Supplementary Information

Reciprocal expression of MRTF-A and myocardin is crucial for pathological vascular remodeling in mice

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Supplementary Methods

Plasmids

-250bp MRTF-A(-184C)-luc (MRTF-A-luc) and -250bp MRTF-A(-184T)-luc, in which DNA fragments from 5' flanking region of the human MRTF-A gene (-184T and -184C, respectively), vinculin-luc and mut CArG vinculin-luc (mutCArG-luc), in which DNA fragments from 5' flanking region of the human vinculin gene (-360 to +63 bp) or corresponding 5' flanking region (FR) containing a mutated CArG box were inserted upstream of luciferase gene respectively, SM22α-luc and 3xCArG-luc were described previously(Hinohara et al, 2009; Kuwahara et al, 2007; Morita et al, 2007). Expression vectors encoding MRTF-A, MRTF-B, myocardin and STARS were described previously(Kuwahara et al, 2007).

MRTF-A 3'UTR-luc or mutMRTF-A 3'UTR-luc was generated by inserting MRTF-A 3'UTR containing wild type or mutated miR-1 target sequences downstream of a gene encoding luciferase in the pMIR-REPORTER kit miRNA reporter expression vector (Applied Biosystems). The MRTF-A 3'UTR was obtained by PCR using a human BAC clone as a template with the following primers5’-CCG CCG CCA CTA GTC TCT GCT CAA GAC GG-3’ Reverse primer: 5’-CCG CCG CCA AGC TTA GTC TCT CTG GCT CAA GAC GG-3’
-5500 bp MRTF-A-luc was generated by inserting 5500 bp of the 5'-FR of MRTF-A gene upstream of the luciferase gene in pGL4 vector (Promega).

Animal experiments.

Mkl1<sup>−/−</sup> mice were kindly provided from Dr. E.N. Olson (The University of Texas, Southwestern Medical Center at Dallas) (Li et al, 2006). ApoE<sup>−/−</sup> and Mkl1<sup>−/−</sup> used in this study were C57BL/6 background. ApoE<sup>−/−</sup> mice and Mkl1<sup>−/−</sup> mice were cross-bred. Genotype analyses of ApoE<sup>−/−</sup> and Mkl1<sup>−/−</sup> mice were performed by PCR using genomic DNA isolated from tail snip samples, as described previously (Kobayashi et al, 2004; Li et al, 2006). The animal care and all experimental protocols were reviewed and approved by the Animal Research Committee at Kyoto University Graduate School of Medicine.

Cell culture and transfection

Primary rat aortic vascular smooth muscle cells (RAVSMC) (Cell Applications, Inc.), A7r5 rat vascular smooth muscle cells (DS Pharma Biomedical), NIH3T3 cells and COS7 cells were maintained in DMEM with 10% fetal calf serum (FCS) with
antibiotics. Co-transfection of RAVSMCs with 3xCArG-luc plus expression plasmids encoding MRTF-A (1ng) and STARS (100ng) were accomplished with FuGene6 (Roche) in DMEM with 10% FCS according to the manufacture's protocol. 6 h after transfection, cells were shifted to DMEM without FCS in the presence or absence of CCG-1423 (1µM). 20 hours later cells were lysed and luciferase gene expression was assayed. Co-transfection of RAVSMCs or NIH3T3 cells with MRTF-A-luc plus expression plasmids encoding myocardin and MRTF-A (0, 1 or 10ng each) was performed with FuGene6 in DMEM with 10% FCS. 6 h after transfection, cells were shifted to DMEM without FCS. 24 hours later cells were lysed and luciferase gene expression was assayed.

For transfection of a miR-1 mimic or inhibitor, a miRIDIAN microRNA mimic for miR-1 or its negative control, or a miRIDIAN microRNA hairpin inhibitor for miR-1 or its negative control (Thermo Scientific) was transfected into RAVSMCs grown in 6 cm dishes in DMEM with 10% FCS using Dharmafect2 transfection reagent according to the manufacture's protocol. For mRNA analysis cells were harvested 24 h after transfection and for protein analysis, cells were harvested 48 h after transfection.

Mouse primary aortic vascular smooth muscle cells were obtained as previously reported(Nakamura et al, 2010). Briefly, abdominal aortas were quickly removed from
anesthetized 6-week-old male C57BL/6 mice, and mouse medial VSMC layers were separated from the adventitia and vascular endothelium. Mouse VSMCs were isolated from the medial VSMC layers by the enzymatic dispersion method (Nakamura et al, 2010). Mouse VSMCs passaged 3 to 6 were cultured in DMEM with 10% FCS. Mouse VSMCs were confirmed to show positive staining for α-smooth muscle actin and vimentin, and negative staining for von Willebrand factor.

**RNA interference.**

For analysis of MRTF-A and myocardin using RNA interference, VSMC grown in 6-cm dishes were transfected with 200 pmol of ON-TARGET plus® siRNA reagent against rat MRTF-A or myocardin or control scrambled siRNA (Thermo Scientific) using Dharmafect 2 transfection reagent and 48 h later they were harvested for real-time RT-PCR analysis.

For luciferase assays, VSMC grown in 24-well dishes were transfected with 100 pmol of siRNA and 500 ng of luciferase reporter plasmid for 12 h using Fugene 6. A pRL-TK containing Renilla luciferase gene under the control of thymidine kinase promoter was included in all transfections as an internal control. The transfected cells were then incubated in serum-free medium for 24h.
**Mouse vascular wire injury**

Vascular injury was induced in femoral arteries of male C57BL/6 wild type or Mkl1−/− mice at 8-10 weeks of age, as described previously (Sata et al, 2000; Takaoka et al, 2009). Briefly, after exposure of either right or left femoral artery and its muscular branch, transverse arteriotomy was performed in the muscular branch with Vannas style iris spring scissors (Fine Science Tools, Inc.). Microsurgical forceps (Napox MB54-1, Natsume Seisakusho Co., Ltd.) were used to extend the arteriotomy through which a straight spring wire (0.38 mm in diameter, No. C-SF-15-15, COOK) was carefully inserted into the femoral artery for more than 5 mm toward the iliac artery. The wire was left in place for 1 min to denude and dilate artery. The wire was then removed, and a silk suture looped at the proximal portion of the muscular branch was secured. In CCG-1423 experiment, mice subjected to femoral artery wire injury were treated with CCG-1423 (0.15 mg/kg intraperitoneally) or control DMSO for 3 weeks.

Locked nucleic acid (LNA)\textsuperscript{TM} oligonucleotide anti-miR-1 microRNA inhibitor or LNA\textsuperscript{TM} microRNA inhibitor negative control (20mg/kg) (5'-FAM prelabeled, Exiqon) was injected into sham-operated or injured femoral arteries from the muscular branch using a syringe with 29 gauge needle (TERUMO).
Mouse carotid artery ligation

Mouse carotid artery ligation was performed as described previously (Spencer et al, 2005). Morphometric analysis was performed on transverse sections 1,400 μm proximal to the ligature in the carotid arteries.

Quantification of neointimal hyperplasia

For morphometric studies, the femoral arteries were harvested 4 weeks after injury. Digitalized images were analyzed using image analysis software (Image J, NIH). The lumen, internal elastic lamina and external elastic lamina were defined. The intimal (tissue between lumen and internal elastic lamina) and medial (tissue between internal elastic lamina and external elastic lamina) areas were recorded. Neointima/media ratio was calculated. The average of neointima/media ratios in 5 serial sections was designated as a value to represent the individual.

Analysis of atherosclerotic lesion area of ApoE-/- mice

Mkl1+/+; ApoE−/− and Mkl1−/−; ApoE−/− mice fed a normal chow from 4 -8 weeks of age were fed a high cholesterol diet (F2HFD1: 1.25% cholesterol, 0.5% cholic acid, 15% fat,
Oriental Biotechnology) for 8 weeks beginning at 8 weeks of age. Atherosclerotic lesions were analyzed by en face analysis of the whole aorta and quantified by cross-sectional analysis of the proximal aorta, as described previously (Kobayashi et al, 2004; Paigen et al, 1987; Palinski et al, 1994). For en face preparations of the aorta, a cannula was inserted into the left ventricle and the aortic tree was fixed by perfusion for 10 min with ice-cold PBS containing 4% paraformaldehyde (PFA), 5% sucrose, 20 μM butylated hydroxytoluene and 2 μM EDTA, as described previously (Kobayashi et al, 2004). The aorta and its main branches were dissected from the aortic valve to the iliac bifurcation. The adventitia was removed as much as possible using microscope. The aortic tree was opened longitudinally with extremely fine microscissors (Napox MB54-1, Natsume Seisakusho Co., Ltd.) and pinned flat on a black wax surface in a dissecting pan using 0.2-mm-diameters stainless steel pins (Fine Science Tool). After overnight fixation with the PFA solution described above and a 12-hour rinse in PBS, the aortas were briefly rinsed in 50% ethanol; immersed for 1 hour in a filtered solution containing 0.5% Sudan III, 35% ethanol, and 50% acetone; and destained for 3 minutes in 50% ethanol. The Sudan III-stained aortas were photographed for analysis.

For cross-sectional analysis of the proximal aorta, hearts and proximal aortas isolated from mice anesthetized and euthanized at 16 weeks of age were washed in PBS
and fixed in 4% PFA at 4°C for 24 hours. The hearts with aortic root were rinsed with PBS and were embedded in OCT compound. The OCT-embedded hearts were sectioned with a cryostat, and 10-µm sections in the proximal aorta were obtained sequentially every 60-µm beginning at the aortic root and extending for 300 µm as described previously (Paigen et al, 1987). Lesions from 5 alternating sections were stained with Oil Red O, counterstained with hematoxylin, and quantified using imaging software (Image J, NIH). The average % area of atherosclerotic lesions in 5 sections from aortic root was designated as a value to represent the individual and the means of the average % area of atherosclerotic lesions from 8 mice in each genotype were compared.

In another experiment, ApoE/− mice fed a normal chow from 4 -6 weeks of age were fed a high cholesterol diet (F2HFD1: 1.25% cholesterol, 0.5% cholic acid, 15% fat, Oriental Biotechnology) for 6 weeks beginning at 6 weeks of age with or without CCG-1423 treatment (0.15 mg/kg intraperitoneally for 6 weeks).

**Immunohistochemical analysis**

Paraffin-embedded sections (4 µm thick) of femoral arteries 4 weeks after wire injury were deparaffinized and blocked using 2% rabbit or horse serum. For Mac3 or CD31 staining, the sections were incubated with rat anti-Mac3 (1:500 dilution, BD
Pharmigen) or anti-CD31 (1:500 dilution, BD Pharmigen). The immunoreaction was detected by AP reaction (Vector red substrate, VECTASTAIN ABC-AP kit, Vector Laboratories) with peroxidase-conjugated anti-rat secondary antibody (Simple Stain mouse MAX-PO(rat), Nichirei Bioscience), and the counterstained with 1% methylgreen. For quantitative analysis of Mac-3-positive cell numbers, ratios of Mac-3-positive cells to total cell number in the intima and the media of arteries 4 weeks after wire injury in Mkl1+/+ and Mkl1−/− mice were quantified (n=4 in each group). For semi-quantitative analysis of CD31-positive cell numbers, the stained preparations were scored as (+), (++), or (+++) depending on the intensity of staining by endothelial cells of vessels. These were assigned arbitrary numerical values of 2, 4, and 8, respectively. The scores for femoral arteries from Mkl1+/+ and Mkl1−/− mice 4 weeks after wire injury are shown (n = 5 in each group). For α-smooth muscle actin (SMA), smooth muscle myosin heavy chain (SM-MHC), MRTF-A and F4/80 staining in sections of aorta, mouse monoclonal anti-α-SMA (Sigma-Aldrich; 1:500 dilution), rabbit polyclonal anti-SM-MHC (BT-562, Biomedical Technologies Inc; 1:100 dilution), rabbit polyclonal anti-BSAC antibodies (1:500 dilution) (Sasazuki et al, 2002) or rat monoclonal anti-F4/80 antibodies (Abcam; 1:1000 dilution) were used. For Ki-67 staining in sections of injured femoral arteries, rat monoclonal anti-mouse Ki-67 antigen
(clone TEC-3, 1:100 dilution, Dako) was used. Apoptotic cells were detected using rabbit polyclonal anti-cleaved caspase-3 antibodies (Cell Signaling; 1:300 dilution) and ApopTag® peroxidase in situ apoptosis detection kit according to the manufacture's instruction (Milipore).

**Immunocytochemical analysis**

RAVSMCs plated on glass coverslips in 6-well dishes in DMEM with 10%FCS were shifted to DMEM without FCS for 18 h and then cells were treated with 20% FCS for 30 min in the presence or absence of 1μM of CCG1423. Cells were rinsed with phosphate-buffered saline (PBS), fixed with 4% PFA for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min. Cells were blocked for 30 min at room temperature with 3% bovine serum albumin in PBS. Cells were then incubated with primary anti-MRTF-A antibody (1: 400 dilution)(Nakamura et al, 2010) in 3% bovine serum albumin in PBS for 60 min at room temperature. Secondary antibody were goat anti-rabbit immunoglobulin G fluorescein isothiocyanate (Vector laboratories). Vectashield medium with DAPI (4',6'-diamidino-2-phenylindole) (Vector laboratories) was used for mounting.
Luciferase assay

Cells were harvested and luciferase activities were measured using Dual-Luciferase® Reporter Assay system (Promega) according to the manufacturer's instructions. All assays were performed at least twice in sextuplicate, unless otherwise indicated.

VSMCs proliferation assay

RAVSMC proliferation capacity was assessed by MTT assay according to the protocol (Roche). After plating of VSMCs ($10^3$ cells/well) in 96-well plates for 36 hours, cells were transfected with MRTF-A siRNA or control random siRNA (100ng/each dish) using Dhramafect2 transfection reagent in serum-free medium for 24 hours. FCS (20% of final concentration) was added to the medium and cells were further incubated for 48 hours. In experiments assessing effects of CCG-1423 (Cayman Chemical), after plating VSMCs in 96-well dishes for 24 h, the medium was changed to DMEM with or without 20% FCS in the presence of 0, 0.1 or 1μM of CCG-1423 and cells were maintained for another 24h. Thereafter, in both experiments, 10 μl of MTT 1 labeling reagent (Roche) was added to each well, and incubated for additional 4 hours. One hundred μL of solubilization solution was added to each well thereafter and cells were incubated for another 24 h in a cell incubator. The absorbance at 595 nm was
determined using an ELISA reader (Perkin Elmer). Each experiment was performed in quadruplicate for three times, unless otherwise indicated.

**VSMC migration assay**

RAVSMC migration capacity was quantitatively evaluated using the 8μm Cyto Select M cell migration assay kit (Cell Biolabs). VSMCs grown in 6-cm dishes for 48 hours were transfected with ON-TARGET plus® siRNA reagent against rat MRTF-A or control random siRNA (100ng/each dish) using Dhramafect2 transfection reagent. After 48 hours, transfected VSMCs were detached and suspended in serum-free medium. DMEM or DMEM with or without 30ng/ml PDGF-BB (Sigma-Aldrich) was added to the lower well of the migration plate and 100 μl of cell suspension was added to the inside of each insert for a final concentration of 5x10^5 cells/well in 96 well plates, followed by further incubated for 24 h in a cell culture incubator. To assess the effect of CCG-1423, serum-free DMEM or DMEM with 30ng/ml PDGF-BB with or without 0.1μM or 1μM of CCG-1423 was added to the lower well of the migration plate and 100 μl of VSMCs suspension was added to the inside of each insert. After 20 h incubation, the medium was carefully aspirated from the transwell inserts. The inserts were transferred to a clean well containing 150 μl of Cell Detachment Solutions and
incubated for 1 hour at 37 °C. The cells from the underside of the membrane were completely dislodged by gently tilting the insert several times in the detachment solution. Then the inserts were removed and discarded. Sufficient 4 × Lysis Buffer/CyQuant GR Dye solution for all samples was prepared by diluting the dye 1:75 in 4× Lysis Buffer. Fifty μl of the 4× Lysis Buffer/CyQuant GR Dye solution was added to each well containing cells and 150 μl of Cell Detachment Solution, and incubated for 20 min at room temperature in order to lyse cells and stain the nucleic acids. One hundred and fifty μl of the mixture was transferred to a 96-well plate suitable for fluorescence measurement. Fluorescence at 480nm/520nm was measured with a fluorescence plate reader (PerkinElmer). Each experiment was performed in quadruplicate, unless otherwise indicated.

**Real-time RT-PCR.**

Total RNA was isolated from cultured RAVSMC, primary mouse aortic vascular smooth muscle cells, mouse femoral arteries or mouse aortas using Sepasol-RNA I (Nakarai) following the manufacturer’s protocol. Real-time one-step RT-PCR was performed with 20-100 ng of total RNA using One-step RT-PCR master mix reagent (Applied Biosystems). MiR-1 expression was determined using Taqman MicroRNA RT kit,
Taqman miRNA assay mix, and Taqman Universal PCR Master Mix II (Applied Biosystems). Taqman primers and probes for mouse myocardin, MRTF-A, MRTF-B, vinculin, MMP9, integrin β1, ACTA2, Myh11, GAPDH, 18S, miR-1 and U6, and rat myocardin, MRTF-A, vinculin, MMP9, integrin β1, ACTA2, GAPDH, 18S, miR-1 and U6 were purchased from Applied Biosystems.

**Western Blot Analysis**

Mouse femoral arteries, mouse aortas, COS1 cells, cultured RAVSMC, or primary MAVSMC were lysed in the lysis buffer (Cell Signaling Technology), and 50 or 100 μg of proteins were resolved on a sodium dodecylsulfate-polyacrylamide gel, transferred to PVDF membrane (Bio-Rad), and immunoblotted with rabbit polyclonal anti-myocardin, anti-MRTF-A and anti-MRTF-B antibodies described previously (Nakamura et al, 2010). Bands were visualized using anti-rabbit IgG conjugated to horseradish peroxisome and Western Blotting Luminal Reagent (Santa Cruz Biotechnology). Densitometry was performed on X-ray film using Image J software.

**Statistical analysis.**

Data are presented as means±SEM. Unpaired t-tests were used for comparison between
two groups, and ANOVA with *post hoc* Fisher’s test was used for comparison among
groups. Values of $p<0.05$ were considered significant. Data obtained from the
two-way factorial design were analyzed with the two-way ANOVA.
Supplementary Table S1.
Medial and neointimal area of carotid arteries after ligation

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<th>Intima (x10^3/μm^2)</th>
<th>Media (x10^3/μm^2)</th>
<th>Intima/Media ratio</th>
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<tr>
<td>Mkl1+/− ligation</td>
<td>n=3</td>
<td>132.0±49.0</td>
<td>110.4±28.8</td>
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<tr>
<td>Mkl1−/− ligation</td>
<td>n=3</td>
<td>45.4±25.0*</td>
<td>97.1±23.0</td>
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The ratio of intima to media was calculated as the intimal area/medial area. Values are means ± SEM. *P<0.01 vs. Mkl1+/− ligated arteries.
Supplementary Table S2.
Luminal and neointimal area of femoral arteries 2 weeks after vascular injury with microRNA inhibitors treatment

<table>
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<th>Intima/Media ratio</th>
<th>Lumen (x10^3/µm²)</th>
<th>Intima (x10^3/µm²)</th>
<th>Media (x10^3/µm²)</th>
<th>IEL (x10^3/µm²)</th>
<th>EEL (x10^3/µm²)</th>
<th>n</th>
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<tr>
<td>Control microRNA inhibitor injury</td>
<td>0.75±0.10</td>
<td>24.4±4.8</td>
<td>20.1±4.6</td>
<td>25.5±3.1</td>
<td>44.5±4.3</td>
<td>70.0±5.8</td>
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<tr>
<td>Anti-miR-1 microRNA inhibitor injury</td>
<td>1.99±0.38*</td>
<td>4.5±5.6*</td>
<td>52.7±10.4*</td>
<td>26.3±2.5</td>
<td>56.2±10.7</td>
<td>82.6±12.2</td>
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</tr>
</tbody>
</table>

The ratio of intima to media was calculated as the intimal area/medial area. Values are means ± SEM. IEL, internal elastic lamina; EEL, external elastic lamina. *p<0.05 vs. control injured arteries.
Supplementary Figure Legends

Figure S1. Increased expression of MRTF-A in femoral arteries after wire injury in mice. (A) Western blots showing the specificity of antibodies for myocardin, MRTF-A and MRTF-B used in this study. Cell lysates of COS7 cells overexpressing FLAG-tagged mouse myocardin, MRTF-A or MRTF-B were subjected to Western blot analysis using either rabbit polyclonal anti-myocardin, anti-MRTF-A or anti-MRTF-B antibodies. (B) Immunohistochemical analysis of MRTF-A expression in wire-injured femoral arteries. Tissues are labeled with anti-BSAC antibodies (MRTF-A) or control rabbit IgG; bar indicates 100 μm. Three independent experiments gave identical results. (C, D) Graph shows the temporal profile of MRTF-A (B) and myocardin (C) mRNA levels (normalized to GAPDH mRNA) in femoral arteries after wire injury (day 0, 1, 14 and 50). MRTF-A mRNA expression was once significantly decreased at 1 day after wire injury, when a disruption of endothelial layer and a loss of medial VSMCs followed by the infiltration of inflammatory cells occur, and then significantly increased by 2 weeks after injury, when dedifferentiated neointimal VSMCs expressing a relatively low α-smooth muscle actin (SMA) are increasing. At 50 days after injury, when a more differentiated population of VSMCs is restored, MRTF-A mRNA levels were decreased to the levels almost comparable to those at day 0. By contrast, myocardin mRNA levels
were continuously declined by 2 weeks after injury, followed by their restoration at 50
days after injury (n=3 samples at each time point).

**Figure S2. Attenuated vascular remodeling in response to wire injury in Mkl1−/−
mice.** (A) Representative images of apoptotic cells detected with ApopTag® Peroxidase
in neointima 4 weeks after femoral artery injury in Mkl1+/− and Mkl1−/− mice. Red arrows
indicate TUNEL-positive cells. (B) Numbers of TUNEL-positive cells in injured vessels
of Mkl1+/− and Mkl1−/− mice 4 weeks after wire injury are shown (n=3 in each group).
(C) Representative images of apoptotic cells stained with anti-cleaved caspase-3
antibodies in neointima 4 weeks after femoral artery injury in Mkl1+/− and Mkl1−/− mice.
(D) Numbers of cleaved caspse-3-positive cells in injured vessels of Mkl1+/− and Mkl1−/−
mice 4 weeks after wire injury are shown (n=3 in each group). (E) Representative
images of vascular fibrosis detected with Sirius red staining in neointima 4 weeks after
femoral artery injury in Mkl1+/− and Mkl1−/− mice. (F) % fibrosis area determined by
Sirius red staining in injured vessels (neointima, media and adventitia) of Mkl1+/− and
Mkl1−/− mice 4 weeks after wire injury are shown (n=3 in each group). (G) Real-time
RT-PCR analysis showing relative levels of Col1a1 and Col3a1 mRNAs (normalized to
GAPDH mRNA) in femoral arteries 4 weeks after wire injury (injury) (n=4 each). The
relative mRNA level in sham-operated arteries (sham) was assigned a value of 1.0. (H) Representative images of neointima 4 weeks after carotid artery ligation in $Mkl1^{+/−}$ and $Mkl1^{−/−}$ mice. Transverse sections at 1,400μm proximal to the ligature are shown. Ligation: ligated arteries. control: non-ligated arteries. Bars are 100 μm. (I) Graphs showing the neointima (NI)-to-media (M) ratio in ligated carotid arteries (n=3 each). In all graphs, values are shown as means±SEM. p**<0.01. NS: not significant.

Figure S3. Atherosclerotic lesions in $Mkl1^{−/−};ApoE^{−/−}$ mice are attenuated, as compared to those in $Mkl1^{+/+};ApoE^{−/−}$ mice.

(A) Graphs showing systolic blood pressure (sBP), heart rate (HR) and cholesterol profile in $Mkl1^{−/−};ApoE^{−/−}$ and $Mkl1^{+/+};ApoE^{−/−}$ mice fed a high-cholesterol diet (n=5 in each group). T-cho: total cholesterol, HDL: high-density lipoprotein-cholesterol, TG: triglycerides. (B) Graphs showing relative accumulation of macrophages within atherosclerotic lesions at the aortic root in $ApoE^{−/−}$ mice, which was estimated based on the size of the F4/80-stained area normalized to the corresponding total atherosclerotic lesion area (n=3 each). In all graphs, values are shown as means±SEM. NS: not significant.
Figure S4. MRTF-A maintains the expression of SRF target genes in dedifferentiated VSMCs.

(A) Real-time RT-PCR analysis showing the relative levels of vinculin, MMP-9 and integrin β1 mRNA (normalized to GAPDH mRNA) in femoral arteries 2 weeks after wire injury (injury) and in sham-operated arteries (sham) in Mkl1+/+ and Mkl1−/− mice. (n=4 each). undetected. (B) Real-time RT-PCR analysis showing the relative levels of αSMA (ACTA2) and smooth muscle myosin heavy chain (Myh11) mRNA (normalized to GAPDH mRNA) in femoral arteries 2 weeks after wire injury (injury) and in sham-operated arteries (sham) in Mkl1+/+ and Mkl1−/− mice (n=4 each). (C) The relative protein levels (normalized to GAPDH) of myocardin, MRTF-A and MRTF-B in wire-injured (injury) and sham-operated (sham) femoral arteries, cultured mouse VSMCs and cultured rat VSMCs (n=4 each). The relative protein level in the sham-operated arteries was assigned a value of 1.0. (D) Real-time RT-PCR analysis showing relative levels of MRTF-B and ACTA2 mRNAs in RAVSMCs transfected with MRTF-A siRNA or control siRNA (n=6 each). (E) Real-time RT-PCR analysis showing relative levels of myocardin and MRTF-A mRNAs in RAVSMCs transfected with myocardin siRNA (n=5) or control siRNA (n=5). (F) Migration in the presence or absence of PDGF-BB of RAVSMCs transfected with myocardin siRNA or control
siRNA (n=5 each). (G) Proliferation in the presence or absence of fetal calf serum (FCS) of RAVSMCs transfected with myocardin siRNA or control siRNA (n=5 each).

In all graphs, values are shown as means±SEM. p*<0.05. p**<0.01. NS: not significant.

**Figure S5. MicroRNA-1 regulates MRTF-A gene expression.**

(A) Cotransfection of a plasmid encoding myocardin or MRTF-A (0, 10 and 100 ng) with the -930bp (-0.9) MRTF-A-luc gene into NIH3T3 cells. Data were obtained from two experiments performed in quadruplicate. (B) Cotransfection of a plasmid encoding myocardin (0, 10 and 100 ng) with the -5500bp (-5.5) MRTF-A-luc gene into RAVSMC and NIH3T3 cells. Data were obtained from two experiments performed in quadruplicate. +: 10ng. ++: 100ng. (C) Real-time RT-PCR analysis showing the relative levels of miR-1 (normalized to U6) in femoral arteries 2 weeks after wire injury (injury) and in sham-operated arteries (sham) in Mkl1+/+ and Mkl1-/- mice. (n=4 each). (D) Real-time RT-PCR analysis showing the relative levels of miR-1 (normalized to U6) in femoral arteries 2 weeks after wire injury (injury), in sham-operated arteries (sham) and in cultured RAVSMCs (n=4 each). In all graphs values are shown as means±SEM. p*<0.05. p**<0.01. (E) Relative mRNA levels of MRTF-A, myocardin and MRTF-B in sham-operated femoral arteries 10 days after injection of LNA™ anti-miR-1 microRNA inhibitor or negative control microRNA inhibitor. (n=4 each). (F) Graphs showing the
neointima (NI)-to-media (M) ratios 2 weeks after wire injury in arteries treated with or without anti-miR-1 microRNA inhibitor (n=4 each). (G) Representative images showing the effect of anti-miR-1 microRNA inhibitor on neointima formation in arteries 2 weeks after wire injury in mice. HE: hematoxylin-eosin staining. MRTF-A: staining with anti-BSAC (MRTF-A) antibody. 5'-FAM labeled oligonucleotide microRNA inhibitors diffusely distributed in the cytoplasm of cells in injured vessels are directly visualized (FAM).

**Figure S6. CCG-1423, a MRTF-A inhibitor, attenuated neointima formation induced by wire injury in mouse femoral arteries.**

(A) Effects of CCG-1423 (0.1 µM) and knocking down MRTF-A on STARS-induced activation of SRF in RAVSMCs. Graphs show the relative luciferase activities of 3xCARG-luc (n=3 each). STARS: expression plasmid encoding striated muscle activator of Rho-signaling. Two independent experiments gave identical results. (B) Representative images of neointima 3 weeks after femoral artery injury stained with anti-Ki-67 antigen from mice treated without (control) or with CCG1423. Red arrows indicate Ki-67-positive cells. (C) Numbers of Ki-67-positive cells (n=3 in each group) in injured vessels 3 weeks after wire injury from mice treated without (control) or with CCG1423 are shown. (D) Cholesterol levels in mice subjected to femoral artery wire
injury and treated with or without CCG-1423. N.S.: not significant. T-cho: total cholesterol, HDL: high-density lipoprotein-cholesterol, TG: triglycerides. In all graphs, values are shown as means±SEM. N.S.: not significant. p**<0.01. (E) The systolic blood pressure (sBP) and heart rate (HR) in mice subjected to femoral artery wire injury and treated with or without CCG-1423 (n=3 in control group and 4 in CCG1423 group). (F) The systolic blood pressure (sBP) and heart rate (HR) in ApoE−/− mice fed a high-cholesterol diet and treated with or without CCG-1423 (n=3 in control group and 4 in CCG1423 group).
Supplementary References


Mol Cell Biol 26: 5797-5808


Figure S1

A

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<thead>
<tr>
<th>WB</th>
<th>Myocardin</th>
<th>MRTF-A</th>
<th>MRTF-B</th>
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<tr>
<td>Myocardin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRTF-A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRTF-B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
<td>50 (kDa)</td>
</tr>
</tbody>
</table>

B

IgG | MRTF-A

C

D

Relative mRNA levels

MRTF-A/GAPDH

myocardin/GAPDH

days after wire injury

0 10 20 30 40 50
Figure S2

A) TUNEL

<table>
<thead>
<tr>
<th></th>
<th>Mkl1&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Mkl1&lt;sup&gt;-/-&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>MRTF-A&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
</tbody>
</table>

B) Cleaved Caspase-3

<table>
<thead>
<tr>
<th></th>
<th>Mkl1&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Mkl1&lt;sup&gt;-/-&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>MRTF-A&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
</tbody>
</table>

C) NI/M ratio

D) Cleaved caspase-3 positive cells

E) Sirius red

<table>
<thead>
<tr>
<th></th>
<th>Mkl1&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Mkl1&lt;sup&gt;-/-&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neointima</td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
</tr>
<tr>
<td>Media</td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>Adventitia</td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
</tr>
</tbody>
</table>

F) % fibrosis area in vessels

G) Relative mRNA levels

<table>
<thead>
<tr>
<th></th>
<th>Mkl1&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Mkl1&lt;sup&gt;-/-&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col1a1/GAPDH</td>
<td><img src="image15.png" alt="Image" /></td>
<td><img src="image16.png" alt="Image" /></td>
</tr>
<tr>
<td>Col3a1/GAPDH</td>
<td><img src="image17.png" alt="Image" /></td>
<td><img src="image18.png" alt="Image" /></td>
</tr>
</tbody>
</table>

H) MRTF-A<sup>+/+</sup>

I) N/I ratio

![Image](image19.png)
Figure S3

A

<table>
<thead>
<tr>
<th>sBP (mmHg)</th>
<th>HR (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mkl1+/+; ApoE−/−</td>
<td>120</td>
</tr>
<tr>
<td>Mkl1−/−; ApoE−/−</td>
<td>60</td>
</tr>
</tbody>
</table>

mg/dl

<table>
<thead>
<tr>
<th>T-cho</th>
<th>HDL</th>
<th>TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mkl1+/+; ApoE−/−</td>
<td>29.5±1.3</td>
<td>27.3±0.5</td>
</tr>
<tr>
<td>Mkl1−/−; ApoE−/−</td>
<td>27.5±0.5</td>
<td>29.1±5.2</td>
</tr>
</tbody>
</table>

B

%F4/80 positive area

<table>
<thead>
<tr>
<th>Mkl1+/+; ApoE−/−</th>
<th>Mkl1−/−; ApoE−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6</td>
<td>0.4</td>
</tr>
</tbody>
</table>

N.S.
Figure S6

(A) Relative luciferase activity in RAVSMC 3xCAR-G-luc cells treated with STARS, CCG1423, and siMRTF-A.

(B) Image of tissue sections showing neointima, media, and adventitia with arrows indicating areas of interest.

(C) Ki67 positive cells in vessels treated with CCG1423.

(D) Comparison of T-cho, HDL, and TG levels between control and CCG1423-treated samples.

(E) Comparison of sBP and HR between control and CCG1423-treated samples.

(F) Comparison of sBP and HR between control and CCG1423-treated samples.