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miR-155-dependent regulation of mammalian sterile 20-like kinase 2 (MST2) coordinates inflammation, oxidative stress and proliferation in vascular smooth muscle cells

Zhan Yang, Bin Zheng, Yu Zhang, Ming He, Xin-hua Zhang, Dong Ma, Ruo-nan Zhang, Xiao-li Wu, Jin-kun Wen *

Department of Biochemistry and Molecular Biology, The Key Laboratory of Neural and Vascular Biology, China Administration of Education, Hebei Medical University, Shijiazhuang, China

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ABSTRACT

In response to vascular injury, inflammation, oxidative stress, and cell proliferation often occur simultaneously in vascular tissues. We previously observed that microRNA-155 (miR-155), which is implicated in proliferation and inflammation is involved in neointimal hyperplasia; however, the molecular mechanisms by which it regulates these processes remain largely unknown. In this study, we observed that vascular smooth muscle cell (VSMC) proliferation and neointimal formation in wire-injured femoral arteries were reduced by the loss of miR-155 and increased by the gain of miR-155. The proliferative effect of miR-155 was also observed in cultured VSMCs. Notably, expression of the miR-155-target protein mammalian sterile 20-like kinase 2 (MST2) was increased in the injured arteries of miR-155$^{-/-}$ mice. miR-155 directly repressed MST2 and thus activated the extracellular signal-regulated kinase (ERK) pathway by promoting an interaction between RAF proto-oncogene serine/threonine-protein kinase (Raf-1) and mitogen-activated protein kinase kinase (MEK) and stimulating inflammatory and oxidative stress responses; together, these effects lead to VSMC proliferation and vascular remodeling. Our data reveal that MST2 mediates miR-155-promoted inflammatory and oxidative stress responses by altering the interaction of MEK with Raf-1 and MST2 in response to vascular injury. Therefore, suppression of endogenous miR-155 might be a novel therapeutic strategy for vascular injury and remodeling.

1. Introduction

In response to vascular injury, vascular smooth muscle cells (VSMCs) undergo a series of characteristic changes including phenotypic modulation, abnormal proliferation, migration, matrix synthesis, and inflammation [1]; these events are crucial for the development and progression of vascular remodeling diseases such as atherosclerosis, hypertension, and restenosis after angioplasty. VSMCs are the principal effector cells in this process; therefore VSMCs coordinate and synchronize extremely complex inflammatory, proliferative, differentiation and oxidative stress programs [2,3]. Although a few pathophysiological mechanisms associated with VSMC proliferation, inflammation and oxidative stress are known, the molecular mechanisms by which the VSMC responses to injury are coordinated remain unclear.

MicroRNAs (miRNAs) regulate gene expression at the post-transcriptional level by promoting mRNA degradation or by inhibiting translation and play a critical role in vascular inflammation and remodeling [4]. Several miRNAs such as miR-21, miR-126, miR-133, miR-143/145, miR-146a, and miR-221/222 have been implicated in vascular inflammation and remodeling [5]. For example, miR-133 inhibits VSMC proliferation by targeting transcription factor Sp1 [6], and miR-221/222 promotes VSMC proliferation by targeting p27 and p57 [7]. miR-143/145 are molecular keys that switch the VSMC phenotype [8]. miR-126 inhibits vascular cell adhesion molecule 1 (VCAM-1) expression and reduces leukocyte adherence to endothelial cells [9]. miR-155 is a target of several inflammatory mediators. Recently, we found that miR-155 is involved in neointimal formation in carotid arteries after angioplasty [10]. miR-155 is a pleiotropic regulator of inflammation-related diseases and is critical for various physiological and pathological processes including inflammation, differentiation, carcinogenesis, oxidative stress, and cardiovascular remodeling [11,12]. However, the role of miR-155 in coordinating inflammation, oxidative stress and vascular remodeling has not been elucidated.

The mammalian sterile 20-like kinase 2 (MST2), also called Ser/Thr kinase 3 (STK3), and its close homolog MST1 (STK4) are members of the germinal center kinase group II family which are mitogen-activated protein kinase (MAPK)-related kinases [13]. As a core component of the Hippo pathway in mammalian cells, MST2 regulates cell proliferation, growth and apoptosis [14]. Proteomic analysis of RAF proto-oncogene

* Corresponding author at: Department of Biochemistry and Molecular Biology, Key Laboratory of Neural and Vascular Biology, Ministry of Education, Hebei Medical University, No. 361 Zhongshan East Road, Shijiazhuang 050017, China. Tel.: +86 31186265563; fax: +86 31186266180.
E-mail address: wjk@hebmu.edu.cn (J. Wen).

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serine/threonine-protein kinase (Raf-1) signaling complexes revealed that Raf-1 interacts with MST2 and counteracts apoptosis by suppressing the activation of MST2 [15]. MST2 binds to Raf-1 at two distinct sites that partially overlap with the mitogen-activated protein kinase kinase (MEK)-binding domain on Raf-1 [16], suggesting that MST2 competes with MEK to bind to Raf-1 and affects the MAPK signaling cascade. Furthermore, the interaction between MST2 and Raf-1 regulates the ERK1/2 pathway and inhibits the pro-apoptotic activation of MST2 [15,17]. Although MST1/2 activates Ser/Thr protein kinases and regulates the Raf-1/ERK pathway activity, the role of MST2 in regulation of inflammation and oxidative stress in VSMCs has not been studied.

In this study, we investigated whether and how MST2 mediates miR-155-promoted inflammation and oxidative stress, which lead to VSMC proliferation and vascular remodeling, through integrating inflammatory and oxidative stress signaling.

2. Materials and methods

2.1. Animal models

All animal studies were approved by the Institutional Animal Care and Use Committee of Hebei Medical University (approval ID: HebMU 20080026) and all efforts were made to minimize suffering. Eight- to 12-week-old male wild-type C57BL/6 mice and miR-155$^{-/-}$ mice (Jackson Laboratory, Bar Harbor, ME) were anesthetized with 1.5% isoflurane. To reproducibly induce vascular remodeling, we performed femoral artery wire injury as previously described [18]. Briefly, we carefully separated the left femoral artery and the accompanying femoral nerve under anesthesia. A small branch of the femoral artery was isolated under the muscles. The femoral artery and the small branch were looped with 6–0 silk sutures to temporarily stop blood flow during the procedure. A spring wire (0.38-mm diameter, Cook Inc., Bloomington, IN) was inserted into the femoral artery more than 5 mm and moved in and out twice. The wire was then removed, and blood flow in the femoral artery and branch was restored by releasing the sutures, and the skin incision was closed with a 5–0 silk suture. The other femoral artery was sham-operated and served as a control.

For the miR-155-overexpression model, femoral artery wire injury was performed as described above. Immediately after injury, the femoral artery was cannulated, and the biclamped segment was incubated with 20 μl of adenovirus (1×10^{10} pfu/ml) encoding miR-155 or GFP for 15 min. After 14 days, all animals were anesthetized and perfused with cold 0.9% NaCl, and the tissues were harvested for analysis of RNA, morphology, and histology.

2.2. Morphometry and histology

Mice were euthanized, perfused and then fixed with 4% paraformaldehyde in 0.9% NaCl for 3 min through the left ventricle under physiological pressure. The femoral arteries were harvested, fixed with formalin and embedded in paraffin. Ten consecutive 5-μm-thick sections were prepared for hematoxylin and eosin staining; the sections were prepared at intervals of 550 μm. Images were acquired using a Leica microscope (Leica DM6000B, Switzerland) and digitized with LAS V.4.4 (Leica). Morphometric analysis of the neointimal area and measurement of the intima/media (I/M) ratio were performed in a blind manner.

2.3. Immunofluorescence staining

Immunofluorescence staining was performed with 5 μm paraffin cross-sections from the femoral artery. After deparaffinized with xylene and rehydrated, the slides were pre-incubated with 10% normal goat serum (710027, KPL, USA) and then incubated with primary antibodies anti-SM22α (ab14106, Abcam), anti-MAC2 (60207-1, Proteintech), anti-MST2 (ab52641, Abcam). Secondary antibodies were fluorescein-labeled antibody to rabbit IgG (021516, KPL, USA) and rhodamine-labeled antibody to mouse IgG (031806, KPL, USA), or fluorescein-labeled antibody to mouse IgG (021815, KPL, USA), rhodamine-labeled antibody to rabbit IgG (031506, KPL, USA). In each experiment, DAPI (157574, MB biomedical) was used for nuclear counter staining. Images were captured by confocal microscopy (DM6000 CFS, Leica) and processed by LAS AF software.

2.4. In situ hybridization

Paraffin cross-sections (5-μm thick) from femoral arteries were deparaffinized and rehydrated for *in situ hybridization* according to user manual of miRCURY LNATM microRNA ISH Optimization Kit (Exiqon). Hybridization was performed using fluorescence-labeled miR-155 probes (50 nM) in hybridization buffer (Exiqon) by incubation at 55 °C for 1 h in a thermoblock (Labnet). After stringent washing with SSC buffer, nonspecific binding sites were blocked with 10% normal goat serum (710027, KPL, USA). The sections were then incubated for 1 h at 37 °C with anti-SM22α primary antibody (ab14106, Abcam) diluted 1:50 in PBS. After washing with PBS, the sections were incubated with a rhodamine-labeled secondary antibody (031506, KPL, USA) at 37 °C for 30 min. Images were acquired using a Leica microscope (Leica DM6000B, Switzerland) and digitized with a software of LAS V.4.4 (Leica).

2.5. Isolation of mRNA and real time PCR

Total RNA was extracted from femoral arteries, which were homogenized with gentle MACSTM Dissociator (Miltenyi Biotec), and cultured VSMCs using the Trizol (Invitrogen^{TM}) according to the manufacturer's instructions. The quality of the RNA was determined using a Biospectrometer (Eppendorf). For microRNA: reverse transcription and qRT-PCR was performed using the Taqman microRNA Reverse Transcription kit and TaqMan Universal Master MixII (Applied Biosystems) with specific primers for mmu-miR-155 (Assay ID: 001806) and internal control RNU6b (U6) (Assay ID: 001093) according to the manufacturer's protocol. For large mRNA: cDNA was synthesized using an M-MLV First Strand Kit (Life Technologies). qRT-PCR of mRNAs was performed using Platinum SYBR Green qPCR Super Mix UDG Kit (Invitrogen), and real-time PCR experiments were carried on a ABI 7500 FAST system (Life Technologies). Relative amount of transcripts was normalized with GAPDH and calculated using the $2^{-\Delta\Delta Ct}$ formula as previously described [19]. Supplementary Table I summarizes the primer sequences.

2.6. Cell culture, siRNA transfection and plasmid constructs

Vascular smooth muscle cells were isolated from the thoracic aorta of male Sprague–Dawley rats (60–80 g) as previously described [19] and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies, Rockville, MD) containing 10% fetal calf serum (ABGENT), 100 units/ml of penicillin, and 100 μg/ml of streptomycin under 5% CO_2 atmosphere at 37 °C. VSMCs from passages 3 to 5 were used in experiments. 293A cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies, Rockville, MD) supplemented with 10% FCS. All cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Small interfering RNAs (siRNAs) against the rat MST2 sequence (accession number Genbank: NM_031735.1) were designed and synthesized by Sigma. The siRNA sequences used in these studies were as follows: MST2 siRNA#1: 5′-GGG UCC GUU UCA GAC AUA Att-3′; 5′-UUA UGU CUG AAA CGG AC CCtt-3′; MST2 siRNA#2: 5′-CGA GGU AAU UCA AGA AAU Att-3′; UAU UUC UUG AAU UAC CUC Gtt-3′; siControl: 5′-UUC UCC GAA CGU GUC ACG UTT-3′; 5′-ACG UGA CAC GUU CGG AGA ATT-3′. Twenty hours after transfection, the VSMCs were treated with 10% FCS. The cells were then harvested and lysed