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MicroRNA-124-3p inhibits collagen synthesis in atherosclerotic plaques by targeting prolyl 4-hydroxylase subunit alpha-1 (P4HA1) in vascular smooth muscle cells

Wei'jia Chen, Fangpu Yu, Mingxue Di, Mengmeng Li, Yifei Chen, Yu Zhang, Xiaolin Liu, Xiaozhen Huang, Mei Zhang*

The Key Laboratory of Cardiovascular Remodeling and Function Research, Chinese Ministry of Education, Chinese National Health Commission and Chinese Academy of Medical Sciences, The State and Shandong Province Joint Key Laboratory of Translational Cardiovascular Medicine, Department of Cardiology, Qilu Hospital of Shandong University, Jinan, China

HIGHLIGHTS

- MiR-124-3p reduces the stability of atherosclerotic plaques by decreasing VSMC content and collagen expression.
- MiR-124-3p could inhibit type I and type III collagen expression in VSMCs.
- MiR-124-3p modulate collagen expression by directly targeting P4HA1.

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ABSTRACT

Background and aims: Collagen synthesis in vascular smooth muscle cells (VSMCs) is very important in atherosclerosis, as it affects plaque stability. In this study, we aim to assess whether miR-124-3p is involved in the collagen synthesis process in VSMCs and the role it might play in atherosclerotic development.
Methods: We modulated the miR-124-3p expression in the aortic root plaques of high-fat-diet fed *ApoE*$^{-/-}$ mice by lentivirus injection. To determine plaque size and the content of plaque-stability-related cells or molecules, stainings, including hematoxylin and eosin, Oil red O, Sirius Red and immunohistochemical staining, were performed. Fluorescence *in situ* hybridization (FISH) was used to locate miR-124-3p in atherosclerotic plaques. Western blotting and RT-qPCR were carried out to determine the level of P4HA1 as well as type I and type III collagen protein and mRNA expression.
Results: Results showed that collagen and VSMC content of plaques was inversely correlated with miR-124-3p levels. By FISH, we identified that miR-124-3p was primarily expressed by VSMCs. We also found that protein levels of type I and type III collagen in aortas and atherosclerotic plaques were decreased by miR-124-3p. We modulated miR-124-3p level *in vitro* and found it could inhibit collagen expression in HASMCs. This might be caused by the downregulation of P4HA1. P4HA1 was predicted as miR-124-3p's direct target, which was verified with a dual luciferase reporter assay and RIP test.
Conclusions: The results presented here provide evidence that miR-124-3p inhibits VSMC collagen synthesis by directly targeting P4HA1, which might decrease atherosclerotic plaque stability.

1. Introduction

Cardiovascular diseases cause over 17 million deaths in 2016, and during the past decade, this number has increased by 14.5% [1]. Atherosclerosis is the primary cause of cardiovascular diseases and is characterized by the accumulation of lipids and fibrous elements in large arteries. Pathological studies have suggested that the composition and vulnerability of plaques play a more important role in the development of thrombus-mediated acute coronary events than the severity of stenosis. Vulnerable plaques tend to exhibit increased lipid and macrophage content as well as thinner fibrous caps, whose maintenance reflects the production and degradation of the extracellular matrix

* Corresponding author. Key Laboratory of Cardiovascular Remodeling and Function Research Department of Cardiology, Qilu Hospital, Shandong University, No.107, Wen Hua Xi Road, Jinan, Shandong 250012, China.
E-mail address: daixh@vip.sina.com (M. Zhang).

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(ECM) [2]. VSMCs play a very important role in the process of atherosclerosis and are the major source of ECM. VSMCs in atherosclerotic arteries tend to have an increased capacity for cell proliferation, migration and production of ECM than those in normal arteries (reviewed by Martin et al.) [3]. ECM primarily consists of different types of elastin fibers and collagen, among which subtype I and III showed the highest expression levels [4]. Many studies investigating the impact of ECM on atherosclerosis development have focused on its degradation, but little is known about the modulatory factors that influence its synthesis. Given the information above, elucidation of the possible mechanisms of VSMC ECM synthesis may help develop strategies to interfere with the process of atherosclerosis.

MicroRNAs (miRNAs), a class of non-coding RNAs that are approximately 21 nucleotides long, function post-transcriptionally by binding to the 3′ or 5' untranslated regions (UTRs) of mRNAs, causing translational repression or mRNA decay [5]. Previous studies have shown that these molecules are involved in many biological processes. Recently, miRNAs were reported to have important roles in atherosclerosis, and many of them were shown to modulate the function of VSMCs. miR-210 is localized in the fibrous cap of the atherosclerotic plaques and can regulate VSMC survival and differentiation in advanced atherosclerotic lesions [6]. miR-181b was reported to negatively regulate the elastin production in VSMCs and tissue inhibitor of metalloproteinase-3 in macrophages; inhibition of miR-181b could promote the stability of atherosclerotic plaques and abdominal aneurysms [7]. miR-132 was reported to directly target LRRFIP1, which induced VSMC proliferation and ERK phosphorylation, repressing neointimal formation [8]. miR-22 could promote the differentiation of smooth muscle cells (SMCs) from stem cells by inhibiting methyl-CpG binding protein 2 [9] and control VSMC phenotypic switch by modulating MECP2, HDAC4, and EVI [10]. miR-125b inhibited VSMC proliferation and migration while enhanced its apoptosis [11]. Another report found that miR-182-3p may regulate SMC phenotypic changes, including inhibition of SMC proliferation and migration and enhancement of phenotype-related genes, by targeting myeloid-associated differentiation marker [12]. Other miRNAs, such as miR-133 [13], miR-663 [14], miR-23b [15] and miR-26a [16] were also reported to take part in the modulation of VSMCs phenotypic switch. However, few studies have investigated the influence of miRNAs on ECM synthesis in VSMCs. Hence, we aimed to identify a miRNA that could regulate the ECM synthesis in VSMCs.

miR-124-3p is a highly conserved miRNA that is detected in many tissues of the human body and plays an important role in brain development and neuronal differentiation [17]. miR-124-3p was shown to be downregulated in different cancers and might act as a tumor suppresser by inhibiting tumor cell proliferation and migration [18–20]. As reported, miR-124 predominantly originates from the 3′ arm of its gene; thus, in most studies about miR-124, the authors are actually referring to miR-124-3p. Recently, miR-124-3p was shown to be heterogeneously expressed among smokers, and increased levels of this molecule might be associated with an increased risk of atherosclerotic disease [21]. Several lines of evidence have suggested that miR-124 could modulate the proliferation and migration of VSMCs [22–25]. In tissues from abdominal aortic aneurysms, a disease strongly associated with disturbed collagen metabolism, miR-124 was significantly upregulated [26]. These results demonstrated that miR-124-3p may be involved in atherosclerosis by affecting VSMC function, especially ECM synthesis.

In the present study, we observed that in an atherosclerotic model of high-fat-diet-fed *ApoE*$^{-/-}$ mice, the level of miR-124-3p was inversely correlated with collagen expression and VSMC content in plaque areas. We modulated the expression of miR-124-3p *in vitro* and found that, in the human aortic smooth muscle cells (HASMCs), miR-124-3p could downregulate type I and type III collagen expression. This result might be explained by the direct binding of miR-124-3p with P4HA1 mRNA, which encodes an enzyme reported to promote collagen synthesis. Hence, we hypothesized that miR-124-3p could inhibit collagen synthesis in VSMCs by targeting P4HA1, thus decrease the stability of plaques in advanced atherosclerosis.

2. Materials and methods

2.1. Animal model

Eight-week-old male *ApoE*$^{-/-}$ mice were obtained from the Beijing University Animal Research Center (China) and were fed a high-cholesterol diet (containing 0.5% cholesterol and 15% lard) for 12 weeks. Lentiviral expression vectors containing green fluorescent protein (GFP) were used to carry miR-124-3p-expressing plasmids or miR-124-3p sponge plasmids (Hanbio, China). The empty vector was used as a negative control. After 8 weeks on a high-fat diet, mice were treated with lentivirus carrying different types of plasmids. Undiluted viral stocks (2×10^7 TU each) were injected into the tail veins of *ApoE*$^{-/-}$ mice (n = 16 for each group) at the indicated time periods. Four weeks after the injection, the mice were euthanized and analyses were performed. All *in vivo* protocols followed the Animal Management Rules of the Chinese Ministry of Health and were approved by the Animal Care Committee of Shandong University.

2.2. Histology and immunohistochemical analysis

The animals were anesthetized and sacrificed by drawing blood from the heart and were then perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde. The aortic arches as well as the hearts were removed from the mice, embedded in OCT compound (Tissue-Tek, Japan) and then stored at −80 °C. Transverse 6 μm cryosections were prepared by Thermo NX50 (Thermo Fisher Scientific, USA).

These cryosections were routinely stained with hematoxylin and eosin (H&E) and Oil red O (Sigma Diagnostics, USA) for lipid visualization and Sirius Red for collagen content. Corresponding sections on separate slides were stained immunohistochemically with antibodies against a macrophage-specific antigen (MOMA-2, diluted 1:200; Abcam, UK), α–smooth muscle cell actin antigen (α-SMA, diluted 1:500; Abcam, UK) and P4HA1 antigen (diluted 1:200; Abcam, UK). The images were collected by a Nikon Ni-U microscope (Nikon, Japan) and analyzed with Image-Pro Plus 6.0 (Media Cybernetics, USA).

2.3. Fluorescence in situ hybridization (FISH)

The staining procedure was performed with an enhanced sensitive ISH detection kit (Bosterbio, China). Six-micron cryosections were prepared ahead of time. We applied fresh pepsin diluted with 3% citric acid on the slice for 1 min to expose the nucleic acid and then washed the slides 3 times with 0.5 M PBS. Following incubation with hybridization buffer at 37 °C for 2 h, the slides were incubated with 20 μl of 40 nM 5′, 3′ double digoxigenin (DIG)-labeled locked nucleic acid (LNA)-modified miRCURY miR-124-3p detection probe or scrambled miRCURY LNA oligonucleotide as a negative control (Biosune, China) in the same buffer at 37 °C overnight. After the samples were washed with different concentrations of SSC buffers and blocked for 30 min (10% FBS), biotin-labeled mouse monoclonal antibody to DIG (from the kit) as well as rabbit monoclonal antibody to α-SMA (Abcam, UK) or rat monoclonal antibody to MOMA-2 (Abcam, UK)/endomucin (Abcam, UK) was added for 2 h at room temperature. After the samples were washed (0.5 M PBS) 3 times, 20 μl SABC–FITC (diluted 1:100; from the kit) and TRITC-labeled anti-rabbit/rat antibodies (diluted 1:200; Proteintech, USA) were added to each section and incubated in the dark at 37 °C for 30 min. The slides were washed 3 times with 0.5 M PBS, and then, they were mounted with DAPI (Invitrogen, USA) for 10 min. After three 5 min washes in PBS for the last time, the slides were detected under a Nikon Ni-U microscope (Nikon, Japan) and pictures were collected.