Effects of down-regulation of microRNA-23a on TNF-α-induced endothelial cell apoptosis through caspase-dependent pathways

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Aims
Endothelial cell injury induced by inflammatory factors plays a critical role in the pathogenesis of numerous vascular diseases. MicroRNAs are well known to be implicated in cell proliferation and apoptosis in inflammatory responses; however, it remains to be determined whether microRNAs are associated with tumour necrosis factor (TNF)-α-mediated endothelial cell injury. The aim of the present study was to investigate the role of microRNAs in TNF-α-induced endothelial cell apoptosis.

Methods and results
Microarrays were used to analyse the global expression of microRNAs in TNF-α-stimulated human primary endothelial cells. Expression profiles of the microRNAs were verified using qRT-PCR. After TNF-α treatment, 12 miRNAs were dramatically up-regulated and nine were down-regulated. LNA-anti-miR-23a and pre-miR-23a were found to modulate one of the markedly down-regulated miRNAs, miR-23a, which could in turn increase or attenuate TNF-α-induced endothelial cell apoptosis. Bioinformatics analysis suggested that caspase-7 and serine/threonine kinase 4 are potential targets of miR-23a. LNA-anti-miR-23a enhanced but pre-miR-23a inhibited the activation of caspase-7, serine/threonine kinase 4, and its related signalling caspase-3 after TNF-α treatment; however, neither pre-miR-23a nor LNA-anti-miR-23a had an effect on TNF-α-induced Bcl-2 activation.

Conclusion
Our results suggest that miR-23a may be involved in TNF-α-induced endothelial cell apoptosis through regulation of the caspase-7 and serine/threonine kinase 4–caspase-3 pathways.

Keywords
MicroRNA • Endothelial cell • Apoptosis • Tumour necrosis factor-α • Caspase

1. Introduction
It is well known that inflammatory cytokines, tumour necrosis factor-α (TNF-α) in particular, can induce endothelial cell injury, which is a hallmark of many vascular diseases, including atherosclerosis, post-angioplasty restenosis, and sepsis.1–3 The mechanism of TNF-α-induced endothelial cell injury has been extensively studied, and its downstream genes are believed to be involved in the procedure.4–6 Gene expression regulated by TNF-α has recently been documented at both the epigenetic and the transcriptional levels.7,8 For example, in endothelial cells, TNF-α modulates cytokine signalling pathways and transcription factors, which may be an important part of TNF-α-mediated changes in the expression of multiple genes.8,9 However, it still remains to be determined whether translational regulation, a process also critical for gene regulation,10,11 is involved in TNF-α-induced endothelial cell apoptosis.

MicroRNAs (miRNAs) regulate gene expression through complementary binding to 3’ UTR sequences of target mRNAs.12–14 About 800 human miRNAs have been identified and sequenced, but the total number of human miRNA genes is estimated to be as high as 1000. A single miRNA can regulate the expression of several genes by binding to different target mRNAs and is therefore functionally comparable to that of transcription factors.15–17 As a group, miRNAs may directly regulate at least 30% of the genes in a cell and are involved in almost all major cellular functions, such as cell differentiation, growth, mobility, and death (apoptosis and necrosis).16,18 A few specific miRNAs known to regulate endothelial cell function and angiogenesis have been described.16,19–21 For example, Let-7 family...
miR-27b can promote angiogenesis by down-regulating antiangiogenic genes. Both miR-221 and miR-222 have been found to modulate the angiogenic activity of stem cell factor and its receptor in human umbilical vein endothelial cells (HUVECs). Early studies have also indicated that miR-126 and miR-21 are implicated in the vascular inflammation response by regulating the vascular cell adhesion molecule-1 or affecting vascular neo-intimal lesion formation. However, the effects of TNF-α on miRNA expression and the roles of miRNAs in TNF-α-mediated gene regulation and endothelial cell apoptosis are unclear. Therefore, the aim of the present study was to use a combination of microarray and quantitative real-time PCR (qRT-PCR) to analyse miRNA expression profiles in TNF-α-stimulated human primary endothelial cells and to explore the possible roles that miRNAs might play in apoptosis signalling pathways in these cells.

2. Methods

This investigation conformed to the principles outlined in the Declaration of Helsinki for use of human umbilical cord and was approved by the Ethics Committee of Experimental Research, XiangYa Medical College, Central South University.

2.1 Cell culture

Primary cultures of HUVECs were performed as previously described. In brief, sterile technique was maintained in all cord manipulations. The cord was severed from the placenta soon after birth, placed in a sterile container filled with cord buffer, and stored at 4°C. The umbilical vein was cannulated and perfused with cold buffer to wash out the blood. It was then allowed to drain. Ten millilitres of 0.1% collagenase type II (Sigma, St Louis, MO, USA) in cord buffer was then infused into the umbilical vein. The umbilical cord, suspended by its ends, was placed in a water bath containing cord buffer and incubated at 37°C for 12 min. After incubation, the collagenase solution containing the endothelial cells was flushed from the cord by perfusion with 30 mL of cord buffer. The effluent was collected, washed, sedimented, and resuspended, and the cell suspension was divided equally between four to six plastic 35 mm Petri dishes (3001; Falcon Plastics, Oxnard, Calif, USA). Endothelial cells were cultured in endothelial cell culture medium (ScienCell, Carlsbad, CA, USA) containing 1% penicillin–streptomycin, 5% fetal bovine serum, and 1% endothelial cell growth supplement, and were stored in a humidified atmosphere containing 5% CO2 and 95% air at 37°C.

2.2 TNF-α treatments for miRNA expression

Cultured endothelial cells were treated with either vehicle or TNF-α (Sigma; 1–100 ng/mL) for 24 h. MiRNAs were then isolated from the cultured cells using the mirVana miRNA isolation kit (Ambion, Inc., Austin, TX, USA).

2.3 uParaflo™ MicroRNA microarray assay

To minimize inter-individual variability, total RNA was isolated from four control and four TNF-α-treated endothelial cells, pooled, and hybridized using a uParaflo™ MicroRNA microarray (Chip ID miRhsa 12.0; LC Sciences, Houston, TX, USA). Data were analysed by first subtracting the background and then normalizing the signals using a locally weighted regression filter locally weighted scatterplot smoothing (LOWESS). Each sample was repeated four times, and significant differences between vehicle and TNF-α 10 ng/mL were calculated for each given detectable miRNA signal. For the two-colour experiments, the ratio of the two sets of detected signals (log2 transformed, balanced) and P values of the t-tests were calculated. Differentially detected signals were those with P values less than 0.01.

2.4 Determination of RNA levels by qRT-PCR

Briefly, RNAs from endothelial cells were isolated with a RNA Isolation Kit (Ambion, Inc.). The six most notably aberrantly expressed microRNAs (miR-126, miR-23a, miR-125b, miR-1280, miR-222, and miR-155) were identified, and qRT-PCR was performed on complementary DNA generated from 50 ng of total RNA according to the manufacturer’s protocol. For caspase-7 and serine/threonine kinase 4 (STK4), qRT-PCR was performed on complementary DNA generated from 200 ng of total RNA using the protocol from a qRT-PCR miRNA detection kit (Roche, Indianapolis, IN, USA). Amplification and detection of specific products were performed with a Roche Lightcycler 480 Detection System. As an internal control, U6 was used for miRNA template normalization and β-actin was used for capase-7 and STK4 template normalization. Fluorescence signals were normalized to an internal reference, and the threshold cycle (Ct) was set within the exponential phase of the PCR. The relative expression level between treatments was then calculated using the following equation: relative gene expression = 2−[Ct sample – Ct control].

2.5 Measurement of TNF-α-induced endothelial cell apoptosis

Briefly, endothelial cells cultured in 5% fetal bovine serum were treated with either vehicle or TNF-α (1–100 ng/mL) for 24 h. Afterwards, cell apoptosis was measured by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining. TUNEL staining was performed using an in situ cell death detection kit (Roche) according to the manufacturer’s instructions. The number of TUNEL-positive cells was counted under a fluorescence microscope. Apoptotic and live cells were visualized with an inverted fluorescence microscope (Nikon, Tallahassee, Florida, USA) equipped with a CCD digital camera. Images were processed with image analysis software (NIS-Elements BR 3.0; Nikon, Tallahassee, Florida, USA).

2.6 Oligo transfection, miR-23a knock-down, and miR-23a overexpression in cultured endothelial cells

All kits and reagents used in oligo transfection were used in compliance with the manufacturer’s instructions. HUVECs from passages two to five were transfected (Ambion, Inc.). The miR-23a inhibitor locked nucleic acid (LNA)-anti-miR-23a was added to the culture media at a final concentration of 50 nM, and comparable LNA-scramble molecules were synthesized. These unconjugated and fully phosphorylated molecules were added to a mixture containing LNA/DNA oligonucleotides with a 6-carboxyfluorescein (FAM) moiety at the 5′ end. The following sequences were synthesized using Exiqon (Woburn, MA, USA): LNA-anti-miR-23a, S′-FAM-AATCCCTGCGAATGTGAT-3′ and its control oligo, LNA-scramble, S′-FAM-GTGT AACACGTCCTATACGCCCA-3′. For miR-23a up-regulation, pre-miR-23a (Ambion, Inc.) was added directly to the complexes at final a concentration of 50 nM. The transfection medium was replaced with the regular culture medium 4 h post-transfection. Vehicle control, oligo control for LNA-anti-miR-23a (LNA-scramble), and oligo control for pre-miR-23a (pre-scramble) were implemented.

2.7 Western blot analysis

Proteins isolated from cultured endothelial cells were assessed by western blot analysis. Equal amounts of protein were subjected to SDS–PAGE. A standard western blot analysis was conducted using Bcl-2 antibody.
The effects of tumour necrosis factor-α (TNF-α) on human endothelial cell apoptosis. Cultured human umbilical vein endothelial cells were treated with vehicle or TNF-α (1, 10, 40, or 100 ng/mL) for 24 h. Cell apoptosis was measured by terminal deoxynucleotide transferase dUTP nick end labelling (TUNEL) staining. (A) Representative fluorescent TUNEL-stained cell photomicrographs, their corresponding total cell photomicrographs (DAPI), and their merged photomicrographs (merge) from cells treated with vehicle or different concentrations of TNF-α. Scale bar represents 25 μm. (B) Quantitative analysis of apoptosis in cells treated with vehicle or with different concentrations of TNF-α. Data are shown as means ± SD (n = 5). ***P < 0.0001 relative to vehicle control (0 ng/mL), or to the 1 or 40 ng/mL groups.
(1:1000 dilution; Cell Signaling, Danvers, MA, USA), caspase-3 (1:1000 dilution; Cell Signaling), cleaved caspase-3 (1:1000 dilution; Cell Signaling), caspase-7 (1:1000 dilution; Cell Signaling), and STK4 (1:1000 dilution; Cell Signaling).

2.8 Statistics

All data are presented as means ± S.D. For relative gene expression, the mean value of the vehicle control group was defined as 1 or 100%. Student's two-tailed unpaired t-tests and ANOVA were used for statistical evaluation of the data. SigmaStat statistical analysis program was used for data analysis. P values < 0.05 were considered significant.

3. Results

3.1 Effects of TNF-α on endothelial cell apoptosis

Representative TUNEL-stained cell photomicrographs, corresponding total cell photomicrographs 4′,6-diamidino-2-phenylindole (DAPI), and merged photomicrographs (merge) from cells treated with vehicle (0 ng/mL), 1, 10, 40, or 100 ng/mL TNF-α are displayed in Figure 1A. Although low concentrations (1 ng/mL) of TNF-α had no effect on cell apoptosis, high concentrations (10–100 ng/mL) of TNF-α increased endothelial cell apoptosis in a dose-dependent manner (P < 0.0001) after 24 h of treatment in experimental conditions, as shown in Figure 1B.

Figure 2 Dysregulation of miRNA expression in endothelial cells after TNF-α treatment. (A) Differentially expressed miRNAs (P < 0.01) were analysed by hierarchical clustering of the log2 value of each miRNA microarray signal in different conditions (vehicle or 10 ng/mL TNF-α). Red indicates up-regulation; green, down-regulation; and black, no change. The bar code on the top represents the colour scale of the log2 values. Each column includes four repetitions at the top of the heat map. (B) The signal value of the three down-regulated miRNAs as determined by microarray analysis. (C) The signal value of the six up-regulated miRNAs as determined by microarray analysis. (D) Confirmation of aberrantly expressed miRNAs after TNF-α treatment (10 ng/mL) as determined by qRT-PCR. Total RNA was isolated from freshly isolated endothelial cells. Relative expression levels of miRNA-126, miRNA-23a, miRNA-125b, miRNA-128, miRNA-222, and miRNA-155 were analysed using real-time PCR. Each bar represents fold changes in miRNA expression in TNF-α-treated HUVECs relative to vehicle control (0 ng/mL). Data are expressed as means ± SD (n = 5). *P < 0.05, **P < 0.01, ***P < 0.0001 relative to vehicle control.
3.2 Up- and down-regulated miRNAs in TNF-α-stimulated endothelial cells

Tissue specificity is one important characteristic of miRNA expression. One miRNA may be highly expressed in one tissue but show little or no expression in others. To evaluate the biological functions of miRNA in vascular disease, we developed an mRNA expression profile for primary endothelial cells through miRNA microarray analysis. This analysis was based on version 12.0 of the Sanger miRBase (http://microrna.sanger.ac.uk/sequences). Overall, 119 miRNAs of the 895 arrayed were found in normal HUVECs; 30 of the 119 were highly expressed in normal primary endothelial cells (see Supplementary material online, Table S1). The most frequently expressed miRNAs were found to be miR-126 and miR-23a.

We used a well-established TNF-α-stimulated endothelial cell apoptosis model to determine changes in miRNA expression in vascular disease. After normalizing the microarray values using the LOWESS filter, values with significant differences \( (P < 0.01) \) were analysed using hierarchical clustering of the log2 value and displayed in a heat map (Figure 2A). The results show 21 miRNAs to be differentially expressed (12 up and nine down) relative to normal, uninjured endothelial cells, as shown in Supplementary material online, Table S2. Figure 2B shows the changes of miRNAs that were highly expressed in endothelial cells and downregulated after TNF-α-treatment. Figure 2C shows the changes of miRNAs that were highly expressed in rat carotid artery and upregulated after TNF-α-treatment.

3.3 Aberrant expression of miRNAs in apoptotic endothelial cells as confirmed by qRT-PCR

miRNAs whose expression was found to be significantly dysregulated under microarray analysis were selected for confirmation by qRT-PCR. The qRT-PCR results were in agreement with the results of microarray analysis; we found that miR-1280, miR-222, and miR-155 were highly up-regulated (Figure 2C and D), whereas miR-126, miR-23a, and miR-125b were significantly down-regulated (Figure 2B and D). Fold changes in miRNA expression in TNF-α-treated HUVECs (relative to vehicle control) are shown in Figure 2D. Remarkably, miR-23a was highly expressed in primary endothelial cells and more than 50% down-regulated after stimulation with TNF-α (Figure 2D). In addition, among the different genes reported to be targets of miR-23a, some have been identified as participants in apoptosis (see Supplementary material online, Table S3). For this reason, miR-23a merits further study to determine its role in apoptotic signalling pathways.

3.4 Modulation of miR-23a expression in endothelial cells

Both gain-of-function and loss-of-function approaches were used to modulate miR-23a in cultured endothelial cells (Figure 3). LNA-anti-miR-23a decreased miR-23 expression and pre-miR-23a increased it, both in a dose-dependent manner. Significant changes in expression were observed at a concentration of 50 nM, and the maximal effect was observed at 100 nM. In contrast, the control oligos (scrambled oligos) had no visible effect on miR-23a levels, even at 100 nM (see Supplementary material online, Figure S1). Furthermore, the effects of both LNA-anti-miR-23a and pre-miR-23a on miR-23a expression were miR-23a specific, because no effects were observed on other miRNAs, such as miR-222 or miR-126 (see Supplementary material online, Figures S2 and S3).

3.5 Effects of miR-23a on TNF-α-induced endothelial cell apoptosis

Apoptosis was evaluated by TUNEL assay. Representative TUNEL-stained photomicrographs from endothelial cells treated with vehicle, control oligo, pre-miR-23a, and LNA-anti-miR-23a are shown in Figure 4A. Pre-miR-23a significantly decreased TNF-α-induced endothelial cell apoptosis \( (P < 0.01) \). In contrast, endothelial cell apoptosis was increased after treatment with LNA-anti-miR-23a, to a striking degree \( (P < 0.0001) \), showing a nearly three-fold increase over the vehicle control (Figure 4B). In addition, control oligos (scrambled oligos) had no observable effects on endothelial cell apoptosis \( (P > 0.05) \). These results indicate that miR-23a may have a protective effect against TNF-α-induced endothelial cell apoptosis.

3.6 Effects of miRNA-23a on transcripts and protein expression of caspase-7 and STK4

To identify mRNA transcripts that might be regulated by miR-23a, we searched through publicly available algorithms (TargetScan 5.2, PicTar, and miRanda). In silico analysis revealed that caspase-7 and STK4, a critical mediator of apoptosis signalling, are potential targets of miR-23a. Accordingly, the 3’ UTRs of caspase-7 and STK4 mRNA were found to contain predicted binding sites for miR-23a (Figure 5A). If they are indeed the targets of miR-23a, TNF-α should increase expression of both in endothelial cells. To confirm this, we incubated endothelial cells with either vehicle or TNF-α (10 ng/mL) for 24 h and then determined protein levels of caspase-7 and STK4 by western blot. As shown in Figure 5B, TNF-α increased caspase-7...
The effects of miR-23a on TNF-α-induced endothelial cell apoptosis. Cultured endothelial cells pre-treated with vehicle, miR-23a inhibitor scramble (LNA-scramble, 50 nM), miR-23a inhibitor (LNA-anti-miR-23a, 50 nM), pre-miR-23a scramble (50 nM), or pre-miR-23a (50 nM) were treated with TNF-α (10 ng/mL) for 24 h. (A) Representative fluorescent TUNEL-stained cell photomicrographs, their corresponding total cell photomicrographs (DAPI), and their merged photomicrographs (merge) from cells treated with vehicle, LNA-scramble, LNA-anti-miR-23a, pre-miR-23a scramble, or pre-miR-23a. Scale bar represents 25 μm. (B) Quantitative analysis of the effects of miR-23a on TNF-α-induced endothelial cell apoptosis. Data are shown as means ± SD (n = 5), **p < 0.01, ***p < 0.0001 relative to vehicle control.
Figure 5  Caspase-7 and serine/threonine kinase 4 (STK4) are potential target genes of miR-23a in endothelial cells. (A) Conserved miR-23a binding sites in 3′ UTRs of caspase-7 (above) and STK4 (below). (B) TNF-α increased the expressions of caspase-7 and STK4. Representative immunoblots (above) and quantitative analysis (below) of caspase-7 and STK-4 from cells treated with vehicle and TNF-α (10 ng/ml) for 24 h. (C) Pre-miR-23a (50 nM) decreased caspase-7 and STK4 expression in endothelial cells compared with vehicle or pre-scramble (50 nM). Caspase-7 and STK4 mRNA levels (above), representative immunoblots (middle), and quantitative analysis (below) of caspase-7 and STK-4 protein levels. (D) The miR-23a inhibitor LNA-anti-miR-23a (50 nM) increased caspase-7 and STK4 expression in endothelial cells relative to vehicle or LNA-scramble (50 nM). Caspase-7 and STK4 mRNA levels (above), representative immunoblots (middle), and quantitative analysis (below) of caspase-7 and STK-4 protein levels. Data are shown as means ± SD (n = 5). *P < 0.05, **P < 0.01, ***P < 0.0001 relative to vehicle control.
and STK4 expression ($P < 0.01$). Both gain-of-function and loss-of-function approaches were employed to further verify caspase-7 and STK4 as the target genes of miR-23a in endothelial cells. Overexpression of miR-23a significantly decreased mRNA expression of both caspase-7 and STK4 (Figure 5C). By contrast, inhibition of miR-23a expression increased mRNA levels (Figure 5D, upper panel). Regulation of the expression of caspase-7 and STK4 by pre-miR-23a and LNA-anti-miR-23a was further confirmed at the protein level by western blotting (Figures 5C and D). Taken together, these results strongly suggest that, in endothelial cells, caspase-7 and STK4 are both target genes of miR-23a.

3.7 Involvement of caspase-3, a downstream signalling molecule of STK4, in miR-23a-mediated anti-apoptotic effects

Initially identified as a caspase substrate, STK4 is a critical mediator of the process of apoptosis, probably by inducing chromatin condensation or activating the downstream caspases. Caspase-3 is a downstream signal molecule in the STK4 pathway. To confirm the involvement of STK4 in miR-23a-mediated protective effects in endothelial cell apoptosis, caspase-3 activity was determined by western blot analysis. Decreasing STK4 expression via pre-miR-23a (Figure 5C) resulted in reduced caspase-3 activity (Figure 6A). In contrast, increasing STK4 expression via LNA-anti-miR-23a (Figure 5D) resulted in increased caspase-3 activity (Figure 6A). In order to further understand the role of miR-23a in the TNF-α-mediated apoptosis signalling pathways seen in endothelial cells, changes in Bcl-2 expression were also determined by western blot. Interestingly, miR-23a had no effect ($P > 0.05$) on Bcl-2 relative to vehicle control (Figure 6B).

4. Discussion

Recent studies have identified specific miRNAs expressed in endothelial cells. Harris et al. used microarray analysis to determine miRNA expression in HUVECs. Of the 30 miRNAs that we found to be most highly expressed in endothelial cells, 10 were among those previously identified by Harris et al. The miRNAs identified by both the present study and that of Harris et al. are miR-126, miR-23a, miR-23b, miR-21, miR-221, miR-222, miR-24, miR-26a, miR-29a, and let-7a. Another study identified nine miRNAs highly expressed in HUVECs, all of which we also found, most notably miR-23a. These three
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studies establish a set of miRNAs that are expressed in endothelial cells. In addition, our analysis detected an additional 12 miRNAs which had not been reported before. These were miR-99b, miR-720, miR-638, miR-214, miR-1979, miR-1975, miR-1974, miR-1826, miR-155, miR-151–5p, miR-1280, and miR-125b. These differences are most likely to be the result of advances in technical support for microarray chips and in miRNA probes. Notably, the miRNA microarray used in the present study was the later version (release 12.0) of the Sanger miRBase sequence database. The miRNA expression profile established by our and previous results may assist future research into the role of miRNAs in endothelial cells.

MiRNAs are part of normal cell processes, and abnormal miRNA expression plays a role in vascular diseases. According to recent studies, vascular dysfunction involves programmed cell death, a process known as apoptosis. As apoptosis has been observed in endothelial cells during normal ageing and in atherosclerotic disease, endotoxic shock, and heart failure, we hypothesized that miRNAs might also play important roles in endothelial cell apoptosis. Therefore, considering that TNF-α has been widely used as an initiator of endothelial apoptosis and because of its known reproducible effects, we chose TNF-α-stimulated human primary endothelial cells as a cell model to determine which miRNAs were associated with apoptosis. In the present study, we found 21 miRNAs to be aberrantly expressed in TNF-α-stimulated endothelial cells and obtained supporting results from qRT-PCR. These data may allow us to identify several miRNAs involved in endothelial cell responses to TNF-α and in the induction of apoptosis. We found that levels of miR-23a, which are highly enriched in endothelial cells, were dramatically decreased after TNF-α treatment. In addition, the results of in silico studies predicted that miR-23a would target several important apoptosis-related genes (selected examples are provided in Supplementary material online, Table S3). This suggests that some of the modifications in the apoptotic signalling pathways observed after TNF-α treatment might be attributable to the regulation of miR-23a. Moreover, the down-regulation of miR-23a may be highly specific for TNF-α-induced apoptosis, because neither ionizing radiation nor growth factor could induce the down-regulation of miR-23a. In addition, there is no evidence showing that general stresses, such as pulsatile or laminar shear stress, played a role in miR-23a regulation. It should, however, be noted that the miR-23a modulation seen in cellular apoptosis may not be restricted to endothelial cells. Thus, it is possible that miR-23a plays a general role in the regulation of TNF-α-mediated apoptotic signalling pathways.

To ascertain the role of miR-23a in TNF-α-mediated endothelial cell apoptosis, miR-23a expression was modulated via a miR-23a inhibitor and pre-miR-23a. Interestingly, overexpression of miR-23a was found to inhibit TNF-α-mediated endothelial cell apoptosis. In contrast, TNF-α-mediated endothelial cell apoptosis became exacerbated after down-regulation of miR-23a expression. These results suggest that miR-23a has an anti-apoptotic effect. This is consistent with the effects of miR-23a on mesenchymal stem cells and human retinal pigment epithelium cells. In addition, microRNA-23a promotes the growth of gastric adenocarcinoma cells and down-regulates interleukin-6 receptor. However, a more recent report showed that up-regulation of miR-23a and caspase-dependent and caspase-independent apoptosis in human embryonic kidney cells. The different effects of miR-23a on different cells suggest that the physiological effect of miRNAs may be cell-type dependent.

MiRNAs modulate biological functions via multiple target miRNAs. With the help of current bioinformatics, we proposed that caspase-7 and STK4, two important signal molecules associated with apoptosis in HUVECs, might be targets of miR-23a. Supporting this assumption, we first confirmed that TNF-α increased caspase-7 and STK4 expression in cultured endothelial cells. In addition, the transcripts and protein expression of caspase-7 and STK4 were regulated by miR-23a as determined by both gain-of-function and loss-of-function techniques. It is well established that caspase-3 is a downstream signaling molecule of STK4 in other types of cells. In the present study, we found that LNA-anti-miR-23a increases STK4 expression in endothelial cells and results in an increase in caspase-3 activity. In contrast, pre-miR-23a decreases STK4 expression and results in a reduction in caspase-3 activity. All of these findings indicate that STK4 and caspase-7 are indeed the functional target genes of miR-23a and are involved in the miR-23a-mediated protective effect that miR-23a has on endothelial cell apoptosis. Our data suggest that miR-23a regulates an apoptotic pathway. However, it does not seem to have any effect on Bcl-2 expression, implying that it regulates apoptosis through the death receptor pathway alone.

In summary, the present study shows that, in endothelial cells, miR-23a is sensitive to TNF-α stimulation. MiR-23a protects against TNF-α-induced injury to endothelial cells via its potential target genes, caspase-7 and STK4. These novel findings may have extensive implications for the diagnosis and treatment of a variety of cardiovascular diseases related to TNF-α, such as myocardial ischemia reperfusion injury, atherosclerosis, and sepsis.

Supplementary material is available at Cardiovascular Research online.

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