

An EG-VEGF-Dependent Decrease in Homeobox Gene *NKX3.1* Contributes to Cytotrophoblast Dysfunction: A Possible Mechanism in Human Fetal Growth Restriction

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Idiopathic fetal growth restriction (FGR) is frequently associated with placental insufficiency. Previous reports have provided evidence that endocrine gland-derived vascular endothelial growth factor (EG-VEGF), a placental secreted protein, is expressed during the first trimester of pregnancy, controls both trophoblast proliferation and invasion, and its increased expression is associated with human FGR. In this study, we hypothesize that EG-VEGF-dependent changes in placental homeobox gene expressions contribute to trophoblast dysfunction in idiopathic FGR. The changes in EG-VEGF-dependent homeobox gene expressions were determined using a homeobox gene cDNA array on placental explants of 8-12 wks gestation after stimulation with EG-VEGF *in vitro* for 24 h. The homeobox gene array identified a greater-than-five-fold increase in *HOXA9*, *HOXC8*, *HOXC10*, *HOXD1*, *HOXD8*, *HOXD9* and *HOXD11*, while *NKX3.1* showed a greater-than-two-fold decrease in mRNA expression compared with untreated controls. Homeobox gene *NKX3.1* was selected as a candidate because it is a downstream target of EG-VEGF and its expression and functional roles are largely unknown in control and idiopathic FGR-affected placentae. Real-time PCR and immunoblotting showed a significant decrease in *NKX3.1* mRNA and protein levels, respectively, in placentae from FGR compared with control pregnancies. Gene inactivation *in vitro* using short-interference RNA specific for *NKX3.1* demonstrated an increase in BeWo cell differentiation and a decrease in HTR-8/SVneo proliferation. We conclude that the decreased expression of homeobox gene *NKX3.1* downstream of EG-VEGF may contribute to the trophoblast dysfunction associated with idiopathic FGR pregnancies.

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INTRODUCTION

Fetal growth restriction (FGR), also known as intrauterine growth restriction, is a pregnancy complication in which a

fetus fails to reach its genetically determined growth potential *in utero*. Impairment in fetal development and growth *in utero* has been associated with lifelong

adverse health sequelae, including diabetes and cardiovascular diseases (1,2), therefore, understanding the molecular mechanism of human FGR is of utmost importance. Obvious maternal, fetal and placental causes of FGR account only for one third of FGR cases (3), while the remaining two thirds are termed idiopathic. Idiopathic FGR is often associated with placental insufficiency (4) where the placentae are often smaller and show numerous morphological defects such as reduced trophoblast proliferation and abnormal villous vasculature with less branched terminal villi (5).

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During the first trimester of human pregnancy, extravillous cytotrophoblasts (EVCT) grow from anchoring villi, invade the maternal decidua and remodel the uterine spiral arterioles (6). On the other hand, the mononuclear villous cytotrophoblasts (VCT) on the floating villi continue to proliferate and differentiate into the multinucleated syncytiotrophoblasts (ST). The ST layer plays a key role throughout pregnancy. It is the site for several important placental functions, including nutrient and ion exchange and the synthesis of hormones required for fetal growth and development (7,8). Thus, a syncytium that functions efficiently requires both fusion and differentiation of cytotrophoblasts (CTB). A dysfunction in CTB differentiation and proliferation leads to deficient EVCT invasion and ST apoptosis, both hallmarks of pregnancy pathologies, including human idiopathic FGR (6,9).

The proliferation, differentiation and invasion of the first-trimester human CTBs are influenced by multiple regulatory factors, including adhesion molecules, growth factors, cytokines, proteases, matrix-derived components, transcription factors and oxygen tension (10). Growth factors such as epidermal growth factor (EGF), hepatocyte growth factor (HGF) and transforming growth factor- β 1 (TGF- β 1), are widely studied as regulators of CTB proliferation, differentiation, migration and invasion (11–13).

In the last decade, we and others have been interested in the study of a new angiogenic factor, endocrine gland-derived vascular endothelial growth factor (EG-VEGF), that is characterized, sequenced and shown to be expressed in testis, adrenal gland, ovary and placenta (14). Human EG-VEGF exerts its effects via G protein-coupled receptors (PROKRs), termed PROKR1 and PROKR2 (15). We have recently shown that EG-VEGF and its receptors are highly expressed in the human placenta during the first-trimester of pregnancy, with the highest expression found in the ST; that its expression is up-regulated by hypoxia; and that its circulating levels are significantly higher dur-

ing early pregnancy and in pregnancy pathologies including FGR and pulmonary embolism (PE) (16–19). In relation to placental development, we have demonstrated that EG-VEGF controls CTB proliferation, survival, differentiation and vascular development during the first-trimester of pregnancy, however, whether EG-VEGF control of these activities is a direct or an indirect process is far from being understood.

Growth factors including EG-VEGF and HGF have been shown to modulate the expression of the placental homeobox gene, *HLX*, and to alter CTB proliferation and migration (20,21). Homeobox genes, among other transcription factors, are known to play key roles in placental development and function (22–24). Also, it is proposed that differential responses to fluctuating hormone, or growth factor signals, such as those influenced by EG-VEGF, may constitute key controlling mechanisms for homeobox gene expressions in the human placenta (20).

In this study, we hypothesize that EG-VEGF-dependent change in placental homeobox gene expressions contribute to trophoblast dysfunction in idiopathic FGR. To investigate this, we employed first-trimester placental explant cultures and treated them with EG-VEGF to identify the candidate homeobox gene that showed a downregulated expression downstream of EG-VEGF in FGR placentae. The candidate gene was then validated in placentae obtained from third-trimester human idiopathic FGR and gestation-matched uncomplicated control pregnancies. Further, the candidate gene's functional role on trophoblast proliferation and differentiation was determined using trophoblast-derived cell lines, *in vitro*.

MATERIALS AND METHODS

First-Trimester Placental Tissues

Human placentae from 8 to 14 wks gestation ($n = 12$) and second trimester pregnancies of 20 wks gestation ($n = 3$) were obtained from elective terminations. First trimester and second trimes-

ter placentae collection and processing were performed with the approval of the University Hospital ethics committees at the Broussais Hospital, Paris, France, and Grenoble University Hospital, Grenoble, France, and with the informed written consent of each patient.

Patient Details and Placental Tissue Sampling from Human Idiopathic FGR and Gestation-Matched Uncomplicated Control Pregnancies

Placentae from pregnancies complicated by idiopathic FGR ($n = 25$) and those from gestation-matched uncomplicated pregnancies as controls ($n = 25$) were collected following Cesarean section or vaginal delivery; gestation times ranged from 27 to 40 wks. Placenta collection was approved by the Research and Ethics Committee of The Royal Women's Hospital, Melbourne. All patients were informed and consented. Table 1 reports the clinical characteristics of both the FGR and gestation-matched control groups included in this study (25,26). As illustrated in Table 2, the inclusion criteria for the FGR study group were a birth weight of less than the 10th percentile for gestation as determined by the Australian fetal growth charts (27), and at least two of the following diagnoses on antenatal ultrasound: abnormal umbilical artery Doppler flow velocimetry, oligohydramnios (Amniotic Fluid Index < 7), or asymmetric growth of the fetus (head circumference-to-abdominal circumference ratio > 95 th percentile for gestation). Exclusion criteria for both cases and control groups were factors that are known causes of FGR and included maternal smoking and drug dependency during pregnancy, other underlying maternal diseases including gestational diabetes, preeclampsia, hypertension, placental abruption, prolonged rupture of membranes, fetal congenital anomalies and suspected intrauterine infection (25,26). Uncomplicated control pregnancies were selected to match FGR cases according to gestation times. All control patients gave birth to normally formed babies with birth weights appropriate for gestational

Table 1. Clinical characteristics of patient samples.

Characteristics	FGR (n = 25)	GMC (n = 25)	Significance
Maternal characteristics			
Gestational age (weeks) ^a	34.4 ± 6.5	35.8 ± 6.6	<i>p</i> = 0.25
Maternal age (years) ^a	33.2 ± 5.7	31.9 ± 6.5	<i>p</i> = 0.4
Placental weight (g) ^a	409.3 ± 110.3	525.0 ± 148.2	<i>p</i> < 0.005
Parity^b			
Primiparous	12	10	<i>p</i> = 0.56
Multiparous	13	15	
Mode of delivery^b			
Vaginal	10	6	<i>p</i> = 0.35
Cesarean section in labor	3	2	
Cesarean section not in labor	12	17	
New born characteristics			
Gender^b			
Male	8	12	<i>p</i> = 0.25
Female	17	13	
Birth weight			
Weight (g) ^a	2,051.4 ± 637.0	2,603.8 ± 857.0	<i>p</i> < 0.05
10th–90th percentile	0	25	
5th–10th percentile	11		
<5th percentile	14		

^aSignificance was determined by the Student *t* test. Results are expressed as mean ± standard error of the mean (SEM).

^bSignificance was determined by the chi-square test. Results are expressed as mean ± standard error of the mean (SEM).

age, and the placentae from these patients were also grossly normal (25,26).

Placental bed samples (n = 10 each) from FGR and control pregnancies were collected with informed patient consent from the Department of Obstetrics and Gynecology at the hospital La Citadelle, Liège, Belgium, with the approval by the local ethics committee. Seventy percent of FGR cases were reported with abnormal umbilical Doppler velocimetry, while 20% FGR cases displayed reverse diastolic blood flow. FGR cases were associated with a significant decrease in both placental and infant birth weight (*p* < 0.05) (28).

All samples were processed within 20 min of placental delivery with tissues excised from randomly selected areas of central cotyledons following the removal by dissection of any attached decidua. Tissue pieces were thoroughly washed in 0.9% phosphate buffered saline (PBS) to minimize blood contamination, and then snap frozen and stored at –80°C for RNA and protein analysis or fixed in 10% formalin for immunolocalization studies.

Tissue and Cell Culture

Human villous explant cultures. First trimester human placentae were used to establish villous explant cultures (10–12 wks gestation, n = 6) as described previously (17). Fragments of placental villi

(15–20 mg) were teased apart and seated on a transparent Biopore membrane (Millipore Corp.) of 12-mm diameter (inserts with a pore size of 0.4 μm). The inserts were precoated with 0.2 mL undiluted Matrigel (Becton Dickinson), polymerized at 37°C for 30 min and transferred to a 24-well culture dish. Explants were cultured in medium containing DMEM-Ham's F-12 (DMEM/F12) (Invitrogen) supplemented with 0.25 μg/mL ascorbic acid, 100 μg/mL streptomycin and 100 IU/mL penicillin at pH 7.4. Villous explants were kept in culture for up to 72 h. The viability of the explants was assessed as described previously (17) by measuring human chorionic gonadotropin (hCG) production in the culture medium every 24 h. After 24 h culture, the medium was changed and explants incubated in the absence or presence of 50 ng/mL or 5 nmol/L recombinant human EG-VEGF (Tebu) (20).

Isolation and purification of villous (VCT) and extravillous (EVCT) trophoblasts. VCTs and EVCTs were prepared from first-trimester placental tissues (n = 12) as described previously (29). Briefly, trophoblasts were enriched by differential sequential trypsin digestion and the use of a Percoll gradient (20% to 60%). EVCTs were plated at a

Table 2. Clinical criteria for FGR-affected pregnancies.^a

Clinical characteristics	Number of samples
Birth weight <10th percentile	25/25
Abnormal umbilical artery Doppler velocimetry	
Elevated	7/25
Reversed	6/25
Absent	8/25
Not recorded	4/25
Head circumference to abdominal circumference ratio	
Asymmetry HC: AC >1.2	21/25
Not recorded	4/25
Amniotic fluid index	
Oligohydramnios, AFI <7	18/25
Normal, AFI ≥7	7/25

^aAll FGR-affected pregnancies in this study met with the first selection criterion of birth weight being less than 10th percentile and at least two of the other ultrasound-determined selection criteria. Seventeen out of 25 FGR-affected pregnancies met all three ultrasound-determined selection criteria.

density of 5×10^4 cells/cm² on Matrigel-coated (5 mg/mL) (BD Biosciences) 35-mm Falcon culture dishes and grown in HAM-F12/DME mol/L (v/v) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mmol/L glutamine. EVCT cell purity was assessed by positive staining for the specific markers of human invasive EVCTs, cytokeratin-7 (CK7) and human leukocyte antigen-G expression (29). VCTs were plated at a density of 1.5×10^4 cells/cm² on 35 mm or 60 mm TPP (Techno Plastic Products) tissue culture dishes using DME mol/L supplemented with 10% FBS, 2 mmol/L glutamine, 100 µg/mL streptomycin and 100 IU/mL penicillin. The purity of VCT cultures was characterized by positive staining for CK7 (95% positive cells) and by microscopic observation of cell aggregates and syncytiotrophoblast formation at 48 to 72 h (29).

Human placental trophoblast-derived cell lines. The human placental trophoblast-derived choriocarcinoma BeWo cells (B30 clone) were grown in RPMI-1640 culture media supplemented with 10% FBS, 100 µg/mL streptomycin and 100 IU/mL penicillin (Invitrogen) and incubated at 37°C, 5% CO₂ and 95% air. The HTR-8/SVneo cells were immortalized with the SV40 virus (30). In the present study, cells between 40–50 passages were used and grown in RPMI 1640 supplemented with 5% FBS, 100 µg/mL streptomycin, 100 IU/mL penicillin and amphotericin B (Invitrogen). Cells were maintained at 37°C in an atmosphere of 5% CO₂ and 95% air.

Total RNA Extraction and cDNA Preparation

Total RNA from placental villous and placental bed tissues and cultured cells was extracted using the RNeasy Midi and Mini kits, respectively, as described previously (25). cDNA was prepared from 2 µg total RNA reverse-transcribed using Superscript III ribonuclease H-reverse transcriptase (Invitrogen) in a two-step reaction also as described previously (25,31).

Homeobox Gene cDNA Array

The Human HOX TaqMan PCR Array (Applied Biosystems) for gene profiling was used to screen for genes that showed differential expressions in EG-VEGF treated first-trimester placental explant cultures compared with untreated placental explant cultures. Briefly, 2 ng/well cDNA was used in a 20 µL reaction by the addition of TaqMan Master Mix (Applied Biosystems) and distributed into a TaqMan Array 96-well plate. The Human HOX Array plate contained 84 gene-specific primer sets with a panel of five housekeeping gene primers for normalization. The housekeeping genes consisted of 18S ribosomal RNA (18S), β-2-microglobulin (B2M), hypoxanthine phosphoribosyltransferase 1 (HPRT1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin (ACTB). PCR was completed in an ABI Prism 7500 Sequencer according to the manufacturer's instructions. The relative gene expression values, or fold changes, were analyzed using the DataAssist Software v3.0 (Applied Biosystems). Genes of interest were identified and prioritized based on the difference in gene mRNA expression in EG-VEGF treated placental tissues when compared with untreated control placental tissues.

Real-time PCR

Relative quantitation of mRNA was performed in an ABI Prism 7500 Sequencer using the Applied Biosystems prevalidated assay mix. Each assay mix consisted of unlabeled PCR primers and a TaqMan FAM-labeled MGB probe (NKX3.1 Hs00171834_m1; or gene that codes for chorionic gonadotropin-β [CGB] Hs00361224_g1, B-cell CLL/lymphoma 2 [BCL2] Hs00608023-m1 and BCL2 associated X-protein [BAX] Hs00180269_m1 [Applied Biosystems]). Gene expressions relative to the eukaryotic 18S rRNA endogenous control (#4319413E, VIC/MGB probe) (Applied Biosystems) were calculated according to the 2^{-ΔΔCT} method (32).

Immunoblotting

Total cellular and tissue protein was extracted as described previously (25).

Immunoblotting was performed with 25 µg of total protein using a 10% SDS/PAGE and electroblotting onto a nitrocellulose membrane (Pal Gelman) The membrane was blocked with 5% (w/v) skim milk for 1 h at room temperature, and followed by an overnight incubation in 0.02 µg/µL rabbit anti-human polyclonal NKX3.1 (H-50, SC25405, Santa Cruz Biotechnology) at 4°C. Antibody binding was visualized using horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Invitrogen/Life Technologies), followed by autoradiography using the ECL-Western Chemiluminescence Detection Kit (GE Healthcare). Total protein load on the gel was determined by Coomassie staining, and the immunoreactive NKX3.1 protein was analyzed by scanning densitometry (ImageQuant, GE Healthcare) as described previously (25,26,33,34).

Immunofluorescence

Spatio-temporal distribution and localization of NKX3.1 protein in first-trimester and third-trimester FGR and control placental tissue sections was by immunofluorescence. Briefly, paraffin-embedded placental tissue sections, cut to 5-µm thickness, were dewaxed in xylene, hydrated in graded ethanol (100% to 50% ethanol) and blocked with 1% bovine serum albumin (BSA) prepared in PBS. Tissue sections were then incubated overnight at 4°C with the primary antibody, either rabbit anti-human NKX3.1 or mouse monoclonal anti-CK7 (RCK 105 clone, Abcam), at a concentration of 0.01 µg/µL in 1% BSA/PBS. Control sections were incubated with 0.02 µg/µL mouse IgG nonimmune rabbit serum prepared in 1% BSA/PBS (Dako). Fluorescence detection was by Alexa Fluor 488 and counterstaining with DAPI (Dako) according to the manufacturer's recommendations.

Forskolin Mediated BeWo Cell Differentiation

BeWo cells were seeded at a density of 2×10^5 cells/well and serum starved in RPMI-1640 medium supplemented with 0.1% BSA (w/v). Forskolin, (FSK)

(Invitrogen/Life Technologies) prepared in 1% dimethyl sulfoxide (DMSO), was added to a final concentration of 10 $\mu\text{mol/L}$ (35) to induce differentiation over 72 h in culture. At the end of the incubation period, the medium was collected and stored at -20°C and the cells were processed for RNA and protein analysis. Untreated cells were maintained in RPMI 1640 medium supplemented with 1% DMSO and used as the control. Each experimental condition was implemented in duplicate, and all cell culture experiments repeated on three independent occasions.

β -hCG Protein Assay

Determination of β -hCG protein levels was by ELISA (α Diagnostic International) and carried out as described previously (36). The assay quantitation was performed on cell culture supernatant collected from $n = 6$ independent experiments. The minimum detection limit of this assay was 1.5 mIU/mL.

Gene Inactivation of *NKX3.1* Expression in BeWo and HTR-8/SVneo Cells

BeWo cells were seeded at a density of 2×10^5 cells/well in 6-well plates and treated with FSK. They were then transfected with *NKX3.1* siRNA (19 to 21 base pairs, Thermo Fisher Scientific Inc.) using the Hiperfect transfection reagent (Invitrogen). The optimum siRNA:RNAiFect ratio and incubation time for the culture was determined to be 1:6 and 72 h, respectively (data not shown).

HTR-8/SVneo cells were also seeded at a density of 2×10^5 cells/well in 6-well plates with supplemented culture medium and transfected as above for 48 h. Negative control (NC) siRNA used in these experiments consisted of an enzyme-generated pool of oligonucleotides (15–19 base pairs) that showed no DNA sequence similarity to any known human gene (AllStars Neg. siRNA AF 488, Qiagen).

Inhibition of EG-VEGF Receptors

To determine the role of EG-VEGF receptor-mediated signaling on *NKX3.1* ex-

pression, BeWo cells grown in either normoxic or in hypoxia (2%) were treated with EG-VEGF receptor antagonists, PC-7 for PROKR1 (37) at 1 $\mu\text{mol/L}$ and PKRA505 for PROKR2 (38) at 1 $\mu\text{mol/L}$, and incubated for 8 h. *NKX3.1* mRNA was determined using real-time PCR as described above. Specificity of both antagonists was recently characterized (37,38).

Human Apoptosis cDNA Array

Human apoptosis cDNA arrays (Applied Biosystems) were used for gene profiling and to screen for genes that showed differential expression in BeWo and HTR-8/SVneo cells treated with *NKX3.1* siRNA and NC siRNA. Briefly, cDNA was prepared and added to a TaqMan Master Mix (Applied Biosystems) and distributed in a TaqMan Array 96-well plate at approximately 2 ng/well in a 20 μL reaction. The array plate contained 84 gene-specific primer sets with a panel of five housekeeping gene primers for normalization. The housekeeping genes consisted of *18S ribosomal RNA (18S)*, *β -2-microglobulin (B2M)*, *hypoxanthine phosphoribosyltransferase 1 (HPRT1)*, *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* and *β -actin (ACTB)*. PCR was performed in an ABI Prism 7500 Sequence Detector under the following cycling parameters: 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and primer extension at 60°C for 1 min. Data (Ct values) were analyzed using the ABI Sequence Detector System software version 2.0 and the relative gene expression values, or fold changes, were analyzed using the DataAssist Software v3.0 (Applied Biosystems) and normalized to the average Ct value of the five housekeeping genes. Genes were prioritized based on the difference in gene mRNA expression in *NKX3.1* siRNA treated cells when compared with NC.

The xCELLigence System

The functional consequence of *NKX3.1* gene inactivation on HTR-8/SVneo cell proliferation was assessed by real-time monitoring of the xCELLigence RTCA DP system (ACEA Biosciences), as described

previously (39). Briefly, the assay was performed in E-plates at 37°C , 5% CO_2 and 95% humidified air. The xCELLigence system measures cell attachment expressed as an arbitrary parameter called the cell index (CI) and each experiment was monitored for at least 48 h.

Data Analysis

The significance of any differences between the clinical characteristics of the FGR-affected and the control pregnancies was by either the chi-square test or Student *t* test with the values described as mean \pm SEM. The differences in mRNA and protein expressions of the FGR-affected placentae compared with gestation-matched controls were assessed by the Mann–Whitney *U* test. The value $p < 0.05$ was considered significant and data analysis was performed using GraphPad Prism 5 (GraphPad Software Inc.).

RESULTS

Homeobox Gene Expression in Placental Explants

Figure 1A shows the homeobox gene expression profile identifying a greater-than-five-fold mRNA expression increase of *HOXA9*, *HOXC8*, *HOXC10*, *HOXD1*, *HOXD8*, *HOXD9* and *HOXD11*, and a greater-than-two-fold decrease of *NKX3.1* in placental explants stimulated with EG-VEGF compared with untreated controls. Homeobox gene *NKX3.1* was the only gene that showed reduced expression in the presence of EG-VEGF, therefore, it was selected as the candidate gene for further gene expression validation and investigation of its functional role in trophoblastic proliferation and differentiation.

Effect of Gestation on *NKX3.1* mRNA Expression in Placental Tissues

NKX3.1 mRNA relative to *18S* rRNA was determined in placental tissues at 8–14 wks gestation, 20 wks gestation and at term (>37 wks). As depicted in Figure 1B, a significant increase in *NKX3.1* expression relative to *18S* rRNA was observed in placental tissues with increasing gestation times.

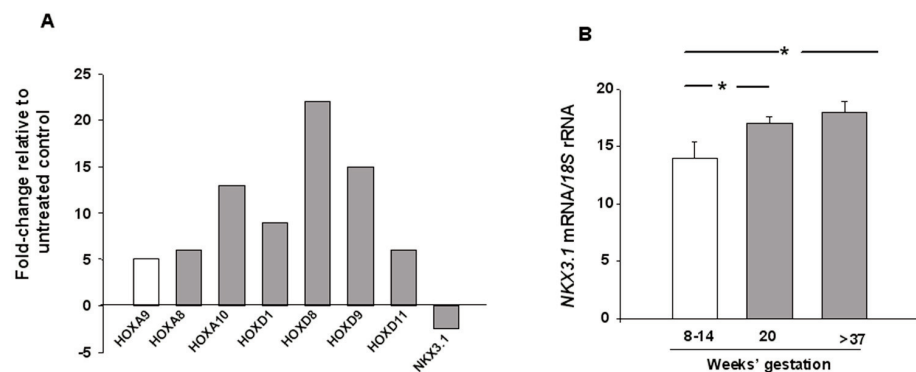


Figure 1. Candidate gene identification and placental expression throughout pregnancy. (A) Effect of EG-VEGF on homeobox gene expression profile in first-trimester placental explants ($n = 6$). Real-time PCR was used to detect homeobox gene mRNA relative to 18S rRNA. Results are reported as fold changes relative to untreated controls. (B) Quantification of *NKX3.1* mRNA expression levels in placental tissues at 8 to 14 wks gestation ($n = 12$), 20 wks gestation ($n = 3$) and at term ($n = 6$). 18S rRNA was used as the internal control. Results are reported as the mean \pm SEM. Data were analyzed using the Mann-Whitney U test (significant differences when compared with the 8 to 14 wks gestation period, $*p < 0.05$).

FGR and Control Patient Characteristics

Table 1 describes the clinical features of FGR and the gestation-matched uncomplicated control pregnancies used in this study. Maternal age, parity, gestational age and mode of delivery were not significantly different between the two groups, conversely, mean placental weights and birth weights were significantly lower in FGR compared with gestation-matched control pregnancies (Table 1, $p < 0.025$). Using these placental samples, we have already demonstrated reliable gene expression differences for other homeobox genes (25,26,31). Table 2 describes the samples that were collected from a clinically well-defined cohort of FGR-affected pregnancies.

NKX3.1 Expression in FGR

NKX3.1 mRNA levels and protein expressions were validated in FGR and control placentae by using real-time PCR and immunoblotting, respectively (Figure 2). The relative level of placental *NKX3.1* mRNA was significantly decreased in placental villous tissues (Figure 2A) and in placental bed tissues (Figure 2B) in FGRs compared with controls. The decrease in *NKX3.1* mRNA level was also further confirmed at the protein level. Figure 2C shows a representative immunoblot for the immunoreactive *NKX3.1* protein (35 kDa) in FGR-affected placentae and in gestation-matched controls. A Coomassie-stained gel demonstrated equal loading of total placental protein, while densitometry showed that the immunoreactive placental *NKX3.1* protein was decreased significantly in FGRs compared with controls (Figure 2D).

Effect of Inhibition of EG-VEGF Receptors on *NKX3.1* mRNA under Normoxic or Hypoxic Conditions

To determine the role of EG-VEGF receptor-mediated signaling on *NKX3.1* expression, we performed an experiment using BeWo cells that were treated with EG-VEGF receptor inhibitors PC-7 for PROKR1 and PKRA505 for PROKR2 at 10 $\mu\text{mol/L}$ concentrations each and incu-

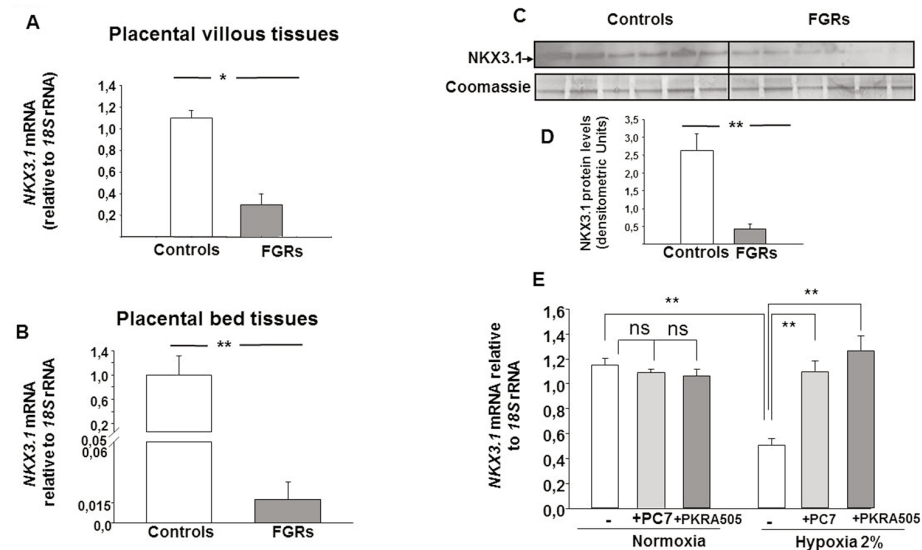


Figure 2. Expression of *NKX3.1* downregulated in FGR pregnancies. (A) Quantification of *NKX3.1* mRNA expression levels in placental tissues from gestation-matched control ($n = 25$) and FGR pregnancies ($n = 25$). 18S rRNA was used as an internal control. (B) Quantification of *NKX3.1* mRNA expression levels in placental bed tissues. 18S rRNA was used as an internal control. (C) Representative immunoblot for *NKX3.1* that compares gestation-matched control ($n = 6$) versus FGR placentae ($n = 6$). Coomassie-stained gel depicting total protein load. Quantification of the intensity of the bands is illustrated in (D). Results are reported as the mean \pm SE mol/L from $n = 3$ independent experiments. Data were analyzed using the Mann-Whitney U test (significant differences, $*p < 0.05$). Panel (E) reports *NKX3.1* mRNA expression levels in BeWo cells that were incubated under normoxic or hypoxic conditions for 8 h, in the absence or presence of PROKR1 (PC7) or PROKR2 (PKRA505) antagonists. Both antagonists were used at 10 $\mu\text{mol/L}$. (significant differences, $*p < 0.05$; $**p < 0.01$).

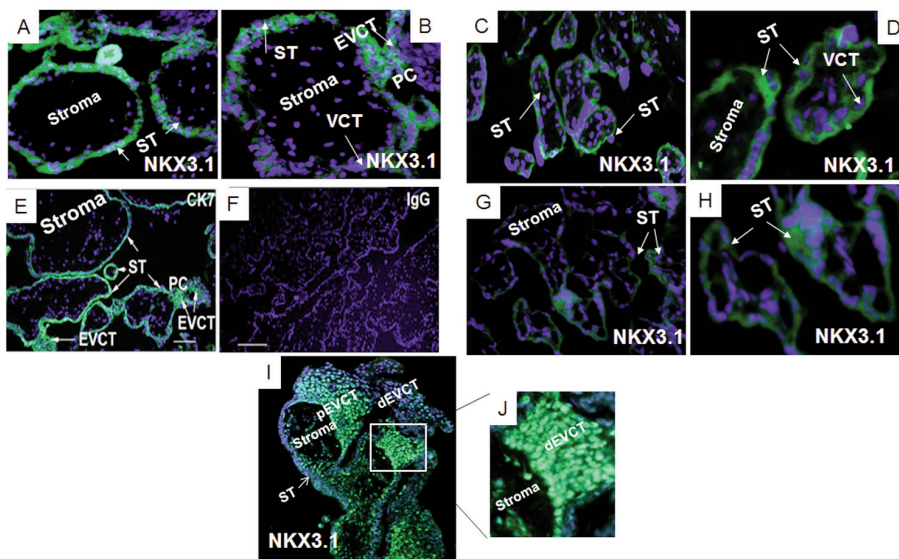


Figure 3. NKX3.1 protein expression in first and third-trimester placental sections. Localization of NKX3.1 protein in a first-trimester placenta (A and B, $n = 6$) in a third-trimester FGR placenta (G and H, $n = 6$) and a gestation-matched control placenta (C and D, $n = 6$). NKX3.1 staining (rabbit anti-human, green). Panels (E) and (F) show representative controls: cytokeratin7 (CK7) used as a positive control in a term placental section (E), rabbit IgG used as a negative control (F). Panels (I) and (J) show representative staining of the human placental column during the first trimester of pregnancy. Panel (J) is a higher magnification of the photograph in panel (I). Blue DAPI stain. Villous cytotrophoblast (VCT), extravillous cytotrophoblast (EVCT), proximal extravillous trophoblast cells (pEVCT) and distal EVCT (dEVCT), syncytiotrophoblast (ST), proximal cell column (PC). Panels (G) and (H), 200x magnification, and the scale bars are 100 μm .

bated for 8 h under normoxic (20%) or hypoxic (2%) conditions (hypoxia being an environment that mimics the FGR pathology and is known to increase EG-VEGF expression and production) (17). Figure 2E shows that under normoxic conditions NKX3.1 mRNA was not significantly altered in the presence of inhibitors of EG-VEGF receptors in both BeWo. Interestingly, hypoxia induced a significant reduction in NKX3.1 mRNA expression that was completely abolished in the presence of PROKR1 and PROKR2 antagonists.

NKX3.1 Expression in Normal and FGR Human Placentae

NKX3.1 protein localization in first-trimester placentae was detected in the nuclei of CTBs, the ST and in proliferating EVCTs of the proximal cell column (Figures 3A, B). In the third-trimester FGR (Figures 3G, H) and gestation-matched

control placentae (Figures 3C, D) immunoreactive NKX3.1 protein was detected predominantly in the cytoplasm of the ST. Immunoreactivity to CK7 was used as a positive control in a term placental section (Figure 3E) while rabbit IgG was used as a negative control (Figure 3F). Figures 3I and 3J show nuclear immunostaining of NKX3.1 in placental columns from first trimester human placenta. NKX3.1 is mainly localized to the nuclei of villi cytotrophoblast cells and in the proximal extravillous trophoblast cells (pEVCT), that is, proliferative proximal cells within the column. NKX3.1 expression was decreased in distal EVCT (dEVCT). Photograph in Figure 3J is a higher magnification of the image reported in Figure 3I.

In Vitro Differentiation and NKX3.1 Expression in Placental Cells

NKX3.1 mRNA levels were detected in cultured VCTs and EVCTs isolated from

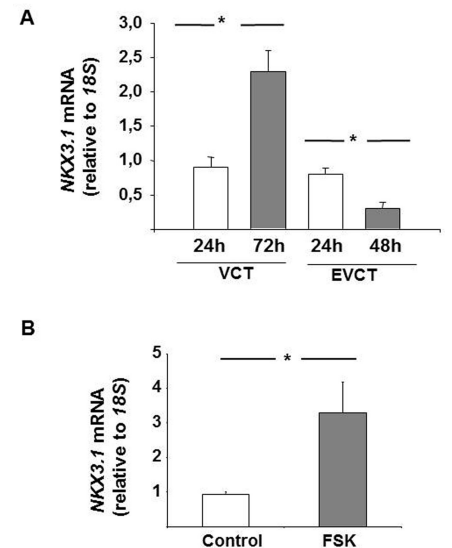


Figure 4. Effect of *in vitro* differentiation on NKX3.1 mRNA expression in placental cells. (A) Quantification of NKX3.1 mRNA expression levels in primary VCT and EVCT cultures from first-trimester placentae ($n = 12$). 18S rRNA was used as an internal control. (B) Effects of forskolin (10 $\mu\text{mol/L}$, 72 h) on NKX3.1 mRNA expression levels in BeWo cells. 18S rRNA was used as an internal control. Results are reported as the mean \pm SE mol/L from $n = 3$ independent experiments. Data were analyzed using the Mann-Whitney *U* test (significant differences, $*p < 0.05$).

first-trimester placentae. *In vitro* differentiation of VCTs into ST occurred at 72 h and was associated with a significant increase in NKX3.1 mRNA, while NKX3.1 mRNA levels were detected in proliferating EVCTs at 24 h and decreased significantly in invasive EVCTs after 48 h (Figures 4A).

Effect of NKX3.1 Gene Inactivation on βhCG Production in BeWo Cells

To determine NKX3.1 expression during differentiation of BeWo cells, FSK was used. This induced a significant increase in NKX3.1 mRNA compared with the vehicle control (Figure 4B).

The functional consequence of a reduced placental NKX3.1 in FGR was modeled in cell culture by inactivating the NKX3.1 expression using NKX3.1 siRNA in FSK-induced BeWo cells. As shown in Figure 5, a significant decrease

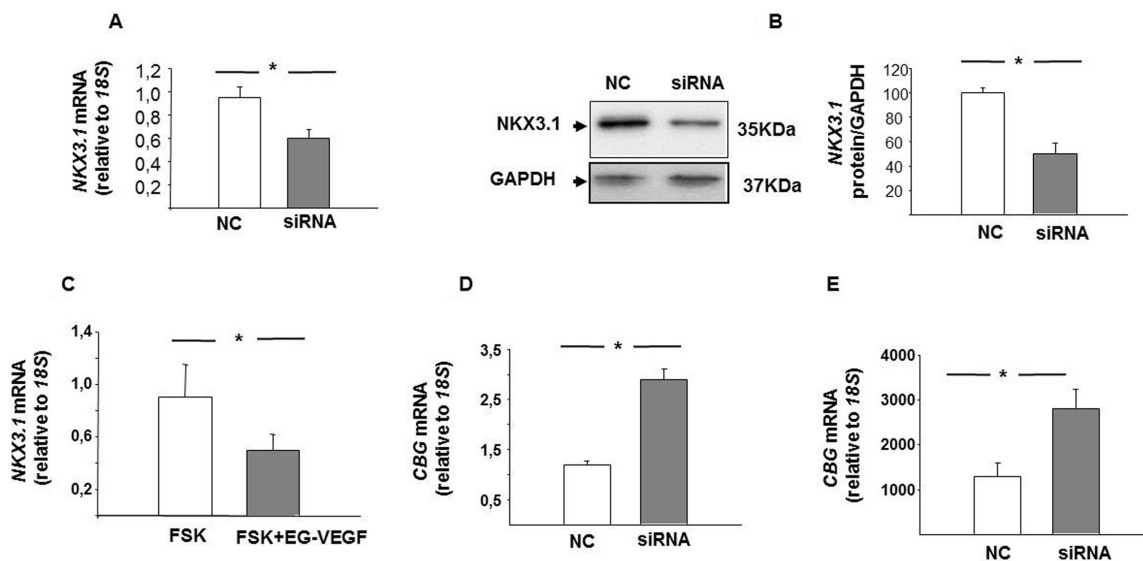


Figure 5. Effect of *NKX3.1* downregulation on cell differentiation in BeWo cells. (A) Quantification of the downregulation of *NKX3.1* mRNA expression following treatment with *NKX3.1* siRNA. (B) Shows a representative Western blot and quantification of the downregulation of *NKX3.1* protein following the treatment with *NKX3.1* siRNA. Cells were analyzed for the downregulation of *NKX3.1* mRNA and protein expressions 72 h after transfection. Nonspecific siRNA served as control siRNA. (C) Expression of *NKX3.1* mRNA in FSK ± EG-VEGF-treated BeWo cells. (D) Effect of *NKX3.1* downregulation on *CGB* mRNA expression and β-hCG protein secretion in BeWo cells. Results are reported as the mean ± SE mol/L from n = 3 independent experiments. Data were analyzed using the Mann-Whitney *U* test (significant differences, **p* < 0.05).

in *NKX3.1* mRNA (Figure 5A) and protein (Figure 5B) was observed in the BeWo cells thus treated compared with the NC control. EG-VEGF treatment in FSK-induced BeWo cells also showed a significant decrease in *NKX3.1* mRNA expression (Figure 5C). The effect of *NKX3.1* inactivation on BeWo cell differentiation, as determined by real-time PCR and immunoassays, was an increased expression of *CGB* mRNA and β-hCG protein in *NKX3.1* siRNA-treated cells compared with NC siRNA-treated cells (Figures 5D, E).

EG-VEGF Stimulation and *NKX3.1* Inactivation Effect on Trophoblast Differentiation

The trophoblast differentiation marker *CGB* was measured following *NKX3.1* siRNA transfection in the presence of EG-VEGF treatment (Figure 6A). Reduced *NKX3.1* mRNA levels had significantly increased the *CGB* mRNA expression in BeWo cells, even in the presence of EG-VEGF stimulation.

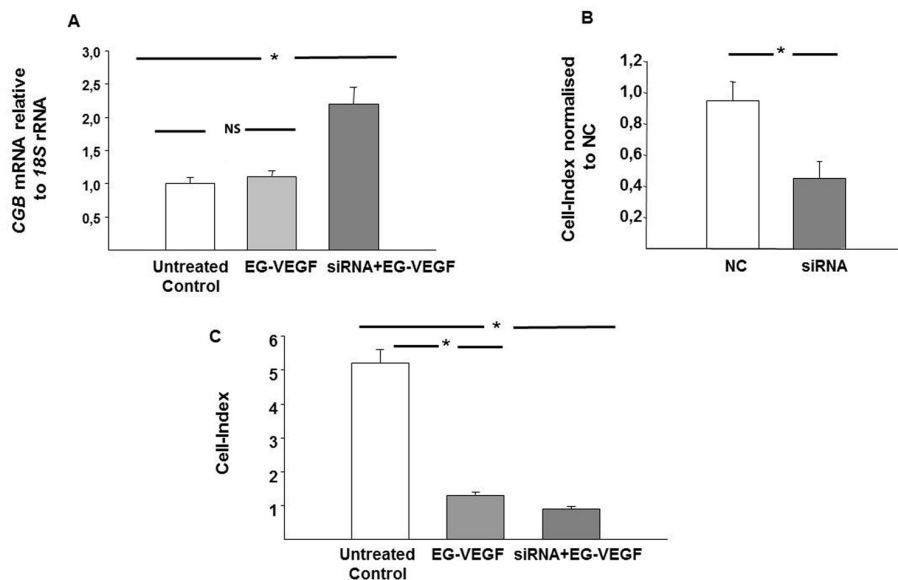


Figure 6. Effect of *NKX3.1* inactivation on trophoblast differentiation and proliferation. (A) Expression of *CGB* mRNA in BeWo cells treated with EG-VEGF ± *NKX3.1* siRNA. (B) Effect of *NKX3.1* downregulation on HTR-8/SVneo proliferation. Results are reported as the mean ± SE mol/L from n = 3 independent experiments. Data were analyzed using the Mann-Whitney *U* test (significant differences, **p* < 0.05).

Effect of *NKX3.1* siRNA Knockdown on Cell Proliferation in HTR-8/SVneo Cells

The effect of *NKX3.1* inactivation on HTR-8/SVneo proliferation was studied using the xCELLigence system in real-time monitoring over 48 h. As shown in Figure 6B, there was a significant decrease in the cell-index, suggesting decreased HTR-8/SVneo proliferation in *NKX3.1* siRNA-treated cells compared with the NC control. In fact, proliferation was significantly decreased in siRNA-treated HTR-8/SVneo cells with and without EG-VEGF stimulation ($p < 0.001$) (Figure 6C).

Figures 7A and 7B report the candidate gene *BCL2* and *BAX* that showed differential expression (greater than five-fold) in human apoptosis array in both BeWo and HTR-8/SVneo cells when treated with *NKX3.1* siRNA compared with the NC control, respectively. Further validation for mRNA expression for *BCL2* and *BAX* showed a significant increase in FGR placentae compared with control (Figures 7C, D), respectively.

DISCUSSION

Placental *NKX3.1* Expression in FGR

In this study, we report that EG-VEGF is an important regulator of homeobox genes in first trimester placental tissues. This was done using a clinically well-defined cohort of idiopathic FGR pregnancies that were carefully selected using strict clinical criteria indicative of placental insufficiency and underlying pathology (6,40–42). Thus, our study excluded pregnancies with delivery of constitutionally small-for-gestation-age babies, who were below the 10th percentile of birth weight but were otherwise healthy, and the clinical criteria for placental insufficiency was normal.

A recent study by Buckberry *et al.*, reports that *NKX3.1* is one of the sex-based transcription factors detectable in the human placenta (43). The human *NKX3.1* gene, located on chromosome 8q21, is expressed at lower to moderate levels in tissues other than the placenta, including, heart, prostate and salivary glands

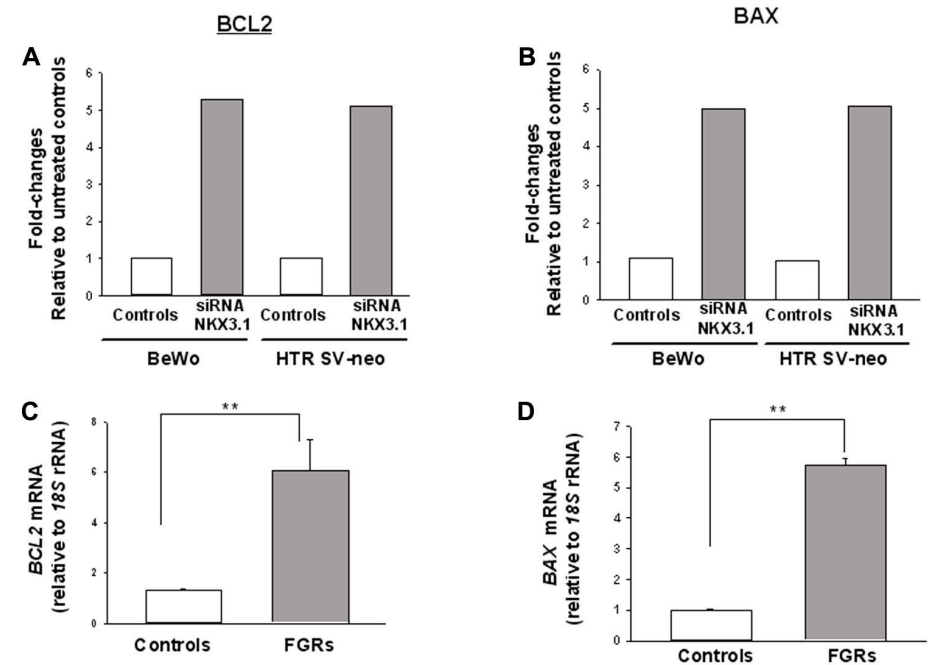


Figure 7. Candidate gene identification using human apoptosis cDNA array and placental expression for candidate gene in FGR and control pregnancies. Effect of *NKX3.1* gene inactivation on apoptosis gene expression profile identified *BCL2* and *BAX* mRNA as candidate genes in BeWo and HTR-8/SVneo (A, B). Real-time PCR was used to detect apoptosis gene mRNA relative to *18S* rRNA. Results are reported as fold changes relative to cells treated with NC. Quantification of candidate genes, *BCL2* and *BAX* mRNA expression levels in FGR ($n = 25$) and control ($n = 25$) (C, D). *18S* rRNA was used as the internal control. Results are reported as the mean \pm SEM. Data were analyzed using the Mann-Whitney *U* test (significant differences when compared with control, * $p < 0.05$; ** $p < 0.01$).

(44). It regulates the proliferation of glandular epithelium and the formation of ducts in prostate and minor salivary glands (45). *NKX3.1* is also known as a tumor suppressor. Its expression appears to be regulated by androgens while loss of its expression is linked to prostate cancer development (46). The data from this study show that growth factor-induced *NKX3.1* regulation on cell proliferation and differentiation is not limited to prostate development but also occurs in placental trophoblast cells. Previous studies in the literature reported the expression of *HOX* genes in the normal and abnormal trophoblastic tissues and their role in the differentiation of trophoblasts (47,48). However until now, no data was reported on the expression and role of *NKX3.1* in the control of placental developmental processes.

NKX3.1 and Trophoblast Differentiation in Primary Trophoblast Cells

This study has demonstrated the presence of *NKX3.1* mRNA and protein in both primary EVCTs from first-trimester placentae and primary villous CTBs. The ST layer is a major site for numerous important placental functions, including nutrient and ion exchange and the synthesis of steroid and peptide hormones required for fetal development and growth (7,8). Here, we have used primary VCT and EVCT cells isolated from first-trimester placentae to determine the effect of *NKX3.1* on *in vitro* differentiation as a fusion phenotype (ST) and as an invasive phenotype (EVCT), respectively, on *NKX3.1* expression. We demonstrated that *in vitro* spontaneous differentiation of trophoblasts is associated with an increased *NKX3.1* expres-

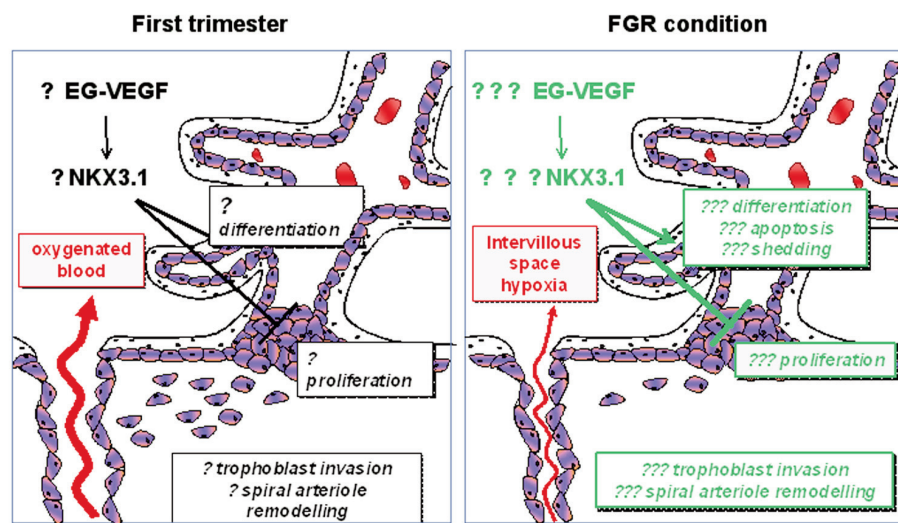


Figure 8. Proposed model for the potential implication of *NKX3.1* in the pathogenesis of fetal growth restriction (FGR) by the end of the first trimester. Lack of maintenance of EG-VEGF has been associated with FGR (16-19). An EG-VEGF increase could lead to abnormal downregulation of *NKX3.1* expression leading to an increase in trophoblast differentiation, apoptosis and shedding as well as sustained inhibition of extravillous trophoblast (EVCT) invasion. The failure in spiral arteriole remodeling, known to be associated with FGR, would therefore contribute to abnormal persistence of placental hypoxia in the intervillous space, and could then act as a positive feedback loop to exacerbate EG-VEGF production.

sion, suggesting *NKX3.1* may have a significant role in villous trophoblast differentiation. A syncytium that functions normally requires both differentiation and fusion of trophoblasts. Thus, the role of *NKX3.1* in the regulation of trophoblast fusion and differentiation will also be of interest in future studies. In this study we have used the independently derived, well-characterized human villous BeWo trophoblast cell line and HTR-8/SVneo extravillous trophoblast cell line to model for first-trimester VCT differentiation and EVCT proliferation, respectively.

***NKX3.1* in the BeWo Cell Model for Differentiation of Trophoblasts**

To study the effect *NKX3.1* expression has on villous trophoblast differentiation, the human choriocarcinoma BeWo cell line was used as described previously (49). ST differentiation can be reproduced *in vitro* using different models (7,49-51), but BeWo cells exhibit a low spontaneous fusion rate that can be increased with FSK treatment (52). We demonstrated

that FSK treatment increased *NKX3.1* expression in BeWo cells, suggesting *NKX3.1* may contribute to an optimum differentiation potential of trophoblasts.

Gene inactivation using *NKX3.1* siRNA in FSK-treated BeWo cells demonstrated that *NKX3.1* is involved in the regulation of BeWo cell differentiation, as demonstrated by a significant increase in *CGB/βhCG* gene expression and secretion. In our previous studies, we reported that FGR placental villi show an upregulated expression of *CGB/βhCG* (34,53). The decreased expression of *NKX3.1* in FGR, therefore, may have clinical significance in contributing to the premature depletion of the pool of proliferating CTBs by favoring their differentiation. This may also be reflected by changes in the ST layer leading to trophoblast shedding and apoptosis in FGR (54).

FGR also is associated with increased EG-VEGF levels (20). This elevation might, in turn, contribute to the decrease in *NKX3.1* expression and to aberrant ST differentiation (Figure 8).

Furthermore, in a recent pilot study, we have observed an increased expression of *TP53* mRNA, a marker of apoptosis in BeWo cells, following siRNA *NKX3.1* inactivation (data not shown). Altogether, our observations support a key role for *NKX3.1* in the regulation of differentiation and its possible role in apoptosis of trophoblasts.

Using BeWo cells that mimic syncytiotrophoblast cells, we demonstrated that EG-VEGF might have a direct effect on the regulation of *NKX3.1* expression, since both PROKR1 and PROKR2 antagonists abolished the decrease in *NKX3.1* expression observed under hypoxia, a condition that mimics FGR and increases EG-VEGF production. Further studies are needed to better dissect the signaling pathways involved in *NKX3.1*-EG-VEGF-dependent regulation.

***NKX3.1* in the HTR-8/SVneo Model for Proliferation of Trophoblasts**

In this study, immunohistochemical localization of *NKX3.1* revealed the presence of the immunoreactive protein expressed primarily in the nuclei of proliferating CTB cell types in the anchoring villi of early pregnancy human placenta, but not in the nuclei of invading distal CTBs in the cell columns. Previous studies using HTR-8/SVneo cells as a model for proliferating trophoblasts confirmed that the *HLX* homeobox gene is an important regulator of EG-VEGF-mediated trophoblast cell proliferation (20). We also investigated whether *NKX3.1* expression is a downstream effector gene in the EG-VEGF signaling pathway of trophoblast function. Upon stimulation of HTR-8/SVneo with EG-VEGF, in the presence of *NKX3.1* siRNA, cell proliferation remained significantly decreased. This suggests that EG-VEGF is a negative regulator of *NKX3.1*-mediated trophoblast proliferation. Therefore, it is plausible that a decrease in *NKX3.1* gene expression levels in cultured trophoblast cells may lead to substantially decreased trophoblast cell migration and invasion. Thus, the reduction in trophoblast proliferation would ultimately

result in a decreased pool of cells acquiring the migratory and invasive phenotype. This may account for the characteristic shallow trophoblast invasion and reduced spiral arteriole remodeling observed in FGR.

Analyses on gene expression for apoptosis in BeWo and HTR-8/SVneo cells following gene inactivation using *NKX3.1* identified proapoptotic genes *BCL2* and *BAX* as direct or indirect downstream targets of *NKX3.1*. Further validation in placental tissues demonstrated a significant increase in both *BCL2* and *BAX* in FGR compared with control, these observations are consistent with previous reports on increased indices of placental apoptosis in FGR (55,56).

CONCLUSION

In summary, EG-VEGF is a negative regulator of *NKX3.1* expression in first-trimester placenta. *NKX3.1* expression is decreased in placenta from pregnancies affected by idiopathic FGR when compared with gestation-matched controls. Furthermore, our functional studies suggest that decreased *NKX3.1* expressions may lead to premature differentiation in the chorionic villous tissue of FGR-affected pregnancies. Our results also provide evidence for a reduced *NKX3.1* expression leading to a decreased pool of extravillous trophoblasts leading, in turn, to the shallow invasion of trophoblasts seen in FGR.

Altogether these data suggest that maintenance of EG-VEGF expression over the first trimester of pregnancy is of vital importance and that abnormally increased levels during that trimester might affect the modulation of expression of homeobox genes such as *NKX3.1*, which in turn may contribute to the etiology of FGR. Nevertheless, it is still to be determined whether EG-VEGF increased levels in FGR are the cause or the consequence of the pathology. The causal hypothesis is currently under investigation by sustaining EG-VEGF production (osmotic pump delivering EG-VEGF) beyond early gestation in the gravid mouse and determining its direct effects on

pregnancy outcome. The second hypothesis is also plausible as FGR condition is associated with the deregulation in multiple other predisposing factors such as hypoxia, a parameter known to increase EG-VEGF levels, which in turn will affect the expression of key homeobox genes. This hypothesis is supported by the *in vitro* experiments presented in this study.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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