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Differential Roles of Membrane-bound and Soluble TNF-α in Regulating Trophoblast Invasion and Migration

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ABSTRACT

Background: Proper invasion and migration of trophoblasts are critical for successful human pregnancy. Tumor necrosis factor-α (TNF-α) has two biologically active forms: the membrane-bound TNF-α (mTNF) and the soluble TNF-α (sTNF).

Objective: To investigate the role of both forms of TNF-α in trophoblasts invasion and migration and to elucidate their underlying mechanisms.

Methods: We exposed HTR-8/SVneo trophoblasts to exogenous mTNF or sTNF and evaluated their cellular behaviors. Proliferation was assessed using a Cell Counting Kit-8, while invasion and migration were assessed through Transwell assays. Additionally, we measured mRNA levels of matrix metalloproteinase-9 (MMP-9), tissue inhibitor of metalloproteinases-1 (TIMP-1), and plasminogen activator inhibitor-1 (PAI-1) using qRT-PCR. The expression of IκB-α was determined by western blot analysis.

Results: Unlike sTNF, mTNF enhances the invasion and migration of trophoblasts. Mechanistic analysis showed that mTNF increased MMP-9 expression while decreasing TIMP-1 and PAI-1 expression, and it inhibited the activation of NF-κB signaling pathway in HTR-8/SVneo cells with or without lipopolysaccharide (LPS) treatment.

Conclusion: This research uncovered a new function of mTNF in regulating trophoblast invasion and migration, offering a new approach to treating pregnancy-related diseases associated with inadequate trophoblast invasion.

Keywords: Membrane-bound TNF-α, Trophoblast, Invasion, Migration, NF-κB signaling pathway

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INTRODUCTION

Normal placental development depends on the appropriate invasion and migration of trophoblast cells at the maternal-fetal interface. This process is crucial for maintaining a successful human pregnancy and ensuring fetal health. During human placentation, extravillous trophoblasts invade the uterine decidua and spiral arteries, remodeling the maternal vascular system to ensure adequate nutrient and oxygen delivery to the fetus (1). Inadequate trophoblast invasion canlead to various pregnancy-related disorders, including spontaneous abortions, intrauterine growth restriction (IUGR), preeclampsia (PE), and stillbirth (2, 3). Therefore, elucidating the molecular mechanisms involved in proper trophoblast invasion is important for identifying new targets to prevent pregnancy complications associated with impaired trophoblast invasion.

Tumor necrosis factor-alpha (TNF-α) is initially expressed as a type II trimeric transmembrane protein and functions in two bioactive forms: membrane-bound TNF-α (mTNF) and soluble TNF-α (sTNF). mTNF is cleaved at its stalk region by the metalloproteinase TNF-alpha-converting enzyme, producing sTNF (4). It regulates various cellular processes, including cell survival, differentiation, proliferation, migration, apoptosis, and cell death through two distinct receptors, tumor necrosis factor receptor (TNFR) -1 and TNFR-2 (5, 6). Previous studies have reported that TNF-α and its receptors are present in numerous cell types throughout the maternal endometrium, decidua, and placental tissue, which play both protective and pathogenic roles during pregnancy (7). sTNF, a well-known inflammatory cytokine, contributes to the development of adverse pregnancy outcomes, such as IUGR, PE, spontaneous abortion, and preterm birth, etc. (8). Previous studies have demonstrated that sTNF limits the invasive and migratory function of trophoblasts by promoting the expression of plasminogen activator inhibitor-1 (PAI-1) (9, 10). To date, most studies have focused on the effects of sTNF on trophoblast functions, whereas the role of mTNF in regulating trophoblast behavior and pregnancy-related disorders remains unclear.

The nuclear factor-kappa B (NF-κB) is crucial in modulating numerous cellular functions, including proliferation, differentiation, apoptosis, angiogenesis, epithelial-to-mesenchymal transition, and oxidative stress. Therefore, NF-κB appears to be an essential regulator in placental development, and dysregulation of this pathway can contribute to pathological pregnancy outcomes (11, 12). sTNF impairs trophoblast invasion and migration by triggering the activation of the NF-κB pathway (13). In contrast to sTNF, which activates NF-κB, previous studies from our group and others have shown that mTNF inhibits NF-κB activation in 3T3-L1 adipocytes (14), macrophages (15), and cardiomyocytes (16). Therefore, we hypothesized that, compared to sTNF, mTNF may exert distinct regulatory effects on trophoblast cells, potentially through suppression of the NF-κB signaling pathway.

Here, we treated the human trophoblast HTR-8/Svneo cell line directly with exogenous mTNF or sTNF to investigate the effects of both forms of TNF-α on trophoblast cells. Unlike the inhibitory effects of sTNF on trophoblasts invasion and migration, mTNF promoted these cellular processes. In addition, the expression of matrix metalloproteinase-9 (MMP-9) was increased, while the expressions of tissue-specific inhibitor of metalloproteinase-1 (TIMP-1) and PAI-1 were decreased when trophoblasts were treated with mTNF. Meanwhile, we found that mTNF could restrict NF-κB pathway activation in trophoblasts with or without lipopolysaccharide (LPS). These data indicate that mTNF may facilitate trophoblast invasion and migration through its negative regulation of the NF-κB pathway.

MATERIALS AND METHODS

Cell Culture

A human trophoblast cell line HTR-8/SVneo was obtained from Procell Life Science & Technology Co., Ltd (Wuhan, China) and maintained in RPMI-1640 medium (HyClone, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Thermo Fisher Scientific, MA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO_2 incubator.

Harvesting of Exogenous mTNF

The HEK 293T cell line was stably transfected with a lentiviral plasmid containing human TNF-α cDNA as previously described (15). To detect the expression of mTNF on the cell surface, cells were incubated with an anti-TNF-α primary antibody (Santa Cruz Biotechnology, TX, USA), followed by an Alexa Fluor 488 chicken anti-mouse IgG secondary antibody (Invitrogen, CA, USA). The fluorescence-stained cells were acquired on a FACS Canto II flow cytometer (Becton Dickinson, CA, USA) and analyzed with FlowJo V10 software. The 293T cells overexpressing mTNF on the cell surface were fixed with 4% paraformaldehyde and used as the source of human exogenous mTNF.

mTNF and sTNF Stimulation

HTR-8/SVneo trophoblasts were used as target cells and co-incubated with mTNF (at a 1:10 ratio of HTR-8/SVneo to fixed mTNF overexpressing 293T cells) or 20 ng/ml recombinant human sTNF (Thermo Fisher Scientific, MA, USA).

Cell Counting kit 8 (CCK-8) Assay

HTR-8/SVneo cells were seeded in a 96-well plate at a density of 5×10^3 cells per well and incubated at 37°C with 5% CO_2 for 24 hours. After incubation, the culture medium was replaced with 100 µl of RPMI-1640 medium supplemented with 10 µl of CCK-8 reagent (Biosharp, Hefei, China). The cells were then incubated for additional 2 hours and the absorbance at 450 nm was measured using a Varioskan LUX microplate reader (Thermo Fisher Scientific, MA, USA).

Transwell Assay

The transwell assay was used to measure cell migratory and invasive capacities. For the invasion assay, we first pre-coated the upper chambers of transwell inserts with 50 µl of diluted Matrigel (20%) (BD Biosciences, Fairleigh, NJ, USA). Then, a density of 4×10^4 HTR-8/SVneo cells per well suspended in 200 µl of serum-free medium were seeded into the upper chambers, while the lower chambers were filled with 500 µl of complete medium. After 48 hour of incubation, the cells on the upper surface of the filters were gently wiped away with a cotton swab, and the invaded cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet in ethanol. For the migration assay, the Transwell inserts were not pre-coated with Matrigel. Images were acquired using a microscope (Nikon, Tokyo, Japan) and analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, MD, USA).

Quantitative Real-time PCR

Total RNA was extracted using TriPure Isolation Reagent (Roche, Basel, Switzerland) and reverse transcribed to cDNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. Real-time PCR was carried on in a 20 µl SYBR Green qPCR Master mix (Roche) using a CFX96 real-time PCR instrument (Bio-rad, CA, USA). Results were analyzed using the $2^{-\Delta\Delta Ct}$ method and normalized to the GAPDH level. Primer sequences are provided in Table 1.

Western Blot Assay

Cells were lysed using RIPA lysis buffer (supplemented with protease inhibitors 0.5 mM PMSF, 5 µg/ml aprotinin, and 5 µg/ml leupeptin; Beyotime Biotechnology, Shanghai, China) on ice for 30 min. Total protein in the supernatant was collected

Table 1. The sequences of primers for real-time PCR

Gene	Forward (5′-3′)	Reverse (5′-3′)
MMP-9	TTGACAGCGACAAGAAGTGG	TCACGTCGTCCTTATGCAAG
TIMP-1	AATTCCGACCTCGTCATCAG	TGCAGTTTTCCAGCAATGAG
PAI-1	CAGACCAAGAGCCTCTCCAC	ATCACTTGGCCCATGAAAAG
VEGF-A	CCCACTGAGGAGTCCAACAT	TTTCTTGCGCTTTCGTTTTT
GAPDH	CAAGGTCATCCATGACAACTTTG	GTCCACCACCCTGTTGCTGTAG

after centrifugation and its concentration was measured using a BCA kit (Pierce, IL, USA). The protein samples were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore, MA, USA). The membranes were then blocked with 5 % BSA in TBS-Tween 20 and incubated with primary antibodies against IκB-α (Cell Signaling Technology, MA, USA) and GAPDH (ABclonal, Wuhan, China) overnight at 4°C. Subsequently, the membranes were incubated with horseradish peroxidase-labeled anti-mouse secondary antibodies (Abcam, MA, USA). The protein bands were visualized using an ECL western blotting reagent (Pierce) on a gel imaging system (Shanghai Peiqing Technology Co., Ltd., Shanghai, China).

Statistical Analysis

All data were presented as means±SEM from at least three independent experiments. Statistical analysis was performed using GraphPad Prism V9 software (La Jolla, CA, USA) using Student's *t*-test. A P value < 0.05 was considered statistically significant.

RESULTS

mTNF Promotes HTR-8/Svneo Cells Proliferation

The mTNF must be present on the cell membrane to exert its characteristic biological activities. To investigate the impact of mTNF on trophoblast function, we used fixed HEK 293T cells overexpressing human TNF-α as the source of exogenous mTNF (Fig. 1a). We evaluated the ability of mTNF to promote HTR-8/Svneo cell proliferation using a CCK8 assay and found that mTNF significantly enhanced the proliferation of HTR-8/Svneo cells (Fig. 1b).

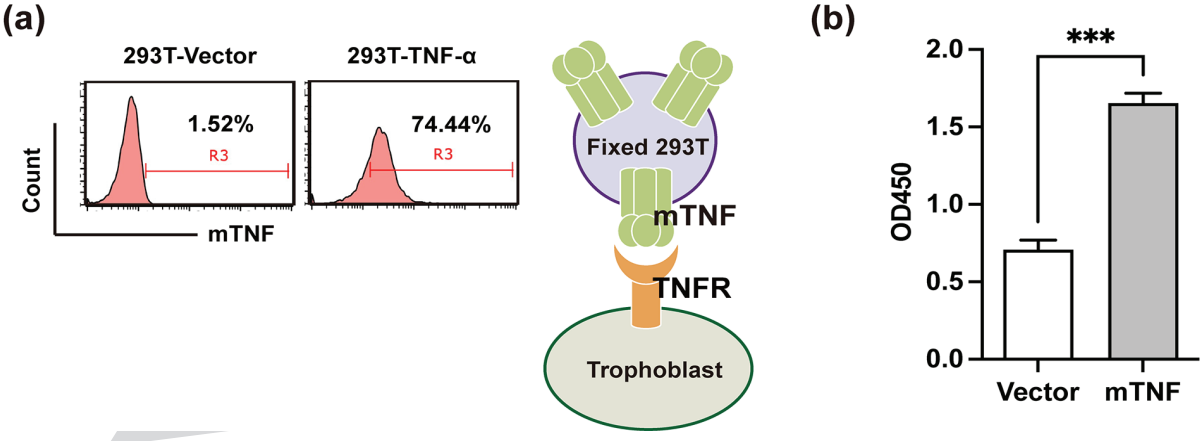

Fig. 1. mTNF promotes the proliferation of HTR-8/SVneo cells. (a) HEK 293T cells were stably transfected with full-length of human TNF-α (293T-TNF-α). Ectopic expression of mTNF on the cell surface was detected by flow cytometry (left). Diagram of mTNF-treated HTR-8/SVneo cells (right): mTNF on 4% paraformaldehyde-fixed 293T-TNF-α cells was co-cultured with HTR-8/SVneo cells at an effector/target ratio of 10:1. Vector transfected cells served as a control. (b) HTR-8/SVneo cells were cultured with mTNF on fixed 293T-TNF-α cells at an effector/target ratio of 10:1 for 24 h. Vector transfected cells served as a control. Cells proliferation was assessed by CCK-8 assay. Results are presented as means±SEM of at least three independent experiments, ***P<0.001.

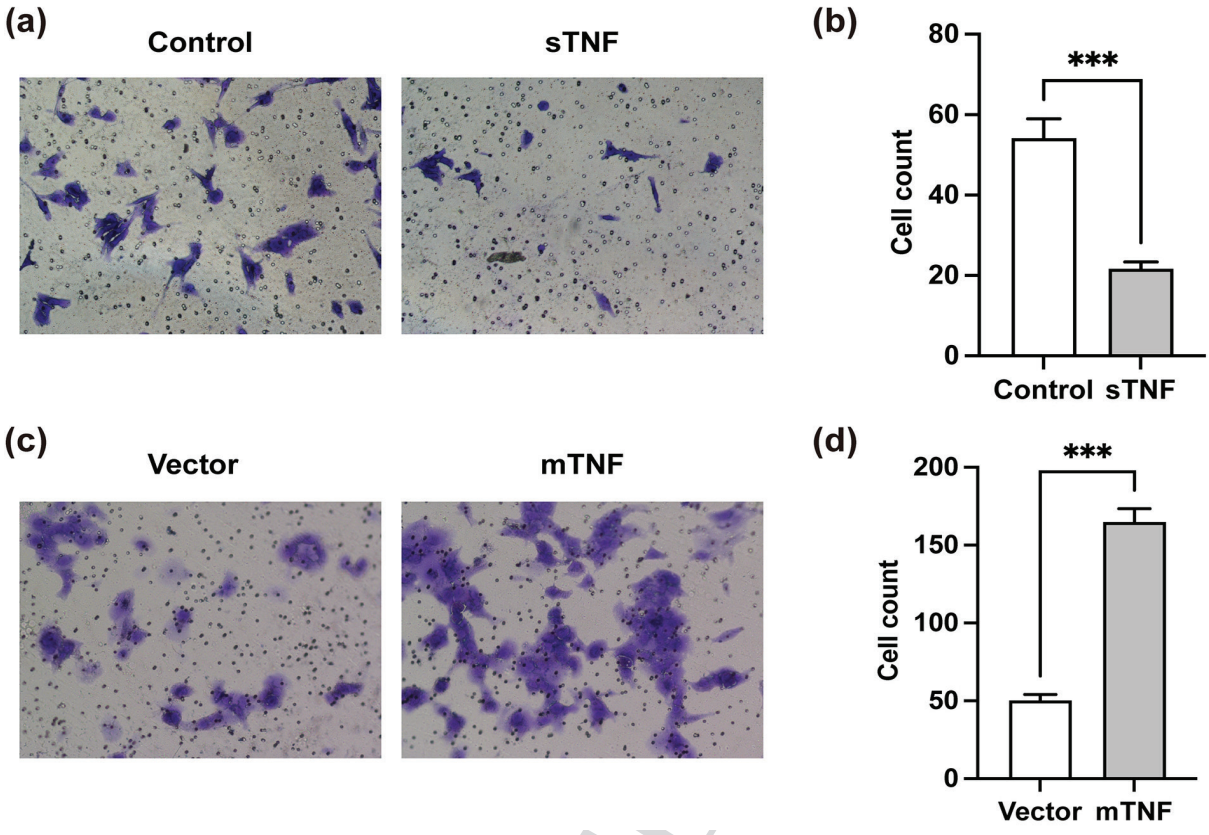

Fig. 2. Differential effects of mTNF and sTNF on the invasion of HTR-8/SVneo cells. (a-d) HTR-8/SVneo cells were treated with or without sTNF (20 ng/mL) (a-b) or mTNF on fixed 293T-TNF-α cells at an effector/target ratio of 10:1 (c-d) and incubated for 48 h. Vector-transfected cells served as a control. Cell invasion was evaluated using a transwell assay. Representative images (a and c) from at least three independent experiments are shown, and quantitative data (b and d) are presented as means±SEM, ***P<0.001.

Differential Effects of mTNF and sTNF on the Invasion of HTR-8/SVneo Cells

We next investigated whether the effects of mTNF on trophoblast invasion differed from those of sTNF. HTR-8/SVneo cells were stimulated with either sTNF or mTNF for 48 hours, and a transwell assay was used to assess the impact of both forms of TNF-α on trophoblast invasion. The results showed that sTNF suppressed the invasive capacity of HTR-8/SVneo trophoblasts (Fig. 2a and 2b). In contrast, mTNF enhanced the invasive capacity of trophoblasts demonstrating an effect opposite to that of sTNF (Fig. 2c and 2d).

Differential Effects of mTNF and sTNF on the Migration of HTR-8/SVneo Cells

To determine whether the effects of mTNF on trophoblast migration differ from that of sTNF, we treated HTR-8/SVneo cells with either sTNF or mTNF. After 48 hours, we assessed the effects of both forms of TNF-α on trophoblasts migration using a transwell assay. We found that sTNF inhibited the migration of HTR-8/Svneo trophoblast cell (Fig. 3a and 3b), whereas mTNF promoted cell migration (Fig. 3c and 3d).

mTNF Promotes Trophoblasts Invasion and Migration by Modulating MMP-9, TIMP-1, and PAI-1 Expression

Previous studies have reported that various factors including MMP-9 and TIMP-1 are associated with the invasive and migratory capacities of trophoblasts (17). Additionally, shown that sTNF has been shown to limit the invasion and migration of trophoblasts by increasing PAI-1 expression (9, 10). To explore the mechanisms underlying the promotional effect of mTNF on HTR-8/SVneo cells invasion and migration, we measured the mRNA levels of MMP-9, TIMP-1, and PAI-1 using real-time PCR.

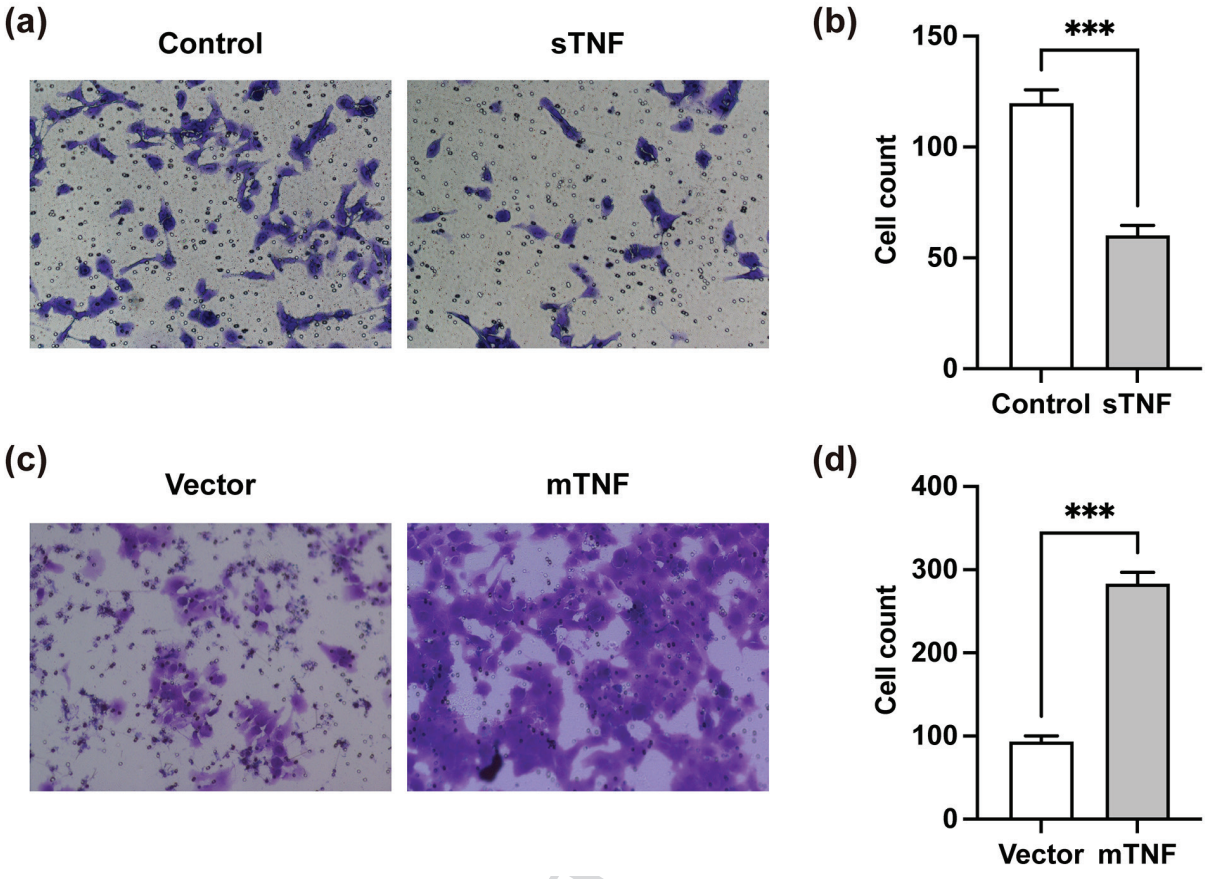

Fig. 3. Differential effects of mTNF and sTNF on the migration of HTR-8/SVneo cells. (a-d) HTR-8/SVneo cells were treated with or without sTNF (20 ng/mL) (a-b) or with mTNF on fixed 293T-TNF-α cells at an effector/target ratio of 10:1 (c-d) and incubated for 48 h. Vector-transfected cells served as a control. Cell migration was evaluated using a transwell assay. Representative images (a and c) from at least three independent experiments were shown, and quantitative data (b and d) are presented as means±SEM, ***P<0.001.

The results showed that sTNF did not affect MMP-9 expression, reduced TIMP-1 mRNA, and increased PAI-1 expression (Fig. 4a-4c). In contrast, mTNF treatment led to an increase in MMP-9 mRNA, and downregulation of TIMP-1 and PAI-1 compared with the control (Fig. 4e-4g). To further investigate the effects of the two types of TNF on gestational vascular remodeling, we measured the mRNA level of the pro-angiogenic factor vascular endothelial growth factor-a (VEGF-A) in trophoblast cells following sTNF or mTNF treatment. We found that sTNF had no effect on the expression of VEGF-A, whereas mTNF significantly upregulated VEGF-A (Fig. 4d and 4h).

mTNF Promotes the Invasion and Migration of Trophoblasts by Inhibiting NF-κB Activation

To further understanding the mechanisms underlying how mTNF affects trophoblast cell invasion and migration, we investigated mTNF's impact on the NF-κB signaling pathway which is crucial for the function of trophoblast cells. We found that unlike sTNF which promotes IκB-α degradation, mTNF inhibits IκB-α degradation (Fig. 5a), suggesting that mTNF suppresses the activation of the NF-κB signaling pathway. Additionally, we induced a PE-like inflammatory response in HTR-8/SVneo trophoblasts using LPS treatment (18). Our results demonstrated that mTNF significantly inhibits the degradation of IκB-α activated by LPS (Fig. 5a). Figure 6 ????

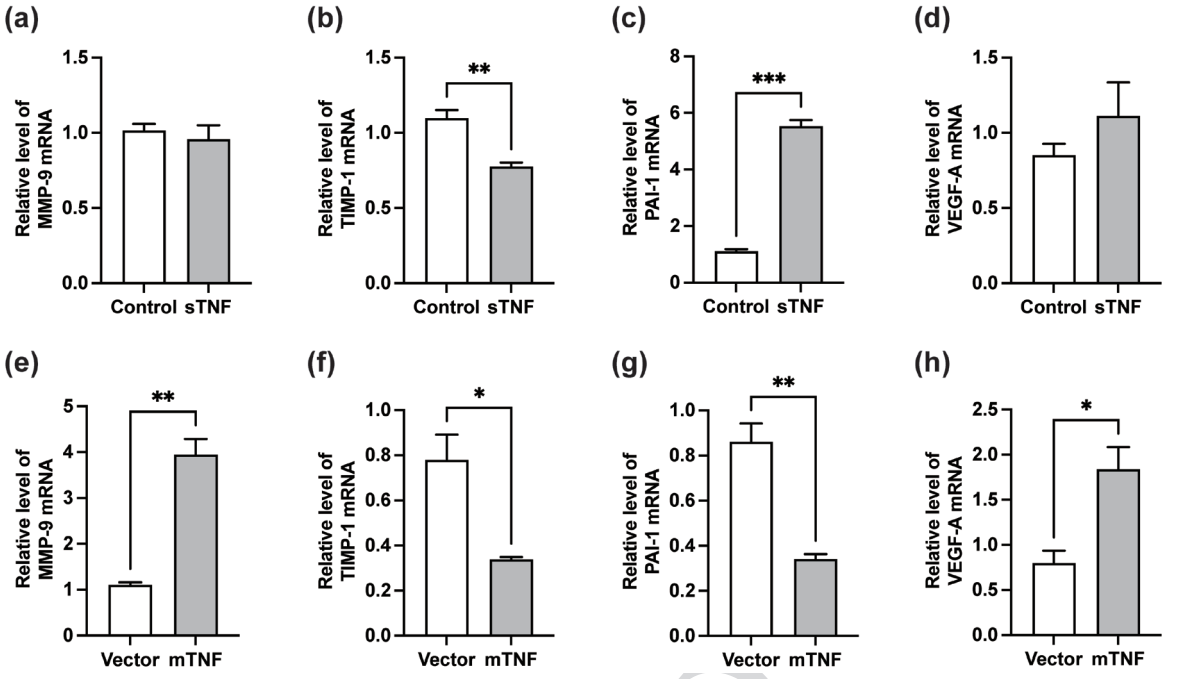

Fig. 4. Differential effects of mTNF and sTNF on MMP-9, TIMP-1, PAI-1 and VEGF-A expression. (a-d) HTR-8/SVneo cells were treated with or without sTNF (20 ng/mL) for 24 h. Relative mRNA levels of MMP-9 (a), TIMP-1 (b), PAI-1 (c) and VEGF-A (d) were assessed by quantitative real-time PCR and normalized to GAPDH. (e-h) HTR-8/SVneo cells were treated with or without mTNF on fixed 293T-TNF-α cells at an effector/target ratio of 10:1 for 24 h. Vector-transfected cells served as a control. Relative mRNA levels of MMP-9 (e), TIMP-1 (f), PAI-1 (g) and VEGF-A (h) were assessed by quantitative real-time PCR and normalized to GAPDH. Results are presented as means±SEM of at least three independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001.

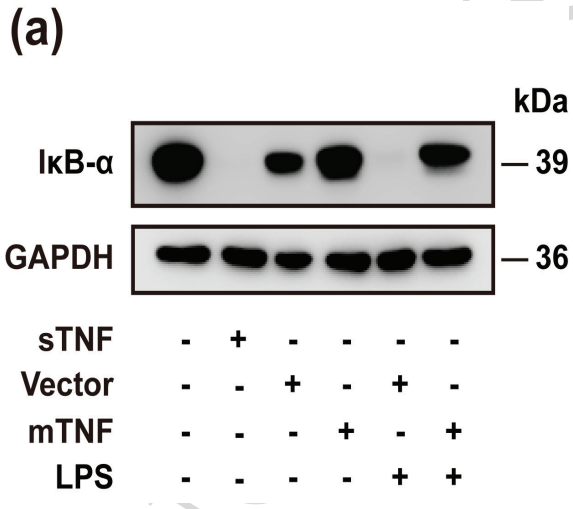

Fig. 5. Differential effects of mTNF and sTNF on the NF-κB pathway. HTR-8/SVneo cells were treated with or without sTNF (20 ng/mL) or mTNF on fixed 293T-TNF-α cells at an effector/target ratio of 10:1 and LPS (100 ng/mL) for 24 h. Vector-transfected cells served as a control. Representative western blot from three independent experiments showing IκB-α levels in total protein is shown.

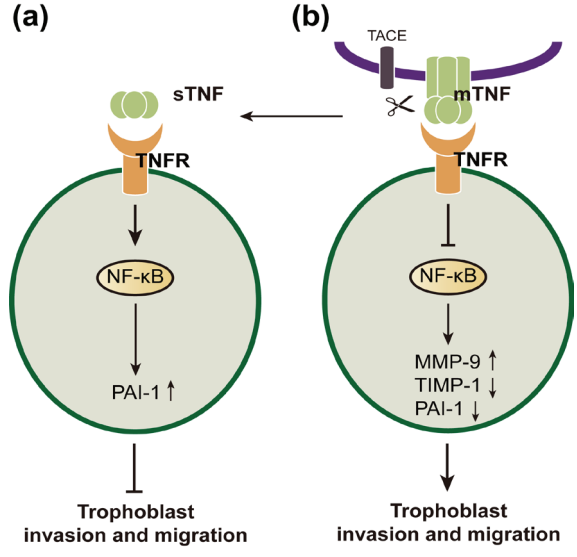

Fig. 6. Differential roles of mTNF and sTNF in the invasion and migration of trophoblasts. Mechanisms by which sTNF (a) and mTNF (b) regulate trophoblast invasion and migration. (a) sTNF inhibits trophoblast invasion and migration by activating NF-κB signaling and upregulating PAI-1 expression. (b) mTNF promotes trophoblast invasion and migration by inhibiting NF-κB signaling, upregulating MMP-9 expression, and reducing TIMP-1 and PAI-1 expression.

DISCUSSION

This study demonstrates that two bioactive forms of TNF-α play opposite roles in regulating trophoblast invasion and migration, which are critical processes for successful placental development. sTNF inhibits trophoblasts invasion and migration, whereas mTNF promotes these processes by upregulating MMP-9 mRNA and downregulating mRNA levels of TIMP-1 and PAI-1. Furthermore, our findings indicate that mTNF enhances the invasive and migratory capacity of trophoblasts, at least in part, by suppressing the NF-κB signaling pathway activation. These findings reveal a novel role of mTNF in the regulation of trophoblast behavior.

Studies have shown that TNF-α and its receptors are widely expressed in the placenta. TNF-α mRNA is expressed in all trophoblast lineages, including cytotrophoblasts, syncytiotrophoblasts and invasive trophoblasts (7, 19, 20). Enhanced TNF-α immunostaining is observed at the tips of anchoring villi and in invading trophoblasts during spiral artery remodeling (21), suggesting that TNF-α may serve as a key regulator of trophoblast invasiveness. Using the HTR-8/SVneo trophoblast cell line, which is widely recognized as a suitable in vitro model of human trophoblasts (22), we investigated whether the two forms of TNF-α exert differential regulatory effects on trophoblast function. In this study, we found that sTNF significantly impaired the ability of HTR-8/SVneo cells to migrate and invade. Our data are consistent with other published results showing that sTNF restricted invasion and migration in trophoblast cells Swan 71, HTR-8/SVneo and in an in *vitro* model of first-trimester villous explants (9, 13, 23). However, limited research examined the role of mTNF in regulating trophoblast biological functions. mTNF-α requires localization on the cell membrane to exert its biological activity (24). In this study, 293T-TNF-α cells displayed high levels of surface mTNF, and fixed 293T-TNF-α cells were used as a source of exogenous mTNF. The vector-transfected control cells theoretically express all membrane molecules present on 293T-TNF-α cells except for mTNF. Our findings demonstrated that, unlike sTNF, mTNF promotes HTR-8/SVneo cell proliferation, invasion and migration. The pleiotropic effects of TNF-α are mediated through two receptors: the 55-kDa TNFR1 and the 75-kDa TNFR2, which are structurally distinct and can activate both overlapping and distinct downstream signaling pathways (25, 26). Both sTNF and mTNF activate TNFR1, which is expressed on nearly all cell types and contains an intracellular death domain. In contrast, mTNF preferentially binds to and activates TNFR2, which lacks a death domain and has a more restricted expression pattern. TNFR2 is predominantly expressed on endothelial cells, mesenchymal stem cells, immune cells and neural cells (27, 28). Furthermore, mTNF transmits signals through cell-to-cell contact, whereas sTNF acts distantly via autocrine/paracrine manner (29, 30). Therefore, sTNF and mTNF can exert different roles under various physiological and pathological conditions, depending on the cellular or tissue context. Thus, sTNF and mTNF can exert distinct roles under various physiological and pathological conditions, depending on the specific context and the cell or tissue environment.

MMP-9 can degrade components of the extracellular matrix to promote the invasion and migration of trophoblasts (31, 32). TIMP-1 is a primary inhibitor of MMP-9, which is also a key mediator of trophoblast invasion and migration (33). PAI-1, the primary inhibitor of plasminogen activators, suppresses extracellular matrix degradation and thereby inhibits trophoblast invasion during implantation and placentation (34). This study showed that sTNF increased PAI-1 expression, whereas mTNF enhanced MMP-9 expression and reduced the expression of TIMP-1 and PAI-1 in HTR-8/SVneo cells. Although our results showed that sTNF did not affect MMP-9 expression and instead reduced

TIMP-1 expression, sTNF still exerted an overall inhibitory effect on trophoblast invasion and migration, likely due primarily to its induction of PAI-1 expression. It aligns with findings from Huber et al. who reported that sTNF restricts trophoblast invasion mainly by activating NF-κB and increasing the expression of PAI-1 (13). Meanwhile, mTNF increased the expression of the pro-angiogenic factor VEGF-A, suggesting that mTNF may contribute to the trophoblast endovascular differentiation (35).

To further investigate the molecular mechanisms by which the two forms of TNF regulate trophoblasts, we found that sTNF activated the NF-κB signaling pathway, a key regulator of trophoblast function (11). In contrast, mTNF inhibited activation of this signaling pathway. This is consistent previous studies showing that mTNF suppresses NF-κB activation via TNFR2 in macrophages and cardiomyocytes (15, 16). However, mTNF expressed on 6-sulfo LacNAc dendritic cells has also been reported to activate NF-κB in NK cells through TNFR2 signaling (36). This suggests that the regulatory effects of mTNF on NF-κB signaling pathway may vary depending on cellular context and physiological conditions. NF-κB is a key regulator of placental development and spiral artery remodeling, and its dysregulation contributes to pathological pregnancy. It is excessively activated in PE, and selective inhibition of NF-κB alleviates the symptoms of PE in rat models (12, 37). We found that mTNF can inhibit NF-κB activation under PE-like inflammatory conditions, indicating a potential new therapeutic direction for PE treatment.

CONCLUSION

In summary, this study demonstrates that mTNF plays a role distinct from sTNF in regulating trophoblast biological functions. mTNF may promote trophoblast invasion and migration by inhibiting the NF-κB signaling pathway, up-regulating MMP-9 expression, and reducing TIMP-1 and PAI-1 expression. However, this study has limitations. It remains unclear whether mTNF acts through TNFR1 or TNFR2, indicating that the molecular mechanisms underlying mTNF-mediated regulation of trophoblast functions require further investigation. Our findings suggest that mTNF has a crucial role in regulating trophoblast invasion and migration, which may provide a novel direction for managing pregnancy-related disorders associated with insufficient trophoblast invasion.

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AUTHOR CONTRIBUTION

Huiming Ye and Xiujuan Li conceived and directed the study. Chenxi Li, Mingxin Lin, Min Zhu and Jianmin Lin performed the experiments. Chenxi Li and Mingxin Lin analyzed data and drafted the manuscript. All authors reviewed and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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