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# Identification of TMIGD1, a novel cell adhesion molecule involved in human trophoblast cell migration

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BOSTON UNIVERSITY  
SCHOOL OF MEDICINE

Thesis

**IDENTIFICATION OF TMIGD1, A NOVEL CELL ADHESION MOLECULE  
INVOLVED IN HUMAN TROPHOBLAST CELL MIGRATION**

by

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B.A., Princeton University, 2014

Submitted in partial fulfillment of the  
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Master of Science

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**IDENTIFICATION OF TMIGD1, A NOVEL CELL ADHESION MOLECULE  
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**CYNTHIA WANG**

**ABSTRACT**

Transmembrane and immunoglobulin domain-containing 1 (TMIGD1) is a newly identified cell adhesion molecule that mediates cell-cell interactions and is mainly expressed in kidney and colon epithelial cells. In renal epithelial cells, TMIGD1 regulates cell proliferation and migration. Human tissue panels showed expression of TMIGD1 in placenta; however, the potential function of TMIGD1 in placenta is not known. We elected to study the expression and function of TMIGD1 during placentation. This is of interest because dysregulation of placental invasion is linked to obstetrical complications such as preeclampsia and intrauterine growth restriction (IUGR). We hypothesized that TMIGD1 is expressed in trophoblast cells and regulates cell migration during placental invasion.

Placental tissues were subjected to immunofluorescence (IF) staining using anti-TMIGD1 antibody and TMIGD1 localization in trophoblast was visualized using a fluorescence microscope. Additionally, we overexpressed TMIGD1 in the immortalized trophoblast cell line, HTR8/SVneo, via a retroviral system. Transduction was verified using IF, Western blot, and qPCR to compare the modified and original cell lines. Migration of TMIGD1-overexpressing HTR8/SVneo cells was assessed using wound-healing and transwell migration assays.

We observed TMIGD1 localization in the apical region of syncytiotrophoblasts. TMIGD1 mRNA expression in the transduced HTR8/SVneo cells was 3-fold greater than that in the control line, and 400-fold greater in first trimester whole placenta. TMIGD1-overexpressing HTR8/SVneo cells exhibited a  $30\pm 5\%$  decrease in migration in the wound-healing assay, compared to the untransduced cells. Similarly, TMIGD1 overexpression in HTR8/SVneo suppressed migration by 36%, compared to control cells in transwell assays. Fluorescent staining showed that increased TMIGD1 expression modifies actin cytoskeleton by redistributing filaments to the peripheries. Additionally, cells overexpressing TMIGD1 exhibit a distinct morphology that lacks filopodia or other motility structures.

Our study demonstrates for the first time that TMIGD1 is expressed in trophoblast cells and acts to inhibit cell migration. The evidence presented in this study supports the idea that TMIGD1 expression in trophoblast may play an important function in regulating placental invasion, and that perturbations in its activity may be associated with obstetrical complications such as preeclampsia and intrauterine growth restriction.

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## LIST OF ABBREVIATIONS

BeWo.....	human placental choriocarcinoma cell line
C1.....	Collagenase step 1
C2.....	Collagenase step 2
cDNA.....	complementary deoxyribonucleic acid
CTB.....	cytotrophoblast
CY3.....	cyanine 3
DAPI.....	4',6-diamidino-2-phenylindole
DMEM.....	Dulbecco's modified Eagle medium
DNA .....	deoxyribonucleic acid
DNase.....	deoxyribonuclease
dNTP.....	deoxynucleotide
DTT.....	dithiothreitol
ECL.....	enhanced chemiluminescence
EDTA.....	ethylenediaminetetraacetic acid
ERK.....	extracellular signal-related kinase
EV.....	empty vector
EVT.....	extravillous cytotrophoblast
FBS.....	fetal bovine serum
GAPDH.....	glyceraldehyde-3-phosphate dehydrogenase
hCG.....	human chorionic gonadotropin

HEK-293.....human epithelial kidney cell line

HK2.....human kidney tubular cell line

HPL.....human placental lactogen

HRP.....horseradish peroxidase

HTR8/SVneo ..... immortalized first trimester trophoblast cell line

IF.....immunofluorescence

IGPR-1.....immunoglobulin-containing and proline-rich receptor-1

IHC.....immunohistochemistry

IRB.....Institutional Review Board

IUGR.....intrauterine growth restriction

mRNA.....messenger ribonucleic acid

PBS.....phosphate-buffered saline

PCR.....polymerase chain reaction

pMSCV.....plasmid murine stem cell virus

qPCR.....quantitative polymerase chain reaction

Rho.....Ras-homologous

RNA..... ribonucleic acid

RNase.....ribonuclease

RPMI.....cell media developed at Roswell Park Memorial Institute

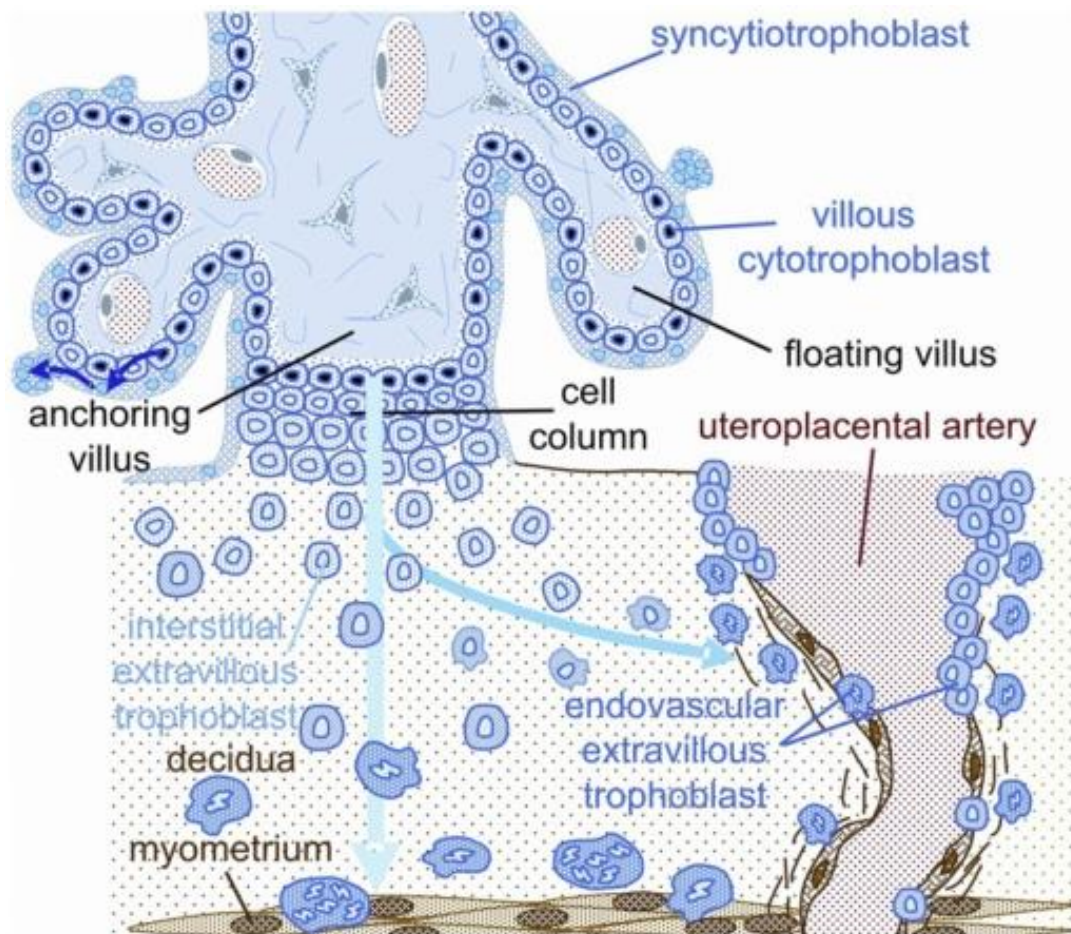
RT.....reverse transcriptase

SFM.....serum-free media

STB.....syncytiotrophoblast  
T1.....Trypsin step 1  
T2.....Trypsin step 2  
T3.....Trypsin step 3  
TLR4.....Toll-like receptor 4  
TMIGD1.....transmembrane and immunoglobulin domain-containing 1  
TMIGD2.....transmembrane and immunoglobulin domain-containing 2

## INTRODUCTION

Through numerous physiological functions, the placenta plays an essential role in supporting the developing fetus during pregnancy. Key changes in placenta structure and activity occur during the first trimester, including placental invasion into the maternal decidua and trophoblast differentiation.



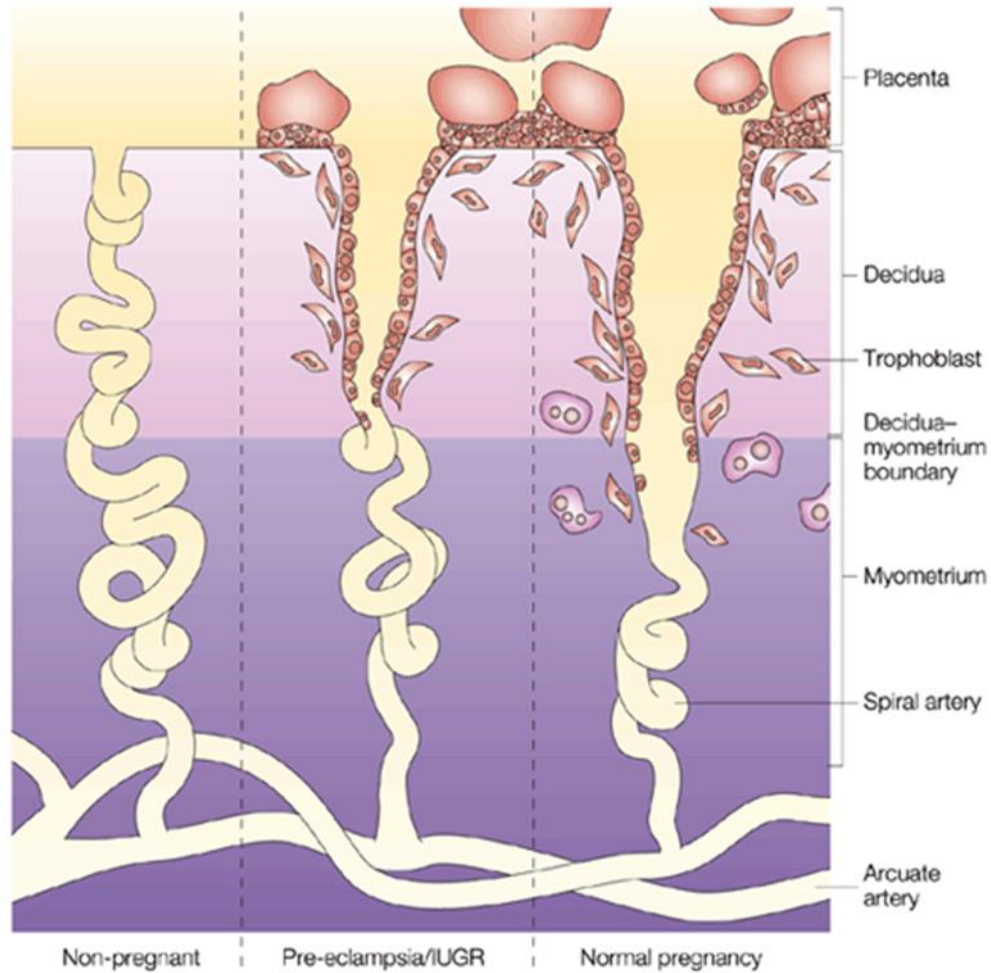
**Figure 1. Interface between placental villi and maternal uterus.** This illustration shows the arrangement of trophoblast layers and cell types. Note that two categories of extravillous trophoblasts are distinguishable: interstitial, which invade the decidua up to the myometrium, and endovascular, which displace smooth muscle cells. Adapted from Kweider et al. (2012).

At the cellular level, the developing placenta is formed of villous cytotrophoblasts (CTBs), which may further differentiate into an epithelial layer of syncytiotrophoblast (STBs), as shown in Figure 1. Inter-trophoblastic communication, possibly achieved via gap junctions or paracrine signaling, allows the two layers to coordinate invasive activity (Bischoff et al., 2000; Cronier et al., 2002). As placenta invasion progresses, CTBs also differentiate into extravillous cytotrophoblasts (EVTs), which migrate through the STB layer into uterine tissue and form attachments to stabilize the growing fetus in a process known as interstitial invasion. Additionally, the EVT's undergo endovascular invasion, during which they remodel the maternal spiral arteries by displacing smooth muscle cells lining the vessel walls (Cartwright et al., 2002). This results in widening of the arteries, lowering resistance and thus allowing greater blood flow to nourish the fetus. These vascular changes ensure sufficient oxygenation throughout pregnancy.

### ***Disorders of deficient placenta invasion: preeclampsia***

Dysregulation of placentation as discussed above may lead to pregnancy complications and/or miscarriage. For example, diminished trophoblast invasion is observed in cases of preeclampsia and intrauterine growth restriction (IUGR). Figure 2 shows that trophoblast invasion is more superficial in these disorders, compared to that of a normal pregnancy. Furthermore, transformation of the maternal spiral arteries is insufficient. Since the placenta fails to initiate the proper changes in vascular resistance,

oxygen delivery to the fetus is compromised. Consequently, fetal growth may be diminished or early pregnancy loss may occur.



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**Figure 2. Remodeling of maternal arteries by invading trophoblasts.** During normal pregnancy, invading trophoblasts reach the myometrium and begin remodeling vasculature. In contrast, cases of preeclampsia and IUGR are characterized by poor trophoblast invasion and a failure to widen spiral arteries into low-resistance, high-flow uteroplacental vessels. Adapted from Moffet-King (2002).

With preeclampsia cases, the mother may suffer from hypertension, edema, and proteinuria. If left untreated, she may progress to eclampsia, a life-threatening condition characterized by the onset of seizures. This necessitates immediate fetal delivery before the condition progresses to multi-organ failure, including renal, hepatic, and cardiovascular complications. A variant of preeclampsia includes “HELLP syndrome”, an acronym for hemolysis, elevated liver enzymes and low platelets that occurs in 10% to 20% of preeclampsia cases (Haram et al., 2012). In North America and Western Europe, preeclampsia occurs in 2-5% of all pregnancies (Ronsmans et al., 2006), but can range as high as 18% in Africa (Villar et al., 2003).

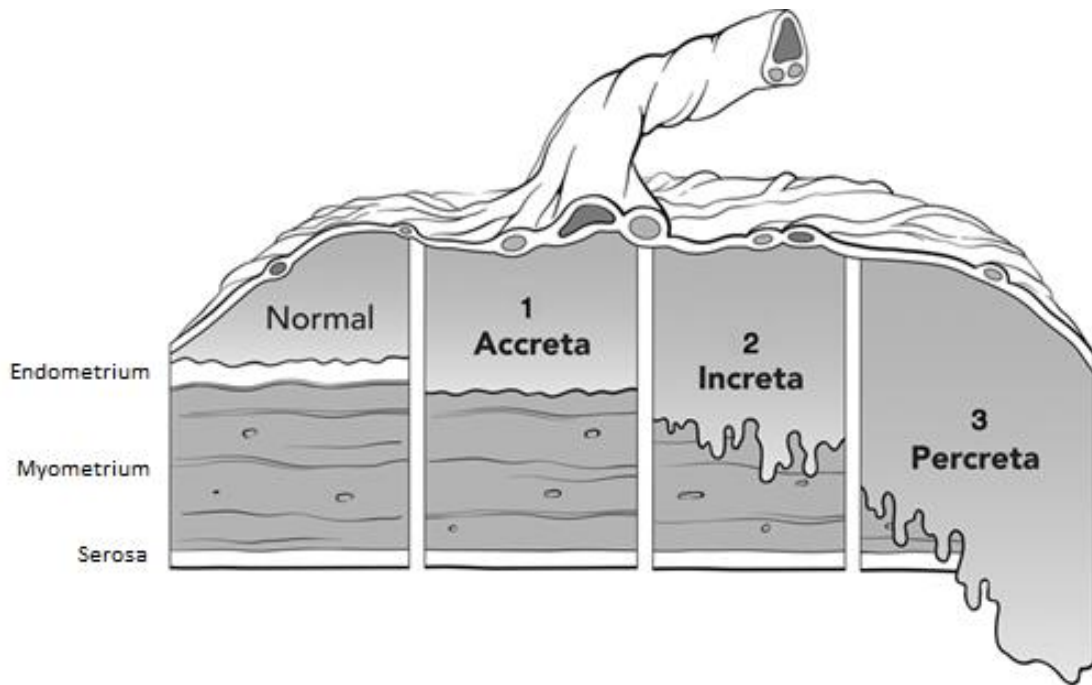
Preeclampsia is a leading cause of global maternal death, and incidence rates have been increasing for the last three decades (Breathett et al., 2014). Advances in maternal and neonatal care have improved clinical outcomes that are even more favorable if symptoms are recognized and managed early on. However, the mechanisms and factors involved in the development of this condition are unclear. Many interdisciplinary explanations have been proposed, and it is unlikely that this disorder is limited to a single cause. For example, studies have linked preeclampsia to a maladaptive immune response (Matthiesen et al., 2005) and inflammation (Redman et al., 1999). One group of researchers performed several meta-analyses of genotype studies from MEDLINE and Embase databases and found significant associations between preeclampsia and several gene groups, including coagulation factors, leptin receptor, and thrombophilic gene groups (Fong et al., 2014). However, more systemic reviews and rigorous study designs

are necessary to draw conclusions about relative risk for carriers. In general, physiological explanations are frequently connected to utero-placental interactions, so the placenta remains an important area of focus. Thus, research into the etiology and risk factors of preeclampsia are crucial for diagnosis and clinical management.

***Disorders of excessive placenta invasion: placenta accreta***

While deficient trophoblast invasion is characteristic of multiple pregnancy disorders, excessive invasion may lead to life-threatening complications as well. Placenta accreta is a serious condition that occurs when the placenta and emerging blood vessels invade too deeply into the uterine wall. More severe degrees of placenta accreta include placenta increta, when the placenta invades into the myometrial layer, and placenta percreta, when the placenta invades beyond the uterine serosa and sometimes into neighboring organs such as the urinary bladder (Figure 3).

The incidence of placenta accreta (and its various forms) has grown in recent years to about 1 in 533 pregnancies, based on a retrospective study of cases from 1982-2002 (Wu et al., 2005). In comparison, the estimated rate in the 1970s was 1 in 4,027 pregnancies (Read et al., 1980). A likely explanation is the increasing incidence of cesarean deliveries (Menacker & Hamilton, 2010); studies have shown that the risk of placenta accreta is directly proportional to the number of prior cesarean deliveries, as well as the presence of placenta previa, a condition where the placenta develops low in the uterus and covers the cervix (Belfort 2010).



**Figure 3. Different levels of excessive placenta invasion.** Compared to a normal pregnancy, the placenta attaches to the myometrium in placenta accreta (1), invades partially into the myometrium in placenta increta (2), and invades through the uterine serosa in placenta percreta (3). Adapted from Reitman et al. (2011).

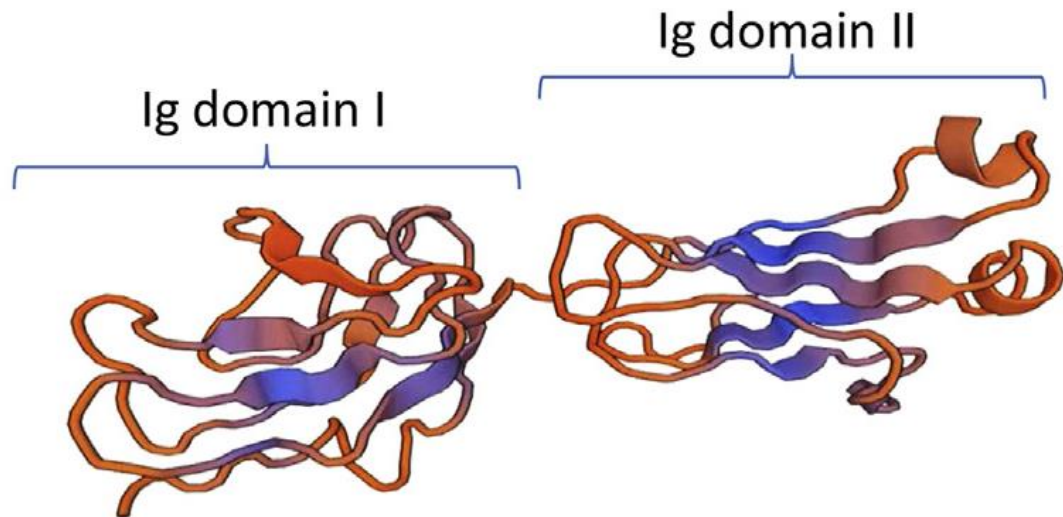
At the time of delivery, the abnormal placenta fails to detach completely from the uterus, resulting in severe hemorrhage. Mothers often require multiple blood transfusions, surgical removal of placenta tissue, and occasionally, gravid hysterectomies. Suspected cases of placenta accreta must be managed with careful preparations to minimize complications during delivery. Although the abnormal placental invasion is frequently associated with previous damage to the uterine lining such as cesarean section or other surgical scarring (Jurkovic et al., 2003), a portion of cases occurs despite no known prior endometrial injury (Clark et al., 1985). Thus, studying invasive properties of trophoblasts

at the molecular level may provide insight into the mechanisms controlling invasion. Identifying regulatory factors will help our understanding of how this condition develops, and will provide potential targets for therapies or diagnostic testing.

### ***TMIGD1 discovery***

Transmembrane and immunoglobulin domain-containing 1 (TMIGD1) was recently characterized in renal tubular epithelial cells as a novel cell adhesion molecule (Arafa et al., 2015). Figure 4 shows the two predicted Ig domains of the TMIGD1 extracellular region, which were found to self-dimerize. This discovery was preceded by the identification of its first protein family member, immunoglobulin and proline-rich receptor-1 (IGPR-1) encoded by transmembrane and Ig domain-containing 2 (*TMIGD2*) gene in human endothelial and epithelial cells. Rahimi et al. (2012) showed that IGPR-1 participates in angiogenesis by regulating capillary tube formation and cell migration. This prompted further investigation into whether TMIGD1 demonstrates similar adhesive properties. Arafa et al. studied protein function by both knocking down and overexpressing TMIGD1 in the human embryonic kidney (HEK-293) and human kidney tubular (HK2) cell lines, and found evidence that TMIGD1 inhibits kidney cell migration and modifies cell morphology by restructuring the actin cytoskeleton. Similarly, IGPR1 was previously observed to reduce cell migration and regulate actin stress fiber remodeling in porcine aortic endothelial (PAE) cells (Rahimi et al., 2012). Additionally, TMIGD1 protects cells against oxidative and metabolic stresses, which is essential

against the frequent injuries experienced by the renal tubular epithelium. Further research is necessary to obtain a clearer picture of mechanisms activating TMIGD1.



**Figure 4. Predicted structure of Ig domains of TMIGD1.** Using a 3D modeling program, this hypothetical protein structure was created based on genomic information. Adapted from Arafa et al. (2015)

## *Objectives*

A tissue panel completed prior to our study (unpublished data) demonstrated high TMIGD1 expression in whole human placenta. Given the presence of TMIGD1 in the placenta and previous evidence that placental invasion shares certain characteristics with the process of tumor metastasis, we sought to examine the potential role of TMIGD1 during placentation. TMIGD1 has been shown to regulate cell proliferation and migration in renal epithelial cells. Specifically, it appears to exhibit inhibitory activity, as observed in overexpression and knockdown experiments in human kidney cell lines. Furthermore, TMIGD1 expression is downregulated in human colon tumors (Cattaneo et al., 2011), suggesting that its reduced expression may be permissive of invasion. We therefore sought to investigate specific TMIGD1 expression in placenta villi layers and observe how overexpression affects trophoblast motility.

The specific aims of our study are:

- 1) To visually localize TMIGD1 expression in human placenta at different stages of development
- 2) To overexpress TMIGD1 in HTR8/SVneo cells using a retroviral vector
- 3) To assess cell motility in our transduced HTR8/SVneo cells and observe the effects of TMIGD1 overexpression
- 4) To analyze actin structure in transduced cells to assess mechanisms underlying changes in migratory/invasive behavior

We hypothesize that TMIGD1 is expressed in trophoblast cells and regulates cell migration during placental invasion. The results of our study would hopefully elucidate the role of TMIGD1 in placental development and provide a target for future studies related to pregnancy disorders.

## METHODS

### *IRB-approved discarded human tissue collection*

De-identified discarded first and second trimester human placental tissue was collected from pregnancy terminations at the family planning unit of Boston Medical Center under IRB approved protocol H-26575.

### *Immunohistochemistry (IHC)*

Human placental tissue: Placental tissue was fixed and dehydrated in a series of ethanol and Pro-Par (xylene substitute) washes before embedding in paraffin wax and mounting 6  $\mu\text{m}$  sections onto Fisherbrand Superfrost Plus slides. Before staining, sections were subjected to a deparaffinization and rehydration treatment consisting of Pro-par, ethanol, and distilled water washes. The tissue sections were then placed in Diva Decloaker 1X (Biocare Medical cat#DV2004MX) for an antigen-retrieval procedure, performed in a pressure cooker to improve epitope recognition by primary antibodies. Tissue sections were washed 5 times in PBST (1X PBS+ 0.4% Triton X-100) for 5 minutes each, blocked for 20 minutes in 4% donkey serum (Jackson ImmunoResearch Laboratories) in PBST, and incubated overnight at 4°C in mouse-anti-HPL (ABCAM cat# AB11396) or rabbit anti-TMIGD1, diluted 1:1000 and 1:150 in PBST with 10% horse serum (Vector Laboratories), respectively. Sections were washed 3 times in PBST for 5 minutes each, blocked for 20 minutes, and incubated for 30 minutes in the dark at room temperature in

secondary antibodies CY3-anti rabbit and AlexaFluor 488-anti mouse (Invitrogen), diluted 1:1500 in PBST. Sections were washed 3 times in PBST for 5 minutes each and coverslipped using VECTASHIELD Mounting Media with DAPI (Vector Laboratories). Images were taken using an Olympus BH2-RFCA microscope and attached Olympus DP73 camera.

Cell lines: HTR8/SVneo cells (Graham et al., 1993) were seeded onto 22mm coverslips in a 6-well plate and incubated at 37°C for 48 hours. Cells were fixed for 15 minutes in 4% paraformaldehyde, washed 3 times in PBS, permeabilized for 10 minutes in PBST, washed 3 times in PBS, blocked for 30 minutes, and incubated for 90 minutes in primary antibody rabbit anti-TMIGD1 diluted 1:400 in blocking agent. Cells were washed 3 times in PBS and incubated for 45 minutes in secondary antibody CY3-anti rabbit (Invitrogen), diluted 1:500 in blocking agent. Cells were washed 3 times in PBS and incubated for 15 minutes in Alexa Fluor 488<sup>®</sup> phalloidin (Thermo Fisher Scientific), diluted 1:500 in blocking agent. Cells were washed 3 times in PBS and the coverslips were mounted onto slides using VECTASHIELD Mounting Media with DAPI (Vector Laboratories). Images were taken using an Olympus BH2-RFCA microscope and attached Olympus DP73 camera.

### ***RNA Extraction***

Cell lines: 500,000 HTR8/SVneo cells were seeded on 6-well plates and incubated at 37°C for 24 hours. Cells were washed with cold PBS and lysed with 350 µl of Buffer RLT (RNeasy Mini Kit, Qiagen, cat # 74104). After vortexing, lysate was mixed with equal volume of cold 70% ethanol and transferred to RNeasy spin column placed in 2 mL collection tube. The sample was centrifuged for 15 sec at 10,000 rpm, then washed with Buffer RW1 (Qiagen) and spun again. DNase I (Qiagen, cat #79254) was added directly onto column membrane to destroy DNA. Sample was washed again with Buffer RW1 and Buffer RPE. Flow-through was discarded after every wash. Purified RNA was eluted from membrane using 30 µL RNase-free water. RNA quantification was performed on a BioTek microplate reader using Gen5™ software.

Placenta tissue: Tissue was collected into cytowash, divided into 30 mg pieces, and transferred to 1 ml of cold Trizol in a 2 ml centrifuge tube. Contents were homogenized for 90 seconds, then incubated on ice for 5 minutes. 200 ul chloroform was added to tubes and vortexed for 15 seconds. After incubating at room temperature for 5 minutes, tubes were centrifuged at 12,000 g for 10 minutes at 4°C. The upper aqueous phase was transferred to a new tube and cell pellet was discarded. The RNA-containing liquid was treated according to the aforementioned protocol, starting with the equal volume addition of 70% ethanol.

### ***Reverse Transcription***

The SuperScript® III First-Strand Synthesis System (Life Technologies, cat#18080051) was used to synthesize complementary DNA to isolated RNA. This kit provided the following reagents and optimized protocol for the reactions. All incubation steps were completed in an Applied Biosystems® GeneAmp® PCR System 9700. 0.5-2 ug of RNA sample was mixed with 1 ul each of random hexamers (50 ng/ul) and dNTP mix (10mM) in a PCR tube, and the solution volume was brought up to 10 ul using RNase-free water. After incubation at 65°C for 5 minutes, the denatured product was placed on ice for at least 1 minute before being gently mixed with 10 ul of cDNA synthesis mix (Table 1).

**Table 1. cDNA Synthesis Mix**

<b>Reagent</b>	<b>Volume for each sample (ul)</b>
10X RT buffer	2
25mM MgCl <sub>2</sub>	4
0.1M DTT	2
40 U/ul RNaseOUT	1
SuperScript III reverse transcriptase	1
<b>Total volume</b>	<b>10</b>

The annealing step was performed at 25°C for 10 minutes before incubation at 50°C for 50 minutes to allow for cDNA synthesis. The reaction was terminated by increasing the temperature to 85°C for 5 minutes, and then placing the PCR tubes on ice. Lastly, 1 ul of RNase H was added to each sample, and the tubes were incubated at 37°C for 20 minutes to remove leftover RNA. An extra tube with RNase-free water replacing the SuperScript III reverse transcriptase was created as a negative control in each experiment. The synthesized cDNA was stored at -20°C until qPCR.

### ***Quantitative Polymerase Chain Reaction (qPCR)***

Reactions were completed with either iTaq™ Universal SYBR® Green Supermix (Bio-rad, cat#:172-5121) or TaqMan® Gene Expression Assays according to Table 2 and Table 3, respectively. For both types of reactions, the housekeeping gene GAPDH was used as an internal control. RNase-free water was used in place of cDNA as a negative control.

**Table 2. qPCR mixture with SYBR® primers**

<b>Reagent</b>	<b>Volume for each reaction (ul)</b>
SYBR® Green Supermix (2X)	8
Primer mix	2
cDNA	2
RNase-free water	4
<b>Total volume</b>	<b>16</b>

**Table 3. qPCR mixture with Taqman® primers**

<b>Reagent</b>	<b>Volume for each reaction (ul)</b>
TaqMan® Gene Expression Master Mix	10
Primer mix	1
RNase-free water	7
cDNA	2
<b>Total volume</b>	<b>20</b>

Reagents were mixed in 96-well plates, which were sealed before placing in an Applied Biosystems 7500 Real-Time PCR System. qPCR reactions performed with SYBR® or TaqMan® followed the thermocycling conditions in Table 4 and Table 5, respectively. Data was collected during the 60°C step of each cycle (marked with asterisks). Cycle thresholds were normalized using GAPDH results and duplicate samples were averaged.

**Table 4. Thermocycling with SYBR®**

Stage	Temperature (°C)	Time
Initialization	95	5 min
35 cycles	95	30 sec
	60*	1 min
	72	30 sec
Hold	72	10 min
Hold	95	15 sec
Hold	60	1 min

**Table 5. Thermocycling with TaqMan®**

Stage	Temperature (°C)	Time
Initialization	95	10 min
40 cycles	95	15 sec
	60*	1 min

***Cell Lines***

The immortalized extravillous trophoblast-derived HTR8/SVneo cell line was cultured at 37°C in RPMI 1640 media (Fisher Scientific) with 10% fetal bovine serum (Life Science) and 1% penicillin/streptomycin (Life Technologies, 100X). Cells were grown in 10 mm cell culture dishes (BD Falcon), dislodged using 0.25% Trypsin-EDTA, and passed at 90-100% confluence.

***Viral transduction***

HTR8/SVneo cells were grown in 6 cm cell culture plates to 70-80% confluence before a 16-hour transduction period by a pMSCV retroviral vector with a cloned in Myc-tagged TMIGD1 or by an empty vector control. 1µg of Polybrene was added to each plate to

increase efficacy of viral uptake. After a 48-hour recovery period, plates were treated with puromycin for 96 hours to select for successfully transduced cells.

### ***Wound-healing assay***

Untransduced and transduced TMIGD1-overexpressing HTR8/SVneo cells were grown to 100% confluence in 12-well plates before scratches were made by 1000  $\mu$ l pipette tips. Quadruplicates of both cell lines were produced, and photographed at 0-hour, 24-hour, and 48-hour timepoints. The wound areas were calculated using ImageJ and the degree of migration was assessed as the percentage change in wound area after 24 and 48 hours. Unpaired t-tests were performed to quantify the relative migration of untransduced HTR8/SVneo cells and transduced TMIGD1-overexpressing HTR8/SVneo cells. The migration of transduced cells was expressed relative to untransduced cells.

### ***Transwell Migration Assay***

10,000 HTR8/SVneo cells were suspended in serum-free RPMI and seeded onto transwell membranes with 8.0  $\mu$ m size pores. 500  $\mu$ l of RPMI with 10% FBS was added to each well to prompt cell migration. Cells were incubated for 16 hours at 37°C, and membranes were washed with PBS. The upper surface of membranes were wiped with cotton Q-tips to remove non-invading cells. Membranes were washed with PBS again, and cells were treated with Diff-Quik fixation and staining solutions for 90 seconds each. Membranes were washed with distilled water and removed from transwells using a razor

blade. Stained cells were viewed under a light microscope, and images were taken using an Olympus BH2-RFCA microscope and attached Olympus DP73 camera.

### ***Invasion Assay***

10,000 HTR8/SVneo cells were suspended in serum-free RPMI and seeded onto Matrigel (diluted 1:8) coated transwell membranes with 8.0  $\mu\text{m}$  size pores. The assay was carried out according to the above protocol for the transwell migration assay.

### ***Western Blot Analysis***

Cells were grown in 10 cm cell culture plates to 100% confluence before lysis. Cell plates were washed twice on ice with H/S buffer, then lysed with 250  $\mu\text{l}$  of lysis buffer. Lysate was collected into centrifuge tube using a cell scraper and centrifuged at 12000 rpm for 15 minutes at 4°C. Supernatant was isolated and diluted 4: 1 using 5X loading buffer, and boiled for 5 minutes at 95°C. 40  $\mu\text{l}$  of cell lysate and 6  $\mu\text{l}$  of ladder were loaded into wells of a 4–20% Mini-PROTEAN<sup>®</sup> TGX<sup>™</sup> Gel, and run at 50 mA until protein bands reached the gel bottom. Proteins were transferred onto a nitrocellulose membrane in a chamber run at 400 mA for 2 hours. The membrane was placed in blocking solution for an hour before incubating overnight at 4°C in rabbit anti-TMIGD1 antibody (diluted 1:1000 in blocking solution). The membrane was rinsed in washing buffer 3 times for 5 minutes each before incubating in horseradish peroxidase (HRP)-anti mouse secondary antibody (Santa Cruz) for an hour. Proteins were visualized by treating the membrane with

Pierce™ ECL Western Blotting Substrate, and the chemiluminescent signal was exposed onto film. As a loading control, the membrane was stripped of antibodies and incubated with mouse anti-ERK antibody (diluted 1:1000 in blocking solution) for an hour.

Membrane treatment was repeated according to the above protocol.

### ***Placental Fractionation***

Placenta tissue was collected and rinsed in a solution of sterile PBS and antibiotics (amphotericin B, gentamycin, and vancomycin) before being placed on ice in sterile DMEM with antibiotics for dissection. Terminal villi were cut off using sterile scissors and collected in a vial to be centrifuged for at 1300 rpm for 5 minutes at 4°C. The isolated pellet was weighed and proportional amounts of digestive enzyme solutions were mixed according to Table 6. After the villi were digested in C<sub>1</sub>, a drop was checked to confirm STB and CTB presence. This was repeated for T<sub>1</sub>, which was expected to contain mostly CTBs and fewer STBs, as well as for T<sub>2</sub>, which contained CTBs and no fibroblasts. The T<sub>3</sub> step was carried out to isolate more CTBs while avoiding fibroblasts.

**Table 6. Enzyme volumes and incubation times**

<b>Enzyme</b>		<b>Amount (mL)</b>	<b>Time</b>
Collagenase <sub>1</sub>	C <sub>1</sub>	mass (grams) × 6	6 min
Trypsin <sub>1</sub>	T <sub>1</sub>	mass (grams) × 6	6 min
Trypsin <sub>2</sub>	T <sub>2</sub>	mass (grams) × 5	4.5 min
<i>Trypsin<sub>3</sub> (if needed)</i>	<i>T<sub>3</sub></i>	<i>20</i>	
Collagenase <sub>2</sub>	C <sub>2</sub>	10 (< 20 grams) 20 (> 20 grams)	2.5 min

After each step, the supernatant was collected through a sterile gauze filter into a vial and diluted with 5 ml of FBS. All supernatants were centrifuged at 1300 rpm for 8 minutes at 4°C. After discarding the supernatant, the pellets were digested by the C<sub>2</sub> step and filtered through a cell strainer before another spin. The pellets were resuspended in serum-free media, layered on top of a Percoll density gradient, and centrifuged for at 2700 x g for 25 minutes at 4°C. CTBs and STBs were isolated by extracting specific layers of the gradient and washing with sterile DMEM. Cells were counted using a hemocytometer and stored in Trizol (ThermoFisher) for future qPCR or in Recovery Cell Culture Freezing Medium (Life Technologies).

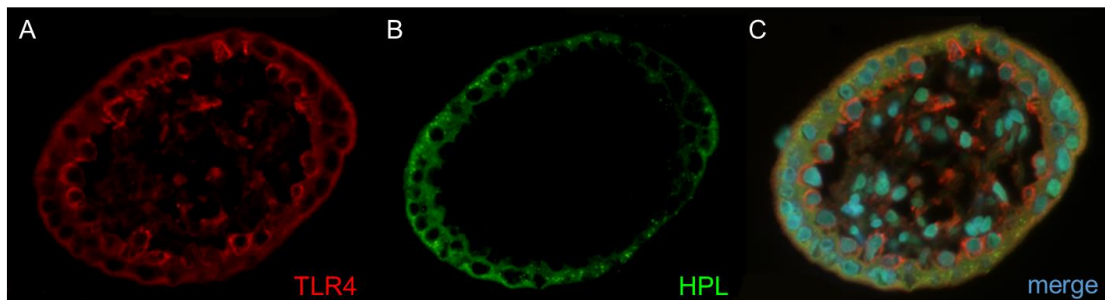
### *Statistical analysis*

Data from qPCR, transwell migration assay, wound healing assay, and invasion assay were analyzed with ImageJ (NIH) and Microsoft Excel. Significance testing was accomplished with un-paired two-tailed t-tests using GraphPad Prism software.

## RESULTS

### *TLR4 is a positive marker of villous cytotrophoblasts*

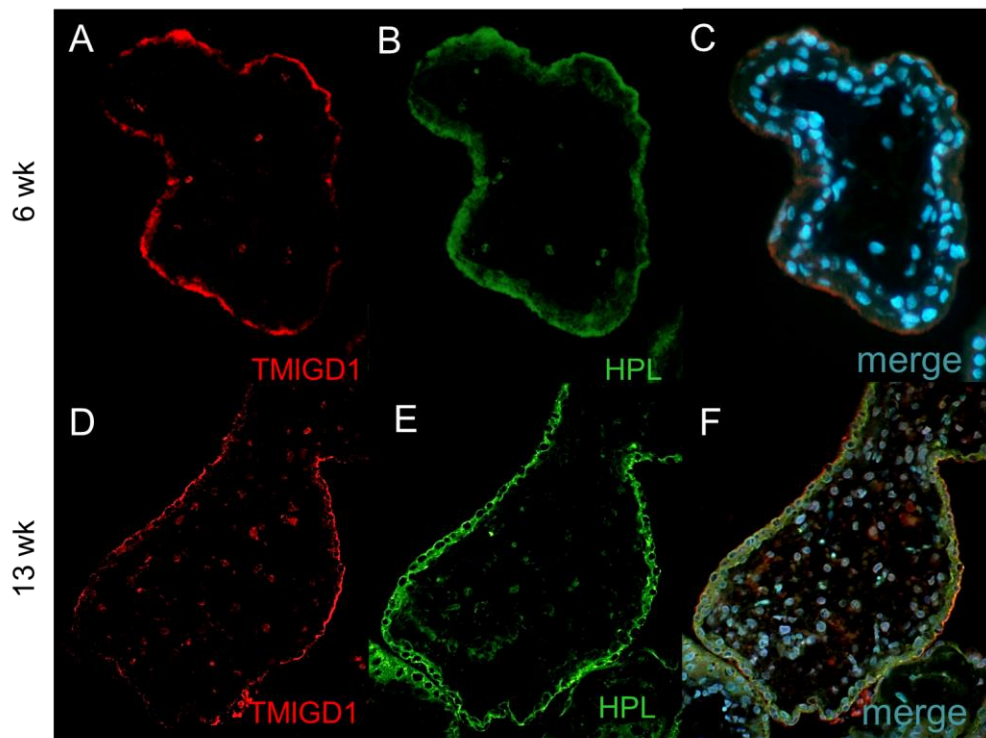
To accurately assess protein localization in trophoblast layers using immunofluorescence, positive markers for the STB and CTB layers are necessary. While the use of human placental lactogen (HPL) staining in STBs has been recorded as early as 1986 (Beck et al., 1986), finding a potential marker for the inner CTB layer has proven more challenging. We tested the specificity of Toll-like receptor 4 (TLR4) that Lye et al. (2015) successfully localized to the CTB layer of first trimester human placental villi. Our antibody against TLR4 showed that its expression is limited to CTB cytoplasm (Figure 5A) in a clearly different layer from HPL that we confirmed as localizing in the STB layer (Figure 5B).



**Figure 5. Fluorescent labeling of chorionic villi with TLR4 and HPL antibodies.** Toll-like receptor 4 (TLR4) specifically localizes to the CTB cytoplasmic region (A), while HPL localizes to the STB layer (B). The merged image shows distinct staining of layers by the two antibodies (C) in 13-week human placenta. Images taken at 20x magnification. Cell nuclei were stained with DAPI.

*TMIGD1 localizes to the apical compartment of syncytiotrophoblasts*

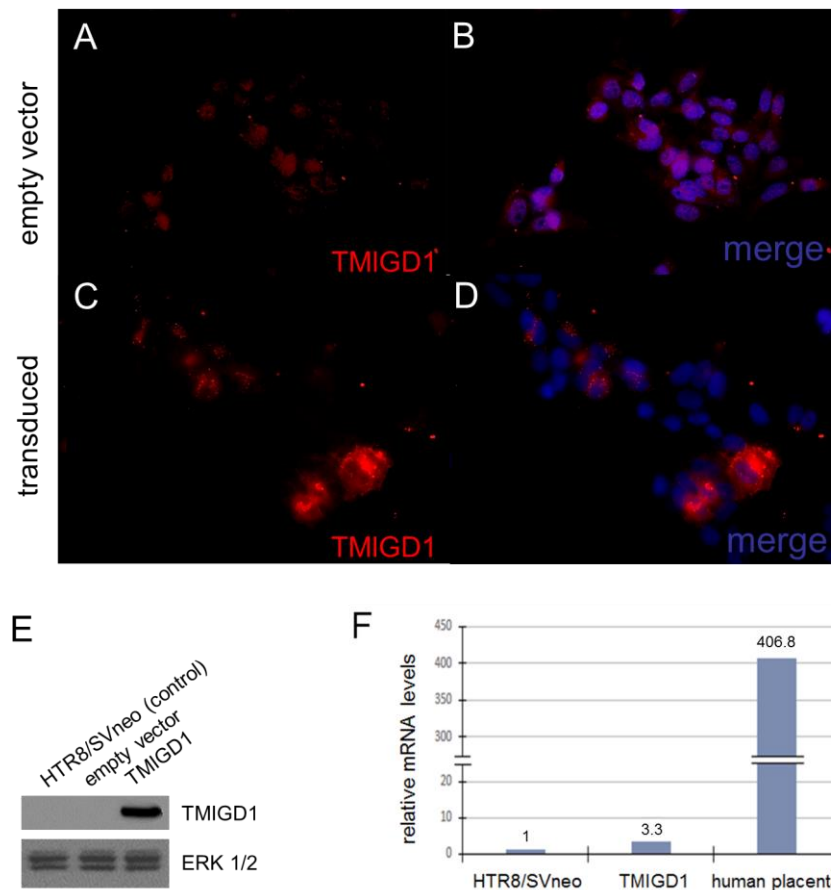
To determine where TMIGD1 activity occurs, we aimed to localize TMIGD1 expression in placental villi using immunofluorescence. 6- and 13-week placental tissue was stained using an antibody against TMIGD1, as well as an antibody against human placental lactogen (HPL) as an STB layer marker. Figure 6 shows that TMIGD1 is co-localized with HPL, indicating that TMIGD1 expression is confined to the STB layer. Furthermore, TMIGD1 staining was less dispersive, with preferential localization at the apical region of STBs as seen in the merged images (panels C and F).



**Figure 6. Fluorescent labeling of chorionic villi with TMIGD1 and HPL antibodies.** Human placental lactogen (HPL) was used as a marker for syncytiotrophoblast cells. TMIGD1 localizes to the apical region of syncytiotrophoblasts in human placenta at 6 weeks (A-C) and 13 weeks (D-F) of gestation. Images taken at 20x (D-F) and 40x (A-C) magnification. Cell nuclei were stained with DAPI.

*Increased TMIGD1 expression in transduced HTR8/SVneo cells*

To examine putative function of TMIGD1 in human trophoblasts, we overexpressed TMIGD1 in HTR8/SVneo cells, which are an immortalized first trimester trophoblast cell line (Graham et al., 1993).

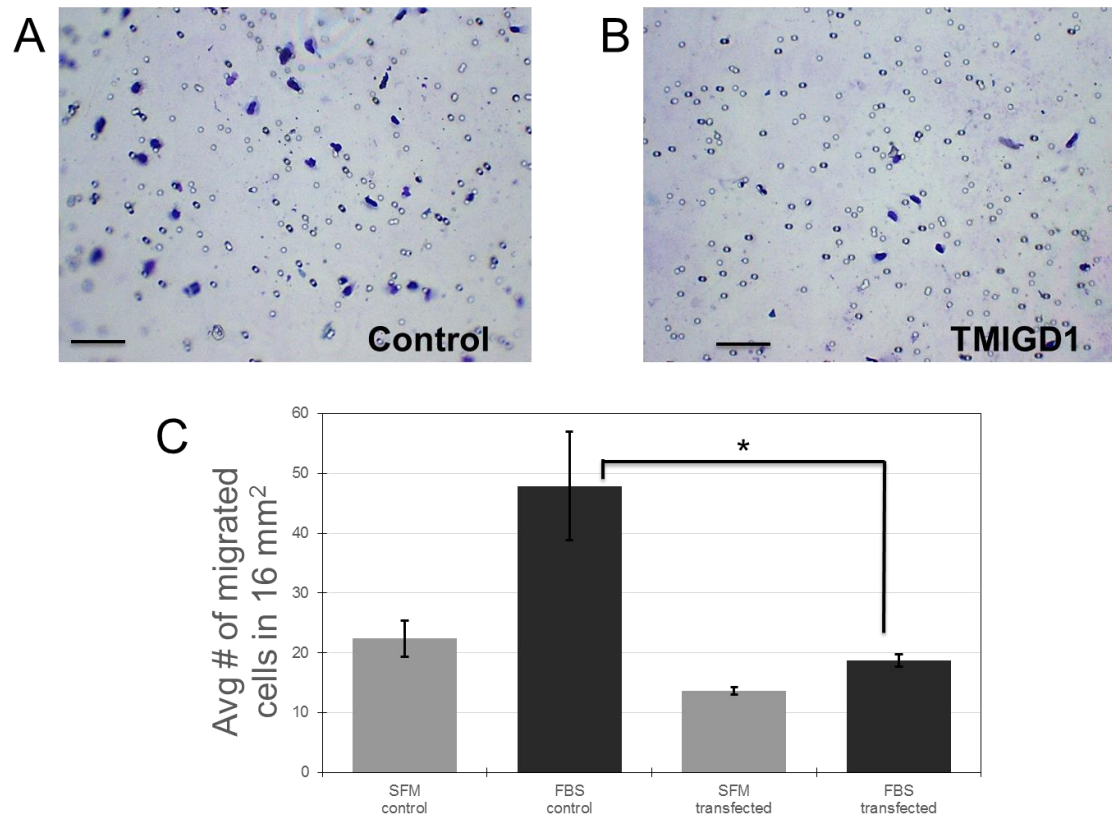


**Figure 7. TMIGD1 overexpression in HTR8/SVneo cell line.** A construct with TMIGD1 cloned into a pMSCV retroviral vector was used to transduce HTR8/SVneo cells. Staining for TMIGD1 showed expression in the transduced HTR8/SVneo cells (C-D) compared to empty vector (A-B) and untransduced cells (not shown). Images were taken at 20x magnification. Whole cell lysates were blotted for TMIGD1 and loading control, ERK 1/2 (E). TMIGD1 mRNA levels were 3-fold higher in the transduced cell line and 400-fold higher in first trimester whole placenta, relative to the untransduced cell line (F).

This was accomplished using a pMSCV retroviral vector containing Myc-tagged TMIGD1. HTR8/SVneo cells were also transduced with an empty vector as a negative control. TMIGD1 overexpression in HTR8/SVneo cells was verified using immunofluorescence; the transduced cell line exhibited more TMIGD1 staining than the empty vector cells (Figure 7A-D). Furthermore, protein and mRNA expression levels were checked using Western blot and qPCR, respectively (Figure 7E-F). The degree of overexpression was quantified using qPCR, which showed that transduced cells displayed ~3-fold increased TMIGD1 expression compared to that of control cells. In addition, human placental tissue expressed more than 400 times more TMIGD1 than the original HTR8/SVneo cell line.

*Increased TMIGD1 expression in HTR8/SVneo cells reduces trophoblast migration*

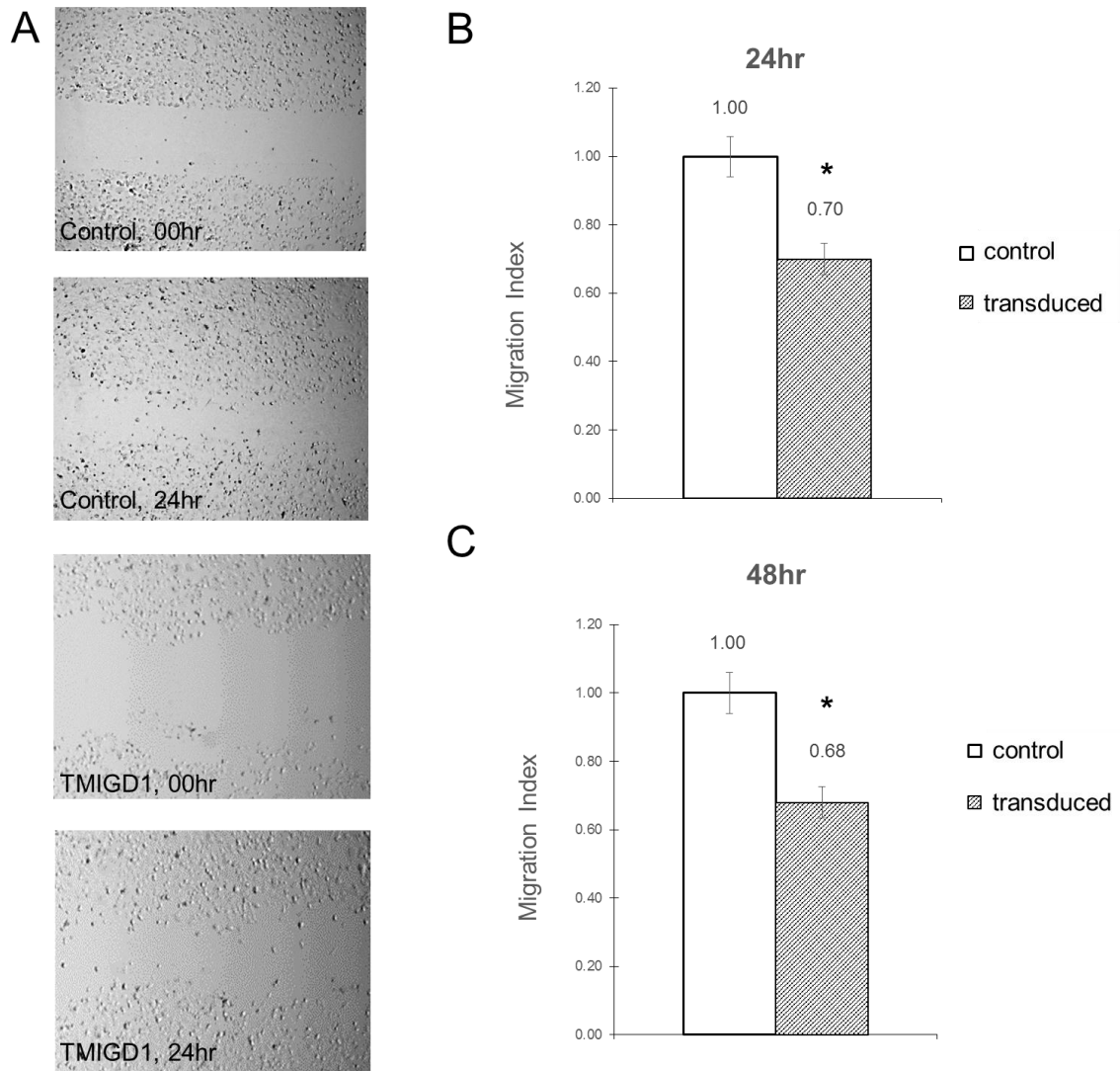
Using the modified TMIGD1-overexpressing cell line, we examined whether increased TMIGD1 expression affects trophoblast migration. Transduced and control HTR8/SVneo cells were seeded onto transwell membranes and allowed to migrate for 16 hours before fixation and staining. To control for random migration, both cell lines were incubated in serum-free media (SFM), while experimental wells contained media with fetal bovine serum (FBS). Figure 8 shows that trophoblasts with increased TMIGD1 expression migrate significantly slower than those with normal TMIGD1 levels. After taking into account random movement, we approximated that the increased TMIGD1



**Figure 8. TMIGD1 overexpression inhibits cell migration in transwell migration assay.** Migrating HTR8/SVneo cells were fixed and stained with Diff-Quik. Representative images were taken of control cells (A) and TMIGD1-overexpressing cells (B). Cells incubated in serum-free media (SFM) were used as negative controls for random migration. Scale bar indicates 500  $\mu$ m. An unpaired t-test showed that increased TMIGD1 expression reduced migration to  $40\pm 10\%$  of controls after 16 hrs (C).  $n=3$ ;  $*p=0.048$

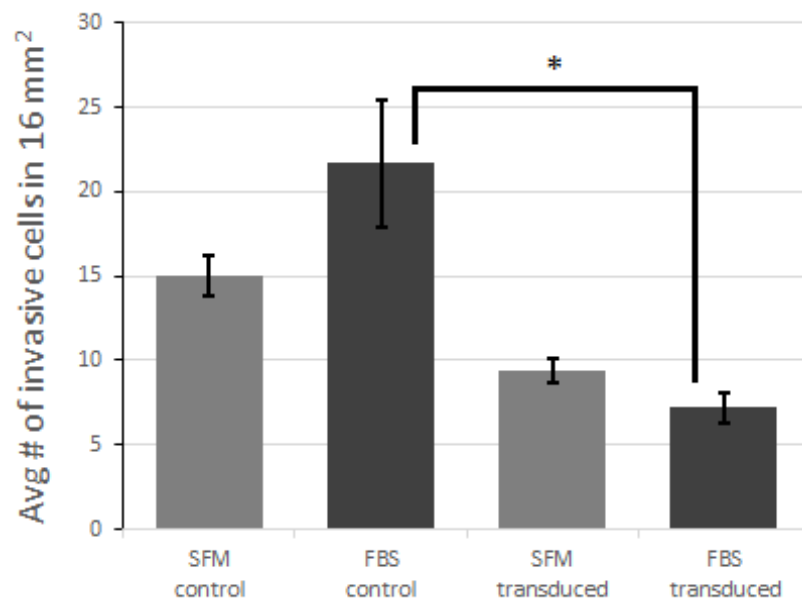
expression reduced migration to  $40\pm 10\%$  of control cell migration. This supports the idea that TMIGD1 may play an inhibitory role in trophoblast invasion.

We further tested the effect of increased TMIGD1 expression on migration using a wound healing assay. Analysis of area changes showed that after 24 and 48 hours, the



**Figure 9. TMIGD1 overexpression inhibits cell migration in wound healing assay.** Scratches were made on confluent cells using 1000 ul pipette tips. Photographs were taken at 0h, 24h, and 48h time points (not shown), and the wound area was measured using ImageJ (A). Unpaired t-tests were performed to quantify the relative migration of untransduced HTR8/SVneo cells and transduced TMIGD1-overexpressing HTR8/SVneo cells. The migration of transduced cells was expressed relative to untransduced cells. The degree of migration was compared separately at 24h (B) and 48h (C). TMIGD1 overexpression reduced migration to  $70 \pm 5.25\%$  ( $p = 0.0001$ ) and  $68 \pm 4\%$  ( $p = 0.0008$ ), respectively ( $n=8$ ).

TMIGD1-overexpressing cell line displayed significantly slower migration than the control cell line (Figure 9). This is consistent with the results of our transwell migration assay, as well as a previous study showing that overexpression of TMIGD1 inhibits migration of kidney epithelial cells (Arafa et al., 2012).



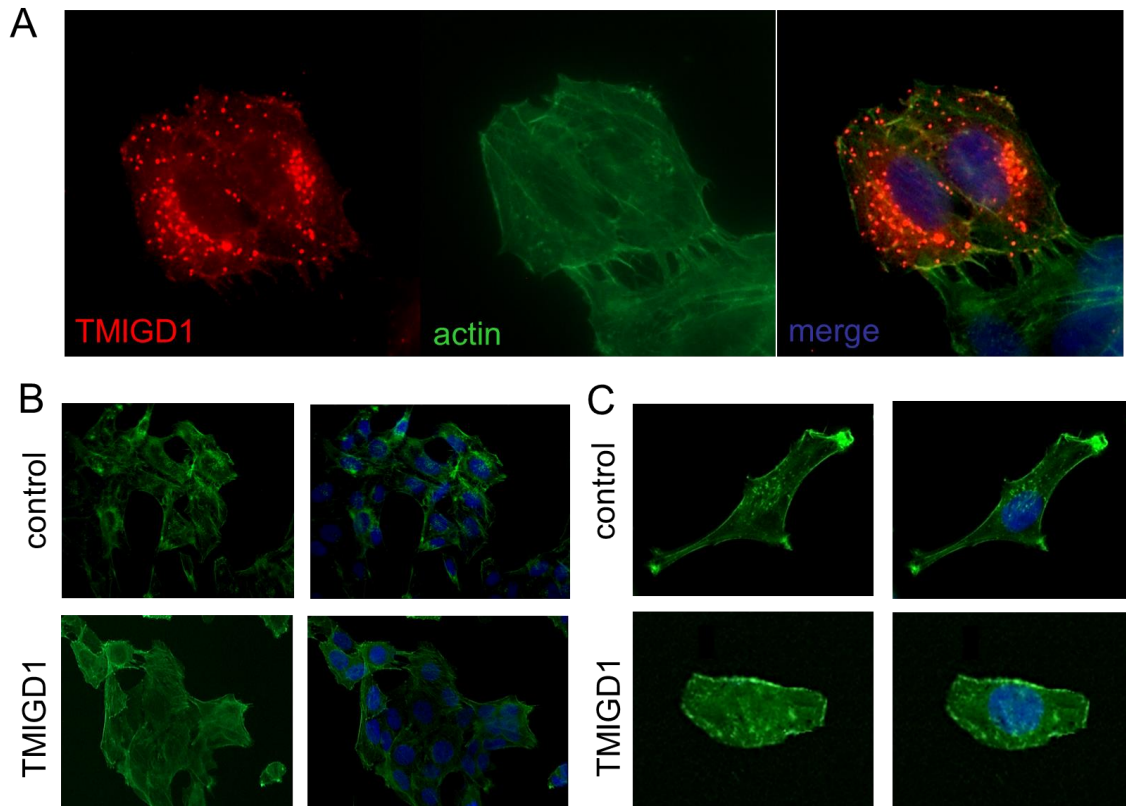
**Figure 10. TMIGD1 overexpression inhibits trophoblast invasion in invasion assays.** Invading HTR8/SVneo cells were fixed and stained with Diff-Quik. Cells incubated in serum-free media (SFM) were used as negative controls for random migration. An unpaired t-test showed that increased TMIGD1 expression reduced migration to  $33 \pm 11\%$  of controls after 16 hrs.  $n=3$ ;  $*p=0.037$

We performed an invasion assay consisting of Matrigel-coated transwells. Matrigel is a gelatinous substance consisting of extracellular matrix components such as laminin, collagen IV, and various other proteins. Our results showed that the increased TMIGD1 expression reduces trophoblast invasion to  $33 \pm 11\%$  of the control cell invasion

(Figure 10). This supports the idea that TMIGD1 may play an inhibitory role in trophoblast invasion during placenta formation.

*TMIGD1 overexpression alters cell morphology and actin formation*

Actin formation is essential for many cellular functions, including motility. Thus, we chose to examine whether TMIGD1 affects the actin cytoskeleton as a possible mechanism by which it affects cell motility. Double labeling of transduced HTR8/SVneo cells using an antibody against TMIGD1 and fluorescently-labelled phalloidin showed organized actin distribution to the peripheries in cells with increased TMIGD1 expression (Figure 11A). In contrast, control cells display actin as filamentous bundles distributed randomly throughout (Figure 11B). Additionally, single cells of the control population frequently exhibit filopodia, actin-rich protrusions that are important for cell migration and wound healing. This was markedly different from the transduced cell population, which contained numerous oval-shaped cells with distinct lack of filopodia and actin localization to the peripheries (Figure 11C). Our visualization of the actin cytoskeleton is consistent with the diminished migration of TMIGD1-overexpressing cells observed in prior experiments.



**Figure 11. TMIGD1 overexpression alters cell morphology and actin formation.** TMIGD1-overexpressing and control HTR8/SVneo cells were stained for actin using fluorescently-labelled phalloidin. Double staining showed actin cytoskeleton of TMIGD1-overexpressing cells (A). Distribution of actin in control cells is filamentous and disorganized (B, top), while actin in TMIGD1-overexpressing cells localizes to the peripheries (B, bottom). Typical structure of lone cells showing that TMIGD1 overexpression modifies morphology (C). In a population with increased TMIGD1 expression, fewer cells exhibited this migratory phenotype, compared to that observed in a control population.

#### *Greater TMIGD1 expression in fractionated STBs*

Comparing expression levels of TMIGD1 in CTBs and STBs is useful for confirming its localization in chorionic villi by IHC and understanding how TMIGD1 activity takes part in regulatory mechanisms. Placental tissue was treated with digestive

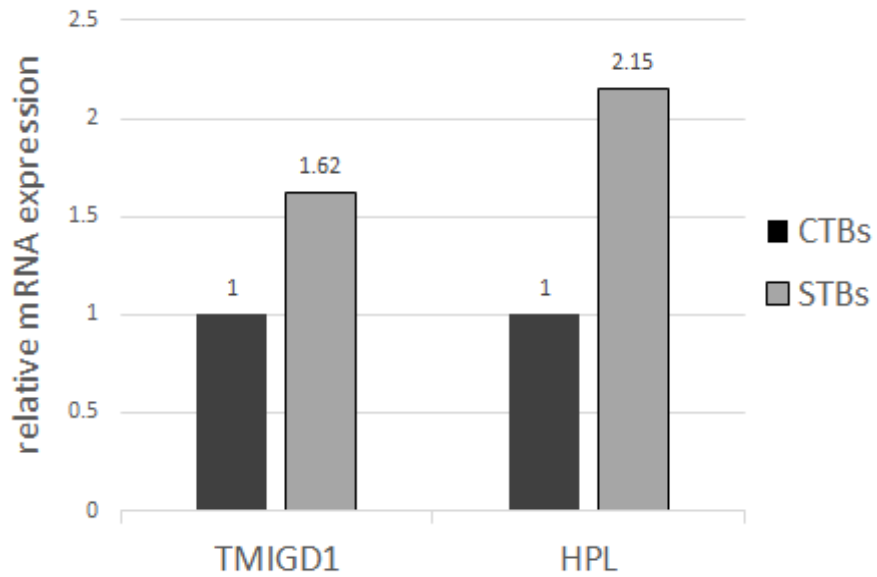
enzymes and centrifuged in a Percoll density gradient to separate the two types of trophoblast cells. This was successfully accomplished with placenta of 13- and 15-week gestational ages. RNA was isolated from CTBs and STBs, for future cDNA synthesis and qPCR analysis. Table 7 shows quantification and purity of these extracts.

**Table 7. Purity and concentration of RNA from fractionated CTBs and STBs**

Gestational Age	CTBs		STBs	
	Concentration (ng/uL)	Purity (260/280)	Concentration (ng/uL)	Purity (260/280)
<b>13 wk</b>	1106.8	1.884	429.2	1.886
<b>15 wk</b>	97.2	1.869	157.2	1.889

\*260/280 represents the ratio of absorbance when RNA is exposed to ultraviolet light at 260 and 280 nm. A sample with ratio of ~2 is generally considered pure RNA.

cDNA was synthesized from RNA extracted from 13-week-old CTBs and STBs, and TMIGD1 expression of the two cell types was compared using qPCR (Figure 12). HPL is known to have greater expression in the STB layer and served as an experimental control. Additionally, the housekeeping gene GAPDH was used as an internal control. Our results showed that TMIGD1 mRNA levels are 1.62 times higher in STBs than in CTBs. This is consistent with our previous fluorescent staining showing localization of TMIGD1 to the apical membranes of STBs. Further analysis of fractionated CTBs and STBs from additional placentas of various gestational ages are necessary to determine statistical significance.



**Figure 12. TMIGD1 mRNA expression in fractionated STBs and CTBs by qPCR.** TMIGD1 mRNA levels were 1.62-fold higher in STBs than in CTBs fractionated from placenta of 13-week gestational age. HPL was used as an experimental control that is known to be preferentially expressed in STBs. mRNA levels were normalized using GAPDH.

## DISCUSSION

### *TMIGD1 localization in STBs*

This research describes characteristics of the novel cell adhesion molecule TMIGD1 in human trophoblast cells. Trophoblast differentiation and invasion into the maternal decidua are highly regulated processes that are crucial for normal placenta development. Dysregulation of placental invasion may cause serious complications for both mother and fetus. The mechanisms for coordinating trophoblast activity are mediated by local factors, including autocrine signals from trophoblasts and paracrine signals from the uterus. Based on previous research, we aimed to investigate whether TMIGD1 plays a regulatory role in placenta invasion by characterizing its expression and activity in human trophoblasts.

Our study demonstrates that TMIGD1 is expressed at the apical membrane of STBs in 1<sup>st</sup> and 2<sup>nd</sup> trimester placenta. TMIGD1 localization on the apical membrane of STBs suggests that TMIGD1 may interact with factors in the intervillous space or the maternal uterine lining. Alternatively, the lack of TMIGD1 in CTBs indicates that its expression may be characteristic of trophoblasts in the differentiated STB state that are no longer invasive.

We plan to explore this idea by inducing syncytialization in BeWo choriocarcinoma cells using forskolin (Wice et al., 1990) and examining changes in TMIGD1 expression post-fusion. Syncytium formation is of utmost importance because

it creates an immunological barrier against components of the maternal defense system and pathogens (Beer & Sio, 1982; Chucru et al., 2010). The STB layer also secretes the hormone human chorionic gonadotropin (hCG), which mediates various processes during pregnancy (Cole, 2010).

qPCR or Western blots may be performed on fractionated STBs and CTBs to confirm our staining observations and to quantify the TMIGD1 expression differences between the two layers. Isolated STBs may also be stained to detect whether the apical localization on STBs is maintained. However, given that the fractionation process involves numerous enzymatic digestions, it is possible that membrane proteins may be degraded or reorganized.

We also showed that TLR4 is a reliable CTB marker that may be used for future experiments to distinguish the two cell types. This will be particularly useful in future immunofluorescence experiments, as well as for checking the purity of isolated CTBs and STBs from placental villi after a fractionation process (described in Methods).

#### *TMIGD1 inhibits migration*

Our immunofluorescence, qPCR, and Western blot data confirmed successful transduction of the HTR8/SVneo cell line, thus providing a way to examine TMIGD1 overexpression. Our wound healing and transwell migration assays showed that increased TMIGD1 expression inhibits trophoblast migration, which is consistent with previous findings from Arafa et al. in 2015 that TMIGD1 inhibits kidney epithelial cell migration.

Our invasion assay further supported the idea that TMIGD1 may play a role in regulating trophoblast invasion that must be tightly controlled to achieve normal placental attachment and function. HTR8/SVneo invasion through the Matrigel substrate provided a more precise assessment of primary trophoblast invasive activity.

*TMIGD1 regulates actin assembly*

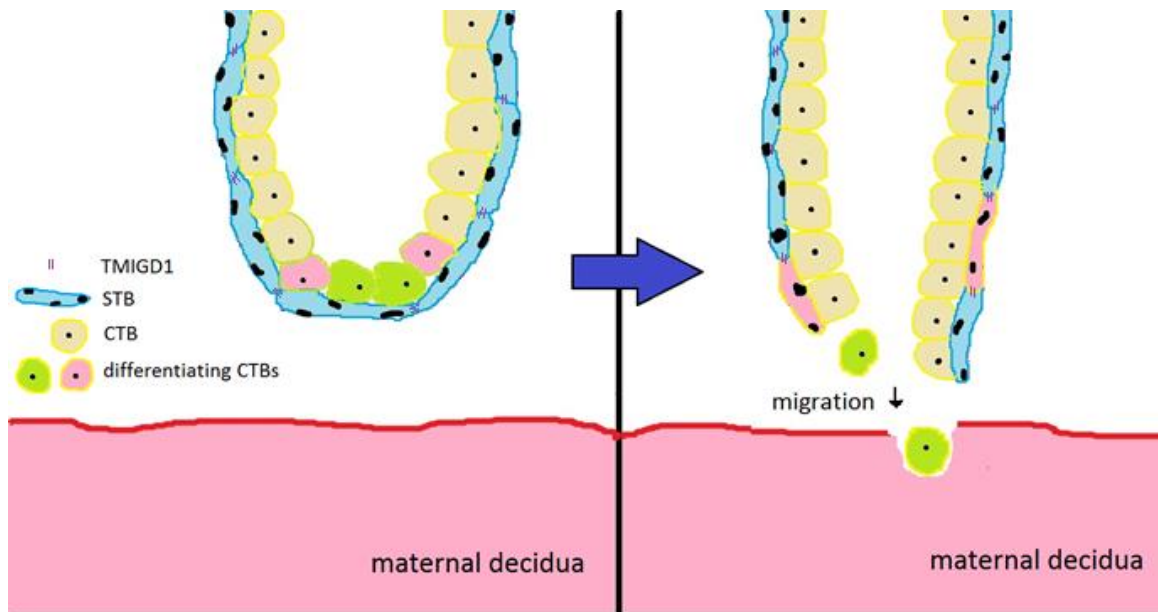
Differences in the actin cytoskeleton of control and TMIGD1-overexpressing HTR8/SVneo cells indicate that TMIGD1 may directly or indirectly regulate actin assembly. One hypothesis is that TMIGD1 is involved in mechanisms controlling cell migration, and downstream effectors may determine whether actin distributes to the peripheries or assembles as stress fibers and filopodia. A comparable study found that epidermal growth factor promotes trophoblast migration, using Rho proteins to mediate actin cytoskeleton remodeling (Han et al., 2013). Alternatively (and less likely), TMIGD1 may directly affect actin expression and promote redistribution to the peripheries, leading to a stable cytoskeletal structure that makes migration difficult.

A recent study showed that extravillous trophoblasts develop invadopodia, which are actin-rich protrusions that assist in invasion (Patel & Dash, 2012). These specialized cellular structures have been characterized in cancer metastasis, and give invading cells the ability to degrade extracellular matrix (Buccionie et al., 2009). Our previous observations that TMIGD1 may be involved in actin remodeling is relevant for studying invadopodia function and the invasive properties of trophoblasts.

A greater proportion of non-motile cells in the transduced HTR8/SVneo population, as revealed by a survey of cell morphology, is consistent with our findings that increased TMIGD1 inhibits migration. This phenotype also suggests mechanical stability, and we may examine the potential mechanism between actin assembly, TMIGD1, and adhesive properties of trophoblasts in the future.

*Proposed model of TMIGD1 expression*

Given the specific localization of TMIGD1 to STB apical membrane and the inhibitory effect of increased TMIGD1 on trophoblast migration and invasion, we theorize that TMIGD1 is involved in regulating placenta invasion according to Figure 13. TMIGD1 may serve as a “stop” signal for cytotrophoblast invasiveness. Villous CTBs (yellow) are progenitors of multinucleated STBs (blue) in the outer layer of placenta villi, as well as of extravillous cytotrophoblasts (green) that invade the maternal decidua. Further studies are necessary to elucidate the significance of TMIGD1 localization to the apical region of STBs, as well as to identify potential molecules that may interact with TMIGD1. Additionally, it is worth analyzing TMIGD1 expression in placenta of different gestational ages. Given the potential role of TMIGD1 in regulating placenta invasion, it may also be involved in the etiology of pregnancy disorders involving abnormal invasion. Further research will examine diseased placenta tissue from preeclamptic and growth-restricted pregnancies to detect altered patterns of TMIGD1 expression.



**Figure 13. Proposed model of TMIGD1 expression during placental invasion.** Inner layer CTBs have the potential to differentiate into STBs (blue) or extravillous cytotrophoblasts (green), which proceed to invade the maternal decidua. The role of TMIGD1 (purple), located at the apical region of STBs, is yet unknown.

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*Taipei, Taiwan*

- Research Assistant *Dec 2009-Jan. 2010*  
Project: Association of two functional polymorphisms of the beta2-Adrenergic Receptor gene, Glu26Glu and Arg16Gly, and heart rate variability (HRV)/physiological complexity

## **ACTIVITIES/VOLUNTEERING**

Rosie's Place, *Boston, MA*

- Server/Food pantry volunteer *Jan. 2015-Current*  
Prepare and serve meals to poor and homeless women at this women's shelter

Princeton First Aid Rescue Squad, *Princeton, NJ*

- EMT volunteer *May 2011-May 2014*  
Respond to 911 calls in town and Princeton University campus and administer pre-hospital emergency care or drive ambulance to hospital
- Recording Secretary (Executive Board) *Jan. 2012-Dec. 2012*

Centre Médical de Forcilles, *Férolles-Attilly, France*

- Summer intern *June 2013-July 2013*  
Clinical intern in ICU, outpatient ward, and oncology departments

Outdoor Action (Princeton University), *Princeton, NJ*

- Freshman orientation backpacking trip leader *Sept. 2011-Sept. 2013*  
Led week-long freshman backpacking orientation trips
- Wilderness first aid coordinator/instructor *Feb. 2012-Jun. 2014*  
Organize group of WFA instructors, manage the WFA curriculum, and teach 30-hour (WFA) course to freshman orientation trip leaders-in-training

## **PUBLICATIONS AND POSTERS**

### **Meeting abstracts:**

Wang C, Pudney J, Meyer RD, Bondzie PA, Zou X, Rahimi N, Kuohung W. Expression of the Novel Cell Adhesion Molecule TMIGD1 in Human Trophoblast Cells. SRI

2016 Annual Meeting. Montreal, Canada: Society for Reproductive Investigation, 2016

Wang C, Pudney J, Meyer RD, Bondzie PA, Zou X, Rahimi N, Kuohung W. Expression of the Novel Cell Adhesion Molecule TMIGD1 in Human Trophoblast Cells. Fifth Annual Translational Research Symposium. Boston, MA: BU Clinical and Translational Science Institute, 2016