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Original Article

MicroRNA-34a suppresses cell proliferation and promotes proinflammatory cytokine secretion in endothelial cells

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Abstract: Background: Endothelial cells play critical roles in the maintenance of vessel wall homeostasis. Nowadays, multiple literatures reported microRNAs (miRs) served as vital regulators in many biological processes of endothelial cells. Nevertheless, the role of miR-34a in endothelial cells has not been well clarified. Thus, this study was aimed to explore the functional effects of miR-34a on endothelial cells proliferation and inflammatory response. Methods: Human coronary artery endothelial cells (HCAECs) was used and transfected with miR-34a mimic or its negative control (NC). After 48 h transfection, cells viability was measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The protein expressions of caspase-3 and B-Cell CLL/Lymphoma 2 (Bcl-2) were detected by Western blot analysis, and the mRNA level expressions of Tumor Necrosis Factor α (TNF-α), Interleukin 6 (IL-6) and Interleukin 1β (IL-1β) were monitored by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Besides, the content of these three factors in the supernatant of miR-transfected cells were detected by ELISA. Results: Overexpression of miR-34a significantly suppressed HCAECs cells viability ($P < 0.05$ or $P < 0.01$). Besides, up-regulation of caspase-3 and down-regulation of Bcl-2 were found in miR-34a overexpressed cells ($P < 0.01$ or $P < 0.001$). Further, miR-34a overexpression significantly increased the production of TNF-α, IL-6 and IL-1β ($P < 0.05$ or $P < 0.001$). Conclusion: MiR-34a could suppress cell proliferation via inducing a caspase-dependent apoptotic pathway. In addition, miR-34a might contribute to proinflammatory cytokine secretion in endothelial cells.

Keywords: microRNA-34a, endothelial cells, proliferation, proinflammatory cytokine

Introduction

Endothelial cells are one of the major cellular components within the vessel wall and they play critical roles in the maintenance of vessel wall homeostasis [1, 2]. Endothelial cells form the inner lining of blood vessels are called vascular endothelial cells, whereas those form the inner lining of lymph are known as lymphatic endothelial cells [3]. Vascular endothelial cells are unique multifunctional cells with critical basal, inducible metabolic and synthetic functions [4]. Besides, vascular endothelial cells are vital in regulating hemostasis, vasomotor tone, immune and inflammatory responses [5-8].

MicroRNAs (miRs) are short non-coding RNAs consisting of 19~22 nucleotide base pairs [9]. MiRs can regulate theirs target gene expressions by interacting with the 3'-untranslated region (3'-UTR) of mRNA, and resulting in either translational repression or transcript degradation [10]. MiRs have versatile roles in diverse processes of cellular biology, including differentiation, growth, proliferation, apoptosis and inflammatory response [11, 12]. In addition, miRs are known as important regulators in endothelial cells biology [11]. For instance, Smits *et al.* demonstrated that suppression of miR-101 in endothelial cells promoted blood vessel formation via regulating enhancer of zeste 2 (EZH2) [13]. Doebele *et al.* showed that miR-17/20 exhibited a cell-intrinsic antiangiogenic activity in endothelial cells [14].

MiR-34 family comprises three processed miRs, *i.e.*, miR-34a, miR-34b and miR-34c. MiR-34a is transcribed from chromosome 1, whereas miR-34b and miR-34c are co-transcribed from chromosome 11 [15, 16]. Nowadays, these three members of the miR-34 family are widely investigated as tumor suppressors [17]. In addition, an increasing number of literatures reported that the expression of miR-

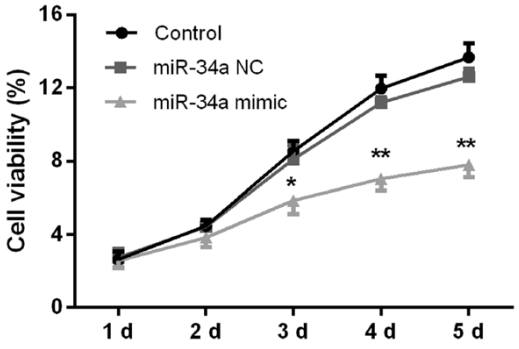

Figure 1. Effects of miR-34a overexpression on HCAECs cells viability. HCAECs cells were transfected with miR-34a mimic or its control, then the miR-transfected cells were cultured for 1-5 days and cell viability were measured by MTT assay. $P < 0.05$, **, $P < 0.01$.

34a was implicated in cell proliferation, apoptosis, cell cycle arrest and differentiation [18, 19]. However, little was known about the functional effects of miR-34a on endothelial cells. Therefore, this study was aimed to explore whether there might be a link between miR-34a expression and the biological processes of endothelial cells. Specifically, human coronary artery endothelial cells (HCAECs) were used and transfected with miR-34a mimic or its negative control (NC). Afterward, miR-transfected cells viability were measured. In addition, the production of three proinflammatory cytokines and the protein expressions of two apoptosis related factors were determined respectively. This study might be helpful for us to better understanding the potential role of miR-34a in endothelial cells.

Materials and methods

Cell culture and transfection

HCAECs were obtained from Cell Applications (San Diego, CA, USA). Cells were cultured in EGB-2MV medium (Lonza, Basel, Switzerland) supplemented with 0.04% hydrocortisone, 0.1% human epidermal growth factor (hEGF), 5% fetal bovine serum (FBS), 0.1% vascular endothelial growth factor (VEGF), 0.4% human fibroblast growth factor (hFGF)-B, 0.1% R3-insulin-like growth factor (IGF)-1, 0.1% ascorbic acid and 0.1% gentamicin/amphotericin-B [20]. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

For cell transfection, cells were plated onto 6-well plates and after cells were grown to about 70% confluence, miR-34a mimic or its control (GenePharma, Shanghai, China) were transfected into cells. Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA) was used in the transfection according to the manufacturers' instructions [21]. After 48 h transfection, cells were collected for the forthcoming analyses.

Cell viability analysis

Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, miR-transfected cells were planted into 96-well plates at a density of 2×10^3 cells per well. After culture for 1-5 days, 20 μL MTT solution (10 mg/mL; Sigma, St Louis, MO, USA) was added into each well. After 4 h incubation, the medium in each well was replaced with 150 μL dimethyl sulfoxide (DMSO; Sigma, USA). Finally, plants were shaken for 10 min and the absorbance was measured by a microplate reader (Bio Rad Laboratories, Hercules, CA, USA) at a wavelength of 570 nm [22].

Western blotting

MiR-transfected cells were lysed with lysis buffer (Beyotime, Shanghai, China). Protein concentration was measured by BCA protein assay reagent (Pierce Biotechnology, Rockford, IL) and the lysates were boiled for 5 min. Proteins were subjected to a sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (GE healthcare). After blocking with 5% skim milk for 2 h at room temperature, membranes were incubated with appropriate primary antibodies: caspase-3 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), B-Cell CLL/Lymphoma 2 (Bcl-2) (1:1000; Cell Signaling Technology, Danvers, MA, USA) or β-actin (1:1000; Cell Signaling Technology) overnight at 4°C. Then the blots were incubated with a horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Finally, the immunoreactive protein bands were visualized by the WEST-ZOL-plus Western Blot Detection System [23].

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA in miR-transfected cells were isolated using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). cDNA synthesis were performed using Transcriptor First Strand cDNA Synthesis Kit (Roche, USA) according to the manufacturer's instructions. RT-PCR was

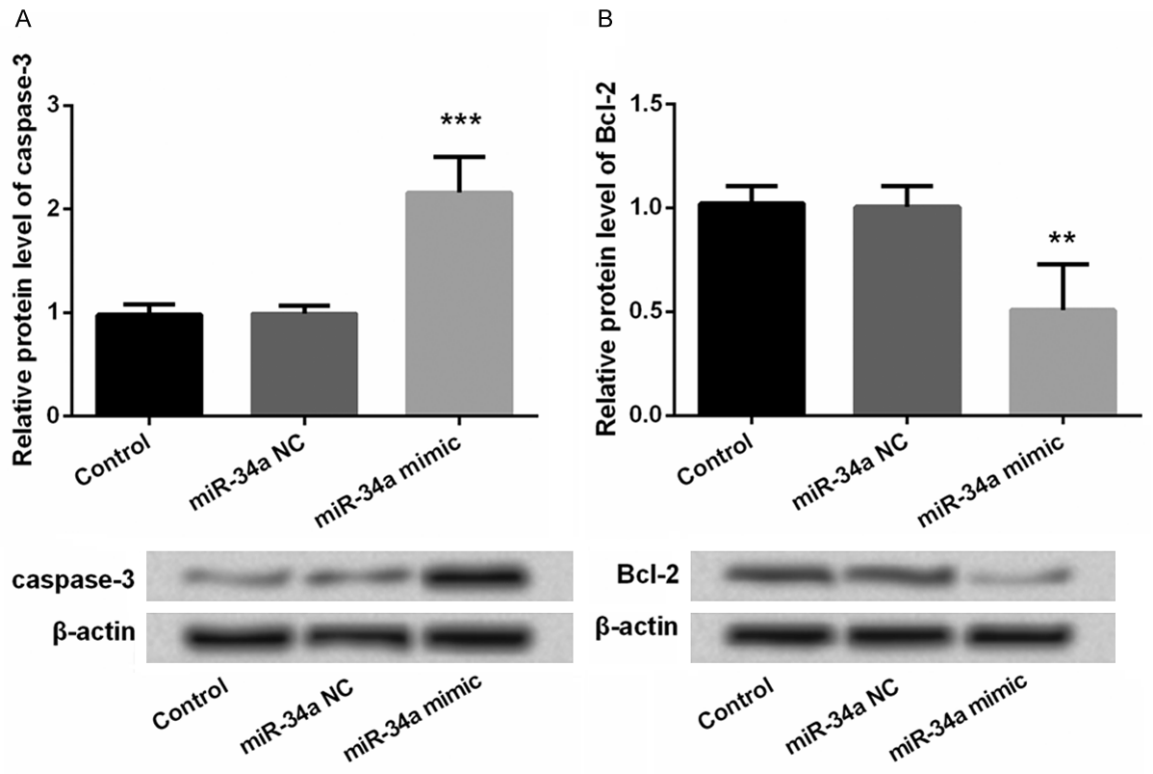

Figure 2. Effects of miR-34a overexpression on the expressions of apoptosis related proteins. After miR-34a mimic or its control was transfected into HCAECs cells, the protein expressions of caspase-3 (A) and Bcl-2 (B) were measured by Western blot analysis. β-actin acted as an internal control. **, $P < 0.01$; ***, $P < 0.001$.

performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). All primers were synthesized by Genepharma (Shanghai, China) and the primer pairs were as follows: Tumor Necrosis Factor α (TNF-α): 5'-AGA CCC TCA CAC TCA GAT CAT CTT C-3', 5'-TTG CTA CGA CGT GGG CTA CA-3'; Interleukin 6 (IL-6): 5'-CGA TGA TGC ACT TGC AGA AA-3', 5'-TGG AAA TTG GGG TAG GAA GG-3'; Interleukin 1β (IL-1β): 5'-TCG CTC AGG GTC ACA AGA AA-3', 5'-ATC AGA GGC AAG GAG GAA ACA C-3'; GAPDH: 5'-GGG AAG CCC ATC ACC ATC T-3', 5'-CGG CCT CAC CCC ATT TG-3' [24].

ELISA

TNF-α, IL-6 and IL-1β concentrations in the culture supernatants of HCAECs were detected by the corresponding ELISA kit (all from Cusabio, Wuhan, China) according to the manufacturer's instructions. Briefly, the culture supernatant was collected by centrifugation at 1000 g for 15 min, and 100 μL was added to each well of 96-well plates for an incubation of 2 h at 37°C. The liquid was removed, after which the Biotin-antibodies and horse radish peroxidase (HRP)-conjugated secondary antibodies were added and incubated for 1 h at 37°C in turn. The color-developing agent was added to the plates, which were incubated in the dark for 30 min at 37°C. After the reaction was stopped, optical density was immediately detected by a microplate reader iMark (Bio-Rad, Hercules, CA, USA) at 450 nm.

Statistical analysis

All data were presented as means ± standard derivations (SD) from at least three independent experiments or analyses. Data were analyzed by GraphPad Prism 5 software (GraphPad, San Diego, California, USA) and *t* tests. A value of $P < 0.05$ was considered statistically significant.

Results

Effects of miR-34a overexpression on HCAECs cells viability

To evaluate the effects of miR-34a on endothelial cells viability, HCAECs cells were transfected with miR-34a mimic or its control, and then miR-transfected cells viability were measured

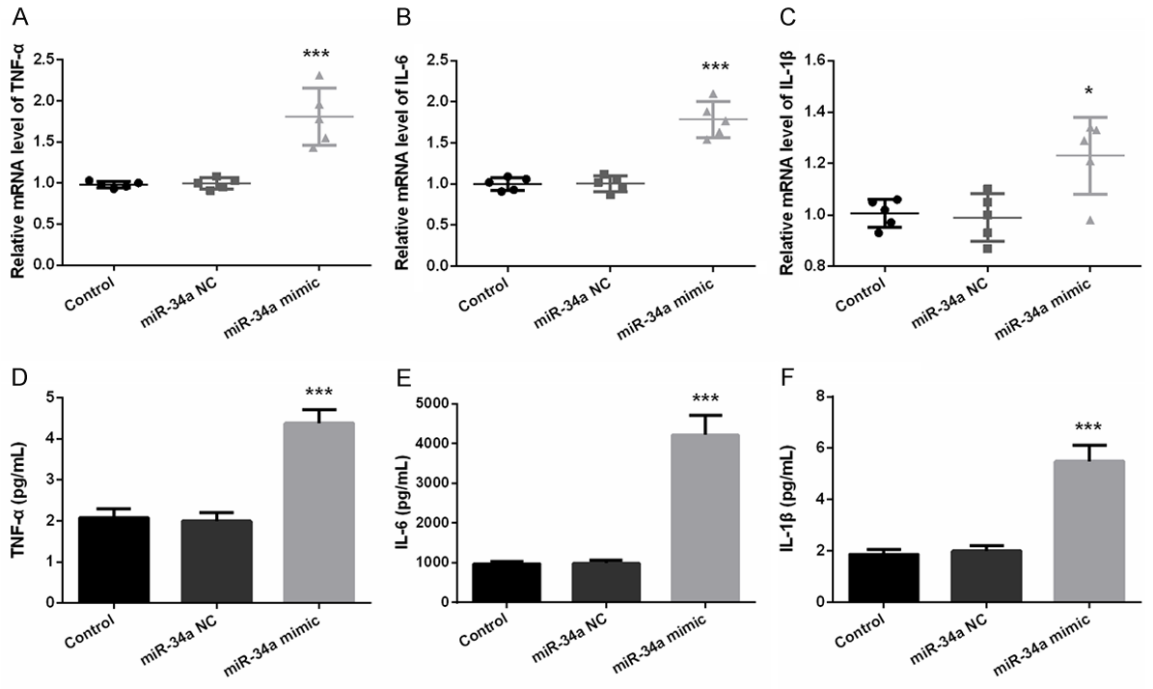

Figure 3. Effects of miR-34a overexpression on proinflammatory cytokines secretion. After miR-34a mimic or its control was transfected into HCAECs cells, the mRNA level expressions of TNF-α (A), IL-6 (B) and IL-1β (C) were detected by RT-PCR. Data were normalized to GAPDH expression. Additionally, the concentrations of TNF-α (D), IL-6 (E) and IL-1β (F) in the supernatant of the miR-transfected cells were detected by ELISA. *, $P < 0.05$; ***, $P < 0.001$.

by MTT analysis. As results displayed in **Figure 1**, overexpression of miR-34a could significantly suppressed cells viability, when cells were cultured for 3-5 days ($P < 0.05$ or $P < 0.01$). These data suggested that miR-34a might be a vital regulator in endothelial cells viability.

Effects of miR-34a overexpression on the expressions of apoptosis related proteins

To explore whether miR-34a could affects endothelial cells apoptosis, the protein expressions of two apoptosis related factors, *i.e.*, caspase-3 and Bcl-2, in miR-transfected cells were determined by Western blot analysis. Results in **Figure 2A** and **2B** showed that, miR-34a overexpression significantly up-regulated the level of caspase-3 ($P < 0.001$), while significantly down-regulated the level of Bcl-2 ($P < 0.01$). These results indicated that miR-34a overexpression might suppress cell proliferation via inducting a caspase-dependent apoptotic pathway.

Effects of miR-34a overexpression on proinflammatory cytokines secretion

To investigate the effects of miR-34a on the production of proinflammatory cytokines, the mRNA level expressions of TNF-α, IL-6 and IL-1β in miR-transfected cells, and the contents of these three factors in the supernatant of miR-transfected cells were respectively monitored by RT-PCR and ELISA (**Figure 3A-F**). We found that, miR-34a overexpression significantly increased the production of these three proinflammatory cytokines ($P < 0.05$ or $P < 0.001$). Thus, we speculated that miR-34a might also act as a regulator in proinflammatory cytokines production.

Discussion

Endothelial cells derive from the umbilical vein and play critical roles in the maintenance of vessel wall homeostasis [1, 2, 25]. Recent studies have revealed that multiple miRs have the abilities of regulating biological processes of endothelial cells [11]. Nevertheless, the role of miR-34a in endothelial cells has not been well clarified. In this study, HCAECs cells were used and transfected with miR-34a or its control. We found that miR-34a overexpression could suppress HCAECs cells viability. Besides, miR-34a overexpression could up-regulate the protein level expression of caspase-3, whereas down-regulate the level of Bcl-2. MiR-34a over-

expression could increase the production of TNF-α, IL-6 and IL-1β.

MiRs play critical roles in multiple cellular processes, including cell proliferation, apoptosis and inflammatory response [26]. To date, miR-34a was found acted as a cell proliferation suppressor in many types of cells, including human pulmonary artery smooth muscle cells, human mesothelial cells and laryngeal carcinoma cells [27-29]. However, our study provided the first evidence that miR-34a can suppress endothelial cells proliferation. In addition, an early literature reported that miR-34a induced apoptosis in neuroblastoma cells [30]. Bommer *et al.* showed that the survival factor Bcl-2 was directly targeted by miR-34a and miR-34a-defective showed a decrease in spontaneous apoptosis [31]. Welch *et al.* revealed that the key executioners of apoptosis, such as caspase-3 and caspase-7, were significantly increased by transfection with miR-34a [30]. Consistent with these previous studies, results in the current study suggested that miR-24a overexpression remarkably up-regulated the expression level of caspase-3 and down-regulated the level of Bcl-2, implying that miR-34a reduced cell proliferation might be though the induction of a caspase-dependent apoptotic pathway.

IL-1β and TNF-α are two of the main cytokines involved in the inflammatory response and cell survival [32]. In addition, the two cytokines induce synthesis of other cytokines, such as IL-6 and IL-8 [32]. Increased IL-1β, TNF-α or IL-6 is commonly associated with a pro-inflammatory status [33]. Nowadays, several miRs have been reported as crucial regulators of vascular inflammation. Ye *et al.* have found that miR-146a is a potential therapeutic target for reducing inflammation in retinal microvascular endothelial cells through inhibition of Nuclear Factor Kappa B (NF-κB) and TNF-α [34]. *In vivo* and *in vitro* studies have demonstrated that miR-24 controls inflammatory activity in vascular endothelial cells by interacting with chitinase 3-like 1 (CHI3L1) [35]. In the current study, the production of IL-1β, TNF-α and IL-6 were increased by miR-34a overexpression, implying that miR-34a could modulate the production of inflammatory modulators in endothelial cells.

In conclusion, the present study indicated that miR-34a might be an antiproliferation gene in endothelial cells, at least in part, through induction of a caspase-dependent apoptotic pathway. In addition, miR-34a overexpression could promote proinflammatory cytokine secretion, suggesting miR-34a might be a key regulator in inflammatory response. However, further study still required to confirm these hypotheses.

Disclosure of conflict of interest

None.

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