

miR-26b-5p regulates hypoxia-induced phenotypic switching of vascular smooth muscle cells via the TGF- β /Smad4 signaling pathway

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Received September 23, 2015; Accepted July 14, 2016

DOI: 10.3892/mmr.2017.6509

Abstract. Hypoxia contributes to the phenotypic switch of vascular smooth muscle cells (VSMCs). Various microRNAs (miRNAs) participate in this process as post-transcriptional regulators, however the mechanism remains unclear. In the present study, mouse VSMCs (mVSMCs) harvested from aortas were cultured in normoxic and hypoxic conditions, and the mRNA levels of miR-26b-5p, desmin, H-caldesmon and smoothelin were quantified using reverse transcription-quantitative polymerase chain reaction. Following treatment with a miR-26b-5p antagonist (agomir) or non-targeting control (scramble), the cell areas of normoxic and hypoxic mVSMCs were analyzed by immunofluorescence staining. In addition, the protein expression levels of collagen I α , Smad2/phosphorylated (p)-Smad2, Smad3/p-Smad3 and Smad4 were determined by western blotting. Potential miR-26b-5p binding sequences in the 3'-untranslated region (UTR) of Smad4 were investigated, and the distribution of Smad4 in mVSMCs was visualized using immunofluorescence methods. Hypoxic mVSMCs exhibited a significant downregulation miR-26b-5p, upregulation of hypoxia inducible factor-1 α mRNA and suppression of desmin, H-caldesmon and smoothelin mRNA levels. Additionally, miR-26b-5p agomir reduced the cell area and decreased collagen I α expression levels in hypoxic mVSMCs compared with normoxic mVSMCs transfected with agomir, and the area was comparable with those of normoxic mVSMCs transfected with agomir or scramble. Furthermore, miR-26b-5p suppressed Smad4 expression in hypoxic mVSMCs, but did not change the expression levels of Smad2 and Smad3, p-Smad2 and p-Smad3, however p-Smad2 and p-Smad3 levels were upregulated in response to hypoxic stimuli. Additionally, the miR-26b-5p agomir caused weak immunoreactivity with Smad4 in hypoxic mVSMCs.

The binding motif of miR-26b-5p in the Smad4 3'-UTR was identified as UACUUGA at position 978-984. These findings suggest that miR-26b-5p regulates hypoxia-induced phenotypic switching of VSMCs via the transforming growth factor β /Smad4 signaling pathway.

Introduction

As a major structural component of the vessel wall, vascular smooth muscle cells (VSMCs) provide vasoactivity by contracting and relaxing, regulating extracellular matrix (ECM) turnover and providing mechanical stability. Physiologically, VSMCs display a characteristic contractile (differentiated) phenotype exhibited as a minimal rate of proliferation, and balance the production and degradation of ECM components in a constant equilibrium. However, VSMCs exhibit remarkable plasticity and are able to switch from a contractile phenotype to a less natural synthetic (proliferative) phenotype in response to various stimuli. During this process, termed phenotypic switching or phenotypic modulation, VSMCs lose their contractile apparatus (myofilaments) and exhibit vigorous proliferation and increased synthesis of ECM components (1-4).

Previous studies have reported that hypoxia can cause phenotypic switching of VSMCs by multiple mediators via different signaling pathways (5,6). Although the molecular mechanisms underlying hypoxia-induced phenotypic switching of VSMCs remain unclear, various microRNAs (miRNAs) have been identified as crucial post-transcriptional modulators that regulate the phenotype of VSMCs. For example, miRNA (miR)-142-3p is understood to be a key regulator of the transforming growth factor β (TGF- β)-mediated contractile phenotype of VSMCs, targeting Dedicator of cytokinesis 6 to inhibit cell migration (7); miR-96 combined with Tribbles-like protein 3 participates in the regulation of the VSMC contractile phenotype via the bone morphogenic protein 4 signaling pathway (8). Additionally, various studies have reported that specific miRNAs, including miR-21 and miR-130a, regulate the behavior of hypoxic VSMCs according to their phenotype (9,10). However, whether and how miR-26b participates in regulating hypoxia-induced phenotypic switching of VSMCs remains unknown, although miR-26b has been confirmed as an important regulator of different cellular processes (11-13).

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Key words: miR-26b-5p, hypoxia, phenotypic switching, vascular smooth muscle cells, TGF- β /Smad4 signaling pathway

In the present study, the expression level of miRNA-26b-5p in VSMCs exposed to low oxygen was detected, and the correlation of this change with the mRNA levels of the specific VSMC biomarkers, desmin, H-caldesmon and smoothelin, was analyzed. A miR-26b-5p agomir was then used, and its effect on cell morphology, collagen Ia expression, and the protein expression levels of Smad2, 3, and 4, and phosphorylated (p)-Smad2 and 3 in hypoxic VSMCs were determined. Additionally, expression changes of cytoplasmic Smad4 in normoxic and hypoxic VSMCs transfected with the miR-26b-5p agomir were examined, and the potential binding sites of miR-26b-5p in the Smad4 sequence were analyzed. The data reported in the current study suggest that miR-26b-5p regulates hypoxia-induced phenotypic switching of VSMCs via the TGF- β /Smad4 signaling pathway.

Materials and methods

Animals. In the current study, a total of 45 male mice (age, 8-12 weeks) were purchased from the Animal Centre of the Second Military Medical University (Shanghai, China). All animals received care in compliance with the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources, National Research Council (Washington, DC, USA). Animals were housed at room temperature, with free access to food and water, and were maintained in a 12 h light/dark cycle. Prior to the experiments, mice were acclimated to laboratory conditions for at least 7 days. The current study was approved by the Medical Ethics Committee of Gongli Hospital (Shanghai, China).

Cell culture. Mice at 8-12 weeks of age were sacrificed by CO₂ overexposure. Using fine-tipped forceps and spring scissors, the sheath around the aorta was opened. The connective fascia and adventitia were then carefully removed, and the aorta (from the aortic arch to the iliac bifurcation) was removed and placed in a 6-cm culture plate containing Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and Fungizone (cat. no. SV30079.01; HyClone; GE Healthcare Life Sciences, Logan, UT, USA). The aorta was subsequently opened with spring scissors and the blood clots were removed. The aortic intima was then scraped with a scissor blade, before it was cut into sections (4-mm in length). Each section was subsequently placed under a plastic cell culture coverslip and maintained in DMEM supplemented with 20% fetal bovine serum (HyClone; GE Healthcare Life Sciences) at 37°C, with 5% CO₂. The coverslip was removed after tissue sections had adhered, and the culture medium was refreshed every 3-4 days. Once cells had reached ~80% confluence, they were dissociated by adding 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.). Following inactivation of trypsin, cells were subcultured at ratio of 1:3. Cells at passages 3-5 were used in downstream experiments. Each experiment was repeated at least 3 times with different cell preparations.

Cells were cultured to 90% confluence in 6 cm² culture dishes and then subcultured at a ratio of 1:2. VSMCs (5-10x10⁶ cells) were then subjected to normoxic or hypoxic conditions. For normoxic conditions, mouse VSMCs (mVSMCs) were incubated at 37°C with 5% CO₂ humidified

atmosphere. For hypoxia, mVSMCs were cultured in normoxic conditions for \geq 24 h until adherent, and then cultured at 37°C for 3 h in a humidified hypoxic chamber supplemented with 1% O₂, 94% N₂ and 5% CO₂.

Transient transfection of miR-26b-5p agomir. The micrON™ miR-26b-5p agomir and micrON™ miRNA agomir control (scramble) were purchased from Guangzhou Ribobio Co., Ltd. (Guangzhou, China) and used to treat mVSMCs using riboFECT CP reagent (RiboBio Co., Ltd.) in accordance with the manufacturer's instructions.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of mRNA levels. According to the manufacturer's instructions, the total RNA was extracted using TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Following quantification of RNA samples using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA), 1 μ g RNA was used to generate cDNA by RT reaction using PrimeScript Reverse Transcriptase kit (Takara Bio. Inc., Otsu, Japan) according to the manufacturer's instructions. The PCR thermal cycling parameters consisted of 1 cycle for 5 min at 95°C, followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, and a final extension step at 72°C for 5 min. A dissociation curve was obtained for each PCR product. qPCR was performed on an ABI PRISM 7000 Sequence Detection System using SYBR Premix Ex Taq™ II kit (Takara Bio. Inc.) with specific primers for hypoxia inducible factor (HIF)-1 α , desmin, H-caldesmon, smoothelin and β -actin. Each sample was analyzed in triplicate and target gene expression levels were normalized to β -actin mRNA levels. The fold change in target gene expression was calculated using the 2^{- $\Delta\Delta C_q$} method (14). The primer sequences were as follows: HIF-1 α forward, 5'-GATGAGGCTTACCATCAGCT-3', and reverse, 5'-ATGTCACCATCATCTGTGAG-3'; desmin forward, 5'-GTTTCA GACTTGACTCAGGCAG-3', and reverse, 5'-TCTCGCAGG TGTAGGACTGG-3'; H-caldesmon forward, 5'-ATGGTA GAGGAGAAAACACCAGA-3', and reverse, 5'-CCATCC CCTTCTATTTTGGACTC-3'; smoothelin forward, 5'-GAG CGGCAAGACAACAAGGA-3', and reverse, 5'-CAGTCT CCCTGCCAATCGT-3'; β -actin forward 5'-CAACCGTGA AAAGATGACCC-3', and reverse, 5'-GTCTCCGGAGTCCAT CACAA-3'.

RNA extraction and RT-qPCR analysis of miRNA levels. Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed at 42°C for 60 min followed by 70°C for 10 min. PCR was performed using a Bulge-Loop™ miRNA RT-qPCR kit (catalogue no. R11067.1; Ribobio, Co., Ltd.). Thermal cycling conditions consisted of 1 cycle for 10 min at 95°C followed by 40 cycles at 95°C for 2 sec, 60°C for 20 sec, and 70°C for 10 sec. A dissociation curve was obtained for each PCR product. The primers used for the detection of miR-26b-5p and control U6 small nuclear RNA were designed and produced by Guangzhou Ribobio Co., Ltd. Each sample was analyzed in triplicate and target gene expression levels were normalized to U6 mRNA levels. The fold change in target gene expression was calculated using the 2^{- $\Delta\Delta C_q$} method.

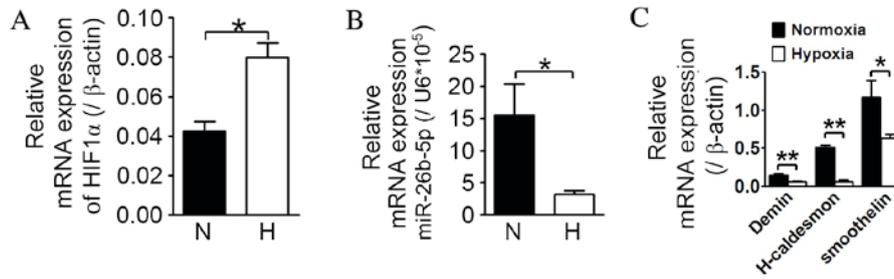


Figure 1. Hypoxia suppresses miR-26b-5p in mVSMCs and downregulates mRNA levels of specific biomarkers of contractile mVSMCs. The hypoxic mVSMCs exhibited (A) a significant increase in HIF-1 α mRNA levels compared with normoxic mVSMCs and (B) miR-26b-5p expression was downregulated in hypoxic mVSMCs compared with normoxic mVSMCs. (C) Downregulated expression of desmin, H-caldesmon and smoothelin mRNA levels, specific biomarkers of contractile VSMCs, was also observed in hypoxic conditions compared with normoxic conditions. Values are presented as the mean \pm standard deviation. * P <0.05 and ** P <0.01 vs. the normoxia group. miR, microRNA; mVSMCs, mouse vascular smooth muscle cells; HIF, hypoxia inducible factor; N, normoxia; H, hypoxia.

Western blotting. Cells were harvested and lysed with radioimmunoprecipitation assay lysis buffer to extract the total protein. Protein concentration was measured by the micro-bicinchoninic acid assay, and 100 μ g protein per lane was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12% gel, then transferred onto polyvinylidene difluoride membranes (Beyotime Institute of Biotechnology, Haimen, China). Non-specific binding sites were blocked by immersing the membrane in tris-buffered saline (TBS) solution containing 5% non-fat milk for 1 h at room temperature and agitating at 50 rpm. The membranes were then washed twice for 2 min in TBS solution. Immune complexes were formed by incubating the membranes overnight at 4°C with primary antibodies against collagen I α (polyclonal rabbit anti-mouse; dilution, 1:1,000; cat. no. ab34710; Abcam, Cambridge, UK), as well as β -actin (monoclonal rabbit anti-mouse; dilution, 1:1,000; cat. no. 8457), Smad2 (monoclonal rabbit anti-mouse; dilution, 1:100; cat. no. 5339) and p-Smad2 (polyclonal rabbit anti-mouse; dilution, 1:1,000; cat. no. 3101), Smad3 and p-Smad3 (monoclonal rabbit anti-mouse; dilution, 1:1,000; cat nos. 9523 and 9520, respectively), Smad4 (monoclonal rabbit anti-mouse; dilution, 1:1,000; cat. no. 38,454; all from Cell Signaling Technology, Inc., Danvers, MA, USA). Blots were then washed and incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:2,000; cat. no. SA00001-2; ProteinTech, Rosemont, IL, USA). Subsequently, immunoreactive protein bands were analyzed using the Pierce ECL Western Blotting Substrate (cat. no. 32106; Pierce Biotechnology, Inc., Rockford, IL, USA), and quantified using ImageJ software (version 1.44p; National Institutes of Health, Bethesda, MD, USA).

Immunocytofluorescence assay. The culture medium was removed and the differently treated cells were fixed in 4% paraformaldehyde for 15 min at room temperature before they were washed three times in TBS. Subsequently, cells were blocked with 5% bovine serum albumin (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) diluted in TBS for 1 h at room temperature on a shaker. The blocking solution was then removed, and a primary antibody against Smad4 (polyclonal rabbit anti-mouse; dilution, 1:1,00; cat. no. ab208804; Abcam) diluted in TBS was added to appropriate wells and incubated at

4°C overnight. The cells were washed with TBS and then incubated with an Alexa Fluor[®]-labeled polyclonal goat anti-rabbit secondary antibody (2 μ g/ml; cat. no. A-11008; Invitrogen; Thermo Fisher Scientific, Inc.) for 3 h at room temperature. Slides were subsequently washed, air dried and mounted on coverslips with DAPI (1 mg/ml; Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. D1306). The samples were mounted on glass slides with ProLong[®] Gold Antifade Reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and visualized using an inverted fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany). A rhodamine phalloidin (Cytoskeleton Inc., Denver, CO, USA) probe was used to label F-actin. The cell area was calculated as: Total area/nuclear number.

miRNA target analysis. A potential miRNA-26b-5p target sequence in the Smad4 3'-untranslated region (3'-UTR) was analyzed using TargetScanMouse 7.1 software (www.targetscan.org/mmu_71). In brief, the mouse 'Smad4' gene and miRNA 'miR-26b-5p' sequence names were entered and submitted. The predicted Smad4 3'UTR-miR-26b-5p target binding regions were then shown.

Statistical analysis. The results presented are the average of at least three experiments and reported as the mean \pm standard deviation. Statistical analyses were performed with one-way analysis of variance or Student's *t*-test using SPSS 11.0 (SPSS, Inc., Chicago, IL, USA). P <0.05 was considered to indicate a statistically significant difference.

Results

Hypoxia causes significant downregulation in miR-26b-5p expression and decreases the mRNA levels of contractile mVSMC biomarkers. As demonstrated in Fig. 1, compared with normoxia, mVSMCs exposed to low oxygen displayed a statistically significant upregulation in HIF-1 α mRNA levels (P =0.0018; Fig. 1A), and a significant downregulation in miR-26b-5p expression (P =0.0275; Fig. 1B). Additionally, significant downregulation of desmin, H-caldesmon and smoothelin mRNA expression levels was detectable in response to hypoxia in mVSMCs compared with the levels in normoxic conditions (Desmin, P =0.0010; H-caldesmon, P =0.0052; smoothelin, P =0.0456; Fig. 1C).

expression of Smad4 in hypoxic mVSMCs compared with normoxic mVSMCs. Furthermore, miRNA target analysis identified the miR-26b-5p target sequence in the Smad4 3'UTR as UACUUGA between 1,030-1,040 bp (Fig. 3B). Additionally, immunofluorescent staining demonstrated that immunoreactivity of Smad4 in the cytoplasm of normoxic mVSMCs transfected with scramble was more extensive and strongly distributed compared with hypoxic mVSMCs transfected with agomir (Fig. 3C).

Discussion

Hypoxia is a common characteristic of various pathological diseases, including hypoxic pulmonary hypertension. It is accepted that cells respond to reduced oxygen availability through changes in gene expression that are mediated by HIFs, which are composed of an oxygen-regulated α subunit (HIF-1 α , HIF-2 α or HIF-3 α) and a constitutively expressed β subunit (15,16). HIF-1 α is distributed extensively in different cell types, therefore increased expression and activity of HIF-1 α is considered to be an indicator of hypoxia. In the present study, mVSMCs exposed to low oxygen demonstrated a significant upregulation in HIF-1 α mRNA levels compared with mVSMCs exposed to normoxia, suggesting that the *in vitro* hypoxic model used was reliable. In accordance with previous reports, the current study demonstrated that hypoxia stimuli resulted in downregulation in the mRNA levels of desmin, H-caldesmon and smoothelin, which are considered to be specific biomarkers of the contractile phenotype of VSMCs (17-19), indicating that hypoxic mVSMCs had lost their differentiated phenotype. Taken together, these findings suggested that miR-26b-5p participates in phenotypic switching of hypoxic mVSMCs.

In order to confirm the aforementioned hypothesis, miR-26b-5p agomir was used in further experiments. The current study demonstrated that hypoxic mVSMCs transfected with scramble were larger than those cultured in normoxic conditions, and exhibited increased expression of collagen I α protein, indicating increased synthesis of ECM components, which is characteristic of phenotypic switching of VSMCs from a contractile to synthetic phenotype. However, miR-26b-5p agomir reversed the changes in cell area and collagen I α expression in hypoxic mVSMCs, resulting in cell size and collagen I α expression comparable with that in normoxic mVSMCs transfected with agomir and normoxic mVSMCs transfected with scramble. The findings of the present study suggested that miR-26b-5p participates in the phenotypic switching of VSMCs caused by hypoxia.

Smad proteins are intracellular proteins that transduce extracellular TGF- β signals to the nucleus where they activate downstream gene transcription to control cellular functions, including in VSMCs (20,21). In the present study, the expression of Smad2, 3 and 4 in mVSMCs transfected with and without miR-26b-5p agomir were detected to validate whether TGF- β /Smad signaling participates with miR-26b-5p to regulate phenotypic switching of hypoxic mVSMCs, and which Smad isoform mediates this effect. Treatment with the miR-26b-5p agomir did not affect the expression levels of Smad2 and Smad3, or the hypoxia-induced upregulation of p-Smad2 and p-Smad3. These preliminary results suggest that the role of miR-26b-5p in hypoxic mVSMCs phenotype

switching may not be associated with Smad2 and Smad3. These proteins are involved in TGF- β signaling transduction (22), and have been previously reported to be correlated with biological functions of mVSMCs (23-25). Taken together, the expression analysis of Smad2, Smad3, p-Smad2 and p-Smad3 in the current study suggests that Smad2 and Smad3 are not targets of miR-26b-5p during its regulation of phenotypic switching of hypoxic mVSMCs. However, the expression of Smad4, a common Smad family member, was altered in hypoxic mVSMCs when transfected with miR-26b-5p agomir. miR-26b-5p agomir caused reduced expression of Smad4 in hypoxic mVSMCs compared with normoxic mVSMCs transfected with scramble, indicating that Smad4 may be a target of miR-26b-5p. The findings of the present study suggested that regulation of miR-26b-5p in hypoxia-induced phenotypic switching of mVSMCs may be mediated by Smad4. The potential binding site of miR-26b-5p in the Smad4 3'UTR was identified as UACUUGA at position 978-984.

In conclusion, miR-26b-5p participates in the regulation of hypoxia-induced phenotypic switching of VSMCs via the TGF- β /Smad signaling pathway. Specifically, miR-26b-5p targets Smad4, not Smad2 nor Smad3, during the regulation process. The results of the current study were in accordance with previous studies demonstrating that the TGF- β /Smad4 signaling pathway contributes to VSMCs differentiation and function (26). Thus, miR-26b-5p may be a potential therapeutic target in diseases associated with hypoxia-induced phenotypic switching of VSMCs.

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