Downstream Targets of the Homeobox Gene DLX3 are Differentially Expressed in the Placentae of Pregnancies Affected by Human Idiopathic Fetal Growth Restriction

Running title: Downstream Targets of DLX3

Amy Chui[1,2], Bill Kalionis[1,2], Mohamed Abumaree[3], Melanie Cocquebert[4,5,6], Thierry Fournier[4,5,6], Daniele Evain-Brion[4,5,6], Shaun P Brennecke[1,2] and Padma Murthi[1,2]

[1,2]Department of Perinatal Medicine Pregnancy Research Centre and University of Melbourne Department of Obstetrics and Gynaecology, Royal Women's Hospital, Parkville, Victoria, Australia, 3052. [3]College of Medicine, King Saud Bin Abdulaziz University for Health Sciences, King Abdullah International Medical Research Center, King Abdulaziz Medical City, National Guard Health Affairs, P.O. Box 22490, Riyadh 11426, Mail Code 1515. [4]INSERM, U767, Faculté des Sciences Pharmaceutiques et Biologiques, Paris F-75006, France. [5]Universite Paris Descartes, Paris F75006, France. [6]PremUp Foundation, Paris, F-75006, France.

Corresponding author:

Dr. Padma Murthi

Department of Obstetrics and Gynaecology

University of Melbourne, Royal Women's Hospital

Parkville, Victoria 3052. Australia.

Tel: 61-03-8345 3747; Fax: 61-03-8345 3746

Email: padma@unimelb.edu.au

Abstract

Human idiopathic fetal growth restriction (FGR) is associated with placental insufficiency. Previously, we reported that the expression of homeobox gene *Distal-less 3* (*DLX3)* is increased in idiopathic FGR placentae and is a regulator of villous trophoblast differentiation. Here, we identify the downstream targets of *DLX3* in trophoblast-derived cell lines. We modelled the high levels of *DLX3* in FGR using an over-expression plasmid construct and complemented this using short-interference RNA (siRNA) for inactivation in cultured cells. Using a real-time PCR-based gene profiling, candidate target genes of *DLX3* over-expression and inactivation were identified as regulators of trophoblast differentiation; *GATA2* and *PPARγ*. The expression of *GATA2* and *PPARγ* were further assessed in placental tissues and showed increased mRNA and protein levels in FGR-affected tissues compared with gestation-matched controls. We conclude that *DLX3* orchestrates the expression of multiple regulators of trophoblast differentiation and that expression of these regulatory genes is abnormal in FGR.

Key words: Homeobox genes, human placenta, trophoblast, fetal growth.

Introduction

Fetal growth restriction (FGR), also known as intrauterine growth restriction (IUGR), is a failure of the fetus to reach its full growth potential compared to that expected for its gestation age. FGR is commonly defined as a birth weight less than the 10th percentile for gestation, together with evidence of fetal health compromise such as oligohydromnios and asymmetric fetal growth involving an increased head to abdominal circumference ratio, as well as evidence of placental pathology (Brodsky and Christou, 2004; Kingdom et al., 2000; Lee et al., 2003). Evidence of such underlying pathology allows clinicians to discriminate between FGR and healthy small for gestation age (SGA) babies that are otherwise normal. FGR is associated with an increased risk of perinatal complications such as prematurity (Gardosi, 1998), stillbirth (Cnattingius and Taube, 1998; Froen et al., 2004; Gardosi et al., 1998), neonatal morbidity (McIntire et al., 1999) and mortality (McIntire et al., 1999). While about 30% of FGR cases can be accounted for by obvious maternal, fetal and placental causes, the remaining 70% are classified as idiopathic.

The characteristics of placental dysfunction in idiopathic FGR include a decreased supply of oxygen to the fetus as well as reduced transfer of nutrients and growth factors to the fetus (Gagnon, 2003; Ghidini, 1996; Godfrey and Barker, 2000). The long-term consequences of FGR can extend well into adulthood (Godfrey and Barker, 2000) and are associated with an increased risk of chronic health disorders such as cardiovascular disease and diabetes (Godfrey and Barker, 2001), asthma (Steffensen et al., 2000), psychiatric disorders such as schizophrenia (Rosso et al., 2000), depression (Gale and Martyn, 2004), decreased intelligence quotient and intellectual developmental delay (Frisk et al., 2002).

Histopathology studies show that idiopathic FGR-affected placentae are typically smaller than gestation-matched controls suggesting that aberrant placental development may be an important contributing factor to FGR (Battistelli et al., 2004; Chen et al., 2002; Salafia et al., 2006). Epidemiological studies have proposed a possible role for genetic and familial factors in human idiopathic FGR (Devriendt, 2000; Ghezzi et al., 2003). Gene expression during normal placental development must be regulated at the transcriptional level to ensure the correct spatial and temporal formation of tissues as well as the production of essential hormones.

Transcriptional regulation of placental development is well documented in mouse knockout studies where targeted disruption of specific transcription factors has demonstrated FGR-like effects including impaired vascular and trophoblast development and placental insufficiency (Cross, 2003; Hemberger and Cross, 2001; Rossant and Cross, 2001; Sapin et al., 2001). Our previous work demonstrated that the expression of the homeobox gene *Distal-less 3* (*DLX3*) is localised to the syncytiotrophoblast of both first trimester and term placentae (Chui et al., 2010). We also showed that *DLX3* is a regulator of villous cytotrophoblast differentiation (Chui et al., 2011a), and that the expression of *DLX3* is significantly increased in human idiopathic FGR (Chui et al., 2011b), suggesting *DLX3* may regulate important differentiation pathways during placental development. In *Dlx3* knockout mice, the embryo dies at E9.5 due to placental and vascular defects (Morasso et al., 1999). Two downstream interrelated gene networks are strongly implicated by the loss of *Dlx3* in the mouse knockout model: the cell to cell signalling gene network such as, *PGF*, *EGFR*, protein kinase C, and *MMP9*, and the haemoglobin gene network (Han et al., 2007). The discovery of these target genes of *Dlx3* is consistent with a role for

Dlx3 in supporting an emerging placenta labyrinth (Han et al., 2007; Morasso et al., 1999; Roberson et al., 2001). However, investigations into the downstream target genes of *DLX3* in the human placenta have not been carried out.

In recent years, many downstream targets have been identified for various homeobox genes. For example, in the central nervous system, cell adhesion molecules and signalling molecules have been identified as candidate target genes of the *HOX* family of homeobox genes (Foronda et al., 2008). As a result, this information was used to determine a role for these targets in processes such as cell migration and differentiation (Akin and Nazarali, 2005). Since target genes seem to have important regulatory roles in basic cellular functions such as differentiation, the identification of downstream targets of *DLX3* in trophoblast cells may reveal the pathways responsible for important placental cell functions that are abnormal in idiopathic FGR.

We hypothesised therefore that downstream target genes of *DLX3* would be involved in villous cytotrophoblast differentiation and that they would be significantly altered in human idiopathic FGR pregnancies compared with uncomplicated control pregnancies. Since homeobox genes regulate and control the expression and function of many different downstream transcription factors, in this study we employed a commercially available human Transcription Factor PCR array. This type of pathway-based array was chosen in preference to a whole genome microarray since the panel of transcriptions factors was chosen for their known importance in regulating developmental pathways. We have previously used the same strategy and identified cell-cycle control genes as target genes of homeobox gene HLX in

trophoblasts (Rajaraman et al., 2010). A further advantage is that the measurement of mRNA levels is carried out by real-time polymerase chain reaction (PCR) technology. We used the well-characterised trophoblast-derived BeWo cell line to model villous trophoblast differentiation into syncytiotrophoblast *in vitro*. *DLX3* mRNA levels were increased with a *DLX3* cDNA plasmid over-expression construct or decreased in BeWo cells using two independent *DLX3*-specific siRNA oligonucleotides. PCR array-determined mRNA levels were compared between plasmid/siRNA treated cells and control cells. Target genes identified as a result of *DLX3* plasmid over-expression or siRNA inactivation were prioritised based on the fold change in expression in mRNA levels determined by real-time PCR, whether mRNA levels changed in a complementary fashion when *DLX3* was siRNA-inactivated or over-expressed, and whether the target gene had a role in cellular differentiation with published evidence identified from the literature. Prioritised *DLX3* target gene expression levels were then assessed by real-time PCR using independent validated probes or by Western immunoblotting in both the treated BeWo cells and in idiopathic FGR-affected placentae compared to controls.

Materials and Methods:

Patient details and tissue sampling

Placentae from pregnancies complicated by idiopathic FGR (total of n=25) and gestation-matched controls (total of n=25) were collected with informed patient consent and approval from the Human Research and Ethics Committees of the Royal Women's Hospital, Melbourne. Table 1 summarises the clinical features of the FGR-affected pregnancies and controls employed in this study (Murthi et al., 2006b; Murthi et al., 2006c; Rajaraman et al., 2010). The inclusion criteria for FGR cases

are listed in Table 2. They have the basic inclusion criterion of a birth weight less than the 10th centile for gestational age using Australian growth charts (Guaran et al., 1994). To exclude small for gestation age (SGA) cases, which are otherwise normal, the classification for idiopathic FGR cases required at least two of the following additional criteria; abnormal umbilical artery Doppler flow velocimetry, oligohydramnios as determined by amniotic fluid index (AFI) <7, or asymmetric growth of the fetus as quantified from the HC (head circumference) to AC (abdominal circumference) ratio (>1.2). Control patients were gestation-matched to FGR cases. Pre-term control patients who presented in spontaneous idiopathic pre-term labour or underwent elective delivery for conditions not associated with placental dysfunction (for example, one patient included in this study delivered pre-term electively by cesarean section for maternal breast cancer) were included to match for pre-term FGR. The exclusion criteria for both control and FGR-affected cases were: multiple pregnancies, chemical dependency, maternal smoking, pre-eclampsia, prolonged rupture of the membranes, placental abruption, intrauterine viral infection, and fetal congenital anomalies. Placental tissue samples were excised by dissection, thoroughly washed in 0.9% phosphate buffered saline (PBS) to minimise blood contamination, snap frozen and stored at –80º C.

Isolation of primary trophoblast cells

Informed patient consent for sampling was obtained from all donors of first trimester pregnancy placental tissues and of term placentae, in accordance with the local research and ethics committee of the Broussais Hospital (Paris, France). First-trimester pregnancy placental tissues (n=8) were obtained at 8-12 weeks of gestation following therapeutic terminations of pregnancy. Normal term placentae

(n=8) were obtained from elective caesarean sections. First trimester villous cytotrophoblasts (VCT) and term villous cytotrophoblasts (TVCT) were isolated and characterised (data not shown) using cytokeratin 7 (CK7) and CD9 as described previously (Handschuh et al., 2007; Tarrade et al., 2001a). Mononuclear VCT were maintained in culture over 72h in DMEM and differentiated *in vitro* into syncytiotrophoblast. Both first trimester and term VCT culture media were supplemented with 10% FCS and 100U/ml penicillin and 100µg/ml streptomycin.

Cell lines

The choriocarcinoma-derived BeWo cell line was a kind gift from Dr. Stephen Rogerson (Department of Medicine, University of Melbourne). BeWo cells were grown in RPMI-1640, supplemented with 10% FCS, 200U/ml penicillin and 200µg/ml streptomycin.

Over-expression of *DLX3* by plasmid transfection

A human cDNA pCMV6-XL4 vector clone of the homeobox gene *DLX3* (catalogue number: SC116854) was purchased together with TurboFectin 8.0 Transfection Reagent (both from Origene Technologies, USA) and used to over-express *DLX3* in confluent cultures of BeWo cells. Experiments were carried out following the manufacturer's instructions. Briefly, BeWo cells were seeded at a density of 2 x 10^5 cells/well in 6-well plates and maintained in culture with *DLX3* cDNA plasmid (1µg/µl) in TurboFectin 8.0 transfection reagent (cDNA plasmid:TurboFection 8.0, 1:3) for 48h. A vector or no cDNA insert is used as a control. Validation of successful over-expression at both the mRNA and protein level was performed as described previously (Chui et al., 2011a).

Down-regulation of *DLX3* expression by siRNA

Two independent *DLX3* siRNA oligonucleotides were designed and obtained as "4-For-Silencing siRNA Duplexes"™ (Qiagen, Australia). The siRNA sequences used were as follows: Hs_DLX3_3_HP siRNA (Catalogue number: SI00073962) and Hs_DLX3_4_HP siRNA (Catalogue number: SI00073969). The *DLX3* siRNAs showed no significant DNA sequence similarity to other genes in GenBank cDNA databases (data not shown). Validation of siRNA inactivation at both the mRNA and protein level was performed as previously described (Chui et al., 2011b).

BeWo cells were grown in supplemented media (2×10^5 cells/well in 6-well plates and 5×10^4 cells/well in 24-well plates) and transfected with individual *DLX3* siRNAs using RNAiFect transfection reagent (Qiagen, Australia). Negative control (NC) siRNAs consisted of a pool of enzyme-generated siRNA oligonucleotides of 15–19 base pairs in length that were not specific for any known human gene (AllStars Neg. siRNA AF 488, Qiagen, Australia) and showed no sequence similarity to *DLX3.*

RNA extraction and cDNA preparation

RNA was extracted from placentae obtained from FGR-affected and gestation-matched control pregnancies; and from cultured BeWo cells using Qiagen RNeasy Midi-kits and the Qiagen RNeasy Micro-kits (Qiagen, Australia) respectively, as described previously (Murthi et al., 2006a; Murthi et al., 2006c). Spectrophotometric analysis and gel electrophoresis was used to determine the yield, purity and integrity of the RNA. RNA was reverse-transcribed using Superscript III ribonuclease H-reverse transcriptase (Invitrogen, Australia) and cDNA was prepared in a two-step reaction using 2µg of RNA as described previously.

cDNA synthesis for PCR Array

The RT^2 First Strand kit was used to perform first strand synthesis of cDNA for RT^2 – profiler PCR array, according to the manufacturer's instructions. Briefly, a mastermix containing 10µl of RT cocktail mix (containing RT buffer, primer, external control mix and RT enzyme mix) and 10µl of genomic DNA elimination mixture (containing 1µg RNA, genomic DNA elimination buffer and RNase-free water) was heated at 42°C for 15 minutes. RNA degradation and reverse transcriptase inactivation was performed by heating samples at 95°C for 5 minutes in a GeneAmp® PCR system 9700.

PCR Array

The "Transcription Factors" RT^2 profiler PCR array (SABiosciences, Australia) for gene profiling was used to identify the downstream target genes of *DLX3* and the methodology was carried out according to the manufacturer's instructions. Briefly, cDNA prepared with the RT^2 First Strand kit was added to a mastermix which contains the fluorescent SYBR green/ROX dye. Equal amounts of this mastermix were distributed evenly to a 96-well pre-prepared plate that contained 84 gene-specific primer sets and each plate contained a panel of five housekeeping gene primers for normalisation. These housekeeping genes consisted of β-2-microglobulin (*B2M*), Hypoxanthine phosphoribosyltansferase 1 (*HPRT1*), Ribosomal protein L13a (*RPL13A*), Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β-actin (*ACTB*). Additional controls such as the no added reverse transcriptase (no reverse transcriptase enzyme) and the no added template (no cDNA) were included in each plate. The PCR reaction was performed on an ABI Prism 7500 Sequence Detector under these cycling parameters: 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 minutes and primer extension at 60°C for 1 minute. Data

(C_t values) were analysed using the ABI Sequence Detector System software version 2.0 and the relative gene expression values, or fold changes, were calculated according to the manufacturer's protocols. Briefly, to calculate the fold changes, values from the control (Vector or NC siRNA) and treated plates (*DLX3*-plasmid construct treated or *DLX3*-siRNA treated) were calculated as $2^{-\Delta\Delta CT}$, normalised to the average C_t value of the five housekeeping genes. Fold changes >1 were considered fold increases, and for fold changes < 1, the negative inverse of the result was reported as fold decreases.

Candidate target genes identified as a result of *DLX3* plasmid over expression or *DLX3* siRNA inactivation, were prioritised based on the fold change in expression in mRNA levels determined by real-time PCR (> 10 fold increase or decrease). Priority was given when a potential role for the target gene in cellular differentiation could be identified from the literature.

Real-Time PCR

Validation of candidate *DLX3* target gene mRNA expression in cell lines and tissues was performed in an ABI Prism 7500 (Perkin-Elmer-Applied Biosystems, Australia) as described previously (Murthi et al., 2006a; Murthi et al., 2006c). Real-time PCR was performed using inventoried assays that consisted of a mix of PCR primers and a TaqMan FAM labeled MGB probe (*GATA2* Hs00231119_m1 and *PPARγ* Hs01115512_m1, Applied Biosystems, Australia). Gene expression quantification for the housekeeping genes *GAPDH* or *18S rRNA* (Applied Biosystems, Australia) was performed in the same well. Levels of gene expression relative to *GAPDH* or *18S rRNA* was calculated according to the $2^{--\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Western Immunoblotting

Immunoblotting was performed using 25µg total protein per well which was electrophoresed under non-reducing conditions by 10% PAGE/0.1% SDS in running buffer (250mM Tris, 1.92mM Glycine, 0.1% SDS) for approximately 1 hour at 150V. The resulting gel was electrically transferred to a nitrocellulose membrane at 75 volts for 1 hour in cold transfer buffer (225mM Tris-HCL, 192mM glycine and 20% methanol). The membrane was blocked with 5% skim milk/TBS for 1 hour at room temperature, and washed in 1 x TBS (0.14M NaCl, 2.7 mM KCL, 0.025M Tris-HCL pH 7.4). The membranes were incubated with GATA2 (0.5µg/ml) and PPARγ (0.5µg/ml) (all from Santa Cruz Biotechonology, INC., USA) antibodies overnight at 4°C. The following day, the membrane was incubated in species specific secondary antibody conjugated to peroxidise (HRP) at a concentration of 1.5µg/ml in 2% skim milk/ TBS for 1 hour at room temperature. Immunoreactive protein was detected using the ECL-Western Chemiluminescence Detection kit (GE Healthcare, Australia) according to the manufacturer's instructions. The resulting immunoreactive protein was normalised to GAPDH. The specificity of the antibodies was determined by omission of the primary antibody.

Data analysis

All data presented in this study are expressed as mean ± SEM, unless otherwise stated. The Student's *t*-test was used to determine the significance of any differences between groups. A probability value of $p<0.05$ was considered significant.

Results

Identification of *DLX3* downstream target genes

Cultured BeWo cells were transfected with a plasmid over-expression construct for *DLX3* mRNA and protein over-expression (Chui et al., 2011a). The human "Transcription Factors" PCR array was used to identify potential downstream target genes of *DLX3*. The relative mRNA expression of the 84 genes after *DLX3* mRNA and protein over-expression is shown in Fig 1. The Y-axis represents the fold change for each of the 84 genes normalised to the average expression of the five housekeeping genes included in the array (*B2M, HX, R13, GAPDH* and *β-actin*).

In addition, to eliminate possible off-target effects of plasmid over-expression, BeWo wells independently treated with either an NC control, siRNA1 or siRNA2 oligonucleotides (Chui et al., 2011b). The fold change for each of the 84 genes after *DLX3* mRNA and protein inactivation, normalised to the five housekeeping genes, is shown in Fig 2. For both figures, genes that had an expression level above the one-fold red line, was classified as a fold increase. Conversely, genes that had an expression level below the one-fold red line was classified as a fold decrease in gene expression. Additionally, for ease of graphical presentation, the fold differences of the genes were divided by 10 or 100 depending on their values.

Several criteria were used for prioritising the 84 genes. The first and most important criterion for prioritisation was published evidence for a role in cellular differentiation from the literature. The second criterion was that mRNA levels had to be either increased or decreased with plasmid over-expression or siRNA inactivation by at least 10 fold. In Fig 3, the *relative up- or down-regulation* values of the candidate

genes were obtained using a calculation template on the manufacturer's website and calculated by: 1/-fold difference obtained from the PCR array (SABiosciences, Australia). Fig 3a shows the prioritised candidate downstream target genes of *DLX3* plasmid over-expression treated PCR array and revealed that the gene *GATA2* (GATA binding protein 2) showed a positive fold change of approximately 45 and *PPARγ* (Peroxisome Proliferator-Activated Receptors γ) with a negative fold change of approximately 500. The siRNA-treated PCR array (Fig 3b) showed *GATA2* with no fold change, whereas *PPARγ* showed a positive fold change of approximately 170. We prioritised the genes *GATA2* and *PPARγ* because these genes are shown to be important and involved in many cellular processes including differentiation in during placental development (Ma et al., 1997; Tarrade et al., 2001b).

Validation of *DLX3* candidate genes in cultured BeWo

The prioritised candidate genes from the PCR array were validated independently in cultured BeWo cells treated with either plasmid over-expression construct or *DLX3* siRNA1/siRNA2, and the appropriate NC or vector controls, as shown in Fig 4. Fig 4a shows the validation results in BeWo cells transfected with a vector control or a plasmid construct for over-expression of *DLX3*. The independent validation showed consistent results with the PCR array where the relative mRNA level of *GATA2* (n=3, $p<0.005$) was significantly increased compared with the vector controls. Conversely, the relative mRNA level of *PPARγ* (n=3, $p<0.005$) was decreased compared with the vector control. Fig 4b shows the validation results when *DLX3* mRNA was inactivated in BeWo cells by transfection with NC control, siRNA1 or siRNA2. The relative mRNA level of *GATA2* (n=3, $p<0.05$) was significantly decreased when compared with the NC control. Conversely, the relative mRNA level of *PPARγ* (n=3,

p<0.05) was significantly increased compared with the NC control. The validation showed consistent results with the PCR array.

Relative mRNA levels of *DLX3* target genes in primary villous cytotrophoblast cells

Primary villous cytotrophoblast cells from first trimester and term were isolated and cultured for 72 hours. After 72 hours, the villous cytotrophoblast cells have differentiated and fused to become the syncytiotrophoblast. RNA was extracted and cDNA was subsequently prepared. Real-time PCR was used to determine the basal mRNA expression level for *GATA2* and *PPARγ* relative to the housekeeping gene 18S rRNA. Fig 5 shows the presence of mRNA expression for *GATA2* (panel a), and *PPARγ* (panel b) in all primary villous cytotrophoblast cells isolated from first trimester and term placentae.

Relative mRNA expression of *DLX3* target genes in idiopathic FGR-affected placentae

The relative mRNA levels of *DLX3* candidate target genes were determined in FGR-affected placentae compared with gestation matched controls. Fig 6a shows that in idiopathic FGR, the mRNA level of *GATA2* (n=25, p<0.005) was significantly increased compared with control. The relative mRNA level of *PPARγ* was also significantly increased when compared with controls (n=25, p<0.005). However, mRNA expression for both *GATA2* and *PPARγ* was not different according to labour status and mode of delivery (data not shown).

Protein level of GATA2 AND PPARγ in human idiopathic FGR-affected placentae

GATA2 and PPARγ were further assessed at the protein level by western immunoblotting. Fig 6b shows a representative immunoblot result for GATA2 (upper panel) and PPARγ (middle panel). The expected size of 50kDa for GATA2 and 65kDa for PPARγ were observed. The housekeeping protein GAPDH (lower panel) shows a constant protein load between all samples. Panel c shows the densitometric protein expression of the genes in idiopathic FGR-affected placentae compared with controls, relative to the housekeeping protein GAPDH. The protein expression of GATA2 was significantly increased by approximately 20% in idiopathic FGR-affected placentae compared with controls ($n=6$, $p<0.05$). Similarly the relative protein expression of PPARγ ($n=6$, $p<0.05$) was also significantly increased in idiopathic FGR-affected placentae compared with the controls. These results followed similar trends to the observed mRNA levels.

Discussion

Transcription factors that are involved in cellular regulatory functions in many systems also play important roles in placental development (Hemberger and Cross, 2001). This study shows for the first time that altering the expression of *DLX3* results in significant changes in the mRNA and protein expression of important downstream target transcription factors required for trophoblast development.

FGR is a clinically significant pregnancy disorder, which compromises fetal health in the short and long term. Idiopathic FGR is frequently associated with utero-placental insufficiency. The molecular mechanism of idiopathic FGR is poorly understood. Our

previous studies have shown that the expression of *DLX3* was increased in idiopathic FGR-affected human placentae compared with gestation-matched controls (Chui et al., 2011b) and that over-expression or inactivation of *DLX3* resulted in increased or decreased BeWo cell differentiation, respectively (Chui et al., 2011a; Chui et al., 2011b). The results from this study show that over-expression of *DLX3* resulted in a positive fold change for *GATA2*, whereas *PPARγ* showed a negative fold change for both mRNA and protein expression. Conversely, inactivation of *DLX3* by siRNA resulted in a negative fold change for *GATA2* and a positive fold change for *PPARγ*. These candidate target genes were then further validated by both plasmid over-expression and siRNA inactivation in the BeWo cell line by independent real-time PCR probes and showed consistent increase or decrease in mRNA expression compared with the PCR array. In order to determine the expression of the candidate genes in idiopathic FGR placental tissue, we showed that the mRNA expression of all the target genes were present in cultured primary villous trophoblasts. Then we validated the mRNA and protein expression of the candidate transcription factors in idiopathic FGR-affected placentae compared with gestation-matched controls. Thus, our study demonstrated that DLX3 is directly or indirectly necessary for mRNA and protein expression of transcription factors required for trophoblast development.

The *GATA* family of transcription factors is responsible for coordinating the transcription of a set of genes in murine placental giant cells, which are thought to be functionally equivalent to extravillous cytotrophoblast cells of the human placenta. This set of genes may be important in trophoblast giant cell differentiation (Ma et al., 1997). In particular, *GATA2* regulates and determines the tissue localisation of

factors required for trophoblast differentiation (Cheng and Handwerger, 2005; Cheng et al., 2004; Ma et al., 1997; Ng et al., 1994). In addition, *GATA* family members regulate steroidogenic genes such as *3β-HSD* (Lavoie and King, 2009), which is suggestive of its role in trophoblast differentiation. In the mouse model, targeted deletion of *Gata2* resulted in embryonic death at E9.5 in most cases, but those pups that survived showed a growth restricted phenotype (Ng et al., 1994).

In this study, we have observed a significant increase in GATA2 mRNA and protein levels in idiopathic FGR-affected placentae compared with controls. We further assessed whether there are potential DLX3 binding sites in the regulatory regions of GATA2 gene. A 5 out of 8 nucleotide exact match (shown by underlining) with the consensus DLX3 binding site 5'-ATAATTAC-3' was found in the genomic DNA within the 5'UTR promoter region of GATA2, at a position 351 nucleotides upstream of exon 1 (Accession Number NM_032638). The matching nucleotides included the highly conserved 5'-TAAT-3' core binding site for homeodomain proteins.

Increased *GATA2* expression may result in increased villous cytotrophoblast differentiation. This increase may, in turn, result in a higher rate of fusion and form a syncytiotrophoblast layer that may not have time to shed into the maternal circulation, affecting the regenerative nature of this layer. Therefore, it is suggested that in order to compensate for this in the idiopathic FGR-affected placenta, increased apoptosis of villous cytotrophoblast cells occur (Huppertz et al., 2006).

The *PPARs* (Peroxisome Proliferator-Activated Receptors) are ligand-inducible transcription factors that belong to the nuclear hormone receptor superfamily (Ogata

et al., 2002). The *PPARs* have been implicated in many normal and disease-related biological processes relevant to the heart and vasculature, insulin sensitivity, adipogenesis and in placental function (Chawla et al., 2001). In the first trimester human placenta, PPARγ is expressed in the villous cytotrophoblast and invading extravillous trophoblast cells (Schaiff et al., 2006). In the second trimester, PPARγ expression is present in the columns of the anchoring villi (Schaiff et al., 2006). In the third trimester, PPARγ is localised to the syncytiotrophoblast and the villous cytotrophoblast cells (Schaiff et al., 2006). *PPARγ* is involved in the invasion pathway during human placental development (Fournier et al., 2007; Fournier et al., 2008) and is also important for regulating the levels of β-hCG, human placental lactogen, placental growth hormone and leptin (Tarrade et al., 2001b), suggestive of its important role in villous cytotrophoblast differentiation. Recently, we have demonstrated co-localisation of DLX3 and PPARγ in differentiating trophoblasts suggesting a possible regulatory role for DLX3 and PPARγ in bovine placental development (Degrelle et al., 2011).

In this study, the relative expression of *PPARγ* was decreased in both the PCR array and validation studies in BeWo cells. However, the relative expression of *PPARγ* was significantly increased in FGR-affected placentae compared with controls, which is consistent with another study (Holdsworth-Carson et al., 2010). Rodie et al (Rodie et al., 2005) have reported the localisation of *PPARγ* within isolated stromal and endothelial cells in FGR-affected placentae. Therefore, the inconsistency in expression of *PPARγ* between cell culture and placental tissue may be due to using isolated trophoblast cells and trophoblast derived cell lines compared with whole placental tissue which consists of multiple cells types. This suggests that changes in

mRNA and protein expression observed in FGR placentae reflect the total placental *PPARγ* expression.

In conclusion, this study identified novel candidate downstream target genes of *DLX3* and showed that they are expressed in the human placenta. Furthermore, this study showed that the candidate genes are significantly altered in human idiopathic FGR and demonstrated the potential value of using *in vitro* models for studies into the functional role/s of genes in the human placenta. The results from this study support the hypothesis that downstream target genes of *DLX3* may be involved in the molecular mechanism underlying the pathogenesis human idiopathic FGR.

Acknowledgements

The authors wish to thank the consenting patients and the clinical and research midwives, Sue Nisbet and Sue Duggan, for the supply of preterm and third trimester placental tissues. Funding support for this work was provided from the Australian National Health and Medical Research Council (NHMRC project grant #509140) to Dr. P. Murthi, and by postgraduate scholarship support to A. Chui from The Royal Women's Hospital, from an Arthur Nylasy Award and from a Felix Meyer Postgraduate Scholarship, the latter two both being from The University of Melbourne.

References

Akin, Z.N., and Nazarali, A.J. (2005). Hox genes and their candidate downstream targets in the developing central nervous system. Cell Mol Neurobiol *25*, 697-741.

Battistelli, M., Burattini, S., Pomini, F., Scavo, M., Caruso, A., and Falcieri, E. (2004). Ultrastructural study on human placenta from intrauterine growth retardation cases. Microsc Res Tech *65*, 150-158.

Brodsky, D., and Christou, H. (2004). Current concepts in intrauterine growth restriction. J Intensive Care Med *19*, 307-319.

Chawla, A., Barak, Y., Nagy, L., Liao, D., Tontonoz, P., and Evans, R.M. (2001). PPAR-gamma dependent and independent effects on macrophage-gene expression in lipid metabolism and inflammation. Nat Med *7*, 48-52.

Chen, C.P., Bajoria, R., and Aplin, J.D. (2002). Decreased vascularization and cell proliferation in placentas of intrauterine growth-restricted fetuses with abnormal umbilical artery flow velocity waveforms. Am J Obstet Gynecol *187*, 764-769.

Cheng, Y.H., and Handwerger, S. (2005). A placenta-specific enhancer of the human syncytin gene. Biol Reprod *73*, 500-509.

Cheng, Y.H., Richardson, B.D., Hubert, M.A., and Handwerger, S. (2004). Isolation and characterization of the human syncytin gene promoter. Biol Reprod *70*, 694-701.

Chui, A., Pathirage, N.A., Johnson, B., Cocquebert, M., Fournier, T., Evain-Brion, D., Roald, B., Manuelpillai, U., Brennecke, S.P., Kalionis, B., *et al.* (2010). Homeobox gene distal-less 3 is expressed in proliferating and differentiating cells of the human placenta. Placenta *31*, 691-697.

Chui, A., Evseenko, D.A., Brennecke, S.P., Keelan, J.A., Kalionis, B., and Murthi, P. (2011a). Homeobox gene Distal-less 3 (DLX3) is a regulator of villous cytotrophoblast differentiation. Placenta *32*, 745-751.

514 Chui, A., Tay, C., Cocquebert, M., Sheehan, P., Pathirage, N.A., Donath, S.,
515 Fournier, T., Badet, J., Evain-Brion, D., Brennecke, S.P., *et al.* (2011b). Homeobox
516 gene Distal-Less 3 is a regulator of villous cytotrophoblast differentiation and its
517 expression is increased in human idiopathic foetal growth restriction. J Mol Med
518 (Berl).

519 Cnattingius, S., and Taube, A. (1998). Stillbirths and rate of neonatal deaths in
520 76,761 postterm pregnancies in Sweden, 1982-1991; a register study. Acta Obstet
521 Gynecol Scand *77*, 582-583.

522 Cross, J.C. (2003). The genetics of pre-eclampsia: a feto-placental or maternal
523 problem? Clin Genet *64*, 96-103.

524 Degrelle SA, Murthi P, Evain-Brion D, Fournier T, Hue I. (2011). Expression and
525 localization of DLX3, PPARG and SP1 in bovine trophoblast during binucleated cell
526 differentiation. Placenta. *32*(11):917-20.

527 Devriendt, K. (2000). Genetic control of intra-uterine growth. Eur J Obstet Gynecol
528 Reprod Biol *92*, 29-34.

529 Foronda, D., de Navas, L.F., Garaulet, D.L., and Sanchez-Herrero, E. (2008).
530 Function and specificity of Hox genes. Int J Dev Biol.

531 Fournier, T., Handschuh, K., Tsatsaris, V., and Evain-Brion, D. (2007). Involvement
532 of PPARgamma in human trophoblast invasion. Placenta *28 Suppl A*, S76-81.

533 Fournier, T., Therond, P., Handschuh, K., Tsatsaris, V., and Evain-Brion, D. (2008).
534 PPARgamma and early human placental development. Curr Med Chem *15*, 3011-
535 3024.

536 Frisk, V., Amsel, R., and Whyte, H.E. (2002). The importance of head growth
537 patterns in predicting the cognitive abilities and literacy skills of small-for-gestational-
538 age children. Dev Neuropsychol *22*, 565-593.

539 Froen, J.F., Gardosi, J.O., Thurmann, A., Francis, A., and Stray-Pedersen, B.
540 (2004). Restricted fetal growth in sudden intrauterine unexplained death. Acta Obstet
541 Gynecol Scand *83*, 801-807.
542 Gagnon, R. (2003). Placental insufficiency and its consequences. Eur J Obstet
543 Gynecol Reprod Biol *110 Suppl 1*, S99-107.
544 Gale, C.R., and Martyn, C.N. (2004). Birth weight and later risk of depression in a
545 national birth cohort. Br J Psychiatry *184*, 28-33.
546 Gardosi, J. (1998). The application of individualised fetal growth curves. J Perinat
547 Med *26*, 333-338.
548 Gardosi, J., Mul, T., Mongelli, M., and Fagan, D. (1998). Analysis of birthweight and
549 gestational age in antepartum stillbirths. Br J Obstet Gynaecol *105*, 524-530.
550 Ghezzi, F., Tibiletti, M.G., Raio, L., Di Naro, E., Lischetti, B., Taborelli, M., and
551 Franchi, M. (2003). Idiopathic fetal intrauterine growth restriction: a possible
552 inheritance pattern. Prenat Diagn *23*, 259-264.
553 Ghidini, A. (1996). Idiopathic fetal growth restriction: a pathophysiologic approach.
554 Obstet Gynecol Surv *51*, 376-382.
555 Godfrey, K.M., and Barker, D.J. (2000). Fetal nutrition and adult disease. Am J Clin
556 Nutr *71*, 1344S-1352S.
557 Godfrey, K.M., and Barker, D.J. (2001). Fetal programming and adult health. Public
558 Health Nutr *4*, 611-624.
559 Guaran, R.L., Wein, P., Sheedy, M., Walstab, J., and Beischer, N.A. (1994). Update
560 of growth percentiles for infants born in an Australian population. Aust N Z J Obstet
561 Gynaecol *34*, 39-50.
562 Han, L., Dias Figueiredo, M., Berghorn, K.A., Iwata, T.N., Clark-Campbell, P.A.,
563 Welsh, I.C., Wang, W., O'Brien T, P., Lin, D.M., and Roberson, M.S. (2007). Analysis

564 of the gene regulatory program induced by the homeobox transcription factor distal-
565 less 3 in mouse placenta. Endocrinology *148*, 1246-1254.
566 Handschuh, K., Guibourdenche, J., Tsatsaris, V., Guesnon, M., Laurendeau, I.,
567 Evain-Brion, D., and Fournier, T. (2007). Human chorionic gonadotropin expression
568 in human trophoblasts from early placenta: comparative study between villous and
569 extravillous trophoblastic cells. Placenta *28*, 175-184.
570 Hemberger, M., and Cross, J.C. (2001). Genes governing placental development.
571 Trends Endocrinol Metab *12*, 162-168.
572 Holdsworth-Carson, S.J., Lim, R., Mitton, A., Whitehead, C., Rice, G.E., Permezel,
573 M., and Lappas, M. (2010). Peroxisome proliferator-activated receptors are altered in
574 pathologies of the human placenta: gestational diabetes mellitus, intrauterine growth
575 restriction and preeclampsia. Placenta *31*, 222-229.
576 Huppertz, B., Kadyrov, M., and Kingdom, J.C. (2006). Apoptosis and its role in the
577 trophoblast. Am J Obstet Gynecol *195*, 29-39.
578 Kingdom, J., Huppertz, B., Seaward, G., and Kaufmann, P. (2000). Development of
579 the placental villous tree and its consequences for fetal growth. Eur J Obstet
580 Gynecol Reprod Biol *92*, 35-43.
581 Lavoie, H.A., and King, S.R. (2009). Transcriptional regulation of steroidogenic
582 genes: STARD1, CYP11A1 and HSD3B. Exp Biol Med (Maywood).
583 Lee, P.A., Kendig, J.W., and Kerrigan, J.R. (2003). Persistent short stature, other
584 potential outcomes, and the effect of growth hormone treatment in children who are
585 born small for gestational age. Pediatrics *112*, 150-162.
586 Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data
587 using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*,
588 402-408.

589 Ma, G.T., Roth, M.E., Groskopf, J.C., Tsai, F.Y., Orkin, S.H., Grosveld, F., Engel,
590 J.D., and Linzer, D.I. (1997). GATA-2 and GATA-3 regulate trophoblast-specific gene
591 expression in vivo. Development *124*, 907-914.
592 McIntire, D.D., Bloom, S.L., Casey, B.M., and Leveno, K.J. (1999). Birth weight in
593 relation to morbidity and mortality among newborn infants. N Engl J Med *340*, 1234-
594 1238.
595 Morasso, M.I., Grinberg, A., Robinson, G., Sargent, T.D., and Mahon, K.A. (1999).
596 Placental failure in mice lacking the homeobox gene Dlx3. Proc Natl Acad Sci U S A
597 *96*, 162-167.
598 Murthi, P., Doherty, V., Said, J., Donath, S., Brennecke, S.P., and Kalionis, B.
599 (2006a). Homeobox gene HLX1 expression is decreased in idiopathic human fetal
600 growth restriction. Am J Pathol *168*, 511-518.
601 Murthi, P., Doherty, V.L., Said, J.M., Donath, S., Brennecke, S.P., and Kalionis, B.
602 (2006b). Homeobox gene ESX1L expression is decreased in human pre-term
603 idiopathic fetal growth restriction. Mol Hum Reprod *12*, 335-340.
604 Murthi, P., Said, J.M., Doherty, V.L., Donath, S., Nowell, C.J., Brennecke, S.P., and
605 Kalionis, B. (2006c). Homeobox gene DLX4 expression is increased in idiopathic
606 human fetal growth restriction. Mol Hum Reprod *12*, 763-769.
607 Ng, Y.K., George, K.M., Engel, J.D., and Linzer, D.I. (1994). GATA factor activity is
608 required for the trophoblast-specific transcriptional regulation of the mouse placental
609 lactogen I gene. Development *120*, 3257-3266.
610 Ogata, T., Miyauchi, T., Sakai, S., Irukayama-Tomobe, Y., Goto, K., and Yamaguchi,
611 I. (2002). Stimulation of peroxisome-proliferator-activated receptor alpha (PPAR
612 alpha) attenuates cardiac fibrosis and endothelin-1 production in pressure-
613 overloaded rat hearts. Clin Sci (Lond) *103 Suppl 48*, 284S-288S.

614 Rajaraman, G., Murthi, P., Pathirage, N., Brennecke, S.P., and Kalionis, B. (2010).
615 Downstream targets of homeobox gene HLX show altered expression in human
616 idiopathic fetal growth restriction. Am J Pathol *176*, 278-287.
617 Roberson, M.S., Meermann, S., Morasso, M.I., Mulvaney-Musa, J.M., and Zhang, T.
618 (2001). A role for the homeobox protein Distal-less 3 in the activation of the
619 glycoprotein hormone alpha subunit gene in choriocarcinoma cells. J Biol Chem *276*,
620 10016-10024.
621 Rodie, V.A., Young, A., Jordan, F., Sattar, N., Greer, I.A., and Freeman, D.J. (2005).
622 Human placental peroxisome proliferator-activated receptor delta and gamma
623 expression in healthy pregnancy and in preeclampsia and intrauterine growth
624 restriction. J Soc Gynecol Investig *12*, 320-329.
625 Rossant, J., and Cross, J.C. (2001). Placental development: lessons from mouse
626 mutants. Nat Rev Genet *2*, 538-548.
627 Rosso, I.M., Cannon, T.D., Huttunen, T., Huttunen, M.O., Lonnqvist, J., and
628 Gasperoni, T.L. (2000). Obstetric risk factors for early-onset schizophrenia in a
629 Finnish birth cohort. Am J Psychiatry *157*, 801-807.
630 Salafia, C.M., Charles, A.K., and Maas, E.M. (2006). Placenta and fetal growth
631 restriction. Clin Obstet Gynecol *49*, 236-256.
632 Sapin, V., Blanchon, L., Serre, A.F., Lemery, D., Dastugue, B., and Ward, S.J.
633 (2001). Use of transgenic mice model for understanding the placentation: towards
634 clinical applications in human obstetrical pathologies? Transgenic Res *10*, 377-398.
635 Schaiff, W.T., Barak, Y., and Sadovsky, Y. (2006). The pleiotropic function of PPAR
636 gamma in the placenta. Mol Cell Endocrinol *249*, 10-15.
637 Steffensen, F.H., Sorensen, H.T., Gillman, M.W., Rothman, K.J., Sabroe, S.,
638 Fischer, P., and Olsen, J. (2000). Low birth weight and preterm delivery as risk

639 factors for asthma and atopic dermatitis in young adult males. Epidemiology *11*, 185-
640 188.

641 Tarrade, A., Lai Kuen, R., Malassine, A., Tricottet, V., Blain, P., Vidaud, M., and
642 Evain-Brion, D. (2001a). Characterization of human villous and extravillous
643 trophoblasts isolated from first trimester placenta. Lab Invest *81*, 1199-1211.

644 Tarrade, A., Schoonjans, K., Guibourdenche, J., Bidart, J.M., Vidaud, M., Auwerx, J.,
645 Rochette-Egly, C., and Evain-Brion, D. (2001b). PPAR gamma/RXR alpha
646 heterodimers are involved in human CG beta synthesis and human trophoblast
647 differentiation. Endocrinology *142*, 4504-4514.

648

649

650 **Table 1 Clinical characteristics of samples used in this study**

Characteristics	FGR (n=25)	Control (n=25)	Significance (t-test)
Gestational age (Weeks) (Mean ± SD)	34.4±6.5	35.8±6.6	p=0.25
Maternal Age (Mean ± SD)	33.2±5.7	31.9±6.5	p=0.4
Placental Weight (g) (Mean ± SD)	409.3±110.3	525.0±148.2	p<0.005
Parity (n)			
Primiparous (n)	12	10	p=0.56
Multiparous (n)	13	15	
Mode of Delivery			
Vaginal Delivery (n)	10	6	
Caesarean in labour (n)	3	2	p=0.35
Caesarean not in labour (n)	12	17	
New born characteristics			
Male (n)	8	12	
Female (n)	17	13	p=0.25
Birth weight(g) (Mean±SD)	2051.4±637.0	2603.8±857	p<0.05
11-90% (n)	0	25	
6-10% (n)	11	0	
<3-5% centile (n)	14	0	

651

652 **Table 2 Clinical criteria of the idiopathic FGR-affected pregnancies**

Clinical Characteristics[a]	**Number of samples**
BW < 10th centile (n/25)	25/25 100%
<u>Abnormal Umbilical Artery Doppler velocimetry</u>	
Elevated (n/25)	7/25
Absent (n/25)	8/25
Reversed (n/25)	6/25
Not Recorded for Doppler velocimetry (n/25)	4/25
<u>Asymmetric Growth</u>	
HC: AC >1.2 (n/25)	21/25
Not recorded for HC: AC (n/25)	4/25
<u>Oligohydramnios</u>	
AFI<7 (n/25)	18/25
AFI >7 (n/25)	7/25

653

654 a. All FGR-affected pregnancies included in this study met with the first criterion for
655 birth weight (BW) less than the 10th centile for gestation and at least two of the other
656 independent ultrasound selection criteria. 17/25 FGR-affected pregnancies met with
657 all three ultrasound selection criteria.

658

659 **Figure Legends**

660 **Fig 1 Identification of *DLX3* downstream target genes after plasmid construct**
661 **over-expression of *DLX3***

662 BeWo cells were transfected with a plasmid over-expression construct for *DLX3*
663 over-expression; The RT^2 PCR Transcription Factors array was used for gene
664 profiling. Relative gene expression values or the fold change of each gene relative to
665 the vector control was calculated by normalisation to the housekeeping gene panel
666 (x-axis). The line shows the threshold value at 1. Values greater than 1 were termed
667 a fold increase and those less than 1 were considered a fold decrease.

668

669 **Fig 2 Identification of *DLX3* downstream target genes after *DLX3* siRNA**

670 RNA was extracted from BeWo cells transfected with *DLX3* siRNA, transcribed into
671 first strand cDNA, and the RT^2 Profiler PCR Transcription Factors array performed
672 for gene profiling. Gene expression values (fold change above or below threshold
673 value of 1) were subsequently calculated for the *DLX3* siRNA-treated plate, relative
674 to the NC control and normalised to the housekeeping gene panel (x-axis). The line
675 shows the threshold value at 1. Values greater than 1 were termed a fold increase
676 and those less than 1 were considered a fold decrease.

677

678 **Fig 3 Prioritised candidate *DLX3* downstream target genes**

679 Candidate downstream target genes of *DLX3* were prioritised according to
680 documented function in cell differentiation. Relative up- or down-regulation of the
681 prioritised genes were calculated using the PCR array template provided by
682 SABiosciences (Australia), such that the inverse of the negative fold difference value

683 from the PCR array generated a up- or down-regulation value for the gene of interest
684 (1/- fold difference).
685
686 **a**. The relative up- or down-regulation of the prioritised candidate downstream
687 targets of *DLX3* after plasmid over-expression.
688
689 **b.** The relative up- or down-regulation of the prioritised candidate genes after siRNA-
690 mediated *DLX3* mRNA and protein inactivation.
691
692 **Fig 4 Validation of candidate *DLX3* downstream target genes in cultured BeWo**
693 **cells**
694 **a.** cDNA from BeWo cells transfected with a plasmid over-expression construct for
695 *DLX3* mRNA over-expression using pre-validated Taqman gene expression assays.
696 Relative quantification of gene expression was normalised to the *18S rRNA*
697 housekeeping gene, according to the $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen (2001).
698 The vector control was used as the calibrator. * = Significance, $p<0.005$, n=3,
699 Student's t-test. The Y-axis represents the gene expression relative to *18S rRNA*.
700 The black bars represent BeWo cells treated with the vector control and the grey
701 bars represent BeWo cells treated with the *DLX3* plasmid over-expression construct.
702
703 **b.** cDNA from BeWo cells transfected with NC control, siRNA 1 or siRNA 2 for *DLX3*
704 mRNA down-regulation using pre-validated Taqman gene expression assays. The
705 *18S rRNA* housekeeping gene was used for relative quantification according to the 2^{-}
706 $^{-\Delta\Delta CT}$ method of Livak and Schmittgen (2001). The NC control was used as the
707 calibrator. * = Significance, $p<0.005$, n=3, Student's t-test. The Y-axis represents the

gene expression relative to 18S rRNA. The black bars represent BeWo cells transfected with the NC control, the grey bars represent cells transfected with siRNA 1 and the clear bars represent cells transfected with siRNA 2.

Fig 5 Basal mRNA levels of *DLX3* candidate target genes in isolated primary villous cytotrophoblast cells

Primary villous cytotrophoblast cells were isolated and cultured for 72 hours from first trimester and term whole placentae. Real-time PCR for the basal mRNA levels of *GATA2* (panel a) and *PPARγ* (panel b) normalised to the housekeeping gene 18S rRNA was performed according to the $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen (2001). The Y-axis represents gene expression normalised to the housekeeping gene *18S rRNA.*

Fig 6 *DLX3* candidate target gene expression in idiopathic FGR-affected placentae

a. Real-time PCR for relative quantification of *GATA2* and *PPARγ* mRNA normalised to the housekeeping gene *GAPDH* was performed in all FGR-affected placentae (n=25) and gestation matched controls (n=25). Statistical data was analysed according to the $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen (2001). * = Significance, $p<0.05$, n=25, Student's t-test. The Y-axis represents the gene expression normalised to the housekeeping gene *GAPDH*. The X-axis represents the control and idiopathic FGR-affected placentae groups. The black bars represent control placentae and the grey bars represent idiopathic FGR-affected placentae.

b. Protein was extracted from human idiopathic FGR-affected placentae (n=6) and gestation matched controls (n=6). Immunoblotting was carried out using commercially available GATA2, PPARγ and GAPDH antibodies. Chemiluminescent detection of GATA2 (50kDa), PPARγ (65kDa) and a representative GAPDH (38kDa) are shown. Lanes 1-6 are control placental samples and lanes 7-12 are FGR-affected placental samples. Lane 13 is the primary antibody omitted negative controls.

c. The percentage densitometric values of GATA2 and PPARγ relative to each individually paired GAPDH are shown. * = Significance, $p<0.05$, n=6, Student's t-test. The Y-axis represents the percentage densitometric values of the GATA2 and PPARγ relative to GAPDH.

Fig 1
Figure 1

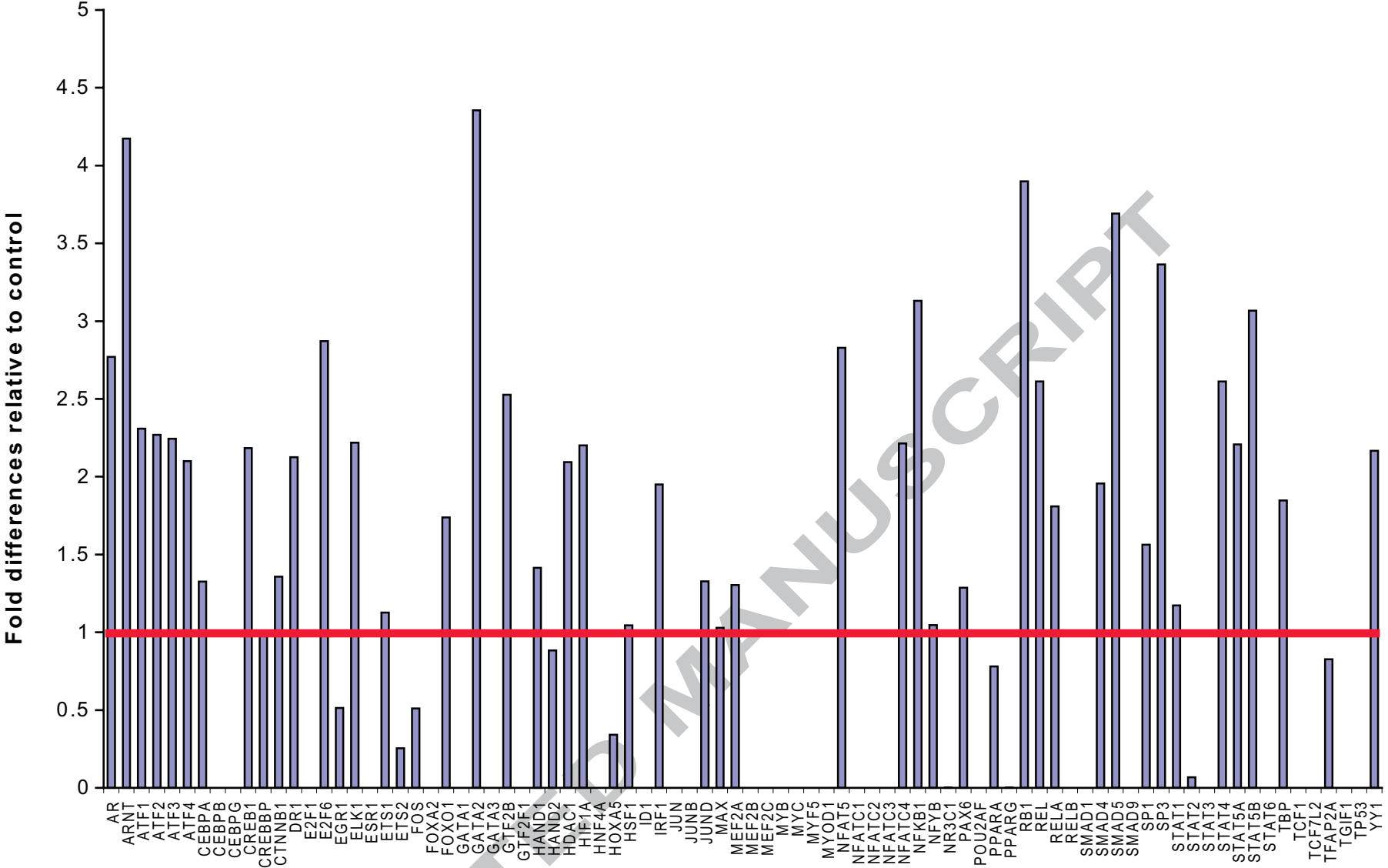
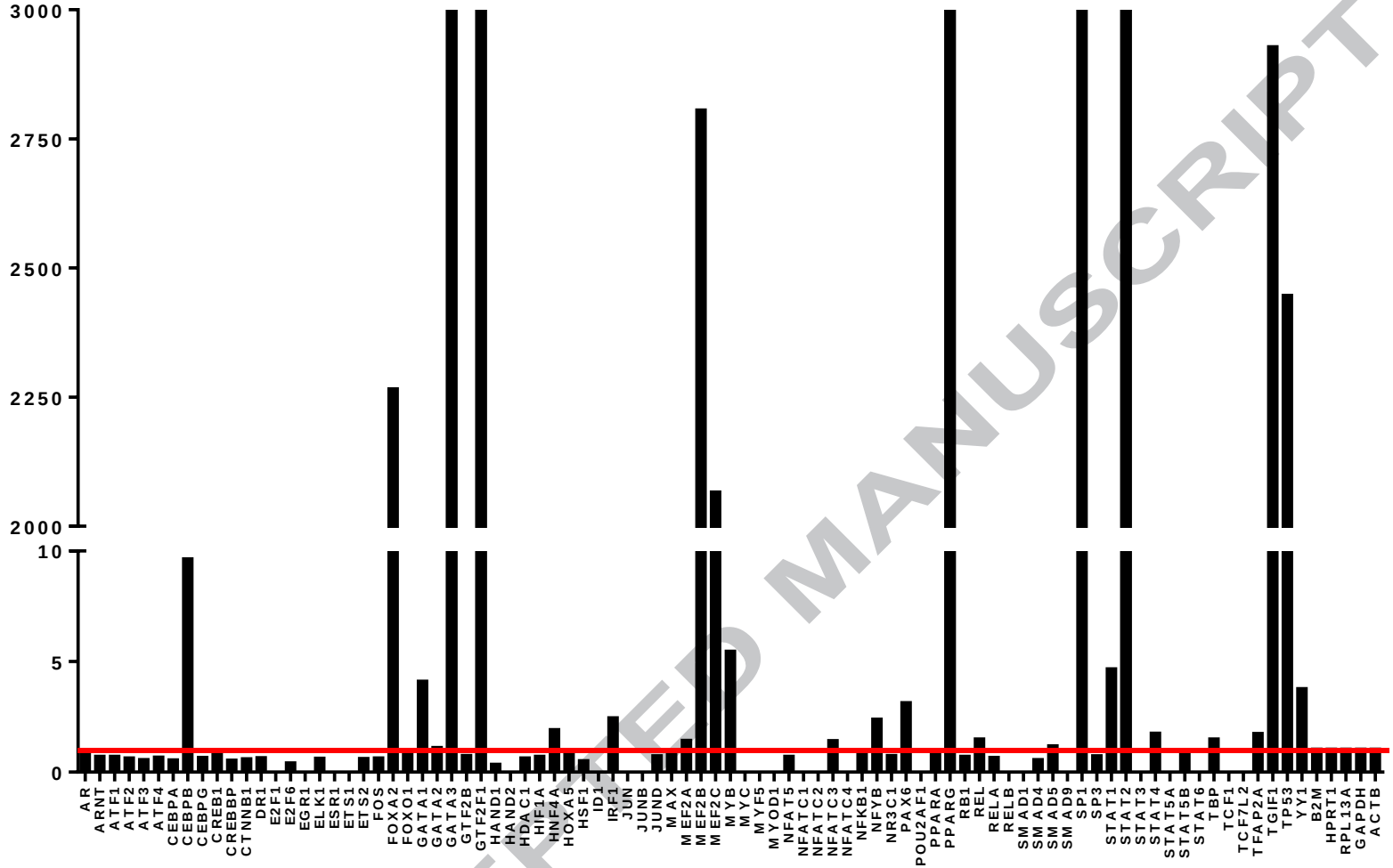
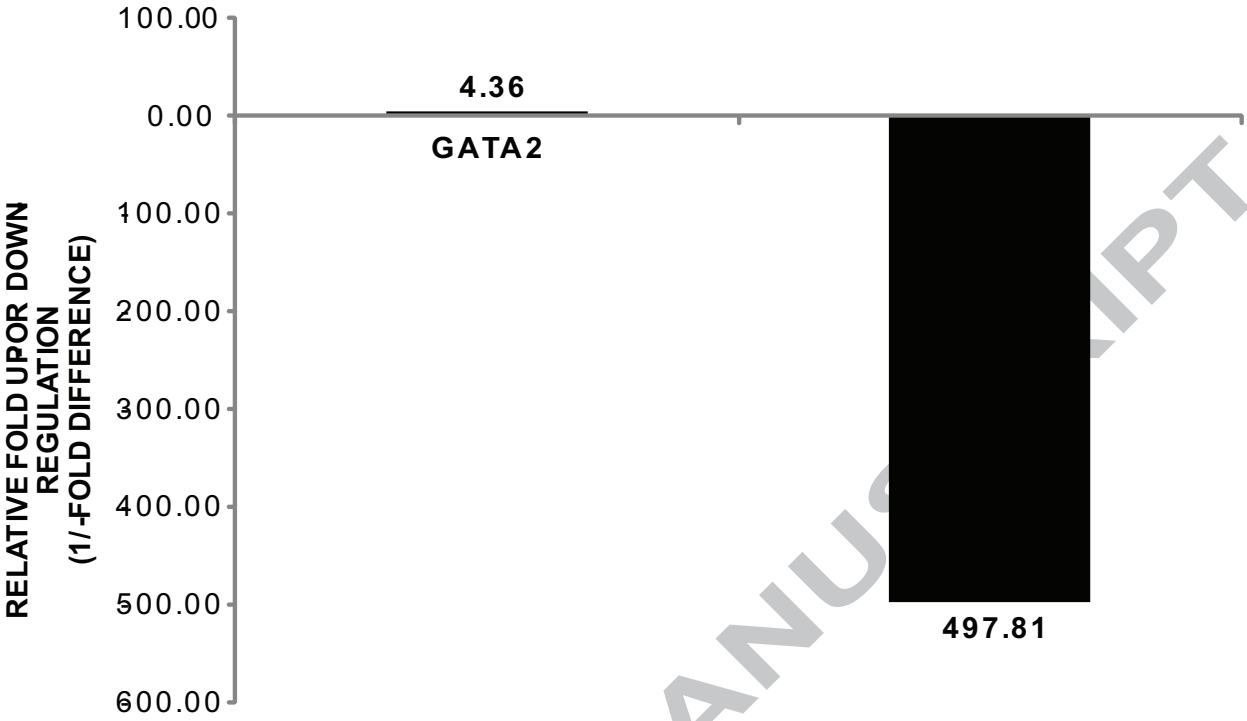


Figure 2

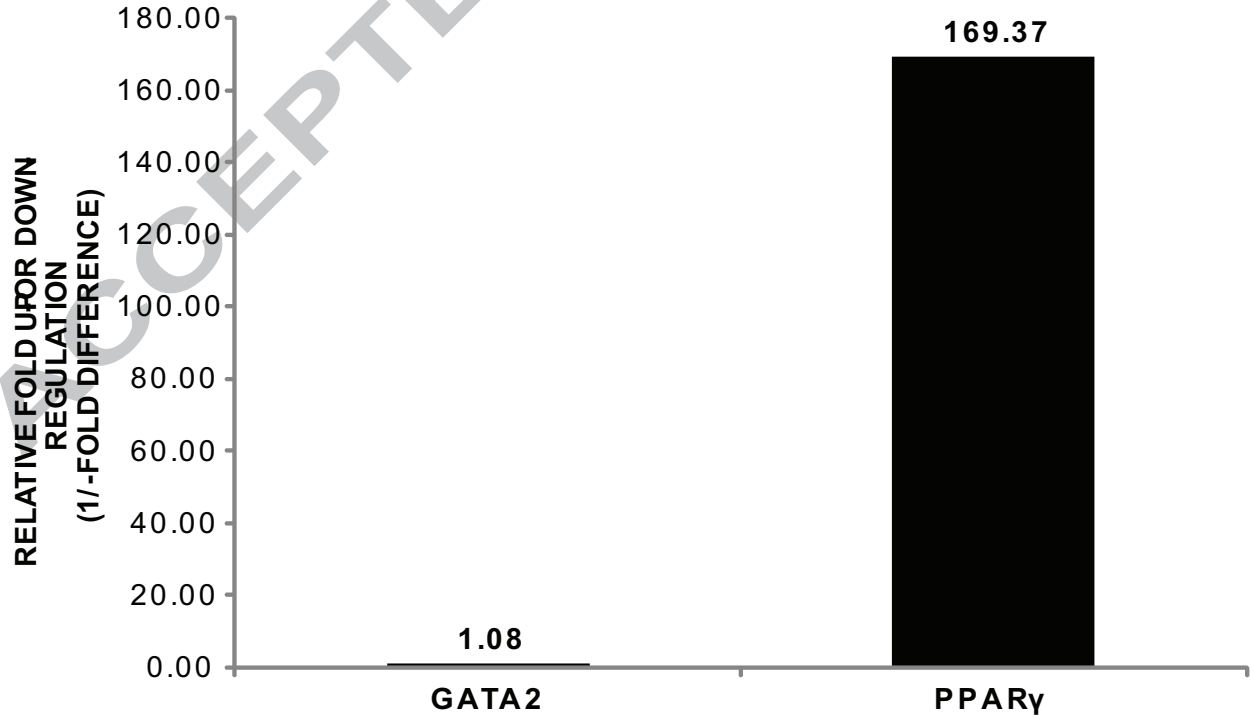
Fig 2



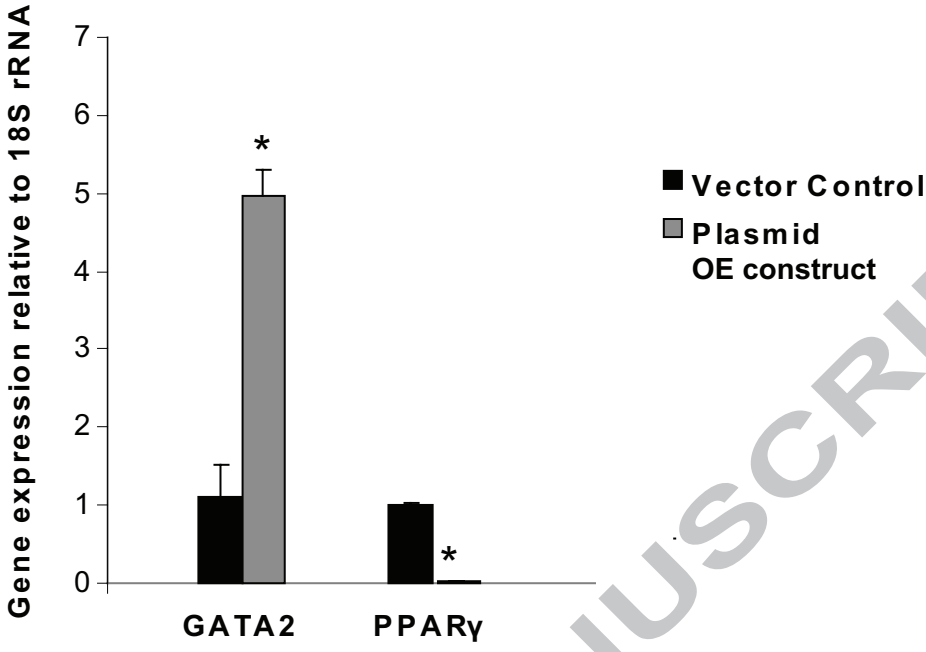
a



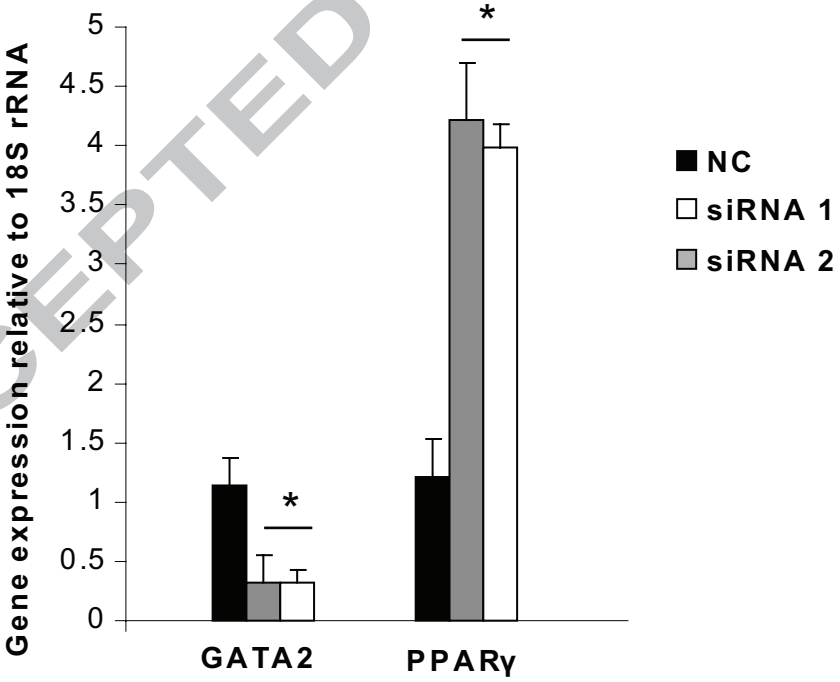
b



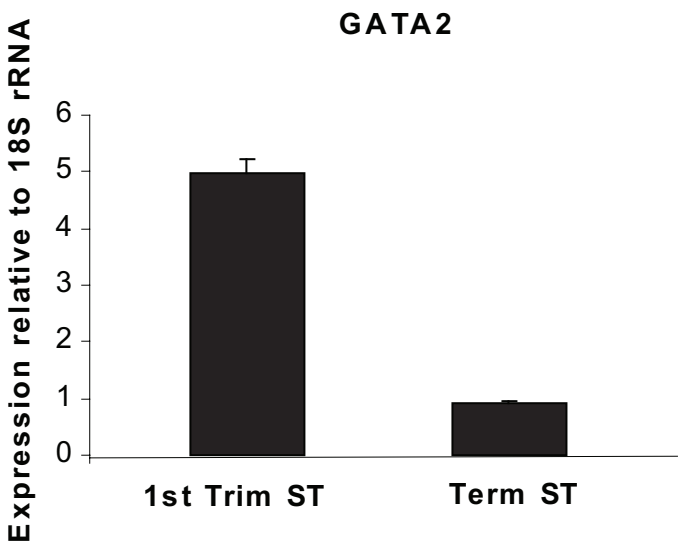
a



b



a



b

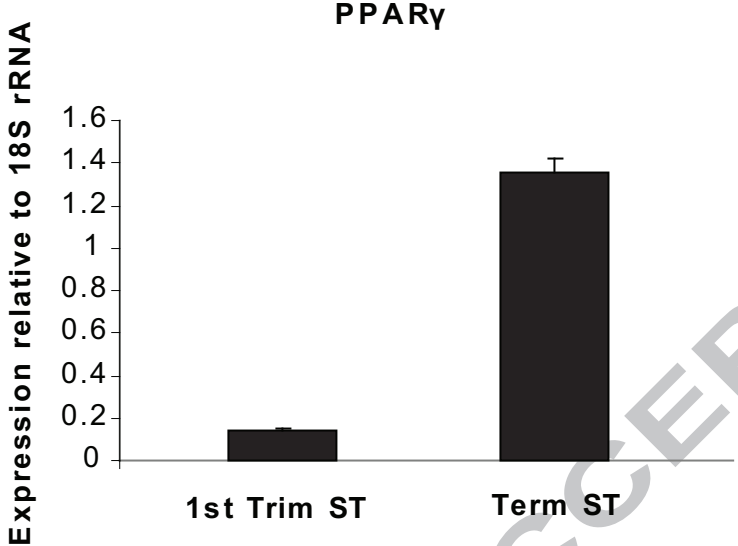
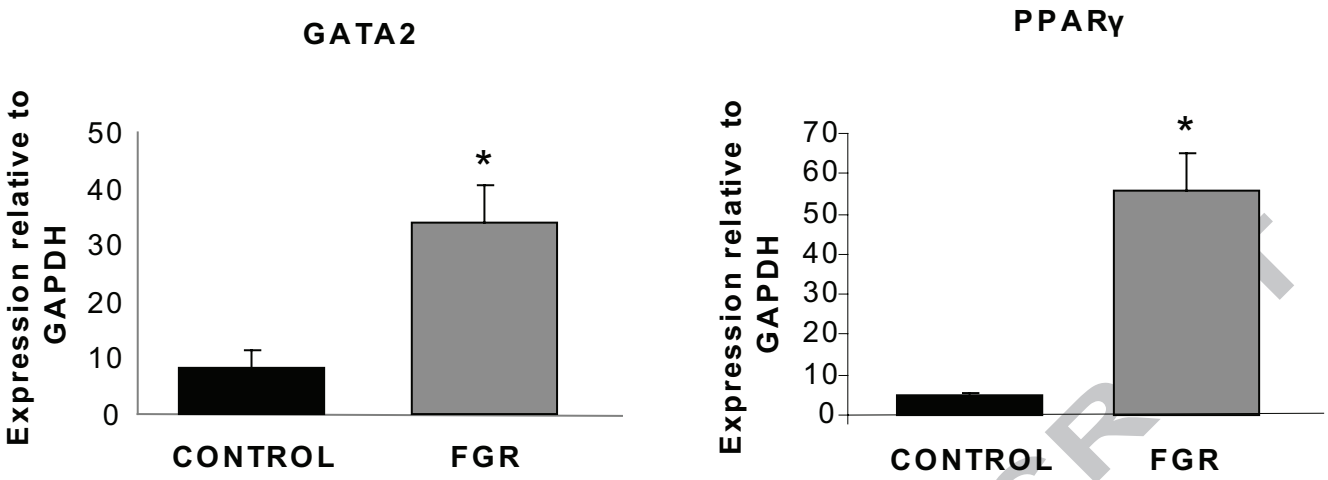
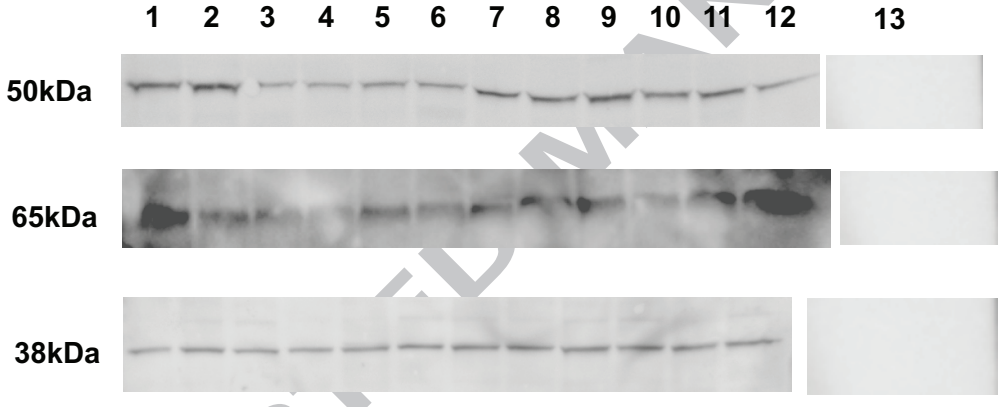


Fig 6
Figure 6

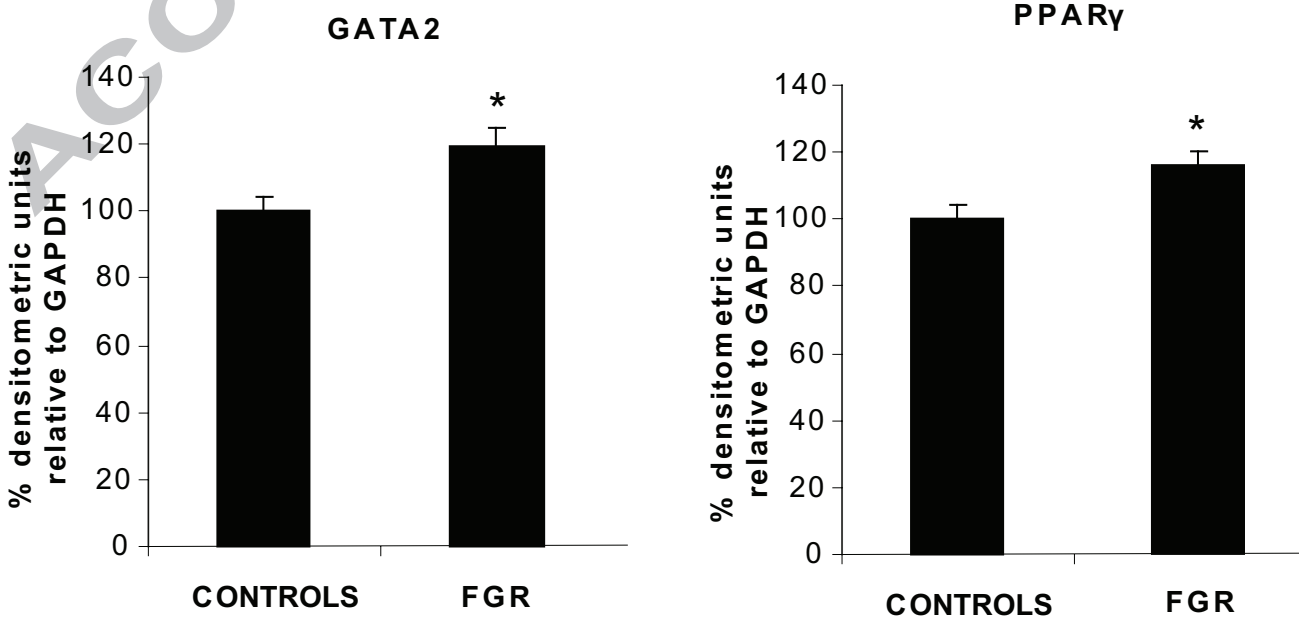
a



b



c



Highlights

- Homeobox genes
- Gene expression
- Trophoblast
- Fetal growth

Author/s:
Chui, A; Kalionis, B; Abumaree, M; Cocquebert, M; Fournier, T; Evain-Brion, D; Brennecke, SP; Murthi, P

Title:
Downstream targets of the homeobox gene DLX3 are differentially expressed in the placentae of pregnancies affected by human idiopathic fetal growth restriction

Date:
2013-09-05

Citation:
Chui, A; Kalionis, B; Abumaree, M; Cocquebert, M; Fournier, T; Evain-Brion, D; Brennecke, SP; Murthi, P, Downstream targets of the homeobox gene DLX3 are differentially expressed in the placentae of pregnancies affected by human idiopathic fetal growth restriction, MOLECULAR AND CELLULAR ENDOCRINOLOGY, 2013, 377 (1-2), pp. 75 - 83

Publication Status:
Accepted manuscript