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MicroRNA-133b regulates the growth and migration of vascular smooth muscle cells by targeting matrix metallopeptidase 9

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ABSTRACT

Atherosclerosis is a systemic disease affecting the whole arterial tree of the human body, and it is the leading cause of cardiovascular diseases.Vascular smooth muscle cells (VSMCs) have been identified to play a key role in the development of atherosclerosis. MicroRNAs (miRNAs) are a group of endogenous small non-coding RNAs, and they play a critical role in many biological processes including regulating cell proliferation, migration and apoptosis. However, till now, the expression and role of miR-133b in atherosclerosis remain largely unknown. Therefore, our purpose was to investigate the expression and role of miR-133b in atherosclerosis and to explore the underlying mechanism. The results showed that miR-133b was down-regulated in the blood and vascular plaque tissues of rabbits with atherosclerosis. Matrix metallopeptidase 9 (MMP-9) was a direct target of miR-133b. In addition, our data indicated that miR-133b mimic could significantly inhibit rVSMC cell proliferation activity, migration ability and induce cell apoptosis compared with the control group, and all these effects were reversed by MMP-9-plasmid. Taken together, these findings highlight an important role for miR-133b/MMP-9 axis in atherosclerosis. And miR-133b might be a valuable clinical marker and therapeutic target for atherosclerosis.

1. Introduction

Atherosclerosis (AS) is a primary cause of major cardiovascular events, accounting for 31% of total deaths worldwide [1]. In 2016, cardiovascular diseases caused 17 million deaths, and in the past decade, the number has increased by 14.5% [2]. Atherosclerosis is characterized by the accumulation of lipids and fibrous elements in large arteries. Pathological researches have demonstrated that the composition and vulnerability of plaques play a more crucial role in the development of thrombus-mediated acute coronary events than the severity of stenosis [3]. Atherosclerosis is a serious threat to the health and safety of patients, and it places a heavy burden on families and society, but so far, the treatment of atherosclerosis is still not satisfactory. Therefore, the search for new and effective diagnostic markers and therapeutic targets for atherosclerosis is significant and imminent.

MicroRNAs (miRNAs), small non-coding RNAs that including 18–23 nucleotides, can regulate gene expression by binding to the 3′-untranslated region (UTR) of target mRNAs and trigger translation repression or mRNA cleavage [4]. Previous studies have shown that miRNAs are involved in various biological processes, such as cellular differentiation, proliferation, apoptosis, as well as stem cell maintenance, and their deregulation are associated with the development of many diseases including atherosclerosis. Vascular smooth muscle cells (VSMCs) have been identified to play a key role in the development of atherosclerosis. Recently, miRNAs were reported to have important roles in atherosclerosis, and many of them were shown to modulate the function of VSMCs. Chen et al. reported that miR-125b inhibited VSMC proliferation and migration while promoted cell apoptosis [5]. Recently, miR-124-3p was shown to be heterogeneously expressed among smokers, and increased levels of miR-124-3p might be associated with an increased risk of atherosclerotic disease [6]. Several lines of evidence have suggested that miR-124 could modulate the proliferation and migration of VSMCs [7–10].

It has been reported that miR-133b participates in myoblast differentiation and myogenic-related diseases, and it is commonly known as a muscle-specific miRNA [11–14]. Previous studies have indicated that miR-133b plays an important role in the development of neuron and fat differentiation [15–18]. MiR-133b was also involved in the development and progress of various cancers [19]. However, there has been no report on the role of miR-133b in atherosclerosis.

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Matrix metalloproteinase 9 (MMP-9), a member of the family of endopeptidases, is involved in extracellular matrix degradation and remodeling. Studies have shown that increased expression of MMP-9 in vascular component cells during vascular injury and inflammation plays a key role in the formation and rupture of atherosclerotic plaques [20]. Besides, MMP-9 has been reported to play important roles in the regulation of vascular smooth muscle cell proliferation and migration [21]. In addition, miR-133 has been revealed to play important roles in cancer cell growth and migration by targeting MMP-9 [22,23]. These findings suggested that miR-133b may play important roles in atherosclerosis development through regulating the function of VSMCs. However, till now, the relationship between miR-133b and MMP-9 in VSMCs and the role of miR-133b in VSMCs remain unclear.

Therefore, the present study aimed to explore the expression of miR-133b in atherosclerosis and to further investigate the mechanism of the effect of miR-133b on atherosclerotic vascular smooth muscle cells. We hope to provide more theoretical basis and treatment strategies for the treatment of atherosclerosis.

2. Materials and methods

2.1. Rabbit atherosclerosis model establishment

The rabbit atherosclerosis model was established as previously described [24,25]. All healthy New Zealand white rabbits of both sexes (˜2.5 kg) were obtained from the Vital River Company (Beijing, China). Animals were maintained in our laboratory under standard conditions. The use of the rabbits and all of the procedures in this study were approved by the Animal Care and Use Committee of the Second Clinical Medical College (Shenzhen People's Hospital), Jinan University.

2.2. Cell culture and cell transfection

The rabbit VSMC cells (rVSMCs) were isolated as previously described and cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco), and incubated at 37 °C with 5% CO_2. MiR-133b mimic and mimic control were synthesized by Genepharma (Shanghai, China). The control-plasmid and MMP-9-plasmid were purchased from Santa Cruz (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The rVSMCs were transfected with miR-133b mimic, mimic control, MMP-9-plasmid, control-plasmid or miR-133b mimic + MMP-9-plasmid by using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

2.3. qRT-PCR

Total RNA from blood, tissues or cells was extracted by using the Trizol Reagent (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's instructions. The RNA concentration was detected by Nanodrop2000. The total RNA was stored at −80 °C to prepare to use. MiR-133b was reversely transcribed into cDNA using the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNAs using the PrimeScript RT Reagent Kit (Takara Bio, Inc.) as per the manufacturer's protocol. SYBR Premix Ex Taq (Takara Bio, Inc.) was applied for PCR analysis. PCR was carried out as follows: 10 min at 95 °C followed by 35 cycles of 15 s at 95 °C and 40 s at 55 °C. The primer sequences used for qRT-PCR were obtained as required and listed as following:

U6 forward, 5′-GCTTCGGCAGCACATATACTAAAAT-3′;
Reverse, 5′-CGCTTCACGAATTTGCGTGTCAT-3′;
GAPDH forward, 5′-CTTTGGTATCGTGGAAGGACTC-3′;
Reverse, 5′-GTAGAGGCAGGGATGATGTTCT-3′.
miR-133b forward, 5′-CTCAGCTTTGGTCCCCTTCAAC-3′;
Reverse, 5′-GTGCAGGGTCCGAGGT-3′.
MMP-9 forward, 5′-AGACCTGGGCAGATTCCAAAC3′;
Reverse, 5′-CGGCAAGTCTTCCGAGTAGT-3′. Relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method [26] after normalization with reference to expression of U6 or GAPDH. All experiments were performed in triplicate.

2.4. Western blot assay

Proteins from cells were extracted by using a modified RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) with 1 mM PMSF for 30 min. The proteins (30 μg/lane) were separated on 10% SDS-PAGE gel and transferred to PVDF membranes (Millipore, Bedford, MA). The membranes were blocked with 5% non-fat milk for 1.5 h, followed by incubation with primary antibodies (dilution rate: 1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4 °C. As a secondary antibody (dilution rate: 1:2000; Cell Signaling Technology, Inc.), the membranes were incubated for 2 h at room temperature. Proteins were detected by using enhanced chemiluminescence (Applygen Technologies, Inc., Beijing, China) and imaged. β-actin was used as an internal control.

2.5. Transwell assay

The cell migration ability was examined by transwell assay using Transwell inserts (Corning Incorporated, Corning, NY, USA) without matrigel (BD Biosciences, Franklin Lakes, NJ, USA). After cell transfection, the transfected cells in 100 μl serum-free medium were placed into the upper chamber, at the same time, 500 μl medium with 20% FBS was added to the lower chamber. Then, the 24-wells were incubated at 37 ℃ for 24 h. The migratory cells were fixed with methanol for 20 min, and then stained with 0.5 ml 0.1% crystal violet (Beyotime Biotechnology, Shanghai, China) for 20 min. Finally, the migratory cells on the underside of the membrane were counted at five random fields under an inverted light microscope (Olympus Corporation, Tokyo, Japan).

2.6. CCK-8 assay

CCK-8 assay was performed to measure the cell proliferation. Logarithmic phase rVSMCs were seeded in a 96-well plate with 1×10^4 cells per well and incubated in the 37 °C, 5% CO_2 incubator for 12 h, after which 10 μl CCK-8 solution (Beyotime Biotechnology, Shanghai, China) was added to each well, and cells were incubated for a further 2 h at 37 °C with 5% CO_2. The absorbance was measured at a wavelength of 490 nm using a micro-plate reader (Thermo Fisher Scientific, Inc.).

2.7. Flow cytometry assay

rVSMC cells were collected in logarithmic growth phase, and the cell suspension density was adjusted and inoculated into 6-well plates at 1×10^5/well. Subsequently, cells were transfected with miR-133b mimic, mimic control, or miR-133b mimic + MMP-9-plasmid. 48 h after cell transfection, we detected apoptotic cells by using the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Cat no. 70-AP101-100; MultiSciences, Hangzhou, China) according to the manufacturer's instructions. Finally, we used flow cytometry (BD Biosciences) to analyze cell apoptosis.

2.8. Luciferase reporter analysis

TargetScan 7.2 (http://www.targetscan.org/vert_72/) was used to predict the potential targets of miR-133b, and we found the binding sites between miR-133b and 3′-UTR of MMP-9. Then, to investigate the relationship between miR-133b and MMP-9, we constructed a luciferase reporter that contains a 3′-UTR sequence of MMP-9. Cells seeded in 24-well plates were co-transfected with miR-133b mimics or mimic

control and the MUT or WT 3′-UTR of MMP-9 using Lipofectamine 2000 for 48 h, together with Renilla luciferase pRL-TK vector as a control. After transfection for 48 h, cells were lysed with RIPA buffer. The relative luciferase activity was detected using the dual-luciferase reporter assay system (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions.

2.9. Statistical analyses

We performed every experiment at least for three times. All data were showed as mean ± SD. The significance of differences between two groups was measured using the Student's *t*-test and differences between multiple groups were determined by one-way analysis of variance with Tukey's post hoc test. Statistical significance was set at $p < 0.05$. Data analyses were performed by using SPSS software version 17.0 (IBM Corp., Armonk, NY, USA).

3. Results

3.1. The expression of miR-133b in the blood specimens and vascular plaque tissues from rabbits with atherosclerosis

To explore the role of miR-133b in atherosclerosis, we firstly performed qRT-PCR to detect the relative expression of miR-133b in rabbits with atherosclerosis. The expression of miR-133b was significantly reduced in the blood of rabbits with atherosclerosis compared with normal controls (Fig. 1A); Moreover, the expression of miR-133b was significantly reduced in atherosclerotic plaque tissues compared to normal vascular tissue (Fig. 1B).

3.2. MMP-9 was the target gene of miR-133b

Next, to predict the targets of miR-133b, TargetScan 7.2 (http://www.targetscan.org/vert_72/) was performed. TargetScan results revealed that miR-133b has thousands of target genes, including MMP-9 (Fig. 2A). MMP-9 has been confirmed to play important roles in the regulation of vascular smooth muscle cell proliferation and migration and in atherosclerosis [20,21]. And And the relationship between miR-133b and MMP-9 in VSMCs remain unclear. Therefore, we hypothesized that miR-133b may be involved in atherosclerosis through regulating the function of VSMCs by targeting MMP-9. Thus, we choose MMP-9 for further studies. Then, we picked for a dual luciferase reporter assay to examine whether miR-133b interacts directly with the target gene MMP-9. The reporter with wild-type MMP-9 3′UTR exhibited markedly lower luciferase activity in rVSMC cells transfected with miR-133b mimic compared with luciferase activity in cells transfected with mimic control. However, the decrease in luciferase activity was abolished by the mutant 3′UTR (Fig. 2B). Taken together, these results showed that MMP-9 was the direct target gene of miR-133b.

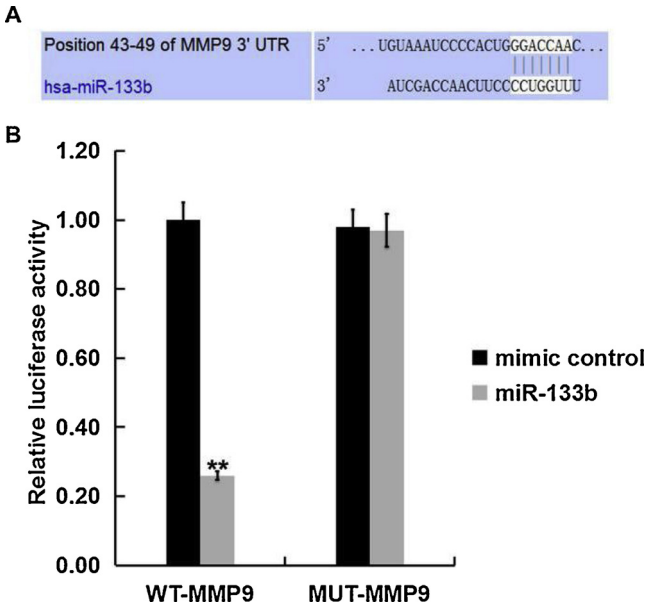

Fig. 2. MMP-9 is the target gene of miR-133b.
TargetScan predicted MMP-9 3′-UTR binding site for miR-133b. (B) miR-133b mimic or mimic control and wild-type or mutant MMP-9 3′UTR were co-transfected into rVSMC cells. Luciferase activity was detected by dual-luciferase reporter assay. Data were displayed as mean ± SD. **p < 0.01 vs. mimic control.

3.3. Transfection efficiency of miR-133b mimic and MMP-9-plasmid in rVSMC cells

The rVSMC cells were transfected with miR-133b mimic, mimic control, MMP-9-plasmid, control-plasmid, or miR-133b mimic + MMP-9-plasmid. We performed qRT-PCR or/and western blot assay to detect the transfection efficiency of miR-133b mimic and MMP-9-plasmid. The results indicated that miR-133b mimic significantly increased miR-133b expression in rVSMC cells (Fig. 3A). MMP-9-plasmid significantly increased MMP-9 mRNA and protein expression in rVSMC cells (Fig. 3B and C). Next, we found that miR-133b mimic significantly reduced

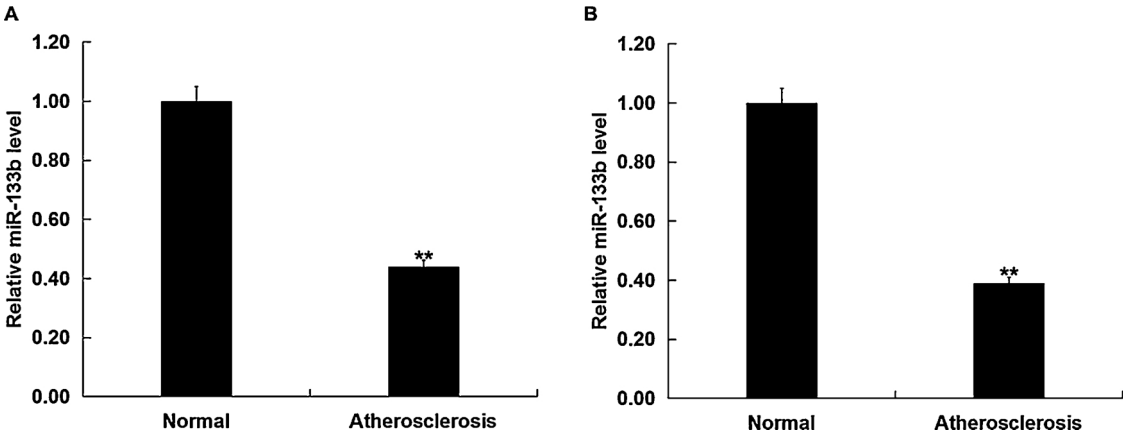

Fig. 1. miR-133b was down-regulated in the blood and plaque tissues of rabbits with atherosclerosis.
(A)qRT-PCR assay detected the relative expression of miR-133b in the blood of rabbits. (B) qRT-PCR assay detected the relative expression of miR-133b in the atherosclerotic vascular plaque tissue of rabbits. Normal: the normal control rabbits; Atherosclerosis: rabbits with atherosclerosis. Data were displayed as mean ± SD. **p < 0.01 vs. Normal.

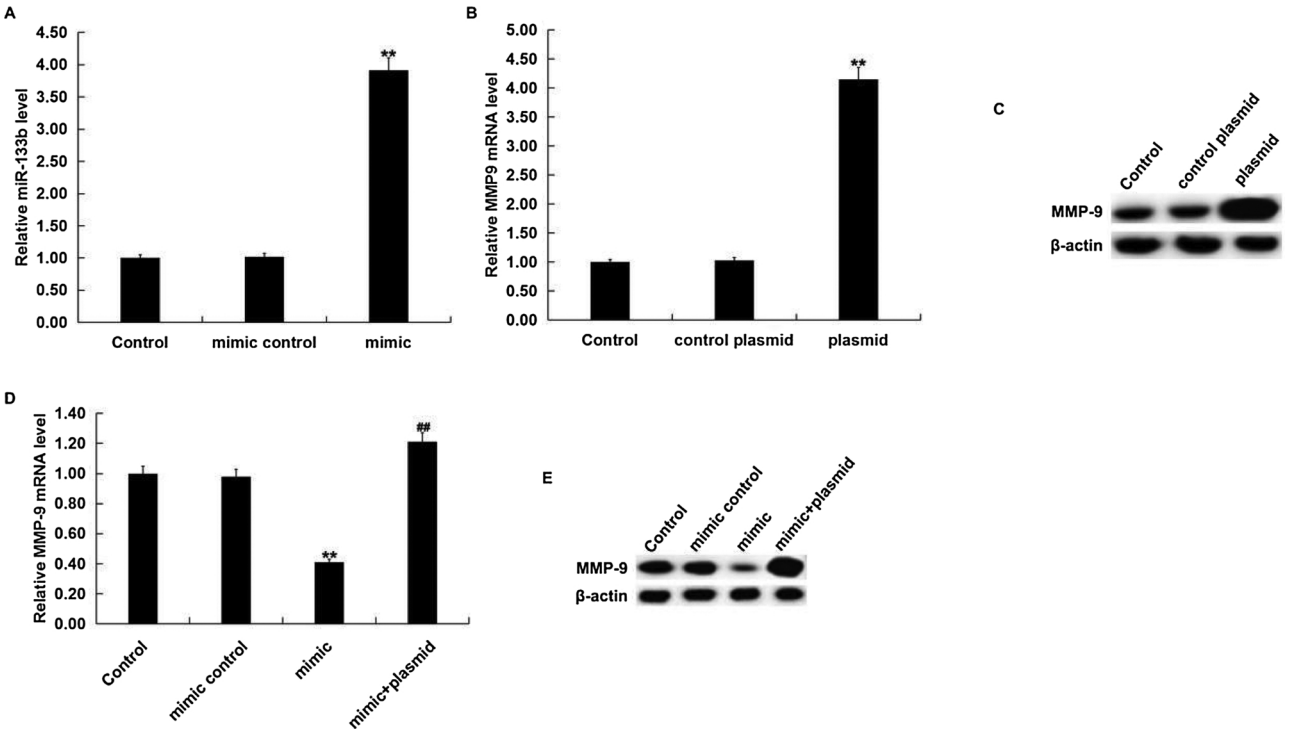

Fig. 3. Transfection efficiency of miR-133b mimic and MMP-9-plasmid in rVSMCs.
(A) qRT-PCR assay measured the relative expression of miR-133b after the rVSMC cells were transfected with miR-133b mimic, mimic control for 48 h. (B) qRT-PCR assay and (C) western blot assay detected the expression of MMP-9 after the rVSMC cells were transfected with MMP-9-plasmid, control-plasmid for 48 h. (D) qRT-PCR assay and (E) western blot assay detected the expression of MMP-9 after rVSMC cells were transfected with miR-133b mimic, mimic control, or miR-133b mimic + MMP-9-plasmid for 48 h. Control: cells without any treatment; mimic control: cells transfected with mimic control; mimic: cells transfected with miR-133b mimic; control-plasmid: cells transfected with control-plasmid; plasmid: cells transfected with MMP-9-plasmid; mimic + plasmid: cells co-transfected with miR-133b mimic and MMP-9-plasmid. Data were displayed as mean ± SD. **p < 0.01 vs. Control; ##p < 0.01 vs. mimic.

MMP-9 mRNA and protein expression in rVSMC cells, and these decreases were significantly reversed by MMP-9-plasmid (Fig. 3D and E).

3.4. Effect of miR-133b on proliferation, migration and apoptosis of rVSMC cells

In order to shed light on the function of miR-133b in rVSMC cells. We then investigated the effect of miR-133b on the proliferation of rVSMC cells. CCK-8 assay results showed that miR-133b mimic could significantly inhibit cell proliferation activity after transfection compared with the control group, and this inhibition was reversed by MMP-9-plasmid (Fig. 4A). Next, we performed transwell assay to detect the ability of cell migration. The results showed that miR-133b mimic significantly inhibited the migration of vascular smooth muscle cells, which was significantly reversed by MMP-9-plasmid (Fig. 4B). To further determine whether miR-133b could regulate apoptosis, we performed flow cytometry assay to detect cell apoptosis. Flow cytometry analysis showed that miR-133b mimic could obviously induce cell apoptosis, and this increase was reversed by MMP-9-plasmid (Fig. 4C and D).

4. Discussion

MiRNAs have been found to have a role in determining the phenotype and modulating proliferation of VSMCs. In the present study, we focused on the in vitro influence of miR-133b on atherosclerotic vascular smooth muscle cells and found that miR-133b was significantly reduced in the blood and atherosclerotic plaque tissues of rabbits with atherosclerosis. Moreover, we demonstrated that MMP-9 was the direct target gene of miR-133b, and the inhibitory effects of miR-133b on VSMC proliferation and migration were reversed by MMP-9 up-regulation.

In recent years, different types of miRNAs were shown to play a crucial role in the development and process of atherosclerosis. MiR-124 is a highly conserved miRNA [3], and it is found in many animal species. Previous studies showed that miR-124 could inhibit VSMC proliferation and migration by down-regulating specificity protein 1 (Sp1) [9] or by directly targeting the IQ motif containing GTPase activating protein 1 (IQGAP1) that is an enzyme that regulates cell proliferation [27]. MiR-155 has been reported to inhibit VSMC differentiation, possibly by decreasing angiotensin II type 1 receptor expression [28], whereas miR-21 stimulates cell proliferation by affecting the expression of phosphatase and tensin homolog and Bcl-2 [29].

Previous researches showed that miR-133 expression was associated with the contractile VSMCs, where it has been connected to the inhibition of cell proliferation and migration and promotion of contractility. However, the role miR-133b in atherosclerosis and vascular smooth muscle cells is still unclear. In our data, we found that miR-133b played a crucial role in the proliferation and migration of atherosclerotic vascular smooth muscle cells by targeting MMP-9.

Matrix metalloproteinase 9 (MMP-9) played a crucial role in the degradation of type IV and V collagens, and its expression levels are raised in individuals with atherosclerosis and acute ischemic stroke [30]. In addition, MMP-9 plays a pivotal role in early atherosclerosis, vascular remodeling, and the development of arterial plaque rupture [31–34]. It has been reported that miR-491-5p confers increased risk for atherosclerotic cerebral infarction in a Chinese population by targeting MMP-9 [35]. However, there was no clear evidence showing the relationship between miR-133b and MMP-9 in VSMCs. We performed TargetScan and dual-luciferase reporter assay to analyze the target gene of miR-133b. Our study gave evidence to demonstrate that MMP-9 was a direct target gene of miR-133b. Moreover, MMP-9 was inversely

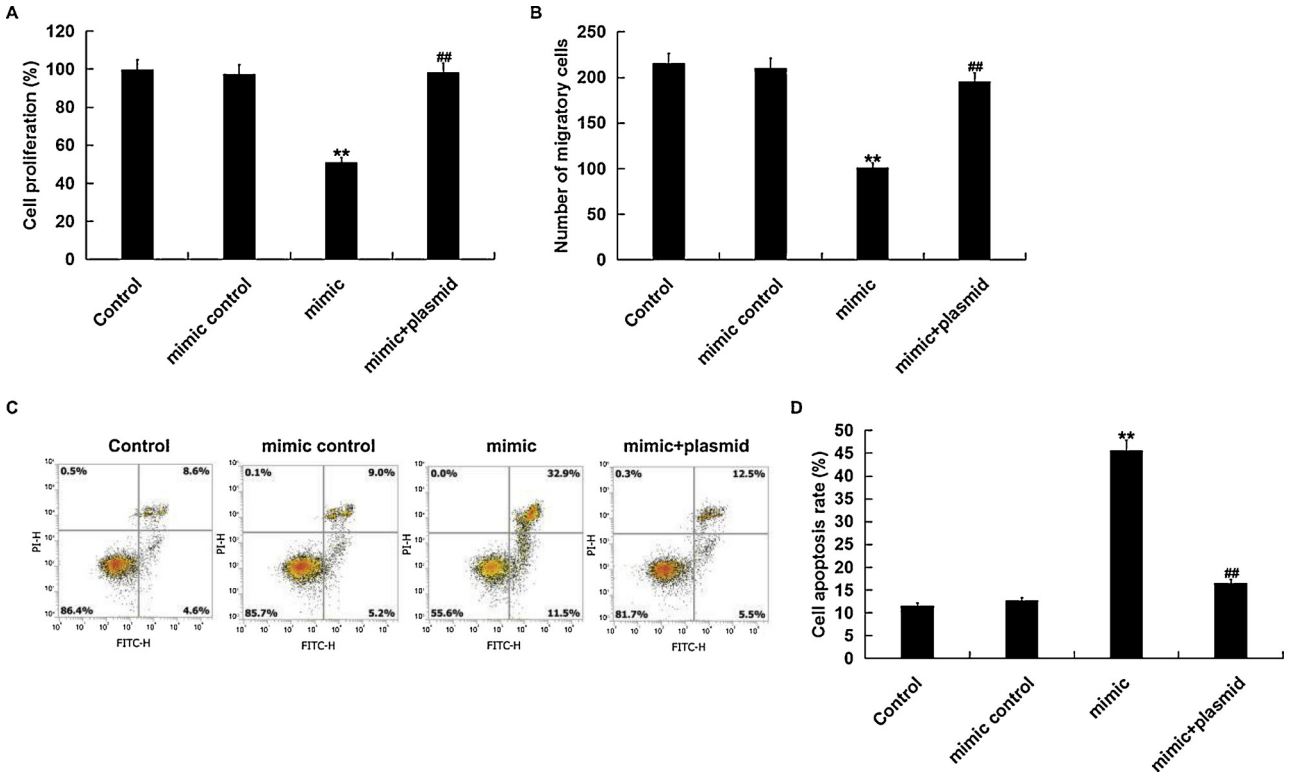

Fig. 4. miR-133b could inhibit cell proliferation, migration and induce cell apoptosis of rVSMCs.
(A) CCK-8 assay determined cell proliferation after rVSMC cells were transfected with mimic control, miR-133b mimic or miR-133b mimic + MMP-9-plasmid for 48 h. (B) Transwell assay detected the cell migration ability after rVSMC cells were transfected with mimic control, miR-133b mimic or miR-133b mimic + MMP-9-plasmid for 48 h. (C and D) Flow cytometry assay analyzed cell apoptosis (early apoptosis + late apoptosis) after rVSMC cells were transfected with mimic control, miR-133b mimic or miR-133b mimic + MMP-9-plasmid for 48 h. Control: cells without any treatment; mimic control: cells transfected with mimic control; mimic: cells transfected with miR-133b mimic; mimic + plasmid: cells co-transfected with miR-133b mimic and MMP-9-plasmid. Data were displayed as mean ± SD. **p < 0.01 vs. Control; ##p < 0.01 vs. mimic.

correlated with miR-133b expression in rVSMCs. Next, to investigate the role of miR-133b in rVSMC cells, rVSMC cells were transfected with mimic control, miR-133b mimic or miR-133b mimic + MMP-9-plasmid for 48 h. And we found that miR-133b inhibited cell proliferation and migration ability and promoted cell apoptosis of rVSMCs, which were reversed by MMP-9-plasmid. Thus, our work showed the inhibitory function of miR-133b on VSMCs and provided a possible therapeutic target for atherosclerotic diseases. However, this study is only a preliminary study of the role of miR-133b in atherosclerosis. To make the role of miR-133b in atherosclerosis more convincing, a lot of research is still needed. For example, there is a huge difference between in vitro experiments and true human atherosclerosis, thus more in vivo and clinical experiments should be performed to reveal the role of miR-133b in atherosclerosis. Besides, the expression of miR-133b and MMP-9 in patients with atherosclerosis, and the correlation of miR-133b/MMP-9 expression with the clinical characteristics of patients with atherosclerosis should be further determined. And we will study these issues in the future.

5. Conclusion

MiR-133b was down-regulated in atherosclerosis, and its over-expression significantly inhibited proliferation and migration, and promoted apoptosis of rVSMC cells by targeting MMP-9. MiR-133b may be a therapeutic target for atherosclerosis treatment.

Disclosures

All authors declare no financial competing interests.

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