

A novel regulator of human villous trophoblast fusion: the Krüppel-like factor 6

Ana Cristina Racca, Magali Evelin Ridano, Soledad Camolotto, Susana Genti-Raimondi, and Graciela María Panzetta-Dutari*

Centro de Investigaciones en Bioquímica Clínica e Inmunología (CIBICI-CONICET), Departamento de Bioquímica Clínica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Haya de la Torre y Medina Allende, Ciudad Universitaria, X5000HUA, Córdoba, Argentina

*Correspondence address. Tel: +54-351-5353851 ex 3142; Fax: +54-351-4333048 ex 3177; E-mail: gpan@fcq.unc.edu.ar

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ABSTRACT: Cell–cell fusion is an essential event during life. Throughout human pregnancy, the syncytiotrophoblast (STB) layer of the placenta is formed by continuous fusion of the underlying villous cytotrophoblasts, thus maintaining placental functionality. Defects in this process are associated with pathologies like pre-eclampsia and intrauterine growth restriction. Krüppel-like factor 6 (KLF6) is a transcription factor highly expressed in human and murine placenta. However, KLF6 functions in trophoblast cells remain largely unexplored. The aim of this work was to address the role of KLF6 during STB formation. KLF6 knockdown through small interfering RNA experiments hindered cell–cell fusion revealed by immunofluorescence microscopy in human primary villous cytotrophoblast as well as in the human placental-derived BeWo cell line. Furthermore, KLF6 silencing led to a decrease in the expression of the fusogenic protein Syncytin-1 and the cell cycle regulator p21^{Cip1/Waf1} measured by quantitative RT-PCR and western blot assays. On the contrary, transcript levels of genes that encode for proteins involved in STB formation such as *Syncytin-1*, *Syncytin-2*, *Connexin-43* and *Zonula Occludens-1* increased when KLF6 was overexpressed in differentiating villous cytotrophoblasts and in non-fusing placental-derived JEG-3 cells. Interestingly, the expression of two trophoblast biochemical differentiation markers, β hCG and PSG3, were not reduced after KLF6 silencing in differentiating trophoblast cells. Present results support the notion that KLF6 is a relevant participant in cytotrophoblast fusion.

Key words: cell fusion / gene expression / human trophoblast / syncytin-1 / transcription factors

Introduction

Cell–cell fusion is a highly regulated and dynamic cellular event required for development and homeostasis, but only few specialized human cell types can fuse together and differentiate into a multinucleated cell (Oren-Suissa and Podbilewicz, 2007). This process is involved in the formation of myotubes, osteoclasts and the syncytiotrophoblast (STB) layer of the human placenta. Additionally, cell fusion participates in tissue repair, viral infections and may be important to cancer development and progression (Larsson et al., 2008).

Placental STB is a continuous, uninterrupted, multinucleated layer derived from and maintained by differentiation and fusion of the mononuclear cytotrophoblasts (CTB). This differentiation process is essential for placental growth and maintenance throughout pregnancy. The syncytial layer controls the exchange of gases, nutrients and other factors between the maternal and fetal circulations, protects the fetus against the maternal immune system and is responsible for the production of many proteins, hormones and growth factors, including human chorionic gonadotrophin (hCG) and pregnancy-specific glycoproteins (PSG; Camolotto et al., 2010; Ji et al., 2013).

The fusogenic protein Syncytin-1 (Syn-1), a member of the syncytin family, has been involved in the fusion between placental trophoblasts (Mi et al., 2000; Frendo et al., 2003b), between cancer cells, between cancer cells and host cells, as well as between skeletal muscle-forming myoblasts (Larsson et al., 2008). Syncytin-2 (Syn-2), another member of the syncytin family, has also demonstrated a potential role as a fusogenic protein in human trophoblast syncytialization (Vargas et al., 2009). Connexin-43 (Cx-43) and zonula occludens-1 (ZO-1) are two other molecules involved in human cell–cell fusion and subsequent trophoblast differentiation as established by morphological and biochemical data (Frendo et al., 2003a; Pidoux et al., 2010). Abnormal CTB fusion and/or reduced Syn-1 expression have been linked to pathologies like pre-eclampsia, intrauterine growth restriction and Down syndrome (Frendo et al., 2000; Kudo et al., 2003; Langbein et al., 2008; Malassine et al., 2010; Ruebner et al., 2010; Vargas et al., 2011).

Although STB formation is a widely studied process, the molecules and mechanisms involved are not fully understood. It comprises two related processes, the morphological differentiation consisting of cell–cell fusion and the biochemical differentiation characterized by the synthesis of placenta-specific proteins and hormones (Castellucci and Kaufmann,

2006; Orendi et al., 2010). So far, only few human transcription factors involved in the regulation or modulation of the syncytium formation have been described. Among them, glial cell missing 1 (GCM1; Baczyk et al., 2009), PPAR γ (Schaff et al., 2000), NR2F2 (Hubert et al., 2010), Ets-1 (Handwerger and Kessler, 2013) and members of the AP2 and Sp families (Loregger et al., 2003) have been implicated in morphological and/or biochemical trophoblast differentiation.

The transcription factor KLF6 is a member of the Sp/KLF family mostly implicated in human carcinogenesis, cell cycle regulation (Andreoli et al., 2010) and in some differentiation processes (Liet al., 2005b; Kremer-Tal et al., 2007; Zhao et al., 2010). Current data strongly suggest that the outcome of KLF6 function depends on the particular cellular environment (Andreoli et al., 2010). KLF6 is a ubiquitous transcription factor highly expressed in human and murine placenta. Importantly, *klf6*^{-/-} knockout mice phenotype is characterized by impaired placental development and a poorly defined liver, contributing to early lethality along with failures in yolk sac vascularization (Matsumoto et al., 2006). In the human placental villi, KLF6 is highly expressed in the underlying CTB, in the STB layer and some stromal cells with a nuclear as well as cytoplasmic localization as reported for several other cells (Gehrau et al., 2010). We have previously demonstrated that KLF6 is expressed throughout the *in vitro* differentiation of human villous CTB (vCTB) and contributes to the transcriptional activation of the *CGB5* gene (coding for the β hCG subunit), as well as *PSG3* and *PSG5* genes, two of the most expressed members of the *PSG* gene family. These findings support a role of KLF6 in human placental development and function (Racca et al., 2011).

The aim of this work was to explore the potential participation of KLF6 in trophoblast differentiation by loss- and gain-of-function studies on vCTB cells isolated from human term placentas and in two human placental-derived cell lines.

Materials and Methods

Placentas collection, cytotrophoblast isolation, cell culture and differentiation

Tissues from normal full-term placentas (37–41 weeks of pregnancy) were obtained after Caesarean delivery and were processed within 30 min after delivery. Placentas were obtained from uncomplicated pregnancies of unidentified anonymous patients with the ethics approval of the local Advisory Committee of Biomedical Research in Humans Córdoba, Argentina (RePIS No. 1202, HP 4-112). After removal of the cord, amniochorion and decidua layer, villous tissue free of visible infarct, calcification or haematoma was sampled from the maternal–fetal interface. The tissue was cut into small pieces and washed with 154 mM NaCl, to remove blood. vCTBs were isolated according to the protocol of Kliman (Kliman et al., 1986), with slight modifications. Briefly, after thorough washing, tissue was subjected to three digestions in Hank's balanced salts solution containing 2.5 mg/ml trypsin (Difco, Chemical Center, Buenos Aires, Argentina), 4.25 mM MgSO₄, 0.5 μ M CaCl₂, 25 mM HEPES, and 50 U/ml DNase I type IV (Sigma-Aldrich, St Louis, MO, USA) for 30 min at 37°C with shaking. The cell pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 100 U/ml penicillin and 0.1 mg/ml streptomycin and separated by centrifugation at 1200 g for 35 min on discontinuous Percoll gradient (5–70% v/v). Isolated trophoblasts ($\geq 97\%$ cytokeratin 7-positive cells) were plated in keratinocyte growth medium (KGM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% v/v fetal bovine serum (FBS, GBO Argentina, Buenos Aires, Argentina) and antibiotics (100 U/ml penicillin/0.1 mg/ml streptomycin). Cells were

maintained in culture for up to 72 h with complete medium change every 24 h. JEG-3 cells were grown in DMEM supplemented with antibiotics and 10% v/v FBS. BeWo (ATCC no. CCL98) cells were cultured in Ham's F12 (Invitrogen) supplemented with antibiotics and 10% v/v FBS and, when necessary, they were induced to *in vitro* differentiate by 10 μ M forskolin (FSK, Sigma-Aldrich) treatment.

Immunofluorescence assays

Isolated CTB and BeWo cells were cultured on coverslips using supplemented medium as described above. The coverslips were washed three times with phosphate-buffered saline (PBS), and cells were fixed for 20 min in 3% w/v paraformaldehyde at room temperature or 10 min in methanol at -20°C according to the antibody used. Cells were incubated 10 min with 10 mM NH₄Cl to inhibit quenching, washed with PBS three times and permeabilized for 20 min with 0.5% v/v Nonidet P-40 in PBS or 7 min with 0.1% v/v Triton 100X in PBS. Cells were rinsed with PBS three times and blocked with 2.5% v/v normal goat serum in 0.2% v/v Tween-20 in PBS (PBS-T) for 15 min and with 0.5% w/v fish skin gelatin in PBS-T for another 15 min. Then, cells were incubated with the following primary antibodies: rabbit polyclonal anti-KLF6 (1:100; R-173 Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-desmosomal protein (1:400; 0.045 mg/ml, ZK-31, Sigma-Aldrich). Cells were washed with PBS-T, blocked as described above and incubated with the appropriate species-specific secondary antibodies, either red Alexa Fluor 594-conjugated goat anti-mouse IgG (Fab) or green Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Fab; Molecular Probes, Carlsbad, CA, USA) in a 1:720 final dilution. Negative controls were performed by replacing the primary antibodies with buffer. All antibody incubations were carried out in a humidity chamber for 1 h at 37°C. Nuclei were counterstained with Hoechst 33342 (Molecular Probes) dye and visualized in an inverted Nikon Eclipse TE2000-U (Nikon Corporation, Japan) optical microscope or a Confocal Olympus Fluoview FV300 microscope (Olympus Latin America, Miami, FL, USA), as indicated. Slides were mounted in Aqueous Mounting Medium with fluorescence tracers (Fluor Save, Calbiochem, Research AG, Buenos Aires, Argentina).

Cell fusion assay

Immunofluorescence microscopy was performed to assess cell fusion. Images obtained for desmosomal protein (desmoplakin) and nuclei counterstaining with Hoechst were merged, and the number of nuclei in syncytia and the total number of nuclei in 20 randomly chosen microscopic fields were counted. The percentage of the nuclei in syncytia was determined as: (number of nuclei in syncytia/total number of nuclei) \times 100, as described (Flores-Martin et al., 2012). Duplicate samples of at least three independent experiments were evaluated.

Western blotting

BeWo and CTB whole protein extracts were prepared in 5X Laemmli buffer containing 60 mM Tris–HCl pH 6.8, 10% v/v glycerol, 2% w/v sodium dodecyl sulphate, 1% v/v 2- β -mercaptoethanol and 0.002% w/v bromophenol blue. Total protein samples were separated on a 10% w/v SDS–PAGE and transferred to a nitrocellulose Hybond-ECL (GE Healthcare, Piscataway, NJ, USA). The membranes were blocked in 5% w/v non-fat milk in Tris-buffered saline (TBS; 20 mM Tris–HCl, 150 mM NaCl pH 7.8) supplemented with 0.2% v/v Tween-20 (TBS-T) for 1 h at room temperature. Blots were incubated with primary antibodies diluted in 5% w/v non-fat milk in TBS-T overnight at 4°C. The following antibodies were used: mouse monoclonal anti-KLF6 (1:3000; clone 2c11, whose specificity was previously determined; Gehrau et al., 2010), mouse monoclonal anti- α -tubulin

(1 : 2000; Sigma-Aldrich), rabbit polyclonal anti-Syncytin-1 (1 : 250; z-25, Santa Cruz Biotechnology), mouse monoclonal anti-p21 (1 : 1000; Cell Signaling, Danvers, MA, USA) and rabbit polyclonal anti- β hCG (1 : 1000; Dako, Tecnolab, Buenos Aires, Argentina). After washing, the blots were incubated with horseradish peroxidase-conjugated donkey anti-rabbit or sheep anti-mouse IgG secondary antibodies (1 : 5000; Amersham Bioscience, Piscataway, NJ, USA) in 5% w/v non-fat milk in TBS-T at room temperature for 1 h. Protein-antibody complexes were visualized using an enhanced chemiluminescence detection system (SuperSignalWest Pico; Pierce, Tecnolab) and exposed to GE Healthcare film. Ponceau staining (0.2% w/v Ponceau, 3% w/v trichloroacetic acid, 3% w/v sulphosalicylic acid) was used to verify protein transference from gel to nitrocellulose membrane.

Real-time quantitative RT-PCR

Total RNA was extracted from cultured cells at the indicated times using Trizol reagent (Invitrogen). One microgram of total RNA was reverse-transcribed in a total volume of 20 μ l using random primers (Invitrogen) and 50 U M-MLV reverse transcriptase (Promega, Biodynamics, Buenos Aires, Argentina). Primers were manually designed with the assistance of the Netprimer software (PREMIER Biosoft International, Palo Alto, CA, USA), UCSC In-Silico PCR (UCSC Genome Browser website), and Primer Express software (ABI, Applied Biosystems, Carlsbad, CA, USA). Primer sequences were compared against the human genomic and transcript database by using the BLAST program (Altschul *et al.*, 1997) at the NCBI website. Specific transcripts were quantified by qRT-PCR (ABI 7500 Sequence Detection System, Applied Biosystems) using the Sequence Detection Software v1.4. Experiments were performed using IX SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences and concentrations are indicated in Table 1. The cycling conditions included a hot start step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. For all reactions, amplification efficiency was between 94 and 98%. Specificity was verified by melting curve analysis. Fold change in gene expression was calculated according to the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). Each sample was analysed in triplicate. No amplification was observed in PCRs containing water or RNA samples incubated without reverse transcriptase as template.

Plasmid DNA and small interfering RNA transfections

For KLF6 overexpression purposes, 1.5×10^6 primary CTB/well and 5×10^5 BeWo cells/well were seeded into six-well tissue culture plates and incubated for 24 and 16 h, respectively. Both cell types were transfected with 4 μ l of lipofectamine 2000 reagent (Invitrogen) and 1500 ng of the KLF6 expression vector containing the human wild-type full-length KLF6 cDNA (Slavin *et al.*, 2004) or the corresponding empty vector (pXJ-41) as a negative control and incubated for 48 additional hours.

For silencing experiments, 1×10^6 CTB cells per well were seeded in six-well plates, cultured for 16 h, and afterwards transfected with 200 nM or 25 nM of the KLF6-specific small interfering RNA (siKLF6, Ambion ID no. 9700; sense 5'-GGAAGAUUCUGGACCAAAAtt-3', antisense 5'-UUUGGUCCACAGAUCUCCtg-3') or a scrambled siRNA (SCR) as control and 4 μ l of lipofectamine 2000. Forty-eight hours post-transfection, coverslips for immunofluorescence assays were collected, mRNA was purified and protein extracts were prepared. Since 200 nM was the most effective concentration for KLF6 silencing (Supplementary data, Fig. S1A), it was selected for conducting the following experiments. A similar assay was performed with 200 nM of a second KLF6-specific siRNA (siKLF6-B, Ambion ID no. 106424; sense-5' GGUGGAUCCUGUAGGCUAAAtt-3'; antisense-5' UUAGCCUACAGGAUCCACCtt-3'). Both KLF6-specific siRNAs had similar KLF6 silencing efficiency and effect on cell fusion (Supplementary data, Fig. S1B). BeWo cells were either non-transfected, mock-transfected or transfected with a 25 nM final concentration of the control SCR or one of three different KLF6-specific siRNAs: siKLF6, siKLF6-B or Silencer® Select KLF6 siRNA (Sel-siKLF6, Ambion ID no. s3374). The last one is a next-generation siRNA designed to minimize off-target effects through the incorporation of strategic chemical modifications. Sel-siKLF6 targets almost the same sequence as the one targeted by the siKLF6 used in most of the experiments performed in the present work. After 24 h, cells were induced to differentiate with FSK as previously described. Forty-eight hours post-FSK treatment, coverslips for immunofluorescence assays were prepared, and protein extracts were obtained. Cell viability was evaluated by using the metabolic dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay.

Table 1 Oligonucleotides used in qRT-PCR assays.

Transcript	Primer name	Sequence (5'–3')	nM
Syn 1	ERVWE1 F	GCAACCACGAACGGACATC	200
	ERVWE1 R	GTATCCAAGACTCCACTCCAGC	
Syn 2	Syn2 F	CGGATACCTTCCCTAGTGCC	200
	Syn2 R	AGCTGAGGTTGCTGGTTCTG	
Cx 43	Cx 43 F	ACTTGCCCTTTTCATTTTACTTC	200
	Cx 43 R	CCTGGGCACCACTCTTTT	
ZO 1	ZO1 F	CAACATACAGTGACGCTTCACA	200
	ZO1 R	CACTATTGACGTTTCCCCACTC	
Cyclophilin A	Cyclo A F	GTC AAC CCC ACC GTG TTC TT	300
	Cyclo A R	CTG CTG TCT TTG GGA CCT TGT	
PSG3	PSG3 F	TGG TCC AGA CCT CCC CAG AAT T	100
	PSG3 R	CCA GGA AGA TGT CCT GTT CC	
β hCG	β hCG F	GCT ACT GCC CCA CCA TGA CC	300
	β hCG R	ATG GAC TCG AAG CGC ACA TC	
p21	p21 F	GCA GAC CAG CAT GAC AGA TTT C	100
	p21 R	CGG ATT AGG GCT TCC TCT TG	

Statistical analysis

Results are presented as mean \pm SEM or as median and interquartile range according to a parametric or non-parametric data distribution, respectively. When two data sets were compared, one-sample t-test or Mann–Whitney test was used to determine statistical significance. One-way analysis of variance (ANOVA) was performed on the densitometric quantification of KLF6 protein expression levels in BeWo cells during the differentiation time course using Kruskal–Wallis ANOVA with the Dunn's multiple comparisons *post hoc* test. Statistical analyses were performed using the Graph-Pad Prism 5.0 software.

Results

KLF6 silencing interferes with human placental villous cytotrophoblast cell–cell fusion

We have previously demonstrated that KLF6 is expressed in vCTB, STB, and throughout the *in vitro* differentiation of vCTB. Herein, we investigated whether KLF6 is required for vCTB fusion. To this end, vCTB cells were isolated from human normal-term placentas, cultured in serum-containing media to allow spontaneous syncytialization, and KLF6 expression was knocked down by transient transfection with specific siRNAs. Transfection efficiency near 90% was confirmed by employing a fluorescent control siRNA (data not shown). In vCTB cells transfected with 200 nM of siKLF6, KLF6 protein expression level was reduced to $50 \pm 10\%$ compared with cells transfected with the control siRNA (SCR; Fig. 1A). Morphological differentiation was monitored by nuclei staining and desmoplakin immunodetection. SiKLF6-transfected cells showed a clear reduction in the formation of syncytium-like structures compared with SCR-transfected control cultures (Fig. 1B). The impact of KLF6 down-regulation on cell fusion was clearly demonstrated by measuring the percentage of cell fusion, which was reduced by more than 50% compared with the control condition (Fig. 1C). Similar results were obtained with siKLF6-B, which recognizes a different sequence in KLF6 transcript (data not shown). Remarkably, this important impairment in cell–cell fusion occurred even though KLF6 expression was not fully silenced.

Since Syn-1 is a highly fusogenic membrane glycoprotein clearly involved in syncytia formation (Frendo et al., 2003b), its expression was analysed after siRNA-induced down-regulation of KLF6. Indeed, KLF6 knockdown led to a significant reduction in Syn-1 protein level compared with SCR-treated cells (Fig. 2). Altogether, these findings strongly suggest that KLF6 is required for human vCTB fusion during spontaneous *in vitro* trophoblast differentiation into the STB pathway.

KLF6 is expressed in BeWo cells and its down-regulation reduces forskolin-induced cell fusion

To further study KLF6 role in trophoblast fusion, we used the BeWo choriocarcinoma cell line. BeWo cells fuse in response to FSK-induced cAMP level and have been extensively employed as an *in vitro* model for studying various placental trophoblast functions (Wice et al., 1990; Fradet et al., 2012). KLF6 protein was already expressed in untreated cells (0 h) and was rapidly up-regulated by FSK treatment after 2 h, decreasing near to basal levels after 48 h (Fig. 3A). Biochemical and

morphological differentiation of BeWo cells into the syncytial pathway were corroborated by evaluation of β hCG expression and the appearance of multinucleated cells, respectively (Fig. 3A and B). These results show that KLF6 expression pattern in BeWo cells under differentiation resembles that observed during spontaneous *in vitro* syncytialization of primary vCTBs (Racca et al., 2011). Thus, the effect of KLF6 silencing on the differentiation of FSK-treated BeWo cells was measured. To this end, cells were transfected with the specific siKLF6 or the SCR and 24 h later differentiated with FSK. After 48 h post-FSK treatment, KLF6 expression and cell fusion were analysed. Densitometric analysis of western blot assays revealed a 50% knockdown of KLF6 protein level in siKLF6 compared with SCR-treated cells (Fig. 4A). Moreover, KLF6 knockdown significantly decreased FSK-induced cell fusion as observed by the reduction in the formation of syncytium-like structures detected by nuclei staining and desmoplakin immunostaining (Fig. 4B). Indeed, the percentage of nuclei in syncytium-like structures was notably reduced from $24 \pm 2\%$ in SCR-transfected cells to $13 \pm 3\%$ in siKLF6-transfected ones (Fig. 4C). To exclude the possibility that impaired cell fusion was due to reduced cell viability or to off-target effect of the siKLF6, similar assays were performed in BeWo cells which were either non-transfected, mock-transfected or transfected with the SCR siRNA or with one of three different KLF6-specific siRNAs: siKLF6, siKLF6-B and Sel siKLF6. Cell viability was not affected in any of the tested conditions (Supplementary data, Fig. S2A). Moreover, a similar KLF6 protein level and cell fusion percentage were observed in non-transfected, mock-transfected or SCR-transfected cells, while KLF6 expression and fusion were clearly reduced when each KLF6-specific siRNA was assayed (Supplementary data, Fig. S2B and C). These results support the notion that KLF6 down-regulation reduces cell fusion without impairing cell viability.

KLF6 knockdown decreases p21^{Cip1/Waf1} expression

It is well known that KLF6 directly transactivates the expression of the cell cycle inhibitor p21 in different cell contexts (Andreoli et al., 2010). Moreover, KLF6 inhibits cellular growth in part by transcriptional activation of p21 (Narla et al., 2001; Benzeno et al., 2004). Trophoblast terminal differentiation is associated with cell cycle arrest and p21 expression. In this regard, it has been described that the percentage of CTB positive for p21 increases with gestational age and p21 is specifically found in STB in third trimester placentas (Genbacev et al., 2000; Korgun et al., 2006). Therefore, we analysed p21 expression during BeWo and primary vCTB *in vitro* differentiation. As revealed in Fig. 5A, syncytialization in both trophoblast cell models was accompanied by a marked increase in p21 protein levels. More interestingly, KLF6 loss-of-function by siRNA transfection in FSK-treated BeWo cells resulted in a reduction in p21 protein and transcript levels compared with the control condition (Fig. 5B). Taken together, these results suggest that KLF6 might contribute to cell fusion in part by increasing p21 expression.

KLF6 overexpression increases the mRNA level of fusion-associated genes

To further support the notion that KLF6 is involved in trophoblast cell fusion, we assessed whether KLF6 is able to up-regulate the expression of genes directly involved in syncytium formation such as *ERVWE1*, *ERVFRD-1*, *GJA1* and *TJP1* which encode for proteins Syn-1, Syn-2,

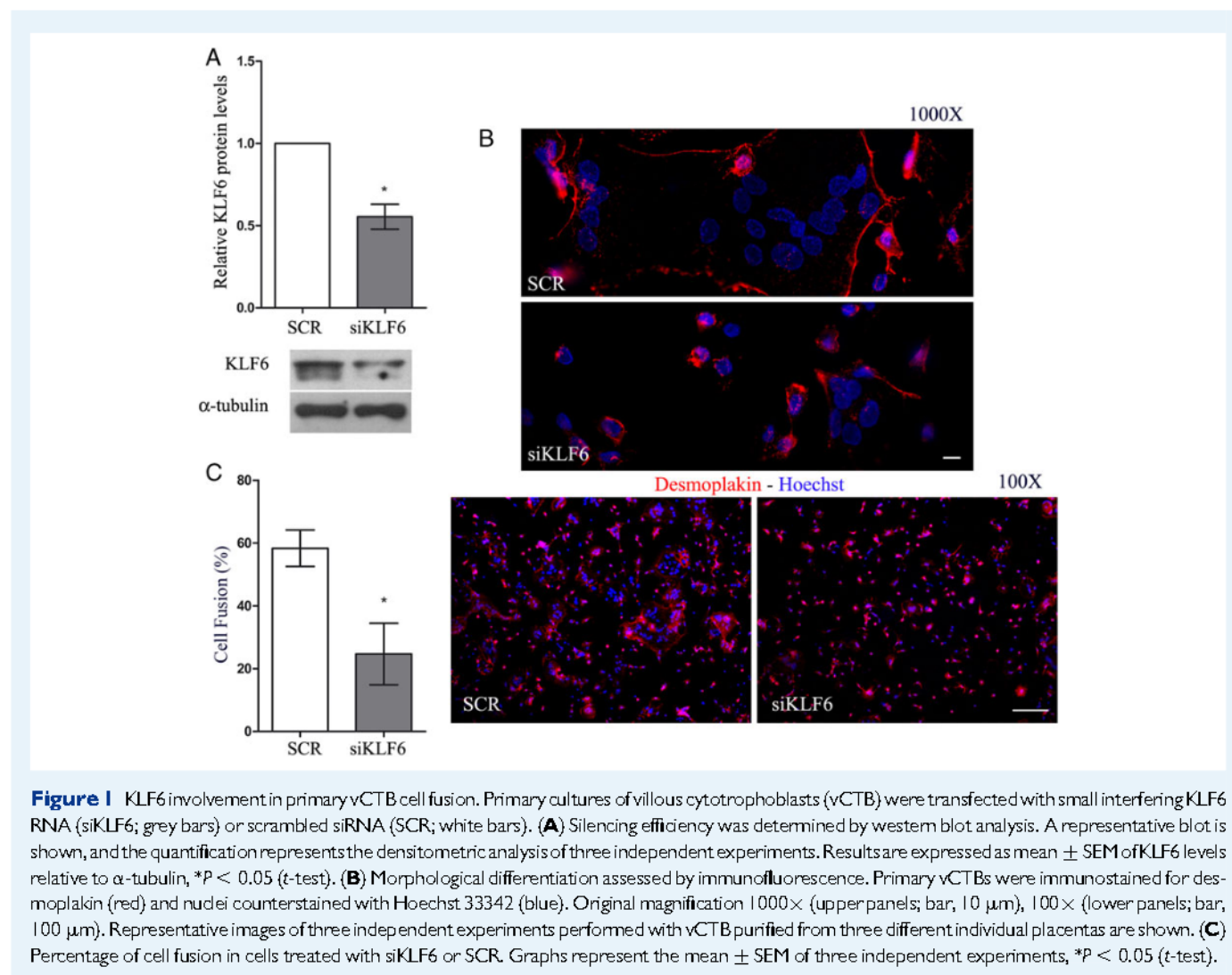


Figure 1 KLF6 involvement in primary vCTB cell fusion. Primary cultures of villous cytotrophoblasts (vCTB) were transfected with small interfering KLF6 RNA (siKLF6; grey bars) or scrambled siRNA (SCR; white bars). **(A)** Silencing efficiency was determined by western blot analysis. A representative blot is shown, and the quantification represents the densitometric analysis of three independent experiments. Results are expressed as mean \pm SEM of KLF6 levels relative to α -tubulin, * $P < 0.05$ (t -test). **(B)** Morphological differentiation assessed by immunofluorescence. Primary vCTBs were immunostained for desmoplakin (red) and nuclei counterstained with Hoechst 33342 (blue). Original magnification 1000 \times (upper panels; bar, 10 μ m), 100 \times (lower panels; bar, 100 μ m). Representative images of three independent experiments performed with vCTB purified from three different individual placentas are shown. **(C)** Percentage of cell fusion in cells treated with siKLF6 or SCR. Graphs represent the mean \pm SEM of three independent experiments, * $P < 0.05$ (t -test).

Cx-43 and ZO-1, respectively. To this end, we studied the effect of KLF6 overexpression in the JEG-3 cell line, which constitutes a suitable model because it has low endogenous KLF6 levels and does not fuse under normal culture conditions. KLF6 overexpression in JEG-3 cells significantly increased the level of the mRNAs coding for Syn-1 (2.6-fold, interquartile range 2.0–2.8), Syn-2 (3.4-fold, interquartile range 2.7–4.5), Cx-43 (2.8-fold, interquartile range 2.5–4.9) and ZO-1 (2.0-fold, interquartile range 1.1–2.1) compared with control cells (Fig. 6A).

Similar experiments were performed in isolated primary vCTB cells that spontaneously fuse in *in vitro* culture. Sixteen hours after plating cells were transfected with the KLF6 expression plasmid or the empty vector and 48 h later the relative transcript levels of the four fusion-related protein-encoding genes were quantified. In these conditions, mRNAs encoding Syn-2 and Cx-43 displayed higher levels compared with control with median values of 1.4-fold (interquartile range, 1.0–8.0) and 1.4-fold (interquartile range, 1.3–2.9), respectively. Transcripts coding for Syn-1 and ZO-1 showed an increasing trend although statistically non-significant (Fig. 6B). These results indicate that KLF6 is able to induce the expression of at least the mRNAs encoding Syn-2 and Cx-43, even in cells undergoing spontaneous syncytialization where they are endogenously up-regulated.

In summary, in both cell models, KLF6 overexpression leads to an increased transcription of genes involved in the fusion process suggesting that KLF6 is positively regulating their expression.

Down-regulation of KLF6 does not hinder β hCG and PSG3 expression

Next, we investigated whether down-regulation of KLF6 expression also impaired vCTB biochemical differentiation. For this purpose, the expression of two trophoblast biochemical differentiation markers, β hCG and PSG3, was determined in *in vitro* differentiating vCTB cells transfected with siKLF6 or SCR. Surprisingly, β hCG mRNA and protein levels were not diminished but modestly induced (Fig. 7A). Similar results were obtained for PSG3 transcript expression (Fig. 7B). These findings indicate that a 50% reduction in KLF6 levels does not inhibit the expression of both biochemical differentiation markers in CTB cells undergoing syncytialization.

Altogether, present results suggest that KLF6 is mainly implicated in the control of trophoblast cell fusion events. In addition, they support the idea that, at least in part, the biochemical differentiation processes can take place although cell–cell fusion is partially impaired.

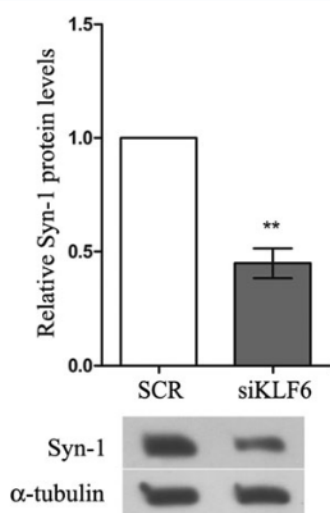


Figure 2 Syn-1 expression in KLF6-silenced primary vCTB. Protein extracts from villous cytotrophoblasts (vCTB) transfected with small interfering KLF6 RNA (siKLF6; grey bar) or scrambled siRNA (SCR; white bar) were subjected to western blot to evaluate Syn-1 protein. One representative blot and densitometric quantification of Syn-1 levels relative to α -tubulin of three independent experiments performed with vCTB isolated from three different placentas are shown. Data are expressed as mean \pm SEM, ** $P < 0.01$ (t-test).

Discussion

STB formation is one of the most important events during human placental development, crucial for growth, maintenance and functionality of placental villi. vCTB differentiation is a complex process that requires the convergence of multiple players in order to assure proper morphological and functional competence (Huppertz and Gauster, 2011). KLF6 is a zinc-finger transcription factor highly expressed in placental tissue, although its function within trophoblast cell physiology has been scarcely addressed. The study of *klf6*^{-/-} knockout mice led to a non-viable phenotype characterized by impaired yolk sack and placental differentiation, revealing that it is essential for placental development (Matsumoto et al., 2006). However, the specific role of KLF6 in this context remains unidentified.

In this work, we show for the first time that siRNA-mediated KLF6 down-regulation decreases trophoblastic cell–cell fusion in human vCTBs isolated from normal term placentas as well as in FSK-induced BeWo cells, suggesting that KLF6 is a novel factor involved in trophoblast syncytialization. The role of KLF6 in this process was supported by the fact that reduction in its protein level resulted in a lower expression of the fusogenic protein Syn-1 in vCTBs undergoing spontaneous syncytialization. In addition, KLF6 overexpression in non-differentiating JEG-3 cells correlated with an increased level of the transcripts encoding for proteins Syn-1, Syn-2, Cx-43 and ZO-1. Moreover, even in differentiating vCTBs where these genes are endogenously activated KLF6 overexpression up-regulated Cx-43 and Syn-2 transcript levels.

Although the molecular mechanisms and factors involved in trophoblast syncytialization are not fully understood, the contribution of the membrane proteins Syn-1, Syn-2, Cx-43 and ZO-1 is well documented

(Frendo et al., 2003a, b; Vargas et al., 2009; Pidoux et al., 2010). The membrane retroviral envelopes, glycoproteins Syn-1 and Syn-2, are *bona fide* fusogenic proteins encoded by the *ERVWE1* and *ERVFRD-1* genes, respectively (Blaise et al., 2003). Both genes are up-regulated by the GCM1 transcription factor, which is critically involved in the maintenance, development and turnover of the human trophoblast (Yu et al., 2002; Baczyk et al., 2009; Liang et al., 2010). Transient transfection of either Syn-1 or Syn-2 promotes cell fusion in a variety of cell types whereas down-regulation of their expression clearly reduces the cell fusion efficiency (Vargas et al., 2009). Moreover, low expression levels of these genes correlate with impaired cell fusion and differentiation in placenta from patients with intrauterine growth restriction and pre-eclampsia (Ruebner et al., 2010; Vargas et al., 2011). Trophoblast cell fusion is also dependent on Cx-43 which mediates both gap junctional cell–cell communication and protein–protein interaction with the Syn-1 receptor, the solute carrier family 1 (neutral amino acid transporter) member 5, as well as with the tight junction protein ZO-1 (Pidoux et al., 2010; Dunker et al., 2012). ZO-1 is one of the proteins involved in the formation of tight and adherens junctions and a significant decrease in syncytium formation and Cx-43 expression is observed in the absence of ZO-1 (Pidoux et al., 2010). In agreement with our previous data regarding the nuclear localization of KLF6 in CTB cells throughout the differentiation process (Racca et al., 2011), present results support the notion that KLF6 contributes to the transcriptional regulation of the genes encoding Syn-1, Syn-2, Cx-43 and ZO-1. However, we cannot rule out some novel participation related to its high protein expression in the cytoplasm. Further studies are required in order to determine if they are direct or indirect transcriptional targets of KLF6.

Another interesting result found in our study is the relation between trophoblast cell fusion and the expression of p21 and KLF6 proteins. Herein, we demonstrate that knockdown of KLF6 led to a diminished cell–cell fusion and a concomitant reduction in p21 expression. We observed that p21 levels increased during spontaneous syncytialization of CTB as well as FSK-induced BeWo cell fusion. These results are in line with previous reports that showed an increase in p21 expression in BeWo cells induced to differentiate by the env-region of the human endogenous retrovirus and by the transforming growth factor-beta1 (TGF β 1; Lin et al., 2000; Rama et al., 2003). Remarkably, TGF β 1, and its type I and II receptors as well as p21 genes are recognized targets of KLF6 (Botella et al., 2002; Li et al., 2005a). Moreover, in several cell contexts, KLF6 inhibits cell growth through various mechanisms including activation of TGF β 1 and p21, repressing cyclin D1 and inhibiting c-Jun function (Slavin et al., 2004; McConnell and Yang, 2010). Differentiation of multiple cell lineages such as, skeletal muscle, cartilage, skin and nasal epithelium correlates with an increase in the expression of the cell cycle inhibitor p21 (Parker et al., 1995). Thus, published data and the findings described herein suggest that KLF6 might contribute to the complex set of events that occur during trophoblast differentiation by activation of CDK inhibitors such as p21. However, since p21 has also been implicated in controlling cell apoptosis (Abbas and Dutta, 2009), it would be important to further address the role of KLF6 in trophoblast turnover in normal and pathological pregnancy.

KLF6 has been implicated in the differentiation process of various cell systems, including corneal development (Nakamura et al., 2004), hematopoiesis and vasculogenesis (Matsumoto et al., 2006). In addition, KLF6 is early up-regulated during the differentiation of pre-adipocytes towards adipocytes and experimental evidence strongly suggest that it

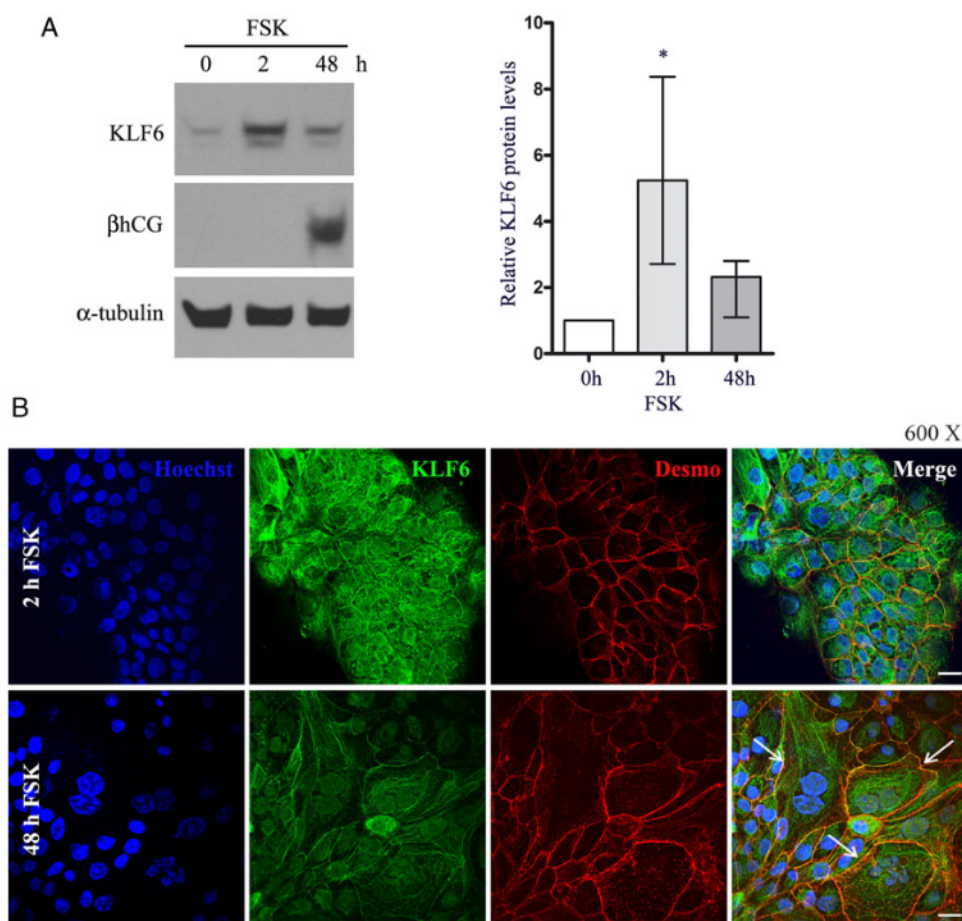


Figure 3 KLF6 protein expression in FSK-treated BeWo cells. **(A)** Protein extracts were prepared from BeWo cells treated with 10 μ M forskolin (FSK) for the indicated times and then subjected to western blot analysis to immunodetect KLF6, β hCG and α -tubulin. Biochemical differentiation was confirmed by the increase in β hCG expression. A representative blot and the quantification of three independent experiments are shown; the bars represent the median and the interquartile range, * $P < 0.05$ (Kruskal–Wallis and the Dunn’s multiple comparison post-test). **(B)** BeWo cells were treated with FSK for 2 (upper panels) or 48 h (lower panels) and immunostained for KLF6 (green) and desmoplakin (red, Desmo). Nuclei were counterstained with Hoechst 33342 dye (blue) and the overlay is shown (merge). Arrows indicate multinucleated structures formed after 48 h of FSK treatment. Bar, 10 μ m. Original magnification 600 \times .

promotes pre-adipocyte differentiation through histone deacetylase 3-dependent repression of the proto-oncogene *Delta-like 1* (Li *et al.*, 2005b). In HepG2 cells, KLF6 decreases hepatocyte growth with the concomitant reduction of cyclin D1 and β -catenin and increases cellular differentiation based on the induction of albumin, E-cadherin and decreased α -fetoprotein (Kremer-Tal *et al.*, 2007). Another proto-oncogene, the *pituitary tumour-transforming gene 1*, is also down-regulated by KLF6 in phorbol 12-myristate 13-acetate-induced myeloid cell lines differentiation (Chen *et al.*, 2013). Present data support the notion that KLF6 participates not only in the differentiation of cells derived from the epiblast lineage but also from the trophoblast.

We had previously reported that KLF6 overexpression in the trophoblast-derived JEG-3 cell line enhanced the expression of human β hCG and PSG. In addition, it transactivated β hCG5, PSG5 and PSG3 gene promoters, supporting a functional role for KLF6 in the transcriptional regulation of these placenta-specific marker genes (Racca *et al.*, 2011). Surprisingly, we found that the expression of neither β hCG5

nor PSG3 was impaired when KLF6 expression was reduced in differentiating vCTBs. Trophoblast fusion has been widely associated with biochemical differentiation reflected by the expression and synthesis of pregnancy hormones, like hCG and hPL, and other STB-specific proteins like PSG (Knöfler *et al.*, 2000; Camolotto *et al.*, 2010). Indeed, in *in vitro* CTB syncytialization as well as in FSK and cAMP-induced BeWo cells, fusion correlates with a marked induction in β hCG expression. Moreover, several studies suggest that morphological differentiation is required before the cells undergo biochemical differentiation, as impaired *in vitro* cell fusion is often accompanied by a diminished synthesis of β hCG (Handwerger, 2010). However, Kao *et al.* (1988) demonstrated that CTB cells cultured under serum-free conditions and in the absence of extracellular matrix proteins do not fuse, but retain their ability to secrete hCG. Since this early report, a growing body of evidence support the notion that both morphological and biochemical differentiation processes are related, but might be dissociated, and are regulated by partially different signalling pathways (Pidoux *et al.*, 2012; Ji *et al.*,

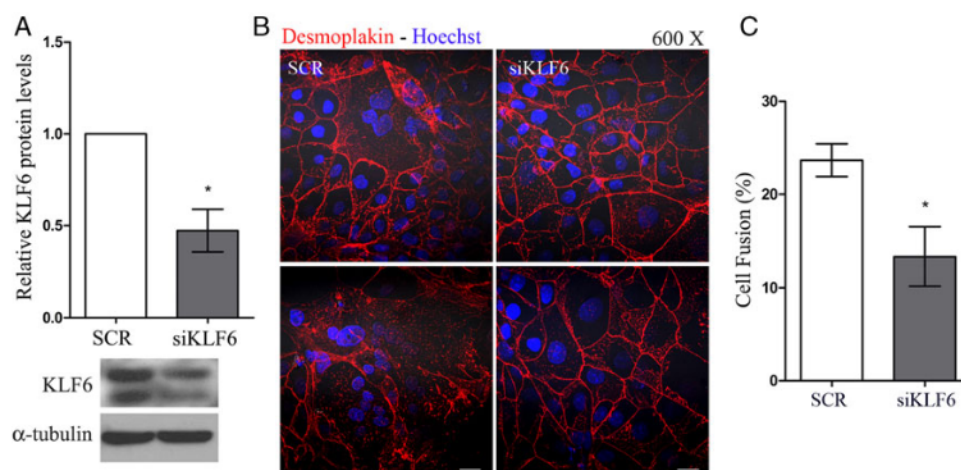


Figure 4 KLF6 involvement in BeWo cell fusion. BeWo cells were transfected with small interfering KLF6 RNA (siKLF6) or scrambled siRNA (SCR) as control and treated with 10 μ M forskolin (FSK) for 48 h. **(A)** Western blot analysis of protein extracts confirmed KLF6 down-regulation in siKLF6 transfected cells. A representative blot is shown; the bar graph represents the densitometric analysis of three independent experiments where KLF6 levels are expressed relative to α -tubulin, * $P < 0.05$ (t -test). **(B)** Morphological differentiation was evaluated by desmoplakin immunostaining (red) and nuclei counterstaining with Hoechst 33342 (blue). Bar, 10 μ m. Original magnification 600 \times . Representative images of three independent experiments are shown. **(C)** Percentage of cell fusion in cells treated with siKLF6 versus SCR. Graphs represent the quantification of three independent experiments. Results are expressed as mean \pm SEM, * $P < 0.05$ (t -test).

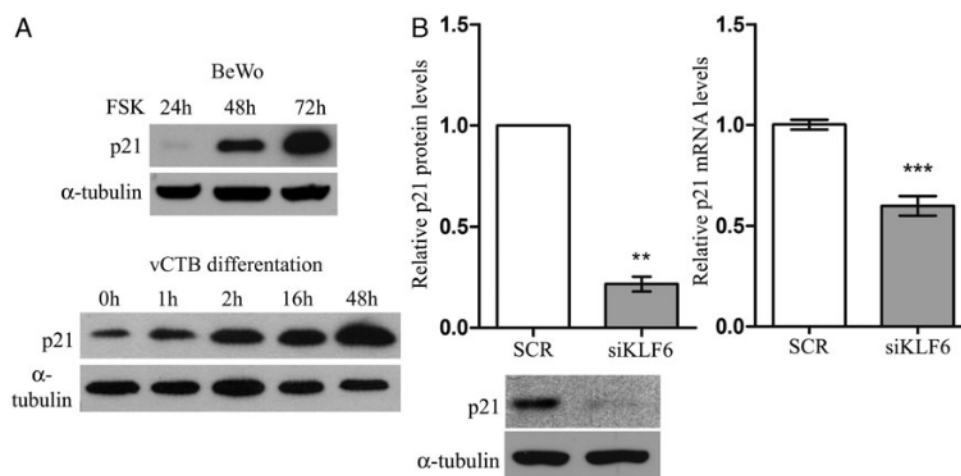


Figure 5 KLF6 knockdown reduces p21 expression in differentiating trophoblast cells. **(A)** Protein extracts from forskolin-treated (FSK) BeWo cells (upper panel) and differentiating primary villous cytotrophoblast (vCTB) cells (lower panel) were subjected to western blot assays to immunodetect p21 and α -tubulin as loading control. One representative blot of each trophoblast model is shown. **(B)** BeWo cells were transfected with small interfering KLF6 RNA (siKLF6) or scrambled siRNA (SCR) and 24 h later treated with 10 μ M FSK for additional 48 h. Cell extracts were subjected to western blot (left panel) and quantitative real-time RT-PCR (right panel) analysis to evaluate p21 expression normalized to α -tubulin or cyclophilin A, respectively. Graphs represent the quantification of three independent experiments performed in triplicate with vCTBs purified from three individual placentas. Results are expressed as mean \pm SEM; ** $P < 0.01$; *** $P < 0.001$ (t -test).

2013). Thus, our results bring further evidence to support that trophoblast fusion is not a prerequisite for functional differentiation.

hCG can act in an autocrine/paracrine manner to increase syncytium formation (Yang et al., 2003), and increased hCG levels have been reported in pre-eclampsia as well as in trisomy 21 pregnancies, where trophoblast fusion and syncytium formation are defective (Pidoux

et al., 2012; Ji et al., 2013). Therefore, it is possible to postulate that when cell fusion is impaired due to KLF6 silencing, hCG expression is maintained in order to preserve some cell functionality. The same should hold true for PSG expression. In this sense, a recent report reveals that PSG mRNA level is increased in placentas and maternal plasma of severe fetal growth restriction pregnancies (Whitehead

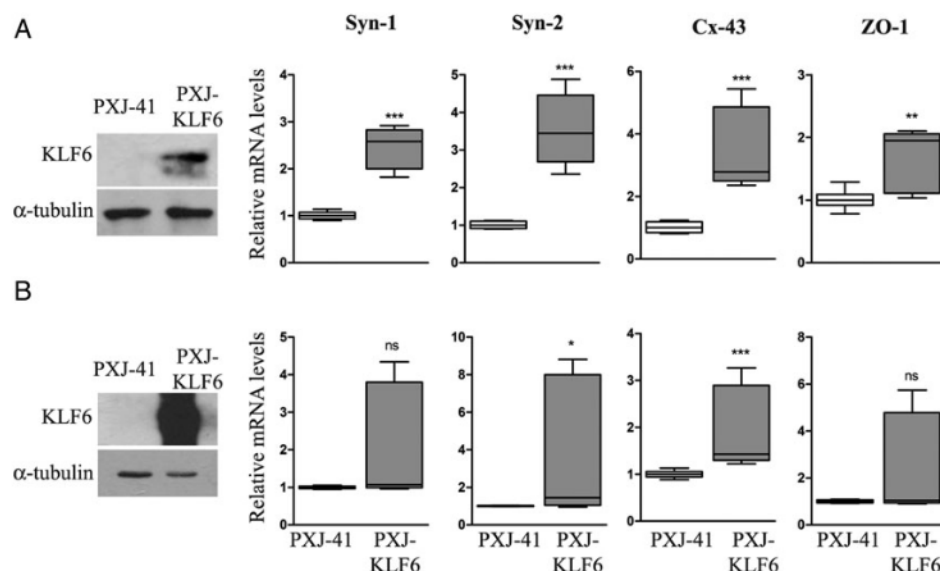


Figure 6 KLF6 overexpression up-regulates mRNA levels of genes involved in trophoblast cell fusion. JEG-3 (**A**) and villous cytotrophoblast (vCTB) (**B**) cells were transfected with the KLF6 expression plasmid (PXJ-KLF6, grey bars) or the empty vector (PXJ-41, white bars). Forty-eight hours after transfection, protein extracts were subjected to western blot analysis to immunodetect KLF6 and α -tubulin. One representative blot for each cell line is shown. Expression level of the mRNAs encoding Syn-1, Syn-2, Cx-43 and ZO-1 was quantified by quantitative real-time RT-PCR. Results were normalized to cyclophilin A and expressed according to the $2^{-\Delta\Delta C_t}$ method. The mRNA level obtained in cells transfected with the empty vector was used as calibrator. Relative transcript levels are depicted as boxplot graphs representing the medians (horizontal bars), the 25–75th percentile interquartile range (box limits) and the lowest and highest values (whiskers) of three independent experiments performed in triplicate using vCTBs isolated from three individual placentas; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns, non-significant (Mann–Whitney test).

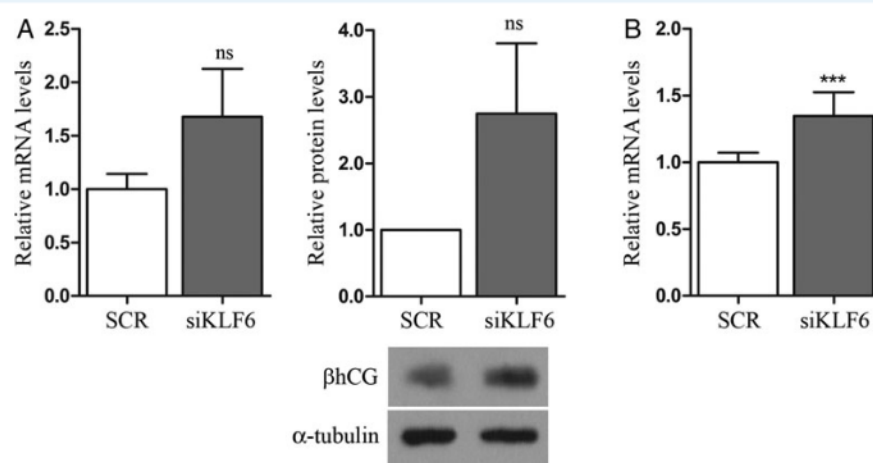


Figure 7 Expression of biochemical differentiation markers in siKLF6-treated vCTB cells. Villous cytotrophoblasts (vCTB) cells were transfected with small interfering KLF6 RNA (siKLF6) or scrambled siRNA (SCR) as control after 16 h of culture. Forty-eight hours after transfection, transcript and protein extracts were prepared and subjected to quantitative real-time RT-PCR (qRT-PCR) or western blot analysis, respectively. β hCG transcript and protein levels (**A**) and PSG3 transcript expression (**B**) were analysed. qRT-PCR results were normalized to cyclophilin A and expressed according to the $2^{-\Delta\Delta C_t}$ method using the mRNA level obtained in cells transfected with the SCR as calibrator. Bar graphs represent the median and interquartile range of three independent experiments performed in triplicate; *** $P < 0.001$, ns, non-significant (Mann–Whitney test). β hCG protein expression was assessed by western blot. One representative blot and the quantification of three independent experiments are shown, ns, non-significant (t-test).

et al., 2013). However, reduced serum levels of the placental PSG proteins in the early second trimester of pregnancies with subsequent pre-eclampsia or with fetal growth restriction have also been reported

(Bersinger and Odegard, 2004). Further studies are needed to fully understand the relation between impaired cell fusion and sustained hCG and PSG synthesis.

Regulation of fusion is a complex process precisely coordinated by a set of multiple factors and essential for the establishment and maintenance of gestation and completion of a successful pregnancy. Indeed, pregnancy complications such as pre-eclampsia and intrauterine growth restriction, among others, are associated with failures in STB formation and turnover. Even though the participation of one single master fusogenic factor has been excluded, there is growing evidence that the deregulation of one of the involved factors might have drastic consequences in trophoblast fusion, placental development and therefore, the pregnancy outcome (Gauster et al., 2009). Present results demonstrate that KLF6 is required for proper trophoblastic cell fusion since KLF6 knockdown resulted in cell–cell fusion impairment accompanied by a decrease in the expression of the fusogenic protein Syn-1 and the cell cycle inhibitor p21. Therefore, we suggest that KLF6 transcription factor is a novel player orchestrating human placental trophoblast differentiation into a functional syncytium.

Supplementary data

Supplementary data are available at <http://molehr.oxfordjournals.org/>.

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Authors' roles

A.C.R.: conception and design of study, experimental work and acquisition of data, analysis and interpretation of data, manuscript preparation, final approval of the manuscript. M.E.R.: experimental work and acquisition of data, analysis and interpretation of data, revising draft article critically, final approval of the manuscript. S.C.: experimental work and acquisition of data, analysis and interpretation of data, revising draft article critically, final approval of the manuscript. S.G.R.: conception and design of study, analysis and interpretation of data, revising draft article critically, contributed reagents, final approval of the manuscript. G.M.P.D.: conception and design of study, analysis and interpretation of data, contributed reagents, manuscript writing, final approval of the manuscript.

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Conflict of interest

None declared.

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