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MicroRNA-204 suppresses trophoblast-like cell invasion by targeting matrix metalloproteinase-9

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ABSTRACT

Preeclampsia is a devastating pregnancy-related syndrome characterized by the onset of hypertension, proteinuria and edema. Insufficient invasion of trophoblasts is well-known to be correlated with preeclampsia development. The present study was performed to investigate the functional role microRNA (miRNA)-204 in trophoblastic invasion *in vitro*. We here found that the invasive capabilities of BeWo and JEG3 trophoblast-like cells were suppressed by miR-204 mimics, whereas enhanced by its inhibitor. Matrix metalloproteinase-9 (MMP9) was first confirmed to play a role in regulating trophoblast invasion through loss- or gain-of-function experiment. Notably, we demonstrated MMP9 as a direct target of miR-204 in BeWo cells by using the dual-luciferase assay. Moreover, forced overexpression of MMP9 was noted to partly attenuate the inhibitory effects of miR-204 on BeWo cell invasion. Taken together, our study indicates that miR-204 may contribute to the development of preeclampsia by inhibiting trophoblastic invasion, and that MMP9 is involved in miR-204-mediated trophoblast cell invasion. Our study suggests miR-204 as a novel therapeutic target for preeclampsia.

1. Introduction

Preeclampsia is a systemic disease that results from placental defects, with hypertension, proteinuria and edema as its major characteristics [1]. This pregnancy-related disease complicates 5–8% of all pregnancies and increases both maternal and neonatal morbidity and mortality worldwide [2]. During normal pregnancy, specialized placental cells known as trophoblasts acquire tumor-like properties and invade deep into the uterine decidua inner myometrium [3,4]. However, trophoblasts fail to invade the uterus properly in the preeclamptic placentas [5]. Investigations on the molecular mechanisms related to insufficient trophoblastic invasion will help to understand the pathogenesis of preeclampsia.

MicroRNAs (miRNAs) are a class of 22–25 nucleotides small non-coding RNAs, that have been predicted to repress gene expression by binding to the 3′ untranslated region (UTR) of certain subsets of messenger RNAs (mRNAs) [6], and thus regulate a broad range of developmental and physiological processes [7]. By using microarray technology, investigators are able to identify tissue or circulating specific miRNAs that are differentially expressed in diseased contexts [8]. Comparison of the expression levels of miRNAs between human normal and preeclamptic placentas reveals that many miRNAs are aberrantly expressed in the preeclamptic placentas [9,10]. Some of them have been experimentally validated to play important roles in regulating trophoblastic invasion *in vitro*, such as miR-210 [11], miR-18a [9] and miR-517a/b/c [12] etc. Preeclampsia is likely a heterogeneous collection of disease [2], a single molecule may never be found to explain its development. Therefore, identification of novel miRNAs implicated in the preeclampsia onset will offer insights into preeclampsia etiology.

Recently, a study from Choi et al. has illustrated that miR-204 is markedly overexpressed in preeclamptic placentas [13], suggesting that miR-204 may be involved in preeclampsia. However, their study did not elucidate the functional role of miR-204 in preeclampsia progress. To our best knowledge, up to date, no studies have addressed the effect of miR-204 on trophoblastic invasion. MiR-204 is belonged to the miR-204/204b/211 cluster, with UCCCUUU as its seed sequence (Targetscan, http://www.targetscan.org). Several previous lines of evidence indicated that miR-204 acts as a tumor suppressor during the carcinogenesis of cancers, such as lung cancer [14] and intrahepatic cholangiocarcinoma [15]. On the basis of these earlier studies, it is likely that the

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overexpression of miR-204 is associated with the shallow invasion of trophoblasts.

The present study was performed to explore the functional role of miR-204 in trophoblastic invasion. We investigated whether and how miR-204 mediated the invasion of trophoblast-like cells (BeWo and JEG3 cells) *in vitro* by using miR-204 mimics or its specific inhibitor. Also, the underlying mechanisms were studied.

2. Materials and methods

2.1. Cell culture

Human trophoblast-like cells BeWo obtained from Cell Bank of Wuhan University (Wuhan, China) and JEG3 cells obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China) were maintained in minimum essential media (MEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Preparation of MMP9 overexpression/silencing plasmids, miR-204 mimics/inhibitor, and cell transfection

For upregulation of MMP9 expression in cells, the complete CDS of MMP9 gene (NM_004994) lacking the 3′-UTR was amplified (Forward: 5′ GTCGGAATTCATGAGCCTCTGGCAGCC 3′, EcoRI; reverse: 5′ TGAACCTCGAGTCCTAGTCCTCAGGGCAC 3′, XhoI) and cloned into pcDNA3.1$^+$ (Clontech Laboratories, Inc., Mountain View, CA, USA; pcDNA3.1-MMP9). Empty vector was used as the negative control (pcDNA3.1-vector). For MMP9 downregulation, a pair of oligonucleotides encoding shRNA targeting MMP9 mRNA (5′ ggttccaactcggtttgga 3′, 670 to 688 bp) was inserted into pRNA-H1.1 vector (Genscript Biotechnology Co., Ltd., Nanjing, China). Plasmid containing scrambled shRNA was used as negative control (pRNA-H1.1-NC). MiR-204 mimics (Forwards: 5′ UUCCCUUUGUCAUCCUAUGCCU 3′), miR-204 inhibitor (anti-miR-204; Forwards: 5′ AGGCAUAGGAUGACAAAGGGAA 3′), and their corresponding controls (miR-NC, anti-miR-204 NC) were purchased from Shanghai GenePharma Company (Shanghai, China). Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) was used for plasmid transfection. At 48-h post transfection, cells were subjected to Western blot, real-time PCR or gelatin zymography analysis.

2.3. Assessment of cell invasion

Cells were first transfected with different plasmids or miRNAs, and 24 h later, their invasion capabilities were detected by using the transwell chambers of 8-μm pores (Corning Incorporated Life Sciences, Tewksbury, MA, USA) coated with matrigel (BD Biosciences, San Jose, CA, USA). In short, the trophoblast-like cells (2×10^4) in 200 μL serum-free MEM medium were seed onto the upper chambers, and the lower chambers were filled with media containing 20% FBS. These cells were allowed to migrate through matrigel-coated membranes for 24 h. Then, the inner surface of the upper chambers was rinsed with PBS, and the invasive cells were fixed in paraformaldehyde (Sinopharm, Shanghai, China) for 20 min, and then stained with 5% crystal violet (Amresco China, Shanghai, China) for 5 min. Thereafter, the stained cells were counted in five random areas in multiple wells.

2.4. Quantitative real-time PCR

Total RNAs, including miRNAs, were extracted from cell lysates with a RNA Simple Total RNA Kit (TianGen, Beijing, China), and then processed for cDNA synthesis with a Super M-MLV Reverse Transcriptase Kit (BioTeke China, Beijing, China). The expression levels of miR-204 and matrix metalloproteinase-9 (MMP9) were determined with SYBR Green (Solarbio, Beijing, China), and normalized to U6 and β-actin, respectively. The relative expression levels were calculated based on the comparative threshold cycle (CT) results ($2^{-\triangle\triangle CT}$). Primers designed for real-time PCR were as follows: miR-204, forward: 5′ GACGCTTTCCCTTTGTCATCCT 3′, reverse: 5′ GTGCAGGGTCCGAGGTATTC 3′; MMP9, forward: 5′ CCAAAACTACTCGGAAGACTTGC 3′, reverse: 5′ GCGACACCAAACTGGATGA 3′.

2.5. Luciferase assays

Online search database microrna.org (http://www.microrna.org/microrna/home.do) was used to predict the sequence in the 3′-UTR region of human MMP9 gene targeted by miR-204. Wide type of MMP9 3′-UTR (MMP9 WT) containing the predicted miR-204 target site and a sequence with mutations (MMP9 MT) were inserted into pmirGLO vector (Promega China, Beijing China) to generate the recombinant constructs, pmirGLO-MMP9-3′UTR-WT and pmirGLO-MMP9-3′UTR-MT, respectively. Whether miR-204 interacted with the 3′UTR of MMP9 was determined with a Dual-luciferase Reporter Kit (Promega) according to standard supplier's protocols.

2.6. Gelatin zymography assay

The activity of MMP9 in cell supernatants was assessed with gelatin zymography assay. In brief, equal volume of each cell culture media was mixed with loading buffer and then loaded on 10% sodium dodecyl sulphate (SDS) with a final concentration of gelatin (Sigma–Aldrich, St. Louis, MO, USA) as 1 mg/ml. After a 2.5-h electrophoresis, the gels were incubated with 2.5% Triton X-100 (Sigma–Aldrich, St. Louis, MO, USA), rinsed, and then incubated in 50 mmol/L Tris–HCl buffer (containing 5 mmol/L $CaCl_2$, 1 μmol/L $ZnCl_2$, 0.2 mol/L NaCl, 0.02% Brij) for additional 40 h. Thereafter, the gels were stained with Coomassie brilliant blue R-250 (Amresco China) for 3 h and the band densities were calculated by a scanning densitometer (BioTeke China).

2.7. Western blot analysis

The MMP9 protein expression was detected with Western blot analysis. Briefly, cell protein samples were extracted by using RIPA lysis buffer (Beyotime, Shanghai, China), separated on SDS-polyacrylamide gel electrophoresis (SDS-PAGE), electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA), and blocked with 5% (w/v) non-fat milk. Then, the membranes were first incubated with rabbit polyclonal antibodies MMP9 (1: 400 diluted; Boster, Wuhan, China) at 4 °C overnight and then with HRP-conjugated secondary antibodies (1: 5000 diluted; Beyotime) for 45 min at 37 °C. Finally, the protein blots were visualized with an enhanced chemiluminescence (ECL) Kit (Sevensea pharmtech Co., Ltd, Shanghai, China). β-actin was served as the endogenous control.

2.8. Statistical analysis

All the results were presented as the mean ± standard deviation (SD). Data from different groups were compared with each other by using one-way analysis of variance followed by the Bonferroni post hoc test. Only a P-value < 0.05 was considered statistically significant.

3. Results

3.1. MiR-204 inhibits the trophoblastic invasion in vitro

In order to investigate the biological effect of miR-204 in trophoblastic invasion, two trophoblast-like cell lines, BeWo and JEG3 cells, were transfected with miR-204 mimics or its inhibitor. Results from quantitative real-time PCR analysis showed that the relative expression level of miR-204 was significantly increased in miR-204 mimics-transfected cells (Fig. 1A), whereas decreased in those transfected with the miR-204 inhibitor (Fig. 1B). Next, transwell inserts coated with matrigel were used to determine the invasiveness of trophoblastic cells. We noted that the invasive BeWo cell number was decreased from 124.80 ± 15.22 in miR-NC group to 81.60 ± 8.73 in miR-204 mimics group ($P < 0.001$) (Fig. 1C and D). Also, JEG3 cell invasion was suppressed when miR-204 was overexpressed (miR-NC group vs. miR-204 mimics group = 151.20 ± 16.33 vs. 89.20 ± 10.57, $P < 0.001$) (Fig. 1C and D). In contrast, more BeWo cells and JEG3 cells were invaded through the membranes of transwell chambers when miR-204 effect was counteracted by its inhibitor (BeWo, anti-miR-NC group vs. anti-miR-204 group = 118.80 ± 13.66 vs. 164.20 ± 18.13, $P < 0.01$; JEG3, anti-miR-NC group vs. anti-miR-204 group = 149.20 ± 15.27

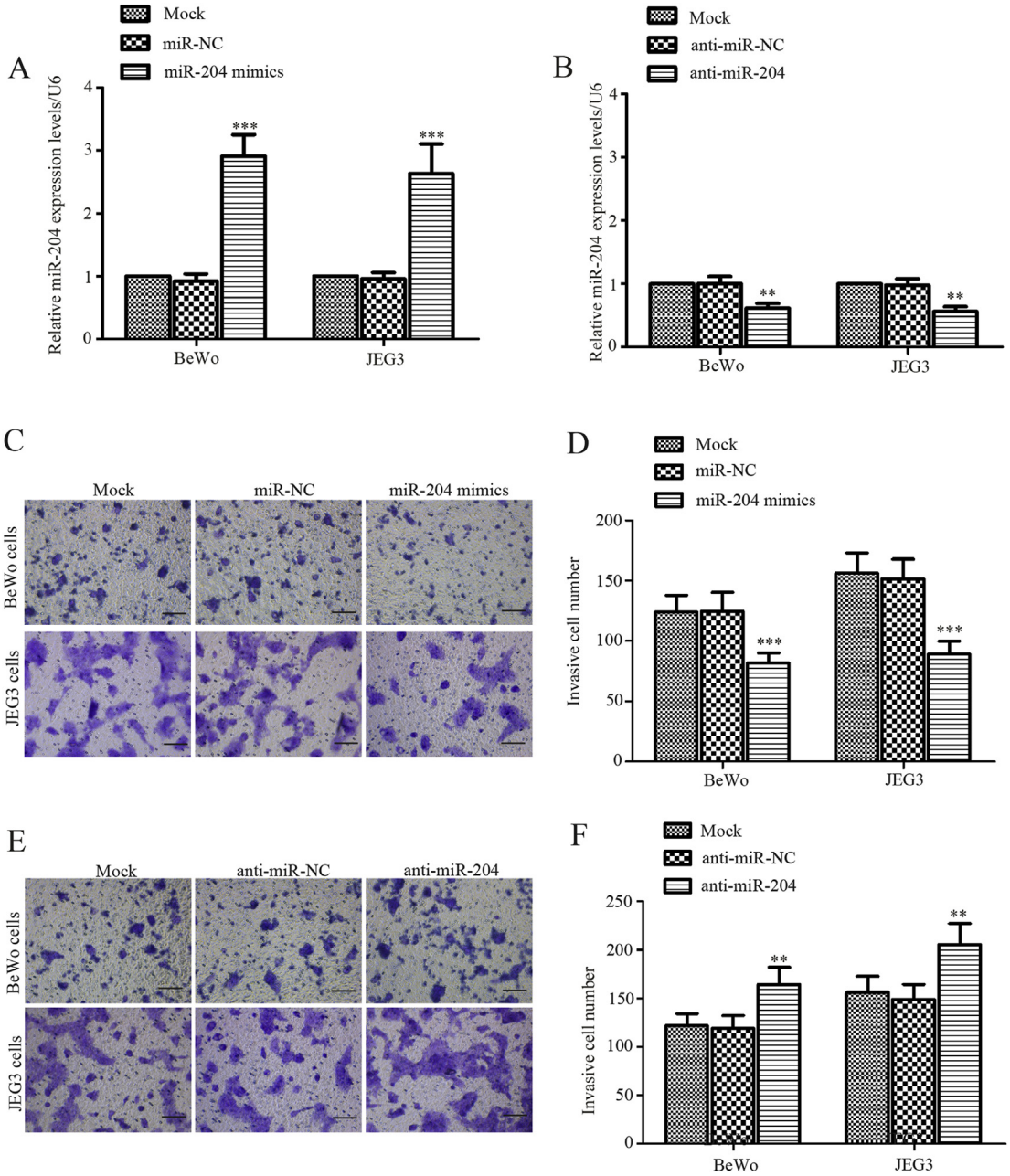

Fig. 1. Effect of miR-204 on the invasiveness of trophoblast-like cells. (A–B) The expression levels of miR-204 in BeWo and JEG3 trophoblast-like cells transfected with miR-204 mimics, miR-NC or mock, and in cells transfected with miR-204 inhibitor (anti-miR-204), anti-miR-NC or mock were determined with quantitative real-time PCR. The relative expression levels of miR-204 in cells were normalized to U6. (C, E) Representative of cell invasion results detected with the matrigel-coated transwell inserts in indicated groups (magnification 200×). (D, F) The invasive cells were counted in five random areas in multiple wells. Data were expressed in mean ± SD. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. the corresponding NC group. SD, standard deviation; NC, negative control.

vs. 205.40 ± 22.10; $P < 0.01$; Fig. 1E and F). The above results suggested miR-204 as a suppressor in trophoblast invasion *in vitro*.

3.2. MMP9 mediates the invasion of trophoblast-like cells

We first tested the role of MMP9 in trophoblastic invasion by using the trophoblast-like cells for loss- or gain-of-function of MMP9. Results from Western blot and gelatin zymography assays confirmed that pcDNA3.1-MMP9 plasmids led to significant upregulation in MMP9 protein expression (Fig. 2A) and activity (Fig. 2B) in trophoblast-like cells. In contrast, MMP9 protein expression (Fig. 2A) and activity (Fig. 2B) in trophoblast-like cells transfected with the MMP9 shRNA showed efficient suppression. As compared with negative control cells, the invasive capabilities of BeWo and JEG3 cells were enhanced when MMP9 was upregulated, whereas inhibited when MMP9 was downregulated (Fig. 2C–E). These results indicated that MMP9 played a role in regulating trophoblastic invasion *in vitro*.

3.3. MMP9 is a direct target of miR-204 in BeWo cells

By using the online database searching for targets of miRNAs, we noted that MMP9 is a potential target of miR-204, and thus examined whether miR-204 mediated trophoblast-like cell invasion through regulation of MMP9. Our results illustrated that both mRNA and protein levels of MMP9 were significantly decreased in BeWo cells transfected with miR-204 mimics (Fig. 3A and B), whereas increased in those transfected with miR-204 inhibitor (Fig. 3A and B). As shown in Fig. 3C, the seed sequence of miR-204 was complementary to the 3′-UTR of MMP9 mRNA. To verify the putative binding site of miR-204 in the 3′-UTR in MMP9 gene, we constructed two luciferase reporter vectors containing wild type MMP9 3′-UTR or mutant MMP9 3′-UTR (Fig. 3C). As shown in Fig. 3D, the relative luciferase activity of the MMP9-3′UTR WT vector was inhibited by miR-204 mimics in BeWo cells, but that of the MMP9-3′UTR MT vector was not (Fig. 3D). These results

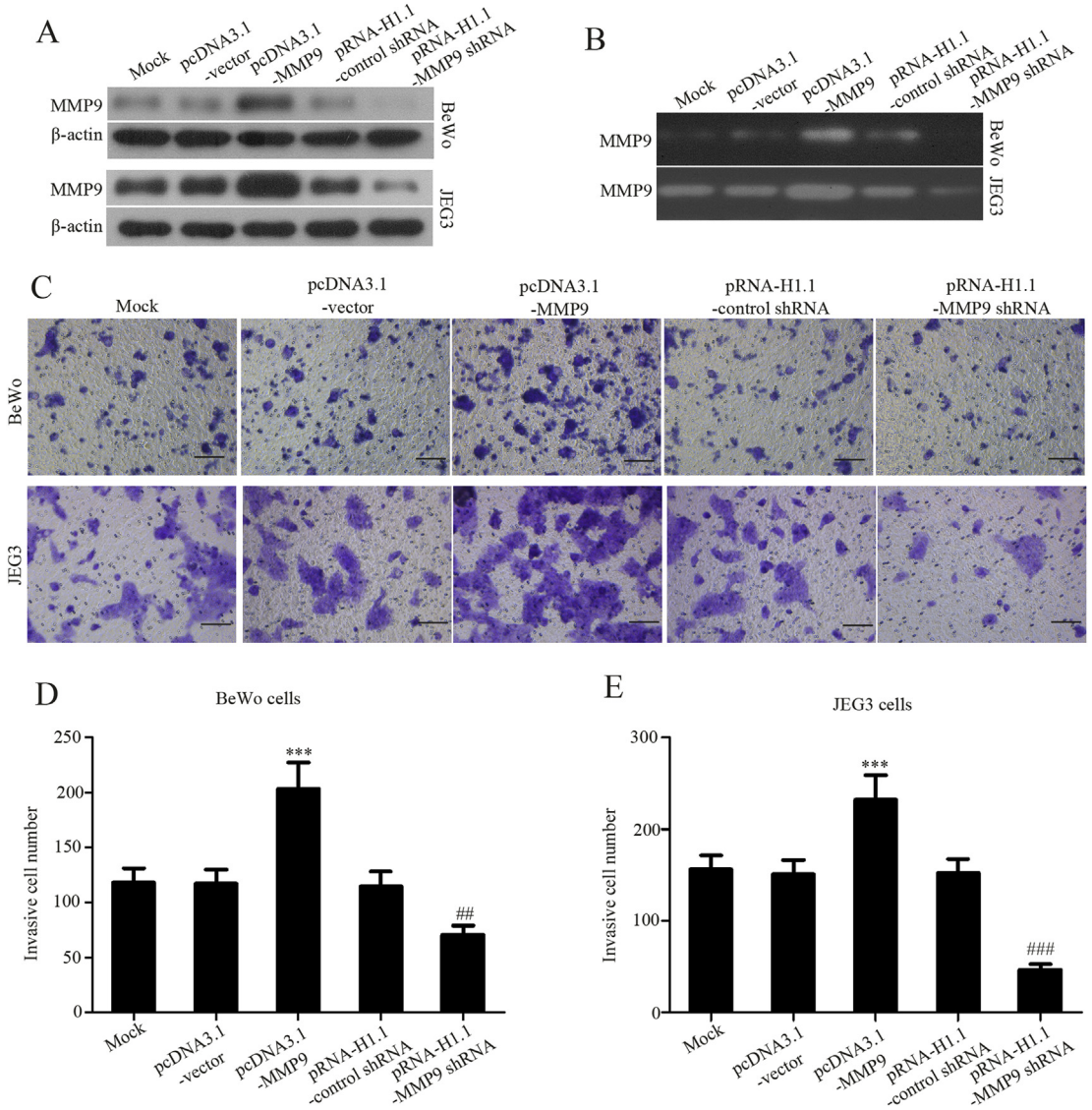

Fig. 2. MMP9 mediates the invasion of trophoblast-like cells. BeWo and JEG3 trophoblast-like cells transfected with pcDNA3.1-vector, pcDNA3.1-MMP9, pRNA-H1.1-control shRNA, pRNA-H1.1-MMP9 shRNA or mock were subjected to (A) Western blot analysis (β-actin, the endogenous control) and (B) Gelatin zymography assay against MMP9, and (C–E) matrigel-coated transwell assay (magnification 200×). The invasive cells were counted in five random areas in multiple wells. Data were expressed in mean ± SD. $^{***}P < 0.001$ vs. pcDNA3.1-vector group, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs. pRNA-H1.1-control shRNA. SD, standard deviation; MMP9, matrix metalloproteinase-9.

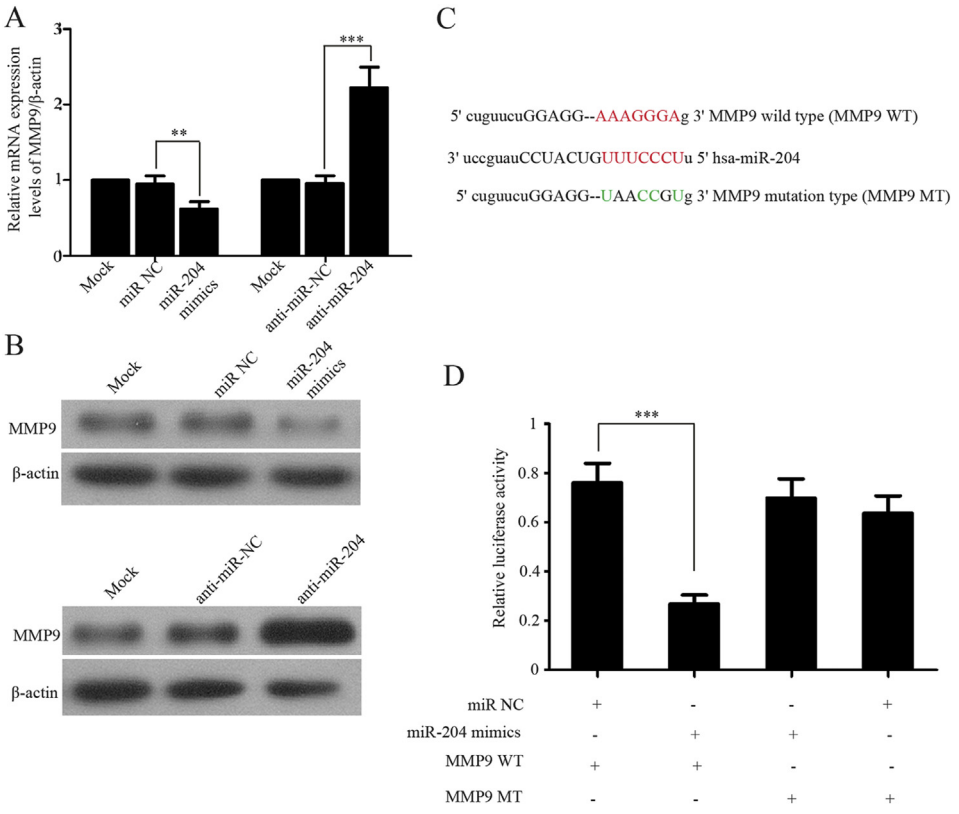

Fig. 3. Validation of MMP9 as a direct target of miR-204 in BeWo cells. The mRNA (A) and protein (B) levels of MMP9 in BeWo cells transfected with miR-204 mimics, miR-NC or mock, and in cells transfected with miR-204 inhibitor (anti-miR-204), anti-miR-NC or mock were determined with quantitative real-time PCR and Western blot analysis respectively. (C) Schematic map of luciferase reporter assay constructs. The sequences indicated putative miR-204 target site on wild-type MMP9 3′-UTR (MMP9 WT), its mutated derivative (MMP9 MT), and the pairing region of miR-204. (D) Results of dual-luciferase assay in BeWo cells transfected with pmirGLO-MMP9-3′UTR-WT or pmirGLO-MMP9-3′UTR-MT reporter together with miR-204 mimics or miR-NC. Data were expressed in mean ± SD. $^{**}P < 0.01$ and $^{***}P < 0.001$. SD, standard deviation; NC, negative control; MMP9, matrix metalloproteinase-9; UTR, untranslated region.

demonstrated that MMP9 could be directly targeted by miR-204 in human trophoblast-like cells.

3.4. Overexpression of MMP9 attenuates the invasion inhibiting effect of miR-204 in BeWo cells

To figure out whether MMP9 was directly involved in the invasion suppressing effect of miR-204, we transfected BeWo cells with miR-204 together with a MMP9 overexpressing vector to perform the rescue experiment. As indicated in Fig. 4, miR-204-induced reduction of BeWo cell invasion was partly counteracted by overexpression of MMP9 (Fig. 4, $P < 0.001$). These results suggested that miR-204 inhibited trophoblast invasion at least partly by decreasing the expression of MMP9.

4. Discussion

It is widely accepted that the limited invasion of trophoblast cells into the uterus is correlated with the development of pre-eclampsia [5]. Trophoblast invasion into the uterine wall is controlled by many factors, and recently, miRNAs are considered as essential regulators [16]. In this study, two trophoblast-like cell lines were transfected with miR-204 mimics or miR-204 inhibitor to explore the role of miR-204 in trophoblastic invasion.

Because of the difficulty in obtaining human placental tissues from which trophoblast cells can be prepared, use of appropriate cell lines enables the investigators to study the trophoblastic invasion *in vitro*. BeWo and JEG-3 cells derived from human choriocarcinomas possess extravillous trophoblast phenotypes and are able to secrete human chorionic gonadotropin, placental lactogen as well as estrogen [17]. Now, these two cell lines are widely used as *in vitro* models of the first trimester human trophoblasts for studies on trophoblast invasion [18]. Members of miR-204/204b/211 cluster have been reported to promote cancer cell metastasis [14,19], however, so far, the role for miR-204 in trophoblastic invasion had not been reported. In this study, we found that the invasive capabilities of both BeWo and JEG-3 cells were suppressed by miR-204 mimics, whereas enhanced by miR-204 inhibitor. These results revealing novel inhibitory effects of miR-204 on trophoblast-like cell invasion suggested that the dysregulated miR-204 might contribute to preeclampsia development.

MiRNAs are able to block the translation or decrease the stability of mRNAs by directly binding to the 3′-UTR of target genes [6,20]. Various online prediction databases have revealed hundreds of genes as potential targets for miR-204. However, relatively few targets have been experimentally validated. We noted that MMP9 is a potential target for miR-204, and examined whether this critical metalloproteinase was involved in miR-204-mediated trophoblastic invasion in the present study.

Invasive trophoblasts acquire characteristics defined by profound alterations in cell–cell and cell-matrix adhesion, and the degradation of extracellular matrix (ECM) enables their invasive behaviors [21]. MMPs, known as zinc-dependent proteases, can mediate cell invasion as reviewed before [22]. Of note, by using a mouse model, Plaks and co-workers have demonstrated that the deficiency of MMP9 causes physiological and placental

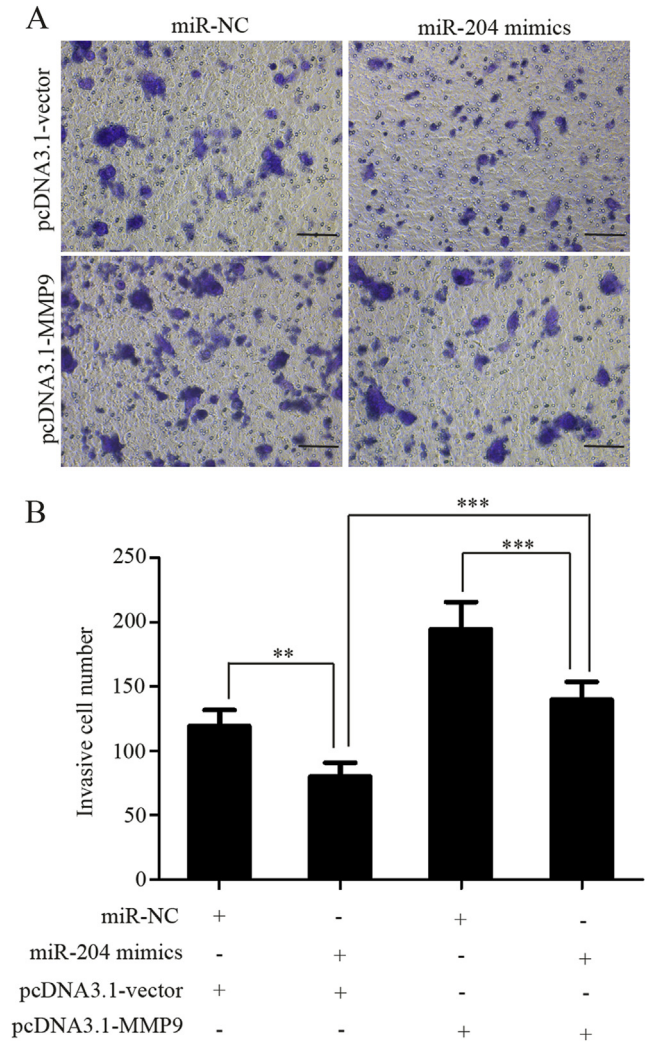

Fig. 4. MMP9 overexpression counteracts the inhibitory effect of miR-204 on BeWo cell invasion. (A) Representives of BeWo cell invasion determined with transwell insert assays (magnification 200×). Cells were transfected with miR-204 mimics/miR-NC and pcDNA3.1-vector/pcDNA3.1-MMP9 as indicated. (B) The invasive cells were counted in five random areas in multiple wells. Data were expressed in mean ± SD. $^{*}P < 0.01$ and $^{***}P < 0.001$. SD, standard deviation; NC, negative control; MMP9, matrix metalloproteinase-9.

abnormalities in mice, which mimic features of preeclampsia [23]. Such precious findings suggest that MMP9 plays an important role in normal placental development. Moreover, MMP9 has been reported to participate in trophoblast cell invasion that is mediated by microRNAs, such as miR-144 [24]. Nonetheless, whether MMP9 is implicated in miR-204-promoted trophoblastic invasion remains unknown. Data shown here for the first time revealed that miR-204 interacted with its partially complementary sequence in MMP9 3′UTR in trophoblast-like cells, and confirmed that miR-204 was able to negatively regulate MMP9 mRNA and protein expression. Our study was in consistent with a previous study showing MMP9 is a direct target of miR-204 in human retinoblastoma cells [25]. In addition, our results also showed that overexpression of MMP9 could mitigate miR-204 mimics-induced BeWo cell invasion, suggesting that miR-204 suppressed the trophoblastic invasion at least partly through inhibition of MMP9. It is worth noticing that MMP9 forced overexpression was not able to completely counteract miR-204 mimics' inhibitory effects, implying MMP9 was not the only effector of miR-204. As a matter of fact, besides MMPs, some other related proteases also play critical roles during trophoblast invasion [26], such as A Disintegrin and Metalloproteinases (ADAMs) [27]. Interestingly, ADAM12 is an important member of ADAMs that is also a potential target for miR-204 as predicted by TargetScan (http://www.targetscan.org/index.html) and microrna.org (http://www.microrna.org). Further experiments should be conducted to explore whether ADAM12 is involved in miR-204-mediated trophoblastic invasion. Additionally, one limitation of our study was that we only tested the interaction between miR-204 and MMP9 in isolated trophoblast-like cells *in viro*. Normal or pre-eclamptic placental tissues are being collected and processed to validate the expression correlation between miR-204 and MMP9 *in vivo* in our lab.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.052.

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