Homeobox gene transforming growth factor β-induced factor-1 (TGIF-1) is a regulator of villous trophoblast differentiation and its expression is increased in human idiopathic fetal growth restriction

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Submitted on December 5, 2011; resubmitted on May 21, 2013; accepted on June 1, 2013

Abstract: Abnormal trophoblast function is associated with human fetal growth restriction (FGR). Targeted disruption of homeobox gene transforming growth factor β-induced factor (TGIF-1) results in placental dysfunction in the mouse. The role of human TGIF-1 in placental cell function is unknown. The aims of this study were to determine the expression of TGIF-1 in human idiopathic FGR-affected placentae compared with gestation-matched controls (GMC), to elucidate the functional role of TGIF-1 in trophoblasts and to identify its downstream targets. Real-time PCR and immunoblotting revealed that TGIF-1 mRNA and protein expression was significantly increased in FGR-affected placentae compared with GMC (n = 25 in each group P, 0.05). Immunoreactive TGIF-1 was localized to the villous cytotrophoblasts, syncytiotrophoblast, microvascular endothelial cells and in scattered stromal cells in both FGR and GMC. TGIF-1 inactivation in BeWo cells using two independent siRNA resulted in significantly decreased mRNA and protein of trophoblast differentiation markers, human chorionic gonadotrophin (CGB/hCG), syncytin and 3β-hydroxysteroid dehydrogenase/3β-honest significant difference expression. Our data demonstrate that homeobox gene TGIF-1 is a potential up-stream regulator of trophoblast differentiation and the altered TGIF-1 expression may contribute to aberrant villous trophoblast differentiation in FGR.

Key words: gene expression / placenta / pregnancy / homeobox genes / fetal growth restriction

Introduction

Fetal growth restriction (FGR) is commonly defined as a birthweight of less than the 10th percentile for gestational age. Evidence of an additional pathology, such as oligohydramnios or asymmetric growth, allows clinicians to discriminate between FGR and small for gestation age babies that are otherwise healthy. FGR is associated with long-term health consequences in the adult such as an increased risk for metabolic syndrome (Gortner, 2007; Miller et al., 2008; Falo, 2009), as well as adverse neonatal neurodevelopmental outcome (Yaney and Marlow, 2004). The etiology of FGR is classified as maternal (e.g. hypertensive disorders, pre-gestational diabetes, malnutrition), fetal (e.g. genetic defects) or placental (e.g. infarcts) (Sankaran and Kyle 2009). Identifiable causes of FGR account for ~30% of cases, and the remainder are idiopathic. Idiopathic FGR is frequently associated with placental insufficiency. Typically, the FGR-affected placenta is smaller compared with gestation-matched control. The morphological differences include a decrease in villous number, diameter and surface area, as well as a decrease...
in arterial number, lumen size and branching (Krebs et al., 1996). At the cellular level, the FGR-affected placenta is characterized by reduced extravillous trophoblast proliferation, migration and invasion in the placental bed (Chen et al., 2002; Kaufmann et al., 2003) and increased apoptosis in cells of the trophoblast lineage (Ishihara et al., 2002; Levy et al., 2002; Murthi et al., 2005).

Trophoblast function is regulated in a spatially and temporally organized manner by specific transcription factors (Morrish et al., 1998; Janatpour et al., 1999). Studies using murine placental development have identified several transcription factors as regulators of trophoblast growth and differentiation (Cross et al., 1994; Cross, 2000; Rossant and Cross, 2001). Among these, members of a large family of transcription factors called the homeobox genes have been identified as master regulators of placental development as well as fetal growth and viability (Li and Behringer, 1998; Quinn et al., 1998; Morasso et al., 1999). For example, targeted deletion of homeobox genes Exstl and Hoxa3 and Hoxa13 resulted in reduced vascular branching in the labyrinthine layer of the placenta and severely growth-restricted embryos (Morasso et al., 1999; Fohn and Behringer, 2001; Shaut et al., 2008). Previous studies have reported the localization of Hlx (formerly known as Hb24), Dlx4 and Msx2 in the human placenta (Quinn et al., 2000). We have previously demonstrated that homeobox genes Hlx, Esx1l and Dlx4 are differentially expressed in human idiopathic FGR-affected pregnancies (Murrthi et al., 2006a, b, c) and more recently have shown that homeobox gene Hlx and Dlx3 are important regulators of trophoblast cell proliferation and differentiation, respectively (Rajaraman et al., 2007; Chui et al. 2011).

TGIF-1, also known as transforming growth factor β-induced factor or TG interacting factor, is a member of the TALE (three-amino-acid loop extension) subfamily of homeobox genes. The clinical relevance of TGIF-1 has been demonstrated in holoprosencephaly, a congenital malformation of the developing human forebrain (El-Jaick et al., 2007) and more recently, in myelogenous leukaemia, where TGIF-1 expression has been shown to correlate inversely with patient survival (Bertolino et al., 1995; Hamid et al., 2008). The biological significance of TGIF-1 is highlighted in murine placental development using knockout studies. Targeted deletion of Tgif-1 in the mice led to decreased labyrinth vascularity and size as well as decreased expression of the gap junction protein connexin 26, resulting in placental insufficiency and severely growth-restricted embryos (Bartholin et al., 2008). Previously, we have reported the expression of TGIF-1 in the feto-placental endothelial cells (ECs) of the term human placenta (Murthi et al., 2008). However, the role of TGIF-1 in the human placenta and in placental pathologies such as FGR is unknown. Therefore, the overall aim of this study was to determine the expression of TGIF-1 in the human idiopathic FGR-affected placenta compared with gestation-matched controls (GMC) and to determine its functional role in the human trophoblast differentiation.

Materials and Methods

Patient samples and tissue collection

Informed consent was obtained from all participating patients. First trimester placentae were obtained following therapeutic terminations of pregnancy, with ethical approval granted by the Southern Health Human Research and Ethics Committee (Melbourne, Victoria, Australia) and the Committee on Clinical Investigation, Broussais Hospital (Paris, France). Placentae from third-trimester FGR-affected pregnancies and GMC uncomplicated pregnancies were collected following approval from the Research and Ethics Committees of the Royal Women’s Hospital (Melbourne, Victoria, Australia). The clinical characteristics of the placentae used in this study are as previously published (Murthi et al., 2006a, b, c). Table I describes the clinical features of the FGR-affected pregnancies and GMC included in this study.

Cell culture

The choriocarcinoma-derived trophoblast cell line, BeWo, which was used as a model for villous trophoblasts was a kind gift from A/Prof Stephen Rogers (Department of Medicine, Royal Melbourne Hospital and University of Melbourne) and was maintained in RPMI-1640 medium supplemented with 10 mM sodium bicarbonate, 50 mg/ml streptomycin, 50 IU/ml penicillin and 10% fetal bovine serum. All reagents were purchased from Invitrogen Corporation, Carlsbad, CA, USA.

Isolation and purification of villous trophoblasts

Villus cytotrophoblasts (VCT) were prepared from first-trimester and term chonic villi (n = 12) as described previously (Handschu et al., 2007). Briefly, trophoblasts were purified by differential sequential trypsin digestion and the use of a discontinuous Percoll gradient (20–60%). Purity of VCT was characterized by positive staining for Cytokeratin-7, CK7 (95% positive cells) and by the observation of cell aggregates and syncytiotrophoblast (ST) from 48 to 72 h (Handschu et al., 2007). Purified VCT was plated at a density of 5 × 10⁵ cells per 30 mm on (Techno Plastic Products) tissue culture dishes and maintained in culture for either 24 h (VCT) or in vitro differentiated for 72 h (ST) in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

Oligonucleotide microarray

Oligonucleotide microarray analysis was performed in placental samples derived from pregnancies complicated by FGR versus healthy, gestational-age-matched controls, as well as primary term trophoblasts cultured in standard conditions for 48 h by Roh et al., (2005). Roh et al. (2005) did not report on the expression profile for homeobox genes, therefore, in this study we have used the homeobox gene data. TGIF-1 expression was analysed by the author (Y.S) using a U95A microarray gene-chip set (Affymetrix) as previously described (Budhraja et al., 2003).

Real-time PCR

Total RNA was extracted using an RNasy midi or microkit (Qiagen®, Valencia, CA, USA). Integrity and purity of RNA was confirmed by agarose gel electrophoresis. RNA was quantified using spectrophotometry. Total RNA (2 µg) was reverse transcribed with random primers (Invitrogen) and SuperScript III reverse transcriptase (Invitrogen) following manufacturer’s instructions. Real-time PCR was performed using FAM and VIC labelled TaqMan probes (TGIF-1 Hs00545233_m1; 3BHSD Hs00426435_m1; CGB Hs00361224_gH; syncytin Hs02341206_m1) on ABI PRISM 7500HT (Applied BioSystems, Foster City, CA, USA). PCR conditions included an activation cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The mRNA expression was normalized to housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or 18S RNA (Murthi et al., 2006a). Data were analysed according to the 2⁻ΔΔCT method (Schmittgen and Livak, 2008).

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**Immunofluorescence**

TGIF-1 localization in the first trimester placentae were also determined using immunofluorescence. Briefly, frozen placental tissue sections cut at 5 μm thickness was fixed in 100% methanol and blocked with 2% non-fat milk/phosphate buffered saline (PBS) for 1 h at room temperature. Tissue sections were then incubated overnight with primary (TGIF-1 H-1) mouse monoclonal IgG antibody (sc-17800, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or mouse monoclonal Ki67, a marker for proliferative cells or mouse monoclonal CK7, a marker of trophoblasts at a concentration of 0.02 μg/μl in 2% (w/v) non-fat milk prepared in PBS. Control sections were incubated with 0.02 μg/μl mouse IgG, 2% (w/v) non-fat milk in PBS (DAKO, Copenhagen, Denmark). Fluorescence detection was performed using Alexa Fluor® 488 Goat Anti-Mouse IgG (H + L) secondary antibody and mounted with 4′, 6-diamidino-2-phenylindole (DAKO, Copenhagen, Denmark) according to the manufacturers’ recommendations.

**Immunohistochemistry**

Paraffin-embedded 5 μm tissue sections were deparaffinized in xylene and dehydrated in graded alcohol or cells were grown on eight-chamber slides to 70% confluency and fixed in 4% paraformaldehyde for 15 min at room temperature. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide for 10 min at room temperature. Antigen retrieval was achieved in the tissue sections by enzymatic digestion for 8 min using 20 mg/ml Proteinase K (Ambion, Austin, TX, USA) in Tris buffer containing 1 M Tris – HCl, 0.5 M EDTA (pH 7.5). Non-specific protein binding was saturated with the blocking agent provided in the Histostain-Plus Broad Spectrum kit (Zymed Laboratories, South San Francisco, CA, USA). Tissue sections were then incubated overnight with primary (TGIF-1 H-1) mouse monoclonal IgG antibody (sc-17800, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a concentration of 0.02 μg/μl, in 2% (w/v) non-fat milk prepared in PBS. Control sections were incubated with 0.02 μg/μl mouse IgG (DAKO, Copenhagen, Denmark), 2% (w/v) non-fat milk in PBS. Immunoreactivity was verified by pre-absorption of the primary TGIF-1 antibody with recombinant TGIF-1 protein (Abcam, Cambridge, MA, USA) (recombinant protein: antibody, 1 : 1) at room temperature for 2 h before incubation with the tissue sections. Staining was visualized by incubating with the biotinylated secondary antibody and streptavidin-conjugated enzyme from the Histostain-Plus Broad Spectrum kit. Chromogenic detection was performed using 3-amino-9-ethylcarbazole (AEC, Zymed). Sections were mounted with 80% glycerol.

**Immunoblotting**

Total tissue or cellular protein was extracted using ice cold lysis RIPA buffer (Radio Immunoprecipitation Assay) containing 50 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate and 0.1% sodium dodecyl sulphate (SDS), supplemented with a complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentration was determined using a bicinecinic acid protein assay kit (Bio-Rad Laboratories, Richmond, CA, USA). Total protein (25 μg) from each sample was electrophoresed on a SDS–PAGE 10% Bis-Tris gel (Bio-Rad) under non-reducing conditions and transferred onto a nitrocellulose membrane (Pall Corporation, East Hills, NY, USA). Non-specific binding was blocked by incubation for 1 h at room temperature with 5% (w/v) non-fat milk in TBS-T (Tris-buffered saline – Tween; 130 mM NaCl, 20 mM Tris – HCl, pH 7.6 and 0.01% Tween 20). Blots were incubated overnight at 4°C with mouse monoclonal TGIF-1 antibody (0.02 μg/μl, sc-17800), goat polyclonal β3-honest significant difference (β3HSD, 0.01 μg/ml, ab48018) and rabbit polyclonal syncytin (0.01 μg/ml, sc-50369) in 2% (w/v) non-fat milk-TBST. The membranes were washed in TBST and incubated for 1 h with horse-radish peroxidase conjugated goat anti-mouse or goat anti-rabbit or rabbit anti-goat IgG ([1 ng/μl] Zymed) at room temperature. Immunoreactive protein was visualized utilizing the enhanced chemiluminescence (GE Healthcare, Piscataway, NJ, USA) and the luminescence detector (LAS-1000; Fujifilm, Tokyo, Japan). To control for loading efficiency, membranes were re-probed with rabbit anti-human GAPDH housekeeping protein (1.25 ng/μl) (Imgenex, San Diego, CA, USA), followed by incubation with horse-radish peroxidase conjugated

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**Table 1 Clinical characteristics of samples included in the study.**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>FGR (n = 25)</th>
<th>GMC (n = 25)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestation age (mean ± SD)</td>
<td>34.4 ± 6.5</td>
<td>35.8 ± 6.6</td>
<td>P = 0.25</td>
</tr>
<tr>
<td>Maternal age (mean ± SD)</td>
<td>33.2 ± 5.7</td>
<td>31.9 ± 6.5</td>
<td>P = 0.04</td>
</tr>
<tr>
<td>Placental weight (g)</td>
<td>409.3 ± 110.3</td>
<td>525.0 ± 148.2</td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td>Parity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primaparous</td>
<td>12</td>
<td>10</td>
<td>P = 0.56</td>
</tr>
<tr>
<td>Multiparous</td>
<td>13</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Mode of delivery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaginal delivery</td>
<td>10</td>
<td>6</td>
<td>P = 0.35</td>
</tr>
<tr>
<td>Cesar in labour</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Cesar not in labour</td>
<td>12</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>New born characteristics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>8</td>
<td>12</td>
<td>P = 0.25</td>
</tr>
<tr>
<td>Female</td>
<td>17</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Birthweight (mean ± SD)</td>
<td>2051.4 ± 637.0</td>
<td>2603.8 ± 857.0</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>10–90%</td>
<td>0</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>5–10%</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5% centile</td>
<td>14</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
goat anti-rabbit IgG (1.5 ng/µl) (Zymed). Immunoreactive protein was semi-quantitated using ImageQuant software (Molecular Dynamics, CA, USA).

Transfection
Gene inactivation was performed using two independent pre-validated TGIF-1-specific siRNAs obtained from Applied Biosystems (TGIF-1 siRNA-1: S14081; TGIF-1 siRNA-2: S14082). Primary and cultured trophoblast cells were grown in supplemented media (2 × 10^5 cells/well in 6-well plates and 5 × 10^6 cells/well in 24-well plates) and transfected with individual TGIF-1 siRNAs at a final concentration of 80 µM using RNAiFect transfection reagent (Qiagen, Australia). Briefly, the siRNA–RNAiFect suspension prepared at a ratio of 1:6 was added drop-wise to cells and incubated at 37°C for 72 h. Non-specific siRNA control (NC) 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 any known mammalian gene.

Trophoblast differentiation
In isolated trophoblasts
Trophoblast differentiation was determined by in vitro differentiation of primary first trimester and term VCT into ST, when maintained in culture for either 24 or 72 h, respectively. Briefly, purified VCT were transfected with TGIF-1 siRNA1 and/or siRNA2, and NC and maintained in culture for either 24 h or in vitro differentiated into ST for 72 h, respectively. Mononuclear VCT in 24 and 72 h cultures were counted using light microscopy.

In cultured BeWo cells
TGIF-1 mRNA as well as mRNA expression of trophoblast differentiation markers such as 3β-hydroxysteroid dehydrogenase (3β-HSD), syncytin and CGB was determined by real-time PCR. Briefly, BeWo cells were seeded on six-well plates (1 × 10^6 cells/well). The cells were serum starved for 24 h in the presence of cultured medium supplemented with 0.5% BSA before treating with 100 µM forskolin (Sigma-Aldrich, Bornem, Belgium) as described by Al-Nasiry et al. (2006) or vehicle, followed by a further incubation for 72 h at 37°C to allow syncytialization. To assess the effect of TGIF-1 inactivation on cell differentiation, BeWo cells treated with forskolin (100 µM) were transfected with either TGIF-1 siRNA1 and/or siRNA2, and NC for 72 h following which CGB, syncytin and 3β-HSD were determined by real-time PCR.

β-hCG protein assay
For determination of β-hCG protein levels, an enzyme-linked immunosorbent assay (ELISA, Alpha Diagnostic International, Australia) was performed following the manufacturer’s instructions using conditioned media collected from cell culture treatments as previously described (Chui et al., 2011). The minimum concentration of human hCG detected using this assay was 1.5 IU/ml.

Data analysis
Statistical analysis was performed using Graph Pad Prism program (GraphPad software, Version 5.01, Inc., San Diego, CA, USA). Statistical differences were calculated using one way ANOVA followed by Tukey’s multiple comparisons test.

### Table II Clinical criteria of the FGR-affected pregnancies included in this study.

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW &lt; 10th centile</td>
<td>25/25 (100%)</td>
</tr>
<tr>
<td>Abnormal umbilical artery Doppler velocimetry</td>
<td></td>
</tr>
<tr>
<td>Elevated</td>
<td>7/25</td>
</tr>
<tr>
<td>Reversed</td>
<td>6/25</td>
</tr>
<tr>
<td>Absent</td>
<td>8/25</td>
</tr>
<tr>
<td>Not recorded for Doppler velocimetry</td>
<td>4/25</td>
</tr>
<tr>
<td>Asymmetric growth</td>
<td></td>
</tr>
<tr>
<td>Head-to-abdominal circumference (HC:AC) &gt; 1.2</td>
<td>21/25</td>
</tr>
<tr>
<td>Not recorded for HC:AC</td>
<td>4/25</td>
</tr>
<tr>
<td>Oligohydramnios</td>
<td></td>
</tr>
<tr>
<td>Amniotic fluid index (AFI) &lt; 7</td>
<td>18/25</td>
</tr>
<tr>
<td>AFI &gt; 7</td>
<td>7/25</td>
</tr>
</tbody>
</table>

All FGR-affected pregnancies included in this study met with the first criterion for birthweight (BW) less than 10th centile and at least two of the other ultrasound determined selection criteria. 17/25 FGR-affected pregnancies met with all three ultrasound determined selection criteria.

### Figure 1 TGIF-1 mRNA and protein expression in normal and FGR-affected placentae. (A) Relative quantification of TGIF-1 mRNA expression of FGR-affected placentae (n = 25) and GMC placentae (n = 25) as determined by real-time PCR. TGIF-1 mRNA expression was normalized to housekeeping gene GAPDH. Data were analysed according to the 2-ΔΔCT method. Data represent the mean ± SEM, *P < 0.05, students t-test. (B) Immunoblot for TGIF-1. Representative immunoblot for TGIF-1 protein (35 kDa) in GMC (n = 6, 37 to 41 weeks) and in FGR-affected placental protein (n = 6, 37 to 41 weeks). Bottom: immunoblot for GAPDH protein expression. (C) Semi-quantification of TGIF-1 protein. Densitometric analysis of the TGIF-1 immunoreactive protein in pooled FGR and GMC is depicted in (C). Data are expressed as relative arbitrary densitometric units (TGIF-1 protein expression corrected for GAPDH protein expression). Values are means ± SEM, *P < 0.05, students t-test.
Figure 2 Immunohistochemical localization of TGIF-1 protein. Red-brown staining represents immune-positivity. (A) and (B) Representative section of first trimester placentae. Immunoreactive TGIF-1 protein was detected in the cytoplasm of EVT in the proximal cell columns of the anchoring villi and in ST. (C) Representative staining in the third-trimester control placentae (n = 6, 37–41 weeks) and (D) third-trimester FGR-affected placentae (n = 6, 37–41 weeks). In the third-trimester placentae, TGIF-1 immunoreactivity was observed in both residual CT, the ST and in ECs of the fetal capillaries. (E) Competition experiments where the primary antibody was pre-absorbed with TGIF-1 recombinant protein (F) Negative IgG control. Scale bars 20 μm. (G) A representative immunofluorescence image for TGIF-1 protein in first-trimester placental villi shows immunoreactive TGIF-1 protein in the mononuclear CT and in ST. (H) Ki67 localization in adjacent section shows the presence of proliferating CT in the first trimester placental villi. (I) Cytokeratin 7 (CK7) was used as a positive control.
Results

Table I describes the clinical features of the FGR-affected placentae and the GMC placentae included in this study. The samples used in this study were collected from a clinically well-defined cohort of FGR-affected pregnancies (Table II). Gestation age, maternal age, parity and mode of delivery were not significantly different between the two groups. However, the mean placental weight and birthweight were significantly lower in FGR compared with GMC (Table I, \( P < 0.005 \) and \( P < 0.05 \), respectively). Using these placental samples, previous studies in our laboratory have demonstrated consistent gene expression differences for homeobox genes (Murthi et al., 2006a, b, c).

**TGIF-1 mRNA and protein expression in FGR and GMC placentae**

Microarray expression data indicated that homeobox gene *TGIF-1* was among the 5% of transcripts exhibiting the highest expression increase in hypoxia, compared with standard culture conditions. Further validation of the microarray data for *TGIF-1* mRNA expression *in vivo* in FGR-affected placentae and GMC was performed by real-time PCR. As shown in Fig. 1A, a significant increase in *TGIF-1* mRNA expression relative to GAPDH was observed in FGR-affected placentae compared with GMC (\( n = 25 \) in each group, \( P < 0.05 \), t-test). *TGIF-1* mRNA expression showed no significant change with gestation age in both FGR-affected and GMC placentae (0.75 ± 0.03 pre-term control placentae versus 0.78 ± 0.12 term control placentae, \( P = 0.74 \)) and (1.24 ± 0.05 pre-term FGR-affected placentae versus 1.31 ± 0.1 term FGR-affected placentae, \( P = 0.65 \)). A representative immunoblot for TGIF-1 expression in FGR-affected placentae and GMC is shown in Fig. 1B. Immunoreactive TGIF-1 protein at 35 kDa was evident in all placental samples tested. As shown in Fig. 1C, semi-quantitative densitometry of the immunoreactive TGIF-1 protein normalized to GAPDH in the pooled FGR and GMC demonstrated a significant increase in TGIF-1 protein expression in FGR-affected placentae compared with GMC (\( n = 6 \) in each group, \( P < 0.05 \), t-test).

To determine the spatial distribution of TGIF-1, immunohistochemistry was performed as shown in Fig. 2A–F. Immunoreactive TGIF-1 protein was localized in the mononuclear cytotrophoblast (CT), ST of the floating villi, extravillous trophoblasts (EVT) in the proximal and distal column of anchoring villi and in few stromal cells in the first-trimester placentae (Fig. 2A and B). In the third-trimester GMC (Fig. 2C) and in FGR-affected placentae (Fig. 2D), immunoreactive TGIF-1 was also observed in the CT, ST, ECs of the fetal capillaries and in scattered stromal cells of the mesenchymal core. Figure 2E shows no immunoreactivity for TGIF-1 confirming the specificity of the TGIF antibody in a competition experiment, where the primary antibody was pre-absorbed with TGIF-1 recombinant protein. Figure 2F shows a representative negative control using an IgG isotype control antibody.

Immunofluorescence was performed to co-localize immunoreactive TGIF-1 with proliferating and differentiating trophoblasts in the floating and anchoring villi of the first-trimester placental tissues. As shown in Fig. 2G, in the first trimester placentae (\( n = 4 \)) immunoreactive TGIF-1 was observed in the nuclei of proliferating villous trophoblasts (CT) and ST of the floating villi and in the proliferating EVT in the proximal column of the anchoring villi. Adjacent section shows immunoreactivity for ki67 (Fig. 2H), a marker of proliferating trophoblasts. Cytokeratin 7 (CK7) was used as a positive control (Fig. 2I).

**TGIF-1 mRNA and protein expression in cultured trophoblasts**

Real-time PCR was used to determine *TGIF-1* mRNA expression in cultured villous trophoblasts derived from first trimester (\( n = 12 \)) and third-trimester placentae (\( n = 12 \)). A significantly increased *TGIF-1* mRNA expression was observed in *vitro* differentiated VCT into ST in both first trimester and third-trimester placentae (Fig. 3A) with a concomitant increase in βhCG in the culture medium (Fig. 3B).

**TGIF-1 inactivation in primary trophoblast cells**

Previously, we have confirmed the fusion ability and syncytialization of *in vitro* differentiated VCT into ST using specific markers of differentiation...
such as connexion 43 and syncytin (Frendo et al., 2000). Using the similar isolation protocol and experimental setup, in this study we have investigated the functional role of TGIF-1 in differentiation of VCT into ST in vitro. Representative images in Fig. 4 depict in vitro differentiated VCT into ST in the presence of TGIF-1 siRNA [sub-panels (B) and (C) for first-trimester (Panel 1) and term trophoblasts (Panel 2), respectively] compared with NC control (sub-panel (A) for first-trimester and term trophoblasts, respectively). As indicated by arrows, down-regulation of TGIF-1 resulted in smaller syncytium compared with the NC control.

**Figure 4** Inactivation of TGIF-1 mRNA was performed in isolated primary VCT using TGIF-1 siRNA1 and siRNA2. Representative phase contrast microscopic images show in vitro differentiated VCT into ST in the presence of TGIF-1 siRNA [sub-panels (B) and (C) for first-trimester (Panel 1) and term trophoblasts (Panel 2), respectively] compared with NC control (sub-panel (A) for first-trimester and term trophoblasts, respectively). As indicated by arrows, down-regulation of TGIF-1 resulted in smaller syncytium compared with the NC control.
TGIF protein following inactivation of TGIF-1 mRNA in trophoblast-derived cell line

TGIF-1 expression in the trophoblast-derived cell line was transiently inactivated using TGIF-1-specific siRNAs (S1 and S2). Non-specific siRNA (NC) was used as a control. As shown in Fig. 5A, a significant reduction in TGIF-1 mRNA was observed, when BeWo cells transfected with S1 (70% reduction) and S2 (65% reduction), compared with cells transfected with NC (n = 3, P < 0.005, ANOVA). This decrease in TGIF-1 mRNA expression was further verified at the protein level (Fig. 5B and C, P < 0.05, ANOVA).

The effect of TGIF-1 inactivation on BeWo cell differentiation was verified by inactivating TGIF-1 expression in the presence or absence of cyclic AMP activator forskolin, a known inducer of syncytialization. BeWo cells treated with forskolin showed significantly increased TGIF-1 mRNA compared with vehicle-treated control cells (2.87 ± 0.57, forskolin-treated BeWo versus 1.08 ± 0.21, untreated BeWo, n = 3, P < 0.05). To investigate the involvement of TGIF-1 in human trophoblast differentiation, TGIF-1 was inactivated in BeWo cells treated with forskolin and mRNA expression of CGB, syncytin and 3BHSD was analysed after 72 h. As shown in Fig. 6A, TGIF-1 silencing by S1 and S2 resulted in a significant reduction in CGB, syncytin and 3BHSD mRNA expression compared with cells transfected with NC (n = 3, P < 0.05, ANOVA). As shown in Fig. 6B, decreased immunoreactive 3BHSD and syncytin protein was observed following TGIF-1 transfection with siRNA1 (S1) and siRNA2 (S2) and forskolin treatment in BeWo cells compared with control (NC). Semi-quantitative analyses of immunoreactive 3BHSD and syncytin protein normalized to house-keeping protein GAPDH demonstrated a significantly decreased 3BHSD and syncytin protein in TGIF-1-transfected cells compared with NC (Fig. 6C). As shown in Fig. 6D, a significantly decreased concentration of hCG was observed in the culture medium from TGIF-1 siRNA1 and siRNA2 transfected cells compared with NC.

Discussion

Micro-array expression data suggested that TGIF-1 exhibited greater expression in hypoxic primary term trophoblasts compared with controls. The microarray data were corroborated by independent real-time PCR on FGR and control placental tissues. Our study is the first to demonstrate that homeobox gene TGIF-1 was significantly increased in the placenta of the human pregnancy disorder idiopathic FGR.

Targeted deletion of Tgf-1 in a mouse model resulted in growth-restricted phenotype in the embryo (Bartholin et al., 2008). However in humans, the real-time PCR and immunoblotting analyses revealed quantitative increase in TGIF-1 expression in human FGR-affected placentae compared with controls. Although the developmentally important genes, including homeobox genes, have often been extraordinarily conserved during evolution, there is increasing evidence for some rapidly evolving homeobox genes that may play important roles in speciation (Ting et al., 1998). We observed increased expression of TGIF-1 in FGR-affected placentae by real-time PCR and immunoblotting, with TGIF-1 detected in the nuclei of ST, CT cells and in the EC of third-trimester FGR-affected placentae and GMC. Furthermore, immunohistochemical analysis suggested that the quantitative differences in TGIF-1 expression observed between FGR and control placentae reflect changes in TGIF-1 expressing cell types in both groups.

FGR is characterized by impaired extravillous trophoblast migration and invasion into the maternal decidua and impaired trophoblast fusion and syncytialization (Kaufmann et al., 2003; Huppertz et al., 2006; Newhouse et al., 2007). In this study, we observed that TGIF-1 was localized in the cytoplasm of VCTs and ST, EC and few stromal cells of third-trimester FGR-affected placentae and GMC. The spatial distribution of TGIF-1 in FGR and control placentae suggest that the quantitative differences in TGIF-1 expression observed between FGR and control placentae reflect changes in TGIF-1 expressing cell types in both groups.

TGIF-1 is a critical regulator of both TGF-β and retinoic acid signalling (Massague and Wotton, 2000; Wotton et al., 1999, 2001; Wotton and Massague, 2001; Zhang et al., 2009), these two pathways have been extensively implicated in trophoblast function. TGF-β has been shown to regulate trophoblast proliferation, invasion and adhesion (Jones et al., 2006; Zhao et al., 2006). Whilst retinoic acid is involved in trophoblast...
proliferation, invasion, apoptosis (Tarrade et al., 2001) and is also a transcriptional regulator of key pregnancy-specific hormones such as hCG, the subunit of hCG (hCG-\(\alpha\)) in trophoblasts (Guibourdenche et al., 1998). Our results indicate that TGIF-1 may be a critical factor that mediates these processes.

Increasing evidence suggests that TGIF-1 affects cellular differentiation, although this is cell-type dependent. TGIF-1 inactivation resulted in reduced differentiation of myeloid leukemia-derived cell lines (Hamid and Brandt, 2009) and forced expression of TGIF-1 regulates retinal progenitor cells differentiation (Satoh and Watanabe, 2008). Earlier studies by Mi et al. (2000) demonstrated that BeWo cells undergo syncytialization in response to cAMP. The expression of specific molecular markers and the production of hCG and progesterone are some of the functional consequences of the process of syncytialization. We show that TGIF-1 mRNA expression is induced in VCT undergoing fusion, concomitant with increased expression of CGB. TGIF-1 silencing in BeWo cells treated with forskolin induced a significant reduction of CGB mRNA, suggesting that TGIF-1 may have a regulatory role in CGB expression. In agreement with the above observation, in vitro differentiation of VCT from FGR-affected pregnancies have been shown to have significantly higher levels of syncytialization and hormone secretion compared with trophoblasts derived from uncomplicated pregnancies (Newhouse et al., 2007). However, the underlying mechanisms of how TGIF-1 is involved in syncytialization remain elusive.

In summary, this study showed that TGIF-1 expression is increased in FGR-affected placentae compared with GMC and demonstrate that TGIF-1 is a regulator of trophoblast differentiation. The precise molecular mechanisms of how TGIF-1 regulates trophoblast functions leading to FGR warrants further investigation.

**Figure 6** The effect of TGIF-1 inactivation on BeWo cell differentiation was determined following treatment with forskolin. (A) CGB, syncytin and 3BHSD mRNA relative to 18S rRNA was determined following TGIF-1 siRNA transfection and forskolin treatment as determined by real-time PCR and calculated according to the \(2^{-\Delta\DeltaCT}\) method. Bars represent fold changes relative to control siRNA (NC) transfected cells. The results are the SEM of three independent experiments performed at least in duplicates \((n = 3, ^* P < 0.05, ^{**} P < 0.005,\) ANOVA). (B) A representative immunoblot to show decreased immunoreactive 3BHSD and syncytin protein following TGIF-1 transfection with siRNA1 (S1) and siRNA2 (S2) and forskolin treatment in BeWo cells compared with control (NC) \((n = 3\) experiments performed at least in duplicates\). (C) Semi-quantitative analyses of decreased immunoreactive 3BHSD and syncytin protein normalized to house-keeping protein GAPDH in TGIF-1-transfected cells compared with NC \((n = 3\) experiments performed at least in duplicates, \(^* P < 0.05,\) ANOVA). (D) Decreased concentration of hCG in the culture medium from TGIF-1 transfected cells compared with NC \((n = 3\) performed at least in duplicates, \(^* P < 0.05,\) ANOVA).
Acknowledgements

The authors wish to thank the consenting patients and the clinical and research midwives, Sue Nisbet and Sue Duggan, for the supply of term control placental tissues.

Authors’ roles

N.A.P. performed all the experiments with established cell lines and produced the initial draft of the paper. P.M. and A.B. performed all mRNA and immunoblotting for placental tissues and cell lines. P.M., T.F. and M.C. performed all the experiments with primary trophoblast cultures. Y.S. provided the microarray data. P.M., B.K. and U.M. assisted with the immunohistochemistry and immunofluorescence experiments and the production of images. R.J.K. and M.A. assisted with data analyses and performed the statistical analyses. S.P.B. and D.E.-B. participated in study design and paper review. All authors contributed to the editing of the paper. P.M. as a principal investigator, designed the experiments, supervised the technical work and produced the final draft of the paper.

Funding

Funding support for this work was provided from the Australian National Health and Medical Research Council (NHMRC project grant #509140) awarded to P.M. U.M. is supported by the Victorian Government’s Operational Infrastructure Program.

Conflict of interest

None declared.

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