Regulation of miR-92a on vascular endothelial aging via mediating Nrf2-KEAP1-ARE signal pathway

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Abstract. – OBJECTIVE: Various human aging-related diseases start with vascular aging, in which the aging of vascular endothelium is the first step to cause a structural and functional deficit of vascular endothelium, leading to vascular disorders. MicroRNA (miR) participates in various processes of body development and pathological processes via mediating cell proliferation, differentiation, and apoptosis. A previous study showed the correlation between cardiovascular disease and miR-92a, whose role and mechanism in vascular endothelial aging has not been reported.

MATERIALS AND METHODS: In vitro, cultured human umbilical vein endothelial cells (HUVECs) were prepared for the vascular endothelial aging model by using 10⁻⁶ M angiotensin II. MiR-92a expression was examined. After transfecting with the miR-92a inhibitor, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) assay was employed to describe cell proliferation, and the Caspase 3 activity assay kit was used to evaluate apoptosis activity. Myeloid peroxidase (MPO) and superoxide (SOD) activity, plus reactive oxygen species (ROS) content were measured. Nrf2, KEAP1 and ARE mRNA expressions were measured by real-time PCR. Nuclear factor erythroid 2 p45 related factor (Nrf2) protein level, inflammatory factors tumor necrosis factor α (TNF-α) and interleukin-2 (IL-2) were tested by western blot or enzyme-linked immunosorbent assay (ELISA).

RESULTS: In model group, miR-92a expression was elevated significantly compared to the control group (p < 0.05). MiR-92a inhibitor transfection facilitated cell proliferation, decreased Caspase 3 activity, ROS or MPO, expressions of TNF-α, IL-2 and KEAP1, and enhanced SOD level and Nrf2, ARE expressions significantly compared to the model group (p < 0.05).

CONCLUSIONS: In aged vascular endothelium, miR-92a was up-regulated. Through inhibiting miR-92a expression and regulating Nrf2-KEAP1-ARE signal pathway, the oxidative stress reaction or inflammation can be suppressed, thus inhibiting endothelial apoptosis and facilitating cell proliferation.

Key Words: Aging, Endothelial cells, microRNA-92a, Oxidative stress, Nrf2-KEAP1-ARE, Inflammation.

Introduction

The world is now in a transition into aged society, which brings increasing incidence of age-related diseases including cardiovascular disease, vascular dementia or Alzheimer’s disease, therefore, severely threatening public health and bringing heavy burdens for the whole world economy¹,². Aging is one of the most complicated biological processes in multi-cellular animals, as its detailed mechanism or counter-measures have not been fully elucidated yet³,⁴. A common knowledge is that aging is the result of accumulation and progression⁵. The occurrence of organ aging and related diseases frequently start from vascular aging, in which vascular endothelial aging occurs firstly, and can cause a structural/functional change of vascular endothelium, thus causing the occurrence of cardiovascular diseases⁶,⁷. As the critical factor for vascular aging, endothelial cell aging leads to increased cell apoptosis and arrested proliferation, further leading to vascular dysfunction⁸. Therefore, initiating step of cardiovascular disease is endothelial cell aging⁹. The regulation of vascular endothelia cells thus effectively affects the aging process.

Various factors regulate aging of vascular endothelium, including inflammatory response, oxidative stress response and angiotensin II schools⁹,¹¹. MiR as one type of non-coding RNA, can regulate the protein-coding gene, and participate in various body development and pathological processes, thus mediating cell proliferation, differentiation, and apoptosis¹². Abundant miR
molecules are expressed in the vascular system, thus may participating in occurrence and progression of vascular systems, reflecting the sensitivity of vessels to injury. MiR can regulate vascular endothelial injury and repair via modulating oxidative stress injury. A previous study reported the correlation between miR-92a and cardiovascular disease. The role and related mechanism of miR-92a in vascular endothelial aging have not been fully reported yet.

Materials and Methods

**Major Equipment and Reagents**

Human umbilical vein endothelial cells (HUVECs) were purchased from ATCC cell bank (Manassas, VA, USA). RPMI 1640 medium, fetal bovine serum (FBS) and streptomycin-penicillin were purchased from HyClone (Logan, UT, USA). Poly-lysine, dimethyl sulphoxide (DMSO) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) powders were purchased from Gibco BRL Co Ltd. (Grand Island, NY, USA). Trypsin-ethylene diamine tetraacetic acid (EDTA) lysis buffer was purchased from Sigma-Aldrich (St. Louis, MO, USA). Angiotensin II was purchased from Sigma-Aldrich (St. Louis, MO, USA). Caspase 3 activity assay kit was purchased from Pall Life Sciences Inc. (Pensacola, FL, US). RNA extraction kit, reverse transcription kit and lipo2000 transfection reagent were purchased from Invitrogen/Life Technologies (Carlsbad, CA, USA). polyvinylidene fluoride (PVDF) membrane was purchased from Pall Life Sciences Inc. (Pensacola, FL, US). The Western blotting reagent was purchased from Beyotime (Beijing, China). Enhanced chemiluminescence (ECL) reagent was purchased from Amersham Biosciences (Piscataway, NJ, USA). NRF2 (D1Z9C) XP rabbit anti-human nuclear factor erythroid 2 p45 related factor 2 (Nrf2) monoclonal antibody (Catalogue No. 12721; 1:3000), and goat anti-rabbit horse-radish peroxidase (HRP)-conjugated IgG secondary antibody (Catalogue No. 7074; 1:2000) were all purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). TaqMan microRNA reverse transcription kit was purchased from Thermo Electron Corp (Burlington, Ontario, Canada). ELISA kits for tumor necrosis factor α (TNF-α) and interleukin-2 (IL-2) were purchased from R&D Systems Inc. (Minneapolis, MN, USA). Myeloid peroxidase (MPO) activity kit and superoxidase (SOD) assay kit were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). MiR-92a inhibitor and negative control (NC) were synthesized by GenePharma (Shanghai, China). Other common reagents were purchased from Sangon (Shanghai, China). Labsystem Version 1.3.1 microplate reader was purchased from Bio-Rad Laboratories (Hercules, CA, USA). ABI7700 Fast fluorescent quantitative PCR cycler was purchased from ABI (Foster City, CA, USA). The ultrapure workstation was purchased from Sutai High-tech Materials Co. Ltd. (Shanghai, China).

**HUVECs Culture and Grouping**

HUVECs stored in liquid nitrogen were resuscitated in 37°C water-bath until fully thawing. Cells were centrifuged at 1000 r/min for 3 min, and were re-suspended in 1 ml fresh medium and were removed into 25 cm² culture flask containing 2 ml fresh culture medium. Cells were kept in a humidified chamber with 5% CO₂ at 37°C for 24-48 h. Cells were seeded in a poly-lysine pre-treated dish at 1×10⁶ per cm². The culture medium contained 10% FBS, and 90% high-glucose Dulbecco’s modified eagle medium (DMEM) medium (containing 100 U/ml penicillin, 100 μg/ml streptomycin). Cells were kept in a humidified chamber with 5% CO₂ at 37°C. Cells at log-phase with 3rd to 8th generation were randomly divided into control group, model group, negative control (NC) group and miR-92a inhibitor group. The model group was prepared for the vascular endothelial aging model by 10⁻⁶ mM angiotensin II. NC and miR-92a inhibitor groups received miR-92a NC or miR-92a inhibitor transfection on model cells.

**Vascular Endothelial Aging Model Preparation and miR-92a Inhibitor Transfection**

Log-growth phased cells were inoculated into 24-well plate at 2.5×10⁴ per well density. Each well was filled with 1 ml normal full medium, which was changed to serum-free medium when cells reached sub-confluence. After 12 h incubation, cells were synchronized. The original medium was discarded and changed for medium containing 10⁻⁶ mM angiotensin II, which was further replenished at 12 h and 24 h. After 48 h continuous stimulus, the model was prepared. MiR-92a inhibitor (5‘-AUCGG GUCGU GAGUU C-3’) or miR-92a NC (5‘-AAUCG
AUUUC GUGCA G-3′) oligonucleotides were transfected into cells with 70%–80% confluence. In brief, miR-92a inhibitor or NC liposome were mixed with 200 μl serum-free medium for 15-min room temperature incubation. Lipo2000 reagent was then mixed with miR-92a inhibitor or NC dilutions for 30-min room temperature incubation. Serum was removed, followed by phosphate-buffered saline (PBS) rinsing gently and the addition of 1.6 ml serum-free culture medium. Cells were then kept in a humidified chamber with 5% CO₂ at 37°C for 6 h, followed by the application of serum-containing medium in 48 h continuous incubation for further experiments.

**Real-time PCR for miR-92a, Nrf2, KEAP1 and ARE Expression**

Trizol reagent was used to extract RNA from HUVECs. Reverse transcription was performed according to the manual instruction of test kit, using primers designed by Primer 6.0 and synthesized by Invitrogen (Shanghai, China) as shown in Table I. Real-time PCR was performed under the following conditions: 55°C for 1 min, followed by 35 cycles each containing 92°C for 30 s, 58°C for 45 s and 72°C for 35 s. Data were collected and calculated for cycle threshold (CT) values of all samples and standards based on fluorescent quantification using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference. A standard curve was firstly plotted using CT values of standards, followed by semi-quantitative analysis by 2-ΔΔCt method.

**MTT Assay for Cell Proliferation**

Cells at log-phase were seeded into 96-well plate which contained Roswell Park Memorial Institute (RPMI)-1640 medium with 10% FBS at 5×10³ density. After 24 h incubation, 20 μl sterile MTT was added into each test well in triplicates every 24 h. After 4 h continuous culture, the supernatant was completely removed, with the addition of 150 μl dimethyl sulfoxide (DMSO) for 10 min vortex until the complete resolving of crystal violet. Absorbance (A) values were measured at 570 nm in a microplate reader. The proliferation rate was calculated in each group.

**Caspase3 Activity Assay**

Caspase 3 activity in all groups of cells was evaluated using test kit. In brief, cells were digested by trypsin, and were centrifuged at 600 ×g for 5 min under 4°C. The supernatant was discarded, followed by the addition of cell lysis buffer and iced incubation for 15 min. The mixture was then centrifuged at 20 000 ×g for 5 min under 4°C, followed by the addition of 2 mM Ac-DECD-pNA. Optical density (OD) values at 450 nm wavelength were measured to reflect caspase 3 activity.

**Enzyme-linked Immunosorbent Assay (ELISA) for TNF-α and IL-2 Expression in Supernatants**

Expression levels of TNF-α and IL-2 in cell culture supernatant were quantified by ELISA following the manual instruction of test kits. In brief, 96-well plate was added with 50 μl serially diluted samples, which were used to plot standard curves. 50 μl test samples were then added into test wells in triplicates. After washing for 5 times, liquids were discarded to fill with washing buffer for 30 s vortex. The rinsing procedure was repeated for 5 times. 50 μl enzyme labeling reagent was then added into each well except blank control. After gentle mixture, the well was incubated for 30 min at 37°C. Chromogenic substrates A and B were sequentially added (50 μl each), followed by 37°C dark incubation for 10 min. The test plate was then mixed with 50 μl quenching buffer as the blue color turned into yellow. Using blank control well as the reference, OD values at 450 nm wavelength were measured by a microplate reader within 15 min after adding quenching buffer. Linear regression model was then plotted based on the concentration of standard samples and respective OD values. Sample concentration was further deduced based on OD values on regression function.

**Table I. Primer sequence.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5′-3′</th>
<th>Reverse primer 5′-3′</th>
</tr>
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<tbody>
<tr>
<td>GAPDH</td>
<td>AGTACCAGTCTGGTTGCTGG</td>
<td>TAATAGCCCGGATGCTCGGT</td>
</tr>
<tr>
<td>Nr2</td>
<td>CTACATCGTAAACATGG</td>
<td>GCACGTCTTAGG</td>
</tr>
<tr>
<td>KEAP1</td>
<td>TAAAGGAGACCGGAATG</td>
<td>ACATCATCTATTCCT</td>
</tr>
<tr>
<td>ARE</td>
<td>TCATCATCTACCTCTC</td>
<td>ACGTCGGTACCCCTC</td>
</tr>
<tr>
<td>Mir-92a</td>
<td>CAGCTCCTACCAGCCTAAAG</td>
<td>GACAGATCGACCTGTG</td>
</tr>
</tbody>
</table>

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Reactive Oxygen Species (ROS) Assay in all Groups of Cells

ROS level change in all groups of cells was measured. In brief, cells were denatured at 95°C for 40 min, iced in tap water, and were centrifuged at 2 000 r/min for 10 min. At 37°C, homogenates were incubated in 2', 7'-dichlorofluorescein diacetate (DCF-DA) for 15 min. After centrifugation at 8000 r/min for 15 min, the supernatant was discarded whilst precipitations were re-suspended in sterilized PBS buffer, and were incubated at 37°C for 60 min. Spectrometry was used to detect the ROS levels expressed as ROS production percentage.

MPO and SOD Activity Assay

SOD activity in all groups of cells was tested following manual instruction. In brief, cells were denatured at 95°C for 40 min, and were centrifuged at 2000 r/min for 10 min. Ethanol-chloroform mixture (5:3, v/v) was used to extract ethanol phase in the homogenate for total SDO activity assay. MPO activity was measured following the manual instruction of test kit. Tissue homogenate was prepared by vortex homogenizer. After 95°C heating for 40 min, tissue lysate was washed by cold water, and was centrifuged at 4 000 r/min for 10 min. Samples were added into phosphate buffer containing 30 mM H₂O₂ (pH 7.0). The mixture was incubated for 10 min. Enzymatic activity was measured by detecting the decrease of absorbance light density at 240 nm wavelength.

Western blot for Nrf2 Protein Expression

Total proteins were extracted from cells. In brief, cells were mixed with lysis buffer on ice for 15-30 min, with ultrasound treatment (5 s, 4 times). After centrifugation at 10 000 ×g for 15 min, the supernatant was saved, quantified for protein content and was stored at -20°C for Western blot assay. Proteins were separated in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to PVDF membrane by semi-dry method (160 mA, 1.5 h). Non-specific binding sites were removed by 5% defatted milk powder for 2 h. Anti-Nrf2 monoclonal antibody (1:1 000) was added for 4°C overnight incubation. After PBS and Tween 20 (PBST) washing, goat anti-rabbit secondary antibody (1:2 000) was added for 30 min incubation at room temperature. ECL reagent was then added for developing the membrane for 1 min after PBST rinsing, followed by X-ray exposure. The film was scanned and analyzed by protein imaging system and Quantity One software for measuring band density. Each experiment was replicated for four times (n=4) for statistical analysis.

Statistical Analysis

SPSS 16.0 software was used for analysis. Measurement data were presented as mean ± standard deviation (SD). Student t-test or Tukey’s post hoc test was used for comparing measurement data between groups. A statistical significance was defined when p < 0.05.

Results

Expression of miR-92a in Aged Vascular Endothelial Cells

Real-time PCR was used to test expression profile of miR-92a in aged vascular endothelial cell model and normal controlled cells. Results showed significantly elevated miR-92a in model group compared to control group (p < 0.05, Figure 1).

MIR-92a Inhibitor Transfection Regulates miR-92a in Aged Vascular Endothelial Cells

Real-time PCR was used to test the effect of miR-92a inhibitor transfection on miR-92a expression in aged vascular endothelial cells. Results showed that transfection of miR-92a inhibitor significantly inhibited expression of miR-92a in aged vascular endothelial cells compared to model group (p < 0.05, Figure 2).

Figure 1. Expression of miR-92a in aged vascular endothelium. *p < 0.05 compared to control group.
Effects of miR-92a Regulation on Proliferation of Aged Vascular Endothelial Cells

MTT assay was used to test the effect of miR-92a inhibitor transfection on the proliferation of aged vascular endothelial cells after 48 h. Results showed significantly decreased endothelial cell proliferation in model group compared to control group ($p < 0.05$). The transfection of miR-92a inhibitor for 48 h significantly facilitated proliferation of aged vascular endothelium compared to model group ($p < 0.05$, Figure 3). These results suggested that inhibition of miR-92a expression facilitated endothelial cell proliferation.

Effects of miR-92a Regulation on Caspase3 Activity in Aged Vascular Endothelial Cells

Caspase3 activity was tested after miR-92a modulation in aged vascular endothelial cells. Results showed significantly elevated Caspase3 activity in aged vascular endothelium compared to control group ($p < 0.05$). Transfection of miR-92a inhibitor for 48 h significantly decreased Caspase3 activity in aged endothelial cells compared to model group ($p < 0.05$, Figure 4). These results suggested that miR-92a exerted protective role via modulating apoptotic proteins to prevent injury of aged vascular endothelium.

Regulation of miR-92a and MPO/SOD Activity in Aged Vascular Endothelial Cells

We then measured the effect of miR-92a modulation on MPO and SOD activities in aged vascular endothelium. Results showed significantly elevated MPO activity in model cells compared to control group ($p < 0.05$). Transfection of miR-92a inhibitor for 48 h remarkably decreased MPO activity ($p < 0.05$ compared to model group). In contrast to MPO activity, SOD activity in aged vascular endothelium was significantly decreased ($p < 0.05$ compared to control group). Transfection of miR-92a inhibitor for 48 h significantly enhanced endothelial SOD activity ($p < 0.05$ compared to model group, Figure 5).

Effects of miR-92a on ROS Content in Aged Vascular Endothelium

The effect of miR-92a on ROS content in aged vascular endothelium was measured. Results showed significantly elevated ROS contents in model group ($p < 0.05$ compared to control group). After 48 h of miR-92a transfection, ROS content was significantly decreased in aged vascular endothelial cells ($p < 0.05$ compared to model group, Figure 6).
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Effects of miR-92a on TNF-α and IL-2 levels in Supernatant of Cultured Endothelial Cells

The level of TNF-α and IL-2 in the supernatant of cultured aged vascular endothelial cells was measured. Results showed significantly elevated TNF-α and IL-2 levels in model group (p < 0.05 compared to control group). 48 h after transfecting miR-92a inhibitor, both TNF-α and IL-2 levels were remarkably decreased (p < 0.05 compared to model group, Figure 7).

Effects of miR-92a on Nrf2 Expression in Supernatant of Cultured Endothelial Cells

Real-time PCR and Western blot were used to test the effect of miR-92a modulation on mRNA and protein expression of Nrf2 in aged vascular endothelial cells, respectively. Results found decreased Nrf2 mRNA and protein expressions in model group (p < 0.05 compared to control group). Transfection of miR-92a for 48h remarkably increased both mRNA and protein expression of Nrf2 (p < 0.05 compared to model group, Figure 8).

Effects of miR-92a Modulation on KEAP1 and ARE Expression in Aged Vascular Endothelial Cells

Real-time PCR was used to test whether miR-92a modulation affected KEAP1 or ARE mRNA expression in aged vascular endothelial cells. Results showed elevated KEAP1 expression and decreased ARE expression in model group (p < 0.05 compared to control group). Transfection of miR-92a inhibitor for 48 h significantly inhibited KEAP1 whilst facilitated ARE expression (p < 0.05 compared to model group, Figure 9).

Discussion

Aging has become a major healthy challenge for the global population, leading to a series of related disorders. Aging of vascular endothelial cells is the prerequisite for the aging of whole vessel tissues, further leading to structural and functional disorder, causing cardiovascular issues such as hypertension and myocardial infarction. A previous study showed the involvement of miRNA in regulating physiological function of vascular endothelial cells. MiR-92a was shown to be related with cardiovascular disease. The functional role and related mechanism of miR-92a in vascular endothelial cell aging have not been reported.

Angiotensin II is the major effector peptide in renin-angiotensin system (RAS). A previous study showed that angiotensin II could induce aging of
vascular endothelial cells and was related with cardiovascular disease\(^7\). This study thus utilized angiotensin II to generate HUVECs aging model, on which miR-92a was up-regulated in vascular endothelial aging. A further work utilized miR-92a inhibitor transfection into aged vascular endothelial cells, and demonstrated that down-regulation of miR-92a significantly facilitated proliferation of vascular endothelial cells, inhibited apoptosis, suggesting the close correlation between miR-92a and vascular endothelial aging.

A further investigated related mechanism of miR-92a for aging effects of vascular endothelium. When body is under oxidative stress status, over-production of free oxygen species may exceed the clearance potency of body, leading to imbalance of oxidation/anti-oxidation system, further causing tissue inflammation and injury. Body redox status is thus closely correlated with aging occurrence\(^8\). During oxidative stress process, reactive oxygen species (ROS) is abundantly expressed, leading to apoptosis and injury of vascular endothelial cells, making it one major reason for endothelial aging and related disease\(^9\). SOD is one important anti-oxidase inside the body for clearing ROS and plays a critical role for oxidation/anti-oxidation homeostasis\(^10\). MPO cannot penetrate membrane by itself, but can be abundantly released under cell injury or death episode\(^11\). We showed that down-regulation of miR-92a facilitated SOD expression in aged vascular endothelial cells, decreased ROS and MPO, further illustrating that miR-92a down-regulation could mediate oxidation/anti-oxidation balance, decreasing Caspase-3 activity and facilitating proliferation and aged cells.

Under normal physiological condition, Nrf2 is tightly bounded with KEAP1, inactivating Nrf2. However, under electric affinitive stress, oxidative stress, toxic substances, and metabolites, Nrf2 may dissociate with KEAP1, leading to the formation of Nrf2-ARE complex\(^22\). The study showed that Nrf2-KEAP1-ARE signal pathway participated in regulating normal human physiological status, antagonizing tumor, detoxifying, and maintaining body homeostasis. Nrf2 is one transcription factor with cell protecting effects\(^23\). Nrf2-KEAP1-ARE signal pathway resists internal/external oxidation and chemical substances via mediating redox balance, further inhibiting oxidative stress and exerting defense effects, and is one critical endogenous anti-oxidation signal pathway of our body\(^24,25\). This study demonstrated up-regulation of KEAP1 in aged model, which also had decreased Nrf2 or ARE expression. Moreover, down-regulation of miR-92a significantly inhibited KEAP1 expression and facilitated Nrf2 and ARE expression, indicating that Nrf2-KEAP1-ARE signal pathway played a critical role in aged oxidative stress response. The down-regulation of miR-92a can affect aging process of vascular endothelial cells via mediating Nrf2-KEAP1-ARE signal pathway.

Figure 8. MiR-92a modulation and Nrf2 expression in aged vascular endothelial cells. (A) Nrf2 mRNA expression in aged vascular endothelial cells after miR-92a modulation; (B) and (C) Effects of miR-92a modulation on Nrf2 protein expression. *\(p < 0.05\) compared to control group; †\(p < 0.05\) compared to model group.

Figure 9. Modulation of miR-92a affected KEAP1 and ARE expression in aged vascular endothelial cells. *\(p < 0.05\) compared to control group; †\(p < 0.05\) compared to model group.
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Conclusions

MicroRNA-92a is up-regulated in aged vascular endothelium. The inhibition of miR-92a expression can inhibit oxidative stress or inflammation via mediating Nrf2-KEAP1-ARE signal pathway, to inhibit endothelial apoptosis and to facilitate cell proliferation. MiR-92a is thus related with endothelial cell aging and probably become a novel target for retarding aging process.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References


