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**TMED2/p24 β 1 is expressed in all gestational stages of human placentas and in
choriocarcinoma cell lines**

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Abstract

Members of the transmembrane emp24 domain (Tmed)/p24 family of proteins are required for transport of proteins between the endoplasmic reticulum and the Golgi. One member of this family, *Tmed2*/p24 β 1, is expressed during placental development in mice and its expression is required for normal development of the labyrinth layer. Although *TMED2* is conserved in humans, little is known about its expression and function in human placenta. We examined *TMED2* expression in human placenta between 5.5 and 40 weeks of gestation and showed that *TMED2* is expressed in syncytiotrophoblast, cytotrophoblast, and stromal cells. We also found a high level of *TMED2* expression in BeWo but not in JEG-3 choriocarcinoma cell line. We used the BeWo cell line to determine *TMED2* subcellular localization in placental cells and we show its co-localization with the endoplasmic reticulum Golgi intermediate compartment. Our findings show conservation of *TMED2* expression in human placenta and suggest that this protein may also play a role during placental development in humans.

Keywords:

BeWo, Jeg-3, *TMED2*/p24 β 1, Chorionic villi, Cytotrophoblast, Syncytiotrophoblast
Placenta

1. Introduction:

Transmembrane emp24 domain (TMED) trafficking protein 2 (p24 β 1, RNP24) is a member of the p24 family involved in protein trafficking between the endoplasmic reticulum (ER) and the Golgi. The p24 protein family is subdivided into four subfamilies (α , β , γ , and δ) based on sequence similarities. Members of the α , β , γ , and δ subfamilies are found in complex with one another in the ER, the ER-Golgi intermediate compartment (ERGIC) and the Golgi compartments (1-5).

Recently, the single members of the β (TMED2) and γ (TMED10) family were found to interact with GPI-anchored proteins and are predicted to aid in the GPI assembly into ER exit sites as well as in their transport from the ER to the plasma (6-8). TMED2 and TMED10 also regulate each other's stability and in the absence of one protein, the level of the second interacting protein is also reduced (9-11). Although little is known about the specific expression and function of the majority of genes in the p24 family during mammalian development, TMED2 and TMED10 are both required for normal development of mouse embryos (9, 11). *Tmed10* homozygous mutant embryos arrest before the blastocysts stage, suggesting that this gene is required very early in development (9) while *Tmed2* homozygous mutant embryos arrest at mid-gestation with numerous morphological abnormalities including a failure to form the labyrinth layer of the placenta (11).

The labyrinth layer of the mouse placenta consists of multiple trophoblast cell types including two types of syncytiotrophoblast cells, called synT-1 and synT-2, which surround the mononuclear trophoblast giant cells of the maternal sinuses (12). The syncytiotrophoblast cells of the mouse placenta are morphologically similar to the syncytiotrophoblast of the human placenta and are predicted to form via similar molecular mechanisms. In fact, genes that are important for formation of the mouse labyrinth layer have also been shown to be expressed in human placenta and to play important roles in trophoblast differentiation both in primary cultures and in established cell lines of trophoblastic origin such as the BeWo and JEG-3 cells (13-17).

In this manuscript we show that TMED2 is expressed in human placenta between 5 and 40 weeks of gestation. We also show that TMED2 expression is higher in the BeWo choriocarcinoma cell line when compared to JEG-3, and that TMED2 co-localizes with the endoplasmic reticulum Golgi intermediate compartment (ERGIC). Our findings reveal conservation of TMED2 expression in human placenta and identify an *in vitro* model, the BeWo choriocarcinoma cell line, that could be used in future studies to address TMED2 function during trophoblast differentiation.

2. Material and methods:

2.1. Tissue collection

A total of 25 placentas were analyzed. First trimester samples: between 5.5 and 5.7 weeks (n=1); between 6 and 6.4 weeks (n= 3); between 7 and 7.5 weeks (n=2); at 10 weeks (n=1); at 11 weeks (n=2); at 12 weeks (n=3); Second trimester: at 13 weeks (n=2); and at term (n=5).

2.2 Cells and cell culture conditions

The human choriocarcinoma cell line BeWo was obtained from American Type Culture Collection (ATCC; Cat. #CCL-98) and cultured in Dulbecco's Modified Eagle's Medium / Ham's F12 (Sigma; Cat. # D2906), containing 10% fetal bovine serum (FBS; Wisent Biocenter; Cat. #080-150) and 1 % streptomycin/penicillin (Invitrogen; Cat. # 15140-163). JEG-3, a trophoblast cell line derived from human choriocarcinoma was kindly provided by Dr. Cathy Vaillancourt and maintained in Minimum Essential Medium Eagle (Sigma; Cat. # M0643), containing 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin. Cells were maintained in culture at 37°C in an atmosphere containing 5 % CO₂ with medium renewal every 2-3 days.

2.3 TMED2 antibody production

A synthetic peptide of TMED2, Ac-VRERIHRAINNTNSRVC-amide, a sequence that is identical in the mouse and human proteins, was used to generate antibodies in rabbit

(21st Century Biochemicals). Serum from one rabbit detected a 23kDa and 13kDa product in lysates from wild type mouse embryos and failed to detect these bands in lysate from *Tmed2* homozygous mutant embryos by Western blot analysis (data not shown).

2.4 Immunohistochemistry

Immunohistochemistry was performed according to standard protocols with TMED2 polyclonal rabbit primary antibody (1:100) and an anti-rabbit secondary antibody (1:500, Jackson ImmunoResearch, 111-035-003). Haematoxylin counterstaining was then performed to visualize the nuclei. Negative controls included incubation with no primary antibody or incubation with antibody pre-blocked with the TMED2 peptide (2µg/ml). Sections were examined under a light microscope and viewed using Axio Vision software.

2.5 Immunofluorescence

BeWo or JEG-3 cells were grown in glass coverslips to confluence, rinsed twice with phosphate-buffered saline (PBS), and fixed with 4% paraformaldehyde (PFA) for 10 min. After washes with PBS, cells were placed in PBS containing 0.25% Triton X-100 for 10 min. Cells were washed three times with PBS, blocked for 1h with 10% goat serum in (PBS/Triton X-100), and then incubated with the primary antibodies, diluted in 1% BSA/PBS/Triton X-100 at 4°C overnight. The following antibodies were used in this study: Anti-TMED2 (1:100), Anti-PDI [R190] (1:100; Abcam; Cat. # ab2792-100), Anti-Giantin [9B6] (1:300; Abcam; Cat. # ab37266-100), Anti-ERGIC-53 (1:100; Santa Cruz Biotechnology; Cat. # Sc-32442), Anti-E-Cadherin (1:200; Invitrogen; Cat. # 13-1900). After washing with PBS, cells were incubated with fluorescein-conjugated anti-IgG antibody diluted in 1% BSA/PBS/Triton for 1h at room temperature. The following secondary antibodies were in a 1:500 dilution: goat anti-mouse IgG 488, Donkey anti-goat IgG 488, and goat anti-Rabbit IgG 568–Alexa-Fluor; Invitrogen). Cells were then washed three times with PBS and mounted in Vectashield Mounting Medium containing DAPI to counterstain nuclei (Vector Laboratories; Cat. # H-1200). Cells were visualized with a Zeiss microscope and viewed using Axio Vision software. All images were

acquired simultaneously once the background had been subtracted using slides stained only with secondary antibody.

2.6 Quantitative RT-PCR

Total RNA was isolated from JEG3 or BeWo choriocarcinoma cells on two different dates with the Total RNA Mini Kit (Bio-Rad) according to the manufacture's instructions, treated with DNase (Invitrogen), and used for cDNA synthesis according to manufacturers direction (Bio-Rad). Quantitative (q) RT-PCR performed on Stratagene Mx3000P was used for mRNA detection and quantification. SYBR green PCR mix (Qiagen; Cat. #05665) was used as a fluorescent dye and the threshold cycle (CT) value of each sample and the melting curve for each primer pair was recorded. Relative gene expression was determined by using $2^{-\Delta\Delta CT}$ method (Applied Biosystem). The cycling conditions included a hot start at 95 °C for 5 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30s. Specific primers to *18S* (CGG CTA CCA CAT CCA AGG AA; GCT GGA ATT ACC GCG GCT), and *TMED2* (-ATG TAT TCC TGT TCG TGC TT; CAC ATG GAT GGA ACA TAC AA), were designed. The *18S* ribosomal RNA was used as a housekeeping gene for qRT-PCR. The data was normalized using the ratio of *TMED2* to that of the *18S* rRNA. A total of 8 qPCRs were performed for the two experimental duplicates. A Student's t-test (Two-Sample Assuming Unequal Variances) was used to calculate significance.

2.7 Western blot analysis:

BeWo and JEG-3 cells were harvested in cell lysis buffer. Equal amounts of protein were subjected to SDS-PAGE electrophoresis on 10% polyacrylamide gels then, transferred to an Immun-Blot PVDF membrane (BIO-RAD Laboratories; Cat. #162-0177). The immunoreactive bands were detected by ECL plus Western Blotting Detection System, (GE Healthcare; Cat. # RPN2132). The blot was incubated with the *TMED2* rabbit polyclonal antibody (1:2500); Goat anti-rabbit secondary antibody (1:10.000, Jackson ImmunoResearch; Cat. #111-035-003); GAPDH antibody (1:5000, Cell Signalling; Cat. # 2118), as a loading control. All Western blots were repeated at least three times.

3. Results:

TMED2 expression in all trophoblast cells during the first trimester of development

We performed immunohistochemistry with a TMED2 polyclonal antibody to localize TMED2 expression in early human placenta (between 5.5 weeks and 13 weeks), (**Fig 1**). TMED2 expression in chorionic villi was variable in these early stages and was found expressed at similar levels in both the cytotrophoblast and syncytiotrophoblast (**Fig 1A; 1B; 1E; 1F**) or at higher levels in the cytotrophoblast (**Fig 1C; 1D; 1E; F**). TMED2 was also highly expressed in stromal cells, in Hofbauer cells (**Fig 1A – 1G**), and in endothelial vessels (**Fig 1C**). Sections incubated without the primary antibody (**Fig 1H**) or with TMED2 antibody pre-incubated with a TMED2 peptide had no signals (**Fig 2D**). Thus, TMED2 is broadly expressed in early stages of human placenta.

3.2. TMED2 protein is restricted to the cytotrophoblast and stromal cells of term placenta

In term placenta, the chorionic villi have a thin syncytial layer, a discontinuous cytotrophoblast layer and represent the final branches of the villous tree (18). To determine if TMED2 is expressed at this stage, we performed immunohistochemistry on five term placentas. We found the highest level of TMED2 expression in the cytotrophoblasts (**Fig 2A**), endothelial vessels (**Fig 2B**), and Hofbauer or macrophage cells of the chorionic villous stroma (**Fig 2B**). Expression of TMED2 was low and barely detectable in syncytiotrophoblast cells at this stage (**Fig 2A; 2C**).

3.3 TMED2 is highly expressed in BeWo but not in JEG-3 choriocarcinoma cells

To identify an *in vitro* cellular model that could be used to study the function of TMED2 during trophoblast differentiation, we examined its expression in two choriocarcinoma cell lines, BeWo (19) and JEG-3 (20). We found high expression of *TMED2* mRNA in BeWo cells (average delta CT = 13.14, n=4) compared to JEG-3 cells (average delta CT =15.02, n=4), which expressed a low amount of TMED2 [p= 0.0045] (**Fig 3A**). We confirmed this data at the protein level using Western blot analysis on whole cell lysates

from BeWo and JEG-3. This analysis revealed a 20kDa band corresponding to the TMED2 protein in BeWo cells but not in JEG-3 cell lysates (n= 6) (**Fig 3B**). Incubations with a GAPDH antibody, as an internal loading control, revealed the expected band at 37 kDa in both BeWo and JEG-3 cellular lysates (**Fig 3B**). To further characterize TMED2 expression in BeWo and JEG-3 cells, we used immunofluorescence and again this analysis confirmed our qRT-PCR and Western blot results demonstrating the presence of TMED2 protein in the cytoplasm of BeWo cells (**Fig 4A – C**), but not in JEG-3 cells (**Fig 4D - F**). TMED2 is therefore more highly expressed in the BeWo choriocarcinoma cells as compared to JEG-3 cells at both the mRNA and protein levels.

3.3 TMED2 co-localized with ERGIC-53 in BeWo choriocarcinoma cells

Endogenous TMED2 was previously reported to localize to the endoplasmic reticulum-Golgi intermediated compartment (ERGIC) in COS-1 cell lines (2), and in the Golgi of HEK293 cells transfected with a Myc-tagged TMED2 construct (4). To determine the localization of TMED2 in placental cells, we performed co-immunofluorescence on BeWo cells with antibodies against TMED2 and markers of the Golgi (Giantin), ER (PDI), or the ER-Golgi intermediate compartment (ERGIC-53). TMED2 (red) did not co-localize with Giantin (**Fig 5A – C**) or PDI, (**Fig 5D - F**) but a clear co-localization was observed with ERGIC-53 (**Fig 5G - I**). Thus, TMED2 protein localization in the BeWo choriocarcinoma placental cells is similar to previously reported data in COS-1 cells (2).

4. Discussion

TMED2 was shown to be expressed and required for normal mouse placental development (11). However, the expression and function of this gene in human placenta have not been investigated. In this study, we report broad expression of TMED2 in trophoblasts of human placenta at all gestational stages. We also identify a choriocarcinoma cell line, BeWo, which expresses a high level of TMED2 and can therefore be used to study the function of this gene during trophoblast differentiation.

In human placenta, the syncytiotrophoblast layer is continuously renewed by the deposition of mRNAs and proteins from fusing cytotrophoblast cells (21). We detected

TMED2 expression in cytotrophoblast and Hofbauer cells in all placental samples examined. However, we observed variable expression of TMED2 in syncytiotrophoblast of villi from first and second trimester placental samples and very low level of this protein in the term placenta samples. Since cytotrophoblast fusion with the syncytiotrophoblast occurs in discrete regions of villi, the TMED2 proteins that we detect could reflect recent fusion events that resulted in varying levels of TMED2 protein or translation throughout the syncytiotrophoblast.

The BeWo and JEG-3 choriocarcinoma cell lines are widely used for studying trophoblast differentiation and although the two cell lines were both derived from choriocarcinomas almost 30 years ago, it is likely that they represent different trophoblast lineages. In fact, microarray analyses by Burelgh et al (14) identified approximately 2700 genes, including *TMED2* (Burleigh et al., 2007 - supplementary data), that were differentially expressed between BeWo and JEG-3 cells. Our data confirm these findings and extend them to show that this difference is also present at the protein level. TMED2 was previously shown to localize to the ERGIC compartment in COS-1 cells (2) and our data show its co-localization with ERGIC-53 in BeWo cells as well. The specific requirement for this gene in the ERGIC is not known although members of the p24 family play a role in the maintenance of the Golgi (22-24). We are currently investigating if TMED2 expression and localization to the ERGIC compartment is involved in the specific differentiation capacity of BeWo and JEG-3 cells.

5. Conclusion

Our studies reveal expression of TMED2 in trophoblast of human placentas at all gestational stages. We also show that TMED2 mRNA and protein is more highly expressed in the choriocarcinoma cell line BeWo when compared to the JEG-3 cell line. We expect that these choriocarcinoma cell lines will be useful for studying the function of TMED2 during trophoblast differentiation.

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Figure Legends:

Figure 1: TMED2 protein expression in first and second trimester human placenta

Representative images showing TMED2 expression in all trophoblast cells (**A – G**) at 11 (C, D), 12 (A, B, G) and 13 (E, F) weeks. In discrete regions of some villi, TMED2 is detected in both cytotrophoblast and syncytiotrophoblast (**A, B, E, F**). TMED2 expression is also found restricted to cytotrophoblast cells (Cyt) (**B - F**) and extravillous cytotrophoblast (EVCT) (**C**). TMED2 is expressed in the endothelial cells (endo), and stromal cells (star) of all villi. Negative control shows no staining in sections incubated with no primary antibody (**H**). Scale bar represent = 20µm a magnification of 40x in A, C, E and G and a 100x B, D and F.

Figure 2: TMED2 protein expression in term placenta

Representative images from 3 term placentas show that TMED2 is expressed in cytotrophoblast cells (Cyt) and Hofbauer cells inside the stroma (pink arrow), but was low and barely detectable in the syncytiotrophoblast layer (Syn) of the terminal villi (**A - C**). TMED2 is also detected in endothelial cells (yellow arrows) (**B**). Immunohistochemistry with TMED2 antibody pre-incubated with a TMED2 peptide revealed no staining (**D**).

Figure 3: TMED2 mRNA and protein expression in BeWo and JEG-3 cells

Quantification of *TMED2* mRNA, to the house keeping gene 18s rRNA was determined using qRT-PCR_(**A**). *TMED2* mRNA levels in BeWo cells are significantly higher compared to JEG-3 cells, $P = 0.0045$. The final result is presented as a relative expression of TMED2 expression in JEG-3 cells compared to BeWo cells. (Mean \pm SEM, $n=8$). Western blot analysis of TMED2 protein in BeWo and JEG-3 cells (**B**). A 20 kDa band, corresponding to TMED2 protein was detected in BeWo cells, but not in JEG-3 cells.

GAPDH was used as an internal control; the band size is 37kDa as shown in both BeWo and JEG-3 cells. Representative immunoblot for one experiment is shown.

Figure 4: TMED2 protein localizes to the cytoplasm of BeWo cells

Immunocytochemical detection of TMED2 protein in BeWo and JEG-3 cells. TMED2 expression (red) was detected in the cytoplasm of BeWo cells (**A-C**); whereas its expression is not observed in JEG-3 cells (**D-F**). E-cadherin (green) shows the cell membrane (**B, C** and **E, F**), and DAPI (blue) indicate the nucleus (**C, F**). Magnification panels are shown at 63x, and the scale bar = 20µm.

Figure 5: TMED2 co-localizes with ERGIC-53

Co-immunofluorescence of TMED2 (**A**) with Giantin (**B**), as a Golgi marker, showed no co-localization (**C**). Co-immunofluorescence of TMED2 (**D**) with PDI (**E**), as an ER marker, also showed no co-localization (**F**). Co-immunofluorescence of TMED2 (**G**) with ERGIC-53 (**H**), as an ER-Golgi intermediate compartment marker, showed (yellow merge) a clear co-localization with the ERGIC compartment (**I**). DAPI (blue) was used to visualize the nucleus (**C, F, I**). Magnification panels are shown at 63x and the scale bar = 20µm.

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