SUPPLEMENTAL MATERIAL

Olfactomedin 2 Regulates Smooth Muscle Phenotypic Modulation and Vascular Remodeling Through Mediating Runx2 Binding to SRF

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Materials and Methods:

Animals

Male Sprague-Dawley rats weighing 450-500 g were purchased from Harlan. Olfm2-/- mice were previously described.[1] All animals were housed under conventional conditions in the animal care facilities and received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals. Animal surgical procedures were approved by the Institutional Animal Care and Use Committee of The University of Georgia.

Cell culture and Trasfection

Rat aortic smooth muscle cells (RASMCs) were cultured by the explant method from rat thoracic aorta as described previously.[2] Rat thoracic aortas were removed and washed with DMEM. Aortic media were carefully dissected from the vessels, cut into pieces (< 1 mm^3), and explanted onto a 0.02% gelatin-coated flask. To obtain a stable attachment of the tissue pieces, the flask was incubated upside down for 1 h, and then DMEM supplemented with 20% FBS, penicillin, and streptomycin was slowly added. Cells were allowed to grow at 37°C in a humidified atmosphere of 5% CO2 for 2 weeks. RASMCs were confirmed by expression of MYH11 and TAGLN. The cultured SMCs from passage 1 to 5 were used for all the experiments. RASMCs were transfected with expression plasmid as needed using the Lipofectamine LTX reagents (Life Technologies) by following the manufacture's recommendation.

Construction of Adenovirus

Adenoviral vectors expressing rat Olfm2 short hairpin RNA (shRNA) (shOlfm2) were constructed, and the viruses were purified as described previously.[3] Rat Olfm2 short hairpin RNA (shRNA) (shOlfm2) coding sequences were as follows: 5'- CGC GTC GCA CGT CCA GTT ACG AGT ACA CGG ACG TGT TCA AGA GAC ACG TCC GTG TAC TCG TAA CTG GAC GTG CTT TTT TCC AAA -3' (top strand) and 5'-AGC TTT TGG AAA AAA GCA CGT CCA GTT ACG AGT ACA CGG ACG TGT CTC TTG AAC ACG TCC GTG TAC TCG TAA CTG GAC GTG CGA -3' (bottom strand). Both strands were annealed and ligated into pRNAT-H1.1/Adeno (Genscript Corp). Recombinant adenoviral vector was produced by homologous recombination in AD-1 competent cells following the manufacturer's instructions (Agilent). The resulting recombinant vector was digested with Pac I and then transfected into AD-293 cells with Lipofectamine LTX (Invitrogen) to package into viral particles expressing shOlfm2 (Ad-shOlfm2). The Ad-shOlfm2 was purified with gradient density ultracentrifugation of cesium chloride and dialyzed in dialysis buffer (135 mmol/L NaCl, 1 mmol/L $MgCl_2$, 10 mmol/L Tris-HCl, pH 7.5, 10% glycerol). Rat Runx2 shRNA adenoviral vector (Ad-shRunx2) was constructed similarly using the following

sequences: 5’- CGC GTC GCT TGA TGA CTC TAA ACC TAG TTT GTT CTT TCA AGA GAA GAA CAA ACT AGG TTT AGA GTC ATC AAG CTT TTT TCC AAA-3’ (top strand) and 5’-AGC TTT TGG AAA AAA GCG CTT GAT GAC TCT AAA CCT AGT TTG TTC TTT CAA GAG AAG AAC AAA CTA GGT TTA GAG TCA TCA AGC GA -3’ (bottom strand). Green fluorescent protein (GFP)-expressing adenoviral vector (Ad-GFP) was used as control.

Western Blot Analysis

Western blot was performed as described previously.[4] Cultured SMCs were washed twice with PBS followed by protein extraction using RIPA buffer (50 mmol/L Tris-HCl, pH 7.4, 1% Triton X-100, 0.25% wt/vol sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EGTA, 0.1% SDS) containing protease inhibitors. SMCs from Rat or mice thoracic aorta were homogenized in RIPA buffer containing protease inhibitors. The protein concentration was measured using BCA Protein Assay Reagent (Thermo Scientific). Lysates were denatured by boiling with SDS and 2-mercaptoethanol solution. Cell lysates or proteins collected from arteries were resolved on a 10% SDS-PAGE and were transferred to PVDF membrane (Bio-Rad). Membranes were blocked with 5% nonfat dry milk for regular antibodies, and then incubated for 1 to 2 hours with primary antibodies in blocking buffer followed by incubation with HRP-conjugated secondary antibody for 1 hour (Sigma). Detection was performed with enhanced chemiluminescence (Millipore). Antibodies against Olfm2 (Abcam, ab101007), TAGLN (Abcam, ab10135), PCNA (Santa Cruz Biotechnologies, sc-56), SRF (Santa Cruz Biotechnologies, sc-335), Myocardin (Abcam, ab22073), MYH11 (Biomedical Technologies Inc., BT-562), KLF4 (Abcam, ab129473), Runx2 (Cell Signaling Technology, 12556), and TUBA1(Sigma, T6074) were used for immunoblotting.

Real-time Quantitative PCR (qPCR)

Quantitative PCR was performed as described previously.[5] Total RNA from cells was extracted using Trizol Reagent (Invitrogen) according to the manufacturer’s protocol. Briefly, add 1 ml of Trizol per well to collect samples to RNase free microcentrifuge tubes and incubate 2 min at room temperature (RT). Next add 0.2 ml chloroform to each tube and shake by hand for 30 seconds. Incubate the samples for 5 min at RT and centrifuge for 15 min at 12,000×g at 4°C. Transfer the upper aqueous phase about 0.5 ml to a new tube and add 0.5 ml isopropyl alcohol to the aqueous phase to mix well. Incubate samples at RT for 10 min and spin for 15 min at 12,000 x g at 4°C. Take off the supernatant and wash pellet with 1 ml 70% ethanol. Air dry the pellet for 10min and dissolve the RNA in 30 μl of RNase free water to do later experiment. cDNA was synthesized from 1μg of total RNA by iScript cDNA Synthesis kit (Bio-Rad). qPCR was performed on a Stratagene Mx3005 qPCR thermocycler using SYBR Green master mix (Agilent Technologies).

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed using ChIP kit (Millipore) as described previously.[6] Briefly, RASMCs were transfected with control, Olfm2 expression plasmid (pshuttle-IRES-GFP-Olfm2) or KLF4 expression plasmid (pcDNA3.1-HA-KLF4) followed by serum starvation for 24 h. Chromatin complexes were immunoprecipitated with 3 μg of SRF antibody or IgG (negative control) according to the manufacturer's instructions. Semiquantitative PCR and qPCR were performed to amplify rat Myh11 and Tagln promoter regions containing CArG box sequence using the following primer sets: Myh11: 5’-CTG CGC GGG ACC ATA TTT AGT CAG GGG GAG -3’ (forward) and 5’-CTG GGC GGG AGA CAA CCC AAA AAG GCC AGG -3’ (reverse). Tagln: 5’-GGT CCT GCC CAT AAA AGG TTT-3’ (forward) and 5’-TGC CCA TGG AAG TCT GCT TGG-3’ (reverse).

Rat Carotid Artery Injury Model and Adenoviral Gene Transfer

Rat carotid artery balloon injury was performed as described previously.[7, 8] Briefly, rats were anesthetized by an intraperitoneal injection of Ketamine (80 mg/kg) and Xylazine (5 mg/kg). A 2F Fogarty arterial embolectomy balloon catheter (Baxter Edwards Healthcare) was introduced through the left external carotid artery and advanced 4 cm toward the thoracic aorta. The method that introduces adenovirus into rat balloon-injured carotid artery has been previously described.[2, 9] The injured artery was washed with saline, and incubated with 100 µl saline or adenovirus ($5x10^9$ pfu) for 20 minutes. 14 days later, the balloon-injured segment of the artery from the proximal edge of the omohyoid muscle to the carotid bifurcation was perfused with saline and excised. The balloon-injured and adenovirus-dwelled segments were fixed with 4% paraformaldehyde (PFA) and embedded in paraffin. Subsequent morphometric analyses were performed in a double-blinded manner.

Mouse Carotid Artery Wire-injury Model

Mouse carotid artery wire injury was performed as described previously.[10] Mice were anesthetized with ketamine hydrochloride (80 mg/kg IP) and xylazine (5 mg/kg IP). The left common carotid arteries were exposed and wire-injured with epon resin probes. The wire-injured segments were collected at 14 days later, fixed with 4% paraformaldehyde and embedded in paraffin for sectioning and histological staining. Subsequent morphometric analyses were performed in a double-blinded manner.

Histomorphometric Analysis and Immunohistochemistry (IHC) Staining

Vessel segments were cut by serial sectioning and 10 sections that were evenly distributed in the vessel segment were collected for analysis. The dissected arteries were stained with modified hematoxylin and eosin (HE) or Elastica van Gieson staining and captured using a Nikon microscope. The circumference of lumen, internal elastic lamina, and external elastic lamina were measured by Image-pro Plus Software. For immunohistochemistry, sections were rehydrated, blocked with 10% goat serum and permeabilized with 0.01% Triton X-100 in PBS, and incubated with Olfm2 or MYH11 primary antibody at 4°C overnight followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody. The sections were counterstained with hematoxylin.

Wound Healing Assay

RASMC migration was evaluated by wound healing assay using the CytoSelect Wound Healing Assay Kit (Cell Biolabs). Wound healing inserts were put into 24-well cell culture plates coated with fibronectin. Cell suspension (250 µl) was added to either side of the insert and incubated overnight to form a monolayer. The inserts were then removed, and then the cells were incubated with mitomycin C (10 µg/ml, dissolved in culture medium), a potent inhibitor of cell proliferation, for 2 h. The medium was replaced with serum-deprived medium containing PDGF (20 ng/mL, R&D), and the cells were incubated for additional 24 h. Images of wound healing were captured using a dissection microscope. Cell migration was quantified by a blind measurement of the migration distances.

Cell Proliferation Assay

5×10^3 cells/well were cultured in 96-well plates. On the following day, 10 µl of MTT reagent (R&D) was added to the medium and incubated in a CO_2 incubator for 3 h, and then 100 µl of Detergent Solution (R&D) was added and incubated at 37°C for additional 2 h. Absorbance at 570 nm wavelength was measured in a plate reader (Bio-Tek). The medium containing no cells was used as blank control.

Coimmunoprecipitation (CoIP) Assay

CoIP assays were performed as described previously.[5] Briefly, RASMCs were washed with ice-cold mild lysis buffer containing protease inhibitor mix (Thermo Scientific). The lysates were incubated with immunoglobulin G (IgG, Santa Cruz Biotechnology), SRF (Santa Cruz Biotechnology), or Olfm2 antibodies using reagents provided in a CoIP kit (Thermo Scientific). The immunoprecipitates were pelleted, washed, and subjected to immunoblotting according to the manufacturer's instructions.

Mouse Genotyping

Genomic DNA was obtained from mouse tails using DirectPCR reagent (Viagen Biotech Inc.). Briefly, the reaction was set up with the PCR mix (Bioline) and genotyping primers as follows: 5'-GCT CTG TGG ATG GGT TCC TA-3' (forward); 5'-GAG GCA AAA GGG AAT GTC AG-3' and CTT GAG CAG CTC CTT GCT G-3' (reverse). The PCR was performed by initial denaturation at 95°C for 1 min followed by 30 cycles with denaturation at 95°C for 30 s, annealing and elongation at 60°C for min and a final elongation at 72°C for 5 min.

Statistical Analysis

All data were evaluated with a 2-tailed, unpaired Student *t* test or compared by 1-way ANOVA followed by the Fisher *t* test and are expressed as mean ± SD. A value of $p < 0.05$ was considered statistically significant.

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