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Original Article

MiR-223 promotes trophoblast cell survival and invasion by targeting STAT3 in preeclampsia

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Abstract: Increasing evidence indicated that the dysregulation of trophoblast cell proliferation and invasion might play a central role in the pathogenesis of preeclampsia. MicroRNAs (miRs) are critical in the regulation of the pathogenesis of preeclampsia and a reduction or increase in the expression of certain miRs can mediate the proliferative and invasive activities of trophoblast cells. In the present study, we aimed to explore the effects and underlying mechanism of miR-223 on the proliferation and invasion of trophoblast cells. Real time PCR results showed that the expression of miR-223 was significantly down-regulated whereas STAT3 was high expression in preeclamptic patients. MiR-223 mimics markedly promoted trophoblast cell survival, proliferation and invasion, while inhibition of miR-223 expression has been shown to impede trophoblast cell survival, proliferation and invasion. Dual-luciferase reporter assay was confirmed that STAT3 is a direct target for miR-223, and miR-223 mimics strikingly inhibited the mRNA and protein expression of STAT3. Overexpression of STAT3 reversed the proliferation and invasion promoting effect of miR-223 in JEG-3 cells. These findings emphasized the essential role of miR-223 and opened new avenues and provide a mew target for diagnosis and treatment for preeclampsia.

Keywords: Preeclampsia, trophoblast cell, miR-223, proliferation, invasion

Introduction

Preecampsia, a life-threatening complication of human pregnancy, is a major cause of maternal and perinatal morbidity and mortality, intrauterine growth restriction, preterm birth and perinatal deaths [1]. It is characterized by maternal hypertensive component and signs of organ dysfunction, such as significant amounts of protein in the urine of a pregnant woman, visual symptoms thrombocytopenia, pulmonary edema, and/or cerebral or impaired liver function [1-3]. The disorder typically occurs in the third trimester and the main reason is chronic placental ischemia which resulted from the invading extravillous trophoblast [4, 5]. According to the published estimates, the molecular mechanism of trophoblast is appearing to limit the invasiveness of trophoblast cells into the uterine wall [6, 7]. Moreover, less trophoblast invasion is closely related to impaired remodeling of the spiral arteries which have been associated with preeclampsia [8-10]. A recent study found that human placenta trophoblast cells proliferate, differentiate and invasion resulting in successful implantation of the embryo [11]. Although some advancement have achieved, the specific molecular mechanism in trophoblast cell proliferation and invasion remains unclear.

Recently, dysregulation of microRNAs (miRNAs) have been identified associated with preeclampsia in placenta [12]. MiRNAs are a class of ~22 nucleotides non-coding, single strand small RNA with repression of proteins by transcriptional and post-transcriptional regulation of gene expression [13, 14].

A host of studies demonstrated that miRNA could seed sequence pairing with the 3'-untranslated region (3'-UTR) of target mRNAs to inhibit gene expression [14]. Evidence suggested that miRNAs play important roles in a burst of human diseases development and pathogenesis through via regulating the protein expression of its target genes [15, 16]. Ample

Table 1. Clinical characteristics of study subjects

Characteristics	Control	PE
Ages	28.3±0.21	28.9±0.15
Gestational age (wks)	30.6±0.65	31.5±0.36
Systolic BP (mmHg)	112.6±2.31	153.9±3.06
Diastolic BP (mmHg)	73.9±1.18	98.6±1.65
Proteinuria (g/24 h)	-	4.19±0.97

previously studies suggested that approximately 600 miRNAs expressed in human placenta and miRNAs genomewide expression in normal and preeclamptic placentas were also profiled [17-19]. For example, ectopic expression of miR-210 in patients with preeclampsia, inhibited the migration and invasion capability of trophoblast cells by targeting ephrin-A3 and homeobox-A9 [20]. High level of miR-20a and miR-125b-1-3p both regulate the invasive behaviors of trophoblast cells by targeting different proteins [11, 21]. These findings suggest that targeting preeclampsia-associated miRNAs may play critical roles in human placentas function and development. However, the detailed role and mechanism of miRNAs in the pathogenesis of preeclampsia remains largely unknown.

Signal transducer and activator of transcription 3 (STAT3), a signaling molecule for a number of inflammatory cytokines, was previously shown to be linked to the invasiveness of tumor cells [22]. Also, emerging studies have revealed that aberrant STAT3 activation was been found in kinds of cancers, such as brain, breast and prostate and has been demonstrated implicated in tumor metastasis [23, 24]. Evidence also indicated that STAT3 was associated with preeclampsia, but is also positively related with trophoblast invasiveness [25]. STAT3 is activated through phosphorylation of its tyrosine 705 and serine 727 sites, and the high phosphorylation in invasive first trimester trophoblast cells was been found [25, 26]. STAT3 has been validated that play roles in trophoblast cells invasion, however, its role in the function of miRNAs in trophoblast cells is still unclear. In the present study, we aimed to elucidate the functional roles and mechanism of miR-223 in human trophoblast cells. We demonstrated that miR-223 promotes trophoblast cell invasion by targeting STAT3 in preeclampsia.

Materials and methods

Sample collection

Placental tissues were obtained from nulliparous women who were admitted to the affiliated hospital of Inner Mongolia medical university in Inner Mongolia, China and all participants gave informed consent. The pregnant women enrolled in this study are Chinese Han. The study was approved by our institutional review board of the Inner Mongolia medical university. Placental tissues were derived from patients with preeclampsia (n=20) and the normal pregnancies control (n=10). Clinical characteristics of preeclamptic patients as shown in **Table 1**. Preeclampsia was defined according to the International Society for the Study of Hypertension in Pregnancy. As shown in **Table 1**, the gestational age of PE patients was around 31.5 weeks and the patients are early-onset PE. Women having other pathologies (exclude preeclampsia) or under medication were excluded. There is no woman with preterm birth or any other clinical disorders in the control group. Placental tissue samples were collected from randomly selected region after removal of the placenta. Samples were cut in small pieces and stored at -80°C until used.

Cell culture and transfection

Human trophoblast cell line JEG-3 and HTR8/SVneo (ATCC) were maintained in RPMI-1640 (Hyclone, USA) medium. Cell-culture medium was supplemented with 10% fetal bovine serum under 5% CO_2 at 37°C. Cells were transfected with miR-223 mimics, negative control (NC), miR-223 inhibitor or anti-negative control (Anti-NC) (Shanghai GenePharma Company, China) using LipofectamineTM 2000 (Invitrogen, CA, USA) according to the manufacturer's protocol. For STAT3 plasmid transfections, JEG-3 cells were transfected with pCDNA3.1-STAT3 or pCDNA3.1 control plasmid using LipofectamineTM 2000 (Invitrogen). Forty eight hours after transfection, cells were harvested for further assays.

Cell viability assay

JEG-3 and HTR8/SVneo cell viability was measured by WST-1 assay according to the manufacturer's introduction. The miR-223 mimics or

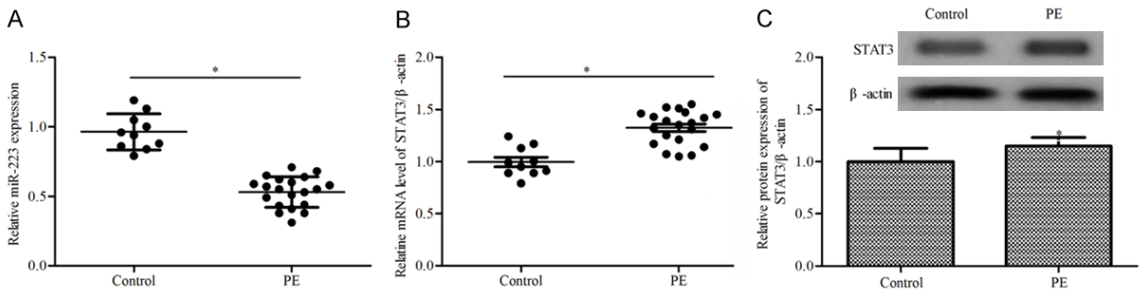

Figure 1. Expression of miR-223 and STAT3 in human placental tissues. A. RT-PCR was used to measure the level of miR-223 in human normal (Control) and preeclampsia (PE) placental tissues. B. Expression of STAT3 mRNA in control and PE placental tissues. C. Expression of STAT3 protein in control and PE placental tissues. Data are expressed as mean ± SEM. N=3. *P < 0.05 vs control group.

miR-223 inhibitor transfected cells were plated into 24-well plates at a density of 1×10^5 cells/well. At 24, 48, 72, and 96 h post-transfection, the cells were exposed to WST-1 reagent for 1 h at 37°C. The absorbance on A450 nm was measured with a spectrophotometer.

Cell survival assay

JEG-3 and HTR8/SVneo cells (1×10^5 cells/well) were plated in 24-well dishes and transfected with miR-223 mimics or miR-223 inhibitor. At 48 h post-transfection, cells were trypsinize and stained with trypan blue. Cell numbers were counted with hemocytometer. All experiments were repeated at least 3 times.

In vitro transwell insert invasion assay

Cell invasion was measured as previously reported with minor modification [27, 28]. Briefly, 1×10^5 cells were transfected with miR-223 mimics or miR-223 inhibitor and seeded into the top of Matrigel-coated (Becton Dickinson; Franklin Lakes, NJ) transwell inserts (Costar, Corning incorporated, NY). After 48 h, the invaded cells were washed with PBS and stained with a Harleco hemacolor stain set (EMD, NJ, USA). Average invaded cell numbers from 15 random fields at 200× magnification in each field were used for statistical analyses.

RNA extraction and real-time qPCR

Total RNA was isolated from JEG-3 and HTR8/SVneo cells using the TRIZOL reagent (Invitrogen, CA, USA) following the manufacturer's protocol. To obtain cDNA, oligo-dT or specific microRNA stem loop RT primers were used to reverse transcription for mRNA and miRNA, respectively. To measure the expression of miR-223, miRcute miRNA qPCR Detection kit (Tiangen, Beijing, China) was used, whereas the SYBR Premix Ex Taq kit (Applied Biosystems, CA, USA) were used to detect the level of STAT3. U6 was used as an internal control for miR-223 detection, whereas GAPDH was used to normalize the expression of STAT3.

Western blotting

Protein samples from JEG-3 cells were isolated using RIPA buffer and western blot analysis was carried out according to the previously reported [28]. Antibodies used in this study including STAT3 (abcam) and β-actin (abcam).

Luciferase assays

Luciferase assay was measured as previously reported [29]. The STAT3 3'UTR cDNA fragment was amplified from human genomic DNA, and cloned into a pGL3 luciferase promoter vector (Promega, Madison, WI, USA). Mutant STAT3 3'-UTR was also generated and used as the control. JEG-3 cells were co-transfected with the miR-223 mimics or controls and pGL3-STAT3 3'-UTR vectors. After 24 h, relative luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega).

Statistical analysis

Results presented in this study were expressed as mean ± SEM. Statistical analysis was performed using SPSS 13.0 statistical software (SPSS, Chicago, IL, USA), statistical comparisons were performed using One-way ANOVA followed by Student t test. $P < 0.05$ was considered as statistically significant.

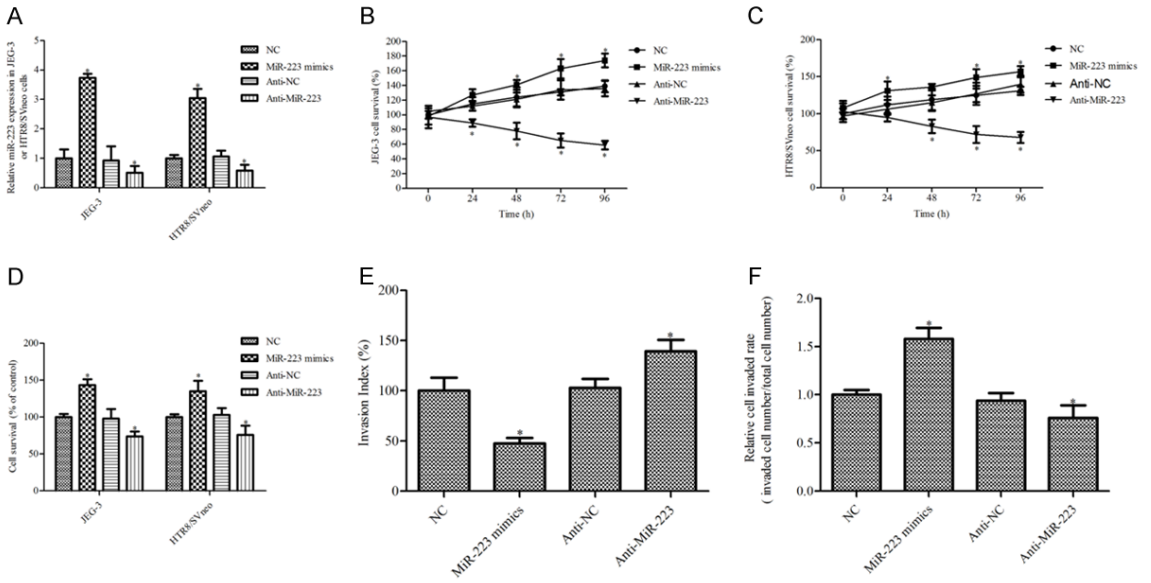

Figure 2. Effect of miR-223 on the growth and invasion in JEG-3 and HTR8/SVneo cells. (A) The expression of miR-223 in JEG-3 and HTR8/SVneo cells transfected with miR-223 mimics, negative control (NC), miR-223 inhibitor or anti-negative control (Anti-NC) was detected by RT-PCR. (B, C) JEG-3 (B) and HTR8/SVneo (C) cell viability was measured by WST-1 assay at different time point (0, 24, 48, 72 and 96 h). (D) JEG-3 and HTR8/SVneo cell survival was tested by trypan blue assay. (E) The invasion of JEG-3 cells was determined using In vitro Transwell insert invasion assay. (F) The ratio of invaded cell numbers/total cell numbers in JEG-3 cells was calculated. Cells were transfected with miR-223 mimics, negative control (NC), miR-223 inhibitor or anti-negative control (Anti-NC) for 48 h. Data are expressed as mean ± SEM. N=3. *P < 0.05 vs control group.

Results

Expressions of miR-223 and STAT3 were negatively associated in preeclamptic patients

Ample evidence indicated that the level of miR-223 was significant decreased in human placentas from preeclamptic pregnancies compared with normal pregnancies [30, 31]. RT-qPCR results showed that the level of miR-223 in preeclamptic placentas was much lower than the normal control placentas (**Figure 1A**). Evidence also indicated that STAT3 is a direct target for miR-223, and the gene play important role in preeclamptic pregnancies [25, 29]. Thus, we next measured the expression of STAT3 in preeclamptic and normal placentas. As shown in **Figure 1B** and **1C**, the mRNA and protein expression of STAT3 was significantly elevated in preeclamptic patients than the normal pregnancies. These results suggested that the expression of miR-223 and STAT3 is negatively correlated in the placenta.

MiR-223 promoted trophoblast survival

To verify the specific function and mechanism of miR-223 in preeclamptic patients, we elevated the role of miR-223 in human trophoblast cell line JEG-3 and HTR8/SVneo. JEG-3 and HTR8/SVneo cells were transfected with miR-223 mimics or inhibitor and the corresponding negative control. RT-qPCR results showed that the level of miR-223 was obviously increased or reduced in JEG-3 and HTR8/SVneo cells transfected with miR-223 mimics or inhibitors, respectively (**Figure 2A**). Next, cell survival was determined by WST-1 assay, we found that miR-223 mimics resulted in an obviously increased JEG-3 and HTR8/SVneo cell survival at different time point (0, 24, 48, 72 and 96 h), whereas miR-223 inhibitor inhibits cell viability (**Figure 2B** and **2C**). Also, trypan blue assay demonstrated that overexpression of miR-223 promoted JEG-3 and HTR8/SVneo cell survival, whereas miR-223 inhibitor inhibited the survival of these cells (**Figure 2D**) as counted by cell number.

MiR-223 promoted trophoblast invasion

Previously reports showed that JEG-3 trophoblast-like cells are widely used to measure trophoblastic cells invasion [32]. Trophoblast invasion was investigated by transwell assay. JEG-3 cells were seeded in 24-well culture plates and

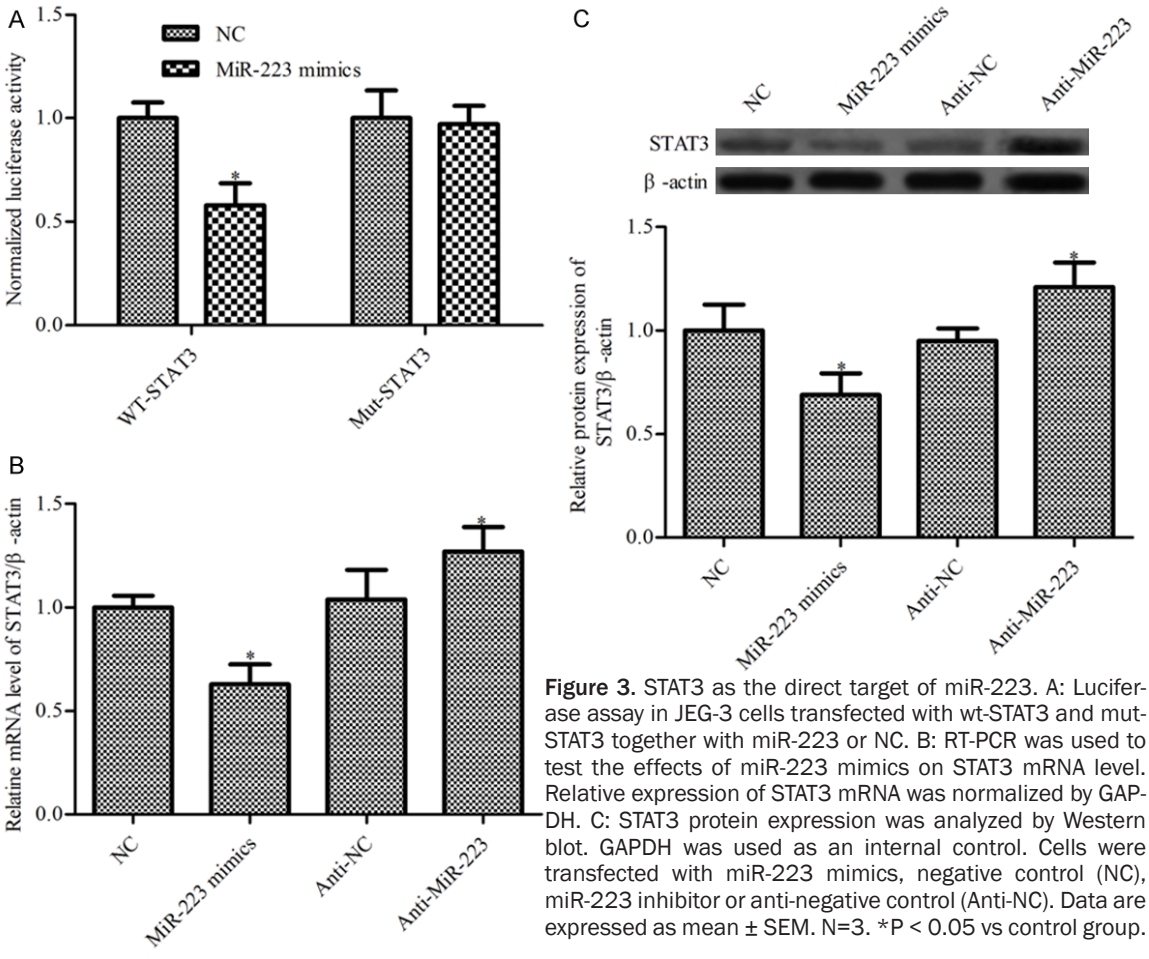

Figure 3. STAT3 as the direct target of miR-223. A: Luciferase assay in JEG-3 cells transfected with wt-STAT3 and mut-STAT3 together with miR-223 or NC. B: RT-PCR was used to test the effects of miR-223 mimics on STAT3 mRNA level. Relative expression of STAT3 mRNA was normalized by GAPDH. C: STAT3 protein expression was analyzed by Western blot. GAPDH was used as an internal control. Cells were transfected with miR-223 mimics, negative control (NC), miR-223 inhibitor or anti-negative control (Anti-NC). Data are expressed as mean ± SEM. N=3. *P < 0.05 vs control group.

transfected with miR-223 mimics or inhibitor. As shown in **Figure 2E**, *in vitro* transwell insert results showed that overexpression of miR-223 significant promoted JEG-3 cells invasion compared with the NC groups, while JEG-3 cells transfected with miR-223 inhibitor lead to a significant reduction of cell invasion compared with anti-NC group. Also, the ratio of invade cell number/total cell number was showed as **Figure 2F**, the results demonstrated that miR-223 mimic significant promoted JEG-3 cells invasion.

MiR-223 down-regulates STAT3 directly in JEG-3 cells

To further investigate the mechanism of miR-223 promotion of proliferation and invasion of JEG-3 cells, we measured the target gene of miR-223 that contributed to JEG-3 cells proliferation and invasion. There has some evidence indicated that STAT3 is a direct target for miR-223, and the gene play important role in preeclamptic pregnancies [25, 29]. To confirm the directly regulation of miR-223 on the level of STAT3, a dual-luciferase reporter assay was carried out. As shown in **Figure 3A**, miR-223 mimics markedly decreased the luciferase activity of pGL3-STAT3 3'-UTR transfected cells, and there has nothing effect on luciferase activity of pGL3-mut STAT1 3'-UTR. We next determined the effects of miR-223 mimics and inhibitor on the expression of STAT3. A significantly decrease in the mRNA and protein levels of STAT3 was seen in JEG-3 cells transfected with miR-223 mimics, whereas miR-223 inhibitor reversed these effects (**Figure 3B** and **3C**). These results suggested that miR-223 negatively regulates the expression of STAT3 in JEG-3 cells.

STAT3 overexpression reversed the survival and invasion promoting effect of miR-223 in JEG-3 cells

To further figure out miR-223 promoted JEG-3 cells proliferation and migration was dependent on STAT3 level, miR-223 together with a

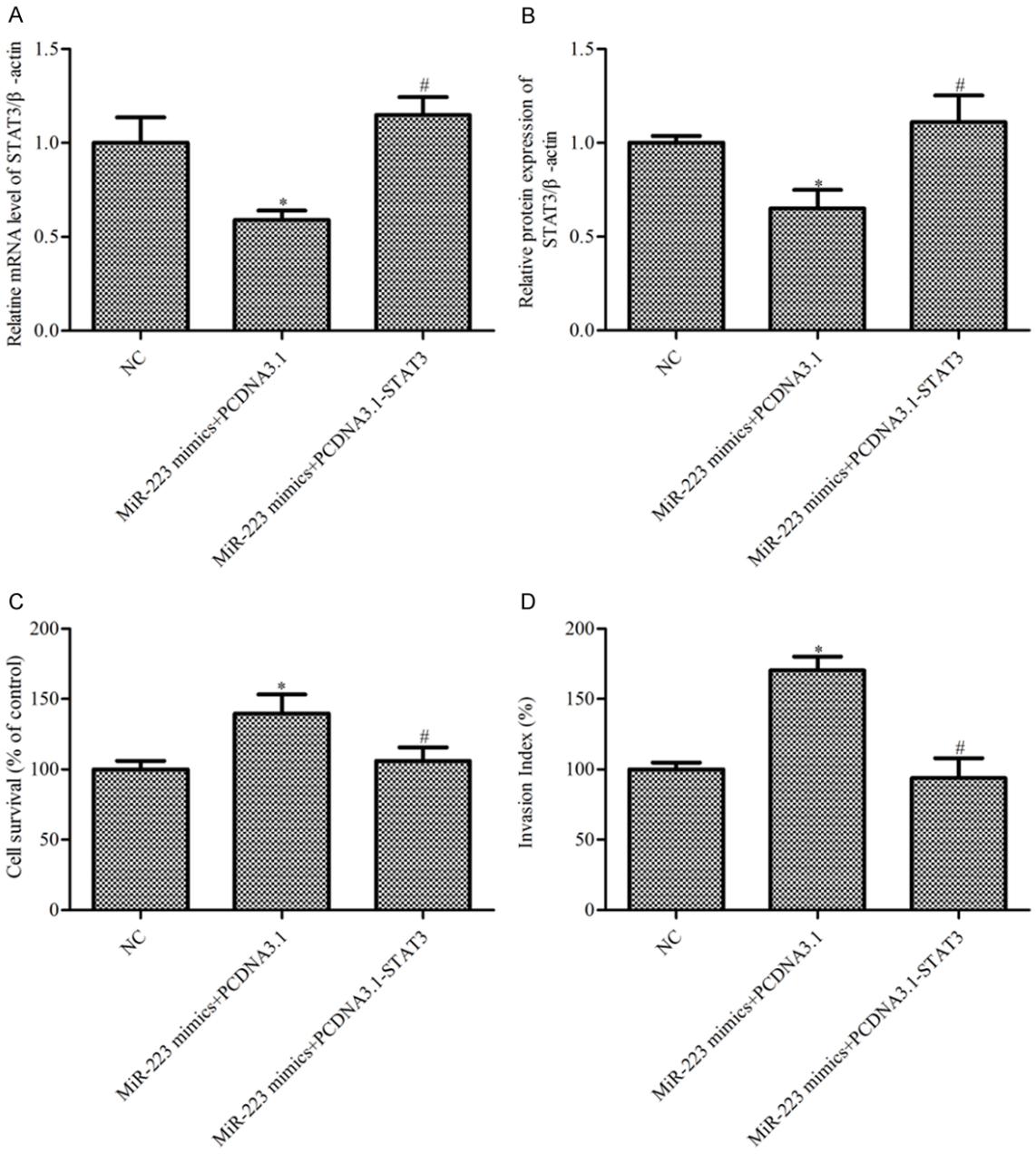

Figure 4. STAT3 overexpression impeded the effect of miR-223 on the survival and invasion of JEG-3 cells. (A, B) The STAT3 mRNA (A) and protein (B) expression in JEG-3 cells co-transfected with miR-223 mimics and pcDNA3.1-STAT3 were measured by RT-PCR and western blot, respectively. (C, D) Overexpression of STAT3 can reverse the cell survival and invasion increased by miR-223 mimics. JEG-3 cells were co-transfected with miR-223 mimics and pcDNA3.1-STAT3. Cell survival and invasion were determined by trypan blue assay and In vitro Transwell insert invasion assay, respectively. Data are expressed as mean ± SEM. N=3. *P < 0.05 vs control group. #P < 0.05 vs miR-223 mimics +pcDNA3.1 group.

STAT3 overexpressing vector (pCDNA3.1-STAT3) was co-transfected in JEG-3 cells (**Figure 4A** and **4B**). The results showed that pCDNA3.1-STAT3 significant inhibited miR-223 induced-cell proliferation and invasion (**Figure 4C** and **4D**). Also, the effect of overexpression of STAT3 alone was also measured, as shown in Supplementary Figure 1, pCDNA3.1-STAT3 significantly increased the level of STAT3 mRNA and protein expression; moreover, markedly inhibits JEG-3 cells survival and invasion. These results suggested that STAT3 is involved in the prolif-

eration and invasion-promoting effect of miR-223 in JEG-3 trophoblast cells.

Discussion

The pregnancy-specific syndrome, preeclampsia, is characterized by *de-novo* onset of hypertension and proteinuria after 20 weeks of gestation [33]. A rapidly growing body of evidence suggested that poor trophoblast invasion has been implicated in the impaired remodeling of the spiral arteries which have been associated with preeclampsia [3, 8-10]. Although some progress has been made, the molecular mechanisms underlying the pathogenesis preeclampsia is largely unknown. It is well accepted that miRNAs are differentially expressed in placenta from preeclamptic and normal pregnancies and played a pivotal roles in the development of placenta via involving in various biological processes [17, 21, 30]. AS a serum biomarker for preeclampsia, miR-210 has been shown to inhibit trophoblast invasion [34]. Another study has also demonstrated that miR-195 was downregulated in preeclamptic placenta and play crucial role in trophoblast cell invasion [35]. Qinghua Li et al showed that abnormal expression of miR-125b-1-3p inhibits trophoblast cell invasion and might contribute to the pathogenesis of preeclampsia [21]. Thus, clarifying the roles and mechanism of miRNAs in trophoblast cells will largely valuable for understanding the pathogenesis of preeclampsia.

MiR-223 is one of the differential miRNAs which significantly down-regulated in preeclamptic placentas reported by Zhu et al and Xu et al [30, 31]. However, the specific role and mechanism of this MiR in preeclampsia is still unclear. The present study provides evidence for the first time to support an essential role for miR-223 in regulating trophoblast proliferation and invasion, which facilitates the development of better intervention of this disease. Notably, miR-223 is differentially expressed in human placentas from preeclamptic pregnancies and normal pregnancies. Also, we found that the aberrant miR-223 expression in human trophoblast cells produced promotion effects on the survival and invasion of trophoblast cells, in part, by suppressing STAT3 expression. MiR-223-deficient trophoblast cells were presented inhibitor effects on cell survival and invasion, and increased the level of STAT3.

MiRs have been demonstrated that paly critical regulation effects in a burst of physiological processes via inhibiting target genes expression [36]. In the present study, we identified STAT3 as a genuine target of miR-223 by using luciferase reporter assays. STAT3, a member of signal transducers and activators of transcription (STAT) families, was previously shown to be related to invasiveness of tumor cells [37]. Dysregulation of STAT3 has been found in multiple cancers, such as breast and prostate cancer and the gene has also been confirmed that play an effective role in tumor metastasis [24]. STAT3 activity was recently found contributes to tumor cell invasive properties and the malignant phenotype of choriocarcinoma cells was demonstrated with STAT3 activity; moreover, this reports indicated that STAT3 is a signaling molecule which associated with PE and trophoblast invasiveness [25]. Weber et al showed that the expression of STAT3 and its activated forms is negatively altered in trophoblast and decidual stroma cells derived from preeclampsia placentae [26]. Evidence indicated that STAT3 was observed in immature trophoblasts and appeared to be lost in term placentae [25]; this indicated that gestational age may effects the expression of STAT3. Our study indicated that STAT3 expression was increased in PE patients, these results was different from Weber et al. In the present study, the gestational ages of control pregnancy women was 30.6 weeks, and gestational ages of PE group was 31.5; whereas the gestational ages of first and third trimester pregnant women was around 9 and 38 weeks, and the gestational ages of PE patient was around 33 weeks. We guess may be different gestational age has different expression STAT3 expression which needs to demonstrate in our further studies. Moreover, our study also indicated that the level of STAT3 was closely related with trophoblast survival and invasion. In summary, this cooperation verified that overexpression of miR-223 is valuable for preeclampsia via promoting JEG-3 trophoblast proliferation and inhibiting its invasion by negatively regulating its target gene STAT3. Thus, our study may be a guide to new diagnostic and therapeutic approaches for preeclampsia, and miR-223 may be a potential targets for preeclampsia.

Disclosure of conflict of interest

None.

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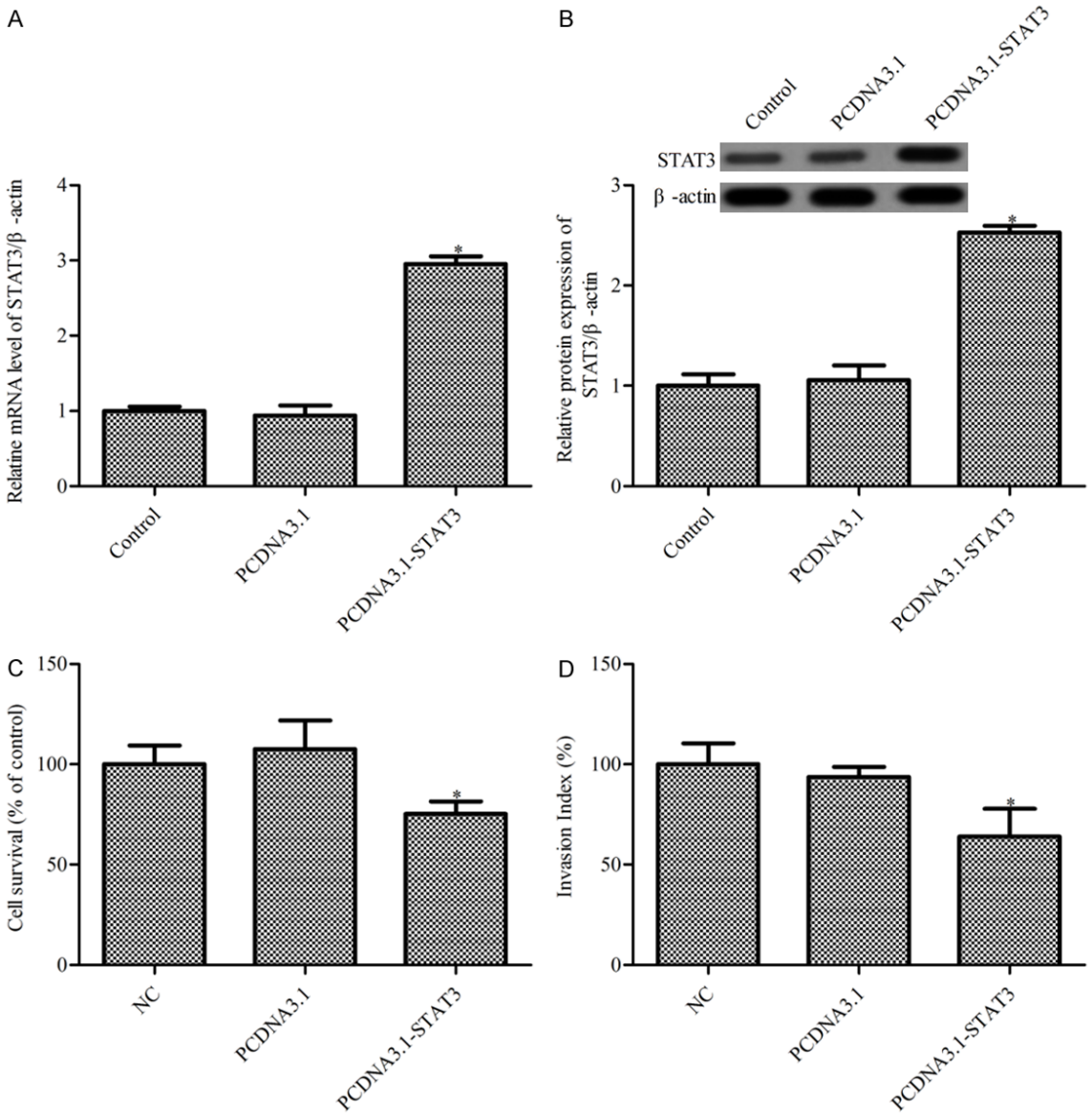

Supplementary Figure 1. STAT3 overexpression inhibits JEG-3 cells survival and invasion. (A, B) The STAT3 mRNA (A) and protein (B) expression in JEG-3 cells transfected with pcDNA3.1-STAT3 were measured by RT-PCR and western blot, respectively. (C, D) Overexpression of STAT3 inhibits the cell survival (C) and invasion (D). JEG-3 cells were transfected with pcDNA3.1-STAT3 or pcDNA3.1 control vector for 48 h. Cell survival and invasion were determined by trypan blue assay and In vitro Transwell insert invasion assay, respectively. Data are expressed as mean ± SEM. N=3. *P < 0.05 vs control group.