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 fetoplacental 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 placentae may contribute to abnormal trophoblast function in idiopathic FGR-affected pregnancies.

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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 syncytium called the 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 fetoplacental 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 fetoplacental growth was investigated using an *in vitro* cell model.

Materials and methods

Patient details and tissue sampling

Placentae 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 >95 th 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.

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

Results are expressed as mean $\pm$ s.d. or frequency (%). * $P<0.05$.

Table 2 Clinical characteristics of FGR pregnancies included in this study.

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

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 infarcts.

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 $\mu\text{g}/\text{ml}$ 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 NH_4Cl , 10 mM NaHCO_3 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 $\mu\text{g}/\text{ml}$ streptomycin in 5% CO_2 humidified atmosphere at 37°C . CTB purity was confirmed by the proportion of CK7-positive trophoblast cells in the culture, which was $95 \pm 3.14\%$ ($n=3$) (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 $\mu\text{g}/\text{ml}$ streptomycin (Invitrogen) at 37°C , 5% CO_2 and 95% air in a humidified chamber.

RNA extraction and cDNA preparation

Total RNA from placental tissues and cultured cells was extracted using the RNeasy 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 house-keeping 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 *18S* rRNA (VIC-labeled Endogenous control, Applied Biosystems) was calculated according to the $2^{-\Delta\Delta CT}$ 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-CK7 (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 488, Qiagen).

Caspase activity assay

Caspases 3 and 8 activity was measured in BeWo cells using the ApoAlert Caspase Colorimetric assay kits specific for caspases 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 (*p*NA) 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

Figure 1 Candidate genes identified from JAK–STAT signalling array: gene expression profiling in FGR ($n=26$) and control placentae ($n=27$) was performed using The ‘JAK–STAT signalling pathway’ TaqMan PCR array as described in the ‘Materials and methods’ section. Gene expression (mRNA) profiling compared with the controls. Y-axis denotes ‘% mRNA decrease in FGR relative to controls’.

cell lysates by the addition of 50 μ M caspase 3 or 8 substrate (DEVD-*p*NA) in the presence or absence of caspase 3 or 8 inhibitor DEVD-fmk, all provided in the assay kit. Chromogen 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 $\pm$ S.E.M. Either the χ^2 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 p*STAT3* (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 p*STAT3* were significantly decreased in FGR-affected placentae compared with controls (Fig. 2C). However, p*STAT3* relative total *STAT3* was not significantly different in both FGR and control placentae (data not shown).

Figure 2 (A) *STAT3* mRNA relative to the house-keeping gene *18S* rRNA in placentae from FGR ($n=26$) and control ($n=27$) was determined using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001). Data are expressed as mean $\pm$ S.E.M. * $P < 0.05$, Mann–Whitney *U* test. (B) Immunoblot of *STAT3* protein: a representative immunoblot for total *STAT3* (79 kDa), phosphorylated *STAT3* (p*STAT3*) at 86 kDa and the loading control β -actin (40 kDa) is depicted. (C) Semi-quantitative analysis of *STAT3* protein: the asterisk (*) value denotes significantly decreased levels for both total *STAT3* and p*STAT3* protein in FGR-affected placentae compared with controls (* $P < 0.05$, ** $P < 0.01$ $n=12$ in each group).

Figure 3 Immunohistochemical localisation of STAT3 protein. Representative sections stained with the STAT3 antibody in a third-trimester control placenta and a third-trimester FGR placenta are shown in A and B respectively. CK7, which is immunoreactive on villous cytotrophoblast and syncytiotrophoblast (ST) in the villi, was used as a positive control (C). Sections stained with mouse IgG were used as negative controls (D). Qualitative analysis shows nuclear expression of STAT3 antigen in the ST in both control (A) and FGR-affected placenta (B).

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 placentae 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 placentae.

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).

Figure 4 (A) Relative quantitation of STAT3 mRNA normalised to 18S rRNA gene was performed in isolated trophoblasts from first-trimester placentae ($n=12$) and term placentae ($n=10$). (B) Immunoblot of STAT3 protein in first-trimester villous cytotrophoblasts. A representative immunoblot for total STAT3 (79 kDa) and the loading control tubulin (49 kDa) is depicted. (C) Semi-quantitation of STAT3 protein normalised to tubulin in cultured CTB from first-trimester and term placentae is shown. The asterisk (*) value denotes significantly increased levels for total STAT3 protein in *in vitro* differentiated ST compared with CTB ($P<0.05$).

Figure 5 (A) Relative quantitation of *STAT3* mRNA normalised to *18S* rRNA gene was performed in BeWo cells treated with either 10 μ M 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-quantitation of *STAT3* protein normalised to tubulin in BeWo cells cultured with FSK or vehicle DMSO control is shown. The asterisk (*) value denotes significantly increased levels for total *STAT3* protein in BeWo cells cultured with FSK compared with DMSO control ($P < 0.05$). 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.

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.

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 p*STAT3* 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.

Figure 6 (A) Relative quantitation of *STAT3* mRNA normalised to *18S* rRNA gene was performed in BeWo cells treated with NC or *STAT3* siRNA. Gene expression was determined using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001). Data are expressed as mean $\pm$ 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 51 and 52) experiments were repeated at least on three independent occasions.

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 ($P < 0.005$, $n = 3$, Mann–Whitney *U* test). (B) Real-time PCR analysis of *TP53* in BeWo cells transfected with NC or *STAT3* siRNA. Data are expressed as mean $\pm$ S.E.M. $*P < 0.05$, Mann–Whitney *U* test. A representative immunoblot of total *TP53* (55 kDa) and the loading control tubulin (49 kDa) is depicted. Semi-quantitative analysis of *TP53* protein in BeWo cells transfected with NC or *STAT3* siRNA was normalised to tubulin. Data are expressed as mean $\pm$ S.E.M. $*P < 0.05$, Mann–Whitney *U* test. (C) Real-time PCR analysis of caspases 3 and 8 in BeWo cells transfected with NC or *STAT3* siRNA. Data are expressed as mean $\pm$ 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 ($P < 0.05$).

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 syncytium 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 choriocarcinoma 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 syncytium 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

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.* 1996, Cetin 2003, Mando *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-mated 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 fetoplacental 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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