Inhibition of microRNA-25 by tumor necrosis factor α is critical in the modulation of vascular smooth muscle cell proliferation

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Abstract. Atherosclerosis and coronary heart disease are characterized by a hyperplastic neointima and inflammation involving cytokines, such as tumor necrosis factor- α (TNF- α). TNF- α is pleiotropic and mediates inflammation and proliferation in various cell types, such as vascular smooth muscle cells (VSMCs). The molecular mechanism for the pleiotropic effects of TNF- α has not previously been fully elucidated. The current study identified that the expression of microRNA-25 (miR-25), a small noncoding RNA, was reduced in response to TNF- α signaling in VSMCs. Restored miR-25 expression inhibited cell proliferation and Ki-67 expression. The present study indicated that cyclin-dependent kinase 6 (CDK6) was the direct target gene of miR-25 using mRNA and protein expression analysis, and luciferase assays. It was also observed that restored CDK6 expression in the miR-25 mimic-treated VSMCs partly reduced miR-25-mediated VSMC proliferation. In conclusion, miR-25 is suggested to be important in TNF- α -induced abnormal proliferation of VSMCs.

Introduction

The vasculature is predominantly comprised of vascular smooth muscle cells (VSMCs), and these cells are involved in the maintenance of vessel tone and blood pressure (1,2). Abnormal proliferation of VSMCs is key in the pathogenesis of cardiovascular diseases, such as coronary heart disease, hypertension and atherosclerosis (3,4). Several growth factors and cytokines have been reported to be capable of stimulating the migration and proliferation of VSMCs, for example leptin, which is vital in restenosis (5-7). A previous study indicated that tumor necrosis factor- α (TNF- α) is a major risk factor in atherosclerosis and coronary heart disease (8). TNF- α is

a pleiotropic inflammatory cytokine that has been reported to serve a pathophysiological role in vascular atherosclerosis (9,10). In addition, TNF- α has been demonstrated to regulate the proliferation, apoptosis, migration and differentiation of VSMCs, which are critical in the pathogenesis of cardiovascular disease (8,11,12). However, the precise mechanism underlying TNF- α -induced proliferation of VSMCs remains to be fully elucidated.

MicroRNAs (miRNAs) are a class of small (22-nucleotide) noncoding RNA molecules that function as endogenous silencers of various target genes (13-15). The majority of mature miRNAs suppress gene expression either by facilitating the cleavage of their target messenger RNAs (mRNAs) or by inhibiting mRNA translation upon imperfect base pairing to the 3'-untranslated region (3'-UTR) of the mRNA (16,17). It has been reported that miRNAs are highly conserved among species and are crucial in various physiological and pathological processes, including age-associated diseases, developmental abnormalities, autoimmune diseases and various types of cancer (18-21). The role of miRNAs in the cardiovascular system has been previously investigated (22,23); however, the function of miRNAs in the proliferation of VSMCs induced by TNF- α remains to be fully investigated.

In the current study, it was hypothesized that the expression of miR-25 was inhibited in TNF- α -stimulated VSMCs and that overexpression of miR-25 in the VSMCs inhibited cell proliferation by targeting cyclin-dependent kinase 6 (CDK6). Thus, the current study aimed to elucidate whether miR-25 was impaired in the pathogenesis of atherosclerosis and coronary heart disease, and may be a potential therapeutic target in atherosclerosis and coronary heart disease.

Materials and methods

Ethical statement. All experiments were approved by the Clinical Research Ethics Committee of The Fourth Affiliated Hospital, Harbin Medical University (Harbin, China).

Vectors and cell culture. The pcDNA-CDK6 vector was purchased from Sigma-Aldrich (Oakville, ON, Canada). The CDK6 3'UTR sequence with the binding site for miR-25 was cloned into the pMIR-REPORT luciferase construct (Ambion Life Technologies, Carlsbad, CA, USA). The

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mutant construct of CDK6 3'UTR was generated using the QuikChange Site-Directed Mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA, USA). The reagents for cell culture (fetal bovine serum, penicillin and streptomycin) were purchased from Gibco Life Technologies (Carlsbad, CA, USA). Human VSMCs were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in the medium 231 supplemented with smooth muscle cell growth supplement (Gibco Life Technologies) at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Cell transfection. The miR-25 mimics and the control were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China) and transfected into the cells to a final oligonucleotide concentration of 20 nmol/l. All cell transfections were introduced using DharmaFECT 1 reagent (GE Healthcare Biosciences, Pittsburgh, PA, USA) according to the manufacturer's instructions.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and the miRNA was reverse transcribed into cDNA (miRNA reverse kit, Promega Corp., Madison, WI, USA). The reaction mixture contained 1 µg purified total RNA, 5X M-MLV buffer (Invitrogen Life Technologies), 200 U/µl (M-MLV; Invitrogen Life Technologies), 1.0 µl dithiothreitol (Invitrogen Life Technologies), 1.0 µl of 10 µmol/l stem-loop RT primer (Invitrogen Life Technologies), 0.5 µl of 40 U/µl RNase inhibitor (Invitrogen Life Technologies) and 1.0 µl of 10 mmol/l deoxyribonucleotide triphosphate (Invitrogen Life Technologies). Relative transcript levels of mRNA were determined by RT-qPCR using the ABI 7300 Real-time PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA). The RT-qPCR reaction was composed of 1X SYBR green fluorescent dye (Takara Biotechnology Co., Ltd., Dalian, China), 1 µl of 10 µM forward primers, 1 µl of 10 µM reverse primers, 1X qPCR mix (Invitrogen Life Technologies) and 1 µl cDNA. Reactions were run with the following thermal cycling parameters: 95°C for 5 min followed by 35 cycles of 95°C for 5 sec and 60°C for 30 sec; melting curve program (60-95°C) with a heating rate of 0.1°C/sec. The relative gene expression was assessed by the $\Delta\Delta C_t$ method. GAPDH or U6 was used as an internal control (Table I).

Cell proliferation assays. Cells were seeded in 96-well plates at a density of 1,000 cells/well with 100 µl complete culture medium. The cells were then cultured for an additional 24, 48 or 72 h. The supernatant was removed and 100 µl medium 231 containing 10 µl Cell Counting kit 8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well for a 2-h incubation at 37°C. The culture plates were then agitated for 10 min and the optical density values were read at a wavelength of 450 nm using a Thermo Fisher Scientific microplate reader (Beijing, China).

Dual luciferase assays. Cells were co-transfected with 0.4 µg miR-25 or negative controls and the reporter construct 0.2 µg pGL-3 control vector (Promega Corp.). Cells were plated at a density of 5x10⁵ cells/well in 24-well plates 24 h prior to

transfection. The cells were harvested 24 h post-transfection and assayed using the Dual-Luciferase Reporter Assay system (Promega Corp.) according to manufacturer's instructions. Firefly luciferase values were normalized to *Renilla* and the ratio of Firefly/*Renilla* luciferase values was reported.

Western blotting. Total cellular protein extraction and western blotting procedures were conducted using standard methods (14). CDK-6 and GAPDH proteins were incubated with rat polyclonal anti-human CDK-6 (1:1,000; Abcam, Cambridge, UK) and mouse polyclonal anti-human GAPDH (1:5,000; ProteinTech Group, Inc., Chicago, IL, USA) primary antibodies, respectively. The membranes were subsequently incubated with horseradish peroxidase-labeled rabbit anti-mouse and goat anti-rabbit antibodies (ZSGB-BIO, Beijing, China). the signal was detected using an enhanced chemiluminescence kit (EMD Millipore, Billerica, MA, USA).

Statistical analysis. Each experiment was repeated a minimum of three times. Statistical analysis was performed using SPSS, version 15.0 (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean $\pm$ standard deviation. Either the analysis of variance or Student's t-test was completed in order to analyze the data and P<0.05 (two-tailed) was considered to indicate a statistically significant difference.

Results

Expression of miR-25 is reduced in VSMCs induced by TNF- α . A significant time-dependent induction of cell proliferation by TNF- α (100 ng/ml) was observed with the maximal response at 72 h (P<0.001) using the CCK-8 proliferation assay (Fig. 1A). Inhibition of miR-25 expression was observed in VSMCs following TNF- α stimulation with RT-qPCR analysis (Fig. 1B).

Overexpression of miR-25 inhibits VSMC proliferation. VSMCs were transfected with miR-25 mimics or control oligo, which were demonstrated to have high transfection efficiency (Fig. 2A; P<0.001). The CCK-8 proliferation assay indicated that cell proliferation was significantly decreased in miR-25 mimic-transfected VSMCs compared with the control oligo-transfected cells or untreated cells (Fig. 2B). This proliferative effect of miR-25 was further confirmed by Ki-67 expression. As demonstrated in Fig. 2C, a significant decrease in the protein expression of Ki-67 was observed in the group transfected with miR-25 mimics as compared with the control group or untreated group.

miR-25 directly regulates the CDK6 gene in VSMCs. The current study suggested CDK6 as a potential target of miR-25 (Fig. 3A). To confirm whether CDK6 is a target, luciferase reporter gene assays were conducted. The miR-25 mimic rather than control significantly suppressed the luciferase activity of the reporter gene containing wild-type 3'-UTR of CDK6 (P<0.001); however, did not affect the activity of the gene containing the mutant 3'-UTR (Fig. 3B). Furthermore, the mRNA and protein levels of CDK6 were detected following transfection of cells with the miR-25 mimic. Notably, mRNA and protein levels of CDK6 were substantially reduced upon transfection with the miR-25 mimic (Fig. 3C and D; P<0.001).

Table I. Primer sequences.

Primer	Sequence (5'-3')
MicroRNA reverse transcription primer	
MicroRNA-25	GTCGTATCCAGTGC GTGTCGTGGAGTCG GCAATTGCACTGGATACGACTCAGAC AAAATATGGAACGCTTCACGAATTTG
U6 snRNA	
Real-time polymerase chain reaction primer sequence	
MicroRNA-25	F: CATTGCACTTGTCTCGGTCTG R: ATTGCGTGTCTGTCGGAGTCG
U6 snRNA	F: CTCGCTTCGGCAGCACATATACT R: ACGCTTCACGAATTTGCGTGTG
GAPDH	F: AATGGGCAGCCGTTAGGAAA R: TGAAGGGGTCATTGATGGCA
Ki-67	F: TCCTTTGGTGGGCACCTAAGACCTG R: TGATGGTTGAGGTCGTTCTTGATG
CDK6	F: GGACTTTCTTCATTACACCG R: GACCACTGAGGTTAGGCCA

F, forward; R, reverse; snRNA, small nuclear RNA.

Figure 1. Expression of miR-25 is reduced in VSMCs induced by TNF- α . (A) The Cell Counting kit-8 proliferation assay demonstrated that TNF- α (100 ng/ml) can promote VSMC proliferation. (B) Reverse transcription-quantitative polymerase chain reaction analysis demonstrated that TNF- α can inhibit the expression of miR-25. ** $P < 0.01$ and *** $P < 0.001$ vs. control. miR-25, microRNA-25; VSMCs, vascular smooth muscle cells; TNF- α , tumor necrosis factor- α ; OD, optical density.

Figure 2. Overexpression of miR-25 inhibits VSMC proliferation. (A) The expression levels of miR-25 were examined following transfection of miR-25 mimics or control or no transfection using reverse transcription-quantitative polymerase chain reaction. (B) The Cell Counting kit-8 proliferation assay demonstrated that the miR-25 mimic was able to inhibit the proliferation of VSMCs. (C) Western blot analysis demonstrated that overexpression of miR-25 inhibited the protein expression of Ki-67. GAPDH was also detected as a loading control. * $P < 0.05$ and *** $P < 0.001$ vs. control. miR-25, microRNA-25; VSMC, vascular smooth muscle cells; OD, optical density; untreat, untreated.

Figure 3. miR-25 directly regulates the CDK6 gene in VSMCs. (A) CDK6 was identified as a potential target of miR-25. CDK6 3'-mut indicates the CDK6 3'-UTRs with six mutation sites (underlined) in miR-25 binding sites. (B) miR-25 mimic but not the control mimic significantly suppressed the luciferase activity of the reporter gene containing WT 3'-untranslated region of CDK6, but did not affect the activity of the gene containing mutant 3'-UTR. (C) Reverse transcription-quantitative polymerase chain reaction analysis demonstrated that overexpression of miR-25 suppressed the mRNA expression of CDK6. (D) Overexpression of miR-25 was observed to inhibit CDK6 protein expression using western blot analysis. GAPDH was also detected as a loading control. *** $P < 0.001$ vs. control. miR-25, microRNA-25; CDK6, cyclin-dependent kinase 6; VSMCs, vascular smooth muscle cells; WT, wild type; MUT, mutant; untreat, untreated.

Figure 4. CDK6 is involved in miR-25-mediated proliferation of VSMCs. (A) Western blot analysis of CDK6 expression in untreated VSMCs or those co-transfected with either miR-25 mimics or control with pCDNA-CDK6 or a pCDNA empty vector; GAPDH was also detected as a loading control. (B) Cell growth curves in the VSMCs transfected with different combinations using CCK8 proliferation analysis. (C) CCK8 proliferation analysis demonstrated that the promotion of cell proliferation upon TNF- α stimulation was significantly attenuated by the re-introduction of miR-25. (D) Western blot analysis demonstrated that TNF- α can enhance the CDK6 protein expression and miR-25 can attenuate this effect. GAPDH was also detected as a loading control. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, vs. untreated. CDK6, cyclin-dependent kinase 6; miR-25, microRNA-25; VSMCs, vascular smooth muscle cells; CCK8, Cell Counting kit 8; TNF- α , tumor necrosis factor- α ; OD, optical density; untreat, untreated.

CDK6 is involved in miR-25-mediated VSMC proliferation. To explore whether miR-25 mediated tumor suppression in VSMCs via directly targeting CDK6, a 'rescue' methodology was used. The expression of CDK6 was restored in cells that had been previously treated with miR-25 mimics. In agreement with the restored expression of CDK6 protein (Fig. 4A), an increase in cell proliferation was observed upon transfection with the CDK6 plasmid (Fig. 4B). Furthermore, miR-25 mimics were able to inhibit cell proliferation and the promotion of cell proliferation following TNF- α treatment was significantly attenuated by the re-introduction of miR-25 (Fig. 4C). In addition, TNF- α was able to enhance CDK6 protein expression and miR-25 attenuated this effect (Fig. 4D).

Discussion

The abnormal proliferation of VSMCs is critical in the pathogenesis of atherosclerosis, coronary heart disease and restenosis (24,25). The presence of chronic or mild inflammation in the arteries is a risk factor for cardiovascular disease (26,27) and TNF- α is a potent inflammatory cytokine. TNF- α has been reported to possess the ability to induce vascular damage and promote the pathogenesis of atherosclerosis (8,28). In addition, TNF- α is known to regulate the proliferation and migration of VSMCs, which results in calcium deposition and an increase in arterial stiffness (12,29,30). However, the precise mechanisms of TNF- α in the regulation of abnormal VSMC proliferation remains to be fully elucidated. The data of the current study suggested that TNF- α inhibits miR-25 expression, overexpression of miR-25 suppresses the proliferation of VSMCs and overexpression of CDK6 impairs the miR-25-induced inhibition of proliferation of VSMCs. These observations suggest that TNF- α may function to promote abnormal proliferation of the VSMCs via the inhibition of miR-25. Thus, miR-25 may be a potential therapeutic target in the treatment of vascular disease.

Previous studies have demonstrated that miR-25 is able to regulate various developmental and cellular processes, and is implicated in a number of human diseases (31-33). miR-25 is known to be important in several types of cancer, including hepatocellular carcinoma, and colon, gastric and lung cancer (34-36). miR-25 has been indicated to be involved in various cellular processes, including cell proliferation, apoptosis and the release of cytokines (37-40). Esposito *et al* (36) demonstrated that miR-25 was reduced in anaplastic thyroid carcinomas, and ectopic expression of miR-25 suppressed the proliferation and colony formation of anaplastic thyroid carcinoma cells via the induction of G₂/M-phase cell cycle arrest (36). However, the precise function of miR-25 in VSMCs remains unknown. In the present study, TNF- α was observed to inhibit the expression of miR-25. Furthermore, overexpression of miR-25 was suggested to inhibit VSMC proliferation. Thus, it is possible that TNF- α induces VSMC proliferation partly via the inhibition of miR-25 expression.

Various previous studies have suggested that cell proliferation may be caused by dysregulation of cell cycle-associated proteins, including cyclins, CDKs and CDK inhibitors (41,42). CDK6 is a member of the family of serine-threonine kinases, which predominantly mediate the regulation of cell cycle progression (43). The CDK6 gene has been observed to often

be amplified or overexpressed in various types of human cancer, including gastric cancer, Ewing's sarcoma and lymphoid malignancies (44-46). A previous study demonstrated that acetylbromide (ABL) treatment inhibited platelet-derived growth factor-induced synthesis and proliferation of DNA in cultured VSMCs. The ABL-mediated inhibition of cell growth was associated with G₁ phase arrest. This was correlated with a reduction in expression levels of cyclins D1, A and E, and CDK2, 4 and 6, in addition to an increase in the expression of the CDK inhibitory protein p21cip1 and enhanced binding of p21cip1 to CDKs (47). The current study demonstrated that CDK8 is the target of miR-25 in VSMCs, as transfection of miR-25 resulted in a substantial reduction of luciferase activity by the luciferase expression constructs that carry the target CDK6 fragment compared with the mutant constructs that lack this site. Furthermore, ectopic expression levels of miR-25 significantly downregulated the transcription of the CDK6 gene and the expression of CDK6 protein. Thus, decreased expression of miR-25 in tumor cells is suggested to contribute to the increasing expression levels of CDK6 at the post-transcriptional level and in atherosclerotic progression.

In conclusion, the present study suggests that TNF- α inhibits the expression of miR-25 and overexpression of miR-25 inhibits the proliferation of VSMC via targeting CDK6. These results aid in the understanding of the pro-atherogenic mechanisms of TNF- α . miR-25 is suggested to serve an important role in the proliferation of VSMCs and atherosclerosis induced by TNF- α . However, in order to further elucidate the precise mechanisms of the TNF- α -mediated regulation of VSMC proliferation, further investigation is required.

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