Original Paper

MiR-495 Promotes Senescence of Mesenchymal Stem Cells by Targeting Bmi-1

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Key Words

MSC • Preeclampsia • MiR-495 • Senescence • Bmi-1

Abstract

Background/Aims: Mesenchymal stem cells (MSCs) play an important role in regulating angiogenesis and immune balance. Abnormal proliferation and function of MSCs were reported at maternal fetal interface in patients with pre-eclampsia (PE). Micro-RNA-495 was known to be upregulated in the MSCs derived from patients with PE. However, it is not clear whether the up-regulated miR-495 is related to the pathogenesis of PE. **Methods:** We analyzed the expression of miR-495 in MSCs and umbilical cords derived from healthy pregnancies (NC) and PE, then we upregulated or downregulated the expression of miR-495 in MSCs derived from NC and tested the proliferation, apoptosis, migration, invasion, tube formation and senescence. **Results:** In the current study, we found that the expression of miR-495 was significantly increased in both umbilical cord tissues and MSCs in patients with severe PE. Overexpressing miR-495 arrested cell cycle in S phase and promoted cell apoptosis. The supernatants from miR-495-overexpressed-MSCs inhibited the migration of MSCs and HTR-8/SVneo, invasion of HTR-8/SVneo and tube formation of HUVEC, while si-miR-495 had the opposite effects. Furthermore, we analyzed the senescence related β -galactosidase activity and CD146 and found that miR-495 induced the senescence of MSCs. Molecular mechanism studies confirmed that Bmi-1 mediated these effects of miR-495 on MSCs. **Conclusion:** Taken together, our data demonstrated that miR-495 induced senescence of MSCs may be involved in the pathogenesis of PE.

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Introduction

Pre-eclampsia (PE) is a disease of pregnancy characterized by hypertension and proteinuria, developing after 20 weeks of gestation. It has been estimated that 5%–7% of pregnancies worldwide are complicated by this disorder, resulting in a very large disease

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burden [1]. Although much research has been devoted toward this topic, its pathogenesis is still incompletely understood yet [2-5].

Maternal-fetal interface is an important source of mesenchymal stem cells (MSCs) [6]. MSCs are a set of cells capable of self-renewal, expansion and multi-lineage differentiation. Previous studies have found that both umbilical cord and decidua derived MSCs were abnormal in PE, showing impaired proliferation, angiogenesis and immunoregulation [7, 8]. Interestingly, when we used MSCs to treat Th-1-induced PE-like mice, the PE symptoms were ameliorated [9]. Fu also reported that human umbilical cord-derived MSCs could release the PE-like symptoms of endotoxin-induced rat model through reducing the levels of pro-inflammatory TNF α and IL-1 β and increasing the levels of anti-inflammatory IL-10 [10]. Additionally, *in vitro* experiments showed that MSCs from PE inhibited angiogenesis through reducing VEGF [7, 11, 12] and modulate the immune response through keeping the balance of pro-inflammatory and anti-inflammatory factors [11, 13-15]. These results indicate that maternal-fetal interface derived MSCs play an important role in the pathogenesis of PE. However, the reasons leading to the abnormal growth and function of MSCs are still unclear.

Aberrant senescence in the placenta may contribute to PE [16]. It was reported that early onset preeclampsia is associated with placental aging [17]. Trophoblasts and in cord blood cells from pregnancies complicated with PE showed increased senescence [18]. It is unknown whether MSCs are senescent in PE. However, it was reported that human umbilical cord-derived MSCs have anti-senescence ability [19, 20]. Additionally, in the senescent state, MSCs show impaired proliferation and differentiation capacity [21, 22]. Therefore, we speculated that aberrant senescence may contribute to abnormal growth and function of MSCs in PE.

MicroRNAs (miRNAs) play important roles in the regulation of cellular senescence [23, 24] and the pathogenesis of PE [25]. MiR-495 was reported as an anti-tumor miRNA which could inhibit cell migration, invasion and proliferation, thus to play negative roles in the carcinogenesis [26-29]. What's more, miR-495 was reported to suppress breast cancer cell proliferation and tumorigenicity via G1-S arrest through targeting the 3'UTR of Bmi-1 [30]. In our previous study, we reported that miR-495 was highly expressed in MSCs [31]. But the role of miR-495 plays in senescence regulation hasn't been reported yet.

In the current study, we aimed to investigate the possible roles of miR-495 in the pathophysiology of PE and its functions in MSCs. We tested the expression of miR-495 in umbilical cords and MSCs derived from healthy donors and PE patients and we found that miR-495 was highly expressed in PE. Furthermore, we found that miR-495 inhibited the proliferation, migration and angiogenesis of MSCs and promoted cell apoptosis and aging through targeting Bmi-1.

Materials and Methods

Umbilical cord collection

PE was defined as gestational hypertension (systolic pressure > 140 mmHg or diastolic blood pressure > 90 mmHg on two or more occasions after gestational week 20) with proteinuria (> 0.3g/day). Umbilical cord tissues were obtained from healthy pregnancies (NC) ($n = 24$) and PE pregnancies ($n = 24$) who underwent caesarean section in Drum Tower Hospital from Sep 2015 to Apr 2016. This study had got approved by the Nanjing Drum Tower Hospital Ethics-Committee and all healthy controls and patients had written consent. Multiple gestations and the presence of maternal chronic hypertension, the HELLP syndrome, chronic nephritis, gestational diabetes mellitus, hepatic disease, *in vitro* fertilization and embryo transfer (IVF-ET) or other infectious and neoplastic disease and fetal congenital defect were excluded. The relevant clinical characteristics of the patients are presented in Table 1 and 2.

MSCs isolation and culture

MSCs were isolated from umbilical cord tissues within 4 hours as we reported previously in our laboratory. MSCs were obtained from healthy pregnancies (NC) ($n = 6$) and PE pregnancies ($n = 8$). Umbilical

Table 1. The clinical characteristics of umbilical cords. Abbreviations: PE, preeclampsia; NS, not significant

Parameter	PE (n = 24)	Control (n = 24)	P value
Age, years	29.5 ± 0.9	28.9 ± 0.5	NS
Primiparae(n)	14 (58.3%)	15 (62.5%)	NS
Gestational age at delivery, weeks	37.8 ± 0.1	38.5 ± 0.2	NS
Body mass index (kg/m ²)	29.3 ± 0.9	27.8 ± 3.1	NS
Systolic blood pressure (mmHg)	158.7 ± 9.1	110.2 ± 4.7	< 0.05
Diastolic blood pressure (mmHg)	117.5 ± 3.4	77.4 ± 5.2	< 0.05
Proteinuria (mg/24h)	1702.6 ± 40.25	0	< 0.05
Blood urea nitrogen (mmol/l)	3.6 ± 0.7	3.4 ± 0.5	NS
Alanine aminotransferase (units/l)	29.7 ± 3.9	30.0 ± 8.3	NS
Placenta weight (g)	579.4 ± 40.2	575.2 ± 37.8	NS
Platelets (n)	(179.1 ± 40.3) × 10 ⁹	(180.4 ± 30.9) × 10 ⁹	NS
Birth weight (g)	2907.8 ± 206.0	3182.2 ± 103.2	NS

Table 2. The clinical characteristics of MSCs. Abbreviations: PE, preeclampsia; NS, not significant

Parameter	PE (n = 8)	Control (n = 6)	P value
Age, years	29.6 ± 0.2	28.7 ± 0.9	NS
Primiparae(n)	4 (50.0%)	4 (66.6%)	NS
Gestational age at delivery, weeks	37.7 ± 0.8	38.4 ± 0.7	NS
Body mass index (kg/m ²)	29.1 ± 0.3	28.2 ± 2.8	NS
Systolic blood pressure (mmHg)	166.8 ± 10.6	105.7 ± 6.8	< 0.05
Diastolic blood pressure (mmHg)	114.9 ± 4.2	78.2 ± 8.4	< 0.05
Proteinuria (mg/24h)	1802.6 ± 39.2	0	< 0.05
Blood urea nitrogen (mmol/l)	4.1 ± 0.2	3.8 ± 0.2	NS
Alanine aminotransferase (units/l)	31.5 ± 3.2	30.8 ± 6.6	NS
Placenta weight (g)	604.2 ± 28.9	592.3 ± 32.9	NS
Platelets (n)	(167.3 ± 39.5) × 10 ⁹	(170.9 ± 29.6) × 10 ⁹	NS
Birth weight (g)	2794.2 ± 328.5	2867.3 ± 97.4	NS

cord tissues were washed with PBS several times until no blood can be seen and the two umbilical arteries and the umbilical vein were dissected before tearing up and cutting the tissues to pieces in PBS. Then the tissues were incubated in an enzyme cocktail for 3h with gentle agitation at 37°C. The digestion mixture was washed with PBS and then washed with Dulbecco's modified Eagle's medium/F12 (DF-12, Gibco, Grand Island, NY, USA). Then the mixture was suspended in fresh DF12 supplemented with 20% fetal bovine serum (FBS) containing 100 IU/mL antibiotics and incubated at 37°C in a 5% CO₂ saturating humidified atmosphere. After two days of incubation, remove the small residues not attached and add fresh complete medium gently. The medium was replaced two times every week. The culture continued until the cells cover the whole plate then cells were detached using 0.25% trypsin/EDTA to transfer to a new culture plate.

The specific phenotypic surface antigens of MSCs were characterized by flow cytometry (BD, FACS Calibur) assay after the 2nd to 4th cell passages. As was reported before, the adherent, fibroblast-like cells were presented as CD105+, CD73+, CD90+, CD44+, CD29+, HLA-DR-, CD31-, CD14-, CD19-, CD106-, CD11b- and CD45-[32]. We also tested the expression of CD146, which was reported to be a marker of MSCs [33, 34], after transfecting si-NC, si-495, mic-NC, miR-495.

Transient transfection

Overexpression of miR-495 in MSCs was achieved by transfecting cells with miR-495 mimic (mic-495) and miR-495 mimic negative control (mic-NC) (RIBO BIO, Guangzhou, China) using lipofectimine-2000 reagent (Invitrogen) until MSCs reached 30–50% confluence and represented a logarithmic growth. A sequence of small interfering RNAs for miR-495 (si-495) and interfering negative control (si-NC) were ordered from RIBO BIO. After transfection for 48 hours, cells and the supernatants were harvested for the following experiments.

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% CO₂.

Cell proliferation, cell cycle and apoptosis assays

A CCK-8 kit (DojinDo) was used to detect the effect of miR-495 on the viability of MSCs. A cell counting kit was used to achieve a qualitative index of cell viability after transfection in our experiments. After transfection for 48 hours, CCK-8 was separately added to each well and incubation for another 3 hours. An OD absorbance at 450 nm was measured by a multi-detection micro plate reader (Bio-Tek). For the cell cycle experiments, the treated cells were harvested, washed once with PBS and fixed in 70% ethanol overnight. Staining of the DNA content was performed with 50 mg/ml PI, 1 mg/ml RNase A and 0.2% Triton-X100 for 30 min. Analysis was performed with MFLT32 software. For apoptosis assay, 48 hours after transfection, cells and their supernatants were harvested, centrifuged and washed with PBS. Then cells were resuspended with 200ul PBS, stained with 5ul Annexin V and incubated for 15 minutes in the dark. Then PI was added (final concentration was 1 µg/ml) and incubated for another 5 minutes in the dark. Cells were detected by FACs Calibur. Analysis was performed with FlowJo-V10 (BD Biosciences).

Quantitative RT-PCR analysis

Total RNA, including miRNA, was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The concentration of RNA was measured using a SmartSpec™ Plus spectrophotometer (Bio-Rad, Hercules, CA, USA). 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 GAPDH was used as an endogenous control. The method to quantify mature miRNAs was performed by stem-loop RT-PCR. 1µg RNA and primers were put at 65°C for 5 minutes to form highly target-specific stem-loop structure, then reverse transcriptase, RNase inhibitor, dNTPs and 5x buffers were added for reverse transcription. The whole procedure was performed on ice. Besides, the miRNAs amplification was performed by using an Applied Bio-Systems step-one detection system with SYBR green dye (Invitrogen). For relative quantification, the expression of U6 snRNA (Applied Biosystems) was used as an endogenous control. All experiments were performed in triplicate (n = 3). Relative expression was performed using the DDCT method.

Western blot analysis

Whole cell lysate for western blotting were extracted with lysis buffer containing 50 mM Tris (pH 8), HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 0.1% Triton X-100, 0.25% sodium deoxycholate and protease inhibitor (Roche). Lysates (50 µg) were resolved by 12% SDS/PAGE (Bio-Rad), and gels were transferred to poly (vinylidene difluoride) membranes (Roche). Membranes were blocked using 5% Bovine Serum Albumin (BSA) for 2 hours at room temperature, and subsequently incubated overnight at 4°C with diluted primary antibodies against Bmi-1 and GAPDH (1:1000 dilution) (Millipore, Billerica, MA). Signals were detected using the appropriate horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology). The blots were visualized using an enhanced Immobilon Western chemiluminescent horseradish peroxidase (HRP) substrate (Millipore, Billerica, MA), according to the manufacturer's instructions.

HTR-8/SVneo invasion assay

The transwell invasion experiments were performed using transwell membrane filters (8 μ m size pore, Millipore). Before the experiments, 80 μ l Matrigel (BD) was added to the upper chamber and incubated at 37°C till the Matrigel coagulated. Then supernatants were added to the lower chamber, and 100 μ l HTR-8/SVneo cells were seeded in the upper chamber at a density of 2×10^5 /ml. After 24 hours' incubation, non-migrating cells on the upper surface of the membrane were removed with a cotton swab. The migrated cells on the lower surface of the membranes were fixed with 4% PFA for 30 min at room temperature, stained with crystal violet solution for 20 min. After washed with distilled water, ten randomly chosen fields were imaged and quantified by blind counting of the migrated cells of ten fields per chamber. All experiments were performed in triplicate (n = 3).

MSC and HTR-8/SVneo migration assay

After 48 hours' transfection, MSCs were scratched with 1ml pipett and washed with PBS to remove the cell debris. Then MSCs were incubated in fresh medium containing 10% FBS. HTR-8/SVneo cells were also scratched as described above and incubated in fresh RPMI-1640 containing 10% FBS and 50% MSC supernatant. Pictures were taken under a microscope at 40 $\times$ magnification and the results were analyzed using ImageJ Launcher.

HUVEC capillary tube and network formation assay on Matrigel

Matrigel (BD Biosciences, San Jose, CA, USA) (100 μ L) was added to 48-well plates and incubated for 40 minutes for gelling. HUVEC (8×10^3) were added to the pre-solidified Matrigel together with equal volume of conditioned MSC supernatants. The cells started the process of forming capillary tubes and networks once seeded on Matrigel. Incubation for 8 hours revealed the most pronounced difference between treated groups in terms of capillary tube and network morphology. Tube-like structures were defined as endothelial cord formations that were connected at both ends, and the mean tube length in five random fields per well was quantified. These results were analyzed using ImageJ Launcher.

β -galactosidase activity assay

Senescent cells were assessed by staining for β -galactosidase activity using MSCs at passage 3. 48 hours after transfection with mic-NC, mic-495, si-NC or si-495, β -galactosidase activity of MSCs was tested using a Senescence β -galactosidase Staining Kit (Beyotime Biotechnology) according to the manufacturers' instructions. Briefly, MSCs were seeded in 12-well plates and after the corresponding treatments. And MSCs were washed twice with PBS and fixed with Fixative Solution for 15 min at room temperature. Then, the MSCs were stained with 1 ml complete β -galactosidase staining solution per well overnight at 37°C. The complete β -galactosidase staining solution contained Solution A, Solution B, Staining Solution C and X-gal solution (final concentration of 1 mg/ml). After cultured at 37°C without CO₂, the cells were washed twice with PBS and pictures were taken under a microscope at 100 $\times$ magnification. Ten randomly chosen fields were imaged and quantified by blind counting of the positive cells of ten fields per chamber. All experiments were performed in triplicate (n = 3).

Dual-luciferase assays

For luciferase assays, MSCs were pre-seeded into 12-well plates 1 day before transfection. Then the firefly luciferase plasmid of Bmi-1 3' untranslated region (UTR) and mutated plasmid of Bmi-1 3' UTR (mut), constructed by GENeRay, as well as si-495, mic-495 or negative control, were co-transfected into each well. 48 hours post-transfection, cell lysates were prepared using reporter lysis buffer (Promega). Luciferase activity was analyzed using a luciferase assay kit (Promega) with GloMaxTM96 Microplate Luminometer w/Dual Injectors (E6521) according to the manufacturers' instructions.

Statistical analysis

All experiments were performed at least three times and data are expressed as mean $\pm$ S.E.M. Graphpad prism version 5.01 was used to perform graphics and the two-tailed student's t-test was used to compare statistical significance. $P < 0.05$ was set as a statistical significance.

Results

MiR-495 was highly expressed in umbilical cords and MSCs derived from patients with PE

Our previous study showed that miR-495 was highly expressed in MSCs derived from decidua tissues of PE patients [31]. In the current study, we tested the expression of miR-495 in umbilical cord tissues and MSCs obtained from umbilical cords. The results showed that miR-495 was highly expressed both in umbilical cords (Fig. 1A) and MSCs (Fig. 1B) derived from PE compared with healthy donors (NC).

MiR-495 could not change the phenotype of MSCs

As miR-495 was highly expressed in MSCs derived from PE compared with NC, we wondered whether miR-495 was involved in the pathology of PE. MiR-495 mimic (mic-495), miR-495 mimic negative control (mic-NC), interfering RNAs from miR-495 (si-495) and interfering negative control (si-NC) were transfected into MSCs using lipofectimine-2000 reagent. After 48h, cells and supernatant were collected and tested. The mic-495 could overexpress miR-495 (Fig. 2A) and the miR-495 interfering RNA could downregulate miR-495 significantly (Fig. 2B).

To test whether miR-495 changed the phenotype of MSCs, we analyzed the expression of CD90, CD44, CD105, CD73, CD29, CD31, CD45, HLA-DR, CD14, CD19, CD106 and CD11b after transfection with miR-495 mimic and interfering RNA. The results showed that these antigens on the surface of MSCs were not changed significantly with the alteration of miR-495 levels (Fig. 2C and 2D).

MiR-495 inhibits cell proliferation and induces apoptosis of MSCs

As miR-495 was highly expressed in PE, we wondered whether miR-495 could affect the growth of MSCs. We tested the cell viability after transfection with mic-495, mic-NC, si-495 and si-NC into MSCs. The results of CCK-8 assay showed that overexpressing miR-495 inhibit cell viability of MSCs while si-495 promoted it (Fig. 3A). To figure out how miR-495 inhibits cell viability, we next analyzed the effects of miR-495 on cell cycle and apoptosis. The cell cycle analysis showed that overexpressed-miR-495 could arrest the cell in S phase (11.56 ± 0.2685 mic-NC and 14.79 ± 0.3883 in miR-495, $p = 0.0024$) while decreased the G0/G1 phase (83.13 ± 0.3500 mic-NC and 79.85 ± 0.6454 in miR-495, $p = 0.0110$) compared with control (Fig. 3B and 3C). The apoptosis test showed that si-495 inhibited cell apoptosis (8.230 ± 0.2184 in si-NC and 6.702 ± 0.3641 in si-495, $P = 0.0049$) (Fig. 3D and 3E) while overexpressing miR-495 promoted cell apoptosis (8.022 ± 0.3164 in mic-NC and 15.08 ± 1.298 in miR-495, $P = 0.0004$) (Fig. 3F and 3G).

MiR-495 inhibits cell migration and invasion

Sufficient trophoblast invasion plays an important role in the formation of placenta and successful pregnancy and insufficient trophoblast invasion is regarded as one of the causes for PE. As described above, miR-495 could inhibit cell proliferation and promote cell apoptosis, so we wondered whether miR-495 had effects on the migration and invasion

Fig. 1. MiR-495 was highly-expressed both in umbilical cord tissues and MSCs in patients with severe PE. (A) The relative expression of miR-495 in the umbilical cord tissues from healthy donors (NC, N = 24) and PE patients (N = 24). (B) The relative expression of miR-495 in MSCs derived from NC (N = 6) and PE patients (N = 8). Values are means $\pm$ S.E.M. * $P < 0.05$.

Fig. 2. MiR-495 could not change the phenotype of MSCs. After transfected the M-NC or M plasmid, the expression of MALAT1 was tested on mRNA level. (B) After transfected si-NC or si-M, the expression of MALAT1 was tested on mRNA level. (C and D) Expression of surface antigens, CD90, CD44, CD105, CD73, CD29, CD31, CD45, HLA-DR, CD14, CD19, CD106 and CD11b was detected using flow cytometry after transfected with si-NC, si-495, mic-NC or mic-495.

of MSCs and trophoblast cells. After changing the expression of miR-495, we tested the migration of MSCs and HTR-8/SVneo cells through scratching with a pipette. The results showed that miR-495-overexpressed MSCs migrated much more slowly (Fig. 4A and 4B) while miR-495-downregulated MSCs migrated faster (Fig. 4C and 4D) compared with

Fig. 3. MiR-495 inhibits cell proliferation and promotes cell apoptosis. (A) CCK8 test was performed after transfection of si-NC, si-495, mic-NC or mic-495 into MSCs. After transfection with mic-NC or mic-495 for 48 hours, cells were subsequently assayed for DNA content by flow cytometry. Images are shown on (B) for cell cycle distribution, and a statistical analysis shown on (C) for percentage of the cells in different phases of cell cycle. (D and E) Apoptosis of MSCs transfected with si-NC or si-495 for 48 hours was detected using flow cytometry. (F and G) Apoptosis of MSCs transfected with mic-NC or mic-495 for 48 hours was detected using flow cytometry. All results are from three independent experiments. Values are means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

control group both at 6 hours and 12 hours. The migration experiments of HTR-8/SVneo cells also showed that cells migrated slower when co-cultured with supernatants of miR-495-overexpressed MSCs than cells co-cultured with supernatants of mic-NC-transfected-MSCs (Fig. 4E and 4F). Besides, HTR-8/SVneo cells migrated faster when co-cultured with supernatants of miR-495-downregulated-MSCs than cells co-cultured with supernatants of si-NC-transfected-MSCs (Fig. 4G and 4H).

The transwell experiments also showed that supernatants of miR-495 overexpressed-MSCs decreased the invasion of HTR-8/SVneo cells (165.5 ± 1.500 in mic-NC and 55.50 ± 5.500 in miR-495, $P = 0.0027$) (Fig. 5A and 5B) while si-495 increased the invasion of HTR-8/SVneo compared with control groups (168.5 ± 7.500 in si-NC and 251.5 ± 8.500 in si-495, $P = 0.0181$) (Fig. 5C and 5D).

MiR-495 inhibits HUVEC capillary formation in vitro

Insufficiency of angiogenesis is regarded one of the causes of PE. We wondered if miR-495 could affect the regulatory roles of MSCs in angiogenesis. We performed the tube formation analysis on Matrigel through co-culturing HUVEC with supernatants of MSCs after changing the expression of miR-495 in MSCs for 48 hours. After co-culturing for 8 hours,

Fig. 4. Supernatants from miR-495-over-expressed-MSCs promote the migration of MSCs and invasion of HTR-8/SVneo. (A) 48 hours after transfection of mic-NC or mic-495 into MSCs, a scratch was performed using a 1 ml pipette and pictures were taken using a microscope at 40× magnification after 0 hour, 6 hours and 12 hours. (B) The migration pictures of mic-NC and mic-495 groups was analyzed. (C) 48 hours after transfection of si-NC or si-495 into MSCs, a scratch was performed using a 1 ml pipette and pictures were taken using a microscope at 40× magnification after 0 hour, 6 hours and 12 hours. (D) The migration pictures of si-NC and si-495 groups was analyzed. (E) A scratch was performed

using a 1 ml pipette and then HTR-8/SVneo cells was co-cultured with supernatants of MSCs overexpressed miR-495 and pictures were taken using a microscope at 40× magnification after 0 hour, 12 hours and 24 hours. (F) The migration pictures of si-NC and si-495 groups was analyzed. (G) A scratch was performed using a 1 ml pipette and then HTR-8/SVneo cells was co-cultured with supernatants of MSCs downregulating-miR-495 and pictures were taken using a microscope at 40× magnification after 0 hour, 12 hours and 24 hours. (H) The migration pictures of si-NC and si-495 groups was analyzed. All results are from three independent experiments. Values are means ± S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

tubes was pictured and analyzed. The results showed that the supernatants of miR-49-overexpressed MSCs could inhibit HUVEC capillary formation and downregulated-miR-495 could promote tube formation (Fig. 5E). The relative tubes length of tube-like capillary (% control) was 1.000 ± 0.05614 in mic-NC group and 0.7485 ± 0.08279 in miR-495 group ($P = 0.0107$) (Fig. 5F), 1.000 ± 0.09101 in si-NC group and 1.524 ± 0.1145 in si-495 group ($P = 0.0014$).

Fig. 5. Supernatants from miR-495-overexpressed-MSCs inhibited the invasion of HTR-8/SVneo and tube formation of HUVEC. (A) Analysis of migrated HTR-8/SVneo cells treated with supernatants from miR-495-overexpressed-MSCs. The number of migrated cells was quantified using a microscope at 100× magnification after 24 hours. A statistical analysis of the migration experiments was shown on B. (C) Analysis of migrated HTR-8/SVneo cells treated with supernatants from miR-495-downregulated-MSCs. The number of migrated cells was quantified using a microscope at 100× magnification after 24 hours. A statistical analysis of the migration experiments was shown on D. (E) HUVEC was treated with supernatant from MSCs transfected with mic-495, mic-NC, si-NC and si-495. The tubes were photographed under a microscope at 40× magnification after 8 hours. (F) A statistical analysis of tube formation was shown. All results are from three independent experiments. Values are means ± S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

MiR-495 promotes cell senescence

Normal MSCs in fetal-maternal interface play important roles in achieving successful pregnancies. We wonder whether miR-495 could affect the survival of MSCs. After changing the expression of miR-495 for 48 hours, we tested the activity of senile related β -galactosidase using a Senescence β -galactosidase Staining Kit. The results showed that miR-495-overexpressed MSCs showed more blue-staining whereas the miR-495-downregulated MSCs showed little β -galactosidase staining (Fig. 6A and 6B) compared with negative controls, indicating that miR-495 could promote the senescence of MSCs.

Previous studies reported that CD146 was lowly expressed in senescent MSCs [33, 34], so we tested the expression of CD146 on MSCs surface after transfecting mic-NC or mic-495 for 48 hours using FACs Calibur both in passage 3 and passage 6. The results showed that MSCs overexpressing miR-495 expressed less CD146 compared with mic-NC (Fig. 6C and 6D).

Besides, P16, P53 and P21 were reported closely related with cell senescence [35, 36]. So we tested the expression of P16, P53 and P21 after changing the expression of miR-495. The results showed that P16, P53 and P21 were increased in miR-495-overexpressed MSCs (Fig. 6E-6G).

Bmi-1 mediates the effects of miR-495 on MSCs

Bmi-1 was reported one of the genes inhibit cell senescence [37, 38] and inhibiting the expression of Bmi-1 could promote cell senescence [39]. Besides, Bmi-1 was a putative target of miR-495 and, so we wondered whether miR-495 correlated with Bmi-1 in MSCs. Then we tested the expression of Bmi-1 and the results showed that Bmi-1 was lowly expressed in umbilical cords derived from PE compared with NC (Fig. 7A). Although there was no significance between NC and PE in MSCs, there was a trend that Bmi-1 was lowly expressed

Fig. 6. MiR-495 could promote cell senescence. (A) β -galactosidase activity was tested after changing the expression of miR-495 in MSCs. After staining for 24 hours, pictures were taken under a microscope at 40 $\times$ magnification and quantitation was achieved by determining the percentage of SA β -gal-positive cells (B). (C) CD146 expression on the surface of MSCs was tested after overexpressing miR-495 using passage 3 MSCs. (D) CD146 expression on the surface of MSCs was tested after overexpressing miR-495 using passage 6 MSCs. (E) P53 expression was tested after overexpressing miR-495 on mRNA level. (F) P21 expression was tested after overexpressing miR-495 on mRNA level. (G) P16 expression was tested after overexpressing miR-495 on mRNA level. All results are from three independent experiments. Values are means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

in MSCs derived from PE (Fig. 7B) compared with MSCs derived from NC. Correlation analysis showed there was a linear positive correlation between miR-495 and Bmi-1 ($R^2 = 0.1503$, $P = 0.0065$ in umbilical cord tissues and $R^2 = 0.3511$, $P = 0.0255$ in MSCs) (Fig. 7C and 7D). Besides, the expression of Bmi-1 was decreased after overexpressing miR-495 in MSCs both at mRNA level (Fig. 7E) and protein level (Fig. 7F). Although there was no significance after silencing miR-495 at mRNA level, there was a trend that Bmi-1 was upregulated by silencing miR-495 at mRNA level (Fig. 7E) and significantly upregulated at protein level (Fig. 7F).

Fig. 7. Bmi-1 was downregulated in the presence of miR-495. (A and B) The relative Bmi-1 expression in umbilical cord tissues and MSCs derived from NC and PE patients. (C and D) There was a negative linear correlation between miR-495 and Bmi-1 both in umbilical cords ($R^2 = 0.1503$, $P = 0.0065$) and MSCs ($R^2 = 0.3511$, $P = 0.0255$). After 48 hours of transfection with mic-495, mic-NC, si-NC or si-495, total RNAs, protein of MSCs were collected respectively and Q-PCR (E) and western blotting (F) were performed to detect the levels of Bmi-1. (G) There are two regions on Bmi-1 mRNA miR-495 can bind. (H) Analysis of luciferase intensity in cells co-transfected with mic-NC or miR-495 and plasmid containing Bmi-1-mut or the 3'UTR of Bmi-1. All results are from three independent experiments. Values are means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

There were two regions on the Bmi-1 mRNA miR-495 could bind (Fig. 7G). Then the dual-luciferase assay was performed to confirm the relationship between miR-495 and Bmi-1. As is shown in Fig. 7H, the relative luciferase intensity was significantly decreased in cells co-transfected with miR-495 and reporter plasmid containing the 3' untranslated regions (3' UTR) of Bmi-1 for 48h compared with that co-transfected with miR-495 and plasmid containing Bmi-1-mut and mic-NC group, indicating that miR-495 could inhibit the expression of Bmi-1 through targeting the 3'UTR of Bmi-1.

Discussion

The umbilical cord is an important source of MSCs. Aberrant levels of cytokines were observed in MSCs from patients with PE and abnormal MSCs are associated with the origin of preeclampsia development [40]. These findings suggest that abnormal MSCs may contribute to PE development. In our previous study, we found that miR-495 was one of

the highly elevated miRNA in decidua-derived MSCs from PE patients [31]. Therefore, we speculated that miR-495 in MSCs may be involved in the pathogenesis of PE. In the present study, we found that overexpressed miR-495 inhibited cell proliferation, migration, invasion and angiogenesis. Besides, overexpressed miR-495 could also promote cell apoptosis and senescence through decreasing the production of Bmi-1. These findings suggest that miR-495 may be involved in the pathogenesis of PE.

Cellular senescence was reported to be triggered by the gradual accumulation of DNA damage and epigenetic alterations that can directly affect the expression of senescence-associated genes [41]. Because of incomplete and erratic DNA replication, irreversible changes take place in the nuclei of senescent cells, which can limit the cell division and induce cell senescence [42-44]. Senescence can be induced through activating various signaling pathways, such as P53, NF- κ B, PI3K/AKT, ERK/JNK/P38-MAPK [45].

Bmi-1 (B cell-specific Moloney murine leukemia virus integration site 1), a member of the polycomb family of transcriptional repressors, was initially identified as a c-myc cooperating oncogene in the induction of B-cell lymphoma [46, 47]. In mice, the absence of Bmi-1 expression results in neurological defects and severe proliferative defects in lymphoid cells, whereas Bmi-1 overexpression induces lymphomas [48, 49]. Besides, Bmi-1 was involved in cell cycle regulation, self-renewal of stem cells and cell senescence [50-54] and lacking Bmi-1 could inhibit cell proliferation and promote cell apoptosis [55, 56]. Bmi-1 was also reported to promote invasion and metastasis [57]. Bmi-1 could also promote glioma angiogenesis by activating NF- κ B signaling [58]. What's more, UCB-MSCs (umbilical cord blood derived MSCs) exhibited the typical senescent phenotype when knocked down Bmi-1 [59]. In our current study, we found that Bmi-1 was lowly expressed in the umbilical cord tissues and MSCs derived from PE compared with healthy pregnancies while miR-495 was highly expressed. What's more, there was a negative liner-relationship between Bmi-1 and miR-495.

Previous studies have shown that miR-495 was broadly involved in a variety of cancers and it plays critical roles in distinct cancer hallmark capabilities to contribute to cancer, such as non-small cell lung cancer (NSCLC), gastric cancer cells, prostate cancer cells and renal cancer cells [26, 60-63] through inhibiting proliferation, migration, invasion, angiogenesis and other ways. MiR-495 was also reported to inhibit chondrogenic differentiation of MSCs [64]. But whether miR-495 could affect the senescence of MSCs was still unknown. In our study, we first demonstrated that miR-495 was an important endogenous regulator in MSCs which could not only affect cell proliferation, migration, invasion and angiogenesis properties, but also inducing cell senescence of MSCs through targeting Bmi-1. Thus, miR-495 might be a promising target to cure PE.

Collectively, in our study, we work on the relationship between MSCs and miR-495, attempting to explain how the highly expressed microRNA plays the role in the development of PE. Overexpressed miR-495 inhibits the growth, migration, invasion and promoting cell senescence and apoptosis by decreasing the expression of Bmi-1. The present study reveals that miR-495 arrests cell cycle at S transition and affects MSCs' proliferation, migration and tube formation functions and may be a novel mechanism in the development of PE.

However, there are some defects and limitations in some respects. First, we reported that there were several miRNAs which were highly expressed in PE, and we just investigated the effects of miR-495 on MSCs and did not explore the combined effect of miR-495 with other changed microRNAs, there is need to study furthermore. Secondly, although miR-495 could affect some interesting phenomena, it is still unknown whether miR-495 is of importance to cause the symptom of PE or inhibition of miR-495 can reverse the development of PE symptom *in vivo*. It needs to be further explored. Thirdly, although the phenomena caused by changing miR-495 can be explained by Bmi-1, whether there are other targets miR-495 plays roles through is still unknown. Besides, after senescence induced by miR-495, what factors changed in the supernatants inhibits the migration, invasion etc. is also needs to be further investigated.

Conclusion

In conclusion, our findings suggest that miR-495 was highly expressed in PE and thus it can attenuates the proliferation, invasion, migration, tube formation ability of MSCs and promote MSCs apoptosis and senescence through downregulating Bmi-1. Therefore, miR-495 may be a potential target for PE treatment.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (81401223), Jiangsu Provincial Key Medical Talent (RC201670) and Six Talent Peaks Project in Jiangsu Province (2016-WSW-063).

We thank NovelBio Ltd., Co for the support of bioinformatics analysis with their NovelBrain Cloud Platform (www.novelbrain.com).

Disclosure Statement

The authors indicate no potential conflicts of interest.

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