Original Article

miR-320 inhibits human coronary artery smooth muscle cell proliferation and migration by targeting Krüppel-like factor 5

Yuhua Sun, Zhenghai Zhang, Haibin Zhang, Lixin Zhang, Shunjin Gan

Department of Cardiovascular Medicine, Beijing Luhe Hospital, Capital Medical University, Beijing, China

Received September 7, 2016; Accepted October 24, 2016; Epub March 1, 2017; Published March 15, 2017

Abstract: Krüppel-like factor 5 (KLF5) plays an essential role in the regulation of vascular smooth muscle cell proliferation and migration. In the present study, a post-translational mechanism of miR-320 in TNF-α-induced proliferation and migration of HCASMCs by targeting KLF5 was investigated. The results indicated that TNF-α could accelerate the proliferation and migration of HCASMCs. Overexpressed miR-320 could suppress the proliferation and migration of HCASMCs in the absence and presence of TNF-α. Moreover, bioinformatic analysis showed the potential miR-320 binding sites within the 3′-UTR of KLF5. KLF5 as a direct target of miR-320 was involved in TNFα-induced proliferation and migration of HCASMCs. KLF5 knockout could reverse the increased proliferation and migration in HCASMCs induced by miR-320 loss-of-function and TNF-α treatment. In conclusion, overexpressed miR-320 was verified to protect against TNF-α-induced proliferation and migration of HCASMCs, and the underlying mechanism was mediated, at least partially, by inhibiting KLF5 expression.

Keywords: miR-320, carotid artery injuries, KLF5, TNF-α

Introduction

Vascular smooth muscle cells (VSMCs) are the major constituents of the vasculature and are crucial to maintain an appropriate blood pressure and flow distribution [1]. However, VSMCs exhibit marked differences in migration and proliferation rate suffering from vascular injury [2]. Abnormal proliferation of coronary artery smooth muscle cells (CASMCs) has been linked to coronary artery disease (CAD) [3]. Human CASMCs (HCASMCs) phenotypic changes ranging from differentiated (contractile) to a dedifferentiated (synthetic, noncontractile) are involved in vascular remodeling, restenosis and atherosclerosis [4]. In the vascular media of healthy arteries, HCASMCs exhibit a non-proliferative contractile phenotype; however, growth factors promote transdifferentiation of HCASMCs, which is thought to cause carotid artery injuries [5]. However, the underlying mechanisms of HCASMCs proliferation and migration in carotid artery diseases have not been clearly delineated.

Krüppel-like factor 5 (KLF5) is characterized by a highly conserved interfinger space sequence and has been identified in mammalian [6]. It is generally known that KLF5 regulates a wide variety of cellular functions such as growth, angiogenesis and proliferation [7, 8]. A growing body of evidence suggests that KLF5 is associated with vascular remodeling [9, 10]. In human pulmonary arterial hypertension, KLF5 contributes to pulmonary artery smooth muscle cell proliferation and resistance to apoptosis [11]. Moreover, KLF5 expression is up-regulated in angiotensin II (Ang II)-induced cell proliferation in rat vascular smooth muscle cells [12]. Squeezing of KLF5 suppresses TNFα-induced phenotypic conversion of VSMCs; conversely, overexpressed KLF5 stimulates phenotypic conversion of VSMCs [13]. Interestingly, KLF5 can be regulated by oxidative stress stimuli, such as endothelin-1 (ET-1), H2O2 and inflammatory cytokines, including TNF-α and IL-1β [14].

Recently, post-translational mechanisms of microRNAs (miRs) by regulating target genes
are involved in phenotypic changes of VSMCs within the vascular lesions [13, 15]. Recent study indicates that miR-328 targeting PIM-1 inhibits proliferation and migration of pulmonary arterial smooth muscle cells [16]. Xie et al have been found that miR-599 suppresses VSMCs proliferation and migration by targeting TGFβ2 [17]. miR-145, as a novel smooth muscle cell phenotypic marker and modulator, controls vascular neointimal lesion formation [18]. In the present study, we had found that miR-320 was the most abundant miR in HCASMCs. However, the post-translational mechanisms of miR-320 in TNFα-treated HVSMCs were completely unknown. Therefore, we hypothesized that UA could improve TNFα-induced proliferation and migration in HCASMCs by targeting KLF5.

Materials and methods

Cell culture

Primary human coronary artery smooth muscle cells (HCASMCs) were commercially obtained (Frederick, MD, USA). HCASMCs were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco Life Technologies) that contained 10% fetal calf serum (Gibco Life Technologies), 10% L-glutamine, 0.5% penicillin/streptomycin, 10% nonessential amino acids and 10% pyruvate, in a 5% CO₂ atmosphere and incubated at 37°C.

Cell viability detection by MTT

HCASMCs proliferation were monitored by a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) Cell Proliferation/Viability Assay kit (R&D SYSTEMS) in according to the guidelines.

Wound healing assay

HCASMCs were trypsinized and counted. 1 x 10⁵ cells were reseeded in each well of a new 6-well plate. With incubation overnight, the confluent cells monolayers were scratched with a 10 μL sterile pipette tip. Then the non-adherent cells were washed off with sterilized PBS and serum-free medium was added into the wells. The gap area caused by the scratch was monitored by the inverted microscope (Olympus, Japan).

Luciferase reporter gene activity assay

The 3’-UTR of KLF5 containing the predicated target sites for miR-320 was obtained by PCR amplification. The fragment was inserted into the multiple cloning sites in the pMIR-REPORT luciferase microRNA expression reporter vector (Ambion, Austin, USA). Cells were co-transfected with 0.1 μg of luciferase reporters containing KLF5 3’-UTR and miR-320 mimics by Lipofectamine2000 (Invitrogen, CA, USA). The cells were harvested after 48 h transfection, and luciferase activity was performed according to manufacturer’s instruction.

Mimics and inhibitor of miR-320

The FAM modified 2'-OMe-oligonucleotides were chemically synthesized and purified by high-performance liquid chromatography (GenePharma, Shanghai, China). The 2'-OMe-miR-320 mimics were composed of DNA duplexes with the following sequence: 5’-AAAGCGUGGCUUGAGACCCGA-3’. The sequences of 2'-OMe-miR-320 inhibitor as follows: 5'-UCGCCCUCUCACCCACUUU-3’. The 2'-Ome-scramble oligonucleotides of miR-320 as follows: 5'-CGAGCAUGGCUCUAAGUGCA-3’. Cells were transfected using Lipofectamine2000 (Invitrogen, CA, USA) at a final concentration of 100 nM.

Lentiviral knockdown of KLF5

Control shRNA and specific sh-RNAs targeting KLF5 were purchased from Invitrogen, and the corresponding sequences were cloned into the pSIREN-RetroQ plasmid (Addgene) for retrovirus production with 293T cells. The targeting sequences as following: 5'-CCGGTAAAGCTCAGCCTAGGACTCATCTGACTGATAGT CCTCAGGTAGGTCCTTTTTTG-3’. After 48 hours, infected cells were selected for with puromycin (2 mg/ml). Then the clones were picked and cultured for further experiment.

Real time-polymerase chain reaction

RNA extraction was performed according to the TRizol manufacturer’s protocol (Invitrogen, Carlsbad, CA, USA). Synthesis of cDNAs was performed by reverse transcription reactions with 4 μg of total RNA using Maloney murine leukemia virus reverse transcriptase (Invitrogen) with oligo dT (15) primers (Fermentas) as described by the manufacturer. MiR-320 level was quantified by the mirVana qRT-PCR miRNA detection kit (Ambion, Austin, USA) in conjunction with real-time PCR with SYBR Green. After cycle reaction, the threshold cycle (Ct) was determined and relative miR-320 level was calculat-
miR-320 inhibits HCASMCs proliferation and migration

Western blotting

Cells were lysed in NP-40 buffer (Beyotime Institute of Biotechnology, Haimen, China). Following 5-10 min boiling, homogenate were centrifuged at ×10000 g, 4°C for 10 min to obtain the supernatant. Protein samples (50 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidine difluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% (w/v) non-fat milk powder in Tris-buffered saline and 0.1% (w/v) Tween 20 (TBST), and incubated with the following primary antibodies: KLF5 and β-actin (Santa Cruz Biotechnology, Inc.; 1:1000, Dallas, TX, USA), at 4°C overnight. After being washed, the membranes were incubated with HRP-conjugated anti IgG at room temperature for 2 hour. Signal detection was carried out with an ECL system (Amersham Pharmacia, Piscataway, NJ, USA).

Statistical analysis

The data from these experiments were reported as mean ± standard deviation (SD) for each group. All statistical analyses were performed by using PRISM version 7.0 (GraphPad). Inter-group differences were analyzed by one-way ANOVA, and followed by Tukey’s multiple comparison test as a post test to compare the group means if overall P < 0.05. Differences with P value of < 0.05 were considered statistically significant.

Results

Effect of TNF-α on HCASMCs proliferation and migration

Previous studies suggest that TNF-α has been associated with proliferation, migration and phenotypic modulation in VSMCs [19-21]. In line with these reports, our findings demonstrated that TNF-α at 10, 20, 30 and 40 ng/ml significantly accelerated HCASMCs proliferation by 58%, 85%, 132% and 153%, respective-
miR-320 inhibits HCASMCs proliferation and migration

Figure 2. TNF-α inhibited miR-320 in HCASMCs. Unsupervised hierarchical clustering of differentially expressed miRs in HCASMCs treated with TNF-α (20 ng/ml) for 24 h were measured by microRNA microarray analysis. The figure is drawn by MeV software (version 4.2.6) (A). Real-time PCR analysis was performed to measure miR-320 levels in HCASMCs with or without TNF-α (20 ng/ml) treatment (B and C). Real-time PCR analysis was performed to measure miR-320 levels in HCASMCs transfected with the miR-320 mimics (D) or inhibitors (E). Values were expressed as mean ± SD, n = 3 in each group. ***P < 0.001 versus control group.

miR-320 inhibits HCASMCs proliferation and migration

miR-320 mimics and inhibitors were transfected into HCASMCs to investigate whether miR-320 mediates cell proliferation and migration in the present of TNF-α. As compared to control group, transfection of miR-320 mimics significantly inhibited HCASMCs proliferation both with and without TNF-α treatment (Figure 3A). In contrast to that transfection of miR-320 inhibitors markedly promoted the proliferation of HCASMCs with and without TNF-α treatment (Figure 3B). In addition, the effects of miR-320 on HCASMCs migration were measured using wound healing assays. The findings indicated
miR-320 inhibits HCASMCs proliferation and migration

Figure 3. miR-320 inhibited TNF-α-induced HCASMCs proliferation and migration. miR-320 mimics (A) and inhibitors (B) were transfected into HCASMCs; the cell viability was measured by MTT assay with or without TNF-α treatment. miR-320 mimics (C) and inhibitors (D) were transfected into HCASMCs; the cell migration was measured by wound-healing assay with or without TNF-α treatment. Values were expressed as mean ± SD, n = 3 in each group. *P < 0.05, **P < 0.01 versus control group.

that transfected miR-320 mimics significantly inhibited HCASMCs migration both in the absence and presence of TNF-α (Figure 3C). However, miR-320 inhibitors resulted in accelerating migration of HCASMCs with and without TNF-α treatment (Figure 3D). These results suggested that miR-320 could suppress the proliferation and migration of HCASMCs in the absence and presence of TNF-α.

KLF5 was a direct target of miR-320

Bioinformatics (miRanda Target database, http://www.microrna.org) revealed KLF5 RNA contains one conserved target site of miR-320 (Figure 4A). The wild type sequence of KLF5 (KLF5-Wild) or mutant sequence (KLF5-Mut) as shown in Figure 4A were subcloned into the pMIR luciferase reporter and then co-transfected with the mimics of miR-320 or mimics-Con into HCASMCs, and luciferase assays were performed 24 hours post-transfection. As shown in Figure 4B, the luciferase activity in KLF5 Wild-type HCASMCs transfected with miR-320 mimics was significantly decreased compared with control group. However, transfection of miR-320 mimics into KLF5 Mut-type HCASMCs, the luciferase activity did not show significant difference compared with control group. In addition, inhibition of miR-320 in HCASMCs resulted in up-regulation of KLF5 protein expression (Figure 4C). On the contrary, overexpressed miR-320 markedly decreased the protein expression of KLF5 in HCASMCs (Figure 4D). Collectively, these findings demonstrated that KLF5 is a direct target of miR-320 in HCASMCs.

KLF5 knockdown rescued HCASMCs proliferation and migration

Next, we examined whether KLF5 knockdown could prevent the increased HCASMCs proliferation by TNF-α treatment and miR-320 loss-of-function. First, we found that infection of sh-KLF5 lentivirus resulted in a remarkable reduction of KLF5 protein expression (Figure 5A). As shown in Figure 5B and 5C, KLF5 loss-of-function could significantly prevent the
miR-320 inhibits HCASMCs proliferation and migration

Figure 4. KLF5 was a direct target of miR-320. The putative binding sites of miR-320 on KLF5 3'-UTR was highlighted (A). In a dual-luciferase reporter assay, HCASMCs were transfected with firefly luciferase reporter inserted with wild KLF5 3'-UTR or the reporter inserted with Mut KLF5 3'-UTR, the relative luciferase activities were evaluated at 24 hours after transfection (B). The protein expression of KLF5 was measured by western blotting transfected with miR-320 inhibitors (C) or mimics (D). Values were expressed as mean ± SD, n = 3 in each group. **P < 0.01 versus control group.

increased HCASMCs proliferation and migration in the absence and presence of TNF-α. Moreover, KLF5 loss-of-function could also reverse the increased HCASMCs proliferation and migration with or without miR-320 inhibitors (Figure 5D and 5E). Taken together, these data suggested that miR-320 could suppress TNF-α-induced HCASMCs proliferation and migration by targeting KLF5.

Discussion

Our experiments demonstrated that miR-320 could inhibit TNF-α-induced proliferation and migration of HCASMCs. To further research of the molecular mechanisms of TNF-α-induced proliferation and migration in HCASMCs, bioinformatic analysis showed that KLF5-encoded mRNA contains a 3'-UTR element that was partially complementary to miR-320, indicating that miR-320 might directly target the corresponding sites to regulate the expression of KLF5. In fact, inhibition of miR-320 resulted in up-regulating, and overexpressed miR-320 markedly decreased the protein expression of KLF5 in HCASMCs. Furthermore, our findings indicated that KLF5 knockdown rescued TNF-α- or miR-320 inhibitors-induced proliferation and migration in HCASMCs.

Previous study has been found that miR-320 expression is down-regulated in several malignancies, and enhanced miR-320 inhibits cancer cells proliferation and migration [22]. miR-320 is involved in platelet-derived growth factor-BB enhancing MSC-mediated cardioprotection [23]. Moreover, miR-320 as an antiangiogenic exosomes from cardiomyocytes can inhibit diabetes mellitus-induced myocardial vascular deficiency [24]. miR-320 loss-of-function suppresses cardiomyocyte apoptosis and protects against myocardial ischemia and reperfusion injury [25]. However, there has been no relevant report about miR-320 regulating HCASMCs functions. Our current study replenished previous studies that miR-320 possessed an inhibitory effect of TNF-α-induced proliferation and migration in HCASMCs. Moreover, our findings had identified a new target gene of miR-320. KLF5 as a direct target of miR-320 was confirmed in HCASMCs, which directly bound to the 3'-UTR region of KLF5 to
miR-320 inhibits HCASMCs proliferation and migration

negatively regulate KLF5 expression expression. These results suggested that overexpressed miR-320 in HCASMCs might suppress proliferation and migration by dysregulating expression of KLF5. miR-320 might act as a new effective target for the treatment of carotid artery injuries.

Previous study indicates that KLF5 is an essential transcription factor in cardiovascular remodeling and a potential therapeutic target for cardiovascular disease [26]. KLF5 has been implicated in the regulation of phenotypic modulation of VSMCs and mediates TNFα-dependent phenotypic change of VSMCs [14]. Specifically, overexpressed KLF5 results in markedly increased cell proliferation and cell cycle progression of VSMCs, which directly binds to the promoter and up-regulates gene expression of cyclin D1 [27]. Our results showed that KLF5 knockout could significantly inhibit HCASMCs proliferation and migration in the absence and presence of TNF-α. Moreover, KLF5 knockout could also reverse the increased

Figure 5. KLF5 knockdown rescued HCASMCs proliferation and migration. KLF5 protein expression was measured by western blotting in HCASMCs infected with sh-KLF5 lentivirus (A). HCASMCs infected with sh-KLF5, the cell viability was measured by MTT assay with or without TNF-α treatment (B), and cell migration was measured by wound-healing assay with or without TNF-α treatment (C). HCASMCs infected with sh-KLF5, the cell viability was measured by MTT assay with or without miR-320 inhibitors (D), and cell migration was measured by wound-healing assay with or without miR-320 inhibitors (E). Values were expressed as mean ± SD, n = 3 in each group. *P < 0.05, **P < 0.01 versus control group.
HCASMCs proliferation and migration induced by miR-320 inhibitors. These results suggest that KLF5 plays an essential role in vascular remodeling. Cytokines stimuli, such as AngII, can induce vascular remodeling and enhance the expression of KLF5 [12]. Moreover, inflammatory cytokine, including TNF-α, can stimulate KLF5 expression [14]. KLF5 is enrichment in fetal blood vessels, but the expression of KLF5 is dramatically decreased in adult vessels [28]. In general, KLF5 is activated in VSMCs in abnormal conditions, such as restenosis and atherosclerosis [29]. Our results demonstrated that enhancement of miR-320 blocked TNFα-induced proliferation and migration in HCASMCs through increase of promoter activity of KLF5.

Taken together, TNF-α, as an important inflammatory cytokine, could regulate the proliferation and migration of HCASMCs. Overexpressed miR-320 could suppress the proliferation and migration of HCASMCs in the absence and presence of TNF-α. KLF5 as a direct target of miR-320 signaling pathway could be a therapeutic target for carotid artery injuries-related vascular remodeling.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yuhua Sun, Department of Cardiovascular, Beijing Luhe Hospital, Capital Medical University, Beijing 101149, China. Tel: (+86)10-69543901; E-mail: sunyuhua5568@163.com

References


miR-320 inhibits HCASMCs proliferation and migration


