TREM-1 Expression Is Increased in Human Placentas From Severe Early-Onset Preeclamptic Pregnancies Where It May Be Involved in Syncytialization

Reproductive Sciences
2014, Vol. 21(5) 562-572
© The Author(s) 2013
Reprints and permission:
sagepub.com/journalsPermissions.nav
DOI: 10.1177/1933719113503406
rs.sagepub.com

Ratana Lim, PhD^{1,2}, Gillian Barker^{1,2}, and Martha Lappas, PhD^{1,2}

Abstract

Preeclampsia, a major cause of maternal and perinatal morbidity and mortality, is thought to be attributable to dysregulation of trophoblast invasion and differentiation. Microarray studies have shown that triggering receptor expressed on myeloid cells (TREM) 1, a cell surface molecule involved in the inflammatory response, is increased in preeclamptic placentas. The aim of this study was to determine the level of TREM-1 expression in severe early-onset preeclamptic placentas and its functional role in trophoblast differentiation. Placenta was obtained from women with severe early-onset preeclampsia ($n = 19$) and gestationally matched preterm controls placentas ($n = 8$). The TREM-1 expression was determined by quantitative reverse transcriptase polymerase chain reaction and Western blotting. The effect of TREM-1 small interfering RNA on cell fusion and differentiation was assessed in BeWo cells. The effect of oxygen tension on TREM-1 levels, in basal or forskolin-treated BeWo cells, was also assessed. The TREM-1 was localized to the syncytiotrophoblast layer, and TREM-1 messenger RNA and protein expression was significantly increased in preeclamptic placentas. The BeWo cells treated with forskolin were associated with increased TREM-1 expression. The TREM-1 knockdown inhibited forskolin-induced expression of the differentiation marker β -human chorionic gonadotropin but had no effect on the cell-fusion marker E-cadherin. The increase in TREM-1 expression in BeWo cells treated with forskolin during normoxic conditions was reduced in forskolin-treated cells under hypoxic conditions. In conclusion, TREM-1 is increased in preeclamptic placentas and by forskolin treatment. Knockdown of TREM-1 by RNA interference inhibits cell differentiation but has no effect on cell–cell fusion. Finally, we show that TREM-1 upregulation is attenuated under hypoxic conditions in which cell differentiation is impaired.

Keywords

TREM-1, preeclampsia, placenta, function

Introduction

Preeclampsia is one of the most serious complications of pregnancy, affecting approximately 5% of all pregnancies. It is a major contributor to maternal morbidity, mortality, preterm birth, and perinatal loss. Preeclampsia is a multisystem disorder affecting the maternal vasculature (causing hypertension), kidneys, liver, the hematological system, and the fetoplacental unit.¹ It can also affect the nervous system, causing cerebral edema and seizures (eclampsia). Preeclampsia is considered to be a 2-stage disorder; abnormal placental implantation in early pregnancy is followed by maternal endothelial dysfunction.²

Fusion of the mononucleate cytotrophoblasts to form a nonproliferative multinucleated syncytiotrophoblast is critical for normal placental function. It has been shown to play a role in a number of processes including nutrient transport, hormone production, and immune tolerance.^{3,4} There are a number of factors that have been shown to regulate trophoblast fusion; these include cytokines, hormones, protein kinases, and transcription factors.⁵ The mechanisms that

control syncytiotrophoblast differentiation are still poorly understood; however, fusion of cytotrophoblasts can be induced by agents that increase cyclic adenosine monophosphate such as forskolin⁶ and can be inhibited under hypoxic conditions.⁷ Fusion of trophoblast cells is characterized by a decrease in the levels of the cell-adhesion molecule E-cadherin,⁸ increased expression and secretion of human chorionic gonadotropin hormone (hCG),⁶ leptin,^{9,10} and adiponectin.¹¹

¹ Department of Obstetrics and Gynaecology, University of Melbourne, Victoria, Australia

² Mercy Perinatal Research Centre, Mercy Hospital for Women, Heidelberg, Victoria, Australia

Corresponding Author:

Martha Lappas, Department of Obstetrics and Gynaecology, Mercy Hospital for Women, University of Melbourne, Level 4/163 Studley Road, Heidelberg, 3084, Victoria, Australia.

Email: mlappas@unimelb.edu.au

Triggering receptor expressed on myeloid cells (TREM) 1 is a cell surface molecule implicated in the propagation of the inflammatory response. It is a 30-kD glycoprotein of the immunoglobulin (Ig) family. The TREM-1 consists of an extracellular domain, a transmembrane region that contains a conserved lysine residue, and a short cytoplasmic domain, which lacks any signaling motif.¹² The TREM-1 gene is mapped to human chromosome 6p21 and has been demonstrated to cluster with 6 other genes with related functions. These genes include *TREM-2* and *TREM-like transcript (TLT)-1* to *TLT-5*.¹³ Engagement of TREM-1 induces the production of inflammatory chemokines and cytokines, such as interleukin (IL) 8 and tumor necrosis factor (TNF) α .¹⁴ In addition, TREM-1 activation induces cancer proliferation¹⁵ and differentiation of primary monocytes into immature dendritic cells.¹⁶ In addition, it is regulated by hypoxia in mature dendritic cells.¹⁷ Preeclampsia is a disease characterized by elevated levels of proinflammatory cytokines, such as C-reactive protein, IL-6, and others¹⁸⁻²¹ and dysregulated trophoblast differentiation.²² Two recent studies using microarray analysis have revealed that TREM-1 is increased in preeclamptic placentas.^{23,24} However, the preterm samples, which were all preterm, were compared to either a combination of term and preterm controls²³ or term controls.²⁴ Thus, the first aim of this study was to confirm the microarray studies showing that TREM-1 is increased in preeclamptic placentas^{23,24} at both the messenger RNA (mRNA) and protein level in samples from severe early-onset preeclamptic and gestationally age-matched control placenta.

Given that TREM-1 plays a role in proliferation and differentiation,^{15,16} we next sought to examine TREM-1 expression in BeWo cells during syncytialization and tested the hypothesis that TREM-1 could facilitate the differentiation and fusion of cytotrophoblast into the syncytiotrophoblast. To do this, we used BeWo cells, a choriocarcinoma cell line that is widely used to study villous trophoblast fusion by adding forskolin.²⁵ The effect of TREM-1 knockdown, using small interfering RNA (siRNA), on trophoblast cell fusion and differentiation (E-cadherin and β -hCG), and proliferation and viability (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide [MTT] reduction test) were also assessed.

Materials and Methods

Study Population

The Research Ethics Committee of Mercy Hospital for Women approved this study. Written informed consent was obtained from all participating women. Placenta was collected prior to labor onset at caesarean section (to account for any effects of labour on the levels of the endpoints) and from singleton gestations. Placenta was obtained from normotensive preterm pregnancies ($n = 8$) and those complicated by severe early-onset preeclampsia ($n = 19$). Severe early-onset preeclampsia was defined as preeclampsia prior to 34 weeks gestational age in association with either blood pressure greater than 160 mm Hg systolic, 110 mm Hg diastolic, or proteinuria greater than 5 g/d. Hemolysis, elevated liver

enzymes and low platelets syndrome was defined as hematological evidence of hemolysis, thrombocytopenia, and elevated liver enzymes. Babies were classified as having intrauterine growth restriction (IUGR) when birth weight was below the fifth percentile for gestational age calculated using www.gestation.net, which uses the principles of the Gestation Related Optimal Weight program. All preeclampsia ($n = 8$) and preeclampsia-IUGR ($n = 11$) cases were matched to control patients who also delivered preterm ($n = 8$). Indications for preterm delivery (in the absence of preeclampsia) were prolonged prelabor rupture of the fetal membranes, placenta previa, or antepartum hemorrhage. All placentas collected from preterm pregnancies were swabbed for microbiological culture investigations and histopathological examination, and patients with chorioamnionitis were excluded from the analyses. The clinical characteristics for these populations have been described previously.²⁶

Preparation of Placental Tissue

To ensure no sampling bias, placental lobules (cotyledons) were obtained from various regions of the placenta. The basal plate and chorionic surface were removed from the cotyledon. Placental tissue was blunt dissected to remove visible connective tissue and calcium deposits. Tissue samples were fixed and paraffin embedded for immunohistochemical analysis or snap frozen in liquid nitrogen and immediately stored at -80°C for RNA and protein analysis.

Immunohistochemistry

Immunohistochemistry in human placenta was performed according to our previously published methods.²⁷ Goat polyclonal anti-TREM-1 (sc-19309; Santa Cruz Biotechnology, Santa Cruz, California) was used at 2 $\mu\text{g/mL}$. Positive controls, which were composite slides with tonsil, breast tumor, and ovarian tumor, were included in each run. Negative control slides, where primary antibody was replaced with normal mouse IgG serum, were also included.

Gene Silencing of TREM-1 in BeWo Cells

The BeWo cells, steroidogenic human placental cell line derived from choriocarcinoma, were used to investigate the effect of siRNA-mediated gene silencing of TREM-1 on trophoblast fusion and differentiation. The BeWo cells were maintained in Dulbecco Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, and 2 mmol/L L-glutamine, under a humidified atmosphere of 5% CO_2 in air. The day before transfection, approximately 1×10^5 cells were seeded in each well of 12-well cell culture plates in DMEM/F-12 without antibiotics and incubated for 24 hours. The BeWo cells were transfected with SilenceMag reagent according to manufacturer guidelines (Oz Biosciences, France). Cells were transfected

with either 150 nmol/L TREM-1 or 150 nmol/L nonspecific siRNA (Ambion, Austin, Texas) in DMEM/F-12 (supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L L-glutamine) for 24 hours. Cells transfected with nonspecific siRNA were used as the vehicle. The medium was then replaced with DMEM/F-12 with 20 µmol/L forskolin (Sigma, St Louis, Missouri) or 0.02% dimethyl sulfoxide (DMSO; as a vehicle for forskolin) and the cells incubated at 37°C for an additional 48 hours. The concentration of forskolin used in this study was based on previously published studies.²⁸ The medium was then replaced with DMEM/F-12 containing 2% heat-inactivated FCS and cell incubated for an additional 24 hours. The MTT cell proliferation assay was performed (as detailed subsequently) or cells were collected and stored at -80°C until assayed for mRNA expression by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and protein expression by Western blotting as detailed subsequently. Medium was collected and stored at -80°C until assayed for hCG, as detailed subsequently. Six independent experiments were performed.

Hypoxic Experiments in BeWo Cells

To determine the effect of oxygen tension on TREM-1 levels, BeWo cells were grown to 70% to 80% confluence, then treated with 20 µmol/L forskolin or 0.02% DMSO (as a vehicle for forskolin) for 48 hours. The cells were then cultured in DMEM/F-12 containing 2% heat-inactivated FCS for a further 24 hours at 21% O₂ (normoxia) or 1% O₂ (hypoxia). Hypoxia was achieved by placing the cells in a multigas incubator (ASTEC, Fukuoka, Japan). Cells were collected and stored at -80°C until assayed for mRNA expression by qRT-PCR and protein expression by Western blotting as detailed subsequently. Medium was collected and stored at -80°C until assayed for hCG, as detailed subsequently. Six independent experiments were performed, in duplicate.

MTT Cell Viability Assay

Cell viability was assessed by the MTT proliferation assay. To determine the viability of TREM-1 knockout cells, cells were transfected as described previously, however the MTT assay was performed after the 48 hours of forskolin treatment. At the end of the culture period, the culture medium was removed, and 10 µL of MTT solution (diluted in PBS at a concentration of 0.5 mg/mL) was added to 90 µL of the fresh culture medium. After a 4-hour incubation at 37°C, the medium was removed and the resulting MTT formazan crystals were dissolved with 100 µL of DMSO by pipetting up and down 30 times, and the absorbance was measured at a wavelength of 570 nm using the xMark microplate absorbance spectrophotometer (Bio-Rad Laboratories, Hercules, California). Each assay also included blank wells, containing only culture medium without cells. Three independent experiments were performed.

RNA Extraction and qRT-PCR

RNA extractions and qRT-PCR were performed according to our previously published methods.²⁷ We used predesigned primers from Qiagen (QuantiTect Primer Assays, Qiagen, Germantown, Maryland). The following primers were used: TREM-1 (catalogue number QT00046284), E-cadherin (catalogue number QT00080143), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; catalogue number QTQT01192646). Average gene C_T values were normalized to GAPDH of the same complementary DNA sample. Fold differences were determined using the comparative C_T method.

Western Blotting

Western blotting was performed according to our previously published studies.²⁷ Goat polyclonal anti-TREM-1 (sc-19309; Santa Cruz Biotechnology) was used at 2 µg/mL, mouse monoclonal anti-E-cadherin (AB8993; Abcam, Cambridge, Massachusetts) was used at 1 µg/mL, and mouse monoclonal anticytokeratin 7 (M7018; Dako) was used at 2 µg/mL. Membranes were viewed and analyzed using the Chemi-Doc system (Bio-Rad Laboratories). Semiquantitative analysis of the relative density of the bands in Western blots was performed using Quantity One 4.2.1 image analysis software (Bio-Rad Laboratories). For protein loading control, blots were stained with 0.1% Ponceau-S in 5% acetic acid (Santa Cruz Biotechnology), rinsed in distilled water to clear background, then imaged using Quantity One software.²⁹

The hCG Measurement in Culture Media

For hCG quantification, an enzyme immunoassay kit was used (DRG Diagnostics, Marburg, Germany), which is based on the sandwich principle and detects both whole hCG molecule and the free β-hCG subunit present in spent media. The assay was carried out according to the manufacturer's instructions. The results are expressed as hCG concentrations (after correction for background optical density) in mIU/mL.

Statistical Analysis

Statistical analyses were performed using a commercially available statistical software package (Statgraphics Plus version 3.1, Statistical Graphics Corp., Rockville, Maryland). Two sample comparisons were analyzed by Student *t* test or Mann-Whitney *U* (Wilcoxon) test. Statistical significance was ascribed to *P* value <.05. Data were expressed as mean ± standard error of the mean.

Results

Expression and Localization of TREM-1 in Human Placenta

The localization of TREM-1 in placental sections was determined by immunohistochemistry in placenta. The TREM-1

Figure 1. Localization of TREM-1 in human placenta. A, Immunohistochemical localization of TREM-1 in placenta. The TREM-1 staining was in the syncytiotrophoblast (syn) layer. There was no TREM-1 staining in the villous stroma. B, No specific staining for TREM-1 is seen in the negative control for placenta. Magnification 250 $\times$. TREM-1 indicates triggering receptor expressed on myeloid cells 1.

was detected only in the syncytiotrophoblast layer (Figure 1A). No staining for TREM-1 was seen in the negative control (Figure 1B).

TREM-1 Is Increased in Preeclamptic Placentas

The gene and protein levels of TREM-1 were determined in 19 women with pregnancies complicated by severe early-onset preeclampsia and 8 women with preterm pregnancies not affected by preeclampsia. The baseline characteristics for these populations have been previously described²⁶ and depicted in Table 1. The TREM-1 mRNA expression was quantified by qRT-PCR and protein expression by Western blot, and data expressed as fold change. The TREM-1 mRNA (Figure 2A) and protein (Figure 2B) expression was significantly higher in the preeclampsia group compared to the control preterm group (6.3-fold higher by mRNA and 2.6-fold by protein). To

Table 1. Relevant Characteristics of the Preeclampsia Study Group.^a

	Preterm Controls (n = 8)	Preeclampsia (n = 19)
Maternal age, years	27.6 (1.6)	28.6 (1.3)
Maternal BMI, kg/m ^{2b}	27.4 (2.5)	31.2 (2.1)
Gestation, weeks	29 ⁺⁵ (1.0)	30 ⁺⁰ (0.7)
Placental weight, g	437 (51)	311 (21) ^c
Fetal birth weight, g	1492 (204)	1154 (106)
Gravida	2.5 (0.4)	2.0 (0.3)
Parity	2.4 (0.3)	1.6 (0.2) ^c
Ethnicity, No. (%)		
Caucasian	5 (62.5)	11 (58)
Asian	1 (12.5)	3 (16)
African	0	2 (10.5)
Middle Eastern	1 (12.5)	2 (10.5)
Pacific Islander	1 (12.5)	1 (5.0)

Abbreviations: BMI, body mass index; SEM, standard error of the mean.

^a Values represent mean ($\pm$ SEM).

^b Based on first antenatal visit at approximately 12 weeks.

^c $P < .05$ versus preterm control group.

Figure 2. Increased TREM-1 expression in preeclamptic placentas. The TREM-1 expression in preeclamptic (n = 19) compared to preterm control (n = 8) placentas. A, Gene expression was analyzed by qRT-PCR. The TREM-1 mRNA expression is displayed as mean $\pm$ SEM. $*P < .05$ versus preterm control. B, Representative Western blots and quantitation for TREM-1. Expression levels were confirmed by densitometry. Data are displayed as the mean $\pm$ SEM. $*P < .05$ versus preterm control. mRNA indicates messenger RNA; SEM, standard error of the mean; TREM-1, triggering receptor expressed on myeloid cells 1; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction.

ensure that the reduction in TREM-1 expression level is not due to the reduced proportion of TREM-1-expressing trophoblasts, Western blotting of cytokeratin 7 was also performed. There

Figure 3. Syncytialization of BeWo cells enhances TREM-1 expression. The BeWo cells were incubated in the absence (DMSO control) or presence of 20 μ mol/L forskolin for 48 hours ($n = 6$ independent experiments). A-D, Syncytialization of BeWo was confirmed by decreased E-cadherin and increased β -hCG expression and secretion. A, Gene expression of E-cadherin was analyzed by qRT-PCR, and gene expression is displayed as mean $\pm$ SEM. * $P < .05$ versus control cells. B, Representative Western blot of E-cadherin in cellular lysates in basal and forskolin-treated cells. C, Gene expression of β -hCG was analyzed by qRT-PCR, and gene expression is displayed as mean $\pm$ SEM. * $P < .05$ versus control cells. D, The protein level of β -hCG secreted in media. Data are expression displayed as mean $\pm$ SEM. * $P < .05$ versus control cells. E and F, Syncytialization of BeWo cells enhances TREM-1 expression. E, The TREM-1 gene expression was analyzed by qRT-PCR, and mRNA expression is displayed as mean $\pm$ SEM. * $P < .05$ versus basal cells. F, Representative Western blot of TREM-1 in cellular lysates in basal and forskolin-treated cells. DMSO indicates dimethyl sulfoxide; hCG, gonadotropin hormone; mRNA, messenger RNA; SEM, standard error of the mean; TREM-1, triggering receptor expressed on myeloid cells 1; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction.

was no difference in cytokeratin 7 protein expression between control and preeclamptic placentas. A representative image is shown in Figure 2B.

Forskolin Induces TREM-1 Expression

Placental trophoblastic differentiation is characterized by the fusion of monolayer cytotrophoblasts into syncytiotrophoblast. In this study, trophoblast-derived BeWo cells incubated with forskolin were used as a model system for trophoblast fusion.

Syncytialization was confirmed by significant downregulation of E-cadherin and upregulation of β -hCG. Cellular E-cadherin was reduced at both the gene (Figure 3A) and the protein (Figure 3B) level. E-cadherin mRNA was reduced by 35% in cells treated with forskolin. On the other hand, forskolin induced both hCG gene expression (Figure 3C) and secretion (Figure 3D). Forskolin-treated cells were 100-fold higher than basal at the mRNA level and 2.6-fold higher at the secreted level. When TREM-1 gene (Figure 3E) and protein (Figure 3F) levels were compared between basal and forskolin-treated cells, a

higher level was detected in cells treated with forskolin (36-fold higher by mRNA).

Knockdown of TREM-1 in Forskolin-Treated BeWo Cells Decreases hCG Expression and Secretion

Since we found that BeWo cells treated with forskolin were associated with an upregulation of TREM-1, we hypothesized that TREM-1 may play an active role in driving the process of syncytialization. To test this hypothesis, we used RNA interference to knockdown TREM-1 expression. The BeWo cells were transfected with siRNA duplexes targeted to human TREM-1 mRNA (TREM-1 siRNA) or a nonspecific (NS) control which has no homology to any known mammalian gene, and then treated with forskolin to stimulate TREM-1 expression. The TREM-1 was efficiently downregulated by transfection with TREM-1 siRNA, as demonstrated by qRT-PCR and Western blot of BeWo cell lysates following forskolin treatment (Figure 4A and B). The TREM-1 mRNA was reduced by 55% and protein by 40%. The MTT reduction is proportional to the number of metabolically active cells and thus used as a marker of cell viability. The MTT cell viability assay showed that there was no effect of forskolin or TREM1 siRNA on cell viability (data not shown).

We next examined the effect of TREM-1 knockdown on BeWo cell fusion and differentiation. We used E-cadherin as a marker of cell fusion and hCG as a marker of biochemical differentiation of BeWo cells. Fusion was confirmed by observing E-cadherin downregulation; however, there was no effect of loss of TREM-1 on E-cadherin expression (Figure 4C and D). Forskolin treatment increased hCG mRNA by a factor of 38 and secretion by a factor of 2.6. Forskolin-induced hCG gene expression and secretion were significantly decreased in TREM-1 siRNA-transfected cells compared to NS control following forskolin treatment (reduction of mRNA by 35% and secretion by 15%; Figure 4E and F).

TREM-1 Expression in Syncytiotrophoblast Is Regulated by Oxygen Tension

Fusion of cytotrophoblasts is inhibited under hypoxic conditions,⁷ and BeWo cells which are grown under hypoxia secrete less hCG than cells cultivated under normoxic conditions.³⁰ We thus propose that TREM-1 induction under hypoxia may be impaired. To test this, cells were grown to confluence, then treated with forskolin, and cultured at 21% O₂ (normoxia) or 1% O₂ (hypoxia) for a further 24 hours. The TREM-1 protein and mRNA levels were compared. Figure 5A and B shows that hypoxia significantly decreased TREM-1 expression by 50%. Furthermore, the induction of TREM-1 by forskolin at 24 hours was reduced under hypoxic conditions compared to normoxia at both the gene (Figure 5A) and the protein (Figure 5B) level. Likewise, and in support of previous studies,³⁰ hCG gene expression (Figure 5C) and secretion (Figure 5D) were also decreased under hypoxia, both in the absence and presence of forskolin.

Discussion

The present study demonstrates, for the first time, that TREM-1 expression, at both mRNA and protein level, is elevated significantly in severe early-onset preeclamptic placentas compared to gestational age-matched controls. The TREM-1 expression was present in the syncytiotrophoblasts. The localization of TREM-1 to the syncytiotrophoblasts correlates well with the expected localization of a protein that may be involved in the fusion process. Thus, in order to determine whether it indeed plays a role in this process, we used BeWo cells to examine the effects of syncytialization and hypoxia on TREM-1 expression. We also determined the effects of TREM-1 knockdown on BeWo cell proliferation and differentiation by studying biochemical markers of the syncytiotrophoblast. We have demonstrated for the first time that TREM-1 is expressed in BeWo cells, is upregulated in forskolin-differentiated BeWo cells, and is involved in BeWo cell trophoblast differentiation.

Syncytialization is an important event that is needed to cover the developing placental villi with healthy syncytiotrophoblast. The syncytiotrophoblast layer plays an important function during pregnancy, including nutrient exchange and the synthesis of hormones necessary for normal fetal growth and development. In keeping with its role in the proper functioning of the placenta, impaired cytotrophoblast proliferation, differentiation, and fusion of cytotrophoblasts with the syncytiotrophoblast fusion are associated with pathological clinical conditions such as preeclampsia or fetal growth restriction.³¹⁻³⁴ Given that we found increased TREM-1 in preeclamptic placentas, we next sought to determine the effect of syncytialization on TREM-1 expression. To do this, we used an in vitro model of cultured villous trophoblastic cells, BeWo cells in which syncytial formation was induced by forskolin.⁶ We found that TREM-1 gene and protein expression was significantly increased in cells treated with forskolin. Although placental anomalies associated with underperfusion and hypoxia are characterized by abnormalities in syncytiotrophoblast formation,³⁵⁻³⁷ hypoxia has been shown to inhibit syncytial formation and reduce hCG production.^{7,30,36} We also found that under hypoxic conditions, this increase in TREM-1 was reduced when compared to normoxic conditions. These data suggest that the inhibition of BeWo syncytialization under hypoxia conditions may be due, at least in part, to impaired TREM-1 induction. It should be noted that only 1 time point was used to look at TREM-1 expression after forskolin treatment; thus, the effect of forskolin treatment on TREM-1 early or late in the syncytialization process is not known. Nevertheless, our data show that TREM-1 is upregulated in the period following forskolin treatment.

Oxygen tension is a key regulator of trophoblast differentiation during placental development. During placentation, cytotrophoblasts localized in floating and anchoring villi can differentiate into 2 pathways; villous cytotrophoblasts fuse to form the syncytiotrophoblast layer, and anchoring villi cytotrophoblasts generate highly invasive extravillous trophoblasts that later migrate into the decidua and myometrium.³⁸ The

Figure 4. Loss of TREM-1 is associated with decreased BeWo cell differentiation. The BeWo cells were transfected with 150 nmol/L NS siRNA or TREM-1 siRNA. After 24 hours, cells were incubated in the absence or presence of 20 μ mol/L forskolin for an additional 48 hours ($n = 6$ independent experiments). A and B, Efficiency of siRNA transfection of TREM-1. A, TREM-1 gene expression was analyzed by qRT-PCR, and mRNA expression is displayed as mean $\pm$ SEM. * $P < .05$ versus forskolin-treated NS siRNA-transfected cells. B, Representative Western blots and quantitation for TREM-1 in cellular lysates in forskolin-treated NS and TREM-1 siRNA-transfected cells. C and D, Loss of TREM-1 does not affect E-cadherin expression in syncytialized BeWo cells. C, Gene expression of E-cadherin was analyzed by qRT-PCR, and gene expression is displayed as mean $\pm$ SEM. * $P < .05$ versus forskolin-treated NS siRNA-transfected cells. D, Representative Western blot of E-cadherin in cellular lysates in forskolin-treated NS and TREM-1 siRNA-transfected cells. E and F, Loss of TREM-1 decreases hCG levels in syncytialized BeWo cells. E, Gene expression of β -hCG was analyzed by qRT-PCR, and gene expression is displayed as mean $\pm$ SEM. * $P < .05$ versus forskolin-treated NS siRNA-transfected cells. F, The protein level of β -hCG secreted in media. Data are expression displayed as mean $\pm$ SEM. * $P < .05$ versus forskolin-treated NS siRNA-transfected cells. hCG indicates gonadotropin hormone; mRNA, messenger RNA; NS, nonspecific; SEM, standard error of the mean; siRNA, small interfering RNA; TREM-1, triggering receptor expressed on myeloid cells 1; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction.

Figure 5. Hypoxia decreases TREM-1 expression in syncytiotrophoblasts. The BeWo cells were incubated in the absence (DMSO control) or presence of 20 μ M/L forskolin for 48 hours followed by incubation at either 1% or 21% O₂ for 24 hours ($n = 6$ independent experiments). A, The TREM-1 gene expression was analyzed by qRT-PCR, and mRNA expression is displayed as mean $\pm$ SEM. * $P < .05$ versus 21% O₂ basal cells. # $P < .05$ versus 21% O₂ forskolin-treated cells. B, Representative Western blot for TREM-1. C, Gene expression of β -hCG was analyzed by qRT-PCR, and gene expression is displayed as mean $\pm$ SEM. * $P < .05$ versus 21% O₂ basal cells. # $P < .05$ versus 21% O₂ forskolin-treated cells. D, The protein level of β -hCG secreted in media. Data are expression displayed as mean $\pm$ SEM. * $P < .05$ versus 21% O₂ basal cells. # $P < .05$ versus 21% O₂ forskolin-treated cells. DMSO indicates dimethyl sulfoxide; hCG, gonadotropin hormone; mRNA, messenger RNA; SEM, standard error of the mean; TREM-1, triggering receptor expressed on myeloid cells 1; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction.

extravillous trophoblasts within the myometrium induce spiral arteriole remodeling, producing the low-resistance vascular system necessary for fetal growth. Characteristic of this period of development is the physiological switch in oxygen tension.

Early placental villous development occurs in a relatively hypoxic environment, stimulating cytotrophoblast proliferation.^{39,40} From 10 to 12 weeks, the intervillous space opens, where increasing oxygen tension allows the trophoblasts to differentiate into an invasive phenotype.⁴¹ This switch in oxygen tension can fail leading to shallow trophoblast invasion which may result in abnormal placental development and preeclampsia. However, recent evidence suggests that in preeclampsia, hypoxia and reoxygenation (H/R) injury may be a possible etiologic factor for preeclampsia.⁴² It is thought that the detrimental effects of H/R are due to its ability to generate high concentrations of free radicals. Oxidative stress has been found to disturb trophoblast invasion and differentiation.⁴³⁻⁴⁵ It is possible that the increase in oxidative stress after 10 weeks gestation alters TREM-1 expression, which interferes with the syncytialization process and thus contributes to the preeclamptic phenotype. Although the effect of oxidative stress in

TREM-1 expression is not known, in this study we report that hypoxia decreases TREM-1 expression in BeWo cells. It would thus be of interest to determine both the effect of H/R and the oxidative stress on TREM-1 expression.

Having established that TREM-1 is upregulated in differentiated BeWo cells, we then sought to determine how the loss of TREM-1, using siRNA, would affect this process. This was assessed by evaluating cell fusion using E-cadherin,⁸ and cell differentiation using hCG.⁴⁶ In keeping with previous studies,⁸ we found a significant decrease in E-cadherin gene and protein expression in forskolin-treated BeWo cells. However, in TREM-1 siRNA-treated cells, expression of the cell adhesion molecule E-cadherin was not affected. These data suggest that the loss of TREM-1 is not necessary for trophoblast cell-cell fusion. Of note, there was no effect of forskolin or TREM-1 siRNA on cell viability, as assessed by MTT assay.

The synthesis and secretion of hCG are considered to be a marker of syncytiotrophoblast differentiation.^{46,47-49} Recent studies have, however, suggested that hCG protein expression is not necessarily linked to syncytial fusion and proliferation.²⁸ In our study, as expected, forskolin induced hCG expression

and secretion from BeWo cells. Of interest, we found that in response to forskolin, TREM-1 deficient cells exhibited significantly lower levels of hCG at the gene and protein level. We have shown that TREM-1 is a new regulator of hCG which is an essential placental hormone and suggest that TREM-1 may be necessary for trophoblast differentiation. Collectively, our data suggest that TREM-1 does not regulate cell–cell fusion and proliferation but regulates proteins that are produced by the multinucleated syncytiotrophoblast.

Our knockout data in BeWo cells suggest that the downregulation of TREM-1 leads to reduced differentiation in the BeWo model, where the active processes of syncytialization are restricted. The mechanisms by which TREM-1 may affect BeWo differentiation may be due to its inflammatory actions. The TREM-1 is expressed by neutrophils, macrophages, and mature monocytes and has been described as an amplifier of the inflammatory response.⁵⁰ During inflammatory conditions, TREM-1 is upregulated and redirects myeloid cell differentiation and function, thus promoting inflammatory responses.⁵¹ Ligation of TREM-1 stimulates production of the proinflammatory cytokines including TNF- α , IL-1 β , and the chemokines IL-8 and monocyte chemoattractant protein 1.⁵⁰ Inflammation is increased in women with preeclampsia, with activated macrophages and monocytes being further increased.⁵² Circulating levels of IL-1 β and TNF- α are elevated in preeclampsia,⁵³ and infusion of a low dose of lipopolysaccharide in rats at 14 days gestation establishes a preeclampsia model with hypertension and albuminuria.⁵⁴ There is evidence to link inflammation with the modulation of differentiation in BeWo cells and trophoblast cells. For example, proinflammatory cytokines can promote trophoblast fusion and induce hCG production in human BeWo cells.⁵⁵ Thus, it is possible that the elevation of TREM-1 in preeclamptic placentas could be due to the increase in associated inflammation; increased cytokine production may regulate the expression of TREM-1. Furthermore, TREM-1 may also promote differentiation by modulating the expression profile of chemokines/receptors and genes involved in syncytialization. For future studies, it would be of great interest to see whether TREM-1 plays a role in this regard by determining the expression of proinflammatory transcription factors such as nuclear factor κ B and activator protein 1 in TREM-1-deficient cells.

A limitation of this study is that first trimester placenta was not available to examine the localization of TREM-1. In addition, the use of primary trophoblast cells would strengthen the data, and some caution needs to be taken when extrapolating the results obtained in the BeWo cells to normal trophoblast populations. Notwithstanding these limitations, BeWo is the most extensively used as a cell culture model to mimic in vivo syncytialization of placental villous trophoblast.

In conclusion, we have shown that TREM-1, which is localized to the syncytiotrophoblasts, is upregulated in placentas from severe early-onset preeclampsia. Further studies are required to determine whether TREM-1 is actively involved in the etiology of preeclampsia or if the increase in TREM-1 expression is a consequence of the disease. However, our studies do reveal a novel function for TREM-1 in possibly

regulating trophoblast syncytialization. Although TREM-1 does not appear to play a role in BeWo cell fusion, it is required for the induction of hCG, a placental-specific protein that is associated with syncytialization. We have also shown that forskolin acts through TREM-1 to induce trophoblast syncytialization. Our results suggest that TREM-1 may play an important role in trophoblast function in normal and/or pathological pregnancies.

Acknowledgments

The authors gratefully acknowledge the assistance of the Clinical Research Midwives Gabrielle Fleming, Astrid Tieffholz, Lyndall Paolucci, Debra Jinks, Asha Ferguson; and the Obstetrics and Midwifery staff of the Mercy Hospital for Women for their cooperation. Marin Poljak (Department of Obstetrics and Gynaecology, University of Melbourne) is thanked for his assistance with the TREM-1 immunohistochemistry.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Dr Martha Lappas was a recipient of a National Health and Medical Research Council (NHMRC) Career Development Fellowship (grant no. 454777 and 1047025). Funding for ChemiDoc XRS and xMark microplate absorbance spectrophotometer was provided by the Medical Research Foundation for Women and Babies. The Mercy Research Foundation also provided some funding for this project.

References

1. Young BC, Levine RJ, Karumanchi SA. Pathogenesis of preeclampsia. *Annu Rev Pathol*. 2010;5:173-192.
2. Roberts JM, Hubel CA. The two stage model of preeclampsia: variations on the theme. *Placenta*. 2009;30(suppl A):S32-S37.
3. Murphy VE, Smith R, Giles WB, Clifton VL. Endocrine regulation of human fetal growth: the role of the mother, placenta, and fetus. *Endocr Rev*. 2006;27(2):141-169.
4. Red-Horse K, Zhou Y, Genbacev O, et al. Trophoblast differentiation during embryo implantation and formation of the maternal–fetal interface. *J Clin Invest*. 2004;114(6):744-754.
5. Huppertz B, Gauster M. Trophoblast fusion. *Adv Exp Med Biol*. 2011;713:81-95.
6. Kliman HJ, Nestler JE, Sermasi E, Sanger JM, Strauss JF III. Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. *Endocrinology*. 1986;118(4):1567-1582.
7. Alsat E, Wyplosz P, Malassine A, et al. Hypoxia impairs cell fusion and differentiation process in human cytotrophoblast, in vitro. *J Cell Physiol*. 1996;168(2):346-353.
8. Coutifaris C, Kao LC, Sehdev HM, et al. E-cadherin expression during the differentiation of human trophoblasts. *Development*. 1991;113(3):767-77.
9. Wyrwoll CS, Mark PJ, Waddell BJ. Directional secretion and transport of leptin and expression of leptin receptor isoforms in

- human placental BeWo cells. *Mol Cell Endocrinol.* 2005;241(1-2):73-79.
10. Magarinos MP, Sanchez-Margalet V, Kotler M, Calvo JC, Varone CL. Leptin promotes cell proliferation and survival of trophoblastic cells. *Biol Reprod.* 2007;76(2):203-210.
  11. Benaitreau D, Dos Santos E, Leneuve MC, De Mazancourt P, Pecquery R, Dieudonne MN. Adiponectin promotes syncytialisation of BeWo cell line and primary trophoblast cells. *Reprod Biol Endocrinol.* 2010;8:128.
  12. Kelker MS, Foss TR, Peti W, et al. Crystal structure of human triggering receptor expressed on myeloid cells 1 (TREM-1) at 1.47 angstrom. *J Mol Biol.* 2004;342(4):1237-1248.
  13. Allcock RJ, Barrow AD, Forbes S, Beck S, Trowsdale J. The human TREM gene cluster at 6p21.1 encodes both activating and inhibitory single IgV domain receptors and includes NKp44. *Eur J Immunol.* 2003;33(2):567-577.
  14. Klesney-Tait J, Turnbull IR, Colonna M. The TREM receptor family and signal integration. *Nat Immunol.* 2006;7(12):1266-1273.
  15. Ho CC, Liao WY, Wang CY, et al. TREM-1 expression in tumor-associated macrophages and clinical outcome in lung cancer. *Am J Resp Crit Care.* 2008;177(7):763-770.
  16. Bleharski JR, Kiessler V, Buonsanti C, et al. A role for triggering receptor expressed on myeloid cells—1 in host defense during the early-induced and adaptive phases of the immune response. *J Immunol.* 2003;170(7):3812-3818.
  17. Bosco MC, Pierobon D, Blengio F, et al. Hypoxia modulates the gene expression profile of immunoregulatory receptors in human mature dendritic cells: identification of TREM-1 as a novel hypoxic marker in vitro and in vivo. *Blood.* 2011;117(9):2625-2639.
  18. Mihu D, Costin N, Mihu CM, Blaga LD, Pop RB. C-reactive protein, marker for evaluation of systemic inflammatory response in preeclampsia. *Rev Med Chir Soc Med Nat Iasi.* 2008;112(4):1019-1025.
  19. Mazouni C, Capo C, Ledu R, et al. Preeclampsia: impaired inflammatory response mediated by Toll-like receptors. *J Reprod Immunol.* 2008;78(1):80-83.
  20. Schiessl B. Inflammatory response in preeclampsia. *Mol Aspects Med.* 2007;28(2):210-219.
  21. Redman CW, Sargent IL. Preeclampsia and the systemic inflammatory response. *Semin Nephrol.* 2004;24(6):565-570.
  22. Gerretsen G, Huisjes HJ, Elema JD. Morphological changes of the spiral arteries in the placental bed in relation to pre-eclampsia and fetal growth retardation. *Br J Obstet Gynaecol.* 1981;88(9):876-881.
  23. Varkonyi T, Nagy B, Fule T, et al. Microarray profiling reveals that placental transcriptomes of early-onset HELLP syndrome and preeclampsia are similar. *Placenta.* 2011;32(suppl):S21-S29.
  24. Nishizawa H, Ota S, Suzuki M, et al. Comparative gene expression profiling of placentas from patients with severe pre-eclampsia and unexplained fetal growth restriction. *Reprod Biol Endocrinol.* 2011;9:107.
  25. Wice B, Menton D, Geuze H, Schwartz AL. Modulators of cyclic AMP metabolism induce syncytiotrophoblast formation in vitro. *Exp Cell Res.* 1990;186(2):306-316.
  26. Lim R, Lappas M. Decreased expression of complement 3a receptor (C3aR) in human placentas from severe preeclamptic pregnancies. *Eur J Obstet Gynecol Reprod Biol.* 2012;165(2):194-198.
  27. Lappas M, Mitton A, Lim R, Barker G, Riley C, Permezel M. SIRT1 is a novel regulator of key pathways of human labor. *Biol Reprod.* 2011;84(1):167-178.
  28. Orendi K, Gauster M, Moser G, Meiri H, Huppertz B. The choriocarcinoma cell line BeWo: syncytial fusion and expression of syncytium-specific proteins. *Reproduction.* 2010;140(5):759-766.
  29. Lanoix D, St-Pierre J, Lacasse AA, Viau M, Lafond J, Vaillancourt C. Stability of reference proteins in human placenta: general protein stains are the benchmark. *Placenta.* 2012;33(3):151-156.
  30. Strohmmer H, Kiss H, Mosl B, Egarter C, Husslein P, Knofler M. Hypoxia downregulates continuous and interleukin-1-induced expression of human chorionic gonadotropin in choriocarcinoma cells. *Placenta.* 1997;18(7):597-604.
  31. Huppertz B, Kaufmann P, Kingdom J. Trophoblast turnover in health and disease. *Fetal Maternal Med Rev.* 2002;13(2):103-118.
  32. Huppertz B, Kingdom JC. Apoptosis in the trophoblast—role of apoptosis in placental morphogenesis. *J Soc Gynecol Investig.* 2004;11(6):353-362.
  33. Ray JE, Garcia J, Jurisicova A, Caniggia I. Mtd/Bok takes a swing: proapoptotic Mtd/Bok regulates trophoblast cell proliferation during human placental development and in preeclampsia. *Cell Death Differ.* 2010;17(5):846-859.
  34. Gauster M, Moser G, Orendi K, Huppertz B. Factors involved in regulating trophoblast fusion: potential role in the development of preeclampsia. *Placenta.* 2009;30(suppl A):S49-S54.
  35. Jones CJ, Fox H. An ultrastructural and ultrahistochemical study of the human placenta in maternal essential hypertension. *Placenta.* 1981;2(3):193-204.
  36. Kudo Y, Boyd CA, Sargent IL, Redman CW. Hypoxia alters expression and function of syncytin and its receptor during trophoblast cell fusion of human placental BeWo cells: implications for impaired trophoblast syncytialisation in pre-eclampsia. *Biochim Biophys Acta.* 2003;1638(1):63-71.
  37. Redman CW. Current topic: pre-eclampsia and the placenta. *Placenta.* 1991;12(4):301-308.
  38. Aplin JD. Implantation, trophoblast differentiation and haemochorial placentation: mechanistic evidence in vivo and in vitro. *J Cell Sci.* 1991;99(pt 4):681-692.
  39. Genbacev O, Joslin R, Damsky CH, Polliotti BM, Fisher SJ. Hypoxia alters early gestation human cytotrophoblast differentiation/invasion in vitro and models the placental defects that occur in preeclampsia. *J Clin Invest.* 1996;97(2):540-550.
  40. Genbacev O, Zhou Y, Ludlow JW, Fisher SJ. Regulation of human placental development by oxygen tension. *Science.* 1997;277(5332):1669-1672.
  41. Caniggia I, Winter J, Lye SJ, Post M. Oxygen and placental development during the first trimester: implications for the pathophysiology of pre-eclampsia. *Placenta.* 2000;21(suppl A):S25-S30.
  42. Hung TH, Burton GJ. Hypoxia and reoxygenation: a possible mechanism for placental oxidative stress in preeclampsia. *Taiwan J Obstet Gynecol.* 2006;45(3):189-200.
  43. Murata M, Fukushima K, Takao T, Seki H, Takeda S, Wake N. Oxidative stress produced by xanthine oxidase induces apoptosis

- in human extravillous trophoblast cells. *J Reprod Dev.* 2013; 59(1):7-13.
44. Hu R, Jin H, Zhou S, Yang P, Li X. Proteomic analysis of hypoxia-induced responses in the syncytialization of human placental cell line BeWo. *Placenta.* 2007;28(5-6):399-407.
  45. Scifres CM, Nelson DM. Intrauterine growth restriction, human placental development and trophoblast cell death. *J Physiol.* 2009;587(pt 14):3453-3458.
  46. Bernischke K, Kaufmann P, Baergen R. *Pathology of the Human Placenta.* New York, NY: Springer Science.
  47. Frendo JL, Olivier D, Cheynet V, et al. Direct involvement of HERV-W Env glycoprotein in human trophoblast cell fusion and differentiation. *Mol Cell Biol.* 2003;23(10):3566-3574.
  48. Kudo Y, Boyd CA, Sargent IL, Redman CW, Lee JM, Freeman TC. An analysis using DNA microarray of the time course of gene expression during syncytialization of a human placental cell line (BeWo). *Placenta.* 2004;25(6):479-488.
  49. Lin L, Xu B, Rote NS. The cellular mechanism by which the human endogenous retrovirus ERV-3 env gene affects proliferation and differentiation in a human placental trophoblast model, BeWo. *Placenta.* 2000;21(1):73-78.
  50. Bouchon A, Dietrich J, Colonna M. Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J Immunol.* 2000; 164(10):4991-4995.
  51. Colonna M. TREMs in the immune system and beyond. *Nat Rev Immunol.* 2003;3(6):445-453.
  52. von Versen-Hoeynck FM, Hubel CA, Gallaher MJ, Gammill HS, Powers RW. Plasma levels of inflammatory markers neopterin, sialic acid, and C-reactive protein in pregnancy and preeclampsia. *Am J Hypertens.* 2009;22(6):687-692.
  53. Conrad KP, Benyo DF. Placental cytokines and the pathogenesis of preeclampsia. *Am J Reprod Immunol.* 1997;37(3): 240-249.
  54. Faas MM, Schuiling GA, Baller JF, Visscher CA, Bakker WW. A new animal model for human preeclampsia: ultra-low-dose endotoxin infusion in pregnant rats. *Am J Obstet Gynecol.* 1994; 171(1):158-164.
  55. Tsukihara S, Harada T, Deura I, et al. Interleukin-1beta-induced expression of IL-6 and production of human chorionic gonadotropin in human trophoblast cells via nuclear factor-kappaB activation. *Am J Reprod Immunol.* 2004;52(3):218-223.