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Expression of Homeobox Gene *HLX* and its Downstream Target Genes are Altered in Placentae From Discordant Twin Pregnancies

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A discordant twin gestation, in which one fetus is significantly growth restricted, compared to the other normal twin, is a unique model that can be used to elucidate the mechanism(s) by which the intrauterine environment affects fetal growth. In many model systems, placental transcription factor genes regulate fetal growth. Transcription factors regulate growth through their activation or repression of downstream target genes that mediate important cell functions. The objective of this study was to determine the expression of the placental *HLX* homeobox gene transcription factor and its downstream target genes in dizygotic twins with growth discordance. In this cross-sectional study, *HLX* and its downstream target genes' retinoblastoma 1 (*RB1*) and cyclin kinase D (*CDKN1C*) expression levels were determined in placentae obtained from dichorionic diamniotic twin pregnancies ($n = 23$) where one of the twins was growth restricted. Fetal growth restriction (FGR) was defined as small for gestational age with abnormal umbilical artery Doppler indices when compared with the normal control co-twin. Homeobox gene *HLX* expression was significantly decreased at both the mRNA and protein levels in FGR twin placentae compared with the normal control co-twin placentae ($p < .05$). Downstream target genes *CDKN1C* and *RB1* were also significantly decreased and increased, respectively, at both the mRNA and protein levels in FGR twin placentae compared with normal control co-twin placentae ($p < .05$). Together, these observations suggest an important association between *HLX* transcription factor expression and abnormal human placental development in discordant twin pregnancies.

■ **Keywords:** homeobox genes, placenta, fetal growth, gene expression, HLX, discordant twins

The regulation of fetal growth is multifactorial and complex. Normal fetal growth is determined by the genetically pre-determined growth potential, and further modulated by maternal, fetal, placental, and environmental factors (Gardosi, 1993). Fetal growth restriction (FGR), also known as intrauterine growth restriction (IUGR), is a failure of the fetus to reach its full growth potential for its gestational age. FGR is commonly defined as a birth weight less than the 10th percentile for gestational age, together with evidence of fetal health compromise such as oligohydramnios, asymmetric fetal growth involving an increased head to abdominal circumference ratio, and abnormal umbilical artery Doppler velocimetry (Chen et al., 2002). FGR is an adverse pregnancy outcome associated with significant perinatal and pediatric morbidity and mortality, with an increased risk of chronic disease later in adult life (Mongelli & Gardosi, 2000).

The majority of cases of FGR are attributed to placental insufficiency, but the placental pathogenesis is poorly understood. The causes of FGR are varied, but the majority of FGR cases are idiopathic and frequently associated with abnormalities of placental function. Previous research

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has demonstrated an abnormal villous architecture in the placentae of growth restricted fetuses (Chen et al., 2002). Typically, the placentae in idiopathic FGR are smaller than gestation age-matched control placentae. Clinical features of idiopathic FGR pregnancies include abnormal umbilical artery Doppler velocimetry (Salafia et al., 1997). Together, these factors suggest a developmental cause for placental insufficiency in FGR (Jackson et al., 1995). Previous studies from our laboratory have shown that in idiopathic FGR-affected singleton pregnancies, there are significant changes in the gene expression for particular types of placental transcription factors, called homeobox genes (Chui et al., 2012; Murthi et al., 2006a, 2006b; Murthi, Said et al., 2006; Pathirage et al., 2013). Homeobox genes comprise a large family of genes that encode transcriptional regulators containing the homeodomain DNA binding motif. Members of the homeobox gene family play important roles in the program of embryonic development (Hombria & Lovegrove, 2003; Rossant & Cross, 2001). Genetic proof of the critical role of homeobox genes in regulating placental development comes from studies on mouse gene knockouts (Cross et al., 2003; Hemberger & Cross, 2001; Morasso et al., 1999; Rossant & Cross, 2001). Homeobox gene knockouts lead to aberrant formation of particular tissue types and/or alter the expression of proteins characteristic of specialized placental cell types (Han et al., 2007; Morasso et al., 1999). In some cases, the mouse mutant gene phenotypes show the hallmarks of major placental disorders, including restricted fetal growth (Li & Behringer, 1998; Sapin et al., 2001). Late-gestation HLX−/− embryos are small and pale compared to their littermates (Bates et al., 2006). Our analysis of HLX−/− mutant placentae showed significant placental defects (Murthi et al., unpublished data). Thus, altered expression of HLX in human placentae may play an important role in the pathogenesis of human FGR.

Conventional human molecular expression studies associate changes in placental gene expression levels with fetal growth. These studies compare placental gene expression levels of small-for-gestation pregnancies to appropriate-for-gestational-age singleton pregnancies as controls. However, this approach has disadvantages, since numerous maternal confounding factors such as body mass index (BMI), weight gain during pregnancy, nutrition, insulin resistance, smoking, and other known, as well as unknown, maternal factors could contribute to the regulation of placental development and consequently associated fetal growth.

Previous studies from our laboratory, along with others, have provided evidence that homeobox genes are important in human placental development (Chui et al., 2012; Knofler et al., 2000; Murthi et al., 2014; Murthi et al., 2006a, 2006b; Murthi et al., 2012; Murthi et al., 2013; Murthi, Said et al., 2006; Pathirage et al., 2013; Quinn et al., 1997; Quinn, Kilpatrick et al., 1998; Quinn, Latham et al., 1998, 2000). Homeobox genes are important players in the genetic hierarchy of development because they are responsible for initiating genetic pathways that regulate cellular differentiation and/or proliferation. Previous studies in our laboratory using placentae from singleton pregnancies have examined the expression patterns for a repertoire of homeobox genes in the human placental trophoblasts, endothelial, and stromal cells (Chui et al., 2011; Chui et al., 2010; Murthi et al., 2008; Murthi et al., 2007; Rajaraman et al., 2008). While there is compelling evidence to suggest that placental homeobox genes influence feto-placental growth, to date, the models have not taken into account potentially confounding factors such as maternal and in utero environmental influences.

The study of discordant twin pregnancies — those consisting of an FGR and normally grown twin — provides a naturally occurring example in which the normally grown twin serves as an internal control for the FGR twin. Furthermore, this scenario allows an evaluation of the association between placental gene expression differences and associated fetal growth, since the disparity in placental development and associated fetal growth within co-twins cannot be easily attributed to confounding maternal or in utero environmental factors. The scenario eliminates gestational age as a confounding factor, which is an issue in studies of singleton FGR cases and controls, given the practical difficulties in matching cases and controls for gestational age. Thus, there can be a more rigorous evaluation of the association between putative genes regulating placental development and associated fetal growth and birth weight.

We previously showed that *HLX* homeobox gene expression is decreased (Murthi et al., 2006a) and its downstream target genes, cyclin kinase D (*CDKN1C*) and retinoblastoma 1 (*RB1*), are increased and decreased, respectively, in placentae from FGR-affected singleton pregnancies compared to gestation-matched singleton controls (Rajaraman, Murthi, Pathirage et al., 2010). Furthermore, we have also suggested a possible role for *HLX* in trophoblast cell proliferation (Rajaraman et al., 2008). The objective of this study was to investigate *HLX* and its downstream target genes, *RB1* and *CDKN1C* expression levels, in placentae from dichorionic, diamniotic (DCDA) discordant twin pregnancies. In these pregnancies, the placentae were exposed to the same intrauterine milieu, gestational age, and maternal and environmental factors. Gene expression differences in placentae from discordant twin pregnancies would support the contribution of placental dysfunction leading to fetal discordancy and FGR.

Materials and Methods

Patients and Tissue Collection

This cross-sectional study included 23 women with DCDA twin pregnancies complicated by fetal growth discordance as determined by antenatal ultrasound evaluations. The

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inclusion criteria for the discordant twins group were dizygotic twin gestations with discordant growth according to estimated fetal weight by an ultrasound scan (Klam et al., 2005), the estimated fetal weight of the smaller twin below the 10th percentile (Fox et al., 2011). Birth weight below the 10th percentile was confirmed at birth, the estimated fetal weight of the larger co-twin was between the 10th and 90th percentiles — birth-weight percentile between the 10th and 90th percentiles was confirmed at birth, and the smaller twin had absent or reverse-end diastolic flow in the umbilical artery in Doppler studies.

Exclusion criteria for both groups included pregnancies complicated with congenital anomalies or chromosomal abnormalities; pre-eclampsia, eclampsia, hemolysis, elevated liver enzymes, and/or low platelet count syndrome, or fetal demise of one twin; pre-term, pre-labor rupture of membranes; clinical signs or symptoms of chorioamnionitis; and birth weight above the 90th percentile.

All placentae were delivered after elective cesarean section and processed immediately. Briefly, placentae were obtained within 20 minutes of delivery, the membranes and cord were removed and the placentae weighed. After removal of any attached decidua, placental tissue samples were excised from central cotyledons, excluding the peripheral margin and infarcted areas. Samples of fresh placental tissues were divided into small pieces and thoroughly washed in 0.9% phosphate-buffered saline to minimize blood contamination. A sample from each placenta was snap-frozen and stored at -80°C for RNA and protein extraction, or fixed in 10% formalin for immunohistochemical analysis.

RNA Isolation and Real-Time PCR

Total RNA from placental tissues was extracted using the RN*easy* midikit (Qiagen, Australia) as described previously (Chui et al., 2012; Murthi et al., 2006a; Murthi, Said et al., 2006; Pathirage et al., 2013). First-strand synthesis was performed on 2 μg of total RNA using Superscript III ribonuclease H-reverse transcriptase (Invitrogen, Australia). Quantitation of *HLX* mRNA expression in placentae was performed in an ABI Prism 7,700 (Perkin-Elmer-Applied Biosystems) using pre-validated, unlabeled *HLX*-PCR primers and FAM dye-labeled TaqMan MGB probes (*HLX, RB1,* or *CDKN1C* inventoried assays, Applied Biosystems) as previously described (Murthi, Doherty et al., 2006a). Gene expression quantitation was performed as the second step in a two-step RT–PCR protocol according to the manufacturer's instructions. The 20 μl PCR reaction mix contained 20× TaqMan Universal PCR master mix, 1× Assays on Demand gene expression assay mix, and placental cDNA (25 ng). As previously described, gene expression quantitation for the *GAPDH* or *18S* rRNA house-keeping gene was performed in the same tube (Murthi et al., 2006a). 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; Murthi et al., 2006a; Murthi, Said et al., 2006). Amplification was for 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 60 s. Relative quantitation of *HLX*, *RB1*, and *CDKN1C* expression normalized to *GAPDH/18S* rRNA was calculated according to the $2^{-\Delta\Delta ct}$ method of Livak and Schmittgen (2001).

Western Immunoblotting

Total protein was extracted from 500 mg of snap-frozen placental tissues in lysis buffer containing 50 mM Tris-HCl, pH 8.0, 0.1% Triton-X-100, 0.1% sodium dodecyl sulfate (SDS), 250 mM NaCl, 5 mM ethylene diamine tetra-acetic acid (EDTA), and 1 mM 4-(2-aminoethyl) benzene sulfonyl fluoride (AEBSF) using an Ultra-Turex homogenizer, as described previously (Chui et al., 2012; Murthi et al., 2006a; Murthi, Said et al., 2006; Pathirage et al., 2013). Briefly, homogenized samples were centrifuged at 2,500 x *g* for 15 minutes at 4°C to sediment any insoluble material. The protein concentration of the supernatant was determined using the BCA protein assay reagent (Pierce, Australia) with bovine serum albumin (BSA; Sigma Aldrich, Australia) as a standard. Approximately, 25 μg of protein per lane was fractionated using 10% SDS-PAGE (Bio-Rad, Australia). Proteins were transferred electrophoretically to nitrocellulose membranes and blocked with 5% non-fat milk in Tris-buffered saline (TBS, pH 7.4). The membranes were incubated with either 1.7 μg/mL of rabbit polyclonal anti-HLX antibody (Aviva, USA) or RB1 (0.5 μg/mL), CDKN1C (1 μg/mL; Sapphire Biosciences, New South Wales, Australia), or GAPDH (0.5 μg/mL; Imgenex, San Diego, CA) antibodies overnight at 4°C. Tyramide Signal Amplification kit (Perkin–Elmer, Australia) was used to amplify the signals following the manufacturer's instructions, following incubation with a biotinylated swine anti-rabbit secondary antibody for 30 minutes at room temperature. Antibody binding was visualized using peroxide-conjugated goat anti-rabbit secondary antibody and by autoradiography using an enhanced chemiluminescence system (Amersham, Australia). Coomassie blue staining of total protein in each well was used to ensure constant protein load.

Immunohistochemistry

HLX protein localization in the placental tissues ($n = 6$) was determined as previously described (Murthi et al., 2006a). Briefly, paraffin-embedded placental tissue sections cut to 5 mm thickness were dewaxed in xylene, hydrated in graded ethanol (100–50% ethanol), and blocked with 1% BSA/PBS for 1 hour at room temperature. Tissue sections were then incubated overnight with 1.7 μg/mL of rabbit polyclonal anti-HLX antibody (Aviva, USA) in 1% BSA/PBS. Control slides included sections that were incubated with mouse

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TABLE 1
Clinical Characteristics of Patient Samples

	Dichorionic diamniotic (DCDA) twins ($n = 23$)
Gestation (wks) at delivery	35.0 ± 7.8
Mode of delivery	Cesarean
% Birth-weight discordance	22.0 ± 5.5
Abnormal/reversed end-diastolic velocimetry (AREDV)	FGR twin — 100%; Control normal twin — 0%

IgG in 1% BSA/PBS (negative control) or with anti-mouse cytokeratin-7 (CK-7, Dako, Australia) as a positive control for trophoblast specific staining. Chromogen detection was performed using the aminoethyl carbazole substrate Zymed® AEC chromogen kit (Thermo Fisher Scientific Corp. USA) and the slides were mounted with 80% (v/v) glycerol. Sections were viewed with a Zeiss Axioscope microscope and images were captured with a Zeiss Axiocam (Carl Zeiss AG, Oberkochen, Germany).

Data Analysis

Birth-weight discordance was calculated as a percentage of the birth weight of the normal control twin within each pair. All data were analyzed using the paired Student's *t* test. A probability value of $p < .05$ was considered statistically significant.

Results

Patient Demography

The clinical parameters of the collected placentae from the twin pregnancies are shown in Table 1. Discordant growth was defined as discordance of more than 10% in birth weight in conjunction with FGR in one fetus and normal growth in the co-twin.

Feto-Placental Analysis

As shown in Figure 1A, the fetal birth weight was significantly decreased in FGR twins compared to the normal twins. Figure 1B depicts the significantly lower placental weights of FGR twins compared with their respective normal co-twins.

Gene Expression Analysis

HLX, *RB1*, and *CDKN1C* mRNA expression was analyzed in the placentae of twins with normal growth compared to twins with FGR growth in each twin pair (Figure 2). As shown in Figure 2A, the mean *HLX* expression for the FGR twin placentae was significantly decreased compared to the normal twin placentae. For the downstream target genes of *HLX*, *CDKN1C* relative to *18S* rRNA (Figure 2B) was significantly decreased, while *RB1* relative to *18S* rRNA showed a significant increase (Figure 2C) in FGR twin placentae compared to the normal twin placentae.

Protein Expression Analysis

The changes of *HLX*, *RB1*, and *CDKN1C* mRNA levels observed in FGR twin placentae compared with normal twin placentae were further investigated at the protein level. A representative immunoblot for HLX, RB1, and CDKN1C proteins in twin placentae is shown in Figure 3A. Immunoreactive HLX protein was detected at 50 kDa, RB1 at 100 kDa and CDKN1C at 57 kDa in all samples tested. GAPDH (40 kDa) was used as a house-keeping control protein for normalization. Semi-quantitative analyses of FGR twin placentae ($n = 23$) and control twin placentae showed that the levels of HLX (Figure 3B) and CDKN1C (Figure 3C) immunoreactive protein normalized to GAPDH were significantly reduced, while RB1 (Figure 3D) immunoreactive protein normalized to GAPDH was significantly increased in FGR twin placentae compared with normal control twin placentae.

Immunohistochemistry

Immunohistochemical localization of HLX (Figure 4) provided a qualitative evidence for decreased immunoreactivity in the FGR twin placentae (Figure 4A) compared with control twin placentae (Figure 4B). Arrows indicate the presence of immunoreactivity for HLX in the residual cytotrophoblasts (CT), endothelial cells surrounding the fetal capillaries (EC), and in some stromal (Str) in the control twin placentas (Figure 4B); however, in FGR twin placentae, an overall qualitative decrease in the immunoreactivity for HLX was observed, and the immunoreactivity for HLX was observed in EC (Figure 4A). CK7 staining was used as a positive control (Figure 4C), which reveals specific immunoreactivity in the syncytiotrophoblast (ST), while IgG2 used as a negative control showed no specific immunostaining (Figure 4D).

Discussion

The definition of FGR is important, because not all small fetuses are growth restricted. Histomorphological studies indicate that true FGR is associated with structural alterations of the placenta such as abnormally developed terminal capillary loops and sparse arrangement of functional terminal villi (Krebs et al., 1996). These morphological alterations may contribute to increased feto-placental vascular resistance, resulting in decreased blood flow and decreased availability of nutrients and oxygen to the fetus.

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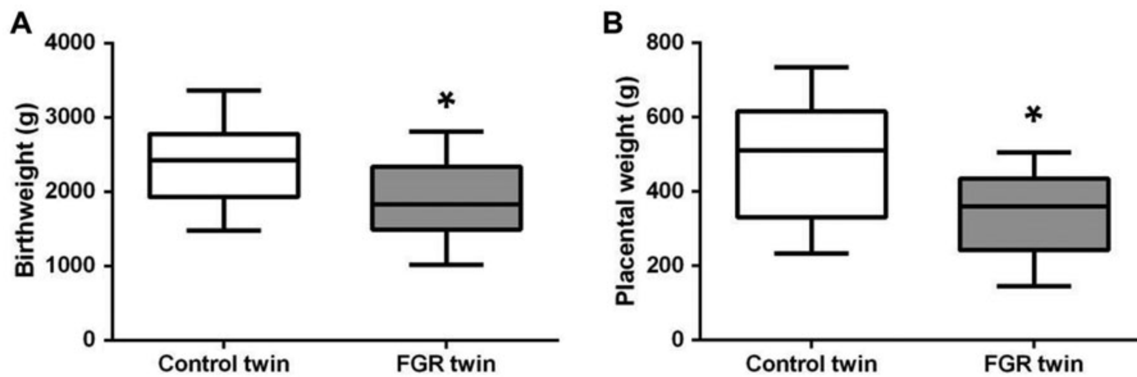

FIGURE 1

Birth weights and placental weights of $n = 23$ growth-discordant twin pairs. A: Fetal birth-weights for FGR twins were significantly lower compared to the normal co-twins. B: Placental weights for FGR twins were also significantly lower compared to the normal co-twins. Results are expressed as box plots. Data were analyzed with paired Student's *t* tests.

Note: *Denotes a significant difference ($p < .05$).

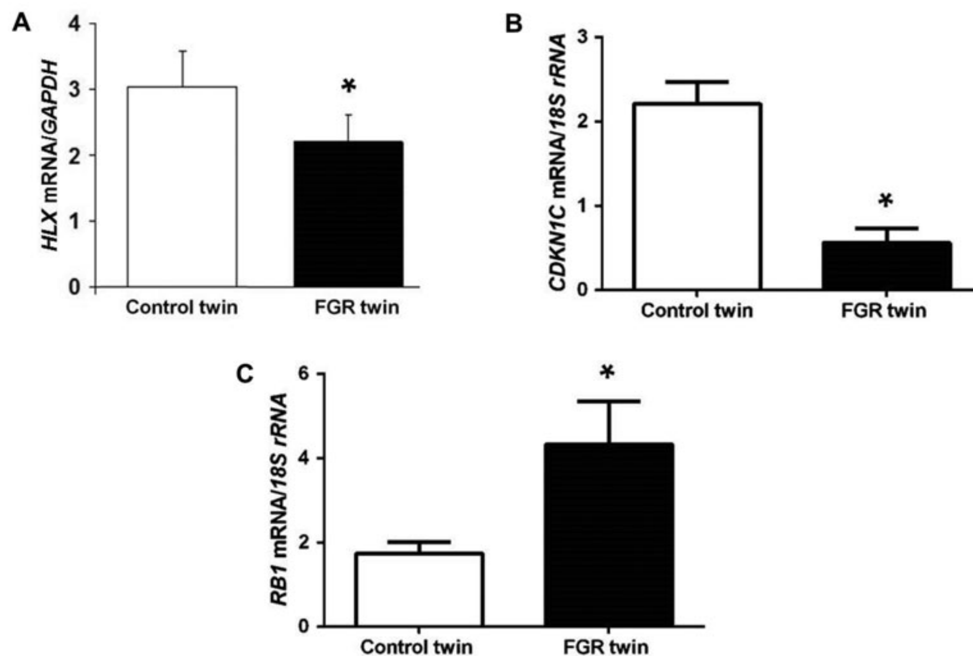

FIGURE 2

Placental mRNA expression of *HLX* and its downstream target genes, *CDKN1C* and *RB1*, in $n = 23$ twin pairs. mRNA expression of *HLX*, *CDKN1C*, and *RB1* was determined by real-time PCR relative to a housekeeping gene (GAPDH/18S rRNA). *HLX* (A) and *CDKN1C* (B) mRNA expression was significantly decreased, while that of *RB1* (C) was significantly increased in FGR twin placentae compared with normal control co-twin placentae. Results are expressed as mean + SEM. Data were analyzed with paired Student's *t* tests.

Note: *Denotes a significant difference ($p < .0001$).

In the present study, we used discordant twin pregnancies as a naturally occurring example where the FGR-affected and normal fetus share the same maternal and in utero environments. Hypothetically, these environments should affect the two placentae and the fetuses in a similar manner. Our data demonstrate that in discordant twin pregnancies, HLX and its target gene mRNA and protein expression levels are altered in the placental tissue from the growth-restricted twin, confirming that the fetal discordance is associated with molecular placental differences.

Studies on molecular differences in discordant twin placentae are limited. Almog et al. (2002) reported increased apoptosis in placentae from FGR-affected discordant twins compared to their normally grown co-twins, indicative of abnormal trophoblast turnover. Thus, in discordant twins, despite being exposed to the same maternal and in utero

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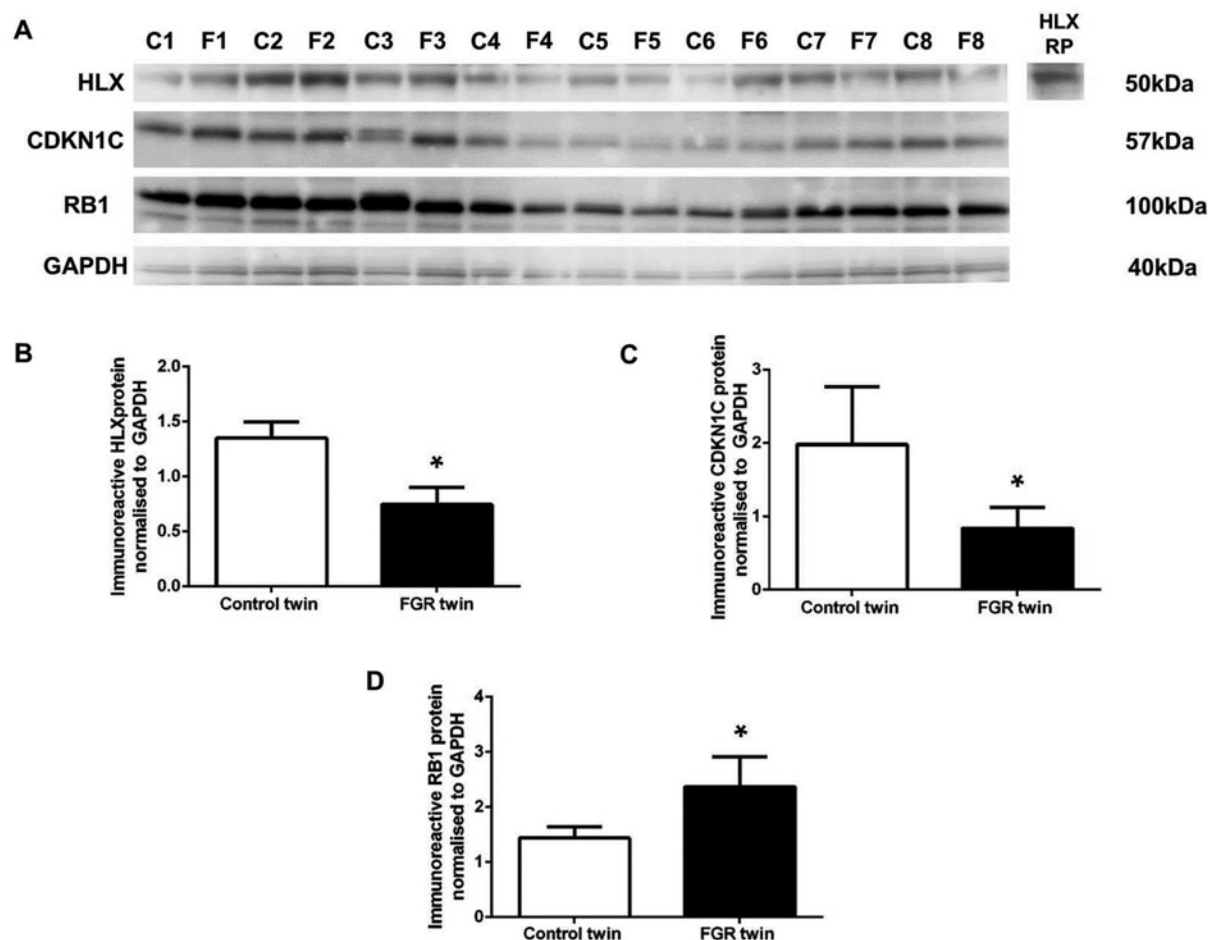

FIGURE 3

Placental protein expression of HLX and its downstream targets, RB1 and CDKN1C, in $n = 23$ twin pairs. Protein expression of HLX, CDKN1C, and RB1 was determined by Western immunoblotting and normalized to GAPDH loading control. A representative immunoblot for HLX protein (50kDa), CDKN1C (57 kDa), RB1 (100 kDa), and GAPDH (40 kDa) in FGR (F) and in normal control (C) co-twin placentae and HLX recombinant protein (referred to as HLX RP) used as a positive control is shown in Panel (A). Significant decreases in immunoreactive HLX (B) and CDKN1C protein (C) and a significant increase in immunoreactive RB1 protein (D) were observed in FGR twin placentae compared with normal control co-twin placentae. Results are expressed as mean + SEM. Data were analyzed with paired Student's *t* tests.

Note: *Denotes a significant difference ($p < .05$).

environments, the two placentae present different morphologies, accompanied by differences in *HLX* expressions and its downstream target genes expression levels.

The clinical data regarding discordant pregnancies suggest the most likely explanation for the IUGR of the small-for-gestational-age twin is abnormal placentation and not a genetic disparity. Several parameters verified in the patient samples support this hypothesis: all small-for-gestational-age fetuses had impaired blood flow and most of them had oligohydramnios; all newborns had a normal antenatal sonographic anatomy scan; and all neonates were healthy, without any abnormal morphological features. Indeed, it is most likely that a placental disorder and not genetic abnormalities is the underlying mechanism for small-for-gestational-age in twins with these clinical characteristics (Blickstein et al., 1989; Blickstein & Lancet, 1988; Blickstein et al., 1988; Blickstein et al., 1987).

The results of the present study are novel. Our study is the first to report a significant change in a placental *HLX* homeobox gene expression and its downstream target genes *CDKN1C* and *RB1* in FGR twin placentae, strongly suggesting an association between homeobox gene expression, placental development, and associated fetal growth. We have previously demonstrated that in singleton FGR-affected pregnancies, *HLX* and its target genes *RB1* and *CDKN1C* expression in FGR-affected placentae were significantly decreased compared to gestation-matched controls (Murthi et al., 2006a; Rajaraman, Murthi, Pathirage et al., 2010).

We have also previously reported the functional roles of *HLX* using in vitro model systems. Altering *HLX*

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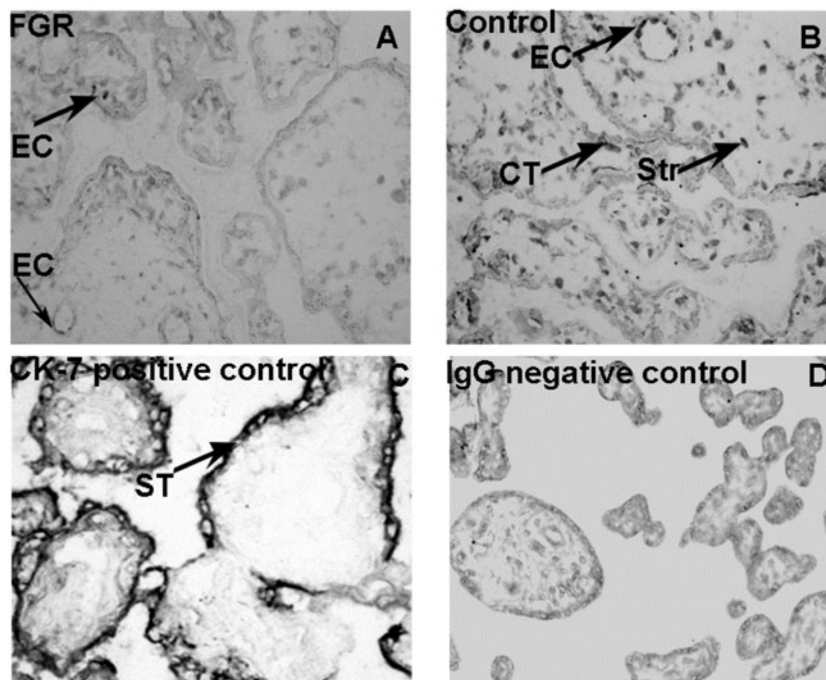

FIGURE 4

Representative section of FGR-twin (A) and in control co-twin (B) placentas stained with HLX antibody to localize HLX expression. The presence of immunoreactive HLX protein is shown in the residual cytotrophoblast (CT) and in endothelial cells of the fetal capillaries (EC) in in the control twin placenta (B). In the FGR twin placentae, a qualitative decrease in HLX immunoreactivity is observed and the immunoreactivity for HLX is observed in endothelial cells of the fetal capillaries (EC) compared with the control twin placenta. The positive control staining for CK-7 immunoreactivity (C) is observed in the syncytiotrophoblast (ST) a control twin placenta. Negative control using IgG shows no specific immunoreactivity as shown in (D).

levels in human placental cells in vitro influenced placental cell proliferation and migration (Rajaraman, Murthi, Brennecke et al., 2010; Rajaraman et al., 2007; Rajaraman, Murthi, Pathirage et al., 2010). Downstream target genes of *HLX*, *CDKN1C*, and *RB1* were identified using RNAi and pathway-specific low-density array in cultured placental cells (Rajaraman, Murthi, Pathirage et al., 2010). Importantly, in this study, we demonstrate that not only was the decreased HLX mRNA and protein expression seen in FGR singleton pregnancies replicated in FGR twin pregnancies, but that parallel changes were also observed for the *HLX* downstream target genes, *CDKN1C* and *RB1,* as compared to their respective controls.

In conclusion, severe growth discordance in twins may reflect an association between homeobox gene *HLX* expression, placental development, and associated fetal growth. Thus, the results of the investigation of dizygotic twins with discordant growth, where maternal and in utero environmental factors are controlled, support our previous data for *HLX* and its target gene expression in singleton gestations complicated by FGR. The use of discordant twin placentae to model for FGR provides a more rigorous model for the complex and intriguing association between placental development and fetal growth. Elucidation of the molecular mechanisms of placental gene expression may lead to more focused and effective therapeutic strategies to improve fetal growth in discordant twin and singleton pregnancies complicated by dysregulated fetal growth.

Limitations

Nearly 10% of dichorionic twins are monozygotic, which can be determined accurately by examination of DNA markers (Sepulveda et al., 1996). Although in the present study, there was no record of DNA marker examinations to confirm zygosity for the samples collected, a mixed newborn gender in all samples collected supports the dizygotic nature of the discordant twins. Furthermore, the relationship of HLX expression was only determined for discordant growth in the FGR twin rather than the association with zygosity. Marginal or velamentous cord insertion are known causes of FGR in twin pregnancies (Visentin et al., 2013); however, this information was not recorded in the pregnancies included in this study.

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Conflict of Interest

None.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

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