OBSTETRICS

Down-regulation of placental neuropilin-1 in fetal growth restriction

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BACKGROUND: Fetal growth restriction (FGR) is associated with adverse outcomes extending from fetal to adult life, and thus, constitutes a major health care challenge. Fetuses with progressive growth restriction show increasing impedance in the umbilical artery flow, which may become absent during end-diastole. Absent end-diastolic flow (AEDF) is associated with adverse perinatal outcomes including stillbirths and perinatal asphyxia. Placentas from such pregnancies demonstrate deficient fetoplacental vascular branching. Current evidence, moreover, indicates an antiangiogenic state in maternal circulation in several pregnancy complications including preeclampsia, small-for-gestational-age births, fetal death, and preterm labor. The angiogenic mediators in maternal circulation are predominantly of placental origin. Information, however, on the role of specific proangiogenic and antiangiogenic mechanisms operating at the placental level remains limited. Elucidation of these placenta-specific angiogenic mechanisms will not only extend our understanding of the causal pathway for restricted fetal growth but may also lead to the development of biomarkers that may allow early recognition of FGR.

OBJECTIVE: We sought to test the hypothesis that fetoplacental angiogenic gene expression is altered in pregnancies complicated with FGR and umbilical artery Doppler AEDF.

STUDY DESIGN: Placental samples were collected from FGR pregnancies complicated with umbilical artery Doppler AEDF (study group, n = 7), and from uncomplicated pregnancies (control group, n = 7), all delivered by cesarean during the last trimester of pregnancy. Angiogenic oligonucleotide microarray analysis was performed and was corroborated by quantitative real-time polymerase chain reaction, Western blot analysis, and immunohistochemistry. The Student *t* test with Bonferroni correction was used with $P < .05$ considered statistically significant. Independent groups *t* test was used to analyze the immunostain intensity scores with a $P < .05$ considered statistically significant.

RESULTS: Our microarray results showed that among several differentially expressed angiogenic genes in the growth-restricted group, only the down-regulation of neuropilin (NRP)-1 was most significant ($P < .0007$). Quantitative real-time polymerase chain reaction confirmed a significantly lower NRP-1 gene expression in the FGR group than in the control group (mean ± SD ˆcycle threshold: 0.624 ± 0.55 and 1.325 ± 0.84, respectively, $P = .04$). Western blot validated significantly lower NRP-1 protein expression in the FGR group than in the control group (mean ± SD NRP-1/β-actin ratio: 0.13 ± 0.04 and 0.34 ± 0.05, respectively, $P < .001$). Finally, immunohistochemistry of placental villi further corroborated a significantly decreased expression of NRP-1 in the FGR group ($P = .006$).

CONCLUSION: The study demonstrated significant down-regulation of placental NRP-1 expression in FGR pregnancies complicated with AEDF in umbilical artery. As NRP-1 is known to promote sprouting angiogenesis, its down-regulation may be involved in the deficient vascular branching observed in FGR placentas suggesting the presence of an antiangiogenic state. Further studies may elucidate such a causal role and may lead to the development of novel diagnostic and therapeutic tools.

Key words: antiangiogenic state, fetal growth restriction, immunohistochemistry, microarrays, neuropilin-1, placental branching angiogenesis, real-time polymerase chain reaction, small for gestational age, umbilical artery absent end-diastolic flow

Introduction

Associated with adverse outcomes spanning fetal to adult life, fetal growth restriction (FGR) constitutes a major obstetrical complication affecting 5-7% of all pregnancies.[1,2] Perinatal mortality is substantially high in FGR and approximately 1 in 4 of the stillborn fetuses is growth restricted. Perinatal morbidities include asphyxia, preterm delivery, neonatal depression, and a spectrum of metabolic, respiratory, and neurological complications. Long-term risks include continuing growth deficit, cerebral palsy, and neurodevelopmental abnormalities.[3-6] Beyond the perinatal period and infancy, adverse consequences of FGR extend into adult life. Experimental and epidemiological evidence indicates that chronic intrauterine deprivation may induce epigenetic programming and a thrifty phenotype setting the stage for subsequent development of adult illnesses including hypertension, stroke, ischemic heart disease, type 2 diabetes, and central obesity.[7] Constrained fetal growth thus constitutes a major health care concern.

Compromised fetal supply line has long been proposed as a major underlying mechanism for limiting fetal growth.[8,9] Consistent with this concept, Doppler ultrasound studies have shown increasing umbilical circulatory impedance associated with progressive fetal decompensation in FGR, eventually leading to absent end-diastolic flow (AEDF) in the umbilical artery.[10] This hemodynamic deterioration is associated with a spectrum of adverse perinatal outcomes including stillbirths and perinatal asphyxia.[11,12] There is morphological evidence, moreover, correlating umbilical artery AEDF to diminished fetoplacental vascular branching in FGR pregnancies.[13,14] The molecular

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mechanisms underlying aberrant feto-placental angiogenesis in FGR, however, require further elucidation.

Angiogenesis is a complex biological process controlled by agonists and antagonists directly or indirectly promoting or inhibiting angiogenic activity. Normal pregnancy represents a balanced angiogenic state. Current evidence, however, indicates the presence of an anti-angiogenic state in several pregnancy complications. Maynard et al[15] first proposed an antiangiogenic state in preeclampsia involving up-regulation of soluble fms-like tyrosine kinase-1 of placental origin in maternal plasma, which opposes proangiogenic vascular endothelial growth factor (VEGF) and placental growth factor (PlGF). Subsequently, the Romero group[16-22] has provided extensive evidence for the presence of an antiangiogenic state in maternal circulation in several pregnancy disorders including preeclampsia, small-for-gestational-age (SGA) births, fetal death, and preterm labor, and in association with placental massive perivillous fibrin deposition, a condition known to be associated with recurrent miscarriage and stillbirths. Others also have documented an antiangiogenic state in pregnancies resulting from in vitro fertilization,[23] obesity,[24] twin transfusion syndrome,[25] and invasive placentation.[26] Moreover, there is evidence that antiangiogenic agents in maternal serum may serve as potential biomarkers for subsequent recognition of fetal growth compromise and adverse pregnancy outcomes.[27-29]

These angiogenic mediators in maternal circulation are predominantly of placental origin. Information, however, on the role of specific proangiogenic and antiangiogenic mechanisms operating at the placental level remains limited.[30,31] Elucidation of these placenta-specific angiogenic mechanisms will not only extend our understanding of the causal pathway for restricted fetal growth but may also lead to the development of biomarkers for FGR that may allow early recognition of FGR and differentiation of constitutionally small fetuses from those that are truly growth restricted. The latter currently remains challenging despite advances in fetal sonography. Such mechanistic understanding may also lead to the emergence of new management strategies for promoting fetal growth.

The purpose of this study, therefore, was to investigate the placental angiogenic mechanisms in FGR pregnancies complicated with fetal hemodynamic compromise as evidenced by umbilical artery AEDF. Specifically, we determined in these pregnancies placental expressions of angiogenic genes utilizing oligonucleotide microarray analysis, and to confirm the significant array findings of differentially expressed messenger RNA (mRNA) transcripts by quantitative real-time polymerase chain reaction (qPCR), Western Blot analysis of protein expression, and immunohistochemistry for localization and quantification in placental tissues.

Materials and Methods

Study design

In this prospective study, angiogenic gene expression was analyzed on placental samples collected from pregnancies complicated with FGR and umbilical artery Doppler AEDF (study group, $n = 7$), and from uncomplicated pregnancies (control group, $n = 7$). FGR was defined as an ultrasound-estimated fetal weight $<$10th percentile for gestational age following the American Congress of Obstetricians and Gynecologists (ACOG) guidelines.[32] We followed these guidelines to restrict the terms "fetal growth restriction" to the fetus and "small for gestational age" to the neonate. Pregnant mothers receiving prenatal care were approached for consent and participation in the study. The inclusion criteria for the study group were: singleton pregnancies, gestational age ascertained according to the ACOG guidelines,[33] gestational age $>$36 weeks, ultrasound biometric diagnosis of FGR, AEDF in the umbilical artery Doppler, and delivery by cesarean delivery. This mode of delivery was chosen to minimize possible placental oxidative stress from labor and delivery as demonstrated by others.[34] The exclusion criteria were labor; pregnancy complications other than FGR such as preeclampsia, multiple gestation, fetal aneuploidy, fetal malformations, prolonged rupture of membranes, and chorioamnionitis; placental pathology such as abruption, placenta previa, and placental accreta; and maternal diseases such as infection, diabetes, and chronic hypertension. The control group included uncomplicated pregnancies, matched by gestational age with the study group, and delivered by elective cesarean delivery before labor indicated by previous cesarean delivery or breech presentation. The birthweight centile was determined utilizing the US national reference values as reported by Oken et al.[35] The institutional review boards of Truman Medical Center/University of Missouri—Kansas City School of Medicine and Winthrop University Hospital approved the study. Informed consent was obtained from each patient according to the institutional review board protocol.

Placental sample acquisition

Within 10 minutes after the placental delivery, placental tissue samples were collected using a sterile scalpel. Three samples each measuring approximately $2 \times 2 \times 2$ cm were removed from a mediobasal location as defined by Wyatt et al.[36] Each sample was then divided into 3 identical pieces, rinsed thoroughly in sterile phosphate buffer solution, and snap-frozen in liquid nitrogen. The samples were stored in a biorepository according to the institutional regulations.

Angiogenesis gene array analysis

Two angiogenesis-oriented arrays were used for gene expression analysis. Angiogenic SuperArray HS-009 contained 96 genes and 16 internal controls and angiogenic SuperArray OHS-024 contained 114 genes and 14 internal controls (SuperArray Biosciences Corp, Frederick, MD).

The total RNA was extracted from placental tissues using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA samples (3 μg) were then reverse transcribed and labeled with biotin-16-dUTP (Roche, Nutley, NJ) by polymerase chain reaction (PCR) using the AmpoLabeling-LPR kit (SuperArray Biosciences Corp). Then, probes were hybridized to either GEArray HS-009 or GEArray OHS-024. The hybridization

signals were generated by GEArray chemiluminescent detection kit (SuperArray Biosciences Corp) and evaluated by the Kodak Image Station 4000R and the ChemiDoc XRS (BioRad Laboratories, Hercules, CA), respectively. The relative expression levels of angiogenesis gene were normalized to a panel of housekeeping genes of the corresponding array and cross-linked using gene symbols.

qPCR analysis

qPCR analysis was used to confirm the significant angiogenic findings of the differentially expressed mRNA transcripts (SuperArray Biosciences Corp). Total RNA (1.5 μg/sample) was reverse transcribed using the First Strand cDNA synthesis kit according to manufacturer's instructions (Roche). The primers were designed using the Primer3 software (MIT) for sense and antisense and purchased from Origene Technologies (Rockville, MD). The sequences of forward and reverse primers were: human GAPDH (GenBank accession no. NM_002046) 5′ CTCTCTGCTCCTCC TGTTCGAC 3′ and 5′ TGAGCGATGT GGCTCGGCT 3′ and human neuropilin (NRP)-1 (GenBank accession no. NM_001024628) 5′ ACG ATG AAT GTG GCG ATA CT 3′ and 5′ AGT GCA TTC AAG GCT GTT GG 3′. Real-time qPCR was performed by using Fast SYBR Green on a StepOne Plus real-time PCR system (Applied Biosystems, Grand Island, NY). Relative expression values were calculated using the 2-delta delta Ct method and were normalized against reference gene GAPDH. In these calculations we accounted for the PCR efficiency of the individual PCR reactions, calculated on the basis of linear regression as described elsewhere.[37] The specificity of amplification was confirmed by evaluation of the melting curve.

Protein extraction and Western blot

The protein was extracted from placental samples and its concentration was measured by the BCA protein assay method per manufacturer's protocol (Pierce, Rockford, IL). Each sample (50 μg) was separated in 10% sodium dodecyl sulfate-polyacrylamide gel. The protein was transferred onto nitrocellulose membrane. The membrane was blocked with blocking serum (Thermoscientific, Waltham, MA) followed by incubation with mouse antihuman NRP-1 antibody (1:1000) (Abcam, Cambridge, MA) overnight at 4°C. After washing with Tris-NaCl-tween 20 buffer, the membrane was incubated with antimouse IgG (1:15000) (Abcam) at room temperature for 1 hour. Immunoreactivity was detected using an enhanced chemiluminescence Western blotting system (Thermoscientific). Qualitative analysis was performed and expressed in relation to β-actin.

Immunohistochemical localization and quantification

The placental tissues were fixed in 4% buffered formaldehyde solution, dehydrated, and embedded in paraffin. The 4-μm thickness sections were transferred onto poly-l-lysin-coated slides, deparaffinized, rehydrated, and immunostained with NRP-1 antibody (1:200) using the Vectastain Immpress Reagent Kit (Vector Laboratories, Burlingame, CA). Two investigators blinded to the sample source independently graded immunostain intensity. Slides were first examined at ×4 magnification to identify NRP-1 immunopositive regions in placental sections. In each section, 5 different areas with 8-12 villi per area were selected at random and were evaluated microscopically with a ×40 objective magnification. All sections were scored in a semiquantitative fashion as described by Hsu et al,[38] which considered both the intensity and percentage of cells staining. Intensities were classified as 0 (no staining), +1 (weak staining), +2 (moderate staining), and +3 (very strong staining).

Statistics

Angiogenic data (SuperArray Biosciences Corp) were normalized to corresponding internal controls and resulting gene expression values were cross-referenced between arrays. The Student *t* test was applied to 67 genes that were common to both SuperArrays and genes with $P < .05$ considered significant. The final candidate selection was done by filtering for the Bonferroni correction for multiple comparisons ($P < .0007$). The values from qPCR were adjusted to GAPDH, and the values from Western blot were adjusted for β-actin, then mean and SD values were determined. The immunostain intensity met the distributional assumptions of a parametric statistical test. Independent groups *t* test was used to compare the control and FGR groups for these values. Differences were considered significant when P was $<.05$.

Results

Clinical characteristics

The basic clinical characteristics of the control and the FGR groups are presented in the Table. There were no differences between the groups regarding maternal age and gestational age. The population was racially diverse and the sample size did not permit any analysis of the impact of race. Notably, this was not an objective of this study. The birthweights and the placental weights were significantly lower in the FGR group than those in the control group. The birthweights were <5th centile for the study group and >20th centile for the control group. There were no fetal deaths in this population.

Down-regulation of placental NRP-1 mRNA expression in FGR

To investigate that placenta-specific angiogenic mechanisms may underlie restricted fetal growth, we first determined expressions of genes in angiogenic pathway using 2 angiogenesis-oriented arrays (SuperArray Biosciences Corp and Qiagen, Valencia, CA). Several angiogenesis-related genes were differentially expressed in placentas from FGR pregnancies. A representative microarray photograph depicts expression of angiogenic genes in control and FGR in Figure 1, A. Densitometric analysis showed significant reduction in NRP-1 in FGR placentas (Figure 1, B). A heat map depicting the results obtained in control and FGR placentas is shown in Figure 1, C. The scatter plot shown in Figure 1, D, demonstrates underexpression and overexpression of genes in FGR placentas.

TABLE
Clinical characteristics of control and fetal growth restriction groups

Parameter	Control (n = 7)	FGR (n = 7)	Significance
Maternal age, y	26.57 ± 2.60	25.71 ± 1.85	NS
Gestational age, wk	39 ± 0.85	37.46 ± 0.86	NS
Birthweight, g	3181.86 ± 234.31	2377.86 ± 72.88	$P < .01$
Placental weights, g	563.66 ± 43.17	217.60 ± 54.58	$P < .001$

Values are mean ± SEM.

FGR, fetal growth restriction; *NS*, not significant.

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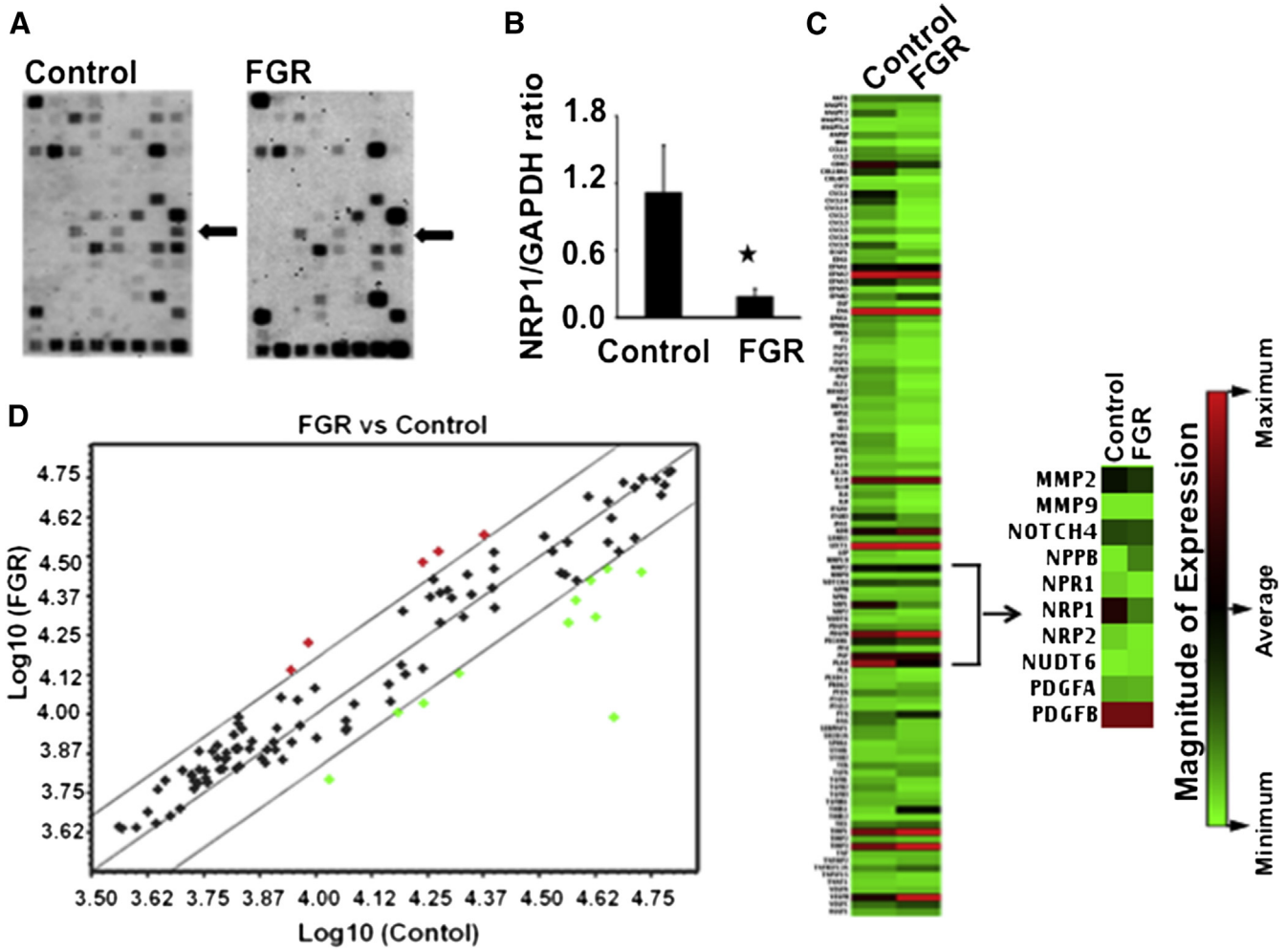
Although several genes in the study group were down-regulated by >30% of those in the control group, only NRP-1 was inhibited by >2-fold (Figure 2). When the gene expression profiles of the 2 microarray platforms, SuperArray HS-009 and SuperArrayOHS-024 (SuperArray Biosciences Corp), were cross-referenced and filtered by Student *t* test, only 3 genes were significantly underexpressed, of which only NRP-1 remained significant ($P < .0007$) when further filtering was performed by

FIGURE 1
Fetal growth restriction (FGR) reduces expression of angiogenesis-related genes in FGR placentas



A, Representative photograph of microarrays from control and FGR placentas; expression of neuropilin (NRP)-1 (arrows). **B**, Histogram showing reduced expression of NRP-1 in FGR placentas compared to controls. Values are mean ± SEM of densitometric ratios of NRP-1 and GAPDH. **C**, Representative heat map of angiogenesis-related genes from control and FGR placentas (left). Magnification of selected area of heat map showing NRP-1 expression status (right). Magnitude of gene expression (far right). **D**, Representative scatterplot of angiogenic gene expression in FGR vs control placentas. Genes were either underexpressed (green) or overexpressed genes (red) in FGR placentas. * $P < 0.05$, as compared with the control group.

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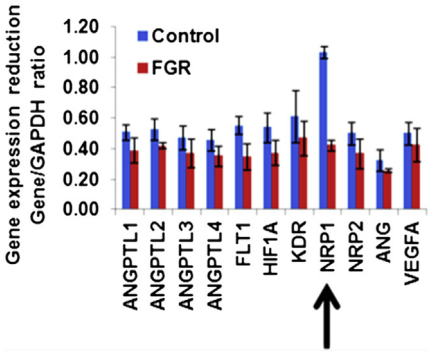
FIGURE 2
Relative expression of 11 down-regulated angiogenic genes in fetal growth restriction (FGR) placentas



Vertical axis shows expression level ratios of each gene to GAPDH in both control and FGR groups. Horizontal axis depicts 11 down-regulated angiogenic genes in FGR placentas. Arrow highlights neuropilin-1 down-regulation.

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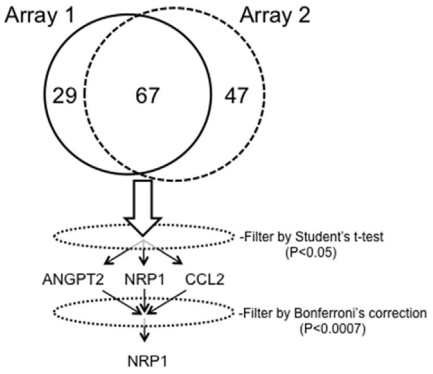
FIGURE 3
Identification of fetal growth restriction candidate genes



Gene expression profiles of 2 microarray platforms were cross-referenced. SuperArray HS-009 is depicted as array 1 and SuperArrayOHS-024 as array 2 (SuperArray Biosciences Corp, Frederick, MD). Student *t* test was used on genes that were common to both microarrays. Final candidate selection was done by Bonferroni correction for multiple comparisons.

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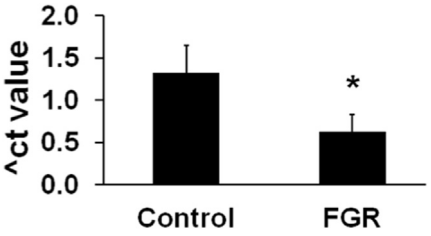
FIGURE 4
Quantitative real-time polymerase chain reaction (qPCR) analysis of neuropilin (NRP)-1 genes in control and fetal growth restriction (FGR) placentas



Differential NRP-1 gene expression by qPCR in placentas from control and FGR groups. Relative expression values were calculated using 2-delta delta Ct method and were normalized against reference gene GAPDH. Data are mean ± SD values of average mean of delta or difference in cycle threshold (ˆct) values in 7 control and 7 FGR placentas. *$P < .05$, significantly different from control group.

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Bonferroni correction for repeated measures (Figure 3).

To confirm the microarray findings by alternative approach, we quantified placental NRP-1 by qPCR. It demonstrated significantly lower NRP-1 gene expression in the FGR group compared to the control group (Figure 4). The ˆcycle threshold mean (±SD) was 0.624 ± 0.55 for the FGR group, and 1.325 ± 0.84 for the control group and the difference was significant ($P = < .05$).

Validation of placental NRP-1 protein expression by Western blot analysis and immunohistochemistry

To validate placental down-regulated NRP-1 protein expression, we performed Western blot and immunohistochemical analysis. Western blot analysis showed that the expression of NRP-1 was reduced in FGR placentas (Figure 5). Loading error was determined with β-actin. Densitometric analysis of Western blot showed reduced expression of NRP-1 in FGR placentas (Figure 5). NRP-1 protein expression, measured as NRP-1/β-actin ratio, was significantly lower in the FGR samples than in the control samples (0.13 ± 0.04 vs 0.34 ± 0.05, $P < .001$).

Immunohistochemistry for the localization of NRP-1 protein in placental tissue revealed strong immunoreactivity in cytotrophoblasts of the placental villi (Figure 6, A and B). NRP-1 immunoreactivity was also found in the vascular endothelium of placental stem vessels, Hofbauer cells, and syncytiotrophoblast cells (Figure 6). In the FGR group the staining intensity was significantly lower in comparison to the control group: 2.6 ± 1.5 vs 8.9 ± 4.2, respectively ($P < .05$). We did not identify NRP-1 immunostaining in syncytial knots in either group. Intensity scoring corroborated significant reduction (<.05) in the tissue expression of NRP-1 in FGR placentas (Figure 6, C).

Comment

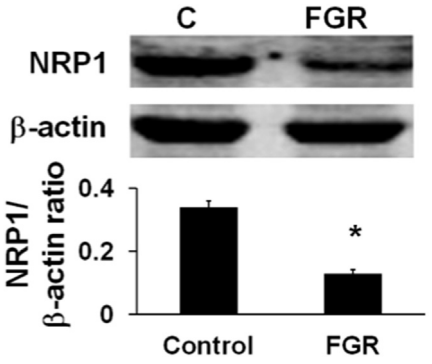
Primary findings

In this study, we demonstrate the down-regulation of NRP-1 in placentas from FGR pregnancies compared to those from pregnancies with appropriate fetal growth. The angiogenesis array data showed that although various angiogenesis and related genes differentially expressed, only the down-regulation of NRP-1 from FGR placentas achieved significance compared to those from the controls. This finding was validated by qPCR and Western blot that showed down-regulation of NRP-1 mRNA and protein, respectively, in the FGR placentas. Decreased immune localization of NRP-1 in the various components of villous tissue in FGR placentas further corroborated this. The relevance of our finding lies with the existing evidence that there is deficient branching angiogenesis in fetal placental vascular system in FGR with hemodynamic compromise[13,14] and with the emerging evidence that NRP-1 plays an important role in modulating sprouting angiogenesis through tip cell formation.[39] These are further addressed below.

Fetoplacental angiogenesis and FGR

In a human placenta, fetoplacental vascular development begins at about 3 weeks postconception with vasculogenesis, which is the de novo formation of first vessels from multipotent angioblastic

FIGURE 5
Western blot of control and fetal growth restriction (FGR) placentas



Upper panel shows representative photograph of Western blot of neuropilin (NRP)-1. β-Actin was included as housekeeping protein control for each blot. Lower panel shows mean ± SD values of densitometric ratio of NRP-1 with β-actin at bottom of each respective gel in 7 control and 7 FGR placentas. *$P < .001$.

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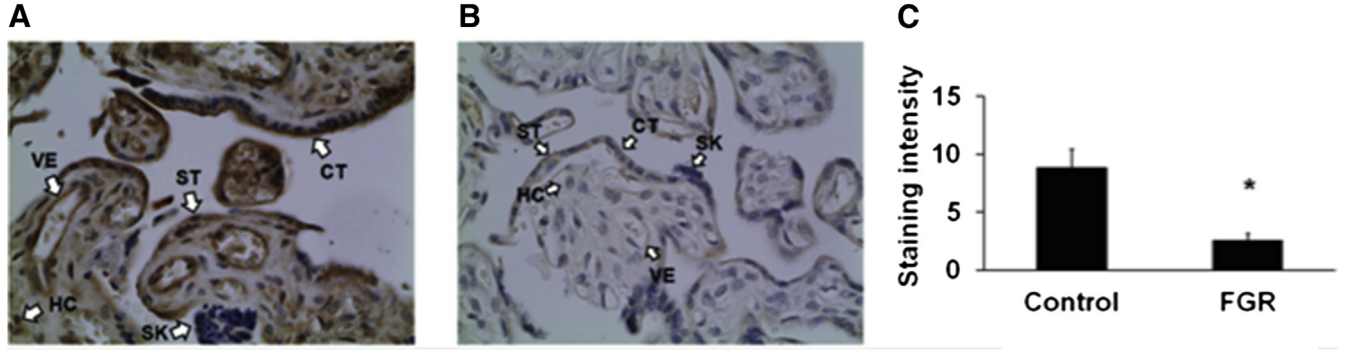
precursor cells, and continues until the 42nd day.[40] Angiogenesis follows with the generation of new vessels from the preexisting ones by either elongation or branching, and the process continues until the end of gestation. As reviewed by Burton et al,[30] there is substantial evidence suggesting continuing branching angiogenesis throughout gestation in human placenta. Our finding of NRP-1 expression in near-term placentas indirectly corroborates this. The expanding fetoplacental vascular tree leads to a progressive decrease in the umbilical artery vascular impedance and a concomitant increase in the umbilical blood flow, which is essential for sustaining fetal growth. As the arterial Doppler waveform is shaped by vascular impedance,[41] these hemodynamic changes are reflected in the rising end-diastolic flow and falling pulsatility indices in the umbilical arterial Doppler waveforms as pregnancy advances.

This pattern of fetoplacental angiogenesis is perturbed in FGR. Placentas from FGR pregnancies show significant reductions in peripheral villous capillary and surface areas.[42] Utilizing scanning electron microscopy of perfusion-fixed villous tissue and vascular plastic cast, it was demonstrated that in FGR pregnancies complicated with umbilical artery AEDF velocity, the terminal villi showed significant paucity in the number of capillaries as well as a decrease in their branching.[14] Further corroboration was provided by morphological examination of the FGR placentas that demonstrated progressive reductions in fetal placental stem artery branching, terminal villous branching, and villous capillarization as the umbilical artery end-diastolic flow progressively deteriorated and became absent or reversed.[15] These and similar observations establish a strong association between defective placental branching angiogenesis, abnormal fetoplacental circulatory dynamics, and fetal growth compromise. The fetoplacental vascular development involves multiple angiogenic agents and pathways involving VEGF and related genes.[43] Discovery of the role of NRP-1 may further elucidate the molecular regulation of fetoplacental branching angiogenesis, especially in pregnancies complicated with FGR.

Role of NRP-1 in angiogenesis

NRP-1, a single-pass transmembrane glycoprotein, is essential for axonal guidance in neuronal development and for angiogenesis.[44-46] Highly conserved in the vertebrates, its molecular structure consists of 3 regions: extracellular, transmembrane, and intracellular. The extracellular region contains 5 domains. Domains a1 and a2 are N-terminal binding CUB (C1r/C1s, urchin embryonic growth factor and bone morphogenic protein 1) domains, and bind class-3 semaphorins, which are essential for axonal guidance. Domains b1 and b2 are coagulation factors V and VII homology domains, act as ligands for

FIGURE 6
Neuropilin (NRP)-1 immunoreactivities in control and fetal growth restriction (FGR) placentas



Representative photomicrograph showing reduced expression of NRP-1 immunostaining in FGR placentas. **A**, Control, **B**, FGR. **C**, Histogram shows staining intensities of NRP-1 in control and FGR placental cells. Placentas were fixed and immunostained with NRP-1 antibody and photographed at ×400 magnification. Bar = 50 μm. Values are mean ± SD of 7 placentas from each group. *$P < .05$, as compared with control group. Arrows indicate different cell types.

CT, cytotrophoblast; *HC*, Hofbauer cells; *SK*, syncytial knot; *ST*, syncytiotrophoblast; *VE*, vascular endothelium.

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VEGF and class-3 semaphorins, and are essential for angiogenesis.[47] The b1 domain forms a complex with VEGF-2 (VEGF receptor 2 [VEGFR-2]), acts as a holoreceptor for VEGF-165, and stimulates angiogenesis. However, the angiogenic role for NRP-1 is not entirely dependent on VEGF. Fantin and associates demonstrated angiogenic capability of NRP-1 without VEGF interaction in a NRP-1 hypomorphic mice knock-in model.[48] Alternative suggested pathways for NRP-1 action include p130 (Cas) tyrosine phosphorylation that involves the intracellular domain of NRP-1.[49] Lanahan et al[50] and others have shown that the intracytoplasmic C domain of NRP-1 molecule containing the PDZ binding site facilitates intracellular VEGFR-2 trafficking, which promotes ERK1/2 signaling and stimulates arteriogenesis. In human, primate, and mouse circulations, soluble NRP-1 molecules, expressing either the complete extracellular domain or parts of the domain, have been identified.[51,52] These soluble forms of NRP-1 offer potential opportunities for developing biomarkers for FGR, although their functional significance in developmental angiogenesis remains to be elucidated.

There is evidence that the NRP-1 molecule may be involved in tip cell formation, which constitutes an essential initial step in sprouting angiogenesis.[53] In response to an angiogenic signal, a subset of dormant endothelial cells of a vessel loses pericyte cover, becomes less adherent to the adjoining cells and the basement membrane, and is ready to migrate. The migration is led by the tip cell, which is an endothelial cell modified by the presence of several angiogenesis-related molecules. Once a tip cell is formed, the adjacent endothelial cells preferentially become stalk cells and proliferate to form the stalk of the vessel branch. Whereas VEGF, VEGFR-2, and NRP-1 promote tip cell formation, NOTCH ligands DLL4 and JAGGED1 act as inhibitors of tip cells and promoters of stalk cells, and their dynamic balance guide tip cell and stalk cell differentiation. Under the effects of vascular endothelium-cadherin, VEGF, and other molecules, the stalks develop lumens and form a vascular sprout.

The above steps in sprouting angiogenesis have not yet been demonstrated in human fetal placental vascular development although there is evidence that NRP-1 plays a role in modulating decidual vascular development in early gestation in a mouse model and in human beings.[54,55] In light of the known role of NRP-1 as a vessel guidance cue in sprouting angiogenesis, our finding that NRP-1 is significantly down-regulated in FGR placentas suggests that it may play a role in deficient fetoplacental vascular branching in FGR pregnancies. Similar to FGR, preeclampsia is also characterized by deficient fetoplacental vascular branching and an antiangiogenic state. The role of NRP-1 in preeclampsia, however, remains to be elucidated. Zhou et al[56] recently demonstrated in an in vitro study that, in severe preeclampsia, cytotrophoblasts overexpress class-3B semaphorins, a transmembrane and secreted protein that competitively blocks VEGF binding to NRP-1 and NRP-2, leading to suppression of cytotrophoblastic invasion and promotion of antiangiogenic activity. However, this finding has been contested in a more recent report.[57] Further studies are needed to clarify this issue.

FGR and antiangiogenic state

Our discovery of placental underexpression of NRP-1, a molecular agent known to promote branching angiogenesis, suggests that an antiangiogenic state prevails also at the placental level in FGR pregnancies complicated with fetal hemodynamic compromise. This observation is consistent with the previous demonstration that an antiangiogenic state exists in several pregnancy disorders including preeclampsia and, more relevantly, SGA births. These are briefly discussed below.

In a longitudinal nested case-control study involving 144 singleton pregnancies, Romero et al[17] observed that the maternal plasma levels of antiangiogenic agent soluble endoglin (s-Eng) was up-regulated and proangiogenic agent PlGF was down-regulated prior to the development of preeclampsia and SGA births. In another longitudinal case-control study involving 402 singleton pregnancies, the same group measured s-Eng, soluble VEGFR-1 (sVEGFR-1), and PlGF and their ratios between the first and second trimesters of pregnancy.[16] Again, an increase in the antiangiogenic state was evident as the up-regulation of s-Eng and the down-regulation of PlGF was associated with a greater propensity to the development of preeclampsia and SGA births. There was, however, no observed association between sVEGFR-1 levels and the risk of SGA. In general the antiangiogenic state was more pronounced in preeclampsia than in SGA. Further corroboration was provided by a cross-sectional study involving 340 pregnant mothers in whom plasma levels of the soluble receptor tyrosine kinase Tie-2 were measured.[58] Tie-2 promotes angiogenesis in conjunction with angiopoietin system, a component of the VEGF family. The levels were lower in preeclampsia and in SGA births suggesting an antiangiogenic milieu in these disorders. Finally, our finding is consistent with a previous report by Chaiworapongsa et al,[59] who demonstrated that in pregnancies with SGA births, an association exists between abnormal uterine and umbilical artery Doppler and a higher maternal plasma concentrations of sVEGFR-1, a known antiangiogenic molecule. Furthermore, the same group more recently demonstrated that in singleton pregnancies between 24-34 weeks with suspected SGA, angiogenic and antiangiogenic factors in maternal plasma could predict subsequent development of preeclampsia or indicated early preterm delivery in most women.[60]

Strengths of the study

There are several strengths to our study. The study was prospective with a well-defined population. The gestational age was determined in early pregnancy; ultrasound biometric determination of FGR was according to national guidelines; and the evidence of fetoplacental hemodynamic compromise as reflected in the Doppler AEDF further defined the population. The study and control populations were matched by gestational

age, and only cesarean deliveries were included. The latter approach minimized the risk of altered placental angiogenic gene expression from periodic placental ischemia and hypoxia caused by uterine contractions, which was reported by Cindrova-Davies et al.[34] We collected tissue samples from the mediobasal location of the placental disk as defined by Wyatt et al[36] who demonstrated that the sampling site significantly affected hypoxia-related genes including VEGF in term placentas. The mediobasal area is well perfused, less likely to express hypoxia-induced angiogenic gene expression, and most likely to have normal branching angiogenesis.

Limitations of the study

There are several limitations of our study. Our findings may not be applicable to early-onset FGR. We did not include these patients because of the difficulty in finding appropriate gestational age-matched control subjects who would be delivered by elective cesarean at a preterm gestational age. In addition, our study demonstrated an association between NRP-1 and FGR but did not actually establish a causal relation between down-regulation of NRP-1 and FGR, nor did it establish the role of NRP-1 in tip cell formation in fetoplacental vascular development in normal and growth-restricted pregnancies. Future studies will address these opportunities both for in vitro and in vivo models as well as the validation of our findings in an independent and larger patient population.

Conclusion

In this study, we demonstrate a significant down-regulation of NRP-1 in placentas from FGR pregnancies complicated with umbilical hemodynamic compromise. To our knowledge this is the first time NRP-1 has been implicated in FGR. The translational significance of our finding resides with the potential of developing NRP-1 measurement in maternal circulation as a biomarker for pregnancies destined to develop FGR. Such a tool may also be able to distinguish FGR fetuses from those that are constitutionally small. Such a distinction is not possible with the current diagnostic approaches. Future potential also exists for therapeutic innovations specifically targeting deficient placental NRP-1 expression. Furthermore, as FGR with umbilical artery AEDF shows deficient placental branching angiogenesis and as NRP-1 is involved in vascular sprouting, we speculate that NRP-1 plays a significant role in fetoplacental branching angiogenesis and its down-regulation may be mechanistically involved in abnormal vascular development in FGR. Future investigations may establish such a causal role advancing our understanding of the complex pathogenic mechanisms underlying FGR. ■

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