NME1 at the human maternal–fetal interface downregulates titin expression and invasiveness of trophoblast cells via MAPK pathway in early pregnancy

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Abstract

Nonmetastatic gene 23-H1 (*NME1*, also known as nm23-H1) is a wide-spectrum tumor metastasis suppressor gene that plays an important role in suppressing the invasion and metastasis of tumor cells. It has been demonstrated that *NME1* is also expressed in human first-trimester placenta, but its function at maternal–fetal interface is not clear. The present study aimed to elucidate the biological function of *NME1* at the maternal–fetal interface, especially on invasion of the human extravillous cytotrophoblasts (EVCTs). *NME1* has been identified in both human trophoblast cells and decidual stromal cells (DSCs) in early pregnancy. We have proved that *NME1* silencing *in vitro* increases the titin protein translation in the invasive EVCTs. Moreover, *NME1* can inactivate the phospho-extracellular signal-regulated kinase 1/2 (P-ERK1/2) in trophoblasts in a time-dependent manner, and U0126, an inhibitor of MAPK/ERK, can inhibit partly the enhanced invasiveness and titin expression in trophoblasts induced by *NME1* silencing. Interestingly, the expression of *NME1* in either villi or decidua is higher significantly in miscarriage than that of the normal early pregnancy. These findings first reveal that the *NME1* expressed in trophoblasts and DSCs controls the inappropriate invasion of human first-trimester trophoblast cells via MAPK/ERK1/2 signal pathway, and the overexpression of *NME1* at maternal–fetal interface leads to pregnancy wastage.

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Introduction

A successful pregnancy requires coordinate program of decidualization, placentation, and embryo development. Embedment of the placenta is one of the most important steps (Ferretti *et al.* 2007). An inappropriate invasive ability of trophoblast cells may lead to spontaneous abortion or trophoblastic disease. Nonmetastatic gene 23-H1 (*NME1*, also known as nm23-H1) is a classical tumor metastasis suppressor gene. A correlation of increased *NME1* expression to low metastatic potential of cancer cells has been established in several malignancies, based on tumor prognostic and transfection studies (Ouatat *et al.* 2003, Balaram *et al.* 2004, Ferenc *et al.* 2004, Sirotkovic-Skerlev *et al.* 2005, Tee *et al.* 2006). In the trophoblastic disease, the expression of *NME1* is closely relative to the prognosis, and thus can be used as a reference indicator to evaluate the tumor progress (Balaram *et al.* 2004).

NME1 plays a key role in majority of tumor metastasis via different pathways. For example, the *NME1* gene may have an impact on the cell adhesion-related signaling pathway, through NDPK-mediated GTP, significantly increase β -catenin, E-cadherin, and CD44 mRNA and protein expression to strengthen intercellular adhesion, further inhibiting tumor invasion and metastasis (Kim & Kim 2006). Also, *NME1* can reduce the expression of matrix metalloproteinase 9 (MMP9) and MMP2, and increase tissue inhibitor of metalloproteinase (TIMP) through the RAS–RAF–MEK–extracellular signal-regulated kinase 1/2 (ERK)–ELK1 pathway, phosphatidylinositol 3-kinase–AKT–nuclear factor- κ B/AP-1 pathway, and RAS–RAC–MKK3/6–p38 pathway, and promote the angiogenesis-related vascular endothelial growth factor (VEGF) expression, finally weakening the metastasis ability of tumor cells (Ohba *et al.* 2005, Horak *et al.* 2007). In addition, *NME1* can activate P53 controlling

cell cycle, differentiation, and apoptosis; the adoption of the P53 pathway is to downregulate growth and metastasis of tumors (Lombardi 2006).

NME1 may play a significant part in the regulation of human trophoblast invasiveness, while the identity as well as regulation mechanism of the associated proteins remains unclear.

Titin, a connectin, is megadalton-sized filamentous molecule which functions as structural, regulatory and movement assemble unit in striated muscle through direct protein–protein interactions. Titin is also involved in cell mitosis and may be critical for functional organization of the nucleus (May *et al.* 2004). Our previous work has demonstrated that titin expression elevation induced by cyclosporin A, an immunosuppressive, promotes human trophoblast invasiveness via the activation of MAPK pathway. Therefore, the titin expression in human trophoblasts is recognized to participate in the process of placentation and embryo development (Du *et al.* 2007).

In the present study, we focus on the possible regulation of NME1 on trophoblast functions, especially invasiveness, and the NME1-mediated cross-talking between trophoblast cells and decidual stromal cells (DSCs) in human early pregnancy. It is noteworthy that multiple regulatory points may exist for important signaling proteins. The identification of the pathway and vital molecules may provide novel targets for the diagnosis and treatment in pathological pregnancy.

Results

The expression of NME1 at human maternal–fetal interface

The expression of NME1 was determined by RT-PCR and immunocytochemistry in both human villi and decidua. The results show that *NME1* is transcribed in both (Fig. 1a). The transcriptional level (0.425 ± 0.09) of *NME1* in villi is lower significantly than that of decidua

Figure 1 NME1 expression at the maternal–fetal interface in human early pregnancy. (a) The mRNA of *NME1* in human first-trimester villus and decidua. The first-trimester human decidua expresses higher *NME1* mRNA (0.425 ± 0.09) than villus (0.775 ± 0.05 , $P < 0.05$). (b and c) Immunocytochemical analysis for NME1 expression in decidua (A) and secretory endometrium (B) (0.0366 ± 0.003 vs 0.104 ± 0.012), and $*P < 0.05$ compared between the both. (d) Immunocytochemical staining of NME1 in the primary human trophoblast cells, DSCs, and BeWo cell line. Magnification $\times 200$. Results are highly reproducible in five independent experiments.

(0.775 ± 0.05 , $P < 0.05$). Moreover, the endometrium of secretory phase translates NME1 (0.0366 ± 0.003) highly significantly than decidua in comparison between nonpregnancy and early pregnancy (0.104 ± 0.012 , $P < 0.05$), which suggests that NME1 downregulation may be involved in cross-talking between embryo and endometrium in implantation (Fig. 1b).

We characterized cytokeratin-7 and vimentin in the isolated trophoblast cells and DSCs. All the isolated human extravillous cytotrophoblasts (EVCTs) are nearly stained for cytokeratin-7, while no cells are found to be stained with anti-vimentin antibody. HLA-G is specifically brown colored in the cytoplasm and on the cytomembrane of invading EVCT. The primary DSCs are polygon cells. The purity of the DSCs characterized by anti-vimentin staining is above 95% (Fig. 1c). Trophoblast, DSCs, and BeWo cells are stained positively for NME1 in cytoplasm and nucleus with the isotypic control. Therefore, both EVCTs and DSCs can express NME1 during first-trimester pregnancy in humans.

NME1 silencing promotes titin expression and invasiveness of human trophoblastic BeWo cell line

Three constructed small-interfering RNAs (siRNAs), targeting different sites of *NME1*, were transfected into BeWo and primary DSCs with negative controls. The fluorescence could clearly be seen under fluorescence microscope in 6 h after transfection. The silence efficiency is 95 and 91% respectively.

At 24, 48, and 72 h after transfection, BeWo cells were harvested. The *NME1* knockdown was demonstrated by in-cell western assay. It has been showed that all the three constructed siRNAs have silencing effects on NME1 protein expression, and at 72 h after transfection, the NME1 expression decreases to the lowest, about 78%, compared to the negative control (0.823 ± 0.038 vs 0.172 ± 0.041 , $P < 0.05$; Fig. 2a).

The in-cell western analysis shows that titin is up-regulated significantly in BeWo cells after *NME1* silencing (0.693 ± 0.032 vs 1.345 ± 0.052 , $P < 0.05$), and that there is no significant change in MMP and TIMP expressions (Fig. 2b). In addition, the immunofluorescence (IF) assay further confirms that *NME1* silencing increases titin protein expression (0.693 ± 0.032 vs 1.385 ± 0.052 , Fig. 2c). To testify the regulation of NME1 on the invasion of trophoblast cells, a matrigel-based transwell assay was carried out. The BeWo cells were added to the upper chamber, and then, the primary DSCs were added to the upper or lower chamber for direct or indirect co-culture respectively. The invasiveness of BeWo cells is significantly enhanced in both direct and indirect co-culture after the *NME1* knockdown (100 ± 6.05 vs 198 ± 15.02 , 102 ± 6.30 vs 222 ± 13.54 , $P < 0.05$; Fig. 2d), which suggests that NME1 can control the invasiveness of human trophoblasts.

Figure 2 *NME1* silencing can promote the invasiveness of BeWo cells. The trophoblast cell line BeWo was transfected with nontargeting siRNA oligonucleotides (control) or with siRNA oligonucleotides targeting *NME1* (silence) respectively. (a) The *NME1* interference efficiency is demonstrated by in-cell western assay with *NME1* antibody (0.823 ± 0.038 vs 0.172 ± 0.041). (b) Expression of the invasion-related molecules in the cells with *NME1* silence by in-cell western assay. *NME1* knockdown significantly promotes titin protein expression in BeWo cells (from 0.693 ± 0.032 to 1.345 ± 0.052), but shows no detectable effect on the protein level of MMPs and TIMPs. The figure is a representative of five experiments. (c) Titin expression in BeWo cells transfected with si-*NME1* by IF. After *NME1* knockdown, the *NME1* expression (red fluorescence) obviously decreases, while the titin protein level (green fluorescence) is apparently up-regulated. The figure is a representative of three experiments. (d) The co-culture was established to evaluate the invasiveness of trophoblasts in co-culture with DSCs. Magnification $\times 200$. *NME1* silencing can promote invasion of BeWo through the matrigel-coated membranes and to the lower surface of the membrane (100 ± 6.05 vs 198 ± 15.02 and 102 ± 6.30 vs 222 ± 13.54). The invasive index is expressed as the ratio of the migrated to the control. Data represent the mean of invasive index. Error bars depict the s.e.m. $*P < 0.05$ compared to the control.

NME1 silencing improves trophoblast invasiveness mainly through MAPK/ERK1/2 signaling pathway

The previous study has proved that the MAPK/ERK1/2 signaling pathway is involved in modulation of invasion and migration of human trophoblasts (McKinnon *et al.* 2001, Balaram *et al.* 2004, Qiu *et al.* 2004, Wu *et al.* 2004, Huang *et al.* 2006). In the present study, the BeWo cells with *NME1* silencing were treated with U0126

(30 $\mu\text{mol/l}$) or vehicle for 30, 60, and 90 min. It has been found that *NME1* silencing can activate Phospho-ERK (P-ERK) (0.42 ± 0.076 vs 0.77 ± 0.14 , $P < 0.05$), but not Phospho-AKT (P-AKT) or Phospho-P38 (P-P38; Fig. 3a and b). U0126, a MAPK inhibitor, significantly but not completely inhibits the enhanced titin translation (control: 0.701 ± 0.029 ; silence: 1.802 ± 0.045 ; control+U0126: 0.456 ± 0.031 ; silence+U0126: 1.203 ± 0.037 , $P < 0.01 \sim P < 0.05$; Fig. 3c), and invasiveness of the EVCTs induced by *NME1* silence (213 ± 15 vs 162 ± 8.9 , $P < 0.05$; Fig. 3d), which suggests that *NME1* downregulates the invasiveness of EVCT by suppressing titin expression via the MAPK pathway.

NME1 silencing in DSCs increases invasiveness of trophoblast cells in co-culture

As the expression of *NME1* in DSCs is stronger than that of trophoblast cells at the human maternal–fetal interface, we propose that the *NME1* expression in DSCs *in vivo* may also engage in the regulation of trophoblast invasion.

After confirming *NME1* silencing in DSCs (Fig. 4a), a matrigel invasive assay was used to evaluate invasiveness of human primary trophoblast cells in co-culture. It has been revealed that the invasiveness of trophoblast cells is apparently up-regulated in both direct and indirect

Figure 3 *NME1* controls MAPK/ERK1/2 signaling and invasion of trophoblast cells. (a) In-cell western assay indicates that P-ERK/T-ERK expression is elevated in BeWo cells after *NME1* silencing (0.42 ± 0.076 vs 0.77 ± 0.14). (b) The BeWo cells were transfected with si-*NME1*, and were then treated with vehicle or U0126 (30 mmol/l) respectively for different durations. The level of P-ERK1/2 was determined by in-cell western assay, and here, the green represents total ERK1/2 and the red represents phospho-ERK1/2, and the overlay of red and green means the ratio of P-ERK1/2 to T-ERK1/2. *NME1* silencing can activate p-ERK1/2, and U0126 can inhibit the p-ERK1/2 activation induced by *NME1* silencing in a time-dependent manner, even completely abolishing it after 90 min (silence+U0126 0 vs 30 vs 60 min): 0.80 ± 0.037 vs 0.26 ± 0.014 vs 0.012 ± 0.005 ; control+U0126 0 vs 30 vs 60 min): 0.39 ± 0.019 vs 0.025 ± 0.016 vs 0.010 ± 0.004). (c) The titin protein expression was viewed using Odyssey Infrared Imaging System, and here, the green represents actin, and the red represents titin. The picture is representative of one of the five individual experiments. The siRNA treated cells were measured by in-cell western assay in 24 h of culture treated with vehicle or 30 $\mu\text{mol/l}$ U0126 respectively. The protein level of titin increases after *NME1* silencing, and U0126 remarkably eliminates the enhanced protein expression. Error bars depict the s.e.m. (control: 0.701 ± 0.029 ; silence: 1.802 ± 0.045 ; control+U0126: 0.456 ± 0.031 ; silence+U0126: 1.203 ± 0.037). * $P < 0.05$, ** $P < 0.01$ compared to the control; (d) The invasive index of cells under different conditions is normalized to the control (silence versus silence+U0126: 213 ± 15 vs 162 ± 8.9 $P < 0.05$). The invasion of trophoblasts through the matrigel-coated membranes was taken by microscopy at 200 $\times$ magnification. The picture is a representative of five individual experiments.

co-culture with the DSCs in *NME1* silencing compared to the si-negative control (100 ± 12.3 vs 252 ± 18.57 , 105 ± 11.7 vs 260 ± 19.03 , $P < 0.05$; Fig. 4b), which suggests that the DSCs with high expression of *NME1* can suppress the invasiveness of the trophoblast cells by secreting some soluble invasion-related molecules at human maternal–fetal interface in early pregnancy.

The DSC-expressed *NME1* controls invasiveness of human first-trimester trophoblast cells by suppressing MAPK/ERK pathway

We wonder which signaling pathway is involved in inhibitory modulation of the DSC-expressed *NME1* on the invasiveness of trophoblast cells. It has been shown that *NME1* silencing in DSCs results in activation of P-ERK (0.42 ± 0.03 vs 0.853 ± 0.05 , $P < 0.05$), but not P-P38 or P-AKT (Fig. 5a), and the activation is abolished completely by U0126 ($P < 0.05$, Fig. 5b). The U0126 treatment can completely inhibit the increased invasiveness of trophoblast cells in co-culture with

NME1 siRNA-treated DSCs (246 ± 20 vs 68 ± 12 , $P < 0.05$; Fig. 5c), which yields a new insight into this signaling pathway involved in the modulation of DSCs on trophoblast invasiveness at the maternal–fetal interface during early pregnancy in humans.

The transcription and translation of *NME1* increases at human maternal–fetal interface of the early pregnancy wastage

We evaluated the *NME1* expression level at the maternal–fetal interface of human miscarriage with normal early pregnancy as a control by real-time PCR, traditional western blot, and immunohistochemistry respectively. It has been found that the villi and decidua from miscarriage show a much higher *NME1* transcription than that of the normal early pregnancy (3.9-fold, $P < 0.05$ and 4.3-fold, $P < 0.05$; Fig. 6a), and similar results have been demonstrated by immunohistochemistry (Fig. 6b) and traditional western blot (0.67 ± 0.046 vs 1.172 ± 0.064 in villus; 0.901 ± 0.145 vs 1.48 ± 0.078 , both $P < 0.05$; Fig. 6c), which suggests that *NME1* plays an important role in controlling the over-invasion of trophoblasts at maternal–fetal interface in human early pregnancy, but overexpression of *NME1* at the interface may lead to early pregnancy wastage.

Discussion

Embryo implantation in humans begins with invasion of trophoblasts into the uterine epithelium and underlying stroma. The trophoblast cells at the human maternal–fetal interface have proliferation and invasion ability the same as the tumor cells (Ferretti *et al.* 2007). However, it is strictly controlled in normal pregnancy, suggesting that the implantation process involves complex and synchronized molecular and cellular events between the uterus and embryo in an autocrine and paracrine manner. In the present study, human endometrium in the implantation window expressed higher *NME1*, which means that blastocyst implantation and development must be controlled by maternal endometrium and decidua. Trophoblast invasion and migration through the uterine wall are mediated by a series of molecular and cellular interactions controlled by the maternal decidua (Staun-Ram & Shalev 2005). Therefore, the related molecules involved in the regulation of trophoblast invasion play vital roles in the physiological state of blastocyst implantation and pathological trophoblastic disease.

Some transfection investigations have demonstrated several biochemical effects of *NME1* knockdown on tumor cellular functions, such as cell motility (Ma *et al.* 2008). In our study, we have investigated for the first time the functional mechanism of the metastasis suppressor *NME1* at the maternal–fetal interface in humans.

Figure 4 DSCs can suppress the invasiveness of trophoblasts via expressing *NME1*. (a) The primary DSCs plated on 96-well plates were transfected with siRNA of *NME1* for 3 days, then treated with vehicle, anti-actin (100 µg/ml), or anti-*NME1* antibody (50 µg/ml) respectively. The green represents actin, and the red represents *NME1* (control versus silence: 0.96 ± 0.020 vs 0.26 ± 0.078). (b) The next day, the invasive index of the primary EVCT cells under different conditions is normalized to the control (direct co-culture: 100 ± 12.3 vs 252 ± 18.57 ; indirect co-culture: 105 ± 11.7 vs 260 ± 19.03). The invasion of EVCT through the matrigel-coated membranes was taken by microscopy at $200\times$ magnification. The *NME1* silencing in DSCs had increased EVCTs invasiveness in either direct or indirect co-culture. The picture is representative one of three individual experiments. Error bars depict the s.e.m. * $P < 0.05$ compared to the control.

Figure 5 The DSC-expressed NME1 inhibits the invasion of trophoblast cells via suppressing ERK1/2 activation. (a) The level of P/T-ERK1/2 was determined by in-cell western assay. P-ERK1/2 expression increases significantly in the si-*NME1* DSCs compared to the control (0.42 ± 0.03 vs 0.853 ± 0.05), but protein levels of P-P38 and P-AKT show no significant change. (b) The interfered cells were treated with vehicle or U0126 (30 $\mu\text{mol/l}$) respectively for different durations. *NME1* silencing can activate p-ERK1/2 in DSCs, which can be inhibited by U0126, MAPK inhibitor, in a time-dependent manner, but not completely (0 vs 30 vs 60 (min) after silence+U0126: 0.75 ± 0.043 vs 0.42 ± 0.024 vs 0.33 ± 0.013 ; 0 vs 30 vs 60 (min) after control +U0126: 0.41 ± 0.038 vs 0.05 ± 0.016 vs 0.02). (c) The invasive index of cells under different conditions is normalized to the control (silence versus silence+U0126: 246 ± 20 vs 68 ± 12 $P < 0.05$). The invasion of EVCTs through the matrigel-coated membranes was taken by microscopy at $200\times$ magnification. Error bars depict the S.E.M. The picture is a representative of five individual experiments.

Our investigations indicate that NME1 expressed at the maternal–fetal interface participates in the regulation of invasion of trophoblast cells. The invasion process of trophoblasts is dependent on a blastocyst-derived signal, and the induction of NME1 also seems to

be an important regulation event and may serve to render the proper trophoblast invasion in the early pregnancy. The trophoblast invasiveness was enhanced after silencing of *NME1* expression in co-culture with the primary DSCs. We have also demonstrated that the expression level of titin increases in trophoblast cells after *NME1* silencing. By serving as a molecular spring, titin has been shown to maintain the structural integrity of contracting myofibrils and is critical for muscle elasticity as well as structural integrity of the sarcomere. Actually, the invasive ability of the trophoblast cells has been thought in a positive relation to their titin expression.

High expression levels of MMP and TIMP in trophoblasts during early pregnancy regulate their invasion (Wernyj *et al.* 2001). TIMPs from decidua and trophoblast are the natural MMP inhibitors, and play an essential role in regulating the process of trophoblast invasion (Zhou *et al.* 2007). Lots of studies have showed that TIMP1, a 28.5 kDa protein, almost formed close

Figure 6 The transcription and translation of *NME1* increase at human maternal–fetal interface in the early pregnancy wastage. (a) *NME1* transcription in villus and decidua was assessed by quantitative real-time PCR (pregnancy versus miscarriage: 1 vs 3.9 ± 0.34 in villus; pregnancy versus miscarriage: 1 vs 4.3 ± 0.37 in decidua). (b) Immunohistochemically specific brown-colored NME1 staining in tissue from miscarriage patients is much stronger than that of the normal early pregnancy. Magnification $\times 200$. (c) The tissue extracts were electrophoresed to detect NME1 translation by western blot (pregnancy versus miscarriage: 0.67 ± 0.046 vs 1.172 ± 0.064 in villus; 0.901 ± 0.145 vs 1.48 ± 0.078 in decidua). * $P < 0.05$ compared to the control.

complexes with all the MMP, in particular MMP2. TIMP2 has a similar amino acid sequence to TIMP1 and also can effectively inhibit the activity of various MMPs, while there is a priority binding with MMP9 (May *et al.* 2004). But in fact, our results have proved that *NME1* silencing has no effect on MMP and TIMP expressions, which suggests that *NME1* controls invasiveness of trophoblasts in a MMP- and TIMP-independent manner.

The MAPK/ERK pathway is in close association with the regulation of cellular proliferation, differentiation, angiogenesis, embryo development, and tumor invasion. P/T-ERK is up-regulated in either trophoblasts or DSCs after *NME1* silencing, and the MAPK/ERK1/2 signaling pathway inhibitor U0126 can efficiently suppress the ERK activation, titin expression, and invasiveness elevation of trophoblast cells. For these reasons, the MAPK/ERK signal pathway is involved in the regulation of *NME1* expression in trophoblast cells.

There must be a complicated cross-talk between DSCs and trophoblast cells, but the extent of our knowledge is limited (Duclos *et al.* 1995). We have found in the present study that DSCs express a higher level of *NME1* than trophoblasts, and the *NME1* silencing of DSCs can promote the invasiveness of trophoblast cells in direct or indirect co-cultures, which suggests that some soluble molecules are involved in the control of the invasion of trophoblasts by *NME1* expressed by DSCs. We have also demonstrated that the MAPK/ERK1/2 pathway is involved in the control of the invasion of trophoblasts by *NME1* expressed by DSCs. Interestingly, there is more expression of *NME1* in miscarriage than in normal early pregnancy. In physiological conditions, there might be a precise modulation in trophoblast invasion to maintain a balance, not only to inhibit or promote but also to contribute to the appropriate and limited invasion of trophoblasts. Collectively, our data imply that *NME1* is involved in the regulation of trophoblast invasiveness, and that higher *NME1* expression in DSCs serves to control over-invasion of trophoblasts, such as trophoblastic disease. Our present investigation is helpful to elucidate the cross-talk mechanism between DSCs and trophoblasts, especially trophoblast invasiveness, at the human maternal–fetal interface in early pregnancy.

Materials and Methods

Human placental tissue collection

All procedures in this study were carried out with the approval of the ethical committee of the Obstetrics and Gynecology Hospital, Fudan University, and all participants completed an informed consent to collect tissue samples.

Villous and decidual tissues were collected from elective termination in the first-trimester pregnancy (gestational age, 7–9 weeks) for nonmedical reasons and spontaneous abortion. Patients who were abortion prone showed no original pulse of the fetal cardiac tube by ultrasonic evaluation, and had

undergone intimate questioning and examination, and those with abnormal chromatin body, anatomy and endocrine secretion abnormality, and infectious and autoimmune disease were excluded. The healthy first-trimester pregnancy women without a history of miscarriage or abnormal pregnancy had no threatened abortion signs such as vaginal bleeding, fever, celiodynia during present gestation, and the embryo was confirmed normal by ultrasonography. There was no significant difference in mean age between the two groups ($P > 0.05$).

All the tissues were immediately put into ice-cold DMEM-high glucose (DMEM/High; Gibco), transported to the laboratory within 30 min after surgery, and washed in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS for further research.

Isolation and primary culture of trophoblasts and DSCs

The villous tissue was treated according to our previous method (Wu *et al.* 2004). Briefly, the tissues from 4 to 5 separate individuals were pooled, and digested with 0.125% trypsin (Bio Basic Inc; BBI, Ontario, ON, Canada) and 0.02% DNase type I (Sigma) at 37 °C with gentle rocking for 30 min. The 10% fetal bovine serum (FBS) was used to stop the enzymatic reaction, and the digested suspension was filtered through sterile gauzes (80, 200, and 300 μm) and centrifuged at 300 *g* for 10 min. After discarding the supernatant, the cellular pellet was resuspended in PBS solution, and carefully layered over a discontinuous Ficoll gradient (20–65%, in 5% steps), then centrifuged at 1000 *g* for 20 min. The cellular sediment at densities between 1.048 and 1.062 g/ml was collected, and washed with DMEM/High. Finally, these cells were diluted in DMEM/High supplemented with 20% FBS (Gibco) to 5×10^5 cells/well and incubated in 5% CO_2 at 37 °C. This method supplied a 98% purity of first-trimester extravillous trophoblast cells.

The DSCs were isolated according to the methods of Nagle (1988) with slight improvement. The decidual tissues from several individuals were carefully washed in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS, minced, and then maintained in a solution of 0.1% Collagen IV (Sigma) for 90 min at 37 °C. Next, the suspension was filtered through sterile gauzes (100, 200, and 300 μm), and centrifuged at 300 *g* for 10 min. After the supernatant was discarded, the cell pellet was resuspended in PBS solution and centrifuged on a discontinuous gradient of 20, 40, and 60% Ficoll for 20 min at 1000 *g*. The cells were collected from the 20 to 40% interface containing mainly DSC, resuspended in RPMI-1640 (Sigma), washed, and cultured in the complete RPMI-1640 medium with 10% FBS. After primary culture for 30 min, the nonadherent lymphocytes were removed, leaving a highly purified population of DSCs. Immunocytochemistry showed that the specific vimentin-positive stained cells made up for above 98%.

BeWo cells, a human trophoblast and choriocarcinoma cell line, were purchased from the China Center for Type Culture Collection (CCTCC, Wuhan, People's Republic of China), and were maintained as monolayer in Kaighn's Modification of Ham's F-12 Medium (Sigma) supplemented with 10% FBS under standard culture conditions of 5% CO_2 at 37 °C with medium renewal every 2–3 days.

RT-PCR

Total RNA was isolated from villous and decidual tissues with Trizol reagent (Invitrogen). The primer sequences of *NME1* are sense, 5'-CAGGAACCATGGCCAACTGTG-3'; antisense, 5'-CGGATGGTCCCAGGCTTGG-3', and those of *GAPDH* are sense, 5'-GGGGAGCCAAAAGGGTCATCATCT-3'; antisense, 5'-GAG GGG CCA TCC ACA GTC TTCT-3'. The cDNA was generated with oligo (dT) primers using Revertra Ace- α First Strand cDNA Synthesis Kit following the protocol (Toyobo, Tokyo, Japan). The 50- μ l PCR amplification of the single-strand cDNA was performed by predenaturation at 94 °C for 5 min and 28 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s, and elongation at 72 °C for 45 s using 2.5 U Taq polymerase. The amplified DNA was fractionated by 2% agarose gel (Oxiod, Basingstoke, UK) electrophoresis, and ethidium bromide-stained bands were photographed. The experiments were performed with five villous and decidual samples. The experiments were repeated three times.

Quantitative real-time PCR

Total RNA was isolated from villous and decidual tissues from early pregnancy and miscarriage with Trizol reagent (Invitrogen). Triplicate samples containing cDNA were prepared as mentioned above. Taqman universal PCR master mix (Applied Biosystems, Foster City, CA, USA), specific primers, and fluorescent dye-labeled Taqman MGB probes for target gene and *GAPDH* were mixed, and analyzed on an ABI7000 thermal cycler (Applied Biosystems).

The primer sequences were synthesized by TaKaRa Biotechnology Co., Ltd (Tokyo, Japan):

NME1: sense, 5'-CAGGAACCATGGCCAACTGTG-3';

antisense, 5'-CGGATGGTCCCAGGCTTGG-3'.

GAPDH: sense, 5'-GGGGAGCCAAAAGGGTCATCATCT-3';

antisense, 5'-GAGGGGCCATCCACAGTCTTCT-3'.

The cycling conditions consisted of a denaturation step at 95 °C for 10 min, 40 cycles at 95 °C for 15 s, a 60-s annealing step at 62 °C, and finally, a holding temperature of 15 °C. To determine the amount of gene product present in the sample, cycle time (C_t) was determined. The average C_t value was calculated from triplicate wells for each sample with each primer set. Most duplicate samples varied by $<0.5 C_t$. The relative gene expression was determined by calculating ΔC_t values (ΔC_t) by subtraction of the C_t value of *GAPDH* from the C_t value of target gene. The relative fold expression of interest was determined compared with control in the experiment. The experiments were carried out in triplicate.

Western blot

Western blot analysis was performed using anti-NME1 antibodies (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After having been ground smoothly, the cells were lysed in mixture of 1% NP-40, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 100 μ g/ml phenylmethylsulphonyl fluoride, 1 μ g/ml Aprotinin, and 0.1% SDS for 10 min at 4 °C. Nuclei were

removed by centrifugation at 12 000 *g* at 4 °C for 10 min, and cell lysate was assayed for protein contents using the Bradford protein assay. Proteins (30 μ g) were resuspended in sample buffer (2% SDS, 62.5 mM Tris-HCl, pH 6.8, 0.1% bromophenol blue and 2.5% 2-mercaptoethanol, 10% glycerol), and were separated on 10% SDS-polyacrylamide gels. The proteins were transferred to a nitrocellulose membrane by electro-transfer for 1 h. After being soaked in blocking buffer (1 $\times$ TBS with blocking reagent (5% milk)) for 30 min, the membrane was incubated with primary antibody overnight at 4 °C. Blots were developed using the HRP-linked secondary antibody and a chemiluminescent detection system. The experiments were repeated three times.

Immunohistochemistry

Immunohistochemical analyses were performed for deciduas, secretory endometrium, and villous tissues. For each sample, 5-mm sections of formalin-fixed, paraffin-embedded tissues were dewaxed in xylene twice for 5 min; rehydrated in 100% ethanol twice for 3 min, followed by 95% ethanol for 3 min, 80% ethanol for 3 min, and 70% ethanol solution for 3 min; then washed in ddH₂O; for each specimen, an antigen retrieval step was required, which was achieved by microwave heating for 20 min in target retrieval solution; and washed twice. Endogenous hydrogen peroxidase activity was quenched using 3% H₂O₂ for 30 min at room temperature, and the specimens were then rinsed in PBS Tween-20 1 $\times$ (PBST). Nonspecific binding of tissue sections was prevented by preincubation with nonimmune block (1% BSA/PBST). Next, the sections were sequentially incubated with mouse anti-human vimentin MAB (GBI, Cincinnati, OH, USA), cytokeratin-7 MAB (GBI), IgG MAB (GBI), or mouse anti-human NME1 MAB (1:50; Santa Cruz Biotechnology) respectively. After washing with PBST, detection of positive binding was performed by application of the appropriate peroxidase-conjugated goat anti-mouse secondary antibody (1:100; GBI). After washing twice in PBS, the slides were subsequently incubated for 10 min in diaminobenzidine (DAB), counterstained by using Harris' hematoxylin (Sigma), washed in tap water for 2 min, and mounted for microscopic examination. The experiments were performed with five placental, secretory endometrial, and decidual samples.

Immunocytochemistry

The cells (BeWo cells, freshly isolated trophoblasts, and DSCs) were plated in dishes. Following washing with PBS, the cells were fixed with 4% paraformaldehyde and then gently rocking in 1% Triton to penetrate the cell membranes for 25 min. Endogenous peroxidase was blocked using 3% H₂O₂ for 30 min, and the dishes were then rinsed in TBS. Blocking was carried out with 10% FBS for 15 min at room temperature. The dishes were incubated for 3 h at 37 °C with mouse anti-human vimentin MAB, CK7, mouse IgG, or mouse anti-human NME1 MAB respectively. After washing for three times with PBS, the dishes were incubated for 15 min at 37 °C with HRP-labeled polymer conjugated to goat anti-mouse immunoglobulin. Lastly, the dishes were subsequently washed three times with

PBS, incubated for 3 min in DAB after washing with PBS and counterstained using hematoxylin solution for 10 s, and then washed for 2 min. Then, they were mounted for microscopic examination. We certified the results five times.

NME1 silence

The cells were grown in culture medium with 10% FCS (Sigma). Hairpin loop was used to produce specific siRNA for *NME1*. The si-*NME1* and the nonspecific control siRNA (Invitrogen) were transfected in cells with Lipofectamine TM 2000 (Invitrogen) according to the manufacturer's recommendations. All transfections in triplication were performed with si-negative as control. One day before transfection, the cells were cultured with medium without antibiotics so that they would be 30–50% confluent at the time of transfection. The siRNA oligomer was diluted by appropriate amount of Opti-MEM I Reduced Serum Medium without FBS, and then mixed with the diluted Lipofectamine 2000. In 20 min at room temperature in order to allow the complex formation, the mixture was added to the cells and incubated at 37 °C in a CO₂ incubator. The condition of transfection was observed in an Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan). Thereafter, in-cell western assay was used to detect the protein level at 24, 48, and 72 h after transfection respectively.

Matrigel invasion assay for human EVCT

The invasion of human trophoblast cells in the upper chamber across Matrigel was evaluated following our previous established methods (Huang *et al.* 2006). EVCTs were seeded at 5×10^4 cells per well into matrigel-coated invasion chambers (8 mm pore size, 6.5 mm diameter; Corning, New York, NY, USA). After culture for 24 h, the cells on the upper surface of each insert were scraped off with a cotton bud. Then, the wells were washed with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. Finally, the inserts were stained with hematoxylin. It was seen that the cells that went through the matrigel to the opposite side of the inserts were almost EVCTs, which had abundant plasma and presented cobblestone-like growth. The number of cells that had migrated to the lower surface of membrane was visualized with Olympus BX51 fluorescence microscope at $\times 200$ magnification. Pictures of five random fields from each condition were obtained, and the numbers of cells that had invaded were quantified in automated fashion using Metamorph analysis software. Each experiment was carried out three times in triplicate.

On one side, considering the vulnerability of the trophoblast cells, we used BeWo cells for *NME1* silencing. The BeWo cells transfected with *NME1* siRNA were seeded at 2×10^5 cells/ml in the upper chamber of 24-well plates with si-negative control, and the lower chambers were filled with freshly isolated DSCs, or the co-culture was treated by MAPK signal inhibitor, U0126. On the other side, the DSCs in the co-culture in lower chamber were silenced for *NME1* with si-negative control, the trophoblast cells were seeded at 2×10^5 cells/ml in the upper

chamber of 24-well plates, or U0126 was added into the medium. The invasiveness of BeWo or trophoblast cells was observed by matrigel invasion assay in the next day.

In-cell western assay

Following the description by Egorina *et al.* (2006), we used a newly established assay called in-cell western to determine the in-cell protein level of titin, MMP2, MMP9, TIMP1, and TIMP2. The procedures were as follows: after 5 days of *NME1* silence, these cells of interest were fixed with 4% paraformaldehyde for 20 min at room temperature, washed with 0.1% Triton, and added of LI-COR Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE, USA) 200 μ l per well for 90 min to block nonspecific binding at room temperature. Then, these cells were respectively incubated with mouse anti-human titin MAB (1:50; Abcam, Cambridge, UK), MMP9, MMP2, TIMP1, TIMP2 MAB (1:300; R&D, Abingdon, UK), or P-ERK, T-ERK, P-P38, P-P38, P-AKT, T-AKT primary antibody (1:200; R&D) with the housekeeping protein, rabbit anti-human actin (1:100; Santa Cruz Biotechnology) as control. After incubation overnight at 4 °C, the wells were incubated with the corresponding second IRDyeTM700DX-conjugated affinity purified (red fluorescence) anti-mouse or IRDyeTM800DX-conjugated affinity purified (green fluorescence) anti-rabbit fluorescence antibody recommended by the manufactures (1:500; Rockland, Inc., Gilbertsville, PA, USA). Images were obtained by using the Odyssey Infrared Imaging System (LI-COR Biosciences). The protein levels were observed by the overlay of the two fluorescence intensity and calculated as the ratio of the intensity of objective molecules to that of actin. The experiments were carried out five times in triplicate.

Immunofluorescence

The cells were fixed with 4% paraformaldehyde for 10 min at room temperature, followed by 10 min incubation with 0.2% Triton X-100. After washing with PBS, nonspecific binding was blocked with PBS containing 10% FBS. Primary antibodies were diluted in PBS and left on the cells for 2 h at 37 °C. After washing three times with PBS, secondary antibody diluted in PBS was added for 1 h avoiding light. Slides were mounted in 0.1% 4,6-diamidino-2-phenylindole (1:50; Invitrogen) for nuclear counterstain for 5 min at room temperature, and observed in a Olympus BX51 fluorescence microscope. The following antibodies were applied: rabbit anti-human *NME1* MAB (1:50; Santa Cruz Biotechnology), mouse anti-human titin MAB (1:50; Abcam), and secondary antibodies were Texas-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG (1:100; Rockland). The experiments were carried out three times duplicate.

Statistical analysis

Data are expressed as mean $\pm$ S.E.M. Statistical significance between groups is assessed by one-way ANOVA followed by Dunnett's multiple comparisons test. The difference is considered statistically significant at $P < 0.05$.

Declaration of interest

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

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