CITED2 modulation of trophoblast cell differentiation: insights from global transcriptome analysis

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Abstract

Trophoblast stem (TS) cells possess the capacity to differentiate along a multi-lineage pathway yielding several specialized cell types. The regulatory network controlling trophoblast cell differentiation is poorly understood. Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain, 2 (CITED2) has been implicated in the regulation of placentation; however, we know little about how CITED2 acts to influence trophoblast cells. Rat Rcho-1 TS cells can be manipulated to proliferate or differentiate into specialized trophoblast lineages and are an excellent model for investigating trophoblast differentiation. CITED2 transcript and protein showed a robust induction during Rcho-1 TS cell differentiation. We used an shRNA knockdown approach to disrupt CITED2 expression in order to investigate its involvement in trophoblast cell differentiation. RNA-sequencing was used to examine the impact of CITED2 on trophoblast cell differentiation. CITED2 disruption affected the differentiating trophoblast cell transcriptome. CITED2 possessed a prominent role in the regulation of cell differentiation with links to several signal transduction pathways and to hypoxia-regulated and coagulation processes. In summary, our findings indicate that CITED2 contributes to the regulation of trophoblast cell differentiation.

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Introduction

The placenta has a diverse set of responsibilities ensuring the survival, growth, and development of the embryo/fetus within the mother's reproductive tract (Amoroso 1968, Cross *et al.* 1994, Geigiades *et al.* 2002). In most primates and rodents, these tasks are accomplished through the formation of a hemochorial placenta where maternal blood directly bathes specialized extraembryonic cells termed trophoblast. The hemochorial placenta, through the activities of trophoblast cells, modifies maternal physiology via intrauterine migration and vascular remodeling and the production of hormones/cytokines (Cross *et al.* 1994, Pijnenborg *et al.* 2006, Soares *et al.* 2014). The hemochorial placenta also regulates the bidirectional transport of nutrients and wastes between the mother and the fetus (Sibley *et al.* 1997, Knipp *et al.* 1999, Watson & Cross 2005, Dilworth & Sibley 2013). Execution of these functions requires coordinated temporal and spatial differentiation of stem and progenitor cells into specialized trophoblast cell types and their organization into a hemochorial placenta (Roberts & Fisher 2011). The rat has proven to be a superb model for investigating hemochorial placentation (Soares *et al.* 2012).

A mature rat hemochorial placenta consists of two major compartments, the junctional zone and the labyrinth zone (Soares *et al.* 1996, 2012). The junctional zone lies at the maternal–placental interface and is composed of progenitor cells, which differentiate into four distinct specialized trophoblast cell types. Trophoblast giant cells are large polyploid cells arising by endoreduplication. They possess a remarkable capacity for steroid and peptide hormone biogenesis (Soares *et al.* 1996, Soares 2004). Spongiotrophoblast cells are a major constituent of the mature junctional zone and produce peptide hormones. Glycogen trophoblast cells accumulate glycogen and are viewed as an energy reservoir for the developing placenta and fetus. Junctional zone progenitors also give rise to invasive trophoblast cells, which exit the placenta and infiltrate the uterine compartment where they target and facilitate uterine spiral artery remodeling and the delivery of nutrients to the placenta (Ain *et al.* 2003, Soares *et al.* 2014). In contrast, the labyrinth zone is situated at the placental–fetal interface and consists of progenitor cells, which can differentiate into trophoblast giant cells or fuse to form syncytial trophoblast (Soares *et al.* 2012). The latter cell layers form the barrier between maternal and fetal compartments and

directly connect with fetal vasculature and promote bidirectional transport (Knipp *et al.* 1999). Trophoblast cell differentiation can be effectively modeled in Rcho-1 trophoblast stem (TS) cells (Faria *et al.* 1991, Sahgal *et al.* 2006). Rcho-1 TS cells were derived from a rat choriocarcinoma (Teshima *et al.* 1983) and can be maintained in the stem/progenitor state or the culture conditions can be modified resulting in their differentiation into specialized trophoblast lineages, including but not limited to trophoblast giant cells (Faria *et al.* 1991, Sahgal *et al.* 2006). Transcriptome profiles of Rcho-1 TS cells in the stem and differentiated states reflect the known behavior of trophoblast cell lineages developing in the rat hemochorial placenta (Kent *et al.* 2010).

Several signaling pathways have been implicated in the regulation of trophoblast development (Soares *et al.* 2014). Among these pathways, hypoxia-inducible factor (HIF) and Fos-like antigen 1/Jun B proto-oncogene transcriptional regulators have been shown to influence the regulation of trophoblast cell differentiation in mice, rats, and humans (Schorpp-Kistner *et al.* 1999, Adelman *et al.* 2000, Caniggia *et al.* 2000, Schreiber *et al.* 2000, Chakraborty *et al.* 2011, Kent *et al.* 2011, Renaud *et al.* 2014, Kubota *et al.* 2015). Both transcription factor complexes recruit histone acetyl transferases, CREB-binding protein (CREBBP), and E1A-binding protein p300 (EP300) in activating their gene targets (Vo & Goodman 2001, Bedford *et al.* 2010, Semenza 2010).

Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain, 2 (CITED2) is a transcriptional co-regulator that regulates interactions between DNA-binding proteins and histone-modifying enzymes, including transcription factor–CREBBP/EP300 interactions (Braganca *et al.* 2003, Freedman *et al.* 2003). CITED2 is widely expressed in embryonic and extraembryonic tissues, is required for normal development of the embryo and placenta (Bamforth *et al.* 2001, Barbera *et al.* 2002, Yin *et al.* 2002, Weninger *et al.* 2005, Withington *et al.* 2006, Moreau *et al.* 2014), and is also expressed in the uterus where it is essential for embryo implantation (Yoo *et al.* 2015). CITED2 has been implicated in the regulation of two transcription factor families: transcription factor-activator protein 2 (TFAP2) and HIF (Bhattacharya *et al.* 1999, Bamforth *et al.* 2001, Yin *et al.* 2002, Braganca *et al.* 2003, Freedman *et al.* 2003). Members of these transcription factor families are significant contributors to the regulation of trophoblast cell differentiation (Simon & Keith 2008, Dunwoodie 2009, Chakraborty *et al.* 2011, 2012, Kuckenberg *et al.* 2012); however, specific roles for CITED2 in regulating trophoblast cell differentiation have not been determined.

CITED2 is dynamically regulated during rat trophoblast cell development (Kent *et al.* 2010), and based on mouse mutagenesis, experimentation contributes to development of both junctional and labyrinthine tro-

phoblast lineages (Withington *et al.* 2006, Moreau *et al.* 2014). In this study, we explore the roles for CITED2 in the regulation of trophoblast cell differentiation. We utilize an *in vitro* loss-of-function approach and RNA-sequencing (RNA-seq) to identify targets downstream of CITED2 action.

Materials and methods

Rcho-1 TS cell culture

Rcho-1 TS cells are an effective model system for interrogating regulatory pathways controlling rat trophoblast cell differentiation (Faria & Soares 1991, Kent *et al.* 2010, 2011, Kubota *et al.* 2015) and are used to investigate a role for CITED2 in the regulation of trophoblast cell differentiation. Rcho-1 TS cells were maintained in Stem State Medium (RPMI-1640 culture medium (Gibco–Life Technologies) supplemented with 20% fetal bovine serum (Atlanta Biologicals, Norcross, GA, USA), 50 µM 2-mercaptoethanol (Sigma–Aldrich), 1 mM sodium pyruvate, 100 µM penicillin, and 100 U/mL streptomycin), as previously reported (Faria & Soares 1991, Sahgal *et al.* 2006). Rcho-1 TS cells were grown to near confluence and differentiation was induced by replacing the Stem State Medium with Differentiation State Medium (NCTC-135 medium (Sigma–Aldrich) supplemented with 1% horse serum (Atlanta Biologicals), 50 µM 2-mercaptoethanol, 1 mM sodium pyruvate, 10 mM HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 38 mM sodium bicarbonate, 100 µM penicillin, and 100 U/mL streptomycin).

CITED2 knockdown

An shRNA knockdown approach was used to disrupt CITED2 expression in Rcho-1 TS cells and to investigate the role of CITED2 in trophoblast cell differentiation. Three different *Cited* shRNAs (shCited2-1: 5'-gaagctcaacaaccagtatttc-3'; shCited2-2: 5'-catcgacgaggaagtgtctatctc-3'; and shCited2-3: 5'-agaagctcaacaaccagtatt-3'; Sigma–Aldrich) and a control shRNA (shCtrl, plasmid no. 1864; Addgene, Cambridge, MA, USA) were packaged into lentiviral vectors and used to produce lentiviral particles in 293FT cells (Kent *et al.* 2011, Asanoma *et al.* 2012). Culture supernatants containing lentiviral particles were harvested, centrifuged to remove cell debris, filter sterilized, concentrated by ultracentrifugation, and stored at –80°C until used. Rcho-1 TS cells maintained in Stem State Medium were exposed to lentiviral particles, selected with puromycin dihydrochloride (2 µg/mL; Sigma–Aldrich) for 2 days, and then maintained in a lower concentration of the antibiotic (1 µg/mL). Puromycin selection was removed during *in vitro* differentiation. Knockdown efficiencies were monitored using quantitative RT-PCR (qRT-PCR) and western blotting.

qRT-PCR

Transcript levels were measured by qRT-PCR. RNA was extracted using TRI reagent (Sigma–Aldrich) according to the manufacturer's instructions. cDNAs were reverse transcribed

using a High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems as instructed by the manufacturer. Power SYBR Green PCR Master Mix (Applied Biosystems) was used in the PCR. Primers utilized in the analyses are provided in Table 1. Amplification and fluorescence detection were carried out using an ABI Prism 7500 Real-Time PCR System (Applied Biosystems). The thermal profile for real-time PCR consisted of an initial hold step (95°C for 10 min) and 40 cycles at 95°C for 15 s and annealing at 60°C for 1 min. Amplification efficiencies of each target and the reference gene, *18S* rRNA, were examined through their calibration curves and found to be comparable. Average cycle threshold values for each target were determined by Sequence Detection System Software v1.2 (Applied Biosystems). Each run was completed with a melting curve analysis to confirm the specificity of amplification and the absence of primer dimer.

Western blotting

Cell lysates were prepared in buffer containing 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, and 50 mM dithiothreitol. Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories). Total proteins were separated by SDS-PAGE and transferred to PVDF membranes. CITED2 was detected using anti-CITED2 antibody (ab108345; Abcam) at a dilution of 1:500.

RNA-seq analysis

Transcriptomic profiles in control shRNA-treated Rcho-1 TS cells and *Cited2* shRNA knockdown Rcho-1 TS cells ($n=3$ each) were performed using RNA-seq analysis. cDNA libraries from total RNA samples were prepared with Illumina TruSeq RNA sample preparation kits (Illumina, San Diego, CA, USA). Five hundred nanograms of total RNA were used as input. Poly-A-containing RNAs were purified with oligo-dT-coated magnetic beads. RNA fragmentation, first- and second-strand cDNA synthesis, end repair, adaptor ligation, and PCR amplification were performed

according to the manufacturer's recommendations. The cDNA libraries were validated for RNA integrity using an Agilent 2100 Bioanalyzer (Agilent Technologies) before sequencing.

cDNA libraries were clustered onto a TruSeq paired-end flow cell and sequenced (100 bp-end reads) using a TruSeq 200 Cycle SBS Kit (Illumina). Samples were run on an Illumina HiSeq2000 sequencer located at the University of Kansas Medical Center Genome Sequencing Facility and sequenced in parallel with other samples to ensure the data generated for each run were accurately calibrated during data analysis. Following generation of sequencing images, the pixel-level raw data collection, image analysis, and base calling were performed by Real-Time Analysis Software (Illumina). The base call files (*.bcl) were converted to *.qseq files by Illumina's BCL Converter, and the *.qseq files were subsequently converted to *.fastq files for downstream analysis. Reads from *.fastq files were mapped to the rat reference genome (Ensembl Rnor_5.0.78) using CLC Bio Genomics Workbench 7.0 (CLC Bio, Aarhus, Denmark). The mRNA abundance was expressed in reads per kilobase of exon per million reads mapped. Statistical significance was calculated by empirical analysis of digital gene expression in the CLC Bio Genomics Workbench. A corrected false discovery rate (FDR) of 0.05 was used as a cutoff for significant differential expression (control vs *Cited2*-knockdown). Functional patterns of transcript expression were further analyzed using Ingenuity Pathway Analysis (Qiagen) and Database for Annotation, Visualization and Integrated Discovery (DAVID v6.7; <https://david.ncifcrf.gov/>). Results from the RNA-seq analysis were validated using qRT-PCR. Primer sets for the qRT-PCR are shown in Table 1.

Statistical analysis

Values are expressed as the mean $\pm$ S.E.M. Comparisons between more than two groups were made using one-way ANOVA following log10 transformation of the raw data and multiple comparisons were done using Dunnett's *post hoc* test. The procedure

Table 1 Primers for qRT-PCR analysis.

Target gene	Forward primer	Reverse primer	Accession number
<i>Apoa4</i>	ACAGCTCAATACCCTCTTCCAG	GGCTCACTTGTGGCATGG	NM_012737
<i>Ass1</i>	AAAAAGGGTCCCTGTGAAG	ATGAGCGTGGTAAAGGATGG	NM_013157
<i>Cited2</i>	GAAGGACTGGAAATGGCAGA	GCGCCGTAGTGTATGTGCT	NM_053698
<i>Egln3</i>	CTCCTATGCCACCAGGTACG	ACAAGGTAGGGAGCCAAACG	NM_019371
<i>F3</i>	GTGTCCTGGGAGAAACACTCAT	CCAGCAGAGGTCTCGGTAAC	NM_013057
<i>Il1r2</i>	CATGGGAGATGCAGGCTATT	TACCAGTCCCAGGAACACC	NM_053953
<i>Krt15</i>	GATCCAGGGGCTCATTACCG	AGCAGCCATCTTAGCATCCTG	NM_001004022
<i>Mest</i>	CAAGCCGAGACCACATCAGT	GTGAGACGGCCAGAACGATT	NM_001009617
<i>Mmp12</i>	ACATGAAGCGTGCGGATGTA	AGGAACAGGTTTGTGCCTTGA	NM_053963
<i>Prl8a5</i>	CCTCCGGAAGCATTTAACAG	GATTTGGGTTTTGCACGATT	NM_173110
<i>Prl8a9</i>	GGCACATTCCTGATGCTTGC	CCTAATCAGTTTTGAGTTAAGAGCC	XM_006253905
<i>Ptprk</i>	ACTACATCGATGGCTACCAGAG	AGCATTTACCCGACCAACT	XM_008758616
<i>Pxdc1</i>	CAACCCAGCTTTCAAAGTCC	CTGGGTCATCACCATCTTCC	NM_001025719
<i>Thbd</i>	CATGGACTCGGGAAGTGAC	TGGTAGCCTGTTTCGCACAT	NM_031771
<i>Timp3</i>	ACAGACGCCAGAGTCTCCTA	ACCTCAAGTCTGTCCGGGTA	NM_012886
<i>Top2a</i>	CAGCGTGTGAGCCTGAATG	TAACTTGGGAGCATGGGCAG	NM_022183
<i>Tpbpa</i>	TGAATTGCAAGAGCAGAAGGGTA	CATCGCCAAGTGACTGTGCT	NM_172073
<i>18S</i> rRNA	GCAATTATCCCCATGAACG	GGCCTCACTAAACCATCCAA	NR_046237

Figure 1 CITED2 expression in Rcho-1 TS cells. Differentiation-stimulated induction of *Cited2* transcript (A) and protein (B) during Rcho-1 TS cell differentiation. Effectiveness of CITED2 shRNAs in the inhibition of CITED2 mRNA (C) and protein (D) in differentiating Rcho-1 TS cells. Rcho-1 TS cells were stably infected with lentiviral vectors containing an shRNA to a control (Ctrl) sequence or three different and independent shRNAs to *Cited2* (shCited2-1, shCited2-2, and shCited2-3) and differentiated for 8 days. *Cited2* sequences targeted by each shRNA are provided in the 'Materials and methods' section. The effectiveness of the *Cited2* shRNAs was compared with an shRNA to a Ctrl sequence, which does not recognize any known mammalian transcript. Statistical comparisons were made using one-way ANOVA and multiple comparisons were done using Dunnett's *post hoc* test. * $P < 0.01$ and ** $P < 0.001$.

for analyzing the RNA-seq data is described in the preceding paragraph.

Results

CITED2 is a transcriptional co-regulator implicated in the regulation of placentation (Withington *et al.* 2006, Moreau *et al.* 2014). We sought to gain insight into the role of CITED2 in trophoblast development using a loss-of-function approach in rat Rcho-1 TS cells.

Cited2 expression in differentiating trophoblast cells

Initially, we profiled the expression of CITED2 transcript and protein in stem and differentiating Rcho-1 TS cells using qRT-PCR and western blotting respectively. Both CITED2 mRNA and protein exhibited a robust increase in expression as Rcho-1 TS cells underwent differentiation (Fig. 1A and B). These observations are consistent with earlier findings correlating *Cited2* expression with trophoblast differentiation (Kent *et al.* 2010) and directed us to examine the developmental consequences of *Cited2*-knockdown in differentiating Rcho-1 TS cells.

Figure 2 Analysis of transcripts affected by *Cited2*-knockdown in trophoblast cells. RNA-seq was performed on Rcho-1 TS cells expressing either control shRNA or an shRNA to *Cited2* (shCited2-2) under differentiating conditions. (A) Pie chart showing the number of transcripts with altered expression levels in Rcho-1 TS cells expressing either control shRNA or shCited2-2. (B) Volcano plot of differentially expressed transcripts. The x-axis shows log₂ fold changes in expression and the y-axis shows corresponding log₁₀ *P* values. Some of the transcripts altered greater than or equal to two-fold ($P < 0.05$) in cells expressing shCited2-2 are highlighted in red.

Identification of CITED2 downstream targets in differentiating trophoblast cells

We utilized an shRNA approach to inhibit the expression of CITED2 in Rcho-1 TS cells and RNA-seq to characterize the transcriptome of the manipulated cells. Rcho-1 TS cells were stably infected with lentiviral constructs containing shRNAs specifically targeting three different and independent sequences within *Cited2* termed shCited2-1, shCited2-2, or shCited2-3 or a control shRNA and exposed to differentiation culture conditions. CITED2 shRNAs effectively inhibited the expression of the differentiation-dependent up-regulation of CITED2 mRNA and protein (Fig. 1C and D). Disruption of CITED2 expression did not interfere with trophoblast cell proliferation or morphological indices of trophoblast giant cell formation. It is important to appreciate that trophoblast cell differentiation is more than the formation of trophoblast giant cells. Trophoblast giant cells can possess distinct phenotypes (Simmons *et al.* 2007) and other distinct differentiated trophoblast cell lineages can arise from TS cells (Gardner & Beddington 1988, Soares

Table 2 Selected transcripts identified by RNA-seq in differentiating control and *Cited2* shRNA-treated Rcho-1 TS cells.

Gene description	Gene symbol	Biological properties	Chromosome	<i>Cited2</i> shRNA/ control shRNA
Trophoblast-specific protein alpha	<i>Tpbpa</i>	Related to cysteine-type endoprotease	17	12.1
Matrix metalloproteinase 12	<i>Mmp12</i>	Metalloelastase, ECM, coagulation	8	8.1
Egl-9 family hypoxia-inducible factor 3	<i>Egln3</i>	Prolyl hydroxylase, hypoxia signaling	6	5.5
Prolactin family 8, subfamily a, member 5	<i>Prl8a5</i>	Cytokine, hormone	17	5.0
Thrombomodulin	<i>Thbd</i>	Thrombin receptor, coagulation	3	4.5
Apolipoprotein A-IV	<i>Apoa4</i>	Lipid metabolism	8	4.4
TIMP metalloproteinase inhibitor 3	<i>Timp3</i>	Inhibitor of matrix metalloproteinases, ECM	7	3.6
Keratin 15	<i>Krt15</i>	Intermediate filaments, epithelial cells	10	3.3
Coagulation factor 3	<i>F3</i>	Coagulation	2	3.2
Prolactin family 8, subfamily a, member 9	<i>Prl8a9</i>	Cytokine, hormone	17	3.2
Interleukin 1 receptor, type II	<i>Il1r2</i>	Decoy receptor for IL1 ligands	9	2.6
PX domain containing 1	<i>Pxdc1</i>	Phosphatidylinositol binding	17	2.5
Argininosuccinate synthase 1	<i>Ass1</i>	Arginine biosynthetic pathway	3	0.37
Mesoderm specific transcript	<i>Mest</i>	Hydrolase superfamily, imprinted, developmentally regulated	4	0.34
Protein tyrosine phosphatase, receptor type K	<i>Ptprk</i>	Signaling pathway controlling cell-cell adhesion, growth control, and invasion	1	0.30
Topoisomerase (DNA) II alpha	<i>Top2a</i>	Nuclear protein regulating chromosome dynamics	10	0.17
Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain 2	<i>Cited2</i>	Transcription co-regulator	1	0.16

ECM, extracellular matrix.

et al. 1996, 2012, Simmons & Cross 2005). Thus, RNA-seq was performed on Rcho-1 TS cells stably expressing control shRNA vs CITED2 shRNA (shCited2-2) on day 8 of differentiation to gain additional insights into the role of CITED2 in the regulation of trophoblast cell differentiation. Results from the RNA-seq analysis are deposited in the Gene Expression Omnibus (accession no. GSE74748). A total of 148 known transcripts (105 up-regulated vs 43 down-regulated) exhibited a significant change in expression (FDR, $P < 0.05$) in control vs CITED2 shRNA cells (Fig. 2A and B). Table 2 includes a list of selected up-regulated and down-regulated transcripts. Canonical pathways sensitive to CITED2 disruption included signal transduction cascades (e.g., ERK/MAPK, peroxisome proliferator-activated receptor, eukaryotic initiation

factor 2, paxillin, integrin-linked kinase, phospholipase C, and integrin) and processes associated with leukocyte extravasation and phagocytosis (Table 3). Additional insights from the pathway analyses were not evident. qRT-PCR was used to validate expression of a set of selected up-regulated and down-regulated transcripts (Fig. 3). Several features characterized subsets of up-regulated transcripts: i) transcripts expressed by non-trophoblast giant cell lineages of differentiated trophoblast cells (*Tpbpa*, *Mmp12*, *Prl8a5*, and *Prl8a9*); ii) known hypoxia/HIF-responsive transcripts (*Tpbpa*, *Mmp12*, and *Egln3*); and iii) transcripts encoding proteins associated with coagulation/thrombosis (*Mmp12*, *F3*, and *Thbd*). A prominent feature associated with several down-regulated transcripts (*Top2a*, *Ptprk*, and *Mest*) is their known linkage

Table 3 Analysis of pathways affected for CITED2 disruption in differentiating Rcho-1 TS cells.

Canonical pathways	–Log (<i>P</i> value)	Ratio	Z-score	Molecules
ERK/MAPK signaling	1.44	3.09×10^{-2}	–0.45	ITGB1, FOS, H3F3A/H3F3B, PRKCI, and SOS1
PPAR signaling	2.53	5.75×10^{-2}	–1.34	IL1R2, FOS, HSP90B1, SOS1, and CITED2
EIF2 signaling	3.68	5.59×10^{-2}	1.34	PABPC1, RPL14, RPL22, SOS1, RPL17, RPL10, EIF4A2, and RPS25
Leukocyte extravasation	2.56	4.19×10^{-2}	1.63	ITGB1, TIMP3, PRKCI, CDH5, MMP12, ACTG1, and ACTN1
Paxillin signaling	4.17	7.78×10^{-2}	1.63	ITGB1, SOS1, ARHGEF6, ITGAV, PTPN12, ACTG1, and ACTN1
Fcy receptor-mediated phagocytosis in macrophages and monocytes	1.78	4.60×10^{-2}	2.00	HMOX1, PRKCI, ARPC2, and ACTG1
ILK signaling	3.39	5.06×10^{-2}	2.12	ITGB1, FOS, FN1, ARHGEF6, FERMT2, TMSB10/TMSB4X, ACTG1, and ACTN1
Phospholipase C signaling	1.64	3.08×10^{-2}	2.24	ITGB1, HMOX1, PRKCI, AHNAK, SOS1, and ARHGEF6
Integrin signaling	2.39	3.91×10^{-2}	2.65	ITGB1, ASAP1, ARPC2, SOS1, ITGAV, ACTG1, and ACTN1

PPAR, peroxisome proliferator-activated receptor; EIF2, eukaryotic initiation factor 2; ILK, integrin-linked kinase.

Figure 3 qRT-PCR validation of RNA-seq analysis. Selected transcripts affected by *Cited2*-knockdown in the RNA-seq analysis were examined by qRT-PCR. qRT-PCR measurements from Rcho-1 TS cells infected with lentiviral vectors containing an shRNA to a control (Ctrl) sequence or three different and independent shRNAs to *Cited2* (shCited2-1, shCited2-2, and shCited2-3) were performed. *Cited2* sequences targeted by each shRNA are provided in the 'Materials and Methods' section. Statistical comparisons were made using one-way ANOVA and multiple comparisons were done using Dunnett's *post hoc* test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

with the Rcho-1 TS cell stem state (Kent *et al.* 2010). Mitogen removal promotes differentiation of Rcho-1 TS cells and is also compatible with the survival of quiescent TS cells, which can be revived by reintroduction of a mitogenic stimulus (Sahgal *et al.* 2006). Collectively, our findings indicate that CITED2 may restrain expansion of TS cells in the stem state, promote TS cell quiescence, and/or direct trophoblast cell differentiation.

Discussion

CITED2 is an established regulator of cell growth and differentiation and has been implicated in a broad range of developmental events, including neural tube, heart, lung, liver, hematopoietic, gonad, adrenal, and placental morphogenesis (Bamforth *et al.* 2001, Barbera *et al.* 2002, Yin *et al.* 2002, Withington *et al.* 2006, Qu *et al.* 2007, Val *et al.* 2007, Xu *et al.* 2008, Combes *et al.* 2010, Du & Yang 2013). In this report, we provide insight into the modulatory actions of CITED2 on trophoblast cell differentiation.

A null mutation at the *Cited2* locus of the mouse leads to defects in placentation (Withington *et al.* 2006, Moreau *et al.* 2014). *Cited2*-null placentas are smaller than those from WT littermates with a profound disruption in the development of the junctional zone (Withington *et al.* 2006). Each differentiated trophoblast cell lineage is present in the *Cited2*-null placenta; however, the absence of CITED2 affects the organization and function of the differentiated trophoblast cell lineages. Differentiating Rcho-1 TS cells exhibit many features of junctional zone trophoblast cell development (Soares *et al.* 1996). Some of the most prominent effects of disrupting CITED2 in differentiating Rcho-1 TS cells was up-regulation of *Tpbpa*, *Mmp12*, *Timp3*, and *Egl3* expression. *Tpbpa* expression identifies a progenitor population of trophoblast cells within the developing junctional zone possessing the capacity to differentiate into spongiotrophoblast, glycogen cells, invasive trophoblast cells, and subsets of trophoblast giant cells (Hu & Cross 2011). MMP12 localizes to invasive endovascular trophoblast lining uterine spiral arterioles (Harris *et al.* 2010, D Chakraborty and M J Soares, unpublished observations) and is viewed as a contributor to pregnancy-dependent structural changes associated with these vessels that are critical for hemochorial placentation (Harris *et al.* 2010). MMP12 is a member of the matrix metalloproteinase family and among its targets is elastin, a prominent constituent of the arterial vasculature (Van Doren 2015). Paradoxically, the transcript for TIMP3, a broad-spectrum inhibitor of MMPs (Brew & Nagase 2010), is also up-regulated by *Cited2*-knockdown, reflecting a level of complexity to CITED2 action in differentiating trophoblast cells. *Tpbpa* and *Mmp12* are known for hypoxia- and HIF-regulated genes in rat TS cells (Chakraborty *et al.* 2011). *Egl3* is a hypoxia/HIF-responsive transcript and encodes for a negative modulator of HIF activities (Lee & Percy 2011). Even though Rcho-1 TS cells were not cultured in hypoxic conditions in the present work, there is some evidence for the involvement of HIF signaling in TS cell differentiation exposed to atmospheric conditions (Maltepe *et al.* 2005). Thus up-regulation of *Tpbpa*, *Mmp12*, and *Egl3* in the present work may reflect removal of CITED2 antagonism of HIF actions.

CITED2-knockdown also led to an increase in the expression of trophoblast cell transcripts possessing ties to blood coagulation (*Mmp12*, *F3*, and *Thbd*). MMP12 has both pro- and anti-coagulant actions via inactivation of tissue factor pathway inhibitor (Belaouaj *et al.* 2000) and the inhibition of circulating fibrinogen levels (Motterle *et al.* 2012) respectively. F3 (also known as tissue factor) is a pro-coagulant (Pawlinski *et al.* 2004) and THBD is an inhibitor of blood coagulation (Isermann *et al.* 2001, 2003, Weiler 2004). Thus, CITED2 negatively regulates multiple transcripts encoding regulatory components of the blood coagulation pathway with apparently opposing outcomes.

Trophoblast differentiation is a multi-lineage process (Gardner & Beddington 1988, Simmons & Cross 2005, Soares *et al.* 2012). *In vitro* differentiation of Rcho-1 TS cells favors trophoblast giant cell differentiation (Faria & Soares 1991, Sahgal *et al.* 2006, Kent *et al.* 2010). Transcripts exhibiting some of the most dramatic changes following CITED2 manipulation are characteristic of trophoblast lineages other than the trophoblast giant cell lineage. Thus, at least in the context of differentiating Rcho-1 TS cells, CITED2 appears to guide/restrict the specialization of specific differentiated trophoblast cell populations, potentially favoring trophoblast giant cell development and inhibiting the development of *Tpbpa*-positive progenitors and their descendants.

A key mechanism of action of CITED2 is its modulation of transcription factor interactions with the histone acetyl transferases, CREBBP and EP300 (Bhattacharya & Ratcliffe 2003, Du & Yang 2013). These histone acetyl transferases govern gene activation of numerous transcription factors (Vo & Goodman 2001, Bedford *et al.* 2010), including many that have been implicated in regulating trophoblast cell differentiation (GATA2, GATA3, AP-1, TFAP2C, ETS2, HIF1, PPARG, and SATB1; Soares *et al.* 2014, Soncin *et al.* 2015). Thus, CITED2 has the potential to modulate a wide spectrum of biological processes associated with trophoblast development. However, at this juncture, only two known modulators of trophoblast development, HIF1 and TFAP2C, have been directly connected to CITED2 (Bhattacharya *et al.* 1999, Bamforth *et al.* 2001, Yin *et al.* 2002, Braganca *et al.* 2003, Freedman *et al.* 2003). The precise mechanism(s) underlying the effects of CITED2 on the differentiated trophoblast cell transcriptome remains to be determined.

Declaration of interest

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

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