miR-146a and KLF4 form a feedback loop to participate in vascular smooth muscle cell proliferation

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SUPPLEMENTARY INFORMATION

METHOD

Synthetic oligonucleotides. The antisense miR-146a oligonucleotide (anti-miR-146a) and the control oligonucleotide for EGFP (anti-miR-ctl) were synthesized by Sangon. The sequences of these oligonucleotides are as follows: 5'-AACCATGGAAATCAGTTCTCA-3' (anti-miR-146a), and 5'-AAGGCAAGCTGACCCGTGAAGT-3' (anti-miR-ctl). The nucleotides underlined were modified by locked nucleic acid (LNA). The miR-146a mimics and miR-ctl were purchased from GenePharma.

Cell culture and transfection. Vascular smooth muscle cells were isolated from the aortic media of male Sprague-Dawley rats (100 g) as previously described (Wang et al, 2008), and maintained and passaged in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum (HyClone), 100 units/ml of penicillin, and 100 µg/ml of streptomycin in a humidified 5% CO₂
atmosphere at 37°C. VSMCs used in experiments were from passages 3–5. 293A cells were cultured in high-glucose Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum. All cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

**Construction of recombinant vectors.** The rat pre-miR-146a gene was amplified by PCR using the primers: 5’-TGGAAGATCTATGACGATAGAGCTATCCCAGCTAA-3’ (Bgl II site underlined), and 5’-TCCCCAAGCTTAAAAATGTGTATCCTCAGCTCTGAGAAGCTG-3’ (Hind III site underlined). The PCR product was digested with Bgl II/Hind III (TaKaRa) and inserted into a mammalian expression vector pBluGFP (kindly provided by Dr. Burton B. Yang, Toronto University, Canada) to construct pBluGFP-miR-146a. The 2.0-kb region of the 5' regulatory sequence of the rat miR-146a gene was amplified by PCR using the following primers: the sense primer is 5’-TCGGGGGTACCCGAGCTGTGAGGGGACTATGTT-3’ (Kpn I site underlined), and the antisense primer is 5’-TCCGGGTCAGAGCTGGGATAGCTCTCATG-3’ (Xho I site underlined). Primers for deletion of the three putative KLF4/5-binding motifs in the 5' regulatory region of the rat miR-146a gene: 5’-TCGGGGGTACCCGCTGTGAGGGGACTATTTT-3’ (sense, Kpn I site underlined) and 5’-TCCGGGTCAGAGCTGGGATAGCTCTCATG-3’ (antisense, Xho I site underlined). The PCR products were digested with Kpn I/Xho I (TaKaRa) and then inserted into the pGL3-Basic vector to generate pGL3-miR-146a-luc or pGL3-miR-146a-luc-Del. The fragment of the rat KLF4 3’-UTR (5’-CTAGTGGGCCAACGAGGGAGCAAAGTTCTCCA-3’) containing the miR-146a-binding site and its mutant sequence (5’-CTAGTGGGCCAACGAGGGAGCAAATCAAGAGCA-3’) were synthesized, respectively, and then inserted into pMIR-Report luciferase reporter vector digested with Spe I/Hind III (TaKaRa).
to construct pMIR-KLF4-UTR-WD or pMIR-KLF4-UTR-MT. All of these clones were verified by sequencing. The construction of pEGFP-KLF4 and Ad-KLF4 was described previously (Wang et al, 2008). The construction of Ad-KLF5 was described previously (Zhang et al, 2009). PMT-KLF5 plasmid was kindly provided by Dr. Mandayam N (Emory University, USA).

**Oligonucleotide pull-down assay.** Biotin-labeled wild-type probe containing three KLF4/ KLF5 binding sites for the miR-146a promoter and the mutant probes were synthesized by Sangon. The sequences of these probes are as follows: 5’-AAGGAGGGTGGCACCCTCCCCTGGGTGGTGTC-3’ (wild-type), 5’-AAGGAACAATGGACAATTCCCCTACAATGTGTC-3’ (mutant 1, 2, 3), 5’-AAGGAGGGTGGACAATTTCCCCTACAATGTGTC-3’ (mutant 2, 3), 5’-AAGGAACAATGCACCCTCCCCTACAATGTGTC-3’ (mutant 1, 3), 5’-AAGGAACAATGACAATTCCCCTGGGTGGTGTC-3’ (mutant 1, 2), 5’-AAGGAACAATGCACCCTCCCCTGGGTGGTGTC-3’ (mutant 1), 5’-AAGGAGGGTGGACAATTTCCCCTGGGTGGTGTC-3’ (mutant 2), and 5’-AAGGAGGGTGGCACCCTCCCCTACAATGTGTC-3’ (mutant 3). Each pair of oligonucleotides was annealed following standard protocols. Cultured VSMCs were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.1% NP-40) containing protease inhibitors. The extracts (100 µg) were precleared with ImmunoPure streptavidin-agarose beads (20 µL, Promega) for 1 hour at 4. After centrifugation, the supernatant was incubated with biotinylated double-stranded oligonucleotides (100 pmol) and poly (dI-dC) (10 µg, Sigma) overnight at 4. The streptavidin-agarose beads (30 µL) was then added and incubated for 1 hour at 4. The protein-DNA-streptavidin-agarose complex was washed three times, and separated by 10% SDS-PAGE, then subjected to Western blotting with anti-KLF4 or anti-KLF5 antibodies.
Site-directed mutagenesis. Site-directed mutagenesis was performed with QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions. In brief, oligonucleotide primers containing mutations were annealed to template DNA (pGL3-miR-146a-luc), and the mutant strand was synthesized and amplified by using PfuTurbo DNA polymerase (at 95°C for 30 sec, 55°C for 45 sec, and 68°C for 3.5 min for 18 cycles). After the reaction products were treated with DpnI endonuclease, the mutated DNA was transformed into XL10-Gold® ultracompetent cells. Plasmid DNA was prepared from the transformants, and the mutated sequences were verified by DNA sequence analysis. Primers used to generate mutation in the putative KLF4/5-binding motif 1 are 5'-CCACCATAAAAGGAGGGCGGCACCCCTCCCTGCGGCTGCTCC-3' (sense) and 5'-GGACACCACCCCAGGGGAGGGTGCGCCCTCCTTTATGTGGG-3' (antisense); for mutation in KLF4/5-binding motif 2, 5'-ACCATAAAAGGAGGGTGCGCCCTCCTTGCGGCTGCTCC-3' (sense) and 5'-CCAGGACACCACCCCAGGGGAGGGCGCCACCCCTCCTTTATGTGG-3' (antisense); and for mutation in the KLF4/5-binding motif 3, 5'-GGAGGGTGCGCCCTCCTTGCGGCTGCTCC-3' (sense) and 5'-CATGCAGTCCAGGACACCACCCCAGGGGAGGGGTGCCACCCCTCC-3' (antisense).

Northern blotting. Total RNA from cultured VSMCs was extracted using TRIZOL (Invitrogen). Northern blot analysis of miR-146a was performed using High Sensitive MiRNA Northern Blot Assay Kit (Signosis) according to the manufacturer’s recommendations. Briefly, 5 µg of RNA was loaded onto a 15% pre-run urea-polyacrylamide gel and run at 60 V for 1 hour, then electrotransferred to a nylon membrane. The RNA was cross-linked to the membrane by exposure to 254 nm ultraviolet radiation for 15 min. After pre-hybridization, the membrane was incubated in 4 ml of hybridization
solution with 10 µl of biotin-labeled miR-146a probe or U6 probe at 42 °C overnight. Finally, the bands on membrane were detected by Chemiluminescence according to the manufacturer’s instructions.

**Western blotting.** The isolation of protein from VSMCs was performed as described previously (Wang et al., 2008). Equal amounts of protein were separated by 10% SDS-PAGE and electrotransferred to a PVDF membrane (Millipore). Membranes were blocked with 5% milk in TTBS for 2 h at room temperature and incubated overnight at 4 °C using the following primary antibodies: KLF4 (1:400 dilution, Santa Cruz), KLF5 (1:400 dilution, Santa Cruz), SM22α (1:400 dilution, Santa Cruz), PCNA (1:500 dilution, Santa Cruz), or β-actin (1:1000 dilution, Santa Cruz), respectively, and then with the respective secondary antibody for 1 h at room temperature. Proteins were detected using the Chemiluminescence Plus Western blot analysis kit (Santa Cruz) according to the manufacturer’s manual.

**Balloon injury model and oligonucleotide treatment.** Animal housing and procedures were approved by the local Animal Care and Use Committee at Hebei Medical University. Carotid artery balloon injury was induced in male Sprague-Dawley rats (300 g) as described in our previous studies. In brief, animals were anesthetized with urethane (600 mg/kg) intraperitoneally. The left common carotid artery was exposed through a midline cervical incision. The catheter was advanced from just under the proximal edge of the omohyoid muscle to the carotid bifurcation three times with a 2F (60 cm) Fogarty catheter (Baxter, McGaw Park, IL). To attain a constant degree of vessel wall injury for each of the animals, we kept the diameter of the balloon and the resistance during withdrawal constant and the same for each of the animals. All procedures were performed by a single operator, three rats were used in each group. One OD (33 µg) of oligonucleotides (anti-miR-146a, or anti-miR-ctl) were
added into the 150 µl of 20% F-127 pluronic gel (Sigma) at 4°C for 2 h. Immediately after balloon injury, the exposed adventitial surface was treated with 150 µl pluronic gel containing oligonucleotides (Zheng et al., 2008).

**Morphology analysis.** At 14 days after balloon injury, six cross-sections from the middle of each carotid artery were stained with Hematoxylin-eosin. For each section, six random, noncontiguous microscopic fields were examined. An algorithm computed the mean thickness (in µm) of the intima (I) and media (M) in each field, from which the I/M thickness ratio was derived. To compute the mean thickness values (intima, media, and I/M= intima-to-media) of an artery, all measurements performed on the six sections of the artery were averaged.

**Immunohistochemistry.** Immunohistochemical staining was performed as already published (Zheng et al., 2008). All primary antibodies for PCNA, SM22α, and SMEMB were purchased from Santa Cruz.

**REFERENCES**


**SUPPLEMENTARY FIGURE LEGENDS**

**Fig S1 |** miR-146a expression in 10% serum-induced VSMCs transfected with antisense miR-146a oligonucleotide (anti-miR-146a, 100 nmol/L) or antisense control oligonucleotide (anti-miR-ctl, 100 nmol/L) for 24 hours was detected by qRT-PCR. *P<0.05 vs anti-miR-ctl control. The experiment was repeated 3 times.

**Fig S2 |** Western blot analysis for KLF4 or KLF5 expression. (A) Western blotting for KLF4 and KLF5 expression in 293A cells in Fig 2F. (B) Western blotting for KLF4 and KLF5 expression in 293A cells in Fig 2G. (C) Western blotting for KLF5 and KLF4 expression in 293A cells in Fig 2H. (D) Western blotting for KLF5 expression in carotid arteries from PBS-, anti-miR-ctl (33 µg)-, and anti-miR-146a (33 µg)-treated rats at 14 days after balloon injury.

**Fig S3 |** Mutation analysis for KLF4/KLF5-binding motifs. (A) Densitometry analysis for KLF4 and KLF5 protein levels in Fig 2E. (B) 293A cells were cotransfected with KLF4 expression plasmid (pEGFP-KLF4) and pGL3-miR-146a-luc (WD), pGL3-miR-146a-luc-MT (MT1, MT2, and MT3), or pGL3-miR-146a-luc-Del (Del). After 24 hours, luciferase activities were measured. *P<0.05 vs pGL3-miR-146a-luc (WD) control. KLF4 expression was examined by Western blotting. All experiments were repeated 3 times. (C) 293A cells were cotransfected with KLF5 expression plasmid (PMT-KLF5) and pGL3-miR-146a-luc (WD), pGL3-miR-146a-luc-MT (MT1, MT2, and MT3), or pGL3-miR-146a-luc-Del (Del). After 24 hours, luciferase activities were measured. *P<0.05 vs pGL3-miR-146a-luc (WD) control. KLF5 expression was examined by Western blotting. All experiments were repeated 3 times.

**Fig S4 |** Immunohistochemical staining analysis for SM22α or SMEMB. (A) SM22α
immunohistochemical staining of carotid arteries from PBS-, anti-miR-ctl-, and anti-miR-146a-treated rats at 14 days after balloon injury. (B) SMEMB immunohistochemical staining of carotid arteries from PBS-, anti-miR-ctl-, and anti-miR-146a-treated rats at 14 days after balloon injury.

**Fig S5** | miR-146a and KLF4 form a feedback loop to regulate negatively each other’s expression and participate in regulation of VSMC proliferation.
Fig S2

A

KLF5
KLF4
β-actin

pGL3-miR-146a-luc (μg) 0.4 0.4 0.4
pEGFP-KLF4 (μg) 0 0 0.4
PMT-KLF5 (μg) 0 0.4 0
pEGFP-N1 (μg) 0.4 0 0
pRL-TK (μg) 0.2 0.2 0.2

B

KLF4
KLF5
β-actin

pGL3-miR-146a-luc (μg) 0.15 0.15 0.15 0.15
PMT-KLF5 (μg) 0.15 0.15 0.15 0.15
pEGFP-KLF4 (μg) 0 0.1 0.2 0.4
pGL3-Basic (μg) 0.4 0.2 0.1 0
pRL-TK (μg) 0.1 0.1 0.1 0.1

C

KLF5
KLF4
β-actin

pGL3-P-miR-146a (μg) 0.15 0.15 0.15 0.15
pEGFP-KLF4 (μg) 0.15 0.15 0.15 0.15
PMT-KLF5 (μg) 0 0.1 0.2 0.4
pGL3-Basic (μg) 0.4 0.2 0.1 0
pRL-TK (μg) 0.1 0.1 0.1 0.1

D

KLF5
β-actin

PBS
anti-miR-ctl
anti-miR-146a

Fig S2
Fig S3
**Fig S4**

**Fig S5**