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Downstream Targets of Homeobox Gene *HLX* Show Altered Expression in Human Idiopathic Fetal Growth Restriction

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Fetal growth restriction (FGR), a clinically significant pregnancy disorder, is poorly understood at the molecular level. This study investigates idiopathic FGR associated with placental insufficiency. Previously, we showed that the homeobox gene *HLX* is expressed in placental trophoblast cells and that *HLX* expression is significantly decreased in human idiopathic FGR. Here, we used the novel approach of identifying downstream targets of *HLX* in cell culture to detect potentially important genes involved in idiopathic FGR. Downstream targets were revealed by decreasing *HLX* expression in cultured trophoblast cells with *HLX*-specific small interfering RNAs to model human idiopathic FGR and comparing these levels with controls using a real-time PCR-based gene profiling system. Changes in candidate *HLX* target mRNA levels were verified in an independent trophoblast cell line, and candidate target gene expression was assessed in human idiopathic FGR-affected placentae ($n = 25$) compared with gestation-matched controls ($n = 25$). The downstream targets *RB1* and *MYC*, cell cycle regulatory genes, showed significantly increased mRNA levels in FGR-affected tissues compared with gestation-matched controls, whereas *CCNB1*, *ELK1*, *JUN*, and *CDKN1* showed significantly decreased mRNA levels ($n = 25$, $P < 0.001$, t -test). The changes for *RB1* and *CDKN1C* were verified by Western blot analysis in FGR-affected placentae compared with gestation-matched controls ($n = 6$). We conclude that cell cycle regulatory genes *RB1*, *MYC*, *CCNB1*, *ELK1*, *JUN*, and *CDKN1C*, which control important trophoblast cell functions, are targets of *HLX*. (Am J Pathol 2010; 176:278–287; DOI: 10.2353/ajpath.2010.090187)

Fetal growth restriction (FGR), also known as intrauterine growth retardation, is a significant pregnancy disorder associated with major perinatal complications including stillbirth, prematurity, fetal compromise, infant morbidity and mortality. Moreover, later in life, FGR-affected individuals also have an increased risk of chronic disorders such as ischemic heart disease, maturity onset diabetes, and psychiatric disorders.^{1,2} A common definition of FGR is a birth weight at or below the 10th percentile for gestational age and gender, failure of the fetus to grow to its genetically determined potential size, and the likely presence of an underlying pathological process that inhibits the expression of the normal intrinsic growth potential. Obvious causes of FGR are fetal (eg, genetic defects), placental (eg, infarcts), and maternal factors (eg, maturity onset diabetes, certain autoantibody disorders, alcohol abuse, and smoking), but these factors account for only 30% of FGR cases. The remaining 70% of FGR cases with no obvious causes are classified as “idiopathic” and are commonly attributed to uteroplacental insufficiency.

Placental insufficiency is associated with abnormal trophoblast function in FGR. Villous outgrowth, which is determined by trophoblast proliferation, is reduced in the FGR-affected placenta together with increased apoptosis of these cells.³ Defective trophoblast function also results in growth restriction of the fetus due to the reduction in transfer of nutrients and growth factors to the fetus.⁴ Another significant defect in FGR is uteroplacental ischemia due to failure of the specialized extravillous trophoblast cells to proliferate, invade, and subsequently trans-

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form and remodel the maternal spiral arteries in the placental bed.⁵ Villous structural defects, vascular abnormalities, and reduced branching of the villous structure are also seen in FGR.^{6–8} These defects also prevent adequate nutrient transfer to the fetus.⁹

Mouse knockout studies of transcription factors provide genetic proof of the critical role of transcription factors in regulating placental development.^{10–14} Some knockout gene phenotypes show the hallmarks of major human placental disorders¹³ and thus may play an important role in the pathogenesis of human idiopathic FGR.

The homeobox gene family of transcription factors is extensively characterized in the human placenta and embryo.^{15,16} These genes are known to play important roles in implantation, placentation, and embryonic development in the mouse.^{11–13,17–20} Only a few studies of human homeobox genes have provided evidence that homeobox genes are important in human placental development and particularly in trophoblast development and differentiation.^{10,16,21,22} Targeted disruption of the *Hlx* homeobox gene in the mouse has shown that *Hlx* also plays a fundamental role in visceral organogenesis.²³ *Hlx* mutant mice resulted in developing gut and liver diverticulum defects. In addition, *Hlx* mutation also showed a defect in proliferation and resulted in embryonic death due to liver failure.²³

Our interest is in the homeobox gene *HLX*, the human homologue of mouse *Hlx* homeobox gene, which is highly expressed in hematopoietic progenitors and shows decreased expression levels in activated lymphocytes.²⁴ *HLX* inactivation impairs CD34⁺ bone marrow cell proliferation in response to stimulation by cytokines while inducing differentiation of these cells. Moreover, *HLX* inactivation reduces the levels of *c-myc*, *c-fos*, *cyclin B*, and *p34^{cdc2}* mRNA expression,²⁴ which are cell cycle regulator genes implicated in trophoblast cell function.²⁵ Therefore, *c-myc*, *c-fos*, *cyclin B*, and *p34^{cdc2}* were the first identified targets of *HLX*.

Slavotinek et al²⁶ have described *HLX* mutations in human congenital diaphragmatic hernia patients. In this study, the *HLX* gene was sequenced in 119 congenital diaphragmatic hernia patients, because *HLX* is in the deleted interval at ch1q41-1q42 for human congenital diaphragmatic hernia, and four amino acid substitution mutations (p.A235V, p.S12F, p.S18L, and p.D173Y) resulted in congenital diaphragmatic hernia phenotype.²⁶ Slavotinek et al²⁶ concluded that *HLX* mutations are etiologically important in human diaphragm formation by interaction with other genetic or environmental factors. Furthermore, Suttner et al²⁷ have determined that *HLX* gene variants influence the development of childhood asthma. Their study identified 19 polymorphisms in the *HLX* gene, and two tagging single nucleotide polymorphisms representing seven polymorphisms were associated with childhood asthma in a study population of 3099 German children. Suttner et al²⁷ concluded that genetic alterations in *HLX* contributed to the pathogenesis of childhood asthma.

Previously, we provided evidence that *HLX* is expressed primarily in the proliferating cytotrophoblast cell

types in early placental development.²⁸ Furthermore, we postulated that reduced levels of *HLX* are required for cytotrophoblast differentiation and that dysregulation of *HLX* expression contributed to the aberrant cytotrophoblast proliferation and differentiation associated with placental pathologies.²⁸ Recently, we also provided evidence of *HLX* regulation by growth factors and cytokines and established that *HLX* is an important regulator for signal transduction-mediated proliferation of human trophoblast cells.²⁹ By using four independent small interfering RNA (siRNA) oligonucleotides, we have reported a significant 60 to 85% decrease in *HLX* mRNA and 73 to 87% decrease in protein expression by *HLX* siRNA transfection compared with the mock-transfected control trophoblast cells.²⁹ Finally, we showed that *HLX* mRNA and protein expression is significantly decreased in human idiopathic FGR,³⁰ suggesting that *HLX* expression may be of pathological significance.

The focus of this study is to identify downstream target genes of *HLX* in cultured trophoblast and to identify the molecular pathways that are affected in human idiopathic FGR.

Signal transduction pathways that regulate trophoblast functions include the phosphatidylinositol 3-kinase-Akt, protein kinase A, protein kinase C, p38/c-Jun N-terminal kinase, Jak-Stat, and mitogen-activated protein kinase (MAPK) pathways.^{31–33} Of these, the two pathways that are frequently used by trophoblast cells are the Jak-Stat and MAPK pathways.³³ The human MAPK signaling pathway PCR array was used in this study.

We hypothesize that placental *HLX* downstream target gene expression will be significantly altered in human idiopathic FGR pregnancies compared with uncomplicated pregnancies. In this study, an *in vitro* cell culture model using *HLX* siRNA, in trophoblast cells, was used. PCR array comparisons were made between *HLX* siRNA-treated cells and control cells transfected with a siRNA that does not target human genes. Candidate *HLX* target gene expression levels were then assessed by real-time PCR, using validated probes, in human idiopathic FGR-affected placentae compared with gestation age-matched control placentae.

Materials and Methods

Patient Details and Tissue Sampling

Human placentae from pregnancies complicated by idiopathic FGR ($n = 25$) and gestation-matched control pregnancies ($n = 25$) were obtained with informed patient consent and with approval from the Research and Ethics committees of The Royal Women's Hospital. These samples have been characterized and used in our previous gene expression studies.^{30,34} Ultrasound was used for the prospective identification of growth-restricted fetuses. The clinical characteristics of FGR-affected pregnancies and the gestation age-matched controls used in this study are as previously published by Murthi et al.^{30,34} As described,^{30,34} the inclusion criteria for this study were a birth weight less than the 10th percentile for

gestation age using Australian growth charts³⁵ and any two of the following criteria diagnosed on antenatal ultrasound: abnormal umbilical artery Doppler flow velocimetry, oligohydramnios as determined by amniotic fluid index <7 on antenatal ultrasound performed before delivery, or asymmetric growth of the fetus as quantified from the head circumference-to-abdominal circumference ratio (>1.2). Placental tissue subjected to maternal smoking, chemical dependency, multiple pregnancies, pre-eclampsia, placental abruption, prolonged rupture of the membranes, fetal congenital anomalies, or suspicion of intrauterine viral infection were excluded from this study. Pre-eclampsia cases with associated FGR were also excluded from this study. The last menstrual period dates were used to calculate the gestation age for both FGR and control patients and further confirmed by early pregnancy ultrasound. Control patients were selected to match FGR cases according to gestation. All control patients presented with either spontaneous labor or required elective delivery by induction of labor/cesarean section. Preterm control patients presented in spontaneous idiopathic preterm labor or underwent elective delivery for conditions not associated with placental dysfunction (eg, elective cesarean section due to maternal breast cancer). Particularly, control patients did not have clinical evidence of FGR, pre-eclampsia, placental abruption, prolonged rupture of the membranes, or ascending infection. All control patients gave birth to normally formed babies with birth weights appropriate for gestation. All control placentae obtained were grossly normal with no observed abnormalities such as infarcts.

Placental tissues were excised from randomly selected areas of central placental cotyledons, after dissecting away any attached decidua. Samples were processed within 10 minutes of delivery of the placenta. Samples of fresh placental tissues were divided into small pieces and thoroughly washed in 0.9% PBS to minimize blood contamination and then snap frozen and stored at -80°C for RNA analysis.

Cell Culture

Human trophoblast cell lines are a well established model for examining trophoblast function *in vitro*. In this study, two well-characterized, human extravillous trophoblast cell lines SGHPL-4 and HTR-8/SVneo were used, because these cell lines retain many features of normal extravillous trophoblast cells, including human placental lactogen, human chorionic gonadotropin, and HLA-Class1.^{36,37} The cell lines used in this study are long term, which senesce after ~30 passages. For this work, SGHPL-4 cells (passages 5 to 12) were cultured in supplemented Ham's F-10 medium, and the HTR-8/Svneo cells (passages 16 to 19) were cultured in supplemented RPMI 1640 medium, as described previously.²⁹

Inactivation of HLX by siRNA

Inactivation of *HLX* by siRNA was performed as previously described.²⁹ Briefly, cultured trophoblast cells

were grown in Ham's F-10 nutrient mixture/RPMI 1640 medium supplemented with 10% fetal bovine serum for 48 hours (2×10^5 cells/well in 6-well plates) and then transfected with two individual *HLX* siRNAs [*HLX*-si1 sequence: r(UGAAUUUCUUGGGUUCGAG)d(TT); *HLX*-si2 sequence: r(AAACCUUUUCUCCAGG CCU)d(TT)], using RNAiFect transfection reagent (Qiagen, Victoria, Australia). The mock-transfected control wells received all reagents, except the siRNA oligonucleotide. Cells were returned to incubation at 37°C in 5% CO_2 for 72 hours. Negative control siRNAs consisted of a pool of enzyme-generated siRNA oligonucleotides of 15 to 19 bp in length, which were not specific for any known human gene (catalog number 0652-13-7000, Superarray Bioscience, Frederick, MD) and showed no sequence similarity to *HLX*.

RNA Extraction

Total RNA from excised placental tissue was isolated as described previously by Murthi et al.^{30,34} Briefly, 300 mg of placental tissue was homogenized, and total RNA was isolated by acid guanidium isothiocyanate-phenol-chloroform extraction and lithium chloride precipitation according to the method of Chomzynski and Sacchi³⁸ and as modified by Puissant and Houdebine.³⁹ Total cellular RNA from cultured trophoblast cells was isolated using the QIAshredder and an RNeasy Microkit (Qiagen) according to the manufacturer's instructions. First-strand synthesis for real-time PCR cDNA preparation was performed on $2 \mu\text{g}$ of total RNA from placental tissue and cultured cells, using Superscript III ribonuclease H-reverse transcriptase (Invitrogen, Victoria, Australia). First-strand synthesis of cDNA for RT²-profiler PCR array was performed using the RT² First Strand kit (catalog number c-03; SuperArray Bioscience). Briefly, $10 \mu\text{l}$ of reverse transcriptase mixture (containing reverse transcriptase buffer, primer, external control mix and reverse transcriptase enzyme mix) was added to $10 \mu\text{l}$ of genomic DNA elimination mixture (containing $1 \mu\text{g}$ of RNA, gDNA elimination buffer, and RNase-free water) and incubated at 42°C for 15 minutes. RNA degradation and reverse transcriptase inactivation were performed by heating at 95°C for 5 minutes.

PCR Array

The downstream target genes of *HLX* were determined using the real-time PCR based "RT² profiler PCR array" for gene profiling (SuperArray Bioscience Corporation, catalog no. PHAS-061). The human MAPK signaling pathway PCR array was used in this study. This commercially available PCR array encompassed the gene expression profiles of 84 genes related to the MAPK signaling pathway, which is an important signaling pathway in the regulation of human trophoblast function.³³ Members of the MAP kinase kinase kinase (MKKK), MAP kinase kinase (MKK), and MAPK families are represented on this array as well as transcription factors and genes whose expression is induced by MAPK signaling. In addition, the array also included genes related to scaffolding and anchoring. Furthermore, the PCR array chosen in this

study contained several cell cycle regulatory genes, including *CCNB1*, *MYC*, *CDKN1C*, and *JUN*, which have been previously identified to be the targets of *HLX* in hematopoietic progenitor cells.²⁴

Procedures were performed according to the manufacturer's protocol. Briefly, diluted cDNA prepared using the RT² First-Strand kit (as described above) was added to a master mix containing the fluorescent SYBR green/ROX dye. Aliquots from this mix were added to a 96-well plate that contained 84 predisposed gene-specific primer sets. The plates (one for each *HLX*-siRNA and controls) were then placed in an ABI Prism 7700 Sequence Detector (PerkinElmer-Applied Biosystems, Victoria, Australia) for quantitation of gene expression under the following cycling parameters: 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and primer extension at 60°C for 1 minute. Each plate contained a panel of five housekeeping gene primers for normalization of the PCR array data, as well as estimation of the linear dynamic range of the assay. The housekeeping gene panel consisted of β_2 -microglobulin (*B2M*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), ribosomal protein L13a (*RPL13A*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and β -actin (*ACTB*). Furthermore, for each reaction, both negative control (no reverse transcriptase enzyme added) and template control (no cDNA added) were included as controls. Raw data were acquired and processed with ABI Sequence Detector System software version 1.0 (PerkinElmer-Applied Biosystems) to calculate the threshold cycle (C_t) value. Relative gene expression values (fold change) were subsequently determined according to the manufacturer's instructions. Briefly, the fold change for each gene from the mock control array and the *HLX*-siRNA-treated array was calculated as $2^{-\Delta\Delta C_t}$, normalized to the average C_t value of the five housekeeping genes. Fold changes >1 were fold increase, and for fold changes <1, the negative inverse of the result was reported as a fold decrease. Candidate gene prioritization was based on the highest positive and lowest negative fold change differences.

Real-Time PCR

Verification of candidate *HLX* target gene mRNA expression (identified from PCR array) in mock control and *HLX* siRNA-transfected SGHPL-4 and HTR-8/Svneo cells was performed on an ABI Prism 7700 (PerkinElmer-Applied Biosystems) using prevalidated TaqMan Gene Expression Assays (*RB1* Assay ID Hs00153108_m1, *EGR1* Assay ID Hs00152928_m1, *MYC* Assay ID Hs00153408_m1, *JUN* Assay ID Hs00277190_s1, *Cyclin B1* Assay ID Hs00259126_m1, *ELK1* Assay ID Hs00428286_g1, and *CDKN1C* Assay ID Hs00175938_m1; Applied Biosystems). Gene expression quantitation was performed as the second step in a two-step RT-PCR protocol according to the manufacturer's instructions. A total of 20 μ l of PCR mix containing TaqMan Universal PCR master mix, TaqMan Gene Expression Assay mix and 3.5 ng of cDNA was used. Amplification was performed for 40 cycles, including denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 60 seconds. Gene expression for the housekeeping gene *GAPDH* was co-amplified with *HLX* in the same reaction well. Murthi et al³⁴ from our laboratory previously showed that *GAPDH* is a suitable endogenous reference gene for relative gene expression studies in placental tissues from human idiopathic FGR, and its expression is not altered in FGR placentae compared with control placentae. The *GAPDH* primers (5'-GCACCACCAACTGCTTAGCA-3' and 5'-GTCTTCTGGGTGGCAGTGATG-3') and TaqMan probe (5'-VIC-TCGTGGAAGGACTCATGACCACAGTCC-TAMRA-3') were designed using Primer Express 1.5 software (Applied Biosystems). The cycling conditions for *GAPDH* were as per the *HLX* target genes. Relative quantitation of *HLX* target gene expression normalized to *GAPDH* was calculated according to the $2^{-\Delta\Delta C_t}$ method of Livak and Schmittgen,⁴⁰ using the mock control as a calibrator (ABI Prism 7700 Sequence detection system, User Bulletin number 2, 2001). These candidate *HLX* target genes were then further tested for expression in FGR-affected

Figure 1. Identification of *HLX* downstream target genes. RNA was extracted from SGHPL-4 cells transfected with *HLX* siRNA, transcribed into first strand cDNA, and the RT² profiler PCR array was performed for gene profiling. Following initial denaturation at 95°C for 10 minutes, the 84 predisposed genes and a panel of housekeeping genes were amplified for 40 cycles including denaturation at 95°C for 15 seconds and primer extension at 60°C for 1 minute. Relative gene expression values (fold change above or below threshold value of 1) were subsequently calculated for the *HLX* siRNA-treated plate, relative to the mock control plate, normalized to the housekeeping gene panel. The red line shows the threshold value at 1. The prioritized candidate *HLX* target genes are indicated by blue dots.

human placental samples compared with gestation age-matched controls, as per the protocol described above.

Western Immunoblotting

Samples were electrophoresed on a 10% PAGE/0.1% SDS gel in running buffer (250 mmol/L Tris and 1.92 mmol/L glycine) and transferred electrophoretically to a nitrocellulose membrane at 100 V for 30 minutes in transfer buffer (255 mmol/L Tris, 1.92 mmol/L glycine, and 0.1% SDS). The filter was blocked with 10% (w/v) nonfat milk powder/TBS for 1 hour. Incubation with the *RB1* (0.5 μ g/ml), *CDKN1C* (1 μ g/ml) (Sapphire Biosciences, New South Wales, Australia) or *GAPDH* (0.5 μ g/ml) (Imgenex, San Diego, CA) antibodies was performed overnight at 4°C. The filter was then washed in Tris-buffered saline and incubated with biotinylated anti-rabbit antibody (1.5 μ g/ml) (Amersham Life Sciences, New South Wales, Australia) in 5% (w/v) nonfat milk powder/TBS for at least 1 hour at room temperature. Streptavidin-HRP (1.5 μ g/ml) in 2% nonfat milk powder/Tris-buffered saline buffer was added and incubation was performed at room temperature for 1 hour. Tyramide signal amplification was then performed, according to the manufacturer's instructions (PerkinElmer), for *CDKN1C* blots. *RB1* and *GAPDH* antibodies did not require tyramide signal amplification. Detection of protein bands was performed using ECL-Lumilyte autoradiography kit according to the manufacturer's instructions (PerkinElmer).

Data Analysis

All parameters for the gestation age-matched, FGR-affected pregnancies and controls were described as mean $\pm$ SEM. Differences between the clinical characteristics of the FGR-affected pregnancies and the control patients were investigated using either χ^2 test or Student's *t*-test where appropriate. The difference in mRNA expression of the *HLX* target genes between siRNA-treated and control-cultured trophoblast cells and between FGR-affected and control pregnancies was assessed by *t*-test. A value of $P < 0.001$ was considered significant.

Figure 2. Prioritized *HLX* downstream target genes. *HLX* downstream target genes, as identified in Figure 1, were prioritized on the basis of most increased and most decreased gene expression with *HLX* siRNA transfection, compared with the control. Fold changes greater than 1 were reported as fold increase, and for fold changes <1 , the negative inverse of the result was reported as a fold decrease.

Results

Identification of *HLX* Downstream Targets

The SGHPL-4 human trophoblast cell line was transfected with two independent siRNA oligonucleotides de-

Figure 3. Validation of target genes that showed increased expression following siRNA-mediated *HLX* silencing. cDNA (3.5 ng) from SGHPL-4 and HTR-8/SVneo cells transfected with *HLX*-siRNA and the mock control were amplified for 40 cycles including denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 60 seconds (using prevalidated Taqman gene expression assays from Applied Biosystems). Gene expression quantitation for the housekeeping gene *GAPDH* was coamplified with each *HLX* target gene. Relative quantification of *RB1*, *MYC*, and *EGR1* (A, B, and C, respectively) expression in SGHPL-4 and HTR-8 cells normalized to *GAPDH* was calculated according to the $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen,⁴⁰ using the mock control as a calibrator. Significance at $*P < 0.001$ ($n = 3$, *t*-test). The black and gray bars represent the SGHPL-4 and HTR-8/SVneo cell lines, respectively.

signed to target different regions of the *HLX* cDNA as described in *Materials and Methods*. *HLX*-si3 and *HLX*-si4 were chosen for this study, because they resulted in a maximal decrease of *HLX* expression in both SGHPL-4 and HTR-8/SVneo cell lines from our previous experiments.²⁹ The *HLX* gene was inactivated at the mRNA and protein levels, in cultured trophoblast cells as described previously.²⁹ PCR array for identification of potential *HLX* downstream targets was performed using the human MAPK pathway specific array. Gene expression (fold change) was calculated relative to five different house-keeping genes included in the array. The relative mRNA expression of the 84 predisposed genes following siRNA-mediated reduction of *HLX* expression is shown in Figure 1. Of the 84 genes in the array, genes that showed significantly altered mRNA levels with *HLX* inactivation using two individual *HLX* siRNAs were prioritized as candidate downstream targets of *HLX*. As shown in Figure 2, retinoblastoma-1 (*RB1*), myelocytomatosis viral oncogene homolog (*MYC*), and early growth response-1 (*EGR-1*) mRNA levels were highly increased with positive fold changes of 35.41, 20.57, and 13.98, respectively. On the other hand, cyclin-dependent kinase inhibitor-1C (*CDKN1C*), *ELK1* (a member of the *ETS* oncogene family), cyclin B1 (*CCNB1*), and *JUN* oncogene mRNA levels were substantially decreased as a result of *HLX* gene

reduction, with negative fold changes of 38.7, 25.29, 24.98, and 15.55 respectively.

Validation of Prioritized *HLX* Target Genes in Independent Trophoblast Cell Lines

The prioritised *HLX* downstream target genes identified from PCR-array were further validated by individual real-time PCR using independent gene-specific PCR primers (validated Taqman Assays), in both trophoblast cell lines SGHPL-4 and HTR-8/SVneo, using the two siRNA oligonucleotides *HLX*-si3 and *HLX*-si4. The independent validation results were consistent with the PCR-array. *RB1*, *MYC*, and *EGR-1* mRNA levels were significantly increased ($n = 3$, $P < 0.001$, t -test) (Figure 3), whereas *CDKN1C*, *ELK1*, *CCNB1*, and *JUN* mRNA levels were significantly decreased ($n = 3$, $P < 0.001$, t -test) with *HLX* inactivation using *HLX*-si3 and *HLX*-si4, in both the cell lines tested (Figure 4).

mRNA Expression of *HLX* Target Genes in Human Idiopathic FGR

Expression of the *HLX* downstream target genes identified in cultured trophoblast cells were tested in FGR-

Figure 4. Validation of target genes that showed decreased expression following siRNA-mediated *HLX* silencing. Relative quantification of *CDKN1C*, *ELK1*, *CCNB1*, and *JUN* (A–D) expression in SGHPL-4 and HTR-8/SVneo cells normalized to GAPDH was calculated according to the $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen,⁴⁰ using the mock control as a calibrator. Significance at * $P < 0.001$ ($n = 3$, t -test). The black and gray bars represent the SGHPL-4 and HTR-8/SVneo cell lines, respectively.

Figure 5. Increased *HLX* target gene expression in FGR-affected placentae. Real-time PCR for relative quantitation of *RB1* (A) and *MYC* (B) normalized to the housekeeping gene *GAPDH* were performed in all gestation-matched controls ($n = 25$) and FGR-affected placentae ($n = 25$). Data were analyzed according to the $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen.⁴⁰ Statistical comparisons were performed using *t*-test. Significance at $*P < 0.001$.

affected placentae compared with gestation age-matched control placentae. As shown in Figure 5, *RB1* and *MYC* mRNA levels were significantly increased ($n = 25$, $P < 0.001$, *t*-test) whereas *CCNB1*, *ELK1*, *JUN*, and *CDKN1C* mRNA levels were significantly decreased ($n =$

25, $P < 0.001$, *t*-test) (Figure 6). However, the mRNA level of *EGR-1* was unchanged in FGR-affected placentae compared with controls (4.8 ± 0.77 control versus 4.55 ± 0.93 FGR, $n = 25$, $P = 0.83$, *t*-test).

Protein Expression of *RB1* and *CDKN1C* in Human Idiopathic FGR

The expression of *RB1* and *CDKN1C*, which showed the highest increase and decrease in mRNA levels, respectively, in FGR-affected placental tissues, was further assessed at the protein level. As shown in Figure 7, *RB1* protein expression was significantly increased in FGR-affected placentae ($n = 6$, $P < 0.001$, *t*-test) (Figure 7A), whereas *CDKN1C* protein expression was significantly decreased in FGR-affected placentae compared with gestation-matched controls, relative to the housekeeping gene *GAPDH* ($n = 6$, $P < 0.001$, *t*-test), which was consistently expressed in all samples used (Figure 7B).

Discussion

Homeobox genes control transcription by binding to the regulatory elements in the promoter regions of their target genes. These downstream targets of homeobox genes often perform specialized roles in controlling various cell functions. Therefore, homeobox target genes are referred to as genes that act downstream of the homeobox, which may be involved, directly or indirectly, in the mediation of homeobox gene function.

In this study, a well-defined group of human pregnancies with severe idiopathic FGR, which is frequently associated with uteroplacental insufficiency, were used. These samples have been used in gene expression studies from this laboratory.^{30,34} The pregnancies complicated by FGR are at risk due to poor placental function⁴¹

Figure 6. Decreased *HLX* target gene expression in FGR-affected placentae. Relative quantitation of *CDKN1C*, *ELK1*, *CCNB1*, and *JUN* (A–D) normalized to the housekeeping gene *GAPDH* were performed in all controls ($n = 25$) and FGR-affected placentae ($n = 25$). Real-time PCR data were analyzed according to the $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen.⁴⁰ Statistical comparisons were performed using *t*-test. Significance at $*P < 0.001$.

Figure 7. HLX target protein expression in FGR-affected placentae. Following protein extraction of human idiopathic FGR-affected placentae ($n = 6$) and control placentae ($n = 6$), protein assays were performed. Protein samples (25 μ g) were then electrophoresed and transferred to a nitrocellulose membrane. Immunoblotting was performed using commercially available *RB1*, *CDKN1C*, and *GAPDH* rabbit polyclonal antibodies. Composite images of autoradiographic detection of *RB1* (100 kDa), *CDKN1C* (57 kDa), and *GAPDH* (40 kDa) are shown in **A**. Lanes 1–6 are control placentae, whereas lanes 7–12 are FGR-affected placental samples. Lanes 13 and 14 are the primary antibody omitted and rabbit serum negative controls, respectively. **B**: The densitometric quantitation of *RB1* and *CDKN1C* in **A**, as the percentage densitometric values relative to *GAPDH*. Significance at $*P < 0.001$, $n = 6$, t -test. Where indicated by white dividing lines, lanes 1 and 2 are from different areas of the same blot, whereas lanes 13 and 14 are from independent blots.

and are characterized by asymmetric growth of the fetus, altered umbilical artery diastolic velocities, and reduced liquor volume.^{6,42} Typically, the placentae are smaller than controls and have a variety of morphological and functional defects. Hence, in this study, the expression levels of *HLX* target genes were determined in placental samples that meet the clinical selection criteria for FGR.³⁰

Previous studies have shown that reduced trophoblast proliferation⁴¹ and increased apoptosis⁴³ occur in the villous core of FGR-affected placentae, whereas decreased trophoblast invasion⁴⁴ and migration⁴⁵ are seen in the FGR placental bed. We have previously shown that siRNA-mediated *HLX* inactivation significantly decreases trophoblast proliferation in the SGHPL-4 and HTR8-SV/neo cell lines by up to 80%, compared with mock-transfected cells, and that *HLX* is a mediator of the cytokine colony-stimulating factor-1-dependent trophoblast proliferation.²⁹ We have also shown that trophoblast migration significantly decreases by 70% with *HLX*-siRNA transfection and that *HLX* is a mediator of hepatocyte growth factor-dependent trophoblast migration, but siRNA-mediated *HLX* inactivation does not affect trophoblast invasion (our unpublished data). Therefore, a functional correlation between *HLX* and trophoblast function is evident and the placental samples used in this study reflect the functional activity of *HLX* in the human placenta (ie, trophoblast proliferation and migration).

A key pathway in trophoblast function is the MAPK pathway.³³ This study has shown that reduced *HLX* expression in cultured trophoblast cells significantly alters genes in the MAPK pathway. Therefore, *HLX* may play an important role in directly signaling trophoblast function via the MAPK pathway, which consists of several tumor suppressor genes and cell cycle regulators.

In this study, we have identified cell cycle regulatory genes as downstream targets of the homeobox gene *HLX* in cultured trophoblast cells, namely *RB1*, *MYC*, *EGR1*, *CDKN1C*, *ELK1*, *CCNB1*, and *JUN*. *RB1* and *MYC* mRNA expression was increased with *HLX* inactivation, whereas *EGR1*, *CDKN1C*, *ELK1*, *CCNB1*, and *JUN* mRNA expression was decreased compared with mock-transfected control cells. These findings are not only consistent in two independent trophoblast cell lines, SGHPL-4 and HTR-8/SVneo, but also reflected in FGR-affected human placental tissue that is associated with abnormal trophoblast function. *RB1* and *MYC* mRNA expression was significantly increased in idiopathic FGR placentae, whereas *CDKN1C*, *ELK1*, *CCNB1*, and *JUN* expression was significantly decreased. Although a significant increase in *EGR-1* mRNA expression in *HLX*-inactivated cultured trophoblast cells was observed, *EGR-1* expression was not significantly altered in idiopathic FGR placental tissue compared with control placentae. This difference in *EGR-1* expression between cultured trophoblast cells and human placental tissue suggests that multiple cell types present in the whole placenta may regulate *EGR-1* expression.

Various studies have shown that *RB1*, *MYC*, *EGR1*, *CDKN1C*, *ELK1*, *CCNB1*, and *JUN* are expressed in human extravillous trophoblast cells and are implicated in

Figure 8. Schematic diagram: Summary of *HLX* downstream target gene expression changes that alter trophoblast cell functions associated with human idiopathic FGR.

the regulation of cell proliferation and migration.^{46–52} This is consistent with our finding of *HLX* expression in the human placenta in actively proliferating and migrating trophoblast cells.²⁸ Results from this study suggest that *RB1*, *MYC*, *EGR1*, *CDKN1C*, *ELK1*, *CCNB1*, and *JUN* are direct or indirect targets of homeobox gene *HLX* and that *HLX*-mediated target gene expression in trophoblast cells may cause the reduction in trophoblast proliferation and in migration associated with idiopathic FGR. The gene expression changes of *HLX* downstream targets in trophoblast cells, and their interrelationship with human idiopathic FGR is summarized in Figure 8.

Mouse knockout studies for *HLX* target genes identified in this study provide evidence that *HLX* target gene mutations directly result in an FGR-like phenotype due to placental defects. Targeted disruption of *c-myc* gene (homolog of *MYC*) in the mouse model system results in severe placental defects and embryonic death due to placental insufficiency.⁵³

The product of the *RB1* gene is a nuclear phosphoprotein that may act as an inhibitor of cell proliferation.⁵⁴ Wu et al⁵⁵ have demonstrated that reduction of *RB1* gene expression in the mouse model system results in excessive proliferation of trophoblast cells and a severe disruption of the normal labyrinth architecture in the placenta. This is accompanied by a decrease in vascularization and a reduction in placental transport function and ultimately embryonic death.⁵⁵ In our results, *RB1* showed the highest increase in mRNA levels in FGR-affected placentae compared with control placentae, and this was shown to be reflected in increased protein levels. Therefore, increased *RB1* expression levels in FGR may reduce trophoblast proliferation and result in a fewer number of trophoblast cells available to migrate and invade into the maternal decidua. This reduction in trophoblast proliferation may lead to the shallow, inadequate remodeling of the maternal spiral arteries associated with FGR.

Studies have shown that targeted disruption of *CDKN1C* in the mouse model system results in severe placental defects.⁵⁶ *CDKN1C* knockout mice have displayed an array of pre-eclampsia-like symptoms, including placental abnormalities, hypertension, proteinuria, and premature labor.⁵⁶ *CDKN1C* is a maternally imprinted gene that is important in the regulation of embryonic implantation and development, placental growth, and the pathogenesis of proliferative trophoblastic diseases.⁴⁸ This is consistent with our data, suggesting that *HLX*-mediated reduction of *CDKN1C* expression may reduce trophoblast proliferation. Because *CDKN1C* was the most decreased gene in FGR out of the prioritized *HLX* target genes, its expression was also confirmed at the protein level. These results show that *CDKN1C* protein expression is also significantly decreased in idiopathic human FGR placentae compared with controls. Therefore, the reduced levels of *HLX* may directly or indirectly cause the reduction in *CDKN1C* mRNA and protein expression seen in idiopathic FGR.

Most importantly, the four *HLX* downstream target genes *CCNB1*, *MYC*, *CDKN1C*, and *JUN*, previously identified as *HLX* target genes in hematopoietic progenitor cells,²⁴ were confirmed as *HLX* targets in cultured trophoblast cells in this

study. Therefore, this study has demonstrated that the *HLX* homeobox gene targets cell cycle regulatory genes in two independent cell types, and these targets are significantly altered in human idiopathic FGR placentae compared with gestation age-matched controls.

In conclusion, this is the first study to identify downstream targets of a homeobox gene in the human placenta. This study shows that candidate downstream target genes of the homeobox gene *HLX* are significantly altered in human idiopathic FGR-affected placentae, compared with gestation-matched control placentae from uncomplicated pregnancies. Most importantly, the findings of this study demonstrate that *in vitro* models for siRNA-mediated knockdown of *HLX* expression in cultured trophoblast cells show consistent changes to those observed in human idiopathic FGR where *HLX* levels are reduced. These results suggest that reduced levels of *HLX* seen in FGR cause direct or indirect effects on target genes that have been shown to be altered in FGR. Therefore, reduced *HLX* levels directly or indirectly cause gene expression changes in targets that have deleterious effects on trophoblast function.

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