

# Glial cell missing-1 mediates over-expression of tissue inhibitor of metalloproteinase-4 in severe pre-eclamptic placental villi

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**BACKGROUND:** Severe pre-eclampsia (sPE) causes significant maternal morbidity and intrauterine growth restriction as a result of severe placental dysfunction. Defects in the formation of both extra-villous and villous trophoblast are characteristic of this disease. The outer syncytiotrophoblast layer covering the placental villi develops syncytial knots and focal necrosis while reduced invasion of the extra-villous trophoblast results in a reduced maternal blood supply and ischemia of the placental villi. The transcription factor glial cell missing-1 (GCM1) regulates formation of both types of trophoblast. GCM1 expression is reduced in placental villi of women with sPE but the functional downstream consequences of reduced GCM1 expression are unknown.

**METHODS AND RESULTS:** In floating first trimester villous explants we demonstrated increased mRNA (2.5-fold,  $n = 12$ ) and protein level (9.8-fold) of tissue inhibitor of metalloproteinase-4 (TIMP4) following repression of GCM1 ( $70 \pm 7\%$ ) by small interfering-RNA, using RT-PCR and western blot, respectively. Similar increases in TIMP4 mRNA (4.2-fold,  $n = 7$ ,  $P < 0.001$  versus control) and protein levels were found following gene silencing of GCM1 in BeWo cells ( $< 90\%$  knock down of protein). TIMP4 protein was increased in placenta from women with sPE ( $3.5 \pm 0.4$  pg/ $\mu$ g,  $n = 8$ ), compared with preterm ( $1.7 \pm 0.17$  pg/ $\mu$ g,  $n = 9$ ) and term controls ( $1.6 \pm 0.16$  pg/ $\mu$ g,  $n = 9$ ;  $P < 0.01$ ; quantified by enzyme-linked immunosorbent assay and visualized using immunohistochemistry) with reduced GCM1 expression, mostly in the pathologic syncytial knots.

**CONCLUSIONS:** TIMP4 is a downstream target of GCM1 that may link the consequences of reduced GCM-1-directed trophoblast differentiation to histologic and functional components of disordered placentation in sPE.

**Key words:** tissue inhibitors of metalloproteinase-4 / pre-eclampsia / glial cell missing-1 / placenta / trophoblast

## Introduction

Severe placental insufficiency occurs in ~5% of human pregnancies and poses a significant risk to both mother and baby for increased morbidity and mortality (Roberts and Perloff, 1977; Rey *et al.*, 2009). In extreme cases the mother develops severe early onset pre-eclampsia (sPE) placing her at risk of seizures, stroke, liver and renal failure. The defective transport functions of the diseased placenta cause co-existent severe intrauterine growth restriction (IUGR) that either leads to stillbirth, or to preterm delivery in order to avoid perinatal death. Presently the only truly effective cure for the disease is the delivery of the fetus and placenta because there is no treatment to

reverse abnormal placental differentiation. Successful placentation is dependent upon sequential growth and differentiation of the two major trophoblast lineages. First the extravillous cytotrophoblasts (EV-CTs) are a population of proliferating cells that invade the uterine wall and differentiate to remodel the maternal spiral arteries and ensure an adequate uteroplacental blood supply to the intervillous space. To match this maternal delivery the chorionic villi rapidly expand and differentiate to create a large surface area for maternal–fetal exchange. These villi are covered by the villous trophoblast compartment, which comprises proliferating villous cytotrophoblasts (VCTs) that are covered by a continuous multinucleated layer of syncytiotrophoblast (SCT). This outer SCT layer forms the fetal–

maternal interface and is responsible for active nutrient and passive gas exchange. The differentiated forms of both the EV-CT and VCT are characterized by exit from the cell cycle.

The development and maintenance of villous and extra-villous human trophoblast are regulated at the proximal level of cell cycle exit by the transcription factor and differentiation marker glial cell missing-1 (GCM1; *Baczyk et al., 2009*). Abnormalities of cell cycle activity, characterized either by excessive proliferation in chorionicarcoma, or by reduced VCT proliferation in sPE with IUGR, underscore the importance of disordered trophoblast differentiation in these diseases (*Okamoto et al., 2002; Huisman et al., 2004; Merchant et al., 2004; Myers et al., 2005; Palei et al., 2008*).

The EV-CTs undergo significant morphologic changes over the course of their development including differentiation, migration and invasion, which are thought to be regulated in part by matrix metalloproteinases (MMPs). The MMPs act to degrade the extracellular matrix (ECM) and contribute to the transformation of the maternal vasculature, however, their role in the villous compartment of the placenta is not as well understood (*Niu et al., 2000; Huisman et al., 2004; Merchant et al., 2004*). The most abundant MMPs that are involved in human placental development are MMP2 and MMP9. MMP2 activity has been shown to decrease over gestation whereas MMP9 increases (*Isaka et al., 2003*). These molecules play significant roles in extra-villous trophoblast invasion and are possibly involved in both villous trophoblast development and in placental insufficiency syndromes (*Polette et al., 1994; Xu et al., 2000; Xu et al., 2001*).

Associated with the MMPs is a group of regulatory proteins known as tissue inhibitors of metalloproteinases (TIMPs). These two protein families act in concert to regulate the breakdown of the ECM and contribute to tissue remodeling. The TIMPs inhibit the action of MMPs, influencing the processes of organogenesis, angiogenesis, cell invasion, migration and carcinogenesis (*Gomez et al., 1997; Li, 2006; Bister et al., 2007; Hilska et al., 2007; Liss et al., 2009*). There is also evidence that TIMPs play a role in cellular proliferation and apoptosis that is modulated by a mechanism separate from MMP inhibition (*Melendez-Zajgla et al., 2008*). The TIMP family consists of four subtypes (TIMP1–4) that have many structural similarities, with the N-terminus containing consensus sequences and 29 amino acid leader sequences which are cleaved during the maturation process. TIMP4 shows 51% homology with TIMP 2 & 3 and about 37% homology with TIMP1. They can be either secreted (TIMP1/2/4) or bound to the ECM (TIMP3) (*Greene et al., 1996; Olson et al., 1998*).

TIMP4 is the most recently described subtype and its function is poorly understood. TIMP4 can regulate the activity of different MMPs with the highest affinity to MMP26 and MMP2 (*Bigg et al., 2001; Hernandez-Barrantes et al., 2001; Zhao et al., 2004; English et al., 2006*). Experiments in cancer cell lines suggest additional roles for TIMP4 in migration but not in angiogenesis (*Fernandez and Moses, 2006*) and as an anti-apoptotic and cytostasis-inducing molecule within different pathways through its interaction with CD63, Bcl-X1 and Bcl-2 (*Stetler-Stevenson, 2008*). TIMP4 has also been described as a major inhibitor of platelet aggregation in humans (*Radomski et al., 2002; Villeneuve et al., 2009*).

TIMP4 expression is inducible and tissue specific (heart, kidney, pancreas, colon, testes, brain and adipose tissue) (*Greene et al., 1996*). Interestingly, TIMP4 seems to be differentially regulated in different types of cancer, with up or down-regulation depending on

the site. Its role as an anti-invasive protein has been suggested, through preventing activation of MMP2 and 9 (*Melendez-Zajgla et al., 2008*). The role of TIMP4 in the invasive process of human placentation has been poorly investigated and functional studies are limited. On the basis of the current literature, we propose a link between trophoblast differentiation and alterations in ECM remodeling via specific proteins, including TIMPs and MMPs. In this study, we employed GCM1 small-interfering RNA (siRNA) to test the hypothesis that defective GCM1-mediated villous trophoblast differentiation exerts its downstream effects via TIMP4. We used the BeWo cell line, first trimester human placental explants and placental villous tissues from control pregnancies and those affected by sPE to investigate TIMP4 expression under physiologic and pathologic conditions.

## Materials and Methods

### Cell culture

The human choriocarcinoma cell line BeWo (passages 10–20) was maintained in six well plates in F12K medium (ATCC, Manassas, VA, USA) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 units/ml streptomycin and 2.5 µg/ml fungizone (Invitrogen, Burlington, ON, Canada), in atmospheric O<sub>2</sub>/5% CO<sub>2</sub> at 37°C. RNA was extracted from the cells using RNeasy kit (Qiagen, Mississauga, ON, Canada) and protein was extracted using RIPA lysis buffer from both GCM1-silenced and non-silenced BeWo cells 48 h post-transfection. Knockdown of silenced BeWos was verified via quantitative RT–PCR (qRT–PCR).

### Placental explant cultures

First trimester placental villous tissues were collected from ultrasound-dated viable singleton pregnancies after elective social termination of pregnancy at 8–12 weeks of gestation. Ethics committee approval was obtained from the Mount Sinai Hospital Research Ethics Board and all subjects gave written informed consent. Individual clusters of villi were dissected in sterile cold phosphate-buffered saline (PBS) with strict avoidance of extra-villous trophoblast (columns). The proximal stem villi were mounted under polystyrene cubes to float the villous trees in serum-free media (Dulbecco's modified Eagle's medium/F12) with 1% liquid media supplement ITS + I (Sigma, St Louis, MO, USA), 100 units/ml penicillin, 100 units/ml streptomycin, 2 mM L-glutamine, 100 µg/ml gentamycin and 2.5 µg/ml fungizone. These floating villous explants were cultured and maintained in 8% ambient oxygen (40 mmHg) as recently described in detail (*Baczyk et al., 2009*). Explants were fixed and wax embedded to assess morphology at time 0 h and throughout the duration of the experiments and to confirm the absence of extra-villous trophoblast (24 and 48 h).

### Placental sampling in pathologic placentas and controls

Samples from diseased and healthy placentas were obtained by the Research Centre for Women's and Infants' Health (RCWIH) BioBank programme of Mount Sinai Hospital, in accordance with the policies of the Mount Sinai Hospital Research Ethics Board (MSH REB #10-0128-E). Immediately after delivery, placental weight was recorded and four 2–3 cm<sup>2</sup> tissue cores through the full thickness of the placenta were obtained from a site within each quadrant, avoiding areas with obvious evidence of infarction or other grossly-visible abnormalities. Chorionic and decidual plate tissues were then removed. The remaining cores were rinsed briefly in chilled PBS to remove residual blood and further dissected to

generate 0.5–1 cm<sup>3</sup> pieces. Pooled samples containing one piece of tissue from each of the four cores were generated, and were snap-frozen in liquid nitrogen, or fixed for histology. Samples for histology were fixed by immersion in 4% paraformaldehyde in PBS, for 24 h at room temperature, and stored in 70% ethanol at 4°C (typically <2 weeks) before being embedded in paraffin. Sections (5 µm thick) were prepared and mounted on Fisherbrand® Superfrost®/Plus microscope slides for immunohistochemistry.

Subjects were classified into the following groups (see Table I): (i) sPE—singleton, 24<sup>+</sup> to 34<sup>+</sup> weeks gestation, birthweight >10th percentile, predelivery blood pressure >140/90, significant proteinuria (>300 mg/day or 1+ on dipstick), (ii) Controls—healthy preterm control and (iii) term (36 completed weeks) control placentas which were matched for gestational age, normal birthweight (>10<sup>th</sup> percentile) and umbilical artery Doppler flow normal or not recorded.

To investigate the involvement of TIMP4 in the phenotype of severe pre-eclampsia, placentas from patients with early onset sPE [*n* = 7, gestational age (GA) 29.5 ± 0.9 weeks] were compared with either healthy term controls (TC) (*n* = 8, GA 38.7 ± 0.3 weeks) and gestational age matched healthy preterm controls (PTC) (*n* = 10, GA 30.6 ± 1.0 weeks). Clinical characteristics of the groups of patients with sPE are listed in Table I.

## GCM1 siRNA and anti-sense oligonucleotide treatment

Two double-stranded siRNA oligonucleotides (21mer, named 201 and 815) against the human GCM1 sequence were purchased from Qiagen and used as described (Baczyk *et al.*, 2009). Phosphothioate

oligonucleotides and controls were designed and manufactured by Biog-nostik (Gottingen, Germany; anti-GCM1 oligo 5'-AGAAGTTGGTGACCGG-3'). BeWo cells and floating villous explants were incubated in the presence of either 1 µM antisense oligonucleotide, scrambled non-silencing oligonucleotide control (performed and validated as described in Baczyk *et al.*, 2009). Results using non-treatment controls showed no differences versus the scrambled non-silencing controls (not shown).

All experiments were monitored for non-specific effects using a human interferon alpha enzyme-linked immunosorbent assay (ELISA) kit (PBL Bio-medical Laboratories) and toxicity was assessed using a lactate dehydrogenase (LDH) cyto-toxicity detection kit (Sigma-Aldrich).

## Reverse transcription and real-time PCR

Total RNA was extracted from BeWo cells or placental explants using the RNeasy kit (Qiagen). DNase treated RNA (1 µg) was reverse transcribed according to manufacturer's instructions (Applied Biosystems Canada, Streetsville, ON, Canada). Samples were incubated at 25°C for 10 min, 42°C for 20 min and 95°C for 5 min. Real-time PCR was performed on an ABI Prism 7700 Sequence Detection System (ABI) in triplicates in 25 µl volume containing 10 ng of template cDNA, 12.5 µl of 2 × SYBR Green PCR Master Mix (ABI) and 50 nM of primers. GCM1 intron-spanning primers were designed using Primer Express 2.0 software (ABI). The PCR program was initiated at 95°C for 10 min, followed by 40 thermal cycles of 15 s at 95°C, 60 s at 60°C. A melting curve for primer validation and a template standard curve were performed to show template-independent amplification results. Comparative CT Method (ABI technical manual) was used to analyze the real-time PCR. The expression of the GCM1 gene was normalized to the geometric mean of the housekeeping genes SDHA and TBP (Bieche *et al.*, 1999). GCM1 primer sequences were: Forward 5'-ATGGCACCTCTAGCCCCTACA-3' and Reverse 5'-GCTCTTCTTGCCCTCAGCTTCTAA-3'; SDHA primers sequences were: Forward 5'-TGGGAACAAGAGGGCATCTG-3' and Reverse 5'-CCACCACTGCATCAAATTCATG-3'. TBP primer sequences have been previously published (Bieche *et al.*, 1999). All data were expressed as fold change relative to non-silenced control.

## Western blotting for GCM1 and TIMP4

Total proteins were extracted from BeWo cells and placental explants as described previously (Baczyk *et al.*, 2009). Protein lysates were resolved in 10% sodium dodecyl sulphate polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore) (100 V constant for 1 h). Membranes were blocked with 5% skimmed milk in 0.1% (v/v) Tween Tris-buffer saline (TBST) for 2 h at room temperature and incubated with anti-GCM1 antibody (0.3 µg/ml stock) (Aviva System Biology, San Diego, CA, USA; P100837) against the specific peptide sequence (DFNSYVQSPAYHSPQEDPFLFTYASHPHQYSLPSKSSKWD-FEEEMTYLG) and rabbit anti-TIMP4 1 µg/ml (Abcam; AB2170) at 4°C overnight. Membranes were washed with TBST and incubated with secondary rabbit horse-radish peroxidase-conjugated antibody for 1 h at room temperature. Antibody reaction was detected using the ECL detection kit (GE Health Care, Baie d'Urfe, QU). Data were standardized by stripping the blot and reprobing it with anti-β-actin antibody [clone I-19 Santa Cruz, #SC-1616 (0.2 µg/ml)]. The results are presented as a ratio of relative optical density of the GCM1 band to the housekeeping gene β-actin, and also compared with the control samples.

## ELISA for TIMP4

We used the R&D systems TIMP4 Duo set to quantify TIMP4 levels in pathologic samples and controls. Proteins were extracted from snap-frozen tissue using rotor homogenizer and RIPA buffer containing protease

**Table I** Clinical characteristics of patients with severe early onset pre-eclampsia, IUGR, TC and PTC for pathologic studies.

	sPE	Preterm control	Term control
<i>n</i>	8	9	9
Maternal age (years)	32.3 ± 2.5 <sup>a</sup>	28.8 ± 2.0 <sup>a</sup>	35.6 ± 2.0 <sup>a</sup>
Gestational age (weeks)	29.5 ± 0.8 <sup>a</sup>	30.6 ± 1.0 <sup>a</sup>	38.7 ± 0.3 <sup>b</sup>
Average maximum systolic blood pressure	169 <sup>a</sup>	127 <sup>b</sup>	122 <sup>b</sup>
Average maximum diastolic blood pressure	103 <sup>a</sup>	75 <sup>b</sup>	75 <sup>b</sup>
BW < 5%ile	0	0	0
BW > 5 to <10%ile	0	0	0
BW > 10 to <50%ile	86 (6/7)	30 (3/10)	38 (3/8)
BW > 50%ile	14 (1/7)	70 (7/10)	62 (5/8)
Umb art normal flow	1/7	2/10	0
Umb art ARED flow	4/7	0	0
Umb art flow not recorded	2/7	8/10	8/8

Data presented as mean ± SEM for maternal age and gestational age, and as mmHg for blood pressure. Birthweight (BW) is shown as the % in the given range (absolute numbers in brackets). Superscripts indicate significant differences from other values in the same category, *P* < 0.01. Umb Art, Umbilical artery; ARED, absent or reversed end diastolic flow. Differences in maternal age, gestational age and blood pressures were determined using analysis of variance with *post hoc* Bonferroni correction for multiple comparisons.

and phosphatase inhibitors (Pierce, Thermofisher) at 4°C. Tissue extract was centrifuged for 10 min at 14 000g in a chilled micro-centrifuge. Supernatant was aliquoted, protein content quantified and stored at minus 80°C. Forty microgram of total protein was used per assay.

## Histology and immunohistochemistry

Paraformaldehyde-fixed placental explants were embedded in wax for hematoxylin and eosin (H&E) histology and immunohistochemistry. Entire floating villous explants were serially sectioned and every fifth section was stained with H&E to determine the three-dimensional structure of the explants. High-temperature sodium citrate antigen retrieval, pH 6.0, was used for all antibodies. Immunohistochemistry was performed for TIMP4 on rehydrated sections with streptavidin–biotin staining procedure, using Peroxidase Dako LSAB kit (Dako Canada Inc., Mississauga, ON) as described previously (Baczyk et al., 2004). Anti-TIMP4 antibody was used at 5 µg/ml (Abcam AB2140; R&D systems MAB974; Millipore AB19168). Immunofluorescence with AB2140 was performed on 3.7% formaldehyde-fixed BeWo cells grown on cover slips. Antigen retrieval was performed as described above. Slides were treated with 0.4% DAPI (4',6-diamidino-2-phenylindole) for nuclear detection. Fluorescence images were viewed using 20× regular and 40× and 100× oil immersion objective lens (NA 1.35) and collected using a DeltaVision Deconvolution microscope (Applied Precision, LLC, Issaquah, WA, USA).

## Tissue microarray for the assessment of pathological and control tissue

In order to further confirm our results in normal and pathological human pregnancies, and to further ensure that staining effects were not influenced by technical differences in exposure time, solutions or antibody concentrations, immunohistochemistry was additionally performed using the RCWIH BioBank Tissue Microarray (TMA) #1—IUGR and pre-eclampsia (sPE) array (<http://biobank.lunenfeld.ca/?page=Tissue%20arrays>). This microarray was developed following a full audit of the clinical profiles by a Maternal–Fetal Medicine specialist. Twenty-eight subjects were selected for inclusion in a villous tissue microarray: 8 subjects with severe IUGR, 8 subjects with sPE, 6 age-matched PTC and 6 TC. Locations for donor core extraction from paraffin-embedded tissue specimens were selected as follows: a 1 mm × 1 mm grid was superimposed on H&E stained sections and four core regions (one from each placental quadrant sampled at delivery) were selected using a random number generator (GraphPad Software). An experienced placental Pathologist confirmed that the selected locations were representative of the complete specimen. An array containing 112 placental cores (1 mm diameter) was constructed by the MSH Pathology & Lab Medicine Department using a Beecher Instruments Manual Tissue Arrayer. The array also contained four cores prepared from umbilical cord tissue, which served as tissue ‘beacons’ for orientation of the array. Sections (5 µm thick) were prepared and mounted on Fisherbrand® Superfrost®/Plus microscope slides, coated in a thin layer of paraffin wax to prevent loss of antigenicity by oxidation, and stored at 4°C until distribution.

Negative controls for all immunohistochemistry included omission of the primary antibody and use of non-specific matched immunoglobulin G (IgG) as well as recombinant 10× TIMP4 (R&D Systems). Slides were counterstained as needed with hematoxylin (Sigma), visualized using a Nikon DMRX light microscope and photographed using a Sony PowerHAD 3CCD colour video camera DXC-970MD (Sony, Toronto, ON, Canada).

## Statistical analysis

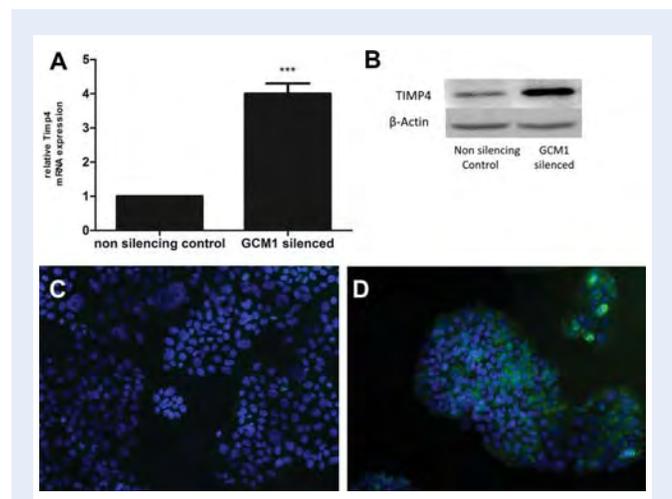
All experiments were performed as a minimum in technical and biological triplicates, unless stated otherwise. Data are presented as mean ± SEM. Unpaired *t*-tests and Mann–Whitney rank sum tests were used to test for significant differences between treatment and the non-silenced controls. Analysis of variance with a Bonferroni *post hoc* test was used to assess differences between pathologic samples and controls. All statistical calculations were performed using Prism® 5.0 software and *P*-values of < 0.05 were considered significant.

## Results

### TIMP4 expression is regulated by GCM1 in BeWo cells

BeWo cells were synchronized in the cell cycle via serum starvation for 1 day and confirmed by propidium iodide staining and analysis in a fluorescence-activated cell sorter (data not shown). GCM1 silencing via siRNA resulted in a consistent and significant (<90%) down-regulation of GCM1.

TIMP4 mRNA levels (assessed by qRT–PCR) were up-regulated 4.2-fold in GCM1-silenced cells compared with the non-silenced controls and western blot analysis confirmed an increase in protein levels (Fig. 1A/B). BeWo cells were grown on coverslips, GCM1 silenced and then stained with rabbit anti-TIMP4 antibody to determine the site of increased TIMP4 expression. GCM1-silenced cells showed intense cytoplasmic immunoreactivity for TIMP4 compared with the non-silenced controls (Fig. 1C/D). Specificity of the TIMP4 immunoreactivity was confirmed by the use of rabbit IgG and the

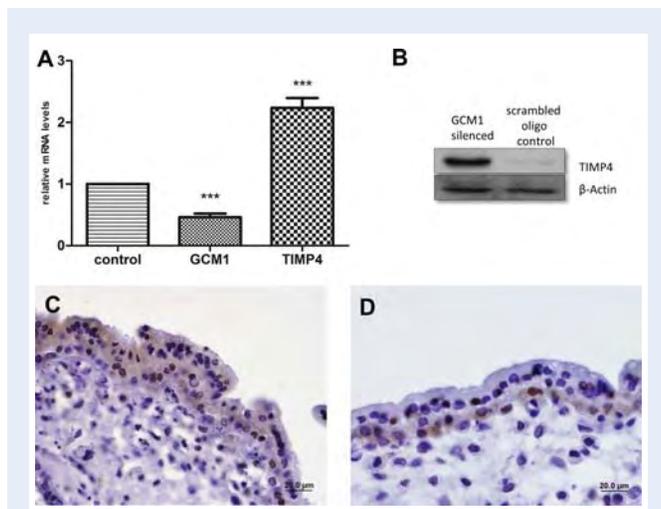


**Figure 1** TIMP4 mRNA and protein expression was significantly elevated after glial GCM1 silencing by siRNA in the choriocarcinoma cell line BeWo as measured by quantitative RT–PCR (A) and western blot (B) analysis, respectively ( $n = 7$ ,  $***P < 0.001$ ) (A/B). This finding was confirmed by immunofluorescence staining with identical exposure times (40×) (C/D). TIMP4 localization (green) in GCM1-silenced cells (D) was mostly found in the cytoplasm and a subset of nuclei (nuclei blue = DAPI stain) (C/D). Low green fluorescence was observed in the non-silencing control group (C). Immunoglobulin G controls showed no non-specific binding (data not shown) (Antibody; Abnova 2140).

competing recombinant 10× TIMP4 protein. Forskolin (a non-specific inducer of GCM1) stimulation did not reduce TIMP4 expression in this model which might be explained by the low baseline expression of TIMP4 (data not shown).

### Effects of GCM1 on TIMP4 expression in first trimester placental villous explants

To confirm these findings in villous trophoblast of intact villi, we used GCM1 siRNA to suppress GCM1 expression in first trimester villous explant cultures. Similar to the findings in BeWo cells, silencing of GCM1 by siRNA caused significant elevation of TIMP4 mRNA levels in the placental villi (Fig. 2A). Both western blot (Fig. 2B) and immunohistochemical analysis (Fig. 2C and D) showed significantly higher levels of TIMP4 protein in the silenced, cultured explants (Fig. 2B/C). Figure 2D represents control tissue which was treated with the scrambled siRNA and cultured for 3 days. TIMP4 protein level in the SCT was reduced compared with time 0 h control/fresh tissue (Fig. 3A) but was no different to the untreated controls which were maintained in culture for 3 days. Culture conditions were therefore the likely cause for reduced expression in SCT under these experimental conditions. LDH assay analysis of the media confirmed that siRNA treatment was not toxic for the explants (data not shown).



**Figure 2** GCM1 regulates TIMP4 expression in healthy control placental villi from first trimester placenta. GCM1 was successfully down-regulated in first trimester tissue (9–13 weeks) with use of anti-GCM1 siRNA after 2 days of culture ( $n = 12$ ;  $***P < 0.001$ ) as reported by our group previously *Baczyk et al.* (2009). This resulted in a significant up-regulation of TIMP4 under these conditions at both the RNA (A,  $***P < 0.001$ ) and protein (B) level. Immunohistochemistry showed increased expression of TIMP4 (C) in the SCT of the silenced villi compared with the scrambled non-silencing siRNA control (D) and the untreated controls (data not shown). Antibody used was Abnova 2140. The low staining in the SCT compared with fresh tissue at time 0 h (Fig. 3A) was a result of the tissue culture and not of treatment with scrambled siRNA, as there was no difference versus the non-treated control in culture (data not shown).

### TIMP4 protein level and localization in normal pregnancy

In the first trimester, TIMP4 protein was predominantly localized in the SCT of the chorionic villi, while some villous cytotrophoblast nuclei and stromal cells also stained positive (Fig. 3A). In the second trimester (Fig. 3B), SCT nuclei showed high immunoreactivity. Cytoplasmic TIMP4 staining was also present in the SCT and CT in second trimester. Reduced overall expression of TIMP4 was observed in placental villous tissues at term where staining was predominantly nuclear in the VCT (Fig. 3C) and in a small subset of endothelial cells of fetal vessels.

GCM1 protein measured by western blot analysis ( $n = 6$  in each group) significantly increased over the course of normal pregnancy and was highest at term (Fig. 3D/E). In contrast TIMP4 levels showed an inverse pattern (i.e. decreased levels) during gestation, consistent with the results of the silencing experiments in BeWo cells and the first trimester explant model (Figs 3D/E and 1/2).

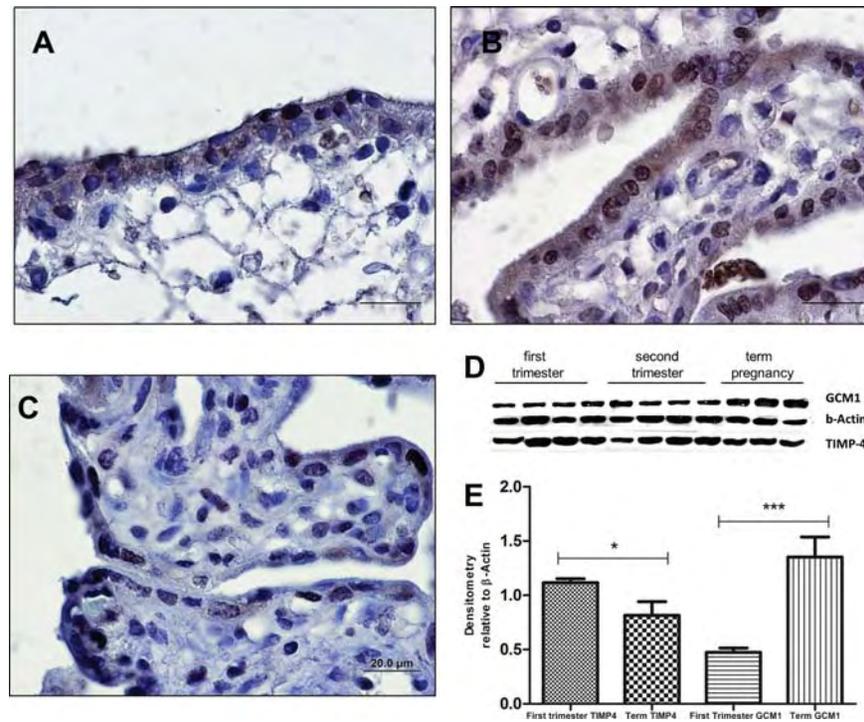
### TIMP4 protein in sPE

GCM1 expression was significantly decreased in sPE and was confirmed by qRT-PCR (data not shown). Medium- and high-power photomicrographs in both control groups showed consistent staining of TIMP4 in villous cytotrophoblast nuclei, with minimal or absent staining of the SCT (Fig. 4A). The sPE group showed high levels of TIMP4 protein in the SCT and a subset of villous cytotrophoblast nuclei, with further accumulation of TIMP4 in the characteristic pathologic syncytial knots (noted by \*, Fig. 4B). These results were confirmed by western blot analysis and ELISA showing a significant increase in TIMP4 protein in these placentas from patients with sPE (Fig. 4C/D). We added the RCWH BioBank Tissue Microarray #1 to our study to semiquantitative extend and confirm our observations. The clinical characteristics of the groups of patients on this microarray are shown in Supplementary data, Table S1. Staining for TIMP4 revealed consistent results with the data presented in this manuscript, and confirmed that the differences found were not related to technical variations in the staining or fixation procedure (data not shown).

## Discussion

Placental insufficiency is a significant cause of perinatal morbidity and mortality and can manifest as a spectrum of disorders, including severe early onset pre-eclampsia, IUGR, abruption and stillbirth. Early detection of placental insufficiency is needed and is crucial for the development of proper intervention strategies, because the only known cure is removal of the placenta. In the current study, we have identified TIMP4 as a downstream effector of the transcription factor GCM1 in both the villous and extra-villous post-mitotic differentiated trophoblast. Under experimental conditions we demonstrate that TIMP4 is negatively regulated by GCM1. Low levels of GCM1 over gestation or pathological repression, as seen in sPE, are associated with significant over-expression of TIMP4.

GCM1 directly regulates terminal differentiation of both the villous and extra-villous trophoblast lineages, as we have shown in both tissue culture and the BeWo cell model (*Baczyk et al.*, 2009). Down-regulation of GCM1 leads to reduced differentiation, such that the active processes of syncytialization (needed to cover the developing



**Figure 3** Expression of TIMP4 during normal placentation. TIMP4 expression in early first trimester (7 weeks) villous tissue was mostly found in the syncytium and the nuclei within this compartment as well in a subset of cytotrophoblasts (A). In the second trimester (19 weeks) a similar expression pattern was seen, with an increased intensity of staining in the nuclei (B). Expression of TIMP4 in villi from term placental was mainly found in the trophoblastic nuclei as well as some stromal cells (C) and a subset of endothelial cells (not shown) with weak staining in the SCT. Term expression ( $n = 6$ ) of TIMP4 protein was significantly reduced in comparison to first trimester ( $n = 6$ ,  $P = 0.027$ ) whereas the differentiation marker GCM1 increased over gestation ( $n = 8$ ;  $P < 0.0001$ ) as shown by western blot analysis (E/D), showing a similar relationship between GCM1 and TIMP4 as in the silenced placental explants and BeWo cells. \* $P < 0.05$  and \*\*\* $P < 0.001$ .

placental villi with healthy SCT) and invasion/remodeling of the maternal spiral arteries (needed to ensure an adequate uteroplacental blood flow) are restricted. These trophoblast deficiencies have been previously demonstrated in placentas from patients with sPE (Chen et al., 2004; Ray et al., 2010) and are accompanied by changes in the expression of multiple proteins including TIMP4 as shown here.

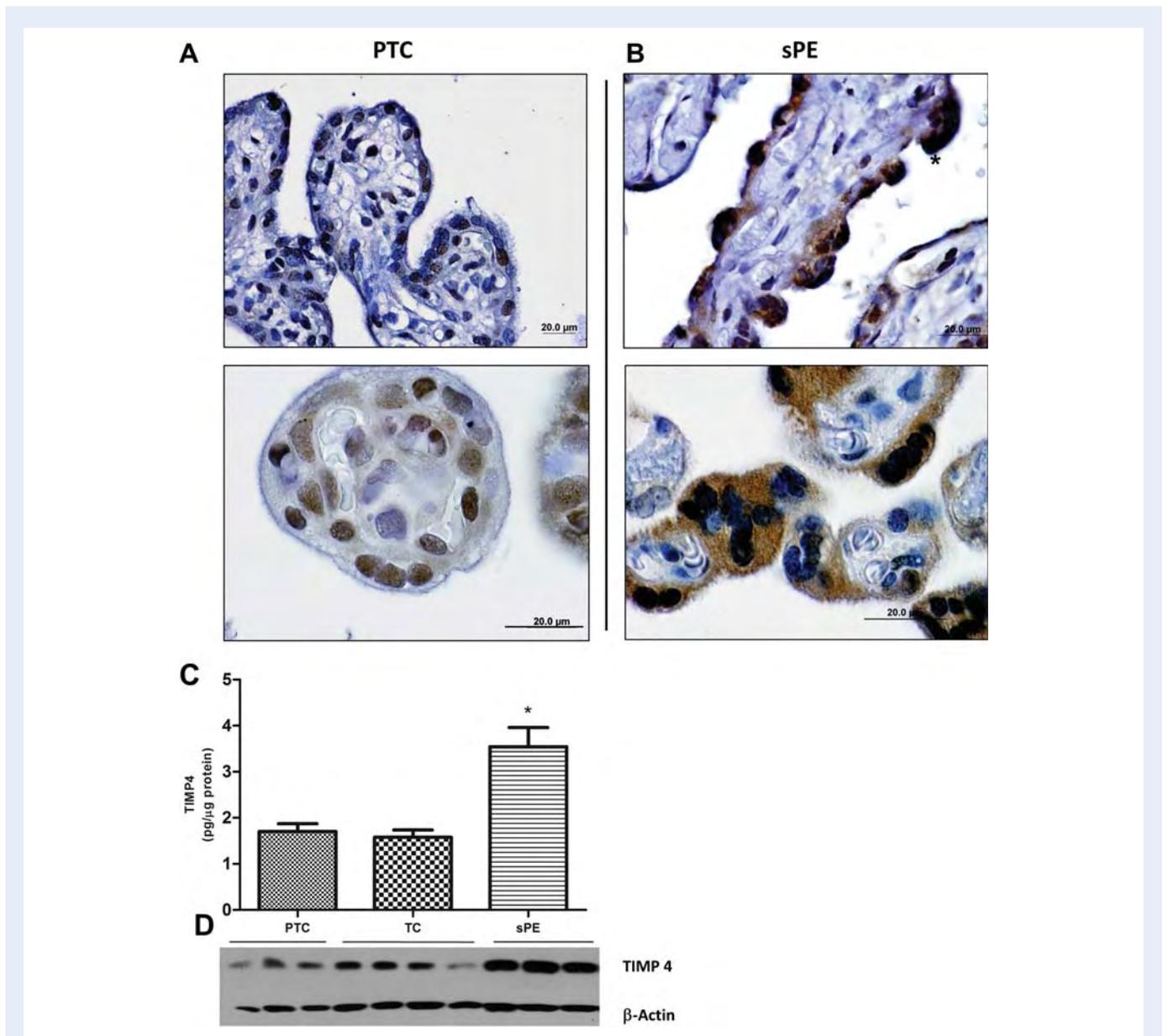
We used three different strategies to demonstrate that TIMP4 is a downstream effector of GCM1, including in the villous trophoblast: (I) a choriocarcinoma cell line (BeWo), (II) a floating human placental villous explant model and (III) assessment of expression in tissues from well-defined human pathology. The data from the BeWo cell line were in line with our findings in the human trophoblast *in vitro* and *in vivo*, showing that silencing of GCM1 results in a significant up-regulation of TIMP4 in all systems (cell line, trophoblast tissue).

To further expand on the role of TIMP4 in placentation, we used a floating placental villous explant model to investigate the effects of GCM1 silencing on TIMP4 expression. This strategy is critical, since it advances observations from a pathologic cell line to normal tissue where villous trophoblast cells interact with their partner cell types within placental villi. These GCM1-silenced explants showed an increase of TIMP4 expression at both the RNA and protein level. Non-silenced controls showed mostly staining in the cytotrophoblast cells indicating a culture-dependent effect (on TIMP4 expression).

GCM1 silencing resulted in a significant up-regulation of TIMP4 in the SCT. Physiologic trophoblast turn over seems to be necessary for the normal suppression of TIMP4 under *in vitro* conditions, and GCM1 silencing interferes with this process leading to increased TIMP4 expression.

We then sought to characterize TIMP4 levels in human tissue over the course of gestation and in a well-defined disease state, i.e. sPE. TIMP4 protein was high in the first and second trimester compared with term tissue and decreased significantly over the course of gestation in healthy pregnancies, an opposite effect to that seen for GCM1 expression. This supports the *in vitro* data which suggest that TIMP4 expression is directly linked to the differentiation status of the villous trophoblast (VT). Although GCM1 levels seem to be directly linked to changes in the trophoblast, it does not explain the expression of TIMP4 in a subset of cells in the mesenchyme as GCM1 is not present in these cells. The effects of GCM1 on TIMP4 in different cell types might be indirect and induced by cell–cell communication in the villi itself. Surprisingly TIMP4 was found consistently in a subset of SCT nuclei, in line with observations from other groups suggesting a potential secondary function of this protein (Zhang et al., 2002; Nakasone et al., 2009).

Furthermore, the current literature suggests an alternative regulation of TIMP4 in EVT that may occur in response to interaction



**Figure 4** Placentas from patients with severe early onset pre-eclampsia (sPE) were investigated for the expression of TIMP4 and compared with both age-matched preterm and term control tissues. Immunohistochemistry showed significantly increased TIMP4 staining intensity in sPE compared with the age-matched preterm control, PTC (**A, B**). Note the focal accumulation of staining in the characteristic pathological syncytial knots (\*), in contrast with the nuclei staining and weak syncytial staining observed in the PTC. Absolute TIMP4 protein level was quantified by enzyme-linked immunosorbent assay, confirming the visual observations of the immunohistochemistry and the western blot data (groups in analysis sPE ( $n = 8$ ,  $P < 0.01$ ) compared with (**C**) age-matched PTC tissue  $n = 9$ ; and term placental tissue (TC, **D**) ( $n = 9$ ). Antibody used: Abnova 2140.

with the maternal decidua, which was not further investigated in this study. MMPs are known to promote invasion of the EVT, with TIMPs acting as potent inhibitors of this process (Bischof *et al.*, 2001, 2002). Abnormalities in remodeling of the ECM may be involved in the pathophysiology of pre-eclampsia, and proteins such as TIMP4 could potentially contribute to this process. There have been a number of studies investigating the role of TIMP4 in cancer invasion and progression as well as the role in apoptosis and angiogenesis, many with conflicting results based on tissue site and stage of disease (Melendez-Zajgla *et al.*, 2008). The current knowledge

suggests that the regulation of TIMP4 itself is complex and somewhat tissue dependant. In order to better examine the expression of TIMP4 in the EVT, further studies using explanted cell columns in Matrigel to investigate their invasive properties need to be performed, however, this was beyond the scope of the current study.

On the basis of the data from the cell and tissue-based models, we decided to characterize the relationship between GCM1-mediated trophoblast differentiation and TIMP4 expression in the pathophysiology of placental disease. We assessed TIMP4 expression in an extreme type of placental insufficiency, namely sPE in women with

babies in the normal size range. GCM1 expression is known to be reduced in sPE across gestation, but the downstream consequences of this molecular defect were not explored (Chen et al., 2004).

In all of our pathologic cases we found a significantly higher protein expression of TIMP4 compared with age-matched controls (150–250%) in parallel with significantly reduced levels of GCM1. The elevation of TIMP4 in sPE was particularly striking in the large syncytial knots, which are characteristic of this disease. We speculate that the mother could be exposed to higher circulating levels of TIMP4 as the syncytial knots are shed into the maternal circulation. Clinical studies on circulating TIMP4 levels during high-risk pregnancies may reveal the potential of TIMP4 as a bio-marker marker for identifying a subset of women at risk of developing sPE. The elevated expression of TIMP4 was also semi-quantitatively confirmed in the second cohort of patients within the TMA#1, proving it as a useful and powerful tool for assessing a well-defined patient cohort with minimal technical variability in staining.

Pathologic thrombosis within the placenta is a hallmark of sPE and IUGR (Toal et al., 2008). One functional consequence of increased TIMP4 expression may be its participation in intra-placental hemostasis. Trophoblast cells secrete anticoagulant substances which prevent platelet aggregation and adhesion to the trophoblast surface (Myatt, 2002). TIMP4 is known to be a major regulator of platelet aggregation, specifically through inhibition of MMP-2 (Radomski et al., 2002). An increased anticoagulant action of TIMP4 at the placental surface could be a protective mechanism to prevent intra-placental thrombosis in the face of reduced uteroplacental blood flow in sPE. Further investigation into the potential anti-platelet properties of TIMP4 in placental as well as its interaction with the drug heparin is warranted, as there is increasing interest in heparin as a possible preventative and therapeutic agent in patients with placental insufficiency (Drewlo et al., 2010).

Second, there has been limited study of the role of MMPs in the pathophysiology and regulation of pre-eclampsia and IUGR. It has been suggested that MMPs are involved in vasodilation and mediating myogenic tone in women with PE. MMPs are known to act on endothelin, leading to activation of the vasodilatory endothelin B receptor (Cockle et al., 2007). The role of the TIMPs/TIMP4 in this process is unknown, however, we can speculate that their inhibitory action may be involved in this process to prevent vasodilatation.

Third, it has been described in the literature that MMPs and TIMPs play a key role in the process of inflammation. Increased expression of MMPs and altered TIMP4 has been noted in many tissue sites where inflammation is present and has been thought to either contribute to, or protect against, the pathological processes involved (Manicone and McGuire, 2008). Pre-eclampsia is considered an inflammatory-like process, with elevated levels of pro-inflammatory cytokines, such as c-reactive protein, interleukin-1 and 6, and others. Therefore, we suggest that the dysregulation of TIMP4 protein expression might contribute to the development of pre-eclampsia (Schiessl, 2007; Challis et al., 2009).

Finally, TIMPs were shown to play different roles in apoptosis: TIMP1 was shown to be anti-apoptotic whereas TIMP2 and TIMP3 were shown to be pro-apoptotic in different systems (Valente et al., 1998; Fata et al., 2001; Lee et al., 2003). A role of TIMP4 in this context has not been shown yet.

In conclusion, elevations in TIMP4 have an established role in a multitude of physiologic and inflammatory processes. On the basis of our findings we demonstrate that TIMP4 is negatively regulated by the transcription factor GCM1. The biologic role(s) of TIMP4 in placental development and diseases sPE remains unknown but could include a role in hemostasis and TIMP4 may act as a possible biomarker for the development of sPE in high-risk pregnancies.

## Authors' roles

S.D.: designed study, executed experiments, analyzed data and drafted manuscript. M.C.: analyzed data, drafted manuscript and critical discussion. J.K.: designed study, analyzed data, edited manuscript and critical discussion. S.L.: designed study and critical discussion. D.B.: performed immunohistochemistry and critical discussion.

## Supplementary data

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

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