

Online Supplementary Material

Online Materials and Methods

Cell Culture and Treatment

Male Sprague-Dawley rats (220-250g) were anesthetized, the aorta was removed, and VSMCs were isolated and cultured as previously described^{1,2}. VSMCs and 293A cells (ATCC, CRL 1573, Manassas, VA) were maintained and passaged in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (HyClone, Logan, UT), 100 units/ml of penicillin and 100 µg/ml of streptomycin. The experiments were initiated when cells reached 70% confluence, unless stated otherwise. For studies on the effects of PDGF-BB, cells were starved for 24 h and then left untreated or treated for 2 h with the indicated concentration of PDGF-BB (R&D Systems, Minneapolis, MN).

Study Subjects

Patients at the second Hospital (Shijiazhuang, China) received routine examinations and detailed assessments of general health preoperatively to guarantee their surgical tolerance. After circumferential dissection of the renal artery and vein and subsequent transection, the kidney was removed (hydronephrosis of kidney, etc) from the renal fossa and placed on a dissecting platform. One-to-two cm segment of proximal renal artery was transected as specimen and immediately immersed into formalin. Exclusion criteria included any inflammatory conditions; infection; liver disease; malignancy; acute coronary event during the last 2 months; or clinically overt heart failure. The study was approved by the local Research Ethics Committee, and each patient gave written informed consent.

Twenty-four patients with CAD were included in the initial study cohort (the derivation cohort) after angiographic documentation of CAD. Patients with an impaired ejection fraction, heart failure, unstable CAD, or acute myocardial injury were excluded. Twenty-four healthy volunteers without any evidence of CAD or inflammatory disorders served as the control group. The general exclusion criteria were a known history of leukopenia, thrombocytopenia, severe hepatic or renal dysfunction, or evidence of inflammatory or malignant disease. All procedures were approved by the Human Research Committee of Hebei Medical University. The nature, benefits, and risks of the study were explained to the volunteers and patients, and their written informed consent was obtained before participation.

DNA Extraction and Genotyping

Genomic DNA was extracted from 2 mL whole blood using standard methods (QIAamp DNA Blood Midi Kit, Qiagen, Germantown, MD).

Adenovirus and Lentivirus Expression Vector and Plasmids

The full-length mouse KLF4 cDNA was cloned into the pAd/CMV/V5-DEST or pEGFP vector (Invitrogen, Carlsbad, CA) to create the KLF4 adenovirus pAd-KLF4 or pEGFP-KLF4, respectively. Various SUMOylation mutants of KLF4 were constructed with

QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Myc-Ubc9 wild-type and dominant negative (DN) mutant (Myc-Ubc9-DN) were cloned into a lentiviral vector pCDH-CMV-MCS-EF1-copGFP, which was kindly provided by Yin-Yuan Mo (Southern Illinois University, Springfield, IL, USA)³. pcDNA-HDAC2 (Kim Young-eui, Sungkyunkwan University School of Medicine, Seoul, Korea.)⁴; pSG-his-SUMO-1 (Stephen P. Goff, Columbia University, New York, NY, USA), pLM-mCherry-KLF4 (Michel Sadelain, Center for Cell Engineering, New York, NY, USA), and Flag-SEN1 (Edward T.H. Yeh, University of Texas, Austin, TX, USA) were ordered from Addgene (Cambridge, MA). KLF4 and Ubc9 siRNAs were designed by custom Accell SMARTpool (Dharmacon, Lafayette, CO). miR-200c adenovirus pAd-miR-200c was constructed by Invitrogen. The Accell siRNA delivery medium (Dharmacon) was used according to the manufacturer's recommendations.

RNA interference

Small interference RNAs (siRNA) targeting human myocardin, human KLF4, human Ubc9, human SRF, and a control siRNA were purchased from Invitrogen. VSMCs and 293A cells were transfected with siRNAs (100nM) using Lipofectamine RNAiMAX transfection reagent (Invitrogen) and cultured for 2 days.

SUMOylation Assay

SUMOylation assays were performed as previously described⁵. Briefly, VSMC or 293A cells were transfected with the indicated plasmids or induced by PDGF-BB and then lysed in buffer I [20 mM Tris-HCl, pH 7.4, 1% NP40, 135 mM NaCl, 20 mM N-ethylmaleimide (Sigma-Aldrich), and complete protease inhibitor cocktail (Roche, Nutley, NJ)], followed by immunoprecipitation and Western blotting with specific primary antibodies, including anti-Myc (Sigma-Aldrich), anti-GFP (Santa Cruz Biotech, Santa Cruz, CA), anti-His (Santa Cruz), and anti-KLF4 (Santa Cruz) antibodies. Alternatively, KLF4 was immunoprecipitated from cells with rabbit anti-KLF4 (Santa Cruz) followed by Western blotting with mouse anti-SUMO-1 (Abcam, Cambridge, UK) or rabbit anti-KLF4 (Santa Cruz).

Reporter Gene Assays

293A cells were transiently transfected with a *miR-200c* promoter-luciferase construct (constructed by PCR), pEGFP-KLF4, pcDNA-HDAC2, Flag-SEN1, and pCDH-Ubc9 using Lipofectamine 2000 (Invitrogen). The cells were harvested after 48 h, and the activities of both firefly luciferase and Renilla luciferase (pGL-TK, Promega, Madison, WI) were measured in the LB 955 Luminometer system using the dual luciferase reporter system (Promega). The activity of firefly luciferase was normalized to that of Renilla luciferase. A minimum of three independent transfections was performed for each experimental group.

Western Blot Analysis

Cells were lysed in radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholic acid, and complete

protease inhibitor mixture tablets (Roche)]. Proteins (70 µg) from the clarified lysates were separated by 8% or 12% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA). The membranes were blocked with 5% milk in TTBS (20 mM Tris, 150 mM NaCl, pH 7.5, 0.1% Tween 20) for 2 h at room temperature and incubated overnight at 4 °C using the following primary antibodies: 1:500 rabbit anti-KLF4 (Santa Cruz), 1:500 rabbit anti-Ubc9 (Abcam), 1:500 rabbit anti-HDAC2 (Santa Cruz), 1:200 anti-p21 (Santa Cruz), 1:400 anti-NCOR (Santa Cruz), 1:800 anti-LSD1 (Abcam), 1:400 anti-GFP (Santa Cruz), 1:400 anti-Myc (Santa Cruz), 1:1000 anti-SRF (Abcam), 1:1000 anti-Myocardin (Abcam), 1:500 anti-c-Fos (Santa Cruz), 1:1000 anti-SUMO-1 (Abcam) and 1:1000 anti-β-actin (Santa Cruz). The membranes were then incubated for 1 h at room temperature with a 1:5000 dilution of anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) and developed with the Chemiluminescence Plus Western blot analysis kit (Santa Cruz).

Quantitative real-time PCR (qRT-PCR)

Small RNAs from cultured VSMCs were extracted using a mirVana miRNA Isolation Kit (Ambion) according to the manufacturer's recommendations⁶. qRT-PCR of miR-200c was performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with the mirVana qRT-PCR miRNA Detection Kit (Ambion). As an internal control, U6 primers were used for RNA template normalization. All PCRs were performed in triplicate.

Immunoprecipitation Assay

Immunoprecipitation was performed as described previously⁷. Briefly, cell extracts were first precleared with 25 µL of protein A-agarose (50% v/v). The supernatants were immunoprecipitated with 2 µg of anti-KLF4 antibodies for 1 h at 4 °C, followed by incubation with protein A-agarose overnight at 4 °C. Protein A-agarose-bound immunocomplexes were collected by centrifugation at 12 000 rpm for 60 s at 4 °C. The pellets were washed five times with 1 mL IPH buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.1 mM PMSF) for 20 min each time at 4 °C. Bound proteins were eluted with Laemmli sample buffer and then resolved by SDS-PAGE, followed by Western blotting. The experiments were replicated at least three times.

Chromatin Immunoprecipitation Assay

HASMCs at 80% confluence were cross-linked with 1% formaldehyde for 10 min, lysed as described above^{8,9}, and sonicated 5-10 times for 10 s each time at 4 °C to reduce the average DNA length to 0.4-0.5 kb. The samples were diluted 10-fold and then precleared with protein A-agarose/salmon sperm DNA for 30 min at 4 °C followed by an overnight incubation at 4 °C with 1:400 anti-KLF4, 1:500 anti-Ubc9, 1:400 anti-HDAC1, 1:800 anti-LSD1 or 1:400 anti-mouse IgG (as negative control). The immune complexes were precipitated with protein A-agarose for 1 h. After reversal of cross-linking, the genomic region of *miR-200c* flanking the potential KLF4-binding sites was

amplified by PCR. The sequence surrounding Sp1 site in the *miR-200c* promoter was amplified from the immune pellets using the primer pair, P1: 5'-CGCTGACCTCAGGGATCTC-3'; P2: 5'-GGCTGGCGCTGGGAGGGAAG-3' and P3: 5'-CCCCTTCGTCTCCCCAGCAC-3'.

Oligonucleotide Pull-down Assay

The sequences of Sp1-1 oligonucleotides of *miR-200c* were as followed: biotin-5'-GGAAGAGCGGAGGCCAGGGCGGGCTCTAGGCCGTGGAATCTGGGG-3' (forward); Sp1-2 oligonucleotides: biotin-5'-GGACACACCTGTGCGCAGGGTGGCAGGCGGGGCCAGGTAAGGAGCCTGCG-3' (forward); Sp1-3 oligonucleotides: biotin-5'-GGCCTGCAGCTCCGCTGTGGGCAGGGTCTGAGGCCACAGAGGAATGGG-3' (forward); Sp1 +2 oligonucleotides: biotin-5'-GCGCAGGGTGGCAGGCGGGGCCAGGTAAGGAGCCTGCGCTGGCTGCCCGG CAGGCGGAGAAGGAAGGAGGAAGAGCGGAGGCCAGGGCGGGCTCTAGGCCG TGG-3' (forward). The sequence of Sp1+2+3 oligonucleotides was obtained by PCR and the primer sequences were 5'-GCCAGCTGACCCCTCGCTG-3' (forward) and 5'-CTTTAAGGCCCCAGATTCCACG-3' (reverse). Oligonucleotides were annealed following standard protocols. VSMCs treated with or without PDGF-BB for 2 h 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. Whole-cell extracts (100 µg) were precleared with ImmunoPure streptavidin-agarose beads (20 µL/sample, Promega) for 1 h at 4 °C. After centrifugation for 2 min at 12 000 rpm, the supernatant was incubated with 100 pmol of biotinylated double-stranded oligonucleotides and 10 µg of poly (dI-dC)·poly (dI-dC) overnight at 4 °C with gentle rocking. 30 µL of streptavidin-agarose beads was then added to sediment the protein-DNA complexes. After a series of washes, proteins were eluted, separated on 10% SDS-PAGE and immunoblotted⁷.

Balloon Injury Model and Drug Treatment

Animal housing and procedures were approved by the local Animal Care and Use Committee at Hebei Medical University. The animals were anesthetized with urethane (600 mg/kg) intraperitoneally. The thoracic-abdominal artery was de-endothelialized as described previously¹⁰. In brief, the catheter was advanced from the left common carotid artery down to the level of the renal arteries 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 for each animal. Immediately after injury, 50 µl (2×10^9 plaque-forming unit/ml) of adenoviral construct (empty, KLF4, Ubc9-DN, Ubc9) was infused and incubated for 15 min. 14 days after operation, rats were euthanized with a lethal dose of pentobarbital (200 mg/kg), and the thoracic-abdominal artery was collected for protein isolation and Western blot. KLF4siRNA, Ubc9 siRNA or Con siRNA dissolved in 200 µL of 25% F127 pluronic gel (Sigma-Aldrich) together with Lipofectamine™ RNAiMAX was also topically administered around the injured carotid

arteries immediately after balloon angioplasty. The uninjured right common carotid artery received F127 pluronic gel vehicle.

Animal model and treatment.

Animal housing and procedures were approved by the local Animal Care and Use Committee at Hebei Medical University. 6–8-week-old male 85 wild-type C57BL/6 mice and miR-200c transgenic mice (Model animal research center of Nanjing University) were anesthetized with 2% isoflurane gas. The left common carotid artery was dissected and ligated near the carotid bifurcation use a 6-0 suture to induce intima formation¹¹. In unligated animals, the suture was passed under the exposed left carotid artery but not tightened. At specified time points after surgery, all animals were anesthetized and perfused with cold PBS and the tissues were harvested for morphology, or histological analysis.

Morphology Analysis

14 days after balloon injury, six cross-sections from the middle of each abdominal artery were stained with hematoxylin/eosin. The neointimal and medial areas were calculated using the Image-Pro Plus Analyzer version 5.1 software (Media Cybernetics, Inc., Silver Spring, MD) in a blind manner. For each section, six random, noncontiguous microscopic fields were examined¹².

Immunohistochemistry

For antigen retrieval, deparaffinized formalin-fixed sections were boiled for 10 min in 10 mM sodium citrate (pH 6). Primary antibody (PCNA or p21 at 1:100 dilutions) was incubated overnight at 4 °C in 1% normal goat serum in phosphate-buffered saline (PBS).

MTT Assay

The cell growth rates were evaluated by the tetrazolium dye-based MTT assay, as described previously¹³. In brief, 1×10^4 cells/well were seeded in triplicate into 96-well plates in Dulbecco's modified Eagle's medium. After 24 h, the cells were treated according to the experimental design. After each treatment, the medium was removed, and the cells were washed in PBS. The MTT reagent was added at 2 mg/ml in Hank's buffer and incubated for 1 h until dark blue crystals could be seen in the cytoplasm under light microscopy. The crystals were dissolved in DMSO, and the absorbance was measured in a Thermo Fluoroscan Ascent spectrometer at 570 nm with background subtraction at 650 nm. The results were expressed as the means \pm SEM of the absorbance relative to time 0 or the control.

Cell proliferation

VSMC proliferation assays were performed with the BrdU Cell Proliferation Assay kit (Millipore) according to the manufacturer's recommendations. VSMCs were labeled for 6 h with BrdU prior to the termination of PDGF-BB (10 ng/mL) incubation. OD readings were performed at 450 nm. All groups were evaluated in a minimum

of three separate wells per experiment¹⁴.

Statistical Analysis

Differences between groups were assessed with Student *t* test, one-way ANOVA and Mann–Whitney. Unless stated otherwise, all data are expressed as mean \pm SEM. A value of $P < 0.05$ was considered to indicate statistical significance.

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Online Table I. Characteristics of study cohort

	Healthy volunteers (n=24)	Patients with CAD (n=24)	P-value
Gender Male	12 (50%)	16 (66.7%)	0.082
Age (years)	37.2 ± 9.16	58.3 ± 8.11	0.000
Stable CAD for at least 4 months	0	100%	0.000
Number of vessels (CAD)	0	I: 13 (54.2%) II: 6 (25%) III: 5 (20.8%)	0.000 0.000 0.000
Active smoker	6 (25%)	11 (45.8%)	0.031
Hypertension	0	17 (70.8%)	0.000
Diabetes mellitus	0	11 (45.8%)	0.000
History of AMI/PCI/ACVB/PTA/Stroke	0	100%	0.000
Concurrent medication	0	20 (83.3%)	0.000
Beta-blocker	0	100%	0.000
Aspirin/Clopidogrel	0	22 (91.6%)	0.000
ACE-inhibitor/ATRB	0		

Online Figure Legend

Online Figure I. Induction of KLF4 SUMOylation by PDGF-BB. (A) Effect of anti-miR-200c (Model), pAd-miR-200c (Model) and Uninjured group on the expression of miR-200c as assessed by quantitative real-time PCR. (B) VSMCs were treated with increasing concentrations of PDGF-BB for 2 h. Anti-KLF4 immunoprecipitates (top and second panels) and cell lysates (third and bottom panels) were immunoblotted with antibodies against SUMO1 (arrows indicate SUMOylated KLF4) and KLF4. (C) 293A cells were transfected with Ubc9-WT and Ubc9-DN (dominant-negative mutant of Ubc9) and the level of endogenous KLF4 SUMOylation was detected in anti-KLF4 immunoprecipitates (top panel). Arrows indicate SUMOylated KLF4. Cell lysates were immunoblotted with the Ubc9 antibody (third panel). (D) SUMOylation of KLF4 was measured *in vitro* with a SUMOylation assay kit. The amount of SENP1, Ubc9 and KLF4 were detected by Western blotting (second to fourth panels). (E) COS-1 cells were co-transfected with pCDH-Ubc9 and one of the following plasmids: pEGFP-KLF4 (WT), pEGFP-KLF4-K275R or pEGFP-KLF4-K225R/K229R/K279R. Anti-GFP immunoprecipitates were immunoblotted with SUMO-1 antibody. Arrows indicate SUMOylated KLF4. Cell lysates were immunoblotted with the Ubc9 antibody (third panel). (F) Normal tissues and vascular hyperplasia specimens were prepared and subjected to anti-KLF4 immunoprecipitation followed by Western blot analysis. All experiments were repeated three times. Representative gel for SUMOylated KLF4 levels (*upper*) (Arrowhead). (G) 293A cells were transiently cotransfected with wild type (wt) or point mutant miR-200c promoter-luciferase constructs together with either pCDH empty vector or pCDH-Ubc9. Twenty-four hours later, luciferase activity was measured. Bars represent the means \pm SEM from three independent experiments. *, $P < 0.05$ vs. pCDH group.

Online Figure II. PDGF-BB increased the interaction between KLF4 and corepressors. (A) HASMCs were treated with PDGF-BB for 2 h. Cell lysates were immunoblotted with the indicated antibodies. (B) HASMCs were infected with pAd-KLF4 or pAd-KLF4-mut (pAd-KLF4-K225R/K229R/K279R) adenovirus for 24 h and then treated with and without PDGF-BB for 2 h. Cell lysates were immunoblotted with the indicated antibodies. (C) VSMCs were infected with pAd-KLF4 or pAd-KLF4-mut in the presence of Ubc9 lentivirus or pcDNA-SENP1 for 24 h. Cell lysates were immunoblotted with the indicated antibodies. (D) HASMCs were treated with PDGF-BB for 2 h. After crosslinking and sonication steps, ChIP assays were performed with the indicated antibodies, and the *miR-200c* promoter region containing Sp1-binding sites was amplified by PCR. Primer 2 (P2, forward primer; P3, reverse primer). (E) HASMCs were infected with pAd-KLF4 or pAd-KLF4-mut (pAd-KLF4-K225R/K229R/K279R) adenovirus for 24 h and then treated with and without PDGF-BB for 2 h. After crosslinking and sonication steps, ChIP assays were performed with the indicated antibodies, and the *miR-200c* promoter region containing Sp1-binding sites was amplified by PCR. (F) VSMCs were infected with pAd-KLF4 or pAd-KLF4-mut in the presence of Ubc9 lentivirus or pcDNA-SENP1 for 24 h. After crosslinking

and sonication steps, ChIP assays were performed with the indicated antibodies, and the *miR-200c* promoter region containing Sp1-binding sites was amplified by PCR.

Online Figure III. KLF4, Ubc9 and SRF are targets of miR-200c in HASMCs. (A) HASMCs were co-transfected with pLuc-KLF4-3'UTR, pLuc-Ubc9-3'UTR, pLuc-SRF-3'UTR or pLuc-HDAC2-3'UTR together with with pre-miR-200c. Twenty-four hours later, the cells were harvested and luciferase assays were carried out. Experiments were performed in triplicate and each value is the mean \pm SEM of three independent experiments. *, $P < 0.05$ vs. pre-control group. (B) Densitometric analyses from three independent experiments. *, # $P < 0.05$ vs. the respective control group. (C) Effect of anti-miR-200c (Model), pAd-miR-200c (Model) and Uninjured group on the expression of KLF4, SRF and Ubc9 as assessed by immunohistochemical staining with the indicated antibodies. (D) HASMCs were transfected with different vectors for 24 h. The KLF4, Ubc9, SRF, and p21 protein levels were detected by Western blotting. (E) 293A cells were transiently co-transfected with a *p21* promoter-luciferase construct (pGL3-p21) together with pEGFP-KLF4 alone with or without different concentrations of pCDH-Ubc9. Twenty-four hours later, luciferase activity was measured. Bars represent the means \pm SEM from three independent experiments. *, $P < 0.05$ vs. pGL3-p21 group.





