MicroRNA-494 inhibits apoptosis of murine vascular smooth muscle cells *in vitro*

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Abstract. Apoptosis of vascular smooth muscle cells (VSMCs) is a process that regulates vessel remodeling in various cardiovascular diseases. The specific mechanisms that control VSMC apoptosis remain unclear. The present study aimed to investigate whether microRNA-494 (miR-494) is involved in regulating VSMC apoptosis and its underlying mechanisms. Cell death ELISA and terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling assays were used to detect apoptosis of murine VSMCs following stimulation with tumor necrosis factor-α (TNF-α). The results indicated that TNF-α upregulated VSMC apoptosis in a dose-dependent manner. Microarray analysis was used to evaluate the expression profile of microRNAs following TNF-α stimulation in murine VSMCs. The expression of miR-494 was downregulated, whereas B-cell lymphoma-2-like 11 (BCL2L11) protein expression levels were upregulated in VSMCs following treatment with TNF-α. Luciferase reporter assays confirmed that BCL2L11 was a direct target of miR-494. Transfection with miR-494 mimics decreased VSMC apoptosis and downregulated BCL2L11 protein levels. Conversely, transfection with miR-494 inhibitors increased cell apoptosis and upregulated BCL2L11 protein levels, suggesting that miR-494 may function as an essential regulator of BCL2L11. The increase in apoptosis caused by miR-494 inhibitors was abolished in cells co-transfected with BCL2L11-targeting small interfering RNA. The findings of the present study revealed that miR-494 inhibited TNF-α-induced VSMC apoptosis by downregulating the expression of BCL2L11.

Introduction

Vascular smooth muscle cells (VSMCs), the major cellular component in the media layer of arteries, are highly specialized cells that perform biosynthetic, proliferative, and contractile functions both in health and disease (1). Apoptosis of VSMCs is closely associated with physiological remodeling of the vasculature and remodeling during disease, including in atherosclerotic plaque rupture, restenosis following angioplasty and abdominal arterial aneurysm (AAA) development (2-5). Increased VSMC apoptosis has been observed in unstable atherosclerotic plaques and is closely associated with plaque vulnerability (6). Excessive apoptosis of VSMCs in AAAs causes thinning and weakening of the medial layer of the aortic wall, leading to loss of tensile forces and aortic dilation (7,8). An incomplete understanding of the molecular mechanisms that regulate VSMC apoptosis has limited the development of diagnostic and therapeutic strategies in these cardiovascular diseases. Therefore, exploring the mechanisms underlying VSMC apoptosis may be useful for the prevention and treatment of cardiovascular diseases. Tumor necrosis factor-α (TNF-α) is an endogenous cytokine involved in the process of inflammation under pathological conditions, including atherosclerosis and vascular calcification (9-11). Although TNF-α has been demonstrated to suppress proliferation and induce apoptosis of VSMCs (12-14), the specific pathways that regulate this process have not been fully elucidated.

In recent years, microRNAs (miRs/miRNAs) have emerged as a group of single-stranded, small, non-coding RNA molecules that exert their biological effects at the post-transcriptional level. They function through base pairing of their seed region (position 2-8) with the 3'-untranslated region (UTR) of target genes (15,16). At present, >1,800 miRNAs (1,881 precursor and 2,581 mature miRNAs) have been identified in humans (www.mirbase.org) and have been reported to be involved in almost all aspects of human physiology and pathology, including stem cell renewal, tumor formation, cardiovascular diseases, metabolic disorders, genetic diseases and neurodegenerative diseases (17-19). Previous studies have investigated the role of miRNAs in regulating VSMC apoptosis under different pathological conditions. miR-487b induces VSMC apoptosis and loss of medial integrity during hypertension-induced remodeling of the aorta via downregulation of insulin receptor substrate 1 (20). miR-138 acts as a negative regulator of pulmonary aortic smooth muscle cell apoptosis during hypoxic pulmonary vascular remodeling via downregulation of serine/threonine kinase 4 (also termed Mst1) (21). miR-92a

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suppresses H_2O_2-induced VSMC apoptosis by targeting the mitogen-activated protein kinase kinase 4-c-Jun N-terminal kinase 1 pathway (22). A cluster of miRNAs can regulate a gene cooperatively, and a single miRNA can have binding sites in several target genes and exhibit different biological effects in different cell types.

In the present study, miRNA expression in murine VSMCs was analyzed by microarray analysis following TNF-α stimulation for 24 h. The present results revealed that miR-494 was one of the most significantly downregulated miRNAs, indicating its potential role in regulating cell apoptosis. Although miR-494 has been reported to regulate apoptosis in several tissues, the specific role of miR-494 in TNF-α-induced VSMC apoptosis remains unclear. The current study aimed to investigate whether miR-494 is involved in regulating TNF-α-induced VSMC apoptosis and the underlying mechanisms by which this miRNA exerts its modulatory effect *in vitro*.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM), TNF-α and pentobarbital sodium were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Fetal bovine serum (FBS) was purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Lipofectamine® 2000 was purchased from Invitrogen (Thermo Fisher Scientific, Inc.). Maxima SYBR-Green/ROX quantitative polymerase chain reaction (qPCR) Master Mix (2X; Thermo Fisher Scientific, Inc.) was used to investigate gene expression by qPCR. A Cell Death Detection ELISA kit was purchased from Roche Diagnostics (Basel, Switzerland; cat. no. 11920685001). B-cell lymphoma-2-like 11 (BCL2L11) and β-actin antibodies, horseradish peroxidase-conjugated goat-anti-mouse secondary antibody and the electrochemiluminescence detection kit for western Blotting was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). miR-494 mimics and miR-494 inhibitors (2'-O-methyl modified) and respective oligo controls were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China).

Cell culture and transfection. The study was approved by The Ethics Review Board of The Second Xiangya Hospital of Central South University (Changsha, China). In total, eight C57BL/6 mice (age, 8 weeks; weight, 25-30 g; female to male ratio, 1:1) were purchased from The Animal Multiplication Centre of Qinglong Mountain (Nanjing, China) and housed in individual cages. The animals were provided with food and water *ad libitum*, in a controlled environment (temperature 20-24˚C; humidity 40-60%) and under a 12-h light/dark cycle. The animals were euthanized using an intraperitoneal injection of 150 mg/kg pentobarbital sodium and primary murine VSMCs were isolated from the abdominal aorta, following an enzymatic dissociation procedure as previously described (23). VSMCs were cultured at 37˚C and 5% CO_2 in DMEM supplemented with glutaMAX™ (Gibco; Thermo Fisher Scientific, Inc.) and 15% FBS. Cells at passage 3-8 were used for subsequent experiments. For induction of cell apoptosis, VSMCs were cultured in serum-free DMEM for 12 h prior to treatment with vehicle (dimethyl sulfoxide) or TNF-α (5-50 ng/ml) for 24 h at 37˚C and 5% CO_2.

For transient transfection of miR-494 mimic (5'-UGAAAC AUACACGGGAAACCUC-3'), miR-494 inhibitor (5'-GGU UUCCCGUGUAUGUUUCAUU-3') and their control oligonucleotides (control miR mimic, 5'-UUCUCCGAACGUGUC ACGU-3'; control miR inhibitor 5'-ACGUGACACGUUCGG AGAA-3'), Lipofectamine® 2000 and 50 nM oligos were mixed according to the manufacturer's instructions and added to the cells for 48 h prior to subsequent experimentation.

Cell death detection. Murine VSMCs were seeded in a 12-well plate at a density of 25,000 cells/well, incubated for 12 h at 37˚C and 5% CO_2 in serum-free DMEM, and then stimulated with 5-50 ng/ml TNF-α for 24 h at 37˚C and 5% CO_2. The cell layers were rinsed twice with PBS and incubated with the lysis buffer provided in the kit for 30 m in at 4˚C. Lysates were extracted and centrifuged at 15,000 rpm and 4˚C for 10 min. The Cell Death Detection ELISA kit was used to quantify the amount of cytoplasmic histone-associated DNA fragments in the cell lysates according to the manufacturer's protocol. Absorbance was measured at 405 nm and apoptotic cell death is expressed as ELISA absorbance units.

To investigate the effects of miR-494 on cell apoptosis, cells were transfected with miR-494 mimics or miR-494 inhibitors for 48 h prior to TNF-α treatment. To determine whether the effects of miR-494 on TNF-α induced VSMC apoptosis were dependent on BCL2L11, VSMCs were cotransfected with scramble small interfering RNA (siRNA) + control miR inhibitor, scramble siRNA + miR-494 inhibitor, BCL2L11 siRNA + control miR inhibitor or BCL2L11 siRNA + miR-494 inhibitor for 48 h, and subsequently stimulated with TNF-α (10 ng/ml) for 24 h at 37˚C. The BCL2L11 siRNA and scramble siRNA were purchased from Sigma-Aldrich (Merck KGaA). BCL2L11 siRNA (5'-ACUUACAUCAGAAGGUUGC-3') or scramble siRNA (5'-UAAGGCUAUGAAGAGAUAC-3') and used at a concentration of 10 nM, and 50 nM miR-494 inhibitor, mimic or control oligos were transfected into cells using Lipofectamine® 2000.

Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) staining. VSMCs were seeded at a density of 60,000 cells/well in 6-well plates and incubated in serum-free DMEM with or without 10 ng/ml TNF-α for 24 h at 37˚C and 5% CO_2. To evaluate the effect of TNF-α on VSMC apoptosis, a TUNEL assay was performed using an *in situ* Cell Death Detection kit (Roche Diagnostics, Indianapolis, IN, USA; cat. no. 11684795910), as previously described (24). Cells were fixed in freshly prepared paraformaldehyde (4% in PBS; pH 7.4) for 1 h at 20˚C. During the labeling reaction, cells were incubated with 50 μl TUNEL reaction mixture at 37˚C for 1 h in the dark. The nuclei were counterstained with 10 ug/ml DAPI for 2 min at room temperature. The coverslips were mounted on slides with antifade mounting medium (50 mM Tris-PO_4, 50 mM NaH_2PO_4, 20% polyvinyl alcohol and 30% glycerol). TUNEL-positive cells were visualized under an inverted florescence microscope (Nikon Corporation, Tokyo, Japan; magnification, x100) equipped with a charge-coupled device digital camera. Images were processed using Imaging Software NIS-Elements BR version 3.0 (Nikon Corporation; magnification, x100). In total, 10 randomly selected fields were analyzed, and TUNEL positive cells and total cells

were counted in each group. Cell death was calculated and is expressed as percentage of apoptotic cells.

To investigate whether miR-494 relies on BCL2L11 to regulate cell apoptosis, four groups of cells were transfected with scramble siRNA + control miR inhibitors, scramble siRNA + miR-494 inhibitors, BCL2L11 siRNA + control miR inhibitors and BCL2L11 siRNA + miR-494 inhibitors for 48 h, and incubated with TNF-α (10 ng/ml) for 24 h at 37˚C and 5% CO_2.

miRNA microarray analysis. Murine VSMCs were pre-cultured in serum-free DMEM for 12 h and then incubated with 10 ng/ml TNF-α for 24 h at 37˚C and 5% CO_2. Total RNA was isolated using an RNA isolation kit (Cells-to-CT Kit; Ambion; Thermo Fisher Scientific, Inc.) and pooled from three control and three TNF-α-treated cell groups and hybridized using a μParaflo® Microfluidic Biochip Technology microarray platform (Chip ID miRhsa 12.0; LC Sciences, Houston, TX, USA), as previously described (25). The microarray values were analyzed following subtraction of the background, were profiled using the LC Science miRNA expression profiling service, and normalized using locally weighted scatterplot smoothing method (25). Each sample was repeated three times and significant signal differences (P<0.01) between TNF-α and control group were analyzed based on miRBase version 17.0 (http://www.mirbase.org/). Hierarchical clustering of the $\log_2$ fold change value was performed using GeneSpring GX software version 7.3 (Agilent Technologies, Inc., Santa Clara, CA, USA) and visualized using a heat map (Heatmap Illustrator; version 1.0; http://hemi.biocuckoo.org/).

Reverse transcription (RT)-qPCR. Total RNA was extracted from VSMCs using the mirVana™ miRNA Isolation kit (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. RNA (1 μg) was reverse transcribed to cDNA using oligo(dT) primers provided in the RT kit (Roche Diagnostics; cat. no. 04897030001) in each RT reaction. qPCR was performed using the Maxima SYBR Green/ROX qPCR Master Mix (2X). The primers used for qPCR are listed in Table I. Each reaction was prepared in a total volume of 25 μl containing 12.5 μl Maxima SYBR-Green/ROX qPCR Master Mix (Roche Diagnostics), 0.3 μM forward Primer, 0.3 μM reverse Primer, template DNA and nuclease-free-water. The thermocycling conditions were the following: Initial denaturation at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec, 60˚C for 30 sec and 72˚C for 30 sec. Expression of miR-494 relative to U6 and BCL2L11 relative to GAPDH were evaluated using the $2^{-\Delta\Delta Cq}$ method (26). Each sample was measured using three technical replicates.

Western blot analysis. To detect protein expression levels of BCL2L11 and β-actin, western blot analysis was performed as previously described (24). Total protein was extracted from VSMCs using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China), and the concentration was determined by bicinchoninic acid assay. The proteins (30 μg per lane) were separated by 12% SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane, and membrane was subsequently blocked with 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature. The primary antibodies, including anti-BCL2L11 (cat. no. sc-374358; Santa Cruz Biotechnology, Inc.) and anti-β-actin (cat. no. sc-47778; Santa Cruz Biotechnology, Inc.), were diluted at 1:1,000 and were incubated at 4˚C overnight. The membranes were washed with TBS for 15 min at room temperature three times. Subsequently, the membrane was incubated with mouse Immunoglobulin Gκ binding protein conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc.; cat. no. sc-516102-CM; 1:1,000) at 37˚C for 1 h. The membrane was washed with TBS at room temperature for 15 min three times. Protein bands were visualized using an electrochemiluminescence detection kit (Santa Cruz Biotechnology, Inc.). The optical density was analyzed by AlphaEaseFC software (version 5.0; ProteinSimple, San Jose, CA, USA).

Table I. Primers for reverse transcription-quantitative polymerase chain reaction.

Gene	Primer sequence (5'-3')
BCL2L11	F: 5'-GGCTCAACTACCGCAGAGTC-3'
	R: 5'-GAGTTAAGTCTACCCGCCCG-3'
GAPDH	F: 5'-AGGTCGGTGTGAACGGATTTG-3'
	R: 5'-TGTAGACCATGTAGTTGAGGTCA-3'
miRNA-494	F: 5'-TGAAACATACACGGGAAACC-3'
	R: 5'-GTGCAGGGTCCGAGGT-3'
U6	F: 5'-CGCTTCGGCAGCACATATACTA-3'
	R: 5'-GCGAGCACAGAATTAATACGAC-3'

BCL2L11, B-cell lymphoma-2-like 11; F, forward; R, reverse.

miRNA target site prediction. In order to predict miRNA target sites, three online software tools, including TargetScan version 7.2 (www.targetscan.org/), DIANA microT-CDS version 5.0 (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=MicroT_CDS/index) and PicTar (pictar.mdc-berlin.de/_update: March 26, 2007), were used to search for base pairing of the miRNA seed sequence with the 3'-UTR region of target genes. The University of California Santa Cruz Genome Browser (http://genome-euro.ucsc.edu/cgi-bin/hgGateway?redirect=manual&source=genome.ucsc.edu; assembly date: December 2013) was used to determine sequence conservation.

Dual luciferase reporter assay. The BCL2L11 luciferase reporter plasmid (Shanghai Yeasen Biotechnology Co., Ltd., Shanghai, China) containing the 3'-UTR of wild-type BCL2L11 (WT-pGL3-BCL2L11) or mutant BCL2L11 (MUT-pGL3-BCL2L11), and miR-494 mimics or control miR mimic were cotransfected into VSMCs with Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Luciferase activities were measured by the Luciferase Assay System and compared with *Renilla* luciferase activity (Promega Corporation, Madison, WI, USA), 48 h after transfection.

To generate MUT-pGL3-BCL2L11, the QuickChange Site-Directed Mutagenesis kit (Agilent Technologies, Inc.) was

Table II. Primers used for plasmid construction.

Gene	Primer sequence (5'-3')
WT BCL2L11	F: 5'-TCTAGAGAGCCAAATGTCTGTGTGCAA-3'
	R: 5'-TCTAGAGAGTGGGAGACAGGGATGTTAAT-3'
MUT BCL2L11	F: 5'-TTTATTAGATTAGAAAGTCATTTATCACTCGTCAACTGAG-3'
	R: 5'-CTCAGTTGACGAGTGATAAATGACTTTCTAATCTAATAAA-3'

MUT, mutant; WT, wild-type; F, forward; R, reverse.

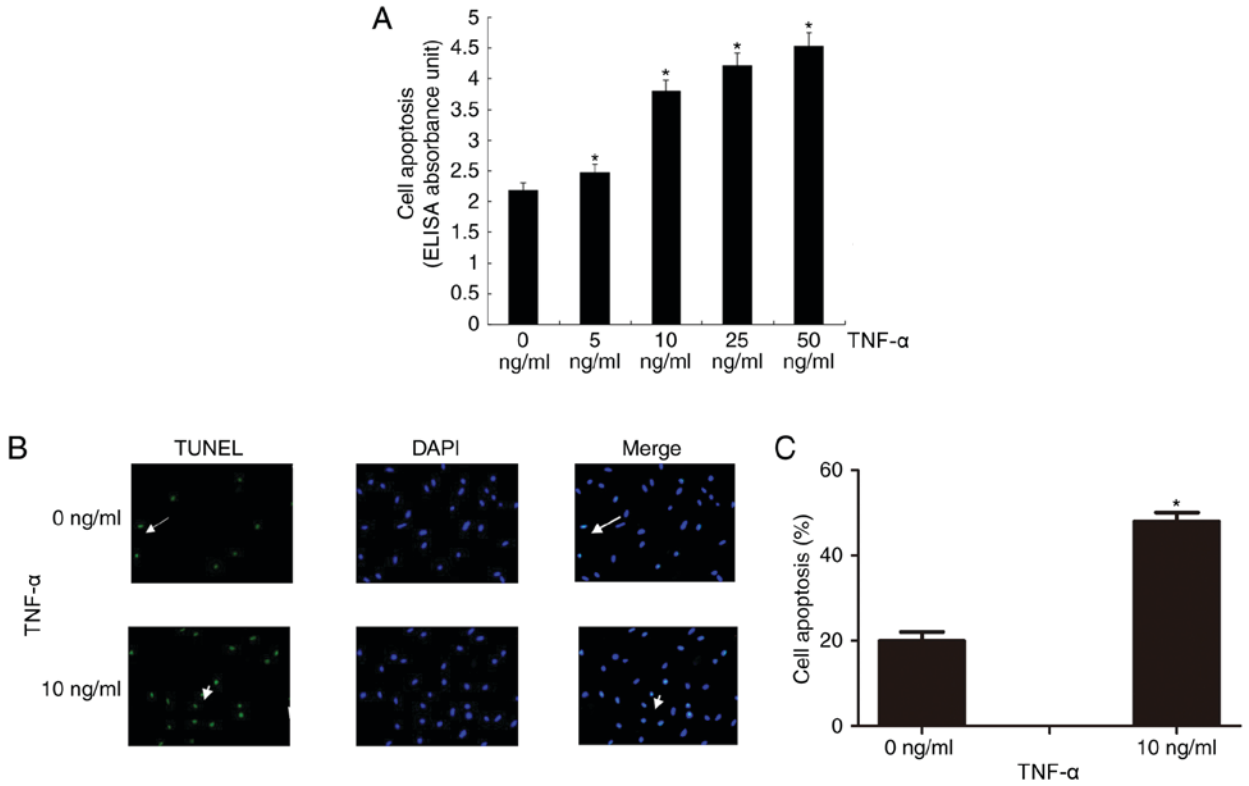

Figure 1. TNF-α induces VSMC apoptosis. (A) Murine VSMCs were stimulated with 0-50 ng/ml TNF-α for 24 h. Cell death was measured using a Cell Death Detection ELISA kit and is expressed as ELISA absorbance units. (B) VSMC apoptosis was determined using the TUNEL assay, following stimulation with 10 ng/ml TNF-α for 24 h. White arrows indicate apoptotic VSMCs (magnification, x100). (C) Quantitative analysis of TUNEL results. n=5. *P<0.05 vs. 0 ng/ml TNF-α. TNF-α, tumor necrosis factor-α; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling; VSMC, vascular smooth muscle cell.

used to induce two point mutations in the 3'-UTR region of WT BCL2L11. The generation of the wild-type and mutant oligos was performed as previously described (27). The sequences of the wild-type and mutant primers are listed in Table II.

Statistical analysis. All experiments were repeated at least three times and data are expressed as the means ± standard deviation. Statistical analysis was performed using SPSS software version 15.0 (SPSS, Inc., Chicago, IL, USA). Statistical significance was assessed using one-way analysis of variance followed by Tukey's test for comparison between two groups. Multiple comparisons between the groups were performed using the Dunnett's test or Student-Newman-Keuls method. P<0.05 was considered to indicate a statistically significant difference.

Results

TNF-α induces apoptosis of VSMCs. The Cell Death Detection ELISA assay revealed that TNF-α induced VSMC apoptosis in a dose-dependent manner (Fig. 1A). Cell apoptosis was significantly increased compared with the control treatment at all concentrations of TNF-α (P<0.05).

In addition, TUNEL staining revealed that 10 ng/ml TNF-α significantly induced VSMC apoptosis compared with the control treatment (P<0.05; Fig. 1B and C).

TNF-α downregulates miR-494 expression in VSMCs. The miRNA expression profile of VSMCs following TNF-α stimulation was evaluated by microarray analysis. The analysis revealed that 13 miRNAs were significantly downregulated

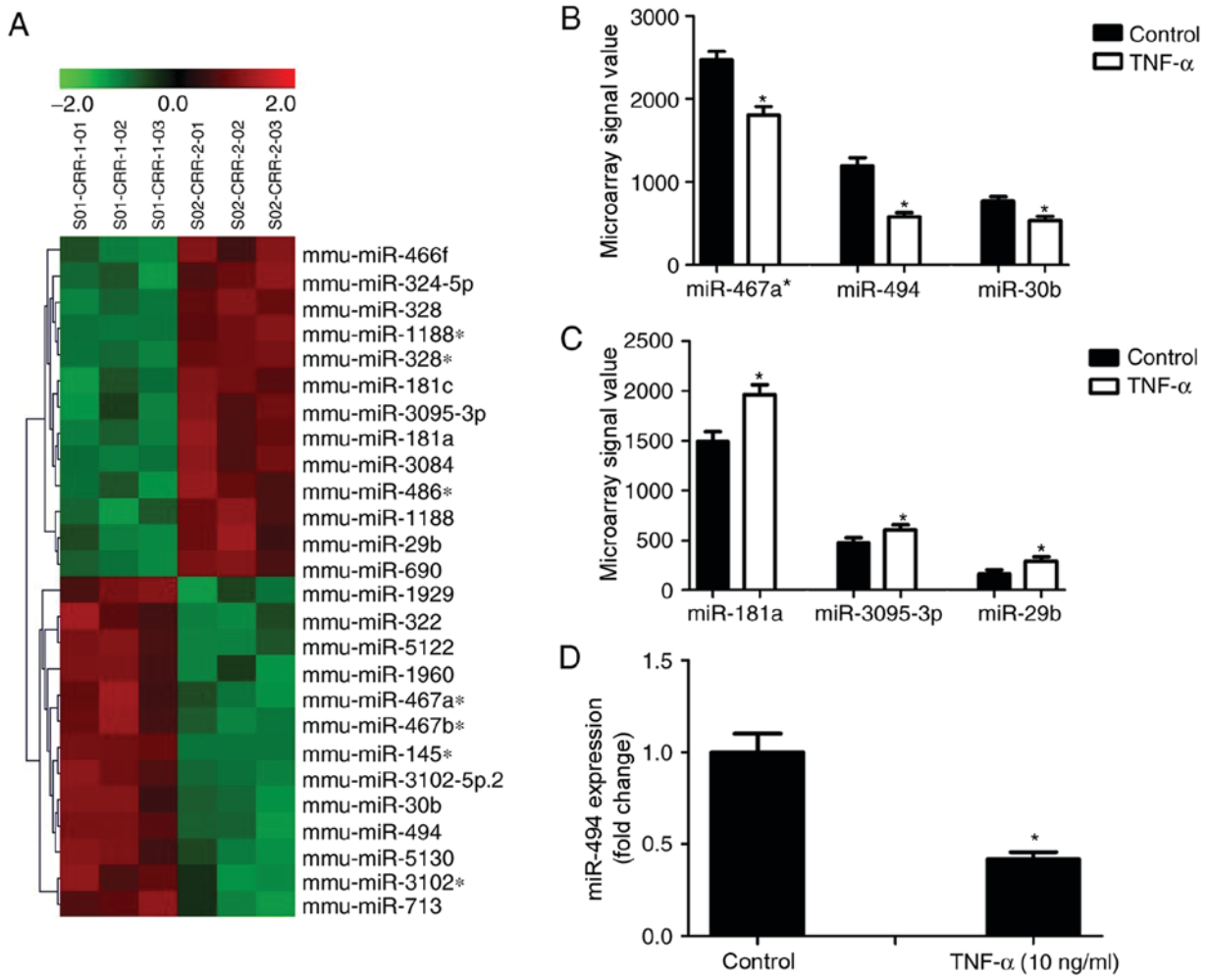

Figure 2. miR-494 expression in TNF-α-treated murine VSMCs. VSMCs were stimulated with 10 ng/ml TNF-α for 24 h. (A) Differentially expressed miRNAs (P<0.01) in three control (S01) and three TNF-α-treated (S02) samples were analyzed by hierarchical clustering of the $\log_2$ fold change value of each microarray signal. Red, upregulation; green, downregulation; black, no change. The microarray signal value of (B) three downregulated miRNAs and (C) three upregulated miRNAs. (D) miR-494 expression levels were confirmed by reverse transcription-quantitative polymerase chain reaction. *P<0.05 vs. Control. miR/miRNA, microRNA; TNF-α, tumor necrosis factor-α; VSMCs, vascular smooth muscle cells.

following TNF-α treatment, including miR-467a-3p, miR-494 and miR-30b; whereas 13 miRNAs were upregulated, including miR-181a, miR-3095-3p and miR-29b (Fig. 2A, Table III). These miRNAs were among the most abundantly expressed miRNAs in VSMCs. The microarray signal values of the three downregulated and three upregulated miRNAs are shown in Fig. 2B and C. Among the genes reported to be targets of miR-494, several have been suggested to be involved in cell apoptosis (28-30). Therefore, miR-494 was further investigated to determine its role in apoptotic signaling pathways. The altered expression of miR-494 was verified by RT-qPCR, which revealed that its expression was downregulated by >50% following treatment with 10 ng/ml TNF-α compared with the control group (Fig. 2D).

TNF-α downregulates miR-494 and upregulates BCL2L11 expression in VSMCs. To explore whether miR-494 is involved in VSMC apoptosis, murine VSMCs were treated with TNF-α and total RNA was extracted for RT-qPCR. The results demonstrated that the expression levels of miR-494 were downregulated by treatment with TNF-α (5-50 ng/ml) in a dose-dependent manner (Fig. 3A).

Three publicly available tools (TargetScan, DIANA microT-CDS and PicTar) were used to identify potential miR-494 target genes. The search results indicated that the 3'-UTR of BCL2L11 contained a predicted miR-494 binding site (Fig. 3B), which was also previously reported by Romano *et al* (31). To investigate whether BCL2L11 was associated with TNF-α-mediated cell apoptosis, western blot analysis was performed to evaluate the protein expression levels of BCL2L11 following TNF-α treatment. The protein expression levels of BCL2L11 were significantly increased following 24 h treatment with TNF-α (Fig. 3C). Since the expression levels of miR-494 were downregulated during VSMC apoptosis, whereas BCL2L11 protein levels were upregulated, this suggested that reduced miR-494 may result in increased BCL2L11 levels during TNF-α-mediated VSMC apoptosis.

miR-494 attenuates BCL2L11 expression. In order to investigate whether miR-494 regulates BCL2L11 expression, VSMCs were transfected with miR-494 mimics or inhibitors and their respective control oligos. RT-qPCR was used to confirm the overexpression of miR-494 in VSMCs transfected

Table III. Differentially expressed miRNAs in VSMCs following treatment with 10 ng/ml TNF-α. P<0.01.

miRNA	Log_2 fold change (TNF-α/control)
mmu-miR-1188-5p	1.92
mmu-miR-486-3p	1.69
mmu-miR-3084	1.61
mmu-miR-1188-3p	1.05
mmu-miR-181c	0.85
mmu-miR-29b	0.84
mmu-miR-466f	0.75
mmu-miR-690	0.72
mmu-miR-324-5p	0.71
mmu-miR-328-3p	0.63
mmu-miR-328-5p	0.39
mmu-miR-181a	0.39
mmu-miR-3095-3p	0.35
mmu-miR-5122	-0.37
mmu-miR-467a-3p	-0.45
mmu-miR-30b	-0.52
mmu-miR-467b-3p	-0.53
mmu-miR-1929	-0.63
mmu-miR-1960	-0.65
mmu-miR-5130	-0.8
mmu-miR-713	-0.81
mmu-miR-322	-0.87
mmu-miR-494	-1.04
mmu-miR-3102-5p	-1.26
mmu-miR-3102-5p.2	-1.85
mmu-miR-145-3p	-11.71

miR/miRNA, microRNA; TNF-α, tumor necrosis factor-α; VSMC, vascular smooth muscle.

with miR-494 mimics and inhibition of miR-494 expression in cells transfected with miR-494 inhibitors (Fig. 4A). Western blot analysis demonstrated that transfection with miR-494 mimics resulted in downregulated BCL2L11 protein levels, whereas transfection with miR-494 inhibitors increased BCL2L11 protein expression (Fig. 4B). However, alteration of miR-494 levels had no obvious effect on BCL2L11 mRNA expression levels (Fig. 4C), indicating that miR-494 attenuated the protein translation of BCL2L11 in VSMCs. Transfection with miR-494 mimics decreased cell apoptosis, whereas transfection with miR-494 inhibitors increased cell apoptosis (Fig. 4D). Collectively, these results indicated that miR-494 modulated VSMC apoptosis and attenuated BCL2L11 expression via post-transcriptional regulation.

miR-494 directly targets BCL2L11 in VSMCs. To determine whether miR-494 directly targets BCL2L11 in VSMCs, luciferase reporter plasmids containing the wild-type or mutant 3'-UTR sequences of BCL2L11 were constructed and co-transfected with miR-494 mimics in murine VSMCs. The results demonstrated that overexpression of miR-494 significantly suppressed the luciferase activity of the WT-pGL3-BCL2L11 reporter plasmid, but did not affect the MUT-pGL3-BCL2L11 reporter plasmid. Control miR mimics did not have any effect on the luciferase activity of the wild-type or mutant plasmids (Fig. 5). These results demonstrated that the BCL2L11 3'-UTR was specifically targeted by miR-494.

miR-494 is dependent on BCL2L11 for inhibition of TNF-α-induced apoptosis in VSMCs. As BCL2L11 was verified as an miR-494 target gene in VSMCs, it was subsequently investigated whether the effect of miR-494 on VSMC apoptosis was dependent on BCL2L11. The efficiency of siRNA knockdown of BCL2L11 was verified by western blot analysis (Fig. 6A). The impact of miR-494 on apoptosis of murine VSMCs with altered BCL2L11 expression levels was detected by TUNEL staining (Fig. 6B and C) and cell death ELISA (Fig. 6D). Cell apoptosis was increased in VSMCs transfected with miR-494 inhibitors and inhibited in cells transfected with BCL2L11 siRNA compared with that of the control. However, the effect of miR-494 inhibitors on cell apoptosis was attenuated in cells co-transfected with BCL2L11 siRNA. Collectively, these results demonstrated that miR-494 was dependent on BCL2L11 to inhibit TNF-α-induced VSMC apoptosis *in vitro*.

Discussion

Apoptosis is a form of programmed cell death that can be induced in all cells. The different stages of this process include blebbing, cell shrinkage, nuclear fragmentation and chromosomal DNA fragmentation (32). Apoptosis of VSMCs, which occurs in various vascular disorders, is an important feature involved in vessel remodeling (33,34). Inhibition of VSMC apoptosis may be a method to halt the initiation and progression of cardiovascular disorders. TNF-α, a cytokine predominantly produced by activated macrophages, is the major extrinsic mediator that induces cell apoptosis. TNF-α binds to TNF receptor 1 and initiates a signaling pathway that leads to caspase activation. The findings in the present study demonstrated that TNF-α induced VSMC apoptosis in a dose-dependent manner, in accordance with previous studies (14).

Previous research on miRNAs expressed in VSMCs demonstrated their fundamental roles in regulating various cell functions, including proliferation, differentiation, calcification and apoptosis (22,27,35,36). However, the role of miRNAs in TNF-α-mediated VSMC apoptosis remains unknown. The current study established an miRNA expression profile, which may assist future research into the role of miRNAs in vascular smooth muscle cells. The expression of miR-494 in murine VSMCs was significantly downregulated during TNF-α-induced apoptosis, indicating that miR-494 may have a role in VSMC apoptosis.

To identify whether miR-494 is directly associated with VSMC apoptosis, the expression of miR-494 was modulated prior to TNF-α-induced apoptosis. Inhibition of miR-494 expression promoted apoptosis of VSMCs, as demonstrated by the increased release of cytoplasmic nucleosomes detected

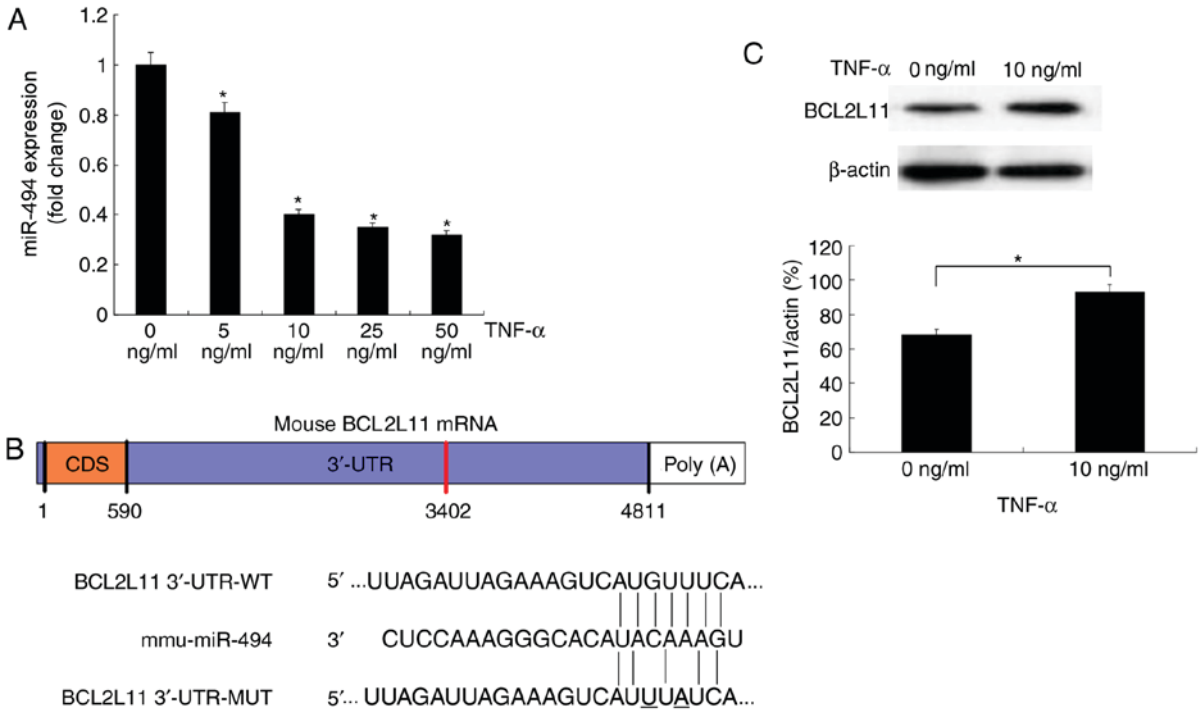

Figure 3. miR-494 and BCL2L11 expression in VSMCs following TNF-α treatment. (A) VSMCs were incubated with 0-50 ng/ml TNF-α for 24 h. miR-494 expression levels were detected by reverse transcription-quantitative polymerase chain reaction. (B) Schematic diagram of the predicted miR-494 binding site in the mouse BCL2L11 3'-UTR. Alignment of miR-494 with 3'-UTR of WT and MUT BCL2L11. The two mutated nucleotides in MUT BCL2L11 are underlined. (C) VSMCs were incubated with 10 ng/ml TNF-α for 24 h. The protein expression levels of BCL2L11 were detected by western blot analysis. n=5. *P<0.05 vs. 0 ng/ml TNF-α. BCL2L11, B-cell lymphoma-2-like 11; CDS, coding sequence; miR-494, microRNA-494; MUT, mutant; TNF-α, tumor necrosis factor-α; UTR, untranslated region; VSMCs, vascular smooth muscle cells; WT, wild-type.

by the cell death ELISA assay. Conversely, overexpression of miR-494 attenuated VSMC apoptosis. These results suggested that miR-494 suppressed VSMC apoptosis *in vitro*. However, these data contradicted the study by Bai *et al* (37), which reported that inhibition of miR-494 leads to overexpression of secretagogin, leading to reduced cell apoptosis and increased chemoresistance in small cell lung cancer. A possible explanation for this phenomenon is that miR-494 may exert different effects in different cell types, and under different stimuli.

Several target genes have been identified for miR-494 in different cells. miR-494 acts as an anti-oncogene in gastric carcinoma by targeting c-Myc (38). Another report demonstrated that miR-494 upregulates hypoxia-inducible factor-1α expression and protects against hypoxia-induced apoptosis in L02 human liver cells (28). miR-494 exerts its cardioprotective effects against ischemia/reperfusion-induced injury by targeting pro-apoptotic genes [Rho-associated protein kinase 1, phosphatase and tensin homolog (PTEN), and calcium/calmodulin dependent protein kinase II δ] and anti-apoptotic genes (leukemia inhibitory factor and fibroblast growth factor receptor 2), and causes activation of the AKT serine/threonine kinase 1 (AKT)-mitochondrial signaling pathway (39). In pancreatic β-cells, miR-494 promotes cell proliferation and inhibits cell apoptosis by targeting PTEN (29). In a rat spinal cord injury model, overexpression of miR-494 was demonstrated to inhibit apoptosis and activate AKT/mechanistic target of rapamycin kinase (mTOR) signaling via inhibition of PTEN (30). miR-494 also inhibits TNF-related apoptosis-inducing ligand-induced apoptosis (40) in non-small-cell lung cancer via downregulation of BCL2L11 (31). Among these target genes identified for miR-494, c-Myc oncogene contributes to the genesis and process of various types of cancer (41,42); PTEN is a dual-specificity phosphatase whose inhibition activates different downstream pathways, including AKT/mTOR signaling that serves a vital role in cell survival and resisting apoptosis, as well as cell regeneration (43-45); BCL2L11 (also known as BIM) is a member of the BH3-only death activator family and is regulated by its interaction with dynein light chain 1. BCL2L11 is one of the most important apoptosis regulators; it induces apoptosis by activating apoptotic proteins (BCL2 associated X apoptosis regulator and BCL2 homologous antagonist/killer) and inactivating anti-apoptotic BCL-2 proteins (46). Under normal physiological conditions, BCL2L11 is sequestered by dynein light chain 1 to form complexes on microtubules. BCL2L11 is released from dynein light chain 1 following phosphorylation in response to a series of apoptotic stimuli, including deprivation of growth cytokines, ionizing radiation and cytotoxic peptides (47-50). BCL2L11 promotes apoptosis of many tumor cell types, including lung cancer, breast cancer, osteosarcoma and melanoma (51). Additionally, increased BCL2L11 protein expression leads to apoptosis in pulmonary arterial smooth muscle cells (52), the critical cells participating in pulmonary arterial hypertension. Therefore, the present study investigated whether BCL2L11 is an miR-494 target in vascular smooth muscle cells in regulating apoptosis. BCL2L11 siRNA inhibited VSMC apoptosis, and miR-494 mimics inhibited VSMC apoptosis by suppressing BCL2L11 protein expression. The current study supported the hypothesis

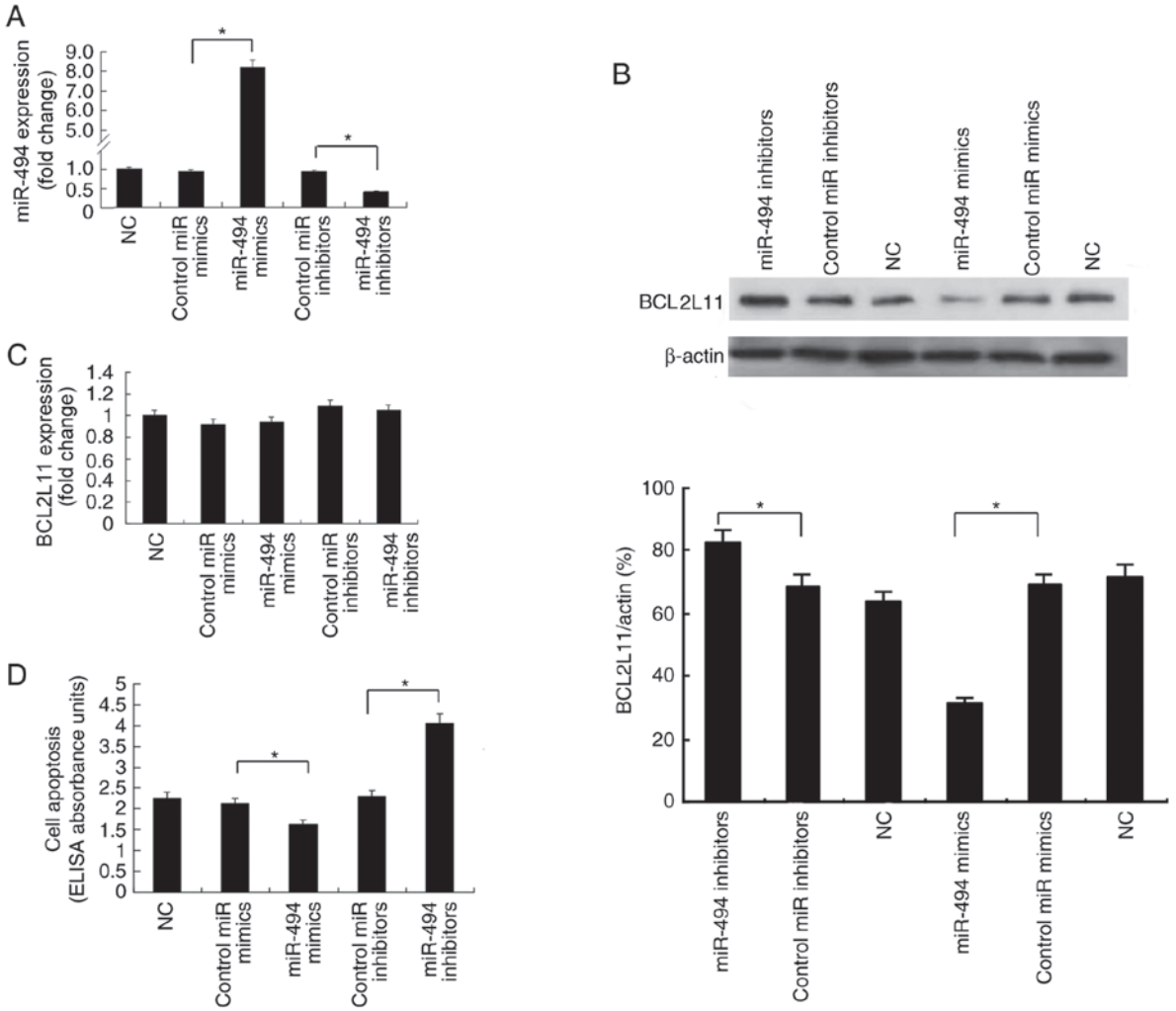

Figure 4. miR-494 inhibits VSMC apoptosis and regulates BCL2L11 expression in VSMCs. Murine VSMCs were transfected with miR-494 mimics or miR-494 inhibitors. (A) miR-494 expression levels were detected by RT-qPCR. (B) Protein and (C) mRNA expression levels of BCL-2L11 were detected by western blot analysis and RT-qPCR, respectively. (D) Cell apoptosis was assessed using a Cell Death ELISA kit and is presented as ELISA absorbance units. *P<0.05. BCL2L11, B-cell lymphoma-2-like 11; miR, microRNA; NC, negative control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; VSMCs, vascular smooth muscle cells.

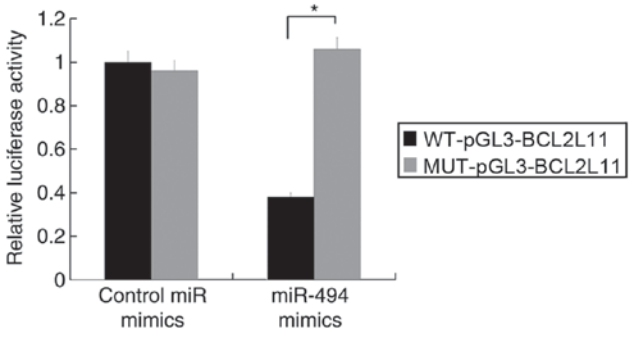

Figure 5. miR-494 targets the BCL2L11 3'-UTR in VSMCs. Murine VSMCs were co-transfected with the WT-pGL3-BCL2L11 or MUT-pGL3-BCL2L11 luciferase reporter plasmid and miR-494 mimics or control miR mimics. At 48 h post-transfection, firefly luciferase activity normalized to *Renilla* luciferase activity was measured. n=3. *P<0.05. BCL2L11, B-cell lymphoma-2-like 11; miR, microRNA; MUT, mutant; UTR, untranslated region; VSMCs, vascular smooth muscle cells; WT, wild-type.

that BCL2L11 is an important direct target of miR-494 in VSMCs. It was confirmed that miR-494 targeted the BCL2L11 3'-UTR through base pairing of the miRNA seed sequence, suggesting miR-494 directly modulated BCL2L11 expression. Overexpression of miR-494 decreased BCL2L11 protein levels, but not mRNA levels, suggesting the effects were mediated by post-transcriptional modulation. Additionally, transfection with miR-494 mimics suppressed the luciferase activity of the WT-pGL3-BCL2L11 reporter plasmid, but not that of the mutant reporter; and finally, BCL2L11 siRNA abolished the VSMC apoptosis that was promoted by miR-494 inhibitors. Taken together, these findings demonstrated that miR-494 inhibited VSMC apoptosis via post-transcriptional modulation of BCL2L11 mRNA.

In conclusion, the present study identified miR-494 as an important inhibitor of VSMC apoptosis, and demonstrated that its effects were mediated by suppression of the target gene BCL2L11. This may be a novel mechanism involved in regulating VSMC apoptosis. Further investigation into miRNA-mediated modulation of VSMC apoptosis may increase our understanding of *in vivo* apoptosis mechanisms and provide novel strategies for prevention and treatment of cardiovascular diseases.

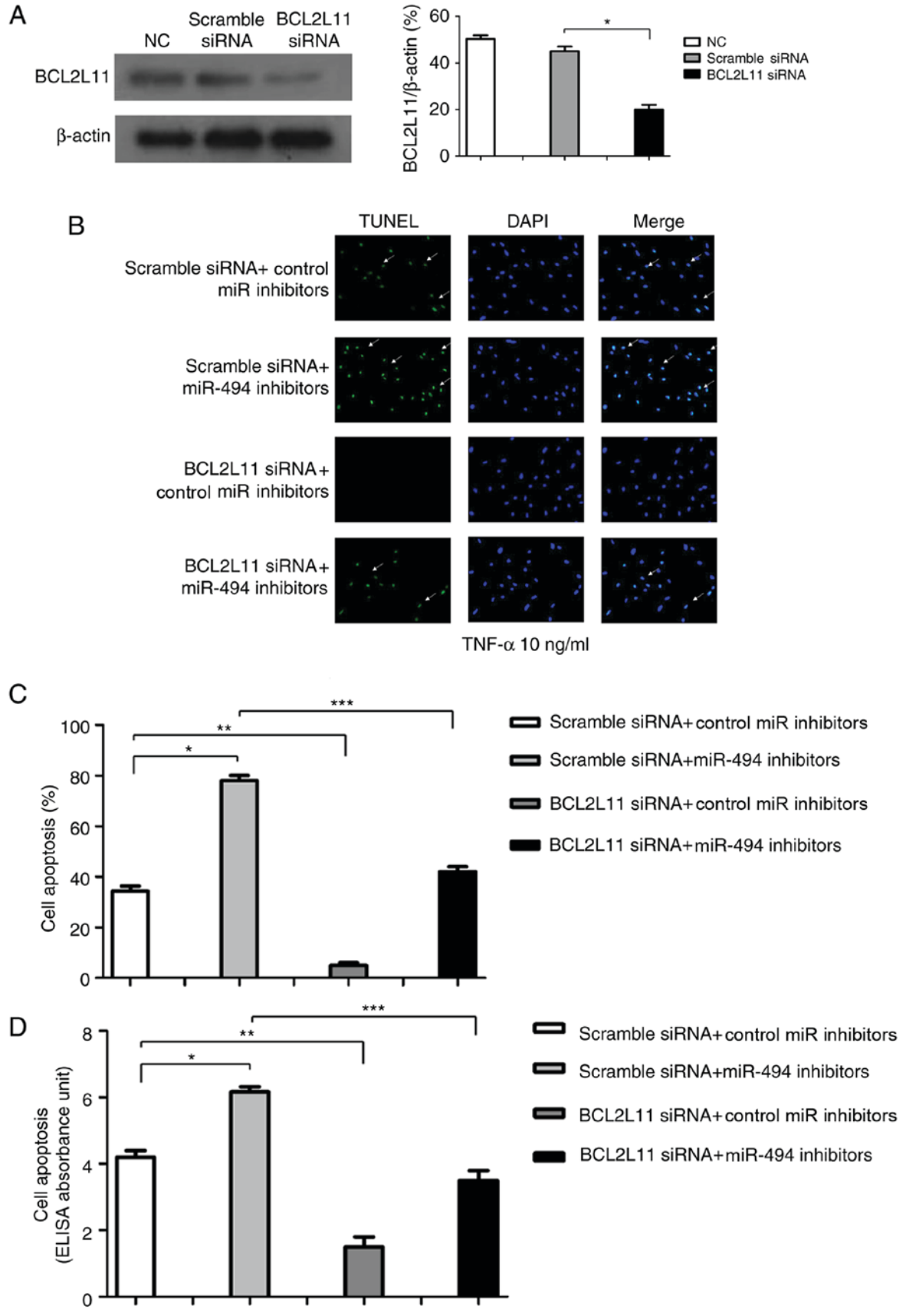

Figure 6. miR-494 inhibits TNF-α induced VSMC apoptosis via BCL2L11. (A) Efficiency of siRNA knockdown of BCL2L11 in murine VSMCs was determined by western blot analysis. Murine VSMCs were transfected with scramble siRNA + control miR inhibitors, scramble siRNA + miR-494 inhibitors, BCL2L11 siRNA + control miR inhibitors and BCL2L11 siRNA + miR-494 inhibitor. Subsequently, 48 h post-transfection, cells were incubated with 10 ng/ml TNF-α for 24 h. (B) Cell apoptosis was measured by TUNEL assay (white arrows indicate apoptotic VSMCs; magnification, x100). (C) Quantitative analysis of TUNEL results. (D) Cell death ELISA was performed and cell apoptosis is presented as ELISA absorbance units. n=5. *P<0.05, **P<0.01, ***P<0.001. BCL2L11, B-cell lymphoma-2-like 11; miR, microRNA; NC, negative control; siRNA, small interfering RNA; TNF-α, tumor necrosis factor-α; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling; VSMC, vascular smooth muscle cell.

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Availability of data and materials

The datasets generated and analyzed during the current study are available in the GEO repository, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE127016.

Authors' contributions

LY and RC conceived the study and designed the experiments. RC, SY, JZ, LL, SL, XL and LY performed the experiments. RC analyzed and interpreted the data, and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The care of animals conformed to the Guide for the Care and Use of Laboratory Animals by the United States National Institutes of Health. The study was approved by The Ethics Review Board of The Second Xiangya Hospital of Central South University (Changsha, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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