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Homeobox gene *TGIF-1* is increased in placental endothelial cells of human fetal growth restriction.

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Short title: TGIF-1 in placental endothelial cells.

Abstract

Aberrant placental angiogenesis is associated with fetal growth restriction (FGR). In the mouse, targeted disruption of the homeobox gene, transforming growth β-induced factor (*Tgif-1*), which is also a transcription factor, causes defective placental vascularisation. Nevertheless, *TGIF-1*'s role in human placental angiogenesis is unclear. We have previously reported increased *TGIF-1* expression in human FGR placentae and demonstrated localisation of TGIF-1 protein in placental endothelial cells (ECs). However, its functional role remains to be investigated. In this study, we aimed to specifically compare *TGIF-1* mRNA expression in placental ECs isolated from human FGR-affected pregnancies with gestation-matched control pregnancies in two independent cohorts from Australia and Canada, and to identify the functional role of *TGIF-1* in placental angiogenesis using the human umbilical vein endothelial cell-derived cell line, SGHEC-7 and primary human umbilical vein ECs. Real-time PCR revealed that *TGIF-1* mRNA expression was significantly increased in ECs isolated from FGR-affected placentae compared with that of controls. The functional roles of TGIF-1 were determined in ECs following *TGIF-1* siRNA transfection. *TGIF-1* inactivation in ECs significantly reduced TGIF-1 at both the mRNA and protein levels, as well as the proliferative and invasive potential, but significantly increased the angiogenic potential. Using angiogenesis PCR screening arrays, we identified *ITGAV*, *NRP-1*, *ANPGT-1* and *ANPGT-2* as novel downstream targets of *TGIF-1*, following *TGIF-1* inactivation in ECs. Collectively, these results show that increased *TGIF-1* in FGR may regulate EC function through mediating the expression of angiogenic molecules and contribute to aberrant placental angiogenesis in FGR pregnancies.

Introduction

Placental angiogenesis is critical for maintaining the highly efficient transport system that facilitate the exchange of nutrients, oxygen and waste between the mother and the fetus (Reynolds and Redmer 2001). Endothelial cells (ECs) play significant roles in coordinating effective angiogenesis and aberrant EC function can lead to pregnancy pathologies such as fetal growth restriction (Bouis, et al. 2001, Kingdom, et al. 2000). Impaired placental angiogenesis is observed in FGR, with reduced tubule length and formation in placental ECs obtained from FGR-affected pregnancies (Su, et al. 2015). Placental angiogenesis is tightly controlled by numerous growth factors, cytokines and signalling pathways that collectively regulate expression of multiple genes through the activation of transcription factors (Hamik, et al. 2006, Latchman 1997).

Transcription factors that regulate angiogenesis include the family of homeobox genes (Gorski and Walsh 2000). Homeobox genes belong to a highly conserved family of transcription factors (Holland, et al. 2007), which control cell and organ differentiation throughout embryonic development (Yaron, et al. 2001) and have pleiotropic effects on cell proliferation, growth arrest and differentiation (Douville and Wigle 2007, Gorski and Walsh 2000). Mouse knock-out studies demonstrate the involvement of homeobox genes in regulating placental functions (Rossant and Cross 2001). Previous studies in our laboratory have identified that several homeobox genes including *DLX3, HOXB6, DLX4, MSX2, GAX* and *HLX1*, are expressed in ECs surrounding the fetal capillaries in the human placenta (Murthi, et al. 2007). We have also demonstrated the expression of several novel homeobox genes in placental ECs including the novel transforming growth β-induced factor (*TGIF-1)* homeobox gene at the mRNA level (Murthi, et al. 2008).

TGIF-1 is a negative regulator of the transforming growth factor beta (*TGF-β*) pathway (Faresse, et al. 2008), which is important for physiological processes such as cell proliferation, differentiation, apoptosis, early development and placental angiogenesis. Missense mutations in the *TGIF-1* gene can lead to holoprosencephaly, which affects cranial development (Hayhurst and McConnell 2003). In addition, mouse studies show that *Tgif-1* null embryos display a severely growth restricted phenotype associated with placental vascular defects (Bartholin, et al. 2008). Our recent study showed significantly increased TGIF-1 expression in FGR placental homogenates at both the mRNA and protein levels, with protein localisation of TGIF-1 in the endothelium lining the fetal capillaries (Pathirage, et al. 2013). However, the role of *TGIF-1* in human placental ECs is largely unknown.

In this study, we hypothesised that *TGIF-1* is an important regulator of placental angiogenesis. A previous microarray analysis on placental endothelial cells (PLEC) from FGR pregnancies performed in Toronto, Canada, demonstrated a trend for increased *TGIF-1* mRNA expression in the FGR PLEC samples as compared with that of the controls (Dunk, et al. 2012). In the current study using the two independent cohorts from Toronto, Canada and from samples collected in Melbourne, Australia, ECs isolated from FGR placentae and control placentae were further investigated and validated for TGIF-1 expression. We aimed to determine the level of *TGIF-1* mRNA expression in placental ECs isolated from FGR and gestation-matched control (GMC) pregnancies in two independent cohorts, and to investigate the functional role of TGIF-1 by gene inactivation in primary human umbilical vein endothelial cells (HUVECs) and the HUVEC-derived cell line, SGHEC-7 (Fickling, et al. 1992).

Materials and Methods

Placental endothelial cell (PLEC) isolation

PLECs were isolated and purified from two independent cohorts of placentae from Melbourne, Australia and Toronto, Canada as described previously (Dunk, et al. 2012). Placentae were collected from n=10 FGR and n=10 GMC patients in the Australian study arm and n=6 FGR and n=4 GMC patients in the Canadian study arm with written informed consent. Patient characteristics of the Australian samples are presented in Table 1. Using these placental samples, previous studies in our laboratory have shown consistent gene expression changes in the placental villi of FGR compared with gestation-matched control pregnancies (Murthi et al., 2006, Pathirage et al., 2013). FGR was defined as birthweight less than the 10th centile for gestational age according to Australian growth charts (Murthi et al., 2006) accompanied by two or more of the following features: abnormal umbilical artery Doppler flow velocimetry; oligohydramnios as determined by amniotic fluid index (AFI) of <7; asymmetric growth of the fetus as defined by a head circumference (HC) to abdominal circumference (AC) ratio >1.2. The exclusion criteria for both FGR and GMC pregnancies were multiple pregnancies, illicit drug dependency, maternal smoking, pre-eclampsia, prolonged rupture of the membranes, placental abruption, intrauterine viral infection, and fetal congenital anomalies. The Australian samples were collected with approval from The Royal Women's Hospital Human Research Ethics Committee (Project # 27/00) in Melbourne, Australia. All Canadian samples were collected by the Research Centre for Women's and Infants' Health BioBank Program (http://biobank.lunenfeld.ca/) with the approval of the Research Ethics Board at Mount Sinai Hospital (04-0018-U) in Toronto, Canada and the FGR and matched control samples were previously characterised by Dunk *et al.* (Dunk, et al. 2012). All FGR cases in the Canadian cohort were presented with abnormal umbilical Doppler velocimetry. Freshly isolated cells were used for gene expression analyses.

HUVEC isolation

Human umbilical vein endothelial cells (HUVECs) were freshly isolated from uncomplicated term pregnancies (n=12) as previously described (Murthi et al. 2008). Briefly, cells were cultured and maintained in M199 tissue culture medium (Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100µg/mL streptomycin and maintained in 5%CO_2/95% air. For gene expression and functional analyses HUVEC cells from passage 2 were used.

SYBR Green qPCR analysis on Canadian cohort

SYBR green q-PCR was performed as previously described (Dunk, et al. 2012). Breifly, 1µg of total RNA from isolated PLECs was reverse transcribed using the I Script cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). Approximately 10 ng of each cDNA was then subjected to Real-Time PCR using primers specific for *TGIF* (Forward 5'-TCGGTGTGGGACAAAACACA-3' and Reverse 5'-TCGGTGTGGGACAAAACACA-3') and housekeeping genes SDHA and YWHAZ (Dunk, et al. 2012). Real-time PCR was performed in a white 96-well plate in a CFX96 real-time PCR system (Bio-Rad Laboratories). The run protocol was as follows: heat activation of Taq and denaturation 95°C for 2min, and 40 cycles of amplification at 95°C for 10s and 60°C for 30s. The mRNA level of the gene of interest from each sample was normalised to the geometric mean of the *YWHAZ* and *SDHA* mRNA expression level and data were analysed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

RNA extraction, cDNA synthesis, real-time PCR of Australian cohort and cell lysates

Total cellular RNA was extracted from the Australian cohort of PLECs and EC lysates using the PureLink RNA Mini kit (Thermo Fisher Scientific Corp, Waltham, MA, USA) following

144 the manufacturer's instructions. RNA yield, purity and integrity were determined by
145 visualising 28S and 18S ribosomal RNA following 1% (w/v) agarose gel electrophoresis.
146 First-strand cDNA was prepared as previously described (Rajaraman, et al. 2010). Real-time
147 PCR was performed using FAM labelled Taqman® probes (*ANGPT-1* Hs00375823_m1;
148 *ANGPT-2* Hs00169867_m1; *ITGAV* Hs00233808_m1; *NRP-1* Hs00826129_m1 and *TGIF-1*
149 Hs00545233_m1) and Eukaryotic *18S rRNA* Endogenous Control (VIC/MGB Probe, Primer
150 Limited) as a housekeeping gene in an ABI PRISM 7500HT thermocycler (Applied
151 Biosystems, Carlsbad, CA, USA). Approximately 12.5 ng/µL of cDNA was amplified in a
152 total reaction volume of 20 µL. PCR conditions included an activation cycle of 50°C for 2
153 minutes and 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for
154 1 minute. Relative mRNA expression for each gene to the *18S rRNA* housekeeping gene was
155 determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).
156
157 *Cell culture*
158 The well characterised HUVEC-derived cell line, SGHEC-7, was cultured as previously
159 described (Fickling, et al. 1992). Briefly, cells were grown in a 1:1 mixture of RPMI 1640
160 and medium 199 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 1% (w/v)
161 L-glutamine, 100 IU/mL penicillin and 100 mg/mL streptomycin. Cells were maintained in
162 controlled, humidified conditions at 37°C in 5% CO_2 and 95% air.
163
164 *Immunocytochemistry*
165 Immunocytochemistry was performed using the Zymed® Histostain-plus Broad Spectrum kit
166 (Thermo Fisher Scientific Corp) as previously described (Lepparanta, et al. 2010). Mouse
167 anti-TGIF-1 monoclonal IgG (0.02 µg/µL; Santa Cruz Biotechnology Inc., Santa Cruz, CA,
168 USA) in 2% (w/v) non-fat milk in phosphate buffered saline was used to detect TGIF-1

169 protein. Mouse IgG2b (Dako, Glostrup, Denmark) was used as a negative control. Colour
170 detection was performed using the Zymed® AEC chromogen kit (Thermo Fisher Scientific
171 Corp) and slides were mounted with 80% (v/v) glycerol. Cells were viewed with a Zeiss
172 Axioscope microscope and images were captured with a Zeiss Axiocam camera and analysed
173 using Axiovision Rel. 4.3 software (Carl Zeiss AG, Oberkochen, Germany).
174
175 *TGIF-1 inactivation in ECs*
176 Two independently validated siRNAs, *TGIF-1* siRNA-1 (S1) and *TGIF-1* siRNA-2 (S2) from
177 Life Technologies Corp. (Carlsbad, CA, USA), were used to silence *TGIF-1* expression in
178 ECs. *TGIF-1* oligonucleotides was diluted to 80 μM with RNAifect transfection reagent
179 (Qiagen, Hilden, Germany) added drop-wise to cells grown in 6 well plates and incubated for
180 72 hours in culture. AllStars Negative Control siRNA (Qiagen) that had no homology to any
181 known mammalian gene was used as a negative control (NC).
182
183 *Protein extraction and western immunoblotting*
184 Whole cell protein was extracted using radio immunoassay precipitation assay buffer
185 containing 50mM TrisHCl, 150mM NaCl, 1% (v/v) Trition-X-100, 1% (w/v) sodium
186 deoxycholate and 0.1% (w/v) sodium dodecyl sulphate supplemented with 1X protease and
187 1X phosphatase inhibitor cocktails (Roche, Basel, Switzerland). Immunoblotting was then
188 performed as previously described (Murthi, et al. 2006). Mouse anti- TGIF-1 monoclonal
189 IgG (0.02 μg/μL; Santa Cruz Biotechnology Inc.) and rabbit anti-GAPDH polyclonal IgG
190 (1.25 ng/μL; Imgenex Corp., San Diego, CA, USA) were used to detect TGIF-1 and GAPDH
191 protein respectively. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1
192 ng/μL; Thermo Fisher Scientific Corp) or HRP-conjugated goat anti-rabbit IgG (1.5 ng/μL,
193 Thermo Fisher Scientific Corp) were used as secondary antibodies. Immunoreactivity was

194 detected using an enhanced chemiliuminescence system (GE Healthcare, Little Chalfont, UK)
195 and the luminescence detector LAS-4000 image reader (Fujifilm Corp., Tokyo, Japan).
196 Immunoreactive protein bands were quantitated using the ImageJ software. Levels of TGIF-1
197 was normalised to that of GAPDH to control for protein loading.
198
199 *Cell proliferation assay*
200 Cell proliferation was assessed using the CellTiter 96® Aqueous One Cell Proliferation Assay
201 (Promega Corp., Madison, WI, USA) following the manufacturer's instructions. Briefly,
202 $2x10^4$ SGHEC-7 cells were transfected with either *TGIF-1* siRNA-1 or siRNA-2 and plated
203 in a 96-well plate in serum-free medium. After 24 hours of culture (72 hours post-
204 transfection) the cell proliferation assay was performed and the plate read at λ_{490}.
205
206 *Zymography*
207 The effect of *TGIF-1* siRNA inactivation on the activity of metalloproteinases was assessed
208 using gelatin-based zymography as described previously (Fitzsimmons, et al. 2007).
209 Dehydrated gels were scanned on an ImageScanner III densitometer (GE Healthcare) and
210 band intensities were quantified using the ImageQuant software provided with the instrument.
211
212 *Network formation assay*
213 To observe the effect of *TGIF-1* inactivation on the network formation ability of ECs, 24-
214 well plates were coated with a thin layer of undiluted growth factor reduced Matrigel™ (BD
215 Biosciences, San Jose, CA, USA). At 48 hours post transfection, $2x10^5$ cells were seeded
216 onto the pre-coated plates and incubated in complete basal medium for a further 24 hours as
217 previously described (Arnaoutova and Kleinman 2010). Images were taken every 4 hours

throughout the 24 hours using the Incucyte™ live cell imaging system (Essen Bioscience, Ann Arbor, MI, USA) at a magnification of 200X.

Angiogenesis array

The effect of *TGIF-1* silencing on angiogenic genes was determined using TaqMan® human angiogenesis signature arrays (Applied Biosystems), which consisted of 92 human genes involved in the regulation of angiogenesis. Gene profiling was used to identify the downstream target genes of *TGIF-1* and the methodology was carried out according to the manufacturer's instructions. Briefly, cDNA was prepared using an RT^2 First Strand kit and added to a TaqMan® Universal mastermix which contained the AmpliTaq Gold DNA polymerase and optimised buffer components (Applied Biosystems). House-keeping genes consisted of β-2-microglobulin (*B2M*), *18S* rRNA, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β-actin (*ACTB*). The quantification of relative gene expressions was performed on an ABI Prism 7700 Sequence Detector (Applied Biosystems) under the cycling parameters: 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and primer extension at 60°C for 1 minute. Data (C_t values) were analysed using the ABI Sequence Detector System software version 2.0 (Applied Biosystems) and the relative gene expression values, or fold changes, were calculated according to the manufacturer's protocols. Briefly, values from the SGHEC-7 control (NC siRNA) and treated plates (*TGIF-1*-siRNA) were calculated as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001), and normalised to the average C_t value of the house-keeping genes. Target genes of *TGIF-1* were identified by calculating the fold-change in gene expression levels for *TGIF-1* siRNA treated cells relative to the NC. Candidate genes that showed either a fold-change increase of >2 or a decrease of <2 in gene expression were identified. Data were analysed and compared with the NC transfected SGHEC-7 cells using Data Assist (Applied Biosystems).

Statistical Analysis

Data are shown as mean ± SEM of $n \geq 3$ independent experiments. Student's *t* tests, 2 X 2 contingency table with Fisher's Exact Test and ANOVA with Bonferroni's post-test were carried out using GraphPad Prism 5 (Graphpad Software Inc., La Jolla, CA, USA). A value of $p<0.05$ was considered to be statistically significant.

Results

Clinical characteristics

Table 1 describes the clinical characteristics of n=10 FGR and n=10 GMC patients, whose placentae were collected and used in the Australian arm of this study. As shown there were no significant differences in the gestational age, maternal age or infant sex between FGRs and controls. FGR cases showed significantly lower birth weights and lower placental weights. In addition, all FGR subjects had a birth weight below the 10th percentile for gestational age (Dobbins, et al. 2012) and either abnormal umbilical artery Doppler findings or oligohydramnios together with evidence of asymmetric growth (head circumference: abdominal circumference >95th centile). The Canadian FGR samples were associated with abnormal umbilical artery Doppler changes in all n=6 cases as previously described by Dunk *et al.* (Dunk, et al. 2012).

Increased TGIF-1 expression in FGR placentae

Real time-PCR was to used validate the initial microarray data which showed a trend towards increased expression in *TGIF-1* mRNA. *TGIF-1* mRNA expression was determined in two independent cohorts of FGR and control PLECs from Melbourne, Australia and Toronto, Canada. As shown in Figure 1, significant increases in *TGIF-1* mRNA of 2.4 fold and 1.5 fold were observed in PLECs isolated from FGR pregnancies compared with those from control pregnancies in the Australian (Figure 1A) and Canadian (Figure 1B) cohorts respectively.

TGIF-1 is expressed in HUVEC and in the SGHEC-7 cell line

Immunocytochemistry was used to demonstrate the expression and localisation of TGIF-1 protein in HUVECs and SGHEC-7 cells, which were used as the *in vitro* cell culture model.

As apparent in Figure 2A (TGIF), TGIF-1 protein localised to the cell nuclei. Substitution of the primary antibody with mouse IgG2b control showed no such immunoreactivity (Figure 2A, IgG control).

TGIF-1 silencing in HUVEC and in the SGHEC-7 cell line

Following transient siRNA transfection of HUVECS and SGHEC-7 cells for 72 hours, both *TGIF-1* specific siRNAs (denoted as S1 and S2) significantly decreased *TGIF-1* mRNA expression in comparison to the NC transfected cells (Figures 2B and 2C). This decrease in mRNA expression was further confirmed by examining the TGIF-1 protein expression in HUVECs SGHEC-7 cells post-transfection. As shown in Figure 2D, a decrease in immunoreactive TGIF-1 protein at 35kDa was evident in *TGIF-1* siRNA treated cells compared with that of NC treated cells, with no change in GAPDH house-keeping protein loading control. Densitometric analysis confirmed a significant decrease in the protein expression of TGIF-1 in both S1 and S2 compared with NC treated cells (Figures 2E and 2F).

TGIF-1 silencing decreases endothelial cell proliferation

Following the siRNA inactivation of *TGIF-1* in HUVECs and SGHEC-7cells, serum starved cells were assessed for their proliferative ability over 24 hours. As shown in Figures 3A and 3B, *TGIF-1* inactivation significantly decreased the proliferative potential of these cells compared with the NC**.**

TGIF-1 silencing increases network formation

The effect of *TGIF-1* inactivation on the angiogenic potential of HUVECs and SGHEC-7 cells was assessed by network formation assays. Angiogenic potential was determined by assessing branch points at the 4 hour time-point. Significantly increased angiogenic potential

was observed in HUVECs and SGHEC-7 cells transfected with either S1 or S2 compared with NC transfected cells (Figures 3C, 3D and 3E).

TGIF-1 silencing decreases SGHEC-7 invasive potential

The matrix metalloproteinase activities of MMP-2 and MMP-9 in SGHEC-7 cells were determined by gelatin zymography and used as proxies for invasive potential. Activities of MMP-2 and -9 were assessed in the culture medium collected from *TGIF-1* inactivated SGHEC-7 cells (S1 and S2) and compared with medium from NC transfected cells (Figures 3F, 3G and 3H). *TGIF-1* inactivation significantly decreased MMP-2 activity (Figure 3F) compared to the NC ($p<0.05$, ANOVA), but did not alter MMP-9 activity (Figure 3G). This was further confirmed in primary HUVEC following TGIF-1 inactivation using S1 siRNA compared to NC treated cells. MMP2 activity in S1 treated cells demonstrated a significant reduction (34%) in MMP2 activity (98.67 ± 1.7 (NC) vs. 33.67 ± 3.8 (S1), n=3, $p<0.05$) compared to NC, while there was no significant difference in MMP9 activity was observed following S1 treated cells compared to NC treated HUVEC cells (100.0 ± 2.5 (NC) vs. 87.67 ± 4.9 (S1), n=3, $p=0.09$).

Downstream targets of TGIF-1 in endothelial cells

The human angiogenesis array consisting of 92 human genes involved in the regulation of angiogenesis was utilised to identify potential downstream targets of *TGIF-1*. Following *TGIF-1* inactivation with siRNA in SGHEC-7 cells, 51 genes were up-regulated, while 19 genes were down-regulated. Changes in gene expression of four prioritised genes (*ITGAV*, *NRP-1*, *ANGPT-1* and *ANGPT-2*) were then validated with real-time PCR in HUVECs and SGHEC-7 cells (Figure 4). *ITGAV* mRNA and *NRP-1* mRNA were significantly increased in *TGIF-1* siRNA transfected HUVECs (Figures 4A and 4C), with a similar trend observed in

the SGHEC-7 cells (Figures 4B and 4D). Expression of *ANGPT-1* mRNA was significantly decreased in both *TGIF-1* inactivated HUVECs (Figure 4E) and SGHEC-7 cells (Figure 4F). In contrast to *ANGPT-1*, *ANGPT-2* mRNA was significantly increased in *TGIF-1* inactivated HUVECs (Figure 4G), although no significant change was observed in that of SGHEC-7 cells (Figure 4H).

330 **Discussion**

331 Homeobox genes are important in the regulation of numerous vascular cell processes such as
332 cell migration, invasion and proliferation (Douville and Wigle 2007). Previous studies in our
333 laboratory reported a range of novel placental homeobox genes expressed in both
334 microvascular and macrovascular ECs (Murthi, et al. 2008). One such example is homeobox
335 gene *TGIF-1,* which is expressed at the mRNA level in both macrovascular ECs and
336 microvascular ECs. Studies from our laboratory demonstrated a significant increase in *TGIF-*
337 *1* in placentae from idiopathic FGR-affected pregnancies compared with uncomplicated
338 control pregnancies. However, the functional role of *TGIF-1* in human placentae, in
339 particular its role in placental angiogenesis, is unclear. Therefore, the focus of this study was
340 to investigate the functional role and the angiogenic downstream targets of *TGIF-1*.
341
342 Nuclear expression of TGIF-1 protein in the primary HUVECs and the SGHEC-7 cell line,
343 which were used as an *in vitro* EC model, was confirmed using immunocytochemistry.
344 Successful knockdown of TGIF-1 expression at both the mRNA and protein levels was
345 achieved using two independent *TGIF-1* siRNAs to inactivate the *TGIF-1* gene expression in
346 ECs. Previous studies have demonstrated the angiogenic potential of the homeobox gene
347 *HEX* in HUVECs (Nakagawa, et al. 2003). This study found over-expression of *HEX*
348 disrupted the ability of the ECs to migrate, proliferate and form tubular structures in response
349 to VEGF stimulation. Apart from this study, limited information exists in the literature about
350 the effects of modulating expression of homeobox genes on EC function in either HUVEC or
351 in human placental microvascular ECs. Therefore, we investigated the functional role of
352 TGIF-1 in placental angiogenesis and examined similar functions.
353

Microvascular ECs, which are found in the fetal capillaries of chorionic villi, exhibit a proliferative phenotype, as they play an important role in the vascularisation of the placenta (Thorin and Shreeve 1998). *TGIF-1* inactivation in HUVECs and SGHEC-7 cells significantly reduced their proliferative ability, while increasing the angiogenic potential of the ECs. This demonstrates that *TGIF-1* plays a role in regulating the ability of ECs to form cell-cell and cell-matrix connections. The effect of *TGIF-1* in vascular development is not surprising as mouse studies have found embryos lacking *Tgif-1* to be extremely growth restricted with placental defects affecting the vasculature (Bartholin, et al. 2008). The overexpression of *TGIF-1* in human FGR placental endothelium suggests that there may be excessive proliferation with insufficient differentiation, resulting in impaired placental angiogenesis.

Another critical aspect of placental angiogenesis is the degradation of the basement membrane by proteases released by ECs (Kaufmann, et al. 2004). Metalloproteinases (MMPs) are proteases that belong to a family of at least 15 secreted and membrane-bound zinc-endopeptidases. The results of the current study indicate a significant difference in MMP-2 activity in HUVECs and SGHEC-7 cells transfected with *TGIF-1* siRNA compared with NC transfected ECs. Other studies have found MMP-1, -2, -3, -9 and TIMP1 enzymatic activity to be associated with ECs (Hanemaaijer, et al. 1993). However, under basal conditions without growth supplementation, MMP1 and MMP2 activity is evident but MMP9 is not, suggesting this is not constitutively secreted (Jackson and Nguyen 1997). Therefore, it is possible due to the use of serum-free medium in our experiments, there was no observable effect on MMP9 activity. Dysfunctional secretion of these enzymes would significantly impact basement membrane degradation and invasive potential, which would affect the vasculature development.

To determine *TGIF-1*'s role as a functional regulator of angiogenesis, we investigated downstream targets of *TGIF-1* in EC angiogenesis by utilising low density angiogenesis-related PCR arrays to profile alterations in gene expression. The array consists of 92 angiogenesis related genes targeting known angiogenic growth factors including VEGF, endostatin and cell adhesion molecules. In addition, the array contains markers and targets for angiogenesis and lymphangiogenesis. From the array, four candidate genes that showed altered expression following *TGIF-1* inactivation were selected for further validation on independent cultures of *TGIF-1* inactivated HUVECs and SGHEC-7 cells. *NRP-1* and *ITGAV* showed increases in gene expression consistent with the array. *ANGPT-1* mRNA expression was significantly decreased in HUVECs and SGHEC-7 cells, while the related *ANGPT-2* showed an opposite increase in mRNA expression.

The TGIF-1 downstream target gene *NRP-1* is an important regulator of angiogenesis particularly in the cardiovascular system (Kawakami, et al. 2002). *NRP-1* acts as a co-receptor for VEGF, which is a principal promoter of angiogenic processes and is involved in the differentiation, tube formation and vascular maturation of ECs (Flamme, et al. 1997). From the low density array, VEGF was also up-regulated as a result of TGIF-1 silencing. Thus, it is speculated that *NRP-1,* in cooperation with VEGF, may help regulate the formation of EC networks. Consequently, with overexpression of *TGIF-1* in FGR placentae, the downstream *NRP-1* targeted gene would be expected to be decreased. A recent study confirms this, demonstrating significantly reduced NRP-1 expression in placentae from human FGR-affected pregnancies with an absent end-diastolic flow in the umbilical artery (Maulik, et al. 2015). Hence, TGIF-1 may be an important upstream regulator of placental angiogenesis.

Our study also identifies *ITGAV* as a downstream angiogenic target of *TGIF-1*. *ITGAV* codes for the αV integrin and is involved in cell adhesion. Overexpression of *TGIF-1* will lead to a reduction in *ITGAV* expression. Deletion of *Itgav* in mice shows impaired vascular development in the central nervous and the ophthalmic systems, similar to that seen in the deletion of *Nrp-1* (Arnold, et al. 2012). A major function of this integrin is to activate TGFβ1 signalling (Arnold, et al. 2012), which is a crucial signalling pathway in placental development. Therefore, impaired placental vascularisation in human FGR may be a result of *TGIF-1* overexpression reducing ITGAV expression.

The *TGIF-1* downstream targets of *ANGPT1* and *ANGPT2* code for angiopoietins 1 and 2 respectively, and are critical mediators of vascular development. Angiopoietin 2 (ANGPT2) is an antagonist for both angiopoietin 1 (ANGPT1) and the TIE-2 receptor (Drenkhahn, et al. 2004). ANGPT1 is known to provide a stabilising signal through the TIE-2 receptor, which can be blocked by ANGPT2 to prevent vascular sprouting only if VEGF is absent (Maisonpierre, et al. 1997). Increased placental expression of *TGIF-1* observed in human FGR is expected to upregulate *ANGPT1* and downregulate *ANGPT2* mRNA. Altered expression of both *ANGPT1* and *ANGPT2* is implicated in an ovine model of FGR (Hagen, et al. 2005). Imbalances in the concentrations of angiopoietins 1 and 2 may contribute to the villous pathology of the FGR microvasculature via the induction of premature maturation of the terminal villi capillaries (Dunk, et al. 2000). Thus, angiopoietin signalling may be another pathway through which pathological *TGIF-1* overexpression impacts placental angiogenesis in human FGR.

In conclusion, this is the first study to report an increased expression of *TGIF-1* in PLECs from FGR-affected pregnancies. *In vitro* functional analyses suggest TGIF-1 regulates

placental angiogenesis through effects on the ability of ECs to proliferate, form networks and invade. Increased expression of TGIF-1 in endothelial cells may contribute to reduced branching angiogenesis observed in FGR placentae. Our study also identified *ITGAV*, *NRP-1*, *ANGPT1* and *ANGPT2* as downstream targets of *TGIF-1*, which are important mediators of placental angiogenesis in FGR. Thus, the increased expression of homeobox gene *TGIF-1* may be involved in the molecular mechanisms underlying the aberrant angiogenesis observed in human FGR.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure legends

Figure 1

Increased *TGIF-1* mRNA expression relative to housekeeping genes in endothelial cells from FGR compared with control placentae was determined by real-time PCR analysis in two independent cohorts from Melbourne, Australia (A) and Toronto, Canada (B). *p<0.05, ***p<0.001, Student's *t* test.

Figure 2

TGIF-1 expression in the primary HUVECs and the SGHEC-7 cell line. Immunoreactive TGIF-1 localised to the nuclei in HUVECs and SGHEC-7 cells as denoted by black arrows, which is absent in the negative IgG control (A). Scale bar represents 100 μm. siRNA inactivation with resulted in reduced *TGIF-1* mRNA expression in HUVECs (B) and SGHEC-7 cells (C), which was validated using western immunoblotting (D) and confirmed at the protein level (E & F). NC denotes the non-specific siRNA used as the negative control, while S1 and S2 refer to the two independent *TGIF*-specific siRNAs used in the siRNA transfection experiments. ***p<0.001, One Way ANOVA with Bonferroni's post test.

Figure 3

Functional analyses of TGIF-1 in HUVECs and SGHEC-7 cells. *TGIF-1* siRNA inactivation significantly reduced proliferation (A & B), increased tube formation (C, D & E) and decreased MMP-2 activity (F), with no effect on MMP-9 activity (G) of ECs. Representative experiments of the tube formation assay and zymography are presented in E and H respectively. NC denotes the non-specific siRNA used as the negative control, while S1 and S2 refer to the two independent TGIF-specific siRNAs used in the siRNA transfection

experiments. *p<0.05, **p<0.01, ***p<0.001, One Way ANOVA with Bonferroni's post-test.

Figure 4

Validation of angiogenesis gene screen following *TGIF-1* siRNA inactivation in HUVECs and the SGHEC-7 cell line. Altered mRNA expression of *ITGAV* (A & B), *NRP-1* (C & D), *ANGPT-1* (E & F) and *ANGPT-2* (G & H) were verified using real-time PCR after normalising to the *18S rRNA* housekeeping gene. NC denotes the non-specific siRNA used as the negative control, while S1 and S2 refer to the two independent TGIF-specific siRNAs used in the siRNA transfection experiments. *p<0.05, **p<0.01, ***p<0.001, Student's *t* test.

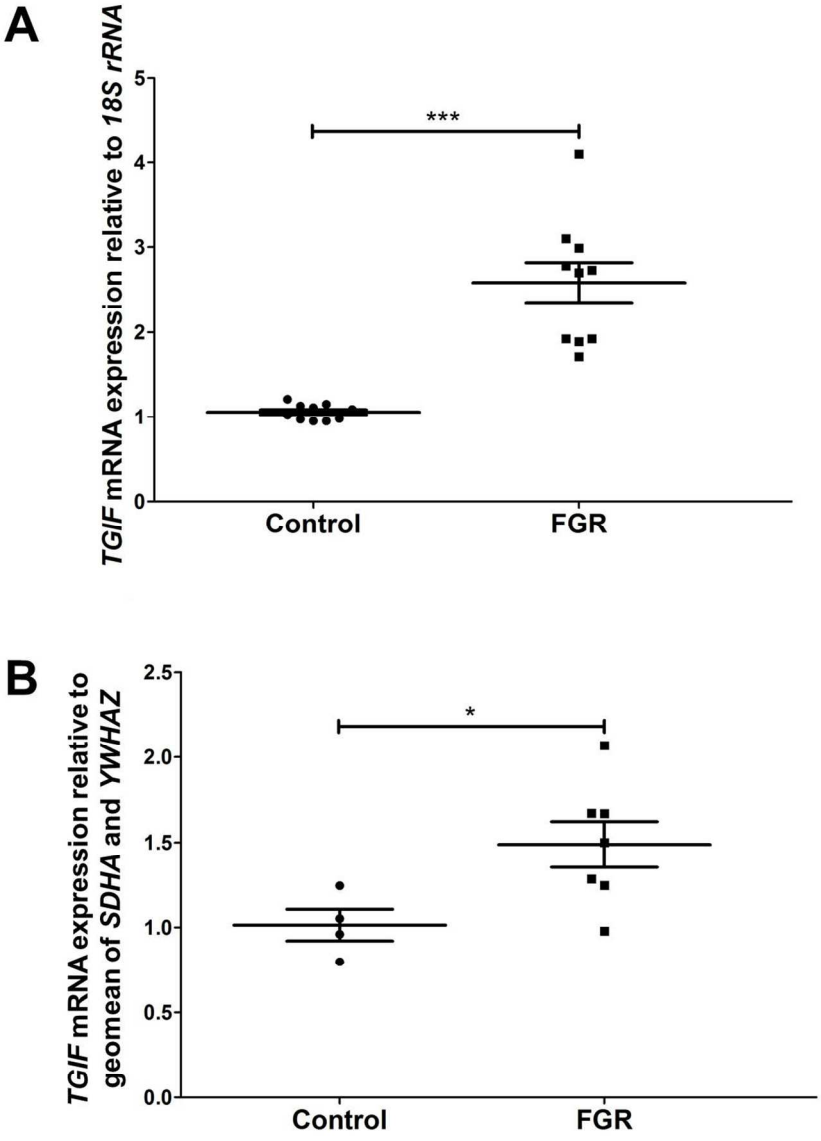

Figure 1: TGIF-1 expression in FGR and control
TGIF-1 mRNA in FGR and control
216x282mm (150 x 150 DPI)

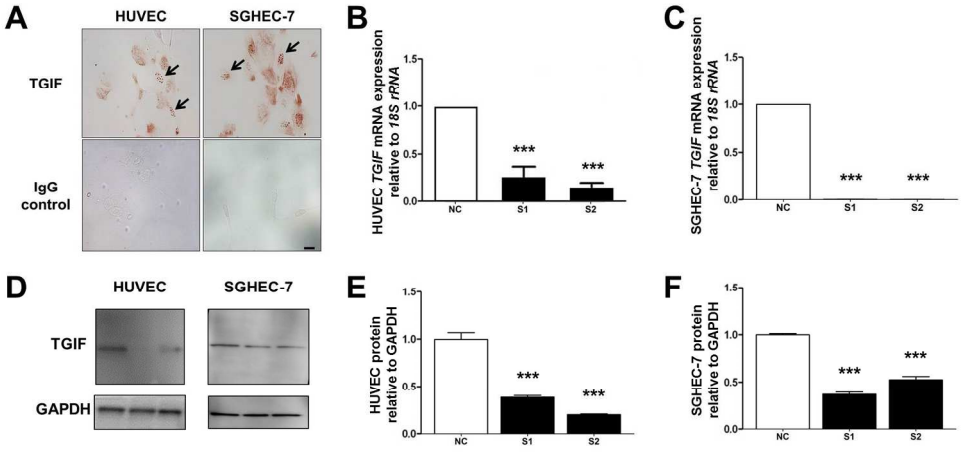

Figure 2. TGIF-1 expression in HUVEC and SGHEC-7 cells.
TGIF-1 mRNA and protein in HUV
358x179mm (150 x 150 DPI)

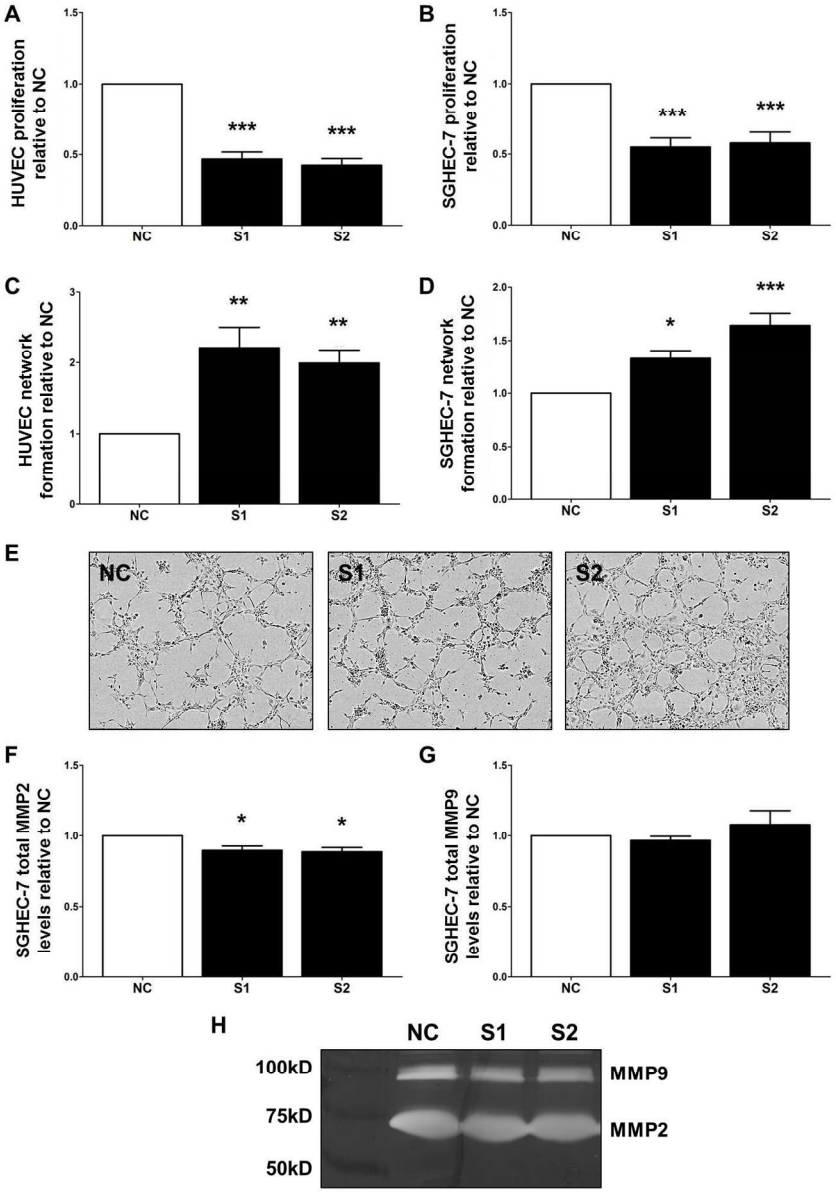

Figure 3: Functional role of TGIF-1 in endothelial cells
TGIF-1 in proliferation, netwo
247x337mm (150 x 150 DPI)

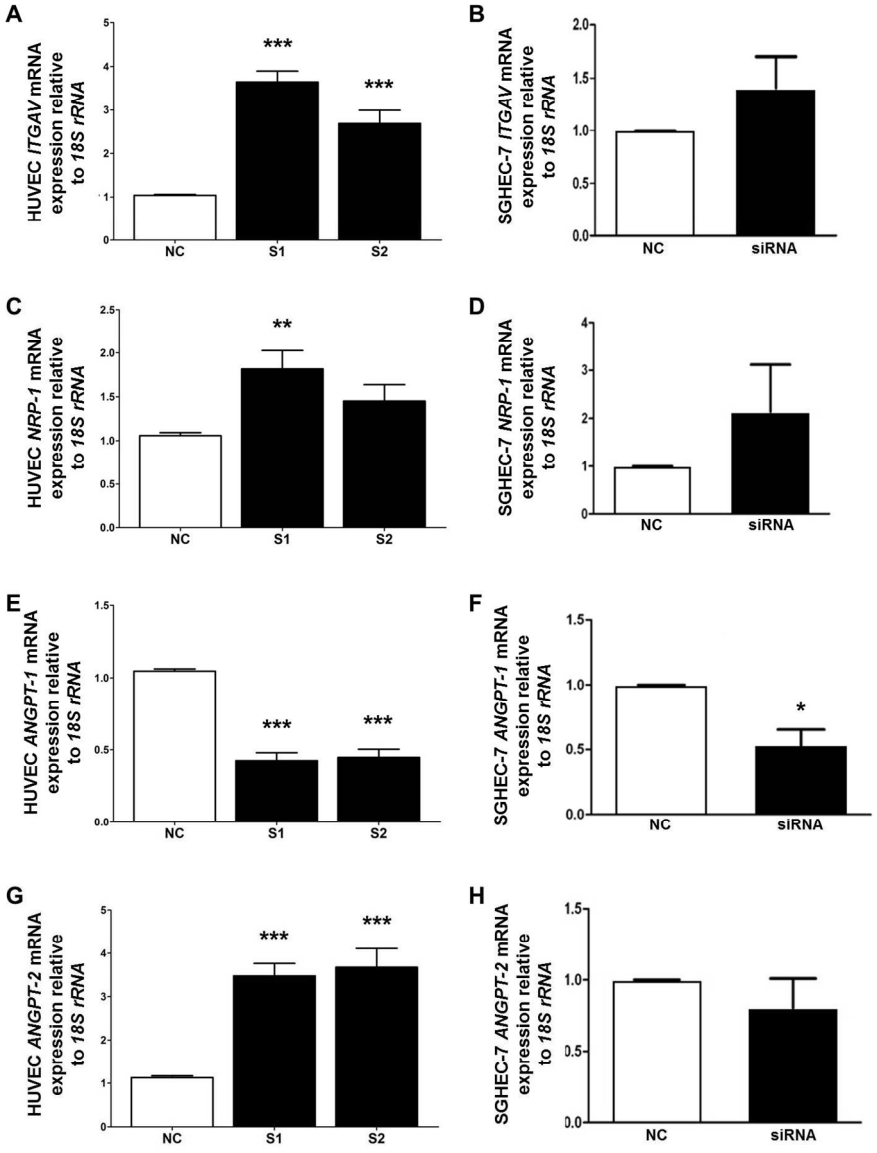

Figure 4: Downstream targets of TGIF-1 in endothelial cells
Downstream target genes of TGI
244x329mm (150 x 150 DPI)

Tables

Table 1

Clinical characteristics of Australian samples

Characteristics[a]	GMC (n=10)	FGR (n=10)	P-value[b]
Maternal Age (years)	34.8±1.6	32.7±2.0	0.41
Gestation at Delivery (weeks)	38.7±0.3	38.4±0.4	0.52
Infant Weight (g)	3406.3±122.8	2448.7±63.3	<0.0001
Placental Weight (g)	662.4±32.8	471.3±28.8	<0.001
Parity	5 primi, 5 multi	4 primi, 6 multi	1.00
Infant Sex	6F, 4M	9F, 1M	0.30

[a] Shown is the mean ± SEM with ranges shown in brackets unless stated otherwise.

[b] Student's *t* test for parametric data and 2 X 2 contingency table with Fisher's Exact Test for categorical data were used where appropriate.