Int J Clin Exp Pathol 2019;12(5):1846-1858
www.ijcep.com /ISSN:1936-2625/IJCEP0089494

Original Article

MiR-210 inhibits hypoxia-induced apoptosis of smooth muscle cells via targeting MEF2C

Qiuhong Bao[1], Haiyu Jia[1], Rong A[2], Zhongchao Cao[1], Yong Zhang[1]

[1]*VIP Healthcare Wards,* [2]*Department of Cardiology, Affiliated Hospital of Inner Mongolia Medical University, Hohhot, Inner Mongolia, China*

Received December 6, 2018; Accepted March 26, 2019; Epub May 1, 2019; Published May 15, 2019

Abstract: Background: Vascular smooth muscle cell (VSMC) apoptosis plays an important role in vascular remodeling and atherosclerotic plaque instability. Growing evidence suggests that microRNAs (miRNAs) play a critical role in VSMC function, however, the underlying mechanism remains unclear. Methods: This study used a hypoxic-induced VSMC apoptosis model. Expression of miR-210, its target MEF2C, and other key factors of apoptosis were detected and measured by real-time PCR and western blot. Luciferase reporter assay was performed to detect the miR-210 target. The function of miR-210 in apoptosis was determined using flow cytometric cell apoptosis assays. The relationship between miR-210 and MEF2C was confirmed and key apoptosis factors were detected. Results: The restoration of miR-210 function in cells transfected with a miR-210 mimic inhibited VSMC apoptosis compared to control. MiR-210 overexpression inhibited the expression of Bax, Bad, cleaved Caspase-3, and promoted the expression of Bcl-2, Caspase-3, Caspase-9 and mitochondrial cytochrome c at both the mRNA and protein levels. Results also found that MEF2C was a direct target of miR-210 in hypoxic VSMCs. Further, miR-210 suppressed MEF2C expression by directly binding to its 3'-untranslated region and the expression of miR-210 was negatively correlated with MEF2C mRNA levels. Conclusions: Results from this study provide the first evidence that miR-210 can inhibit apoptosis by targeting MEF2C in hypoxic VSMCs and may support the development of new biomarkers and therapeutic targets for atherosclerosis.

Keywords: miR-210, VSMCs, hypoxia-induced apoptosis, MEF2C

Introduction

Atherosclerosis is a disease of the arteries, which can result in stroke and acute coronary syndrome such as myocardial infarction and other conditions with high mortality rates [1]. The pathological process of atherosclerosis is initiated by endothelial dysfunction and structural alterations, followed by aortic media change and vascular smooth muscle cell (VSMC) modification [2]. Among these processes, accumulating evidence shows that VSMC apoptosis plays an important role in vascular remodeling. A study by Zhao et al found that ClAPIN1 siRNA promotes VSMC apoptosis by regulating Bax and Bcl-2, which may provide a novel therapeutic strategy for vascular diseases [3]. Another study found that VSMC apoptosis is an early trigger for hypothyroid atherosclerosis [4].

MicroRNAs are short, single-stranded, non-coding RNAs that play critical regulatory role in many biological processes such as proliferation [5], migration [6], differentiation [5] and apoptosis [7-9]. Recent studies have found that miRNA expression is modified by various stimuli involved in different stages of atherosclerosis [10, 11]. Further, there are numerous studies that indicate miRNAs may play regulatory role in VSMC apoptosis regulation. For example, miR-92 inhibited VSMC apoptosis by regulating the MKK4-JNK pathway under oxidative stress [12]. Research has also shown that miR-21 has an anti-apoptotic effect in VSMCs both in vitro and in vivo [13]. Furthermore, PTEN has been found to regulate VSMC apoptosis through the inhibition of AKT activation which is regulated by miR-21 [14]. In Leeper's study, miR-26 was found to promote apoptosis in VSMCs while inhibiting proliferation and migration [15]. Finally, miR-210 gathers more light in hypoxic induced cell apoptosis [16, 17]. Given these findings, there is interest in this field to explore the pathological mechanism and novel treatment options for atherosclerosis.

MiR-210 is a robust target of hypoxia-inducible factor (HIF)-1 target gene, which participates in hypoxia-dependent disease states, among many other diseases. For example, miR-210 is upregulated in most tumors and correlated with poor clinical outcomes. In a study by Li et al, upregulated miR-210 promoted epithelial ovarian cancer cell proliferation and inhibited apoptosis by targeting PTPN1 [18]. In hypoxic human hepatoma cells, miR-210 downregulation significantly inhibited cell viability, induced cell arrest in the G0/G1 phase, increased the rete of apoptosis and enhanced radiosensitivity [19]. In addition, miR-210 regulates human U251 glioma cells apoptosis and proliferation by directly down-regulating SIN3A protein expression [20]. Under hypoxic conditions, miR-210 increased colon cancer cell proliferation and further reduced radiosensitivity by the HIF-1α/miR-210/Bcl-2 pathway [21]. BNIP3, a target of miR-210, was found to be suppressed by miR-210 and over-expression of miR-210 decreased apoptosis in neural progenitor cells [22]. Furthermore, except in cancer cell lines, brain tissue is rich in miR-210 and has anti-apoptosis functions in cerebral ischemia/reperfusion [23]. Although studies exist that focus on the relationship between miR-210 and atherosclerosis, the comprehensive mechanism of VSMC apoptosis remains unclear.

The aim of this study is to identify the role of miR-210 and its target in VSMC apoptosis and further describe its underlying mechanisms. This may also provide a theoretical basis for clinical treatment.

Materials and methods

Cell culture and hypoxia treatment

The VSMCs line was purchased and cultured and maintained in DMEM (Invitrogen) containing 10% FBS (Invitrogen) in 5% CO_2 at 37°C. At the same time, 100 U/ml penicillin and 100 μg/ml streptomycin, 25 ng/ml epidermal growth factor, and 10 ng/ml basic fibroblast growth factor were added as supplement. The culture medium was changed every 3 days. After 7-10 days, the cells were grown to confluence, and passaged using 0.25% trypsin, and grown in DMEM containing 10% FBS plus antibiotics. At each time of isolation, cells were stained positively with monoclonal antibody to smooth muscle actin, which is characteristic of VSMCs. For hypoxia treatment, VSMCs were exposed to a stable flow of low-oxygen gas mixture (1% O_2, 5% CO_2, 94% N_2) in a modular incubator chamber. The treated VSMCs were incubated and collected for the following experiments. The HEK293 cells were cultured and grown in DMEM supplemented with 10% FBS according to the manufacturer's specifications followed in a previous study [24].

Plasmids and miRNA transfection

Transfections with miR-210 mimic, miR-210 inhibitor or scrambled control at a final concentration of 50 nmol/L were performed using Lipofectamine 2000 reagent following the manufacturer's recommendations. Lipofectamine 2000 was diluted and plasmids were added to the solution. The solution was then added into a 6-well-plate and incubated for 6 hours at 37°C, 5% CO_2. The siRNA was utilized to block the expression of MEF2C, siRNA duplexes and siRNA transfection reagent were mixed and added into 100 mL siRNA transfection medium for 45 minutes at 20°C. The medium was then changed to conventional medium and cultured sequentially for 48 hours. After 48 h of transfection, the transfection efficiency of the VSMCs was measured using SYBR Green quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR).

Luciferase reporter assay

For Luciferase assay, 1×10^5 HEK293 cells were cultured, and a segment of the 3'-UTR of MEF2C was cloned into pMiR-Report. A mutated 3'-UTR of MEF2C was then introduced to the potential miR-210 binding site. Reporter vectors containing the wild type or mutant of MEF2C and miR-210 mimic were then co-transfected into HEK293 cells. Luciferase activities were detected using a Dual-Luciferase Assay System (Invitrogen, USA) 48 h after transfection.

Flow cytometric cell apoptosis assays

VSMCs apoptosis was conducted by flow cytometry using the Annexin V-FITC Apoptosis Detection Kit (Sigma, MO, USA) according to the manufacturer's specifications [25]. VSMCs were incubated with state culture medium in hypoxia precondition or normal precondition for 24 h after transfection with miR-210 mimics, inhibitor or control. VSMCs were then collected, washed with fresh PBS, resuspended in 100 μl

Table 1. Primer sequences for RT-PCR analysis

cDNA	Forward primer	Reverse primer
miR-210	5'-GTCCAGTTTTCCCAGGAATCC-3'	5'-TCGCTTCGGCAGCACATAT-3'
Bcl-2	5'-GTGGATGACCTAGGCAAGTCG-3'	5'-GTCTCCTCCTTGTTGTTCTGC-3'
Caspase-3	5'-AGAAGTCAGAGTTCAGAGGCGTCC-3'	5'-AGTAGAAGGCTGTCACCAAGCCAAC-3'
c-Caspase-3	5'-TCCAACCTGAGTGACATAGCGA-3'	5'-CTGACCTCCAACTCCAACGAAT-3'
Caspase-9	5'-CCGGAATTCGAGCCTACAGCAGAACC-3'	5'-CCGCTCGAGTTTGATCCCCTCTGTTA-3'
Mito-cyt c	5'-GAC GCG GAC GAT GAT GTG AAC-3'	5'-TTG TAC TGT TGT GGA TTG AAG-3'
Cyto-cyt c	5'-CACCCAACA TGT TTA CAA ACA ATG AGA-3'	5'-CTGCAGCAACAG TAA GGA ACA TCC TAT-3'
Bad	5'-GGA AGA GAA CTT TGC CGT TGA A-3'	5'-GTG ACG AGC CAT TTC CTC CTT-3'
Bax	5'-CACGACACCGGACAAACCA-3'	5'-GCCGGGAGCTATCTTTCTTAAGTG-3'
MEF2C	5'-AAGACG GAGAGGCATCATCGAG-3'	5'-CAGATCCCTGCTTCTCAAAGGCAC-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'
β-actin	5'-ACAACTTTGGTATCGTGGAAGG-3'	5'-GCCATCACGCCACAGTTTC-3'

c-Caspase-3, cleaved Caspase-3; Mito-cyt c, mitochondrial cytochrome c; Cyto-cyt c, cytoplasmic cytochrome c.

of 1X binding buffer and stained with Annexin V-FITC for 10 min and 5 μl of PI (Becton-Dickinson) at room temperature for 15 min in the dark. Finally, propidium iodide was added at a final concentration of 1 mg/L. A flow cytometer was conducted to evaluate the apoptotic levels and cell cycle in each sample following the manufacturer's specifications. Stained cells were analyzed using a FACScalibur.

Western blot

The Western blot analysis was conducted as previously described [26]. VSMCs were lysed with M-PER Protein Extraction Reagent (Pierce, Rockford, IL) and supplemented with a protease inhibitor. The total cellular protein concentration was then assessed using the BCA protein assay. Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes. After blocking for 4 h in 5% skim milk, the 1:1000 dilution of primary antibodies against Bcl-2, Caspase-3, cleaved Caspase-3, Caspase-9, Bad, Bax, cytochrome c and MEF2C (rabbit; Abcam; UK) were added and incubated with the membrane at 4°C overnight. After being washed with Tris buffer solution, the secondary antibody was added in the solutions and incubated for 1 h. Chemiluminescent detection was performed using an ECL kit. Gray value of the bands was analyzed by Image-J software.

RNA isolation and qRT-PCR

For RNA isolation and qRT-PCR detection, the primers used for amplification are described in **Table 1**. Total RNA from VSMCs were extracted by TRIzol™ Reagent following the manufacturer's instructions. The isolated RNAs were then polyadenylated using the miRNA first-strand cDNA synthesis kit (Invitrogen). The cDNA was then prepared using a Reverse Transcription System. The cDNA synthesized was used to perform quantitative PCR on an ABI 7500 real-time PCR system thermocycler using SYBR Premix Taq. Amplification was performed as follows: 95 C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The results were analyzed using the relative standard curve method of ΔΔCt method. Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II were used for miR-210 detection in turns for reverse transcription and qRT-PCR, respectively. U6 acted as an internal control.

Statistical analyses

Statistical analysis was performed using Graph Pad Prism 5.1 software. Results are expressed as the mean ± standard deviation. When only two groups were compared, the two-tailed Student's t-test was used to assess statistical differences. Binding levels of in vitro autoradiography experiments were compared using one-way ANOVA. *P* value less than 0.05 ($P< 0.05$) was considered statistically significant.

Results

MiR-210 expression in hypoxia model

The morphology of VSMCs was demonstrated under hypoxia conditions at different time points (0.5 h, 1 h, 1.5 h, 2 h and 3 h). The mor-

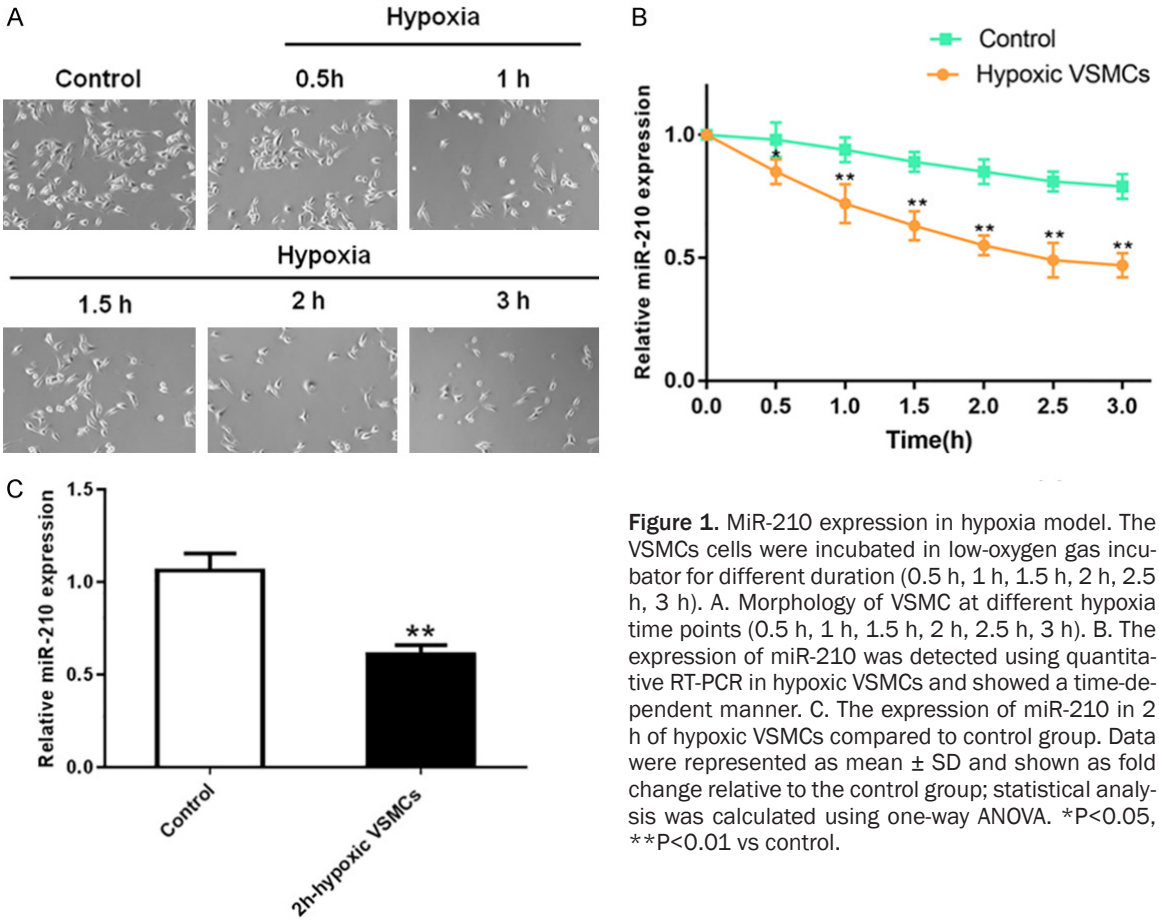


Figure 1. MiR-210 expression in hypoxia model. The VSMCs cells were incubated in low-oxygen gas incubator for different duration (0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, 3 h). A. Morphology of VSMC at different hypoxia time points (0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, 3 h). B. The expression of miR-210 was detected using quantitative RT-PCR in hypoxic VSMCs and showed a time-dependent manner. C. The expression of miR-210 in 2 h of hypoxic VSMCs compared to control group. Data were represented as mean ± SD and shown as fold change relative to the control group; statistical analysis was calculated using one-way ANOVA. *P<0.05, **P<0.01 vs control.

phology of different VSMC groups is shown in **Figure 1A**, with the morphology of VSMCs changing markedly with increased hypoxia condition time. MiR-210 expression was detected and analyzed at different time points (0.5 h, 1 h, 1.5 h, 2 h, 2.5 h and 3 h) to investigate its change in hypoxia conditions using RT-PCR. The results of qRT-PCR showed that miR-210 expression was significantly decreased in hypoxic VSMCs in a time-dependent manner, as shown in **Figure 1B** and **1C**. These results demonstrate that miR-210 is downregulated in hypoxic VSMCs in time-dependent manner.

Effect of hypoxia on the expression of apoptotic factors in hypoxic VSMCs

Previous studies have shown that under hypoxic conditions, the expression of apoptotic factors, such as Bcl-2, Bad, Bax, Bad Caspase-3 and Caspase-9 resulted in changes in different cell lines [21, 27, 28]. It has been demonstrated that mitochondrial damage is critical to enable the transmission of mitochondrial pro-apoptotic factors into the cytoplasm/nucleus in hypoxia conditions [28]. Furthermore, as an anti-apoptotic factor, Bcl-2 is decreased in hypoxic conditions [17, 29]. However, Bax and Caspase-3 show the opposite effect [30]. In order to understand the expression of these apoptotic factors in hypoxic VSMCs, and the relationship between hypoxia and mitochondrial dysfunction (i.e. whether hypoxia condition amplifies mitochondrial apoptosis in VSMCs), qRT-PCR and western blotting were sued to detect the mRNA and protein levels of Bcl-2, Bax, Bad, and Caspase-3, cleaved Caspase-3, Caspase 9, mitochondrial cytochrome c (Mito-cyt c) and cytoplasmic cytochrome c (Cyto-cyt c) respectively. Results found that VSMCs incubated in hypoxic conditions showed significant apoptosis activity compared to controls (**Figure 2A** and **2B**). The level of mito-cyt c mRNA and protein expression was significantly decreased, whereas the expression of cyto-cyt c was increased in hypoxia condition (**Figure 2C-E**). In addition, results showed that mitochondrial apoptotic proteins such as cleaved Caspase-3

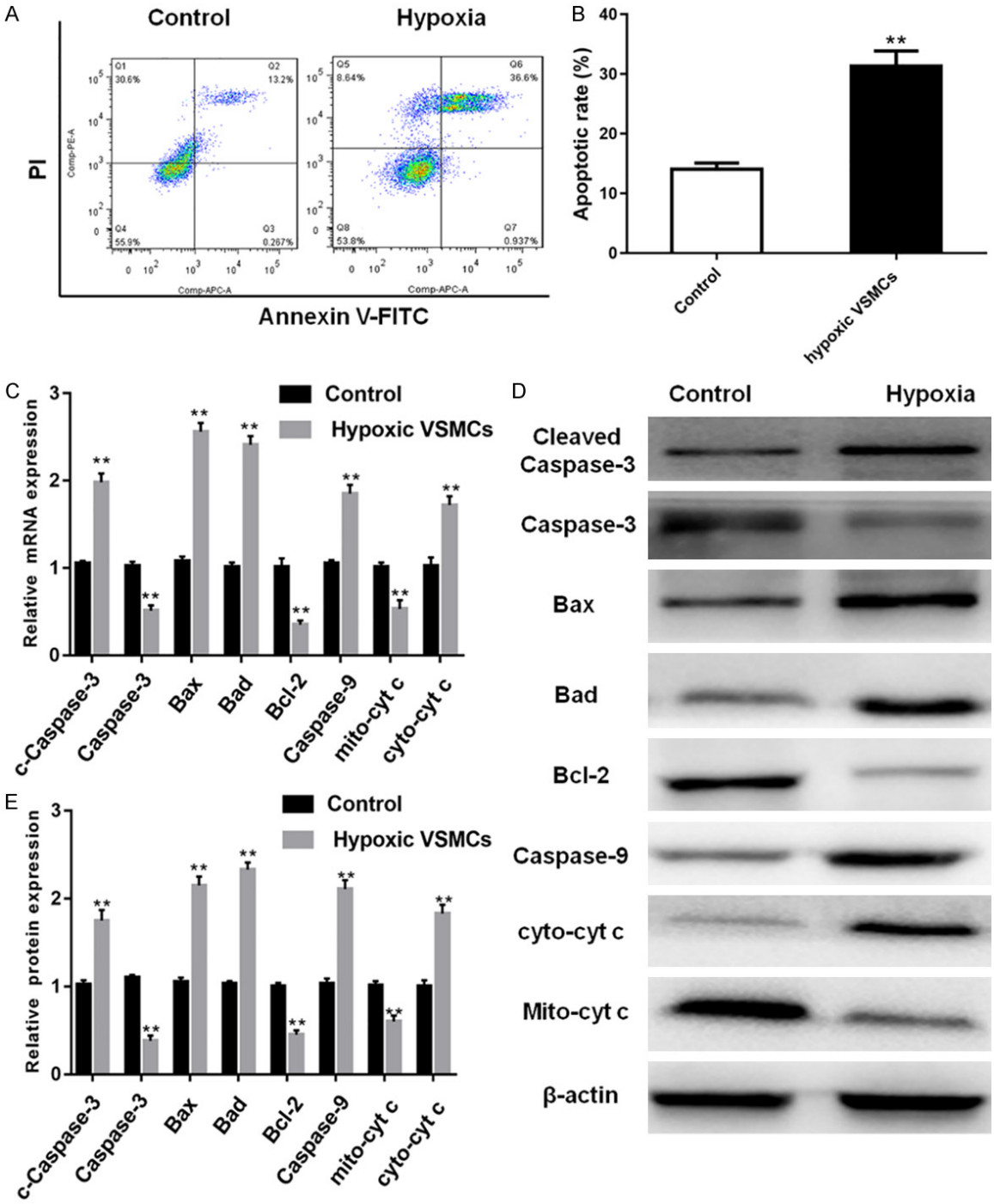


Figure 2. Apoptosis rate and apoptosis-related factor expression in hypoxic VSMCs. A and B. Cell apoptosis rate of VSMCs incubated in hypoxia condition and normal condition; C. The mRNA of mitochondrial apoptotic proteins were analyzed by RT-PCR in hypoxic VSMCs and control; D and E. The protein of mitochondrial apoptotic proteins were analyzed by western blot in hypoxic VSMCs and control. Data were represented as mean ± SD and shown as fold change relative to the control group; statistical analysis was calculated using two-tailed Student's t test. *P<0.05, **P<0.01 vs control.

Bad, Bax and caspase-9 increased. Conversely, the expression of Bcl-2 and Caspase-3 was decreased in the hypoxia treatment group (**Figure 2C-E**). Taken together, results suggest that mitochondrial apoptosis of VSMCs is amplified by hypoxia treatment.

MiR-210 overexpression attenuates hypoxia-induced apoptosis in VSMCs

MiR-210 mimics and inhibitors were used in order to investigate the role of miR-210 in hypoxic VSMCs. Results found that miR-210 expression was increased in VSMCs transfected with miR-210 mimics and decreased in VSMCs transfected with miR-210 inhibitor (**Figure 3A**). As shown in **Figure 3B** and **3C**, transfection of miR-210 mimic into VSMCs inhibited apoptosis, whereas transfection of miR-210 inhibitor promoted apoptosis in the hypoxia treatment compared to the control group. Although the rate of apoptosis was reduced by the transfected miR-210 mimic, it was still higher than the normal control (**Figure 3B** and **3C**). In order to determine if intrinsic apoptotic signaling was involved, the expression of apoptotic factors (Bcl-2, Bax and cleaved Caspase-3) was measured in miR-210 mimics and inhibitors respectively. The result indicated that miR-210 mimic significantly inhibited Bax and cleaved Caspase-3 expression, and promoted Bcl-2 expression in hypoxic VSMCs measured by PCR and western blot. Conversely, hypoxic VSMCs transfected with miR-210 inhibitors reversed the above results (**Figure 3D-F**). In addition, the mRNA and protein level of Bax and cleaved Caspase-3 was reduced by transfected miR-210 mimic, but it was still higher than the normal control (**Figure 3D-F**). These results demonstrate that miR-210 attenuates apoptosis of hypoxic VSMCs.

MiR-210 is confirmed as a direct target of MEF2C

Further experiments aimed to identify the target of miR-210 to better understand its underlying mechanism. Bioinformatic algorithms (TargetScan, Pictar and miRBase Targets) predicted that the target of miR-210 was MEF2C (**Figure 4A**). In order to confirm that result, dual-luciferase reporter assay was used. Wild type pGL3-MEF2C-WT-3'-UTR or pGL3-MEF2C-Mut-3'-UTR was co-transfected with miR-210 mimics or scramble in HEK-293T cells. As shown in **Figure 4B**, after MEF2C-3'-UTR-mut group was transfected with miR-210 mimics or scramble, the luciferase activity between MEF2C-3'-UTR-Mut and MEF2C-3'-UTR-Mut + mimics groups showed no statistically significant difference. Furthermore, to identify whether miR-210 regulates the expression of MEF2C in VSMCs, the mRNA and protein levels of MEF2C were measured using qRT-PCR and western blotting, respectively. As shown in **Figure 5C-E**, overexpression of miR-210 suppressed both the mRNA and protein levels of MEF2C. These data suggested that miR-210 negatively regulates MEF2C expression in VSMCs, and MEF2C may be a direct target of miR-210.

MiR-210 overexpression inhibits VSMCs hypoxia-induced apoptosis by targeting MEF2C

After determining that MEF2C is a direct target of miR-210 in VSMCs, we investigated whether apoptosis is mediated by the down regulation of MEF2C. MEF2C plasmid, pcDNA-MEF2C and miR-210 mimic were co-transfected into hypoxic VSMCs. Results found that the miR-210 mimic suppressed the expression of MEF2C in hypoxic VSMCs, which was reversed by pcDNA-MEF2C (**Figure 5A-C**). Results also indicated that overexpression of MEF2C increased apoptosis rate, which was rescued by co-transfected miR-34a with the pcDNA-MEF2C (**Figure 5D**, **5E**). To further identify whether overexpression of miR-210 mediates hypoxia-induced apoptosis in VSMCs by targeting MEF2C, we performed qRT-PCR and western blot analysis to detect the mRNA level and protein expression of Bcl-2, Bax and cleaved Caspase-3 in different transfected groups. As shown in **Figure 5F**, **5G** the pcDNA-MEF2C significantly reduced the effects of miR-210 mimics on the expression of Bcl-2, Bax and cleaved Caspase-3 in hypoxic VSMCs at both mRNA and protein levels. Taken together, these data indicate that miR-210 mimics protect hypoxic VSMCs from apoptosis by targeting MEF2C.

Discussion

Atherosclerosis is a chronic progressive inflammatory disease and is the leading cause of death worldwide [31]. VSMCs are involved in the remodeling of arterial walls in affected vessels due to atherosclerotic changes [32], and VSMC proliferation in atherosclerosis seems to be predominantly reparative [33]. More recent studies have shown that VSMC phenotypic switching results in less-differentiated forms and promotes the process of atherosclerosis [34-36]. Apoptosis of VSMCs in atherosclerotic lesions may be related to plaque rupture, triggered by oxidative stress present in the diseased vascular milieu [37]. This evidence sh-

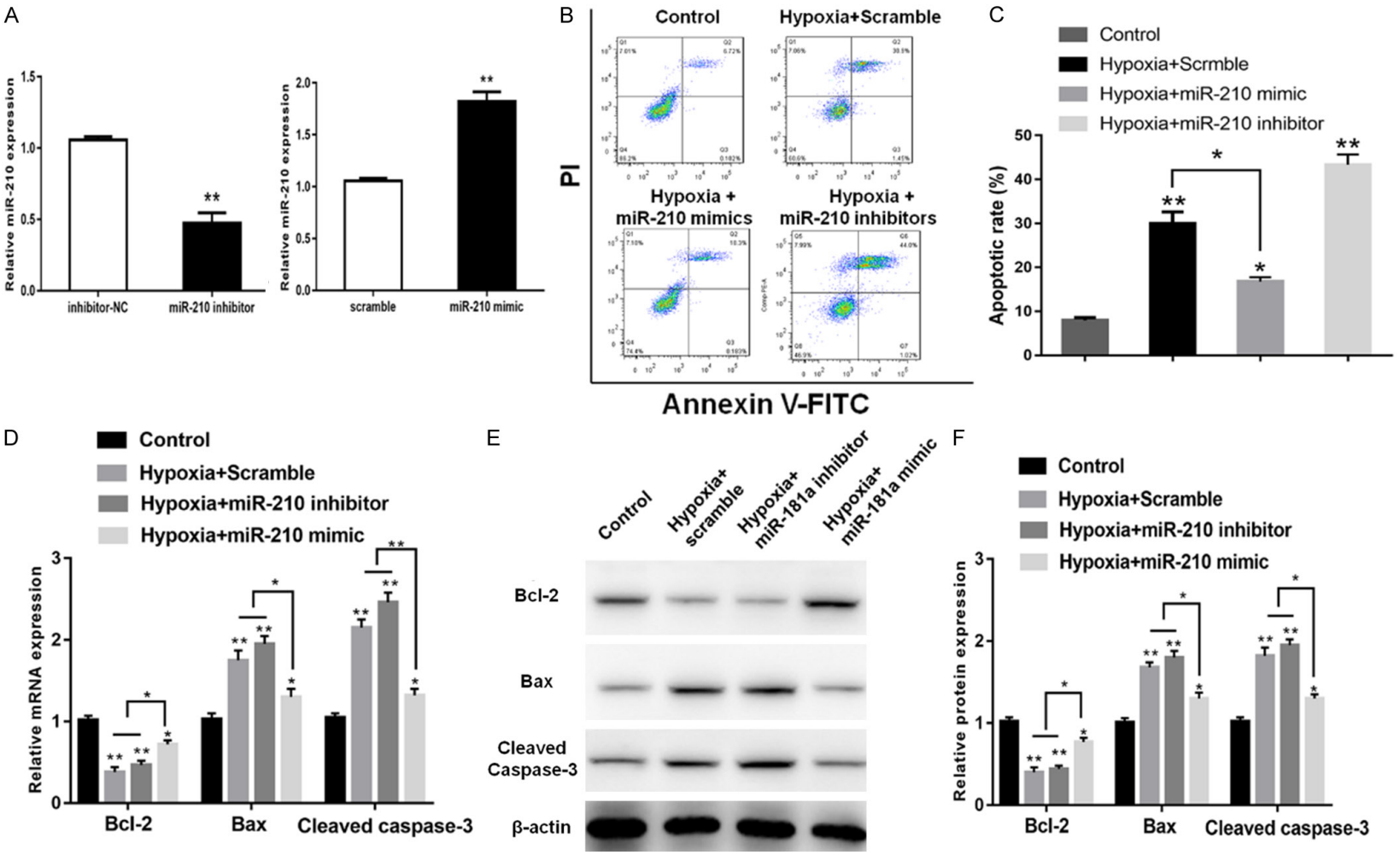


Figure 3. MiR-210 overexpression attenuates hypoxia-induced apoptosis in VSMCs. A. miR-34a mimic and inhibitor were used to achieve miR-34a overexpression and downregulation which was verified by RT-PCR. B, C. The flow cytometric cell apoptosis assays in miR-210 mimics and inhibitors group in hypoxic VSMCs and control. D. The mRNA of Bcl-2, Bax and cleaved Caspase-3 were detected by quantitative RT-PCR in miR-210 mimics and inhibitors group. E, F. The protein expressions of Bcl-2, Bax and cleaved Caspase-3 were detected by western blotting assay in miR-210 mimics and inhibitors group. Data were represented as mean ± SD and shown as fold change relative to the control group; Statistical analysis was calculated by one-way ANOVA. *P<0.05, **P<0.01 vs control.

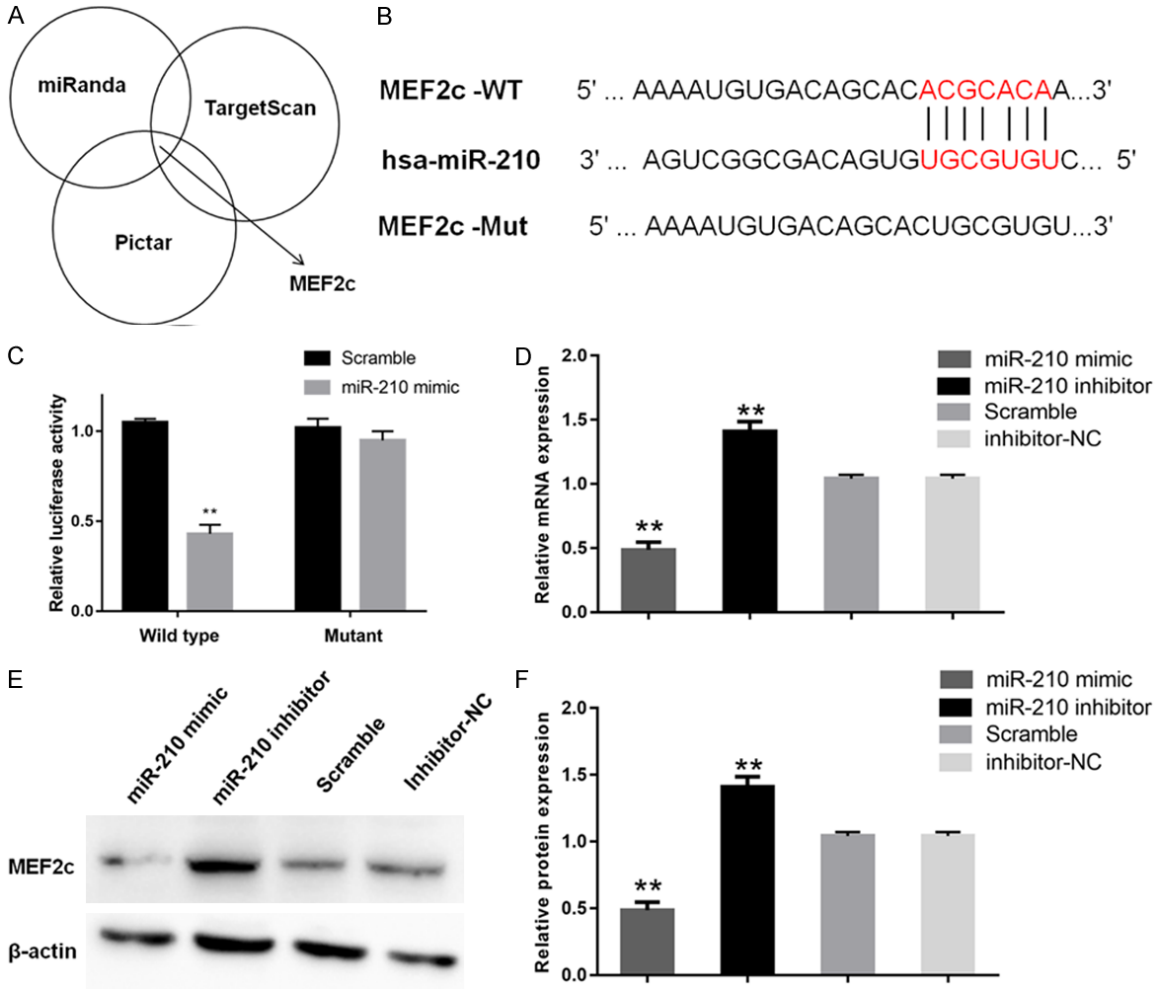
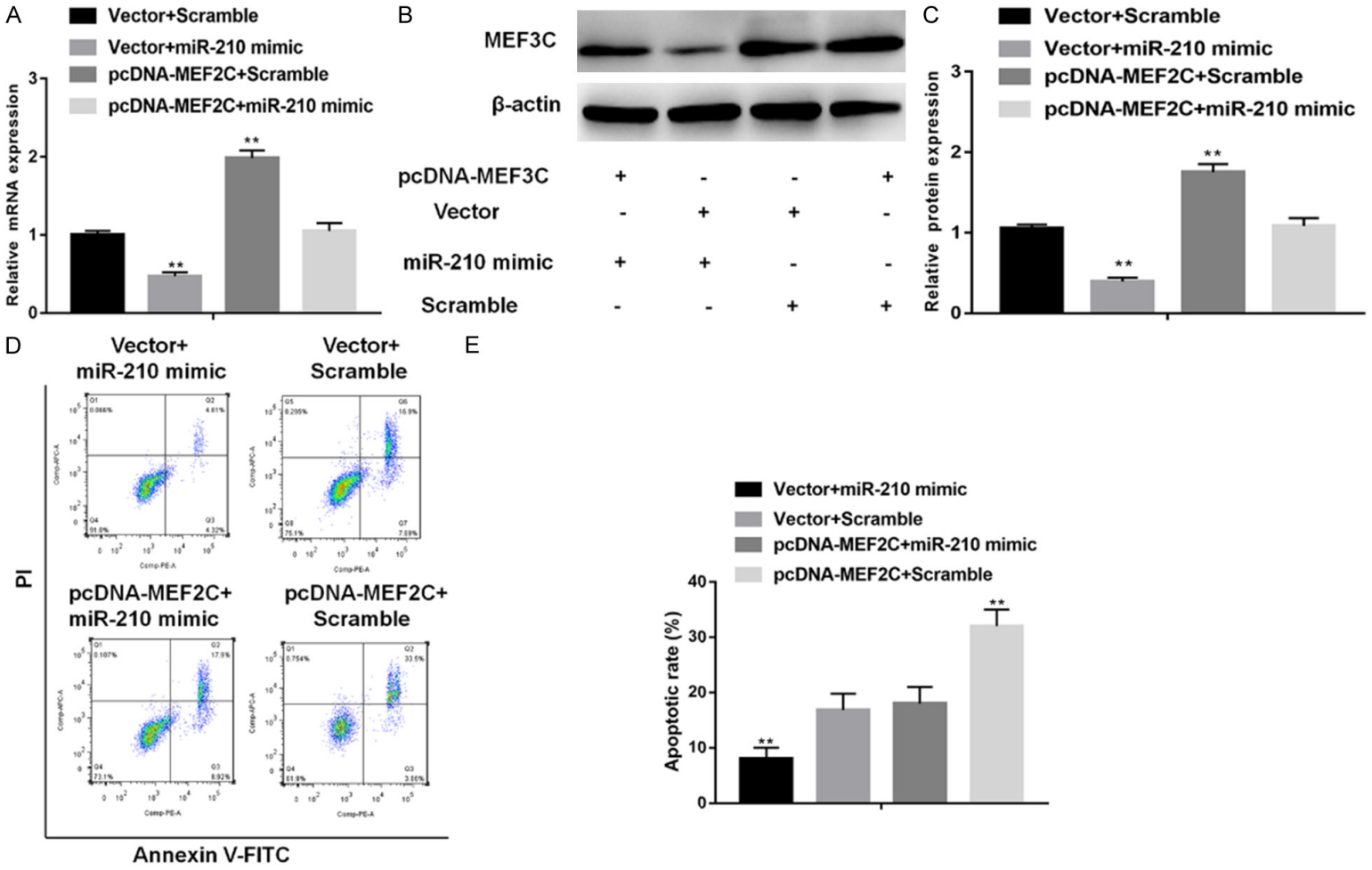


Figure 4. MiR-210 is confirmed as a direct target of MEF2C. A. Complementary sequences of miR-210-MEF2C were obtained from publicly available websites. The mutant of SYT1 3'UTR muted three bases was also shown; B. The predicted binding sequences of the 3'-UTR of MEF2C. C. Relative luciferase activity was analyzed after wild-type or mutant 3'-UTR reporter plasmids were co-transfected with miR-210 in VSMCs. D. qRT-PCR detection of MEF2C mRNA expression in VSMCs transfected with miR-210 mimics or miR-210 inhibitors; E and F. Western blot detection of MEF2C mRNA expression in VSMCs transfected with miR-210 mimics or miR-210 inhibitors. U6 was used as an internal control. Data were represented as mean ± SD and shown as fold change relative to the control group; statistical analysis was calculated using one-way ANOVA. *P<0.05, **P<0.01 vs control.

ows that numerous miRNAs have been found to play a regulatory role in VSMCs apoptosis.

Previous studies revealed that mitochondria play a crucial role in cellular energy generation and metabolism, oxidative stress, fatty acid oxidation, calcium transport and cellular proliferation and apoptosis [34-36]. Many studies have reported the role of mitochondrial dysfunction in various human diseases and that apoptosis helps protect mitochondria from stress damage and promotes mitochondrial renewal [38, 39]. Activation of the apoptotic pathway is caused by multiple stimuli, such as oxidative stress, cytoplasmic Ca^{2+} overload, UV damage and various drugs [39]. These stimuli enhance the permeability of the outer mitochondrial membrane (OMM) and promote the release of apoptotic proteins such as cytochrome c from the intermembrane space to the cytosol [40]. The above process can accelerate caspase cleaved targeted proteins and cause apoptosis [40]. Abnormal regulation of apoptosis is correlated with neurodegenerative diseases and cardiovascular diseases [37]. Results from the present study found that miR-210 promoted apoptosis via mitochondrial pathway apoptosis, which was verified by the expression of


A
Vector+Scramble
Vector+miR-210 mimic
pcDNA-MEF2C+Scramble
pcDNA-MEF2C+miR-210 mimic
Relative mRNA expression
B
MEF3C
β-actin
pcDNA-MEF3C + - - +
Vector - + + -
miR-210 mimic + + - -
Scramble - - + +
C
Vector+Scramble
Vector+miR-210 mimic
pcDNA-MEF2C+Scramble
pcDNA-MEF2C+miR-210 mimic
Relative protein expression
D
Vector+ miR-210 mimic
Vector+ Scramble
pcDNA-MEF2C+ miR-210 mimic
pcDNA-MEF2C+ Scramble
PI
Annexin V-FITC
E
Vector+miR-210 mimic
Vector+Scramble
pcDNA-MEF2C+miR-210 mimic
pcDNA-MEF2C+Scramble
Apoptotic rate (%)

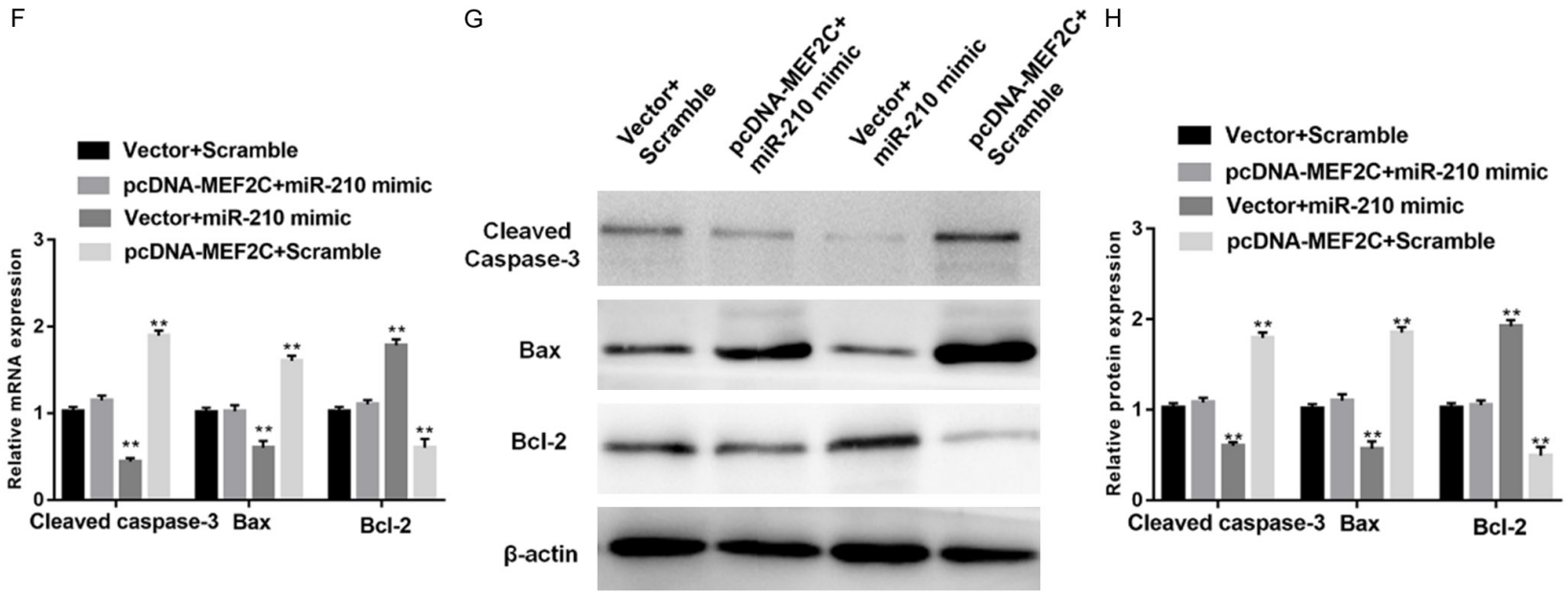


Figure 5. MiR-210 overexpression inhibits VSMC hypoxia-induced apoptosis by targeting MEF2C. The VSMCs were co-transfected with pcDNA-MEF2C and miR-210 mimics before exposure to the hypoxic condition. (A) The mRNA and (B, C) protein expression of hypoxic VSMCs co-transfected with either miR-210 mimic or scramble and pcDNA-MEF2C or pcDNA empty vector; (D) and (E) Apoptosis rate in hypoxic VSMCs co-transfected with either miR-210 mimic or scramble and pcDNA-MEF2C or pcDNA empty vector; (F) The miRNA levels of Bcl-2, Bax and cleaved Caspase-3 in hypoxic VSMCs were measured by RT-PCR; (G) and (H) The protein levels of Bcl-2, Bax and cleaved Caspase-3 in hypoxic VSMCs were measured by western blot. Data are represented as mean ± SD and shown as fold change relative to the control group; statistical analysis was calculated using one-way ANOVA. *P<0.05, **P<0.01 vs control.

apoptotic related factors such as Bcl-2, Bax, Bad, Caspase-3, Caspase-9 and cytochrome c at both the mRNA and protein levels.

Myocyte-specific enhancer factor 2c (MEF2C) is a ubiquitously expressed transcription factor with important functions in the cardiovascular system [41]. This gene is involved in cardiac morphogenesis, myogenesis and vascular development [41]. Evidence suggests that there are many miRNA that regulate MEF2C and that it plays an important role in atherosclerosis. For example, MEF2C has been found to be the direct target of miR-135-5p and miR-4991-3p and plays an important role in cell proliferation and migration in atherosclerosis [42]. Another study found that miR-448 directly targets MEF2C and promotes VSMCs proliferation and migration [42]. There is currently limited evidence that shows the relationship between MEF2C and VSMCs apoptosis.

This study aimed to explore the relationship between miR-210 and VSMCs apoptosis. A hypoxic model of VSMCs was established and the expression of miR-210 at different time points under hypoxic conditions was measured. Results showed that the expression of miR-210 in hypoxic VSMCs demonstrated a time-dependent relationship. Results also showed that miR-210 reduces hypoxic-induced apoptosis in VSMCs and apoptotic factor expression was detected at both the mRNA level and protein levels. To further investigate the molecular mechanism, potential target genes of miR-210 were predicted using microRNA.org, TargetScan and miRDB. Using luciferase reporter assay, results found that MEF2C was a direct target of miR-210. Moreover, western blotting assay and RT-PCR revealed that overexpression of miR-210 resulted in the down-regulation of MEF2C at both the protein and mRNA levels. It was found that miR-210 overexpression inhibits VSMC hypoxia-induced apoptosis by targeting MEF2C.

Conclusions

In conclusion, these results indicate that miR-210 may be involved in apoptosis inhibition induced by hypoxic damage. The downregulation of miR-210 attenuated the inhibited apoptosis effects induced by hypoxia in VSMCs. In summary, these findings provide valuable insight into the association between miR-210 and hypoxic-induced VSMCs protection.

Disclosure of conflict of interest

None.

Abbreviations

VSMC, Vascular smooth muscle cell; MEF2C, Myocyte-specific enhancer factor 2c; MiR, MicroRNA.

Address correspondence to: Yong Zhang, Cadre Healthcare Wards, Affiliated Hospital of Inner Mongolia Medical University, Tongdao North Road, No. 1, Hohhot 010059, Inner Mongolia, China. E-mail: researcherzhang@126.com

References

[1] Weber C and Noels H. Atherosclerosis: current pathogenesis and therapeutic options. Nat Med 2011; 17: 1410-1422.

[2] Usman A, Ribatti D, Sadat U and Gillard JH. From lipid retention to immune-mediate inflammation and associated angiogenesis in the pathogenesis of atherosclerosis. J Atheroscler Thromb 2015; 22: 739-749.

[3] Yang Z, Wang WE and Zhang Q. CIAPIN1 siRNA inhibits proliferation, migration and promotes apoptosis of VSMCs by regulating Bcl-2 and Bax. Curr Neurovasc Res 2013; 10: 4-10.

[4] Wang P, Xu TY, Guan YF, Zhao Y, Li ZY, Lan XH, Wang X, Yang PY, Kang ZM, Vanhoutte PM and Miao CY. Vascular smooth muscle cell apoptosis is an early trigger for hypothyroid atherosclerosis. Cardiovasc Res 2014; 102: 448-459.

[5] Zhang X, Zuo X, Yang B, Li Z, Xue Y, Zhou Y, Huang J, Zhao X, Zhou J, Yan Y, Zhang H, Guo P, Sun H, Guo L, Zhang Y and Fu XD. MicroRNA directly enhances mitochondrial translation during muscle differentiation. Cell 2014; 158: 607-619.

[6] Wojtas B, Ferraz C, Stokowy T, Hauptmann S, Lange D, Dralle H, Musholt T, Jarzab B, Paschke R and Eszlinger M. Differential miRNA expression defines migration and reduced apoptosis in follicular thyroid carcinomas. Mol Cell Endocrinol 2014; 388: 1-9.

[7] Ambros V. The functions of animal microRNAs. Nature 2004; 431: 350-354.

[8] Tetreault N and De Guire V. miRNAs: their discovery, biogenesis and mechanism of action. Clin Biochem 2013; 46: 842-845.

[9] Li C, Hashimi SM, Good DA, Cao S, Duan W, Plummer PN, Mellick AS and Wei MQ. Apoptosis and microRNA aberrations in cancer. Clin Exp Pharmacol Physiol 2012; 39: 739-746.

[10] Madrigal-Matute J, Rotllan N, Aranda JF and Fernandez-Hernando C. MicroRNAs and ath-

erosclerosis. Curr Atheroscler Rep 2013; 15: 322.
[11] Sun X, Icli B, Wara AK, Belkin N, He S, Kobzik L, Hunninghake GM, Vera MP, Blackwell TS, Baron RM and Feinberg MW. MicroRNA-181b regulates NF-kappaB-mediated vascular inflammation. J Clin Invest 2012; 122: 1973-1990.
[12] Zhang L, Zhou M, Wang Y, Huang W, Qin G, Weintraub NL and Tang Y. miR-92a inhibits vascular smooth muscle cell apoptosis: role of the MKK4-JNK pathway. Apoptosis 2014; 19: 975-983.
[13] Ji R, Cheng Y, Yue J, Yang J, Liu X, Chen H, Dean DB and Zhang C. MicroRNA expression signature and antisense-mediated depletion reveal an essential role of MicroRNA in vascular neointimal lesion formation. Circ Res 2007; 100: 1579-1588.
[14] Sedding DG, Widmer-Teske R, Mueller A, Stieger P, Daniel JM, Gunduz D, Pullamsetti S, Nef H, Moellmann H, Troidl C, Hamm C and Braun-Dullaeus R. Role of the phosphatase PTEN in early vascular remodeling. PLoS One 2013; 8: e55445.
[15] Leeper NJ, Raiesdana A, Kojima Y, Chun HJ, Azuma J, Maegdefessel L, Kundu RK, Quertermous T, Tsao PS and Spin JM. MicroRNA-26a is a novel regulator of vascular smooth muscle cell function. J Cell Physiol 2011; 226: 1035-1043.
[16] Chang Z, Huo L, Wu Y and Zhang P. HIF-1 alpha had pivotal effects on downregulation of miR-210 decreasing viability and inducing apoptosis in hypoxic chondrocytes. ScientificWorldJournal 2014; 2014: 876363.
[17] Chio CC, Lin JW, Cheng HA, Chiu WT, Wang YH, Wang JJ, Hsing CH and Chen RM. MicroRNA-210 targets antiapoptotic Bcl-2 expression and mediates hypoxia-induced apoptosis of neuroblastoma cells. Arch Toxicol 2013; 87: 459-468.
[18] Li L, Huang K, You Y, Fu X, Hu L, Song L and Meng Y. Hypoxia-induced miR-210 in epithelial ovarian cancer enhances cancer cell viability via promoting proliferation and inhibiting apoptosis. Int J Oncol 2014; 44: 2111-2120.
[19] Yang W, Sun T, Cao J, Liu F, Tian Y and Zhu W. Downregulation of miR-210 expression inhibits proliferation, induces apoptosis and enhances radiosensitivity in hypoxic human hepatoma cells in vitro. Exp Cell Res 2012; 318: 944-954.
[20] Shang C, Hong Y, Guo Y, Liu YH and Xue YX. MiR-210 up-regulation inhibits proliferation and induces apoptosis in glioma cells by targeting SIN3A. Med Sci Monit 2014; 20: 2571-2577.
[21] Sun Y, Xing X, Liu Q, Wang Z, Xin Y, Zhang P, Hu C and Liu Y. Hypoxia-induced autophagy reduces radiosensitivity by the HIF-1alpha/miR-210/Bcl-2 pathway in colon cancer cells. Int J Oncol 2015; 46: 750-756.
[22] Wang F, Xiong L, Huang X, Zhao T, Wu LY, Liu ZH, Ding X, Liu S, Wu Y, Zhao Y, Wu K, Zhu LL and Fan M. miR-210 suppresses BNIP3 to protect against the apoptosis of neural progenitor cells. Stem Cell Res 2013; 11: 657-667.
[23] Jiang Y, Li L, Tan X, Liu B, Zhang Y and Li C. miR-210 mediates vagus nerve stimulation-induced antioxidant stress and anti-apoptosis reactions following cerebral ischemia/reperfusion injury in rats. J Neurochem 2015; 134: 173-181.
[24] Shepard BD, Natarajan N, Protzko RJ, Acres OW and Pluznick JL. A cleavable N-terminal signal peptide promotes widespread olfactory receptor surface expression in HEK293T cells. PLoS One 2013; 8: e68758.
[25] Zhang W and Liang Z. [Comparison between annexin V-FITC/PI and Hoechst33342/PI double stainings in the detection of apoptosis by flow cytometry]. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 2014; 30: 1209-1212.
[26] Hnasko TS and Hnasko RM. The western blot. Methods Mol Biol 2015; 1318: 87-96.
[27] Aliparasti MR, Alipour MR, Almasi S and Feizi H. Ghrelin administration increases the Bax/Bcl-2 gene expression ratio in the heart of chronic hypoxic rats. Adv Pharm Bull 2015; 5: 195-199.
[28] Ferrer I, Pozas E, Lopez E and Ballabriga J. Bcl-2, Bax and Bcl-x expression following hypoxia-ischemia in the infant rat brain. Acta Neuropathol 1997; 94: 583-589.
[29] Wang W, Wang D and Li H. Initiation of premature senescence by Bcl-2 in hypoxic condition. Int J Clin Exp Pathol 2014; 7: 2446-2453.
[30] Knowlton AA and Gupta S. HSP60, Bax, and cardiac apoptosis. Cardiovasc Toxicol 2003; 3: 263-268.
[31] Virmani R, Kolodgie FD, Burke AP, Farb A and Schwartz SM. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. Arterioscler Thromb Vasc Biol 2000; 20: 1262-1275.
[32] Bennett MR, Sinha S and Owens GK. Vascular smooth muscle cells in atherosclerosis. Circ Res 2016; 118: 692-702.
[33] O'Brien ER, Alpers CE, Stewart DK, Ferguson M, Tran N, Gordon D, Benditt EP, Hinohara T, Simpson JB and Schwartz SM. Proliferation in primary and restenotic coronary atherectomy tissue. Implications for antiproliferative therapy. Circ Res 1993; 73: 223-231.
[34] Libby P, Ridker PM and Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature 2011; 473: 317-325.

[35] Tabas I, Garcia-Cardena G and Owens GK. Recent insights into the cellular biology of atherosclerosis. J Cell Biol 2015; 209: 13-22.
[36] Alexander MR and Owens GK. Epigenetic control of smooth muscle cell differentiation and phenotypic switching in vascular development and disease. Annu Rev Physiol 2012; 74: 13-40.
[37] Larroque-Cardoso P, Swiader A, Ingueneau C, Negre-Salvayre A, Elbaz M, Reyland ME, Salvayre R and Vindis C. Role of protein kinase C delta in ER stress and apoptosis induced by oxidized LDL in human vascular smooth muscle cells. Cell Death Dis 2013; 4: e520.
[38] Wallace DC. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. Annu Rev Genet 2005; 39: 359-407.
[39] Doran E and Halestrap AP. Cytochrome c release from isolated rat liver mitochondria can occur independently of outer-membrane rupture: possible role of contact sites. Biochem J 2000; 348: 343-350.
[40] Long Q, Li L, Wang H, Li M, Wang L, Zhou M, Su Q, Chen T and Wu Y. Novel peptide dermaseptin-PS1 exhibits anticancer activity via induction of intrinsic apoptosis signalling. J Cell Mol Med 2019; 23: 1300-1312.
[41] Xu Z, Yoshida T, Wu L, Maiti D, Cebotaru L and Duh EJ. Transcription factor MEF2C suppresses endothelial cell inflammation via regulation of NF-kappaB and KLF2. J Cell Physiol 2015; 230: 1310-1320.
[42] Zhang R, Sui L, Hong X, Yang M and Li W. MiR-448 promotes vascular smooth muscle cell proliferation and migration in through directly targeting MEF2C. Environ Sci Pollut Res Int 2017; 24: 22294-22300.