Decreased STAT3 in human idiopathic fetal growth restriction contributes to trophoblast dysfunction

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Abstract

Abnormal trophoblast function is associated with fetal growth restriction (FGR). The JAK–STAT pathway is one of the principal signalling mechanisms by which cytokines and growth factors modulate cell proliferation, differentiation, cell migration and apoptosis. The expression of placental JAK–STAT genes in human idiopathic FGR is unknown. In this study, we propose the hypothesis that JAK–STAT pathway genes are differentially expressed in idiopathic FGR-affected pregnancies and contribute to abnormal feto-placental growth by modulating the expression of the amino acid transporter SNAT2, differentiation marker CGB/human chorionic gonadotrophin beta-subunit (β-hCG) and apoptosis markers caspases 3 and 8, and TP53. Expression profiling of FGR-affected placentae revealed that mRNA levels of STAT3, STAT2 and STAT5B decreased by 69, 52 and 50%, respectively, compared with gestational-age-matched controls. Further validation by real-time PCR and immunoblotting confirmed significantly lower STAT3 mRNA and STAT3 protein (total and phosphorylated) levels in FGR placentae. STAT3 protein was localised to the syncytiotrophoblast (ST) in both FGR and control placentae. ST differentiation was modelled by in vitro differentiation of primary villous trophoblast cells from first-trimester and term placentae, and by treating choriocarcinoma-derived BeWo cells with forskolin in cell culture. Differentiation in these models was associated with increased STAT3 mRNA and protein levels. In BeWo cells treated with siRNA targeting STAT3, the mRNA and protein levels of CGB/β-hCG, caspases 3 and 8, and TP53 were significantly increased, while that of SNAT2 was significantly decreased compared with the negative control siRNA. In conclusion, we report that decreased STAT3 expression in placenta may contribute to abnormal trophoblast function in idiopathic FGR-affected pregnancies.


Introduction

Fetal growth restriction (FGR) is a serious pregnancy complication where the fetus fails to reach its full growth potential in utero. FGR affects up to 5% of all pregnancies and is associated with a number of significant perinatal complications, including stillbirth and prematurity, and is associated with an increased risk of cardiovascular disease and glucose intolerance in adult life (Barker 2004). A proportion of FGR cases can be attributed to obvious maternal (e.g., hypertensive disorders, pre-gestational diabetes and malnutrition), fetal (e.g., genetic defects) or placental (e.g., infarcts) causes, but the aetiology remains uncertain in up to 70% of cases and hence these are termed ‘idiopathic FGR’. Idiopathic FGR is frequently associated with abnormal placental development and/or function, and as a consequence, with suboptimal delivery of oxygen and nutrients to the fetus (Gagnon 2003). Furthermore, there is increasing evidence that perturbations in placental development related to abnormal trophoblast function may lead to the compromised pregnancy outcomes characteristic of FGR (Bernstein & Divon 1997).

During early pregnancy, mononuclear villous cytotrophoblast (CTB) cells differentiate and fuse to form a multinucleate syncytiotrophoblast
(ST). For continual maternal–fetal exchange, the ST must be refreshed by differentiation and fusion of underlying, proliferating CTBs. It is important that CTB differentiation is tightly controlled, because inadequate differentiation would compromise the production and function of the ST, and reduce the efficiency of the exchange system (Pidoux et al. 2012). CTB differentiation is accompanied by increased expression of human chorionic gonadotrophin beta-subunit (β-hCG) that is primarily expressed in the ST (Potgens et al. 2004) and used as an in vitro marker for trophoblast fusion.

Multiple signalling pathways control crucial processes involved in CTB proliferation and differentiation (Fitzgerald et al. 2005). One such pathway is the JAK–STAT pathway (Park et al. 2012, Yu et al. 2012). Cytokines and growth factors activate JakS, which subsequently phosphorylate STATs (Rawlings et al. 2004), leading to the regulation of cell proliferation, differentiation, cell migration and apoptosis. Notably, these cellular events are critical for placental development (Bernstein & Divon 1997, Chui et al. 2012, Pidoux et al. 2012).

STATs are latent transcription factors that reside in the cytoplasm until activated. The seven mammalian STATs contain a conserved tyrosine residue near the C-terminus that is phosphorylated by JakS (Rawlings et al. 2004). Tyrosine-phosphorylated STATs (pSTATs) form dimers or multimers through their SH2 domains and are transported from the cytoplasm into the nucleus, where they bind to the cognate DNA sequences to activate gene expression (Hoey & Schindler 1998, Levy & Lee 2002). Activated STATs mediate the expression of a variety of genes in response to cell stimuli, and thus play a key role in many cellular processes including cell growth (Akira 1999, Horvath 2000). Predictably, mutations that reduce the JAK–STAT pathway activity affect these processes. Conversely, mutations that constitutively activate, or fail to regulate JAK signalling properly, cause inflammatory disease, erythrocytosis, gigantism and a range of leukaemias (Igaz et al. 2001, O’Shea & Plenge 2012).

The expression of genes in the JAK–STAT signalling pathway in feto-placental development is largely unknown. In this study, we propose the hypothesis that genes in the JAK–STAT signalling pathway would be differentially expressed in placentae from human idiopathic FGR pregnancies and contribute to the aberrant trophoblast differentiation in FGR. Using a pathway-specific cDNA array approach, we profiled JAK–STAT signalling-related gene expression in third trimester FGR-affected placentae compared with gestation-matched controls. STAT3 was identified as a gene of interest based on its low level of expression in FGR-affected placentae compared with the controls.

STAT3 regulation of trophoblast invasion and the expression and activity of placental amino acid transporters is known in vitro, however, the expression of STAT3 in placentae from idiopathic human FGR is yet to be investigated. Therefore, in this study, using a clinically well-defined cohort of idiopathic FGR and gestation-matched control pregnancies, placental expression for STAT3 and its functional role on feto-placental growth was investigated using an in vitro cell model.

### Materials and methods

#### Patient details and tissue sampling

Placenta from pregnancies complicated by idiopathic FGR (n=26) and placentae from gestation-matched uncomplicated pregnancies as controls (n=27) with gestation times ranging from 27 to 40 weeks were collected following Caesarean section or vaginal delivery. Placenta collection was approved by the Research and Ethics Committee of The Royal Women’s Hospital, Melbourne, and included informed patient consent. Table 1 summarises the clinical characteristics of both the FGR and gestation-matched control groups included in this study (Swan et al. 2010).

As illustrated in Table 2, the inclusion criteria for the FGR study group were a birth weight of less than the 10th percentile for gestation as determined by the Australian fetal growth charts (Guaran et al. 1994), and at least two of the following diagnoses on antenatal ultrasound: abnormal umbilical artery Doppler flow velocimetry (Salafia et al. 1997); oligohydramnios (Vik et al. 1997, Volante et al. 2004) indicated by an amniotic fluid index <7 and fetal growth asymmetry (Vik et al. 1997) as determined by head circumference-to-abdominal circumference ratio > 95th percentile for gestation. Exclusion criteria for both cases and control groups included underlying maternal diseases, maternal chemical dependency, maternal smoking, multiparous pregnancy, placental abruption, hypertension, pre-eclampsia, prolonged rupture of membranes, fetal congenital anomalies and suspected intrauterine infection, all of which are known causes of FGR. Thus, only normotensive patients, with and without idiopathic FGR, were included (Swan et al. 2010).

As described previously, control patients were selected to match FGR cases according to weeks of gestation, which were based on last menstrual period dates and confirmed by first- or second-trimester ultrasound (Swan et al. 2010). Control patients were included if they required elective delivery by induction of labour, Caesarean section, or presented in spontaneous labour (including idiopathic preterm labour) without prolonged rupture of membranes beyond 24 h, or if

### Table 1 Clinical characteristics of FGR and control samples.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control</th>
<th>FGR</th>
<th>Significance (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=27)</td>
<td>(n=26)</td>
<td></td>
</tr>
<tr>
<td>Gestation (weeks)</td>
<td>34.4±4.1</td>
<td>35.7±3.5</td>
<td>0.24</td>
</tr>
<tr>
<td>Maternal age (years)</td>
<td>32.4±5.6</td>
<td>31.0±5.7</td>
<td>0.36</td>
</tr>
<tr>
<td>Mode of delivery</td>
<td></td>
<td></td>
<td>0.42</td>
</tr>
<tr>
<td>Vaginal delivery</td>
<td>7 (26%)</td>
<td>11 (42%)</td>
<td></td>
</tr>
<tr>
<td>Caesarean in labour</td>
<td>3 (11%)</td>
<td>3 (12%)</td>
<td></td>
</tr>
<tr>
<td>Caesarean not in labour</td>
<td>17 (63%)</td>
<td>12 (46%)</td>
<td></td>
</tr>
<tr>
<td>Sex of newborn</td>
<td></td>
<td></td>
<td>0.50</td>
</tr>
<tr>
<td>Male</td>
<td>10 (37%)</td>
<td>12 (46%)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>17 (63%)</td>
<td>14 (54%)</td>
<td></td>
</tr>
<tr>
<td>Birth weight of newborn (g)</td>
<td>2488±927</td>
<td>2028±665</td>
<td>0.04*</td>
</tr>
<tr>
<td>Placental weight (g)</td>
<td>494±146</td>
<td>413±119</td>
<td>0.03*</td>
</tr>
</tbody>
</table>

Results are expressed as mean±s.d. or frequency (%). *P<0.05.
Table 2 Clinical characteristics of FGR pregnancies included in this study.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number of FGR subjects (n = 26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight percentile</td>
<td></td>
</tr>
<tr>
<td>&lt;3rd percentile</td>
<td>11 (42%)</td>
</tr>
<tr>
<td>3rd–&lt;5th percentile</td>
<td>3 (12%)</td>
</tr>
<tr>
<td>5th–&lt;10th percentile</td>
<td>12 (46%)</td>
</tr>
<tr>
<td>≥10th percentile</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Umbilical artery Doppler velocimetry</td>
<td></td>
</tr>
<tr>
<td>Elevated</td>
<td>8 (31%)</td>
</tr>
<tr>
<td>Absent</td>
<td>10 (38%)</td>
</tr>
<tr>
<td>Reversed</td>
<td>8 (31%)</td>
</tr>
<tr>
<td>Normal</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Amniotic fluid index</td>
<td></td>
</tr>
<tr>
<td>Oligohydramnios (AFI &lt; 7)</td>
<td>8 (31%)</td>
</tr>
<tr>
<td>Normal (AFI ≥ 7)</td>
<td>17 (65%)</td>
</tr>
<tr>
<td>Not recorded</td>
<td>1 (4%)</td>
</tr>
<tr>
<td>Head circumference-to-abdominal circumference ratio</td>
<td></td>
</tr>
<tr>
<td>Asymmetry (HC:AC &gt; 1.2)</td>
<td>23 (88%)</td>
</tr>
<tr>
<td>Normal (HC:AC ≤ 1.2)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Not recorded</td>
<td>3 (12%)</td>
</tr>
</tbody>
</table>

Results are expressed as frequency (%).

There was evidence of placental abruption. All control patients gave birth to normally formed babies with birth weights appropriate for gestational age, and the placentae from these patients were grossly normal with no obvious in- fists. All samples were processed within 20 min of placental delivery and excised from randomly selected areas of central cotyledons with any attached decidua carefully removed by dissection. Tissues were divided into small pieces, thoroughly washed in PBS 0.9% to minimise blood contamination, and then snap-frozen and stored at −80 °C for RNA and protein analysis, or they were fixed in 10% formalin and embedded in paraffin for immunolocalisation studies.

First-trimester placental tissues and isolated CTBs

The collection and processing of human placentae from first-trimester pregnancies were approved by the local research and ethics committee of the Broussais Hospital, Paris, France. Informed patient consent was obtained in all cases. First trimester human placentae (n = 12) from 8 to 14 weeks’ gestation were obtained from elective terminations. The placental tissues were snap-frozen and stored at −80 °C for RNA analysis. First-trimester CTBs were isolated and characterised for cytokeratin 7 (CK7) and trophoblast-specific expression (data not shown) as described previously (Tarrade et al. 2001, Handschuh et al. 2007). Mononuclear CTBs were maintained in culture over 72 h in DMEM supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin, and differentiated in vitro into STs.

Third-trimester placental CTBs

This study was approved by the Human Research and Ethics Committee of The Royal Women's Hospital (Melbourne, Australia) and the Northern Regional Ethics Committee (Auckland, New Zealand). Placentae (n = 10) were obtained with informed consent from women after delivery by Caesarean section at term, and CTBs were isolated using trypsin digestion as described previously (Evseenko et al. 2007a). Briefly, villous tissue from term placentae were subjected to eight consecutive digestions in 0.25% trypsin and cells were isolated by centrifugation at 300 g for 7 min. Erythrocytes were removed by incubation of the cell pellet in red cell lysis buffer (50 mM NH4Cl, 10 mM NaHCO3 and 0.1 mM EDTA) and CTBs were purified by centrifugation at 1200 g for 20 min on a discontinuous Percoll gradient (20–60%). Cells were collected as mononuclear CTB at 2 h and grown for a further 72 h in M199 media supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin in 5% CO2 humidified atmosphere at 37 °C. CTB purity was confirmed by the proportion of CK7-positive trophoblast cells in the culture, which was 95 ± 3.14% (n = 3) (Evseenko et al. 2007a).

Trophoblast-derived cell line

The human trophoblast derived-choriocarcinoma BeWo cell line of passage 32 was a kind gift from A/Prof. Stephen Rogerson (Department of Medicine, The Royal Melbourne Hospital, The University of Melbourne, Victoria, Australia). Cells were grown in RPMI-1640 medium, supplemented with 10% FCS, 200 U/ml penicillin and 200 µg/ml streptomycin in 5% CO2 and 95% air in a humidified chamber.

RNA extraction and cDNA preparation

Total RNA from placental tissues and cultured cells was extracted using the RNasy Midi and Micro kits, respectively, according to the manufacturer’s instructions (Qiagen) and as described previously (Murthi et al. 2006a). Total RNA was reverse-transcribed to synthesise cDNA using Superscript III ribonuclease H-reverse transcriptase (Invitrogen) in a two-step reaction as described previously (Murthi et al. 2006a,b).

JAK–STAT signalling pathway-specific cDNA array

The ‘JAK–STAT signalling pathway’ TaqMan PCR array (Applied Biosystems) for gene profiling was used to screen for genes that showed differential expression in placentae obtained from idiopathic FGR and control pregnancies. Briefly, placental cDNA obtained from idiopathic FGR (n = 26) and control (n = 27) pregnancies was prepared and pooled in two independent reaction tubes. TaqMan Master Mix (Applied Biosystems) and ~2 ng/well in a 20 µl reaction volume were distributed into two independent TaqMan Array 96-well plates, that contained 84 gene-specific primer sets and a panel of five house-keeping gene primers for normalisation. The housekeeping genes consisted of 18S rRNA, β-2-microglobulin, hypoxanthine phosphoribosyltransferase 1, glyceraldehyde-3-phosphate dehydrogenase and beta-actin (ACTB). The PCR was performed in an ABI Prism 7500 Sequence Detector using the following cycling parameters: 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and then primer extension at 60 °C for 1 min. Data (CT values) were analysed using the ABI Sequence Detector System Software version 2.0. The relative gene expression values, or fold changes, were analysed using the DataAssist Software v3.0 (Applied Biosystems). Candidate genes were prioritised based on the
difference in gene mRNA expression in FGR placentae when compared with control placentae.

**Real-time PCR**

Relative quantitation of mRNA in placental tissues and in cultured cells was performed in an ABI Prism 7500 using Applied Biosystems inventoried assays. The assay mix consisted of unlabelled PCR primers and a TaqMan FAM-labelled MGB probe (either STAT3: Hs00374280_m1; CGB: Hs00361224_g1; SLC38A2/SNAT2: Hs01089954_m1 or TP53/p53: Hs00153349_m1, Applied Biosystems). Gene expression relative to 185 rRNA (VIC-labeled Endogenous control, Applied Biosystems) was calculated according to the 2^(-ΔΔCT) method (Livak & Schmittgen 2001).

**Immunoblotting**

Total placental protein (n=12 from each of the FGR and control groups) and cellular protein (n=12) were extracted as described previously (Murthi et al. 2006a). Protein concentration was determined using the Pierce Protein Reagent (Pierce Biotechnology, Waltham, MA, USA). Total protein (25 μg) was electrophoresed on a 10% SDS–PAGE and electroblotted onto a nitrocellulose membrane (Pal Gelman, Ann Arbor, MI, USA). The membrane was blocked with 5% (w/v) skim milk for 1 h at room temperature before overnight 4°C incubation with either 0.02 μg/μl rabbit anti-human total STAT3 (cat #9139; Cell Signaling Technologies, Danvers, MA, USA), or 0.02 μg/μl mouse anti-human pSTAT3 at 86 kDa (cat #9136; Cell Signaling Technologies), or 0.05 μg/μl rabbit anti-human p53 (Abcam, Cambridge, MA, USA), or 0.025 μg/μl goat anti-human SNAT2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Antibody binding was visualised using 0.01 μg/μl HRP-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (Invitrogen/LifeTechnologies) followed by autoradiography using the ECL Western Chemiluminescence Detection Kit (GE Healthcare, Bucks, UK). Rabbit and mouse IgGs were used as negative controls (NC). The membranes were stripped and reprobed with rabbit anti-human tubulin (Imgenex, San Diego, CA, USA) for cultured cell-derived protein, or with ACTB for tissue protein extracts (Imgenex) to allow relative protein levels to be determined. Immunoreactive total STAT3, pSTAT3, p53 and SNAT2 protein relative to ACTB or tubulin were determined using scanning densitometry (ImageQuant, GE Healthcare) as described previously (Murthi et al. 2006a, 2006b).

**Immunofluorescence**

STAT3 protein localisation in third-trimester FGR (n=12) and control (n=12) placental tissue sections was determined using immunofluorescence. Briefly, paraffin-embedded placental tissue sections cut to 5 μm thickness were dewaxed in xylene, hydrated in graded ethanol (100–50% ethanol) and blocked with 1% BSA/PBS for 1 h at room temperature. Tissue sections were then incubated overnight with primary antibody mouse anti-human STAT3 that recognises total STAT3 (Cell Signaling Technologies), or mouse monoclonal anti-C7 (RCK 105 clone, Abcam), at a concentration of 0.01 μg/μl in 1% BSA/PBS. Control sections were incubated with 0.02 μg/μl mouse IgG in 1% BSA/PBS (Dako, Copenhagen, Denmark). Fluorescence detection was performed using Alexa Fluor 488 and counter-stained with the nuclear counterstain 4’,6-diamidino-2-phenylindole, dihydrochloride (Dako) according to the manufacturers’ recommendations.

**Forskolin-mediated BeWo cell differentiation**

BeWo cells were seeded in six-well culture dishes at a density of 2 × 10^5 cells/well and serum starved with RPMI-1640 medium supplemented with 0.1% BSA (w/v) overnight. Forskolin (FSK; Invitrogen/LifeTechnologies) was added to a final concentration of 10 μM (Green et al., 2006, Evseenko et al. 2007b) to induce differentiation and then cells were incubated for 72 h. FSK was prepared in 1% DMSO. At the end of the incubation period, the medium was collected and stored at −20°C and the cells were processed for RNA and protein analysis. Untreated control cells were maintained in RPMI-1640 medium supplemented with 1% DMSO and used as the vehicle control. Each experimental condition within an experiment was performed at least in duplicate, and all cell culture experiments were repeated at least on three independent occasions.

**β-hCG protein assay**

Determination of β-hCG protein levels was by the ELISA (Alpha Diagnostic International, San Antonio, TX, USA) and was carried out according to the manufacturer’s instructions using the culture supernatant collected from cell culture treatments (n=3 independent experiments) as described previously (Chui et al. 2011). The minimum concentration of hCG detected using this assay was 1.5 mIU/ml.

**Gene inactivation of STAT3 expression in BeWo cells**

BeWo cells were grown in supplemented media (2 × 10^5 cells/well in six-well plates), treated with FSK and transfected with STAT3 siRNA (19–21 bp, Invitrogen) using RNAiFect Transfection Reagent (Qiagen). The optimum siRNA:RNAiFect ratio and incubation time for the culture was 1:6 and 72 h respectively (data not shown). NC siRNA consisted of a pool of enzyme-generated siRNA oligonucleotides of 15–19 bp that showed no DNA sequence similarity to the STAT3 gene or to any known human gene (AllStars Neg. siRNA AF 468, Qiagen).

**Caspase activity assay**

Caspases 3 and 8 activity was measured in BeWo cells using the ApoAlert Caspase Colorimetric assay kits specific for caspasases according to the manufacturer’s instructions (Clontech Laboratories, Inc., Mountain View, CA, USA). The colorimetric assay is based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) after its cleavage by caspases from the labelled caspase-specific substrates. Briefly, BeWo cells grown to confluence in six-well plates (2 × 10^5 cells/well) were treated with FSK (10 μM). ApoAlert Caspase activity for caspase 8 was measured following STAT3 or NC siRNA gene inactivation, while apoptosis was determined on via free access.
cell lysates by the addition of 50 μM caspase 3 or 8 substrate (DEVD-pNA) in the presence or absence of caspase 3 or 8 inhibitor DEVD-fmk, all provided in the assay kit. Chromagen detection and absorbance was measured at 405 nm using microplate reader SPECTRAmax PLUS (Molecular Devices Corp., Sunnyvale, CA, USA).

**Statistical analysis**

Data analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). All parameters of the FGR-affected placentae compared with gestation-matched controls were described as mean ± S.E.M. Either the χ² test or Student’s t-test was used where appropriate to analyse the significance of any differences between the clinical characteristics of the FGR-affected and the control pregnancies. The difference in mRNA and protein expressions between FGR-affected placentae and control placentae was assessed by the Mann–Whitney U test. P<0.05 was considered significant.

**Results**

**Patient characteristics**

Table 1 summarises the clinical features of the FGR group and gestation-matched controls. Maternal age, mode of delivery and sex of the newborn were not significantly different between the two groups. Mean birth weight and mean placental weight were significantly lower in the FGR group compared with controls with a mean birth weight difference of 461 ± 222 g (P=0.04) and mean placental weight difference of 81 ± 37 g (P=0.03).

Table 2 shows that all FGR placentae were collected from pregnancies that resulted in a newborn with a birth weight less than the 10th percentile for gestation, with 11 (42%) samples from pregnancies with a newborn birth weight less than the 3rd percentile. Most of the FGR subjects (54%) had all three ultrasound selection criteria while 46% had only two criteria due to incomplete medical records.

**STAT expression in FGR placentae**

Figure 1 shows that in FGR-affected placentae, the gene expression profile for the JAK–STAT signalling pathway identified STAT3, STAT2 and STAT5B with decreased expressions of 69, 52 and 50%, respectively, compared with the gestation-matched controls, while JAK genes were unchanged (data not shown). STAT3 was selected as the candidate gene because it had the greatest percentage decrease in relative mRNA levels in FGR samples relative to the gestation-matched controls.

Further validation of the STAT3 mRNA levels and protein expression in FGR and control placentae was performed using real-time PCR and immunoblotting respectively. The data are shown in Fig. 2. The relative level of STAT3 mRNA was significantly decreased in FGR-affected placentae compared with gestation-matched control placentae (Fig. 2A). The decrease in STAT3 mRNA level was also reflected at the protein level. Immunoreactive total STAT3 protein (79 kDa) and pSTAT3 (86 kDa) showed reduced protein expression in FGR-affected placentae compared with gestation-matched controls (Fig. 2B). No specific immunoreactivity was observed in the presence of rabbit or mouse IgG (data not shown). Both immunoreactive total STAT3 and pSTAT3 were significantly decreased in FGR-affected placentae compared with controls (Fig. 2C). However, pSTAT3 relative total STAT3 was not significantly different in both FGR and control placentae (data not shown).
STAT3 protein localisation was determined in third-trimester FGR and gestation-matched control placentae using immunofluorescence. As shown in Fig. 3, immunoreactive STAT3 was present in the nuclei of the ST and in the stroma of third-trimester controls (Fig. 3A) and FGR placentae (Fig. 3B). CK7 was used as a positive control in a term placental section (Fig. 3C) to identify immunoreactive trophoblasts. No specific immunoreactivity was detected when the sections were stained with the NC mouse IgG (Fig. 3D).

Effect of in vitro differentiation on STAT3 expression in placental cells

STAT3 mRNA level was determined in cultured CTBs isolated from first-trimester placentae, term placenta and following spontaneous in vitro differentiation of the ST. STAT3 mRNA (Fig. 4A) and protein (Fig. 4B and C) were detected in both first-trimester and term CTBs. In vitro differentiation of CTBs to ST was associated with a significant increase in STAT3 mRNA (Fig. 4A) and protein (Fig. 4B and C) expression from both first-trimester and term placenta.

To determine the effect of differentiation on STAT3 in BeWo cells, FSK was used. As shown in Fig. 5, FSK induced a significant increase in both the mRNA (Fig. 5A) and protein (Fig. 5B and C) expression of STAT3.

Effect of STAT3 siRNA knockdown on cell differentiation marker, β-hCG expression, amino acid transporter, SNAT2 expression and apoptosis in BeWo cells

The functional consequence of a reduced STAT3 expression in FGR-affected placentae was modelled in cell culture by reducing STAT3 expression using STAT3 siRNA in FSK-induced BeWo cells. The efficiency of transfection is depicted in Fig. 6. There was a significant decrease in STAT3 mRNA (Fig. 6A) and protein (Fig. 6B and C) expression in BeWo cells treated with STAT3 siRNA compared with NC control siRNA transfected cells.

The effect of STAT3 inactivation on BeWo cell differentiation, amino acid transporters and apoptosis was investigated using real-time PCR, immunoblotting and caspase activity assays. The following significant increases were observed in STAT3 siRNA-treated cells compared with NC siRNA-treated cells: CGB mRNA and β-hCG protein (Fig. 7A); TP53 mRNA and TP53 protein (Fig. 7B); and caspases 3 and 8 mRNA activity (Fig. 7C).

In contrast, mRNA and protein expressions of the amino acid transporter SNAT2 were significantly decreased in STAT3 siRNA-treated cells compared with NC siRNA transfected cells (Fig. 7D).
Among members of the STAT family, STAT3 has garnered particular interest due to its role in development and cancer. Constitutive activation of STAT3 has been reported in various tumours and cell lines, which makes STAT3 an attractive molecular target in cancer therapy (Aggarwal et al. 2009). In several mouse malignancies, Stat3, the murine homolog of human STAT3, has been identified as an important regulator of genes that are central to cellular survival, proliferation and apoptosis (Johnston & Grandis 2011). Stat3 plays an important role in early embryogenesis by fostering the metabolic exchange between the embryo and placenta (Garcia et al. 2007, Fitzgerald et al. 2010). The biological effects of Stat3 have also been evaluated by targeted gene ablation in transgenic mice (Horvath 2000, Jiang et al. 2009). Targeted deletion of Stat3 led to early embryonic lethality, whereas targeted ablation of all other Stat family genes produced viable mice with limited phenotypes (Takeda et al. 1997).

Chan et al. (2008) had previously reported the localisation of pSTAT3 protein in first-trimester placentae in both cytoplasm and nuclei of the ST, CTB and villous intermediate trophoblasts, though an expression profile was undetectable at term. However, in this study, we demonstrated the presence of immunoreactive total STAT3 in the nuclei of the ST in both FGR and control third-trimester placentae.

Discussion

Alterations in fetal development and growth have been associated with life-long adverse health sequelae. As fetal growth and placental development are closely linked, a comprehensive understanding of the molecular regulation of feto-placental growth will bring us closer to understanding the mechanisms underlying altered fetal growth.

In this study, we report that genes in the JAK–STAT pathway are differentially expressed in placentae from idiopathic FGR pregnancies compared with those from gestation-matched control pregnancies. Specifically, using a JAK–STAT signalling pathway PCR array, we found that STAT3, STAT2 and STAT5B were expressed at significantly lower levels in FGR placentae compared with gestation-matched controls. STAT3 had the greatest decrease in relative mRNA levels in FGR compared with control placentae. The strength of our study reported herein was that the cohort of placentae from idiopathic FGR was carefully selected using strict clinical criteria indicative of placental insufficiency and underlying pathology (Salafia 1997, Regnault et al. 2002, Chaddha et al. 2004, Garcia et al. 2007). All the FGR study cases had at least two of the three clinical selection criteria diagnosed on ultrasound and comprised cases from the severe end of the FGR spectrum. This ensured that constitutionally small-for-gestational-age infants, who were below the 10th percentile of birth weight but were otherwise normal, were excluded from the study.

Figure 5 (A) Relative quantitation of STAT3 mRNA normalised to 18S rRNA gene was performed in BeWo cells treated with either 10 μM forskolin (FSK) or DMSO vehicle control. (B) STAT3 protein in FSK-induced BeWo cells. A representative immunoblot of total STAT3 (79 kDa) and the loading control tubulin (49 kDa) is depicted. (C) Semi-quantification of STAT3 protein normalised to tubulin in BeWo cells cultured with FSK compared with DMSO control shown. The asterisk (*) value denotes significantly increased levels for total STAT3 protein in BeWo cells transfected with either 10 μM forskolin or vehicle DMSO control. (D) Immunoblot of total STAT3 (79 kDa) and the loading control tubulin (49 kDa) is depicted. (E) Semi-quantitative analysis of STAT3 protein in BeWo cells cultured with NC and/or STAT3 siRNA. Gene expression was determined using the 2−ΔΔCT method (Livak & Schmittgen 2001). Data are expressed as mean ± S.E.M. *P<0.05, Mann–Whitney U test. (B) Immunoblot of STAT3 protein in BeWo cells transfected with STAT3 siRNA. Immunoblotting of STAT3 protein in BeWo cells cultured with NC and/or STAT3 siRNA as described in ‘Materials and methods’ section. A representative immunoblot of total STAT3 (79 kDa) and the loading control tubulin (49 kDa) is depicted. (C) Semi-quantitative analysis of STAT3 protein in BeWo cells transfected with STAT3 siRNA was normalised to tubulin. The asterisk (*) value denotes significantly decreased levels of total STAT3 protein in STAT3 siRNA-treated cells compared with NC (P<0.05). Each experimental condition within an experiment was performed at least in duplicate, and all cell culture (passages 42–44) experiments were repeated at least on three independent occasions.
Furthermore, we have also demonstrated the presence of STAT3 mRNA and protein in primary villous CTBs isolated from both first-trimester and term placentae. The ST layer plays a major role throughout pregnancy. The ST layer is the site for many important placental functions, including ion and nutrient exchange and the synthesis of steroid and peptide hormones required for fetal growth and development (Pidoux et al. 2012, 2014). In this study, we used primary cells isolated from both first- and third-trimester placentae to determine the effect of spontaneous in vitro differentiation on STAT3 expression. We found that in vitro spontaneous differentiation of trophoblasts was associated with increased STAT3 expression, suggesting STAT3 may play a significant role in villous trophoblast differentiation. A syncytiotrophoblast that functions efficiently requires both differentiation and fusion of trophoblasts to be at optimum levels. Thus, the role of STAT3 in the regulation of trophoblast fusion will also be of interest in future studies.

ST formation can be reproduced in vitro using different models (Fitzgerald et al. 2010, Orendi et al. 2010, Pidoux et al. 2012). In this study, we used the human chorioncarcinoma BeWo cell line as described previously (Orendi et al. 2010) to study the effect of villous trophoblast differentiation on STAT3 expression. BeWo cells show a low spontaneous fusion rate, which can be increased with FSK treatment (Wice et al. 1990). We found that FSK treatment significantly increased STAT3 expression in BeWo cells. Loss-of-function studies, using STAT3 siRNA in FSK-treated BeWo cells demonstrated that STAT3 is involved in BeWo cell differentiation, as indicated by a significant increase in β-hCG gene expression and secretion. Worthy of note is that a recent study has implicated the JAK–STAT pathway in leukaemia inhibitory factor regulation of BeWo cell differentiation (Leduc et al. 2012). These findings have potential biological and clinical significance. Placental chorionic villi from idiopathic FGR pregnancies show up-regulated expressions of CGB/β-hCG (Chui et al. 2012). Decreased expression of STAT3 in FGR could prematurely deplete the pool of proliferating CTBs by favouring differentiation. This may be suggested by changes in the ST layer that reflect increased terminal differentiation, such as increased ST shedding and apoptosis; increased ST shedding and apoptosis are hallmarks of the FGR-affected placenta (Huppertz et al. 1998).

Apoptosis is an integral component of villous CTB trophoblast turnover and ST formation (Huppertz et al. 1998, 2006). Indeed, differentiating CTBs that are destined to fuse with the syncytiotrophoblast express multiple apoptotic protein markers (Kaufmann et al. 1987, Huppertz et al. 1998, 2006, Benirschke 2006). Apoptosis and differentiation occur continually throughout gestation and these processes are important for the turnover of the trophoblast bi-layer (i.e. the CTB and ST layers) until term (Huppertz et al. 2006). However, the molecular pathways leading to differentiation and apoptosis of the ST are only partially understood. Targeted STAT3 inactivation in BeWo cells

**Figure 7** (A) Relative quantitation of CGB mRNA normalised to 18S rRNA was performed in BeWo cells treated with NC and/or STAT3 siRNA. β-hCG production in BeWo cells transfected with STAT3 siRNA. The protein level of β-hCG secreted in media was determined by ELISA. Asterisk (*) denotes significance (P<0.05, n=3, Mann–Whitney U test). (B) Real-time PCR analysis of TPS3 in BeWo cells transfected with NC or STAT3 siRNA. Data are expressed as mean ± S.E.M. *P<0.05, Mann–Whitney U test. A representative immunoblot of total TPS3 (55 kDa) and the loading control tubulin (49 kDa) is depicted. Semi-quantitative analysis of TPS3 protein in BeWo cells transfected with NC or STAT3 siRNA was normalised to tubulin. Data are expressed as mean ± S.E.M. *P<0.05, Mann–Whitney U test. Caspase activity assays were performed in BeWo cells cultured with NC and/or STAT3 siRNA. Data are expressed as mean ± S.E.M. *P<0.05, Mann–Whitney U test. Caspase activity assays were performed in BeWo cells cultured with NC and/or STAT3 siRNA as described in ‘Materials and methods’ section. The asterisk (*) value denotes significantly increased levels of caspases 3 and 8 activity in STAT3 siRNA-treated cells compared with NC (P<0.05). (D) Real-time PCR analysis of SNAT2 in BeWo cells transfected with NC or STAT3 siRNA. Statistical comparisons were performed using the Mann–Whitney U test. A representative immunoblot of total SNAT2 (59 kDa) and the loading control tubulin (49 kDa) is depicted. Semi-quantitative analysis of SNAT2 protein in BeWo cells transfected with STAT3 siRNA was normalised to tubulin. The asterisk (*) value denotes significantly decreased levels of SNAT2 protein in STAT3 siRNA-treated cells compared with NC (P<0.05).
resulted in increased apoptosis, as demonstrated by the increased TP53 expression. Furthermore, we have demonstrated increased relative mRNA levels and protein activities of caspases 3 and 8 in STAT3 siRNA-treated BeWo cells. These results are consistent with previous studies, where villous trophoblasts from FGR placentae were associated with increased expression of TP53 and caspase activity (Endo et al. 2005, Sharp et al. 2010). Our observations collectively support a key role for STAT3 in the regulation of differentiation and apoptosis in trophoblasts.

Fetal growth is dependent on nutrient availability, which in turn is related to the capacity of the placenta to transport these nutrients. Previous studies have demonstrated that the placental transport of amino acids is reduced in human FGR fetuses, and the activity of placental amino transporters, SNAT2, is also reduced in placentae obtained from FGR pregnancies when compared with uncomplicated pregnancies (Glazier et al. 2005, Sharp et al. 2014). STAT3 regulation on placental amino acid transport was demonstrated in chorionic villous explants (Jones et al. 2009, 2010). In this study, we have demonstrated that STAT3 is necessary for SNAT2 relative mRNA and protein expression in STAT3 inactivated cultured BeWo cells. Collectively, these data suggest that lower STAT3 expression observed in the FGR placentae may contribute, directly or indirectly, to the development of abnormal fetal growth via mis-regulation of nutrient transporters in the placenta.

In conclusion, STAT3 is lower in placentae from pregnancies affected by idiopathic FGR when compared with gestation-matched controls. Furthermore, our functional studies suggest that STAT3 may have several potentially important roles in the normal and pathological development of the human placenta. There may be significant deleterious consequences of reduced STAT3 levels leading to premature differentiation and apoptosis, thereby decreasing placental amino acid transporters in FGR. Although the origins of altered feto-placental growth in FGR are likely to be multifactorial and controlled at the transcriptional level, how such factors orchestrate and modify placental function and regulate fetal growth remains yet to be established.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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