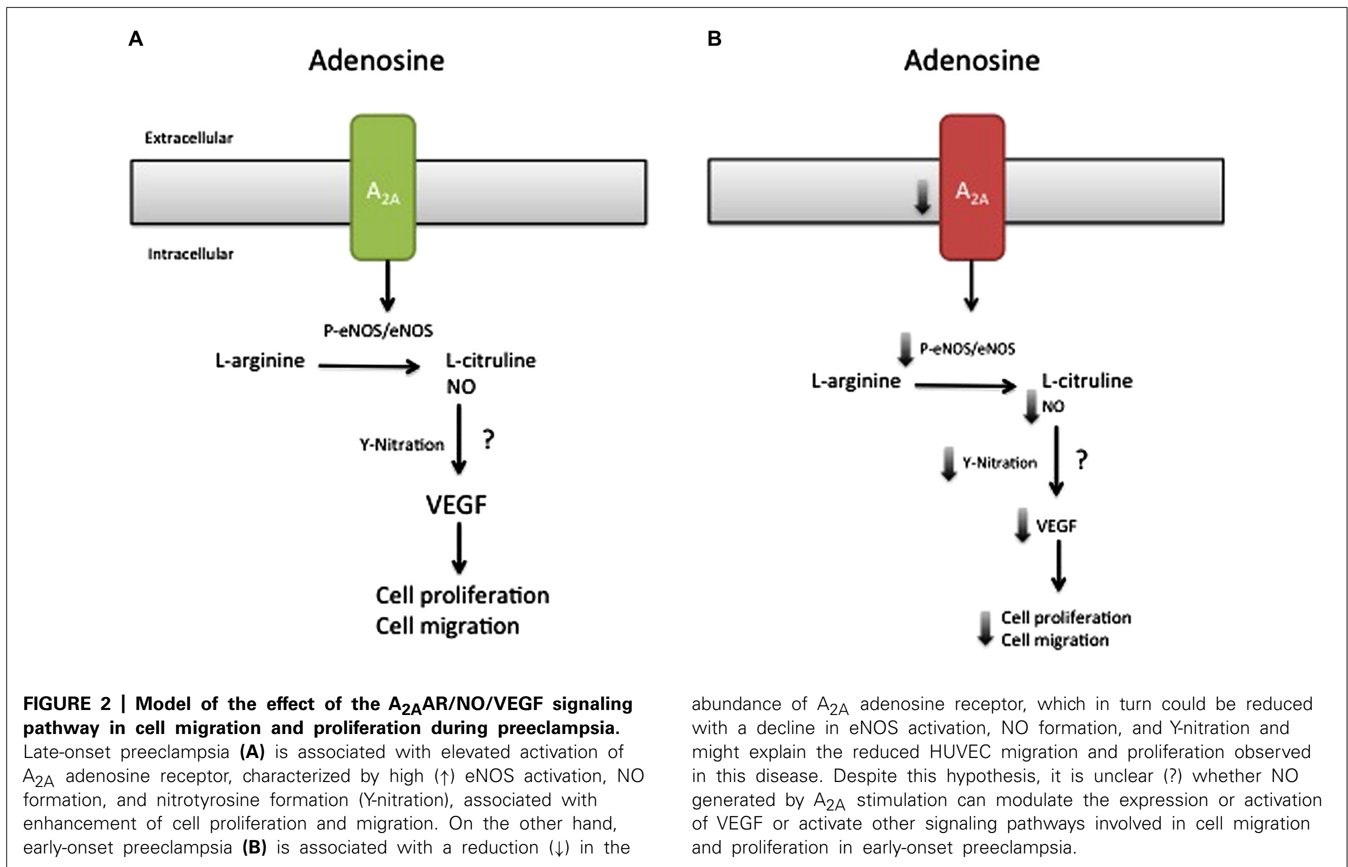

FIGURE 2 | Model of the effect of the A_{2A}AR/NO/VEGF signaling pathway in cell migration and proliferation during preeclampsia. Late-onset preeclampsia **(A)** is associated with elevated activation of A_{2A} adenosine receptor, characterized by high (↑) eNOS activation, NO formation, and nitrotyrosine formation (Y-nitration), associated with enhancement of cell proliferation and migration. On the other hand, early-onset preeclampsia **(B)** is associated with a reduction (↓) in the abundance of A_{2A} adenosine receptor, which in turn could be reduced with a decline in eNOS activation, NO formation, and Y-nitration and might explain the reduced HUVEC migration and proliferation observed in this disease. Despite this hypothesis, it is unclear (?) whether NO generated by A_{2A} stimulation can modulate the expression or activation of VEGF or activate other signaling pathways involved in cell migration and proliferation in early-onset preeclampsia.

pro-angiogenic factors like VEGF (Kimura et al., 2002; Feoktistov et al., 2004). Considering that NO can cause nitration of tyrosine residues on HIF-1α (Riano et al., 2011), and may contribute to stabilization, we propose that the reduced adenosine-mediated NO synthesis observed in EOPE might be associated with impaired HIF-dependent VEGF expression (see **Figure 3**). Clearly, more studies are necessary to understand all the processes involved in these alterations.

Another question that needs to be answered is whether impaired adenosine-mediated angiogenesis in the feto-placental circulation of preeclamptic pregnancies persists after birth. In this context, Coney and Marshall (2010) have demonstrated that prenatal hypoxia has long-lasting effects on vascular function in the skeletal muscle of adult male rats. In particular, in a group of adult males, they investigated how chronic systemic hypoxia *in utero* (CHU) affects the cardiovascular response evoked by acute, systemic hypoxia. One of the most intriguing results was the fact that the overall magnitude of vasodilator response evoked in muscle by acute systemic hypoxia is similar in CHU and normoxic rats, but the mechanisms underlying the response appear to be different. Thus, they conclude that, whereas in normoxia, vasodilatory response is associated with the activation of endothelial A_1AR and NO-dependent effects, in CHU, participation of A_1AR is limited, and vasodilatory response in the muscle is replaced by factors other than adenosine. Moreover, it has been reported that mice deficient in A_{2A}AR (KO-A_{2A}AR) exhibit no significant difference in systemic blood pressure compared to wild-type animals, but they do develop pulmonary artery hypertension and pulmonary vascular remodeling (Xu et al., 2011). Thus, these studies demonstrate how the adenosine-impaired angiogenesis and vascular remodeling observed in pathological pregnancies such as preeclampsia may be related to future cardiovascular risk.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

That fetuses exposed to preeclampsia are at increased risk to develop hypertension later in life has been associated with the occurrence of endothelial dysfunction. Since the endothelium is one of the main factors in the normal process of angiogenesis, an impaired endothelial/angiogenic response in offspring from preeclamptic pregnancies may constitute the underlying mechanism associated with hypertension. On the other hand, preeclampsia is associated with elevated levels of adenosine and low expression and response of A_{2A}AR, but high expression of A_{2B}AR, defects that might be involved in abnormal placental and newborn angiogenic processes. Then, adenosine may constitute a potential new target for improving placental angiogenesis. Furthermore, impairment of those mechanisms may contribute to susceptibility to cardiovascular diseases, including hypertension, in children exposed to preeclampsia.

As presented in this review, there are many questions that need to be answered regarding adenosine-mediated angiogenesis in preeclampsia. Therefore, future studies should consider at least the following inquires. Why A_{2A}AR expression is reduced in

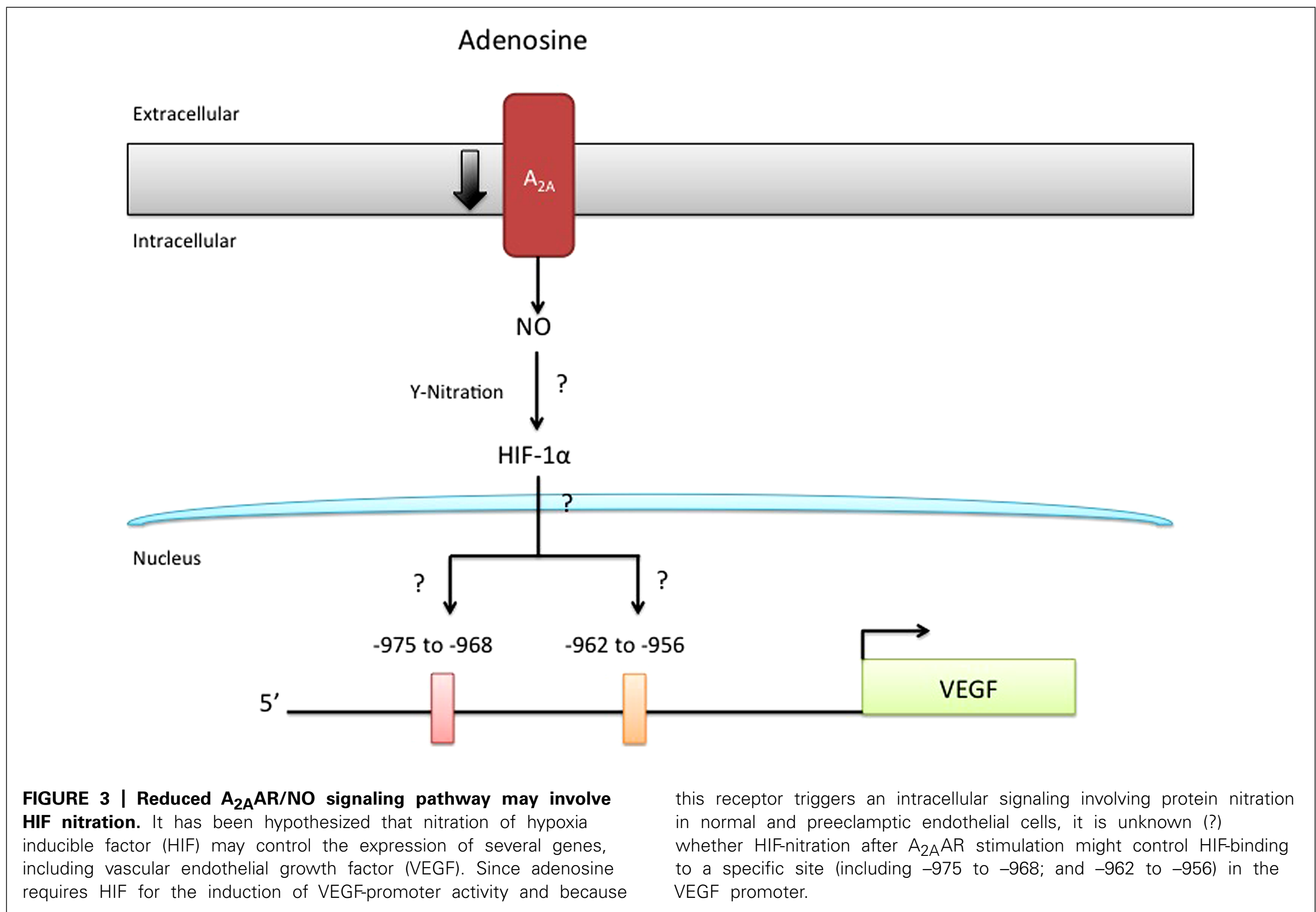

FIGURE 3 | Reduced A_{2A}AR/NO signaling pathway may involve HIF nitration. It has been hypothesized that nitration of hypoxia inducible factor (HIF) may control the expression of several genes, including vascular endothelial growth factor (VEGF). Since adenosine requires HIF for the induction of VEGF-promoter activity and because this receptor triggers an intracellular signaling involving protein nitration in normal and preeclamptic endothelial cells, it is unknown (?) whether HIF-nitration after A_{2A}AR stimulation might control HIF-binding to a specific site (including –975 to –968; and –962 to –956) in the VEGF promoter.

preeclampsia? Future studies should consider analysis of translational and transcriptional regulation of A_{2A}AR expression in endothelial cells derived from preeclampsia. Also, it should be determined whether the reduction in total A_{2A}AR levels observed in preeclampsia, leads to a reduction in the cell surface expression and in the activation of this receptor. Studies are also needed to investigate how cross-talk between intracellular pathways related to adenosine receptor activation might change during preeclampsia; and how these phenomena might generate a compensatory response via other adenosine receptors including A_{2B}AR. As highlighted in this review, it is also necessary to determine whether adenosine-mediated angiogenesis is present after birth in newborns and children exposed to preeclampsia. These studies are difficult to perform in humans due to ethical and technical issues; but certainly animal models might help. Mice deficient in each one of the adenosine receptors have been developed, providing an excellent model to address these last questions. We hope that this review will contribute to awareness, within the scientific community, of this important issue and stimulate further investigation in this area.

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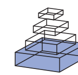

Endothelial heterogeneity in the umbilico-placental unit: DNA methylation as an innuendo of epigenetic diversity

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The endothelium is a multifunctional heterogeneous tissue playing a key role in the physiology of every organ. To accomplish this role the endothelium presents a phenotypic diversity that is early prompted during vascular development, allowing it to cope with specific requirements in a time- and site-specific manner. During the last decade several reports show that endothelial diversity is also present in the umbilico-placental vasculature, with differences between macro- and microvascular vessels as well as arterial and venous endothelium. This diversity is evidenced *in vitro* as a higher angiogenic capacity in the microcirculation; or disparity in the levels of several molecules that control endothelial function (i.e., receptor for growth factors, vasoactive mediators, and adhesion molecules) which frequently are differentially expressed between arterial and venous endothelium. Emerging evidence suggests that endothelial diversity would be prominently driven by epigenetic mechanisms which also control the basal expression of endothelial-specific genes. This review outlines evidence for endothelial diversity since early stages of vascular development and how this heterogeneity is expressed in the umbilico-placental vasculature. Furthermore a brief picture of epigenetic mechanisms and their role on endothelial physiology emphasizing new data on umbilical and placental endothelial cells is presented. Unraveling the role of epigenetic mechanisms on long term endothelial physiology and its functional diversity would contribute to develop more accurate therapeutic interventions. Altogether these data show that micro- versus macro-vascular, or artery versus vein comparisons are an oversimplification of the complexity occurring in the endothelium at different levels, and the necessity for the future research to establish the precise source of cells which are under study.

Keywords: endothelial, epigenetics, artery, vein, placenta, umbilical

INTRODUCTION

Since the discovery of the role of endothelium on vascular tone regulation at the beginning of 1980s, a countless number of studies have shown the plethora of remarkable functions that this tissue has in vascular physiology. Notably significant advances in understanding the role of endothelium have used human umbilical and placental vessels as experimental models, which is also applied to the knowledge regarding endothelial diversity. The diversity of functions that the endothelium exerts (i.e., regulation of vessel tone, angiogenesis, immune cell adhesion and migration, exchange, and haemostasis) associates with specific "zones" of the vasculature, suggesting that endothelial cells present a phenotypic heterogeneity that supports this functional diversity (Atkins et al., 2011). From the molecular point of view endothelial cells *in vivo* express several proteins which allow to distinguish between arterial and venous endothelial cells and some of these patterns are preserved *in vitro*, suggesting that long term endothelial physiology is importantly influenced by epigenetic mechanisms (Matouk and Marsden, 2008; Aird, 2012).

ORIGINS OF ENDOTHELIAL CELLS

Vasculogenesis is the process by which vessels are formed from mesenchymal-derived hemangioblasts which differentiate into endothelial cells (Demir et al., 2007). Current evidence shows that initial stages of vascular development are determined by genetic factors (le Noble et al., 2008; Atkins et al., 2011). These processes require the expression of VEGF (Shalaby et al., 1995) and activation of downstream mitogenic effectors (Parenti et al., 1998; Shizukuda et al., 1999). However, the site from which the vascular progenitors for placental and embryo vasculogenesis emerge is still debated. It is accepted that in the embryo vascular progenitors emerge from intra- and extra-embryonic mesodermal tissues (Jin and Patterson, 2009), whilst in the placenta they arise from the extra-embryonic mesoderm (Chaddha et al., 2004). However, there is growing evidence for a crucial role of the yolk sac in embryo and placental vascular development (Freyer and Renfree, 2009). Indeed, using a sodium-calcium exchanger (*Ncx-1*) knockout mice which fails to initiate cardiac contraction Lux et al. (2008) showed that all the hematopoietic progenitor cells emerge from the yolk sac. The origin of placental endothelial cells

could have an important impact on its vascular physiology because arterial-venous identity is early established by environmental cues which could have diverse effects depending on the localization in the embryo.

Growth and consolidation of the placental vascular tree occurs by angiogenesis. In this process single vessels are formed by endothelial precursor cells (EPCs) which differentiate into endothelial cells, and/or proliferate from endothelial cells. These vessels can spread in two ways, (1) non-branching angiogenesis, which implies an increase in the length of the villous vessels, and (2) branching angiogenesis, in which multiple short capillary loops are formed (Demir et al., 2007), increasing the vascular surface area. After these processes have taken place, the vessels mature and their structures stabilize. Additional maturation and specialization in the vascular system are influenced by environmental signals, such as blood flow, oxygen tension, oxidative stress, and epigenetic factors (le Noble et al., 2008; Atkins et al., 2011). All these factors have been implicated in the development and function of the human placenta (Fowden et al., 2008; Burton, 2009; Dennery, 2010). Thus, angiogenesis is a complex process which involves genetic, epigenetic and environmental commands in the development and establishment of the vasculature.

EPIGENETICS OVERVIEW

During the last decade, the study of genome-environment interactions has revealed a plethora of mechanisms that modulate short and long term cellular physiology. These mechanisms involve mainly epigenetic processes which control chromatin accessibility in a gene- and cell-specific manner. Definition of epigenetics is still under debate mainly due to the several molecular mechanisms that it comprises and the heritability of these changes in an organism and its progeny; however, a simple and broad definition considers epigenetic mechanisms as "chromosome-based mechanisms that change the phenotypic plasticity in a cell or organism" (Krause et al., 2009; Gibney and Nolan, 2010).

Epigenetic mechanisms affect chromatin structure and gene expression regulating DNA and histone interactions, and the translation and stability of mRNA. Epigenetic markers such as DNA methylation, histone deacetylation, and other repressive histone post-translational modifications (PTMs) alter the structure of the chromatin, generating regions with a "closed chromatin" conformation. Conversely, DNA demethylation (potentially driven by the oxidation of methylated cytosines and their replacement by base excision repair; Kohli and Zhang, 2013), ATP-dependent chromatin remodeling, histone acetylation (Ac), and other permissive histone PTMs, convert the closed chromatin into an "open chromatin" conformation allowing binding of transcription factors and the RNA polymerase II (**Figure 1**). As an additional epigenetic mechanism, the presence of non-coding RNAs can post-transcriptionally repress gene expression. Detailed reviews of the diverse epigenetic mechanisms and their effects on gene expression are available (Klose and Bird, 2006;

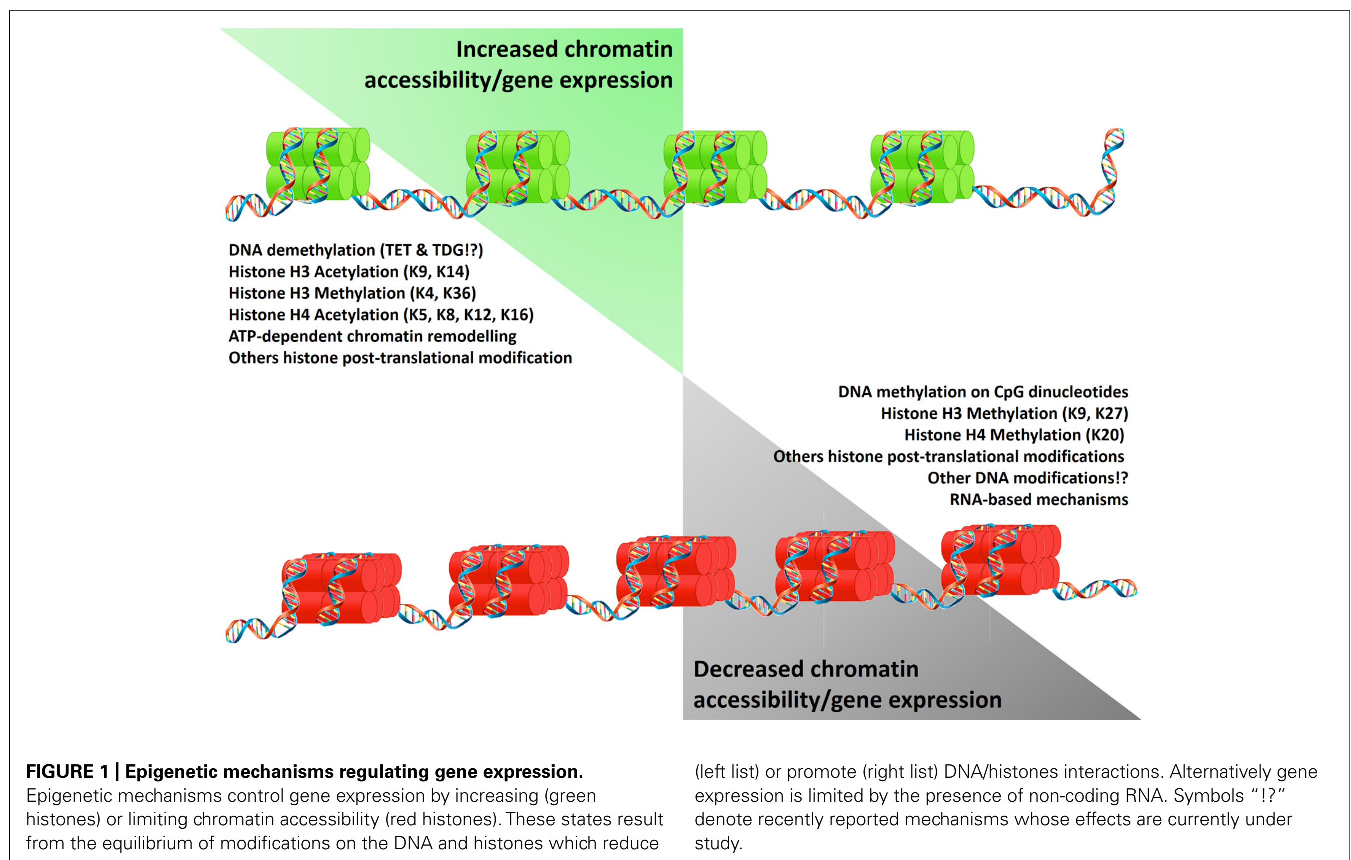

FIGURE 1 | Epigenetic mechanisms regulating gene expression. Epigenetic mechanisms control gene expression by increasing (green histones) or limiting chromatin accessibility (red histones). These states result from the equilibrium of modifications on the DNA and histones which reduce (left list) or promote (right list) DNA/histones interactions. Alternatively gene expression is limited by the presence of non-coding RNA. Symbols "!?" denote recently reported mechanisms whose effects are currently under study.

Kouzarides, 2007; Wang et al., 2007a,b; Kaikkonen et al., 2011; Kohli and Zhang, 2013).

From a developmental perspective epigenetic mechanisms allow the generation of diverse cell phenotypes and functions of an organism from a single genome, and respond to a range of environmental fluctuations. This issue is especially evident in organs and tissues whose structure and function are under constant change across lifespan, such as the cardiovascular system (Aird, 2012). Nonetheless, placental vasculature may also be programmed by epigenetic mechanisms, which are currently under restless research.

EPIGENETICS IN ENDOTHELIAL PHYSIOLOGY AND PATHOPHYSIOLOGY

Vascular development, endothelial differentiation and function require a fine epigenetic tuning (**Table 1**). Initial steps of vascular development in the embryo seem to be influenced by both genetic and environmental stimuli which drive the emergence of two different populations of endothelial cells (Atkins et al., 2011). Differentiation of embryonic stem cells and EPCs into endothelial cells requires the participation of histone deacetylases (HDAC), lysine demethylases (KDM) and reduced DNA methylation in the promoter region of endothelial-specific genes (Rossig et al., 2005; Zeng et al., 2006; Lagarkova et al., 2008; Banerjee and Bacanamwo, 2010; Ohtani et al., 2011). Conversely, differentiated endothelial cells can be reprogramed to a pseudo-embryonic stem cell phenotype increasing the DNA methylation status of endothelial-specific genes (Lagarkova et al., 2010). In endothelial cells, activating histone PTM, such as acetylation of H3 and H4 and methylation of H3K4, control the basal expression of vWF (Peng and Jahroudi, 2003), NOTCH4 (Wu et al., 2005), VEGF receptor 1 (Dutta et al., 2008), endomucin (Kanki et al., 2011), and eNOS (Fish et al., 2005; Gan et al., 2005).

On the other hand, HDAC activity is required for an adequate vascular integrity (Chang et al., 2006) preventing short term endothelial proliferation and angiogenesis (Ha et al., 2008; Jin et al., 2011), whilst calmodulin-lysine *N*-methyltransferase (KMT) activity has the opposite effect (Diehl et al., 2007). However, long term HDAC activity promotes angiogenesis in response to VEGF (Deroanne et al., 2002) and hypoxia (Kim et al., 2001) increasing the expression of VEGF (Ruchko et al., 2009) and eNOS (Rossig et al., 2002). Similarly, HDAC activity is increased in response to shear stress (Illi et al., 2003) improving cell survival (Zampetaki et al., 2010) and eNOS expression (Wang et al., 2010). Noteworthy, the epigenetic regulation of NOS3 gene has been extensively studied in endothelial and non-endothelial cells, showing that endothelial cells have a distinctive pattern of DNA methylation and histone PTMs (Fish and Marsden, 2006). Fish et al. (2007) reported that the decreased expression of eNOS in HUVEC exposed to acute hypoxia is controlled by the overexpression of a natural *cis*-antisense non-coding RNA called sONE, and changes in histone PTMs which occur specifically at the eNOS promoter (Fish et al., 2010). Additionally, abrogation of NOS3 promoter DNA methylation increases basal eNOS mRNA expression *in vitro*, and protects against hind-limb ischemic injury *in vivo* (Rao et al., 2011).

Several studies show that epigenetic mechanisms participate in the increased risk of developing vascular diseases. In humans, endothelial cells from atherosclerotic plaques have decreased levels of estrogen receptor β along with increased DNA methylation at the promoter region of this gene, compared with those from non-atherosclerotic plaque regions (Kim et al., 2007). Deficiency of a specific KDM, lysine-specific demethylase-1 (LSD1, KDM1a), associates with decreased expression of eNOS and NO-dependent vasodilation, as well as, salt sensitive hypertension (Pojoga et al., 2011). In newborn rats with persistent pulmonary hypertension, the increased expression of eNOS mRNA is accompanied by augmented levels of acetylated H3 and H4 in the NOS3 gene promoter (Xu et al., 2010). Alternatively, cultured endothelial cells exposed to elevated levels of homocysteine, which relates with increased cardiovascular risk, present decreased proliferation and increased levels of oxidative stress. In both cases homocysteine acts inducing specific hypomethylation of the gene promoters for the cell cycle regulator cyclin A (Jamaluddin et al., 2007) and the pro-oxidant protein p66shc (Kim et al., 2011). Additionally, high glucose-induced endothelial dysfunction requires the participation of HAT (Chen et al., 2010) and KMT (El-Osta et al., 2008), generating important epigenomic changes (Pirola et al., 2011), which can persist several days after the exposure to the noxa (El-Osta et al., 2008).

Notably, vascular physiology is also influenced by epigenetic mechanisms occurring in smooth muscle cells (SMCs). Development of vascular dysfunction is accompanied by changes in SMC phenotype, which shift from a "contractile" to a "synthetic" and

Table 1 | Effect of DNA methylation and histone post-translational modifications (PTMs) on endothelial cell physiology.

Mechanism	Process	Reference
DNA methylation	*In vitro* and *in vivo* progenitor endothelial cells differentiation Activation of tissue-specific genes Ischemia-induced neo-vascularization	Chan et al. (2004), Lagarkova et al. (2008), Ohtani et al. (2011), Rao et al. (2011)
Histone acetylation	Hypoxia-, VEGF- and shear stress- induced angiogenesis VEGF-induced progenitor endothelial cells differentiation Basal endothelial cell-specific genes	Kim et al. (2001), Deroanne et al. (2002), Rossig et al. (2002), Peng and Jahroudi (2003), Illi et al. (2005), Zeng et al. (2006), Wu et al. (2005)
Other histone PTMs	Progenitor endothelial cells differentiation Hypoxia induced eNOS down-regulation	Ohtani et al. (2011), Fish et al. (2010)

"pro-inflammatory" phenotype with long term consequences in the contractile properties of vessels (Owens et al., 2004; Orr et al., 2010). Increasing data shows that this "phenotypic switching" requires the participation of epigenetic mechanisms which establish an altered SMC function (Alexander and Owens, 2012).

PHENOTYPIC AND EPIGENETIC DIVERSITY IN THE UMBILICO-PLACENTAL ENDOTHELIUM

Pioneer studies by Lang et al. (1993) demonstrated that micro- and macrovascular umbilico-placental endothelium present different immunoreactivity to diverse molecular markers for endothelial cells, suggesting the presence of a phenotypic endothelial diversity in the placenta. Additional evidence from cultured human endothelial cells isolated from the placental microcirculation (PLEC) and the umbilical vein (HUVEC) show that microvascular endothelial cells express higher levels of vascular mediators (angiotensin II, endothelin, and thromboxane; Lang, 2003). Also a differential pattern of homeobox genes (Murthi et al., 2007, 2008) and higher cholesterol transport capacity (Stefulj et al., 2009) in PLEC compared to HUVEC has been shown.

Notably, studies on endothelial cells from arteries and veins have revealed important differences between arterial and venous cells at the same vascular level. In fact the higher mitogenic response observed in PLEC (Lang, 2003) may reflect the combination of a high response to VEGF present in arterial PLEC (PLAEC) and to PIGF in venous endothelial cells (PLVEC; Lang et al., 2008). A transcriptomic analysis between PLAEC and PLVEC showed that they have differential expression of more than 3,000 genes (Lang et al., 2008). Similarly there is a differential expression of eNOS, a key vascular gene, between micro- and macrovascular, and venous and arterial endothelium (Andersen et al., 2009; Krause et al., 2012) being more homogenous at the arterial side (Andersen et al., 2009). This opens the queries about the differences initially reported between micro- and macrovascular endothelium reflecting an endothelial diversity between large and small vessels, and whether they include variances between arteries and veins.

Several studies comparing simultaneously umbilical arterial (HUAEC) and venous (HUVEC) endothelium support the concept that these cells are not a homogenous population, and the necessity of clarifying the precise source of cells when the term "macrovasculature" is used. A general characterization shows that there is a different profile of phospholipids with higher levels of arachidonic acid-related species and heterogeneous expression pattern of selenoproteins (Miller et al., 2002) in HUAEC compared to HUVEC (Takamura et al., 1990). Alongside the classical molecular markers for arterial endothelium, cultured HUAEC express higher levels of PAI 1 (Gallicchio et al., 1994), Cx40 (Van Rijen et al., 1997), 17β-HSD2 (Simard et al., 2011), and VCAM-1 (Egorova et al., 2012); and lower levels of von Willebrand Factor (Shahani et al., 2010) and estrogen receptors beta (ERβ; Simard et al., 2011) compared with HUVEC. On the other hand expressions of pro-constrictive mediators such as angiotensin converting enzyme (Ito et al., 2002) and ET-1 (Egorova et al., 2012) are different in HUVEC relative to HUAEC. Furthermore, expression and activity of eNOS are higher in freshly isolated HUVEC than HUAEC (Andersen et al., 2009) and this expression pattern is also observed in cells cultured up to third passage (Krause et al., 2012). Whether these differences reflects the physiology of umbilical (and potentially placental) arteries and veins, and how they are preserved *in vitro* need further examination. Two recent reports show that the differential gene expression between HUAEC and HUVEC is partially controlled by specific transcription factors. Overexpression of the venous-specific nuclear receptor COUP-TFII in HUAEC decreases the expression of arterial markers (i.e., Hey2, EphrinB2 and NICD4), and its down-regulation in HUVEC increases the expression of arterial markers such as VEGF-A, Dll and EphrinB2 (Korten et al., 2013). Moreover, *in vitro* simultaneous overexpression of eight arterial-specific transcription factors turns the HUVEC transcriptome into a HUAEC-like pattern (Aranguren et al., 2013).

Therefore, the phenotypic diversity in the umbilico-placental circulation is apparently commanded, at least in part, by an equivalent diversity in epigenetic mechanisms.

ENDOTHELIAL DIVERSITY AND ANGIOGENESIS

In terms of angiogenesis, microvascular endothelial cells present a higher mitogenic response to VEGF, PIGF (Lang, 2003; Lang et al., 2008), and prokineticin 1 (Brouillet et al., 2010) compared with HUVEC, along with an increased expression of pro-angiogenic HOX genes (i.e., TLX1, TLX2, and PHOX1; Murthi et al., 2008). These data are in agreement with the notion that placental angiogenic capacity is augmented in microvascular vessels compared to endothelial cells from larger vessels. However, it is also possible to find significant differences in the angiogenic response in endothelial cells from umbilical arteries and veins. *In vivo* VEGFR3, which is commonly expressed in lymphatic endothelium or during active angiogenesis (Koch and Claesson-Welsh, 2012), is absent in HUAEC but expressed in HUVEC (Veikkola et al., 2003). Moreover *in vitro* chemotaxis induced by VEGFA or FGF2 is higher in HUVEC compared to HUAEC (Barkefors et al., 2008), and netrin-1 prevents the VEGF-induced migration in HUAEC without effect on HUVEC (Lu et al., 2004). Further studies are needed to address the effects and the role on placental physiology of this increased angiogenic response observed in HUVEC.

ENDOTHELIAL DIVERSITY IN RESPONSE TO STRESS

Placental vascular and endothelial physiology, similar to adult vasculature, are importantly influenced by stimuli such as altered shear stress and oxygen levels whose effects are apparently different between arteries and veins. Normally arterial endothelium is exposed to higher shear stress and therefore it is plausible to predict a stronger response to increasing stress. In fact pulsatile shear stress increases the expression of arterial markers (i.e., Hey1, Hey1, and ephrinB2) in HUAEC but decreases the expression of venous markers (COUP-TFII) in HUVEC (Buschmann et al., 2010). Laminar shear stress have similar effects on the expression of arterial-venous markers in these cells, and increases the levels of *S*-nitrosylated proteins (Hoffmann et al., 2003) endothelin-1, VCAM, and vWF (Egorova et al., 2012) in HUAEC compared to HUVEC. Whether these differences are observed in microvascular endothelial cells remains to be determined.

Some evidence regarding the effects of low oxygen levels on endothelial function in placental large and small vessels, as well

as arteries and veins, show a differential vascular response to hypoxia throughout the placenta (Krause et al., 2011, 2012). On the other hand placental endothelium is importantly exposed to low oxygen levels and oxidative stress which are negative regulator of placental angiogenesis (Burton et al., 2009). A reduction in oxygen levels from 21 to 12% O_2decreases placental venous microvascular endothelial cells viability with no effect on their arterial counterparts (Lassance et al., 2012), and PLAEC exposed to 3% O_2 show an increased mitogenic response to VEGFA and FGF2 compared to cells cultured at 21% O_2 (Wang et al., 2009). This higher response to VEGFA and FGF2 is also observed in HUAEC exposed to physiological levels of oxygen (3–5% O_2; Jiang et al., 2013). Additionally, hypoxia (1% O_2) increases the expression of the pro-angiogenic factor protease-activated receptor 2 in HUVEC and this effect is higher in HUAEC (Svensson et al., 2011).

Altogether these data show that venous-arterial endothelial phenotypic diversity occurs among umbilical and placental vessels (**Figure 2**). Further studies should include control comparison between arterial and venous endothelial cells from the same branching level to rule out potential differences attributable to arteries and veins rather than micro- and macrovascular vessels. It is worth to note that most of the differences occurring among these cells types could be reverted by genetic manipulation. However, its persistence *in vitro* suggests that additional mechanisms controlling gene expression should be operating, arguing for a crucial role for epigenetics in this process.

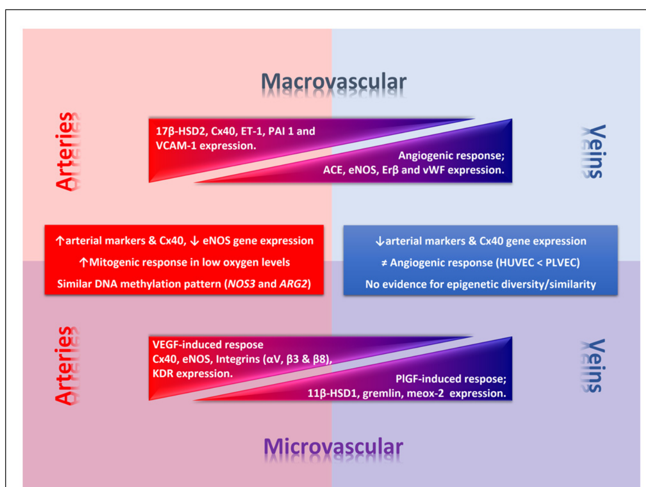

FIGURE 2 | Phenotypic diversity in the umbilico-placental endothelium. Umbilical (macrovascular) and placental (microvascular) endothelial cells present a phenotypic diversity characterized by a differential response to angiogenic factors and gene expression of key endothelial genes. In the figure thickness of the triangle denotes deferential relative expression (or response) regarding arteries or veins at each level. Square text box includes common traits between macro- and micro-vascular arterial or venous endothelial cells. 11β-HSD1, 11β-hydroxysteroid dehydrogenase 1; 17β-HSD2, 17β hydroxysteroid dehydrogenase 2; ARG2, arginase-2 gene; Cx40, conexin-40; eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; KDR, vascular endothelial growth factor (VEGF) receptor 2; NOS3, eNOS gene; PAI 1, plasminogen activator inhibitor-1; PlGF, placental growth factor; VCAM-1, vascular cell adhesion molecule 1.

EPIGENETIC AND PLACENTAL ENDOTHELIAL DIVERSITY

Compelling evidence shows the fundamental role of epigenetics controlling the endothelial-specific gene expression, however, the next frontier is to determine how epigenetic mechanisms influence the endothelial functional diversity. Two recent reports studying placental and umbilical endothelial cells suggest the presence of significant differences in the DNA methylation of gene promoters which could be responsible for the differential gene expression present in these cells.

A comparison of the genome-wide DNA methylation profile in PLAEC and PLVEC show that venous endothelial cells present lower levels of global methylation compared to PLAEC (Joo et al., 2013) which could reflect the immature phenotype of PLVEC (Lang et al., 2008). Further analysis show the presence of several genes which are differentially methylated between PLAEC and PLVEC, and some of them present an inverse correlation between the level of methylation and the gene expression. Notably those genes are considered endothelial markers and play a key role in vascular physiology, such as eNOS, vWF, Conexin40, VEGFR1, VEGFC, and angiopiotein-1. However, there are endothelial genes whose promoters do not present any correlation between methylation levels and gene expression, such as VEGFR2, Hey2, NOTCH, EphB2, and EphB4 (Joo et al., 2013).

Conversely, the comparison of DNA methylation status of NOS3 (eNOS) and ARG2 (arginase-2) promoters by pyrosequencing in HUAEC, PLAEC and HUVEC, suggest the presence of site-specific differences between these cells. Methylation status at NOS3 promoter in umbilical and placental endothelial cells showed differences in three specific CpG between arterial and venous endothelial cells (Krause et al., 2013). Two of these differentially methylated CpGs correspond to the reported hypoxia response element (−5369 and −5375; Coulet et al., 2003) which regulates the response to hypoxia and show lower methylation levels in PLAEC and HUAEC compared to HUVEC. Whether this variation participates in the differential regulation of eNOS expression in response to hypoxia that has been reported between HUAEC and HUVEC (Krause et al., 2012) needs to be addressed. An additional differentially methylated CpG is located at −352 from the transcription starting site, showing a higher methylation pattern in arterial relative to venous cells. Moreover the methylation status at this CpG suggests an inverse correlation between DNA methylation and eNOS expression, which is higher in HUVEC (lower methylation levels) compared to HUAEC (Krause et al., 2012, 2013). It is also reported that CpG −352 is differentially methylated between HUVEC and human dermal microvascular endothelial cells (Chan et al., 2004), having the later a methylation status comparable to that found in HUAEC and PLAEC, which suggest that CpG −352 might play a role in the differential regulation of basal eNOS expression in arterial and venous endothelial cells. Krause et al. (2013) compared the NOS3 promoter DNA methylation status between control and endothelial cells isolated from pregnancies with intrauterine growth restriction (IUGR). Remarkably changes in DNA methylation in IUGR cells are restricted to those CpGs that are differentially methylated in normal endothelial cells. In fact, IUGR HUAEC and PLAEC present similar changes at CpGs −5375 (increased methylation) and −352 (decreased methylation) compared with normal cells,

and these methylation levels are comparable to that find in normal HUVEC. Conversely, changes in the DNA methylation status of IUGR HUVEC where at CpGs −5369 (decreased methylation) and −352 (increased methylation), and they are comparable to those find in normal HUAEC and PLAEC. The methylation levels at CpG −352 in IUGR HUAEC and HUVEC are also related with the levels of mRNA for eNOS (Krause et al., 2013), reinforcing the potential importance of CpG −352 in the regulation of basal eNOS expression. Finally analysis of methylation status of ARG2 promoter in HUAEC, PLAEC, and HUVEC show a single difference between PLAEC and HUVEC, however, it is still unknown if there is a correlation with arginase-2 expression and activity.

DNA methylation is one of the main epigenetic mechanisms that controls long term gene expression, showing a high reproducibility after every cellular replication and this characteristic is driven by the activity of DNA methyltransferase-1 (DNMT1). In IUGR HUAEC and HUVEC DNMT1 silencing shows a differential effect, reducing and increasing basal eNOS expression, respectively (Krause et al., 2013). Silencing of DNMT1 restores to normal eNOS mRNA levels in IUGR HUAEC and HUVEC, and this effect is not observed on arginase-2 expression where it further increases its expression in IUGR HUVEC, without any effect in IUGR HUAEC (Krause et al., 2013). This suggests that DNA methylation (Jamaluddin et al., 2007; Banerjee and Bacanamwo, 2010; Kim et al., 2011) and other epigenetic mechanisms (Kim et al., 2001; Deroanne et al., 2002; Fish et al., 2010) control gene expression in endothelial cells in a gene-specific manner.

Although the studies in PLAEC and PLVEC (Joo et al., 2013), and in HUAEC and HUVEC (Krause et al., 2013) used two different approaches to analyze the DNA methylation patterns, there are some similarities in the outcomes. First, both studies show that methylation status of NOS3 proximal promoter inversely correlates with the levels of mRNA for eNOS, and this occurs in cells exposed for several days to culture conditions. Second, PLAEC and PLVEC show differential levels arginase-2 expression without differences in the DNA methylation in ARG2 promoter, whilst in control and IUGR HUAEC differences in DNA methylation are not associated to difference in arginase-2 expression. Finally, DNMT1 silencing in IUGR cells normalize eNOS expression but not arginase-2 expression.

CONCLUSION

Altogether these seminal data show that epigenetic mechanisms could be responsible for the phenotypic diversity of endothelial cells in the umbilico-placental unit, and these mechanisms would be operating in a cell- and gene-specific manner. The current research on the area is offering novel data about potential mechanisms but still further studies are required to have a comprehensive picture of the additional epigenetic mechanisms controlling the gene expression in physiological and pathophysiological conditions and its consequences in umbilico-placental functions.

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