Homeobox gene ESX1L expression is decreased in human pre-term idiopathic fetal growth restriction

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Fetal growth restriction (FGR) is a clinically significant pregnancy disorder in which the fetus fails to achieve its full growth potential in utero. This study involved idiopathic FGR, which is frequently associated with placental dysfunction. Here, we investigated mRNA levels of the human placental homeobox gene ESX1L in pre-term and term idiopathic FGR pregnancies compared with gestation-matched controls. Real-time PCR quantitation showed ESX1L levels in control placentae decreased between pre-term and term [0.7 ± 0.20 (27–35 weeks, n = 13) versus 0.2 ± 0.06 (36–41 weeks, n = 12), t-test, P < 0.005]. ESX1L levels in FGR-affected placentae were significantly lower than in gestation-matched controls, and there was no significant change between pre-term FGR and term FGR [0.32 ± 0.04 (27–36 weeks, n = 11) versus 0.31 ± 0.02 (36–41 weeks, n = 14), t-test, P = 0.82]. Multiple linear regression analysis revealed a rapid decline in ESX1L expression in control placentae [0.075-fold of the calibrator for each week of gestation (95% CI = –0.105 to –0.045, P < 0.0005)]. In FGR-affected placentae, ESX1L levels were lower than in gestation-matched controls, and the decline in ESX1L levels with gestation was not significant [0.001-fold of the calibrator for each week of gestation (95% CI = –0.030 to 0.010, P < 0.3)]. The linear relationship between ESX1L mRNA levels in FGR-affected placentae and gestation-matched controls during gestation was significantly different (likelihood ratio test for interaction, P = 0.0005). Our findings were consistent with a potential role for the ESX1L gene within the growth control mechanism of the fetus, through its effect on placental function.

Key words: ESX1L/fetal growth restriction/homeobox/IUGR

Introduction

Fetal growth restriction [FGR, also known as intrauterine growth restriction (IUGR)] is a common and significant disorder of human pregnancy. FGR can be defined as a birthweight at or below the 10th centile for gestational age and gender, failure of the fetus to grow to its genetically determined potential size and implies the presence of an underlying pathologic process that inhibits the expression of the normal intrinsic growth potential. FGR causes a variety of serious perinatal complications (Illanes and Soothill, 2004), but increasing numbers of human longitudinal and animal model studies suggest that there are also long-term health consequences of FGR reaching into adulthood (Godfrey and Barker, 2000). These consequences include an increased risk of chronic somatic disorders such as diabetes, cardiovascular disease (Godfrey and Barker, 2000) and asthma (Steffensen et al., 2000), as well as intellectual impairments such as a decreased intelligence quotient (Frisk et al., 2002) and an increased risk of psychiatric disturbances such as schizophrenia (Rosso et al., 2000) and depression (Gale and Martyn, 2004). Understanding the molecular mechanism of human FGR is therefore of increasing importance.

Obvious maternal, fetal and placental causes of FGR account for only a third of FGR cases (Brokdy and Christou, 2004), the remainder being idiopathic. Idiopathic FGR is often characterized by asymmetric growth of the fetus, abnormal umbilical artery diastolic velocities and reduced liquor volume (Chang et al., 1993) and is frequently associated with placental insufficiency (Gagnon, 2003). Typically, the placentae in idiopathic FGR patients are smaller than in controls and show various morphological defects such as reduced trophoblast proliferation and abnormal villous vasculature with shorter, less branched terminal villi (Kingdom et al., 2000). Another significant defect is uteroplacental ischaemia because of the failure of placental extravilous cytotrophoblast cells to effectively carry out the critical processes of invasion, transformation and remodelling of the spiral arteries in the maternal decidua (Chadha et al., 2004). A consequence of altered placental function is reduced transfer of nutrients and growth factors to the fetus that restricts its growth (Mayhew et al., 2004). The changes observed in the placenta are consistent with developmental defects (Chadha et al., 2004), but the genes involved and their molecular mechanism of action are not known. Several longitudinal studies have demonstrated a possible causative role for genetic and familial factors, as yet unidentified, in human FGR (Devriendt, 2000; Ghezzi et al., 2003).

Various attempts to understand the molecular basis of FGR using microarray and proteomic approaches have revealed significant differences between FGR-affected and control placentae (Page et al., 2002; Roh et al., 2005; Okamoto et al., 2006). However, these studies have...
have been carried out on term placentae and have shed little light on the regulatory mechanisms underlying the earlier onset of FGR. Animal model systems are therefore of crucial importance in revealing potentially important regulatory genes that may play a role in the early stages of human FGR.

Homeobox genes *Esx1*, *Pem*, *Psx1* and *Psx2*, localized to the mammalian X chromosome, are expressed in the murine placenta and have been identified as regulators of placental development that influence fetal growth and viability (Branford et al., 1997; Han et al., 1998, 2000; Fohn and Behringer, 2001; Singh et al., 2004). *Esx1* is of interest, because its expression is restricted to a subset of extra-embryonic tissues and male germ cells of the adult testes (Li et al., 1997). *Esx1* is essential for normal murine placental development and is paternally imprinted and always silenced (Li and Behringer, 1998). Targeted gene disruption of *Esx1* causes, in homozygous mutant mice, defects in vascular branching in the labyrinth layer of the placenta and subsequent vascularization abnormalities at the maternal–fetal interface that result in FGR (Li and Behringer, 1998). Evidence for parent-of-origin effects consistent with imprinting can be seen when heterozygous female mice inherit a mutant *Esx1* allele from their father. They develop normally, but when heterozygous females inherit the *Esx1* mutation from their mother (*X<sup>Esx1</sup>* X, where X<sup>Esx1</sup> designates the X chromosome with the mutant allele; the maternally derived X chromosome is designated first), they are born 20% smaller than normal and are identical in phenotype to hemizygous mutant males (*X<sup>Esx1</sup>* Y) and homozygous mutant females (*X<sup>Esx1</sup>* X<sup>Esx1</sup>) (Li and Behringer, 1998).

The human counterpart of murine *Esx1* is therefore of considerable interest in understanding the molecular mechanism of FGR. In humans, *ESX1*-like protein (ESX1L) is located on the X chromosome and is orthologous to murine *Esx1* (Fohn and Behringer, 2001). ESX1L (also referred to as *ESX1*-related protein EXR1) is one of the few homeobox genes restricted in expression to the human placenta and testes (Fohn and Behringer, 2001). RT–PCR analysis reveals that ESX1L is expressed during all stages of human placental development (Figueiredo et al., 2004). Figueiredo et al. (2004) also provided evidence by mRNA in situ hybridization that in the normal term placenta, ESX1L is localized equally in both the non-proliferating syncytiotrophoblast and the residual proliferating cytotrophoblast cells and at lower levels in endothelial and stromal cells of the villi. As described above, reduced trophoblast proliferation is a feature of FGR-affected placentae (Kingdom et al., 2000), and therefore ESX1L is expressed in cell types known to be affected in FGR.

Recent studies have shown that the human *ESX1L* gene, unlike murine *Esx1*, is not imprinted in human third-trimester placentae, and there is no parent-of-origin effect of chromosome X associated with FGR (Grati et al., 2004). Imprinting of *Esx1* on the X chromosome is essential for murine placental development and function but not in the human.

Genetic analyses of the *ESX1L* gene identify two allelic variants of *ESX1L* in the human placenta that are predicted to code for an aberrant ESX1L protein, but the allelic variants do not contribute to genetic susceptibility to idiopathic FGR (Guan et al., 2005). Moreover, no single-nucleotide polymorphisms have been found, and there are no differences in *ESX1L* mRNA and protein expression between control and FGR placentae at term (Figueiredo et al., 2004; Grati et al., 2004; Guan et al., 2005).

In this study, we measured *ESX1L* mRNA expression in pre-term and term placentae obtained from a clinically well-defined group of idiopathic FGR-affected pregnancies compared with gestation-matched controls. We have used semi-quantitative PCR and real-time PCR methods to investigate the levels of *ESX1L* mRNA in the placenta, earlier in pregnancy and at term.

### Materials and methods

#### Patient details and tissue sampling

Placenta 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, Melbourne. Growth-restricted fetuses were identified prospectively using ultrasound. Table I summarizes the clinical characteristics of FGR-affected pregnancies and the gestation-matched controls that were included in this study. As summarized in Table II, the inclusion criteria for this study were a birthweight less than the 10th percentile for gestation age using Australian growth charts (Guaran et al., 1994) and any two of the following criteria diagnosed on antenatal ultrasound: abnormal umbilical artery Doppler flow velocimetry, oligohydramnios as determined by amniotic fluid

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>FGR (n = 25)</th>
<th>Control (n = 25)</th>
<th>Significance</th>
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</thead>
<tbody>
<tr>
<td>Gestation age (weeks, mean ± SD)</td>
<td>34.4 ± 6.5</td>
<td>35.8 ± 6.6</td>
<td>P = 0.25</td>
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<tr>
<td>Maternal age (years, mean ± SD)</td>
<td>33.2 ± 5.7</td>
<td>31.9 ± 6.5</td>
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<td>Placental weight (g)</td>
<td>409.3 ± 110.3</td>
<td>525.0 ± 148.2</td>
<td>P &lt; 0.005</td>
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<tr>
<td>Parity</td>
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<tr>
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<td>10</td>
<td>P = 0.56</td>
</tr>
<tr>
<td>Multiparous</td>
<td>13</td>
<td>15</td>
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<tr>
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<tr>
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<td>6</td>
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<td>3</td>
<td>2</td>
<td>P = 0.35</td>
</tr>
<tr>
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<td>17</td>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>8</td>
<td>12</td>
<td>P = 0.25</td>
</tr>
<tr>
<td>Female</td>
<td>17</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Birthweight (mean ± SD)</td>
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<td>2603.8 ± 857.025</td>
<td>P &lt; 0.05</td>
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<td>&lt;3–5 percentile</td>
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<tr>
<td>&lt;3 percentile</td>
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<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number of samples</th>
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<tr>
<td>BW &lt;10th percentile</td>
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<td>Abnormal umbilical artery Doppler velocimetry</td>
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<tr>
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<tr>
<td>Reversed</td>
<td>6/25</td>
</tr>
<tr>
<td>Absent</td>
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<tr>
<td>Not recorded for Doppler velocimetry</td>
<td>4/25</td>
</tr>
<tr>
<td>Asymmetric growth</td>
<td></td>
</tr>
<tr>
<td>HC : AC &gt;1.2</td>
<td>21/25</td>
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<tr>
<td>Not recorded for HC : AC</td>
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<tr>
<td>Oligohydramnios</td>
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<tr>
<td>AFI &lt;7</td>
<td>18/25</td>
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<tr>
<td>AFI ≥7</td>
<td>7/25</td>
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*AFI, amniotic fluid index; BW, birthweight and FGR, fetal growth restriction.

*All FGR-affected pregnancies included in this study met with the first criterion for BW less than the 10th percentile for gestation and at least two of the other independent ultrasound selection criteria. 17/25 FGR-affected pregnancies met with all three ultrasound selection criteria.*
were used for (1990). Adjusted volumes of the control and the FGR-affected placental cDNA conditions for the ACCACAGTCCATGCCATCACT-3 control, and the cDNA volumes were adjusted using scanning densitometry. was amplified in an ABI PRISM 9700 thermocycler (Applied Biosystems, C, 10 min), 35 cycles of denaturation at 94 °C for 60 s, annealing and extension at 60 °C for 60 s. GAPDH mRNA levels for the control samples, versus 0.3 ± 0.025, n = 25, t-test). ESX1L mRNA expression was analysed in control placentae with mRNA expression was calculated according to the 2 –ΔΔCT method (Livak and Schmittgen, 2001) employing a term control as a calibrator. A standard curve generated to validate the amplification efficiencies for both the target and the housekeeping genes demonstrated that the amplification efficiencies of ESX1L and GAPDH were 99 and 90%, respectively (ABI Prism 7700 Sequence detection system, User Bulletin 2, 2001).

Data analysis
All parameters for the FGR-affected pregnancies and gestation-matched controls were described as mean ± SEM. Differences between the clinical characteristics of the FGR-affected pregnancies and the control patients were investigated using either chi-squared test or Student’s t-test where appropriate. The relationship between mRNA expression of ESX1L and gestation, for FGR-affected and control placentae, was modelled using multiple linear regression. The likelihood ratio test for interaction was used to assess whether there was a significant difference between ESX1L mRNA expression in FGR-affected placentae and gestation-matched control placentae. Results were considered statistically significant if P < 0.05.

Results
Table I summarizes the clinical features of the idiopathic FGR-affected pregnancies and the controls included in this study. Gestation, maternal age, parity and mode of delivery were not significantly different between the two groups. However, mean placental weight and mean birthweight were significantly lower in FGR-affected pregnancies compared with controls (P < 0.025, n = 25, t-test).

ESX1L mRNA expression was observed in control placentae with gestation age ranging from 27 to 41 weeks. Figure 1A shows representative RT–PCR products for ESX1L (162 bp) relative to GAPDH (452 bp) in placentae of 27- to 39-week gestation in the control group. A reduction in the level of ESX1L expression relative to GAPDH was observed with gestation.

Figure 1B shows representative RT–PCR products for ESX1L relative to GAPDH from 27 to 39 weeks of gestation in FGR-affected pregnancies. The amount of ESX1L product was generally lower through gestation in the FGR-affected placentae when compared with controls in Figure 1A. The reduction in ESX1L expression with gestation seen in the controls was not observed in the FGR-affected placentae.

Using initial real-time PCR analysis, ESX1L mRNA levels relative to GAPDH levels were compared between all control placentae (n = 25) and all FGR-affected placentae (n = 25). ESX1L expression was significantly decreased in the FGR-affected placentae [0.5 ± 0.1, controls, versus 0.3 ± 0.03, FGR], t-test, P < 0.01]. The change in ESX1L mRNA level with gestation was further quantitated using real-time PCR (Figure 2), by analysing the samples in two separate groups: pre-term (27–35 weeks) and term (36–41 weeks). In the control samples, ESX1L mRNA expression significantly decreased between pre-term and term [0.7 ± 0.2 (27–35 weeks, n = 13, white bar) versus 0.2 ± 0.06 (36–41 weeks, n = 12, black bar), t-test, P < 0.05]. In the FGR-affected placentae, however, there was no significant change in ESX1L mRNA expression between the pre-term FGR and term FGR [0.32 ± 0.04 (27–35 weeks, n = 11, white bar) versus 0.31 ± 0.02 (36–41 weeks, n = 14, black bar), t-test, P = 0.82]. Comparing pre-term controls

ESX1L expression in human idiopathic FGR

RT–PCR
Total RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform extraction and lithium chloride precipitation according to the method of Chomczynski and Sacchi (1987) and as modified by Puisant and Houdebine (1990) or by Qiagen RNeasy midi-kits (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. First-strand synthesis was performed under 2 μg of total RNA using Superscript II/III ribonuclease H-reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Approximately, 50-ng cDNA was amplified in an ABI PRISM 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) using PCR-Platinum Supermix (Invitrogen). GAPDH was used as a housekeeping gene, and the primers were forward primer, 5′-ACCACTACCAATCCATCACC-3′ and reverse primer, 5′-GTCCACCAAGTGTAGTGA-3′ (Hollington et al., 2004). PCR amplification and the conditions for the GAPDH gene were initial enzyme activation and denaturation (94°C, 10 min), 35 cycles of denaturation at 94°C for 60 s, annealing at 60°C for 45 s and primer extension at 72°C for 45 s. The GAPDH levels for both the control and the FGR-affected placentae were normalized to a internal control, and the cDNA volumes were adjusted using scanning densitometry. The oligonucleotide primers used for the amplification of the ESX1L gene were forward primer, 5′-GGCTTCACCGATTCAG-3′ (exon 2) and reverse primer, 5′-CTGATTTCGTTTCCACTT-3′ (exon 4) (Fohn and Behringer, 2001). Adjusted volumes of the control and the FGR-affected placentae cDNA were used for ESX1L PCR amplification, and the conditions were initial activation and denaturation (94°C, 10 min), 35 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 45 s and primer extension at 72°C for 60 s. Amplified products of GAPDH (452 bp) and ESX1L (162 bp) were fractionated on a 2% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV illumination. The PCR reaction for each sample was performed on both cDNA and corresponding RNA that was not reverse transcribed, to control for contaminating genomic DNA.

Real-time PCR
Quantitation of ESX1L mRNA expression in placentae from FGR-affected pregnancies and gestation-matched controls was performed in the ABI Prism 7700 (Perkin-Elmer-Applied Biosystems) using pre-validated Assays on Demand™ (consisting of 20X mix of unlabelled ESX1L PCR primers and TaqMan® MGB probe (FAM™ dye labelled) ESX1L Assays on Demand Cat No. Hs00538057_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-μl PCR reaction mix containing TaqMan Universal PCR master mix, 1X Assays on Demand™ gene expression assay mix and placental cDNA (3.5 ng) was amplified for 40 cycles, including denaturation at 95°C for 15 s, annealing and extension at 60°C for 60 s. Gene expression quantitation for the housekeeping gene GAPDH was carried out in a separate reaction. The GAPDH primers (5′GCACACCAACCTGTTTCAGCA-3′ and TaqMan probe (5′VIC-TCTGGGAGGCATCATGACCAGTCC-TAMRA′) were designed using Primer Express 1.5 Software (Applied Biosystems). The cycling conditions were identical to ESX1L. Relative quantitation of ESX1L expression normalized to GAPDH was calculated according to the 2 –ΔΔCT method (Livak and Schmittgen, 2001) employing a term control as a calibrator. A standard curve generated to validate the amplification efficiencies for both the target and the housekeeping genes demonstrated that the amplification efficiencies of ESX1L and GAPDH were 99 and 90%, respectively (ABI Prism 7700 Sequence detection system, User Bulletin 2, 2001).
Total RNA was isolated from the control placentae, and the levels of cDNA was carried out with ranging from 27 weeks to term. Semi-quantitative RT–PCR using 50-ng cDNA was performed using the housekeeping gene GAPDH. Relative quantitation of ESX1L mRNA expression were determined in control placentae of gestations ranging from 27 weeks to term. Semi-quantitative RT–PCR using 50-ng cDNA was carried out with ESX1L primers as described in the Methods section. The PCR products were visualized following 2% agarose gel electrophoresis. A representative RT–PCR for control placentae from gestation week 27–36 is shown. The ESX1L product (upper panel) was at the expected size of 162 bp. GAPDH levels (lower panel), as detected by the expected product size of 452 bp, showed a constant level of gene expression for all the samples analysed. (B) Representative RT–PCR of ESX1L mRNA in fetal growth restriction (FGR)-affected placentae. Total RNA was isolated from FGR-affected placentae, and the levels of ESX1L mRNA expression were determined on placentae of gestations ranging from 28 weeks to term. RT–PCR conditions and visualization of the products was as described in Figure 1A. A representative RT–PCR for FGR-affected placentae from gestation week 28–36 is shown. The ESX1L (upper panel) and GAPDH (lower panel) product sizes were as described in Figure 1A.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** (A) Representative RT–PCR of ESX1L mRNA in control placentae. Total RNA was isolated from the control placentae, and the levels of ESX1L mRNA expression were determined in control placentae of gestations ranging from 27 weeks to term. Semi-quantitative RT–PCR using 50-ng cDNA was carried out with ESX1L primers as described in the Methods section. The PCR products were visualized following 2% agarose gel electrophoresis. A representative RT–PCR for control placentae from gestation week 27–36 is shown. The ESX1L (upper panel) and GAPDH (lower panel) product sizes were as described in Figure 1A.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Real-time PCR for ESX1L mRNA expression in pre-term and term placentae. Relative quantitation of ESX1L mRNA expression normalized to housekeeping gene GAPDH (Y-axis) was performed in pre-term (white bars) and term-placentae (black bars, 36–41 weeks) obtained from both FGR-affected placentae (28–35 weeks, n = 11; 36–41 weeks, n = 14) and gestation-matched controls (27–35 weeks, n = 13; 36–41 weeks, n = 12) (X-axis). Real-time PCR probes and conditions were as described in the Methods section. Data were analysed according to the 2−ΔΔCT method (32). Statistical comparisons were performed using the t-test. The values for ESX1L mRNA relative to GAPDH are expressed as mean ± SEM, and the lines above the bars connect values that are statistically significant as denoted by the ‘+’ for P < 0.005. with pre-term FGR placentae, there was a significant decrease in ESX1L mRNA expression [0.7 ± 0.1 (27–35 weeks, n = 14, white bar) versus 0.32 ± 0.04 (27–35 weeks, n = 11, white bar), t-test, P = 0.0001]. However, when comparing the term controls with the term FGR placentae, ESX1L mRNA expression levels were not significantly different from each other [0.2 ± 0.06 (36–41 weeks, n = 11 black bar) versus 0.31 ± 0.02 (36–41 weeks, n = 14, black bar), t-test, P = 0.12]. ESX1L mRNA expression was lower in both term groups than the corresponding pre-term samples.

Multiple linear regression analysis showed that in the control placentae, ESX1L mRNA levels declined rapidly with gestation (Figure 3); the average decrease was 0.075-fold of the calibrator for each week of gestation (95% CI = –0.105 to –0.045, P < 0.0005). In the FGR-affected placentae, ESX1L mRNA expression was low at 27 weeks and remained low until term ESX1L expression was reduced by only 0.001-fold of the calibrator for each week of gestation (95% CI = –0.030 to –0.010, P < 0.3). Thus, in the FGR-affected placentae, there was no evidence that the rate of ESX1L expression changed significantly during gestation. The linear relationship between mRNA expression for ESX1L and gestation for control placentae differed significantly from that of FGR-affected placentae (likelihood ratio test for interaction, P = 0.005).

**Figure 3.** Regression analyses for ESX1L expression in the control and the fetal growth restriction (FGR)-affected placentae. The relationship between ESX1L mRNA expression normalized to GAPDH (Y-axis) and gestation (X-axis) for FGR-affected (+) symbol) and control placentae (‘o’ symbol) was modelled using multiple linear regression. In the control placentae (n = 25), ESX1L mRNA expression decreased with gestation in control placentae (n = 25); the average decrease was 0.0075-fold of the calibrator per week (95% CI = –0.105 to –0.045, P < 0.0005). ESX1L mRNA expression decreased with gestation in FGR-affected placentae (n = 25), the average decrease was 0.001-fold of the calibrator per week, but the decrease was not significantly different from zero (95% CI = –0.030 to –0.010, P = 0.3). The likelihood ratio test for interaction showed that the mRNA expression of ESX1L during gestation in control placentae differed significantly from that of FGR-affected placentae (P = 0.0005).

**Discussion**

The FGR-affected pregnancies chosen in this study were carefully defined in clinical terms to include the severe end of the spectrum of idiopathic FGR, with the fetuses showing reduced growth by the late second and early third trimester. The placentae from pregnancies with severely growth-restricted infants and abnormal end-diastolic blood flow in the umbilical artery show altered characteristics such as reduced villous tree elaboration and diminished surface area (Rosso et al., 2000; Steffensen et al., 2000). Attempts to show gene expression changes employing looser clinical definitions of FGR (e.g. just...
birthweight less than the 10th percentile for gestation) could provide inconsistent data. We have found that mRNA analysis of placentae from well-defined groups that include gestation-matched controls yields reproducible gene expression changes (Murthi et al., 2006).

In this study, we observed a gestation-dependent decrease in mRNA expression for ESXIL in term control placentae compared with the pre-term controls. The decrease was detected using semi-quantitative RT–PCR analysis as well as real-time PCR analysis. Regression analysis confirmed that the linear relationship between mRNA expression for ESXIL and gestation for control placentae differed significantly from that of FGR-affected placentae.

Figueiredo et al. (2004) have shown a decline in ESXIL expression levels between 24 weeks and term placentae using qualitative RT–PCR. We confirmed the results of previous studies (Figueiredo et al., 2004; Grati et al., 2004; Guan et al., 2005) and showed that at term, the expression levels of ESXIL for both FGR-affected placentae and gestation-matched controls were low and did not show any significant differences. However, in our study, we report that in pre-term samples, ESXIL mRNA expression was significantly lower in FGR-affected placentae compared with gestation-matched controls.

The rate of decline of ESXIL mRNA expression in the controls after 27 weeks is greater than that of HLX1, another homeobox gene we have investigated in FGR-affected placentae and gestation-matched controls (Murthi et al., 2006). Using the same samples, quantitative real-time PCR analysis and the method of linear regression analysis showed, the average decrease after 27 weeks for ESXIL was 0.075-fold of the calibrator for each additional week of gestation (95% CI = −0.105 to −0.045, P < 0.0005), whereas the decrease with HLX1 was substantially less at 0.043-fold for each additional week of gestation (95% CI = −0.058 to −0.028, P < 0.0005) (Murthi et al., 2006).

Figueiredo et al. (2004) demonstrated that ESXIL mRNA expression is equally expressed in proliferating residual villous cytotrophoblast cells and non-proliferating syncytiotrophoblast cells at term; therefore, it is unlikely that the decrease in ESXIL expression observed in this study could be attributed solely to the reduced numbers of proliferating villous cytotrophoblast cells at term.

Furthermore, using immunohistochemistry, we have shown a reduced level of homeobox gene HLX1 protein expression in cytotrophoblast cells of FGR-affected term placentae compared with matched controls (Murthi et al., 2006). Thus, despite the reduced number of proliferating cytotrophoblast cells at term, there is a detectable decrease in HLX1 expression in this cell type. Therefore, to confirm that the decrease in ESXIL levels between pre-term and term is because of an overall decrease in ESXIL expression, we await the preparation of a human ESXIL-specific antibody for immunohistochemical analysis.

The pattern of normal human fetal growth is complex. Increases in the rates of fetal weight gain and length increase are not parallel throughout pregnancy. Evidence suggests that the maximal growth rate for length is seen in the second trimester, whereas the maximal rate of weight gain is early in the third trimester (Guiraud-Costa and Larroche, 1992; Morris and Trudinger, 1992; Guiraud-Costa et al., 2000). Guiraud-Costa et al. (2000), in a longitudinal study of human fetal growth, have reported a linear growth rate until 26 weeks, and thereafter the growth rate decreased. In this study, a rapid decline in the levels of ESXIL mRNA expression was observed from 27-week gestation, which may correspond to the decline in the growth rate of the fetus (Morris and Trudinger, 1992; Guiraud-Costa et al., 2000) seen in the third trimester.

Qualitative RT–PCR studies detect ESXIL mRNA expression in the placenta as early as 5-week gestation and throughout the first and second trimester (Li and Behringer, 1998). It is therefore possible that the deleterious effects of reduced ESXIL expression on placental function precede, and are responsible for, the growth-restriction effects on the fetus that are only detectable later in the pregnancy. Consistent with this notion is that X<sup>−</sup>ESXIL-<sup>−</sup>Y and X<sup>−</sup>ESXIL-<sup>−</sup>Y mutant mouse embryos are comparable in size and weight with their wild-type siblings at day 13.5, but the placenta of the mutant embryos are edematous and larger and heavier than their wild-type siblings at day 13.5 (Li et al., 1997). The difference in placental weights peaks at day 14.5, but growth restriction of the X<sup>−</sup>ESXIL-<sup>−</sup>Y and X<sup>−</sup>ESXIL-<sup>−</sup>Y mutant mouse embryos is not detected until day 16.5 and is more severe at day 18.5 (Li et al., 1997). Therefore, placental defects can precede the growth-restriction effects on the fetus.

Our findings in this study are consistent with a potential role for the ESXIL homeobox gene within the growth control mechanism of the fetus through its effect on placental function. Although the imprinting mechanism seen in the mouse placenta does not appear to be conserved in humans, ESXIL may still play a critical role in the human placenta and in human fetal growth and development.

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