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# MicroRNA-494 inhibits the growth and angiogenesis-regulating potential of mesenchymal stem cells

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ABSTRACT

**Mesenchymal stem cells (MSCs) play an important role in the pathology of preeclampsia (PE). Our previous microarray analysis found that microRNA-494 (miR-494) is highly expressed in decidua-derived MSCs (dMSCs) from PE. We hypothesized that aberrant expression of miR-494 in dMSCs is involved in PE development. In the present study, we found that miR-494 arrests G1/S transition in dMSCs by targeting CDK6 and CCND1. We also found that supernatant from miR-494-overexpressing dMSCs reduces HTR-8/SVneo migration and impairs HUVEC capillary formation by suppressing VEGF. Taken together, we report an unrecognized mechanism of miR-494 affecting dMSC proliferation and function in the pathology of PE.**


## 1. Introduction

Preeclampsia (PE) is a pregnancy-specific multi-organ dysfunctional disease and a leading cause of maternal and fetal morbidity and mortality worldwide [1,2]. Its multifactorial pathogenesis is still not clearly elucidated [3–5]. It was reported that insufficiency of angiogenesis might be one of the most important factors [6]. A fine balance between pro-angiogenic and anti-angiogenic factors was pivotal in vascular development of angiogenic processes at maternal–fetal interface, which determines the success of pregnancy [7]. However, the expression of angiogenic growth factors was significantly altered in placenta and serum of pregnancies complicated with PE [8–11].

Mesenchymal stem cells (MSCs), capable of self-renewal, expansion and multilineage differentiation [12,13], are thought to be multifunctional stem cells and can be derived from various tissues [14]. Many studies reported that MSCs play various roles both in immune-regulatory potential and in vasculogenesis and angiogenesis [15,16]. MSCs regulate the immune system by affecting the proliferation, maturation and function of immune cells [17,18]. MSCs also secret a broad range of bioactive molecules, such as vascular endothelial growth factor (VEGF), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1), to display angiogenesis [16,19,20]. Interestingly, recent studies reported that MSCs in maternal–fetal interface could maintain successful pregnancy. The changes in MSCs' function might cause gestational related diseases and be involved in the imbalanced angiogenesis in maternal–fetal interface of PE [15]. However, it is still remains unclear on the molecular mechanism of angiogenic regulation of MSCs in maternal–fetal interface.

MicroRNAs (miRNAs or miRs) are a class of evolutionarily conserved and widespread small non-coding RNAs through inhibiting translation or direct cleavage of target mRNAs to regulate various

*Abbreviations:* microRNAs, miRNAs or miRs; miR-494, microRNA-494-3p; MSCs, mesenchymal stem cells; dMSCs, decidua-derived MSCs; PE, preeclampsia; mi-494, miR-494 mimic; mi-NC, miR-494 mimic negative control; si-RNA, small interfering RNA; VEGF, vascular endothelial growth factor; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; CDK6, cell cycle dependent kinase 6; CCND1, cyclin D1; CCND2, cyclin D2; CCNE1, cyclin E1; HELLP, a syndrome of hemolysis (H), elevated liver enzymes (EL), low platelets (LP); IVF-ET, in vitro fertilization and embryo transfer.

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cellular biological properties in almost all diseases such as proliferation, cell cycle, differentiation and apoptosis [21]. It was found that the expression profiles of miRNAs were significantly altered in MSCs, placenta tissues, maternal serum, and umbilical cord blood from PE pregnancies [22–25]. Our previous studies showed that miR-16 inhibited the angiogenesis potential of MSCs and miR-181a modulated local immune balance in PE [26,27]. And our miRNA array analysis results showed that microRNA-494-3p (miR-494) is one of the most high expressed miRNAs in decidua-derived MSCs (dMSCs) from patients with PE [26,28]. miR-494 was reported to suppress arterial endothelial cell proliferation [29] and be involved in neovascularization [30]. These data suggest that miR-494 may act as an important regulator to be involved in angiogenesis in maternal–fetal interface of PE.

Thus, in the present study, we are engaged to explore the function of miR-494 on dMSCs. We found that miR-494 affected cell growth and the paracrine activity of dMSCs and inhibited trophoblastic cell migration and tube formation of endothelial cells, suggesting that miR-494 may be involved in the pathology of PE.

## 2. Materials and methods

### 2.1. Decidua collection

Decidua tissues were obtained from placental site decidua basalis of healthy pregnancies ($n = 10$) and PE pregnancies ($n = 5$) who underwent Caesarean section in Drum Tower hospital from Jan 2014 through Sep 2014. Nanjing Drum Tower hospital ethics-committee approved and the patients had written consent. Criteria for exclusion were multiple gestations and the presence of maternal chronic hypertension, chronic nephritis, hepatic disease, the HELLP syndrome, gestational diabetes mellitus, in vitro fertilization and embryo transfer (IVF-ET) and mild preeclampsia or other infectious and neoplastic disease and fetal congenital defect.

### 2.2. dMSCs isolation and culture

dMSCs were isolated from fresh decidua tissues. The process was operated within 4 h as we previously reported in our laboratory [31]. Decidua tissues were washed with PBS several times and mechanically broken to pieces and incubated in an enzyme cocktail (Sigma, St. Louis, MO) 1 h with gentle agitation at 37 °C. The digestion mixture was washed with PBS and then washed with Dulbecco's modified Eagle's medium/F12 (DF12, Gibco, Grand Island, NY, USA). Then they were suspended in fresh DF12 supplemented with 20% fetal bovine serum (FBS) plus antibiotics and incubated at 37 °C in a 5% $CO_2$ saturating humidified atmosphere. Two days after the incubation, the small digested residues were removed and the culture was continued. The medium was replaced two times every week. When numerous colonies were observed, cells were detached using 0.25% trypsin/EDTA to transfer to a new culture plate supplemented with 10% fetal bovine serum. After the 2nd to 4th cell passages, the specific phenotypic surface antigens of MSCs were characterized by flow cytometry assay. The adherent, fibroblast-like cells were presented as CD105+, CD73+, CD90+, CD29+, CD44+, CD106-, HLA-DR-, CD11b-, CD14-, CD34-, CD31- and CD45- (Fig. S1). dMSCs also could differentiate into adipose, chondrocyte and bone as we previously reported [31].

### 2.3. Cell line and culture

HTR-8/SVneo cells and human umbilical vein endothelial cells (HUVEC) were cultured in RPMI-1640 (Gibco) supplemented with 10% FBS and 100 IU/mL antibiotics, at 37 °C in a humidified atmosphere with 5% $CO_2$.

### 2.4. Transient transfection

Overexpression of miR-494 in dMSCs was achieved by transfecting cells with miR-494 mimic (mi-494) and miR-494 mimic negative control (mi-NC) (RIBO BIO, Guangzhou, China) using lipofectimine® 2000 reagent (Invitrogen) until dMSCs reached 30–50% confluence and represented a good growth state. Three sequences of small interfering RNAs for VEGF (si-VEGF) were ordered from RIBO BIO. After transfection for 48 h, cells and the supernatants were harvested for the following experiments.

### 2.5. Cell viability analysis

Cell viability was performed by using a CCK-8 kit (DojinDo) to detect the effect of miR-494 on dMSCs' viability. A cell counting kit was used to receive a qualitative index of cell viability after transfection in our experiments. After transfection for 48 h, CCK-8 was separately added to each well and incubation for another 3 h. An OD absorbance at 450 nm was measured by a multi-detection micro plate reader (HynergyTM HT; Bio-Tek, Winooski, USA). All experiments were performed in triplicate ($n = 3$).

### 2.6. Cell cycle and apoptosis analysis

Cell cycle and apoptosis analysis were carried out in order to detect the effect of miR-494 on dMSCs'. Briefly for cell cycle, 48 h after transfection, cells were fixed in cold 70% ethanol for incubation overnight at 4 °C. After washed with PBS twice, cells were incubated with 50 μg/mL propidium iodide (PI) and 20μg/mL RNase A for 30 min at room temperature and were detected by FACs. Analysis was performed with ModFit 3.0 software. For apoptosis assay, 48 h after transfection, cells and their supernatants were harvested and washed with PBS. Annexin V was added to the suspended cells and incubated at 4 °C for 15 min in the dark. Then PI was added for incubation for 5 min in the dark. Cells without addition of Annexin V were used as a negative control. And cells were detected by FACs. Analysis was performed with Cell Quest software (BD Biosciences). All experiments were repeated ($n = 5$).

### 2.7. Proliferation analysis

Proliferation analysis was performed to test whether miR-494 had an effect on dMSCs. dMSCs were pre-treated with or without 5,6-carboxyfluorescein diacetate, succinimidyl ester (CFSE). After 72 h transfection, dMSCs were harvested and washed with PBS to be detected by FACs. Proliferation index analysis was performed with ModFit 3.0 software. The proliferation index, a statistic generated by ModFit that relates to the number of population doublings the dMSCs had undergone following CFSE loading, was used [32,33]. All experiments were performed in triplicate ($n = 3$).

### 2.8. Total RNA isolation and q-PCR analysis

Briefly, total RNA, including miRNAs, was extracted using Trizol reagent (Invitrogen). The concentration of RNA was measured using a SmartSpecTM Plus spectrophotometer (Bio-Rad, Hercules, CA, USA). Then 1 μg total RNA was reverse-transcribed into cDNA using reverse transcriptase, reverse transcriptase buffer, dNTPs, RNase inhibitor and OligodT in the Thermoscript (TaKaRa). The cDNA obtained was used for real-time quantitative PCR (q-PCR) by using an Applied Bio-Systems step-one detection system with SYBR green dye (Invitrogen). For relative quantification of the mRNA expression, the expression of tubulin was used as an endogenous control [34]. The method to quantify mature miRNAs was performed by stem-loop RT-PCR. 1 μg miRNA and primers were put at 65 °C for 5 min to form highly target-specific