Supporting Information for

Original article

Endothelial METRNL determines circulating METRNL level and maintains endothelial function against atherosclerosis

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Methods

Table S1 Information of myocardial infarction (MI) patients with atherosclerosis and their controls.

Item	Control	MI patient
Number of subjects (Male/Female)	11 (8/3)	21 (17/4)
Overall age, years (mean)	42–79 (62.5)	40–89 (64.7)
Male age, years (mean)	42–79 (58.4)	40–83 (61.9)
Female age, years (mean)	69–78 (73.7)	69–89 (76.5)
Overall cTn I, ng/mL (mean)	0.012–0.019 (0.0126)	0.062–80 (18.4653)
Male cTn I, ng/mL (mean)	0.012 (0.012)	0.062–80 (22.3)
Female cTn I, ng/mL (mean)	0.012–0.019 (0.0143)	0.068–7.68 (2.4)
Overall METRNL, pg/mL (mean)	446.5–1065.7 (645.19)	12.2–922.5 (227.54)
Male METRNL, pg/mL (mean)	446.5–673.9 (577.5)	12.2–835.6 (211.40)
Female METRNL, pg/mL (mean)	588.9–1065.7 (825.7)	60–922.5 (296.12)

cTn I, cardiac troponin I.

Table S2 Protein presence and gene expression of METRNL among different human and murine tissues.

Tissue type	Human METRNL		Mouse METRNL	
	Protein	mRNA	Protein	mRNA
Large intestine	+++	ne	+++	+++
Small intestine	+++	ne	ne	+++
Stomach	+++	ne	ne	+++
Skin	+++	ne	ne	ne
Kidney	++	ne	ne	++
Thyroid	++	ne	ne	ne
Testis	++	ne	ne	++
Spleen	+	+	ne	+
Lung	++	ne	ne	+++
Liver	+	+	ne	+
Adrenal	+	ne	ne	ne
Brain	–	ne	ne	+
Cerebellum	–	ne	ne	ne
Skeletal muscle	–	+	ne	-
Ovary	–	ne	ne	ne
Prostate	–	ne	ne	ne
Heart	–	ne	ne	+
Pancreas	–	ne	ne	–
Uterus	–	ne	ne	ne
White adipose	ne	+++	+++	+++
Brown adipose	ne	ne	ne	+
Thymus	ne	ne	ne	–

Protein levels were detected by immunohistochemistry and mRNA levels by real time PCR. The samples are human multi-organ tissue microarray or human tissue obtained during surgical interventions or mouse tissue from normal animals. +++, strong positive; ++, positive; +, weak positive; –, negative; ne, no examination.

Table S3 Primers used for real time PCR.

Gene name	Forward primer	Reverse primer
Mouse *Metrnl*	CTGGAGCAGGGAGGCTTATTT	GGACAACAAAGTCACTGGTACAG
Mouse *Vcam-1*	TGCCGGCATATACGAGTGTGA	CCCGATGGCAGGTATTACCAAG
Mouse *Icam-1*	CAATTCACACTGAATGCCAGCTC	CAAGCAGTCCGTCTCGTCCA
Mouse *Tnf-α*	AAGCCTGTAGCCCACGTCGTA	GGCACCACTAGTTGGTTGTCTTTG
Mouse *Il-6*	CCACTTCACAAGTCGGAGGCTTA	GCAAGTGCATCATCGTTGTTCATAC
Mouse *Il-1β*	TCCAGGATGAGGACATGAGCAC	GAACGTCACACACCAGCAGGTTA
Mouse *F4/80*	GAGATTGTGGAAGCATCCGAGAC	GATGACTGTACCCACATGGCTGA
Mouse *Cd68*	CATCAGAGCCCGAGTACAGTCTACC	AATTCTGCGCCATGAATGTCC
Mouse *Il-10*	CTTACTGACTGGCATGAGGATCA	GCAGCTCTAGGAGCATGTGG
Mouse *MCP-1*	TAAAAACCTGGATCGGAACCAAA	GCATTAGCTTCAGATTTACGGGT
Mouse *Gapdh*	GTATGACTCCACTCACGGCAAA	GGTCTCGCTCCTGGAAGATG
Human *METRNL*	GAGCTGGTTAGGAGGCACAG	AGGCTCGTGGGTAACTTGC
Human *VCAM-1*	GGGAAGATGGTCGTGATCCTT	TCTGGGGTGGTCTCGATTTTA
Human *ICAM-1*	TTGGGCATAGAGACCCCGTT	GCACATTGCTCAGTTCATACACC
Human *TNF-α*	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
Human *IL-6*	CCTGAACCTTCCAAAGATGGC	TTCACCAGGCAAGTCTCCTCA
Human *IL-1β*	AGCTACGAATCTCCGACCAC	CGTTATCCCATGTGTCGAAGAA
Human *F4/80*	ATCGTGGGGCTTCTCATGTTT	TTAGTCATGCCCAACTTCACTTT
Human *CD68*	GGAAATGCCACGGTTCATCCA	TGGGGTTCAGTACAGAGATGC
Human *MCP-1*	CAGCCAGATGCAATCAATGCC	TGGAATCCTGAACCCACTTCT
Human *E-SEL*	GCCTGCAATGTGGTTGAGTG	ACGAACCCATTGGCTGGATT
Human *GAPDH*	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

Table S4 Antibodies used for Western blotting.

Antigen/Target	Source	Stock	Dilution
GAPDH	Beyotime	AG019	1:2000
Actin	Beyotime	AA128	1:5000
METRNL	Abcam	ab235775	1:1000
eNOS	Santa Cruz	sc-136977	1:1000
Phospho-eNOS (Ser1177)	Cell signaling	9570s	1:1000
Phospho-eNOS (Thr495)	Cell signaling	9574s	1:1000
Phospho-eNOS (Ser114)	Signalway Antibody	12472	1:1000
Phospho-eNOS (Ser633)	Signalway Antibody	12473	1:1000
Phospho-eNOS (Ser615)	Signalway Antibody	12137	1:1000
Caveolin 1	Boster	BA2440	1:800
Calmodulin	Boster	BM1614	1:800
HSP90	Beyotime	AH732-1	1:1000
NFκB p65	Beyotime	AN365	1:1000
Phospho-NFκB p65 (Ser536)	Beyotime	AF5881	1:1000

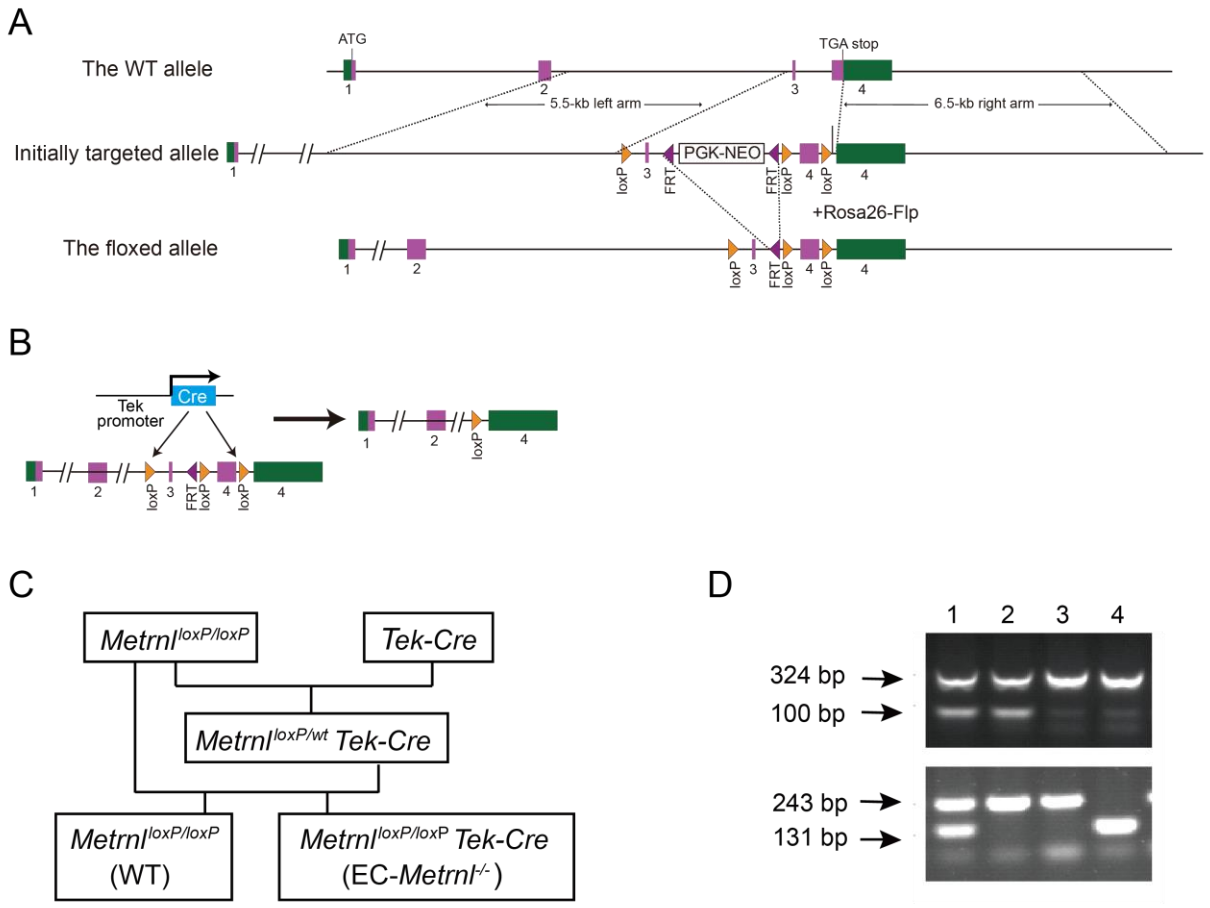

Figure S1 Generation and identification of EC-*Metrnl*$^{-/-}$ mice. (A) Scheme describing the generation of mice with floxed *Metrnl* (*Metrnl*$^{loxP/loxP}$) by gene targeting. (B) Scheme describing the generation of knockout mice with vascular endothelial cell-specific ablation of *Metrnl* (EC-*Metrnl*$^{-/-}$). (C) Breeding strategy for EC-*Metrnl*$^{-/-}$ mice. (D) Genotyping the mice. 1, *Metrnl*$^{loxP/wt}$*Tek-Cre*; 2, *Metrnl*$^{loxP/loxP}$*Tek-Cre* (EC-*Metrnl*$^{-/-}$); 3, *Metrnl*$^{loxP/loxP}$ (WT); 4, *Metrnl*$^{wt/wt}$. Sizes of PCR products: *internal control*~324 bp, *Cre*~100 bp, *Metrnl-floxed*~243 bp, *Metrnl-wt*~131 bp.

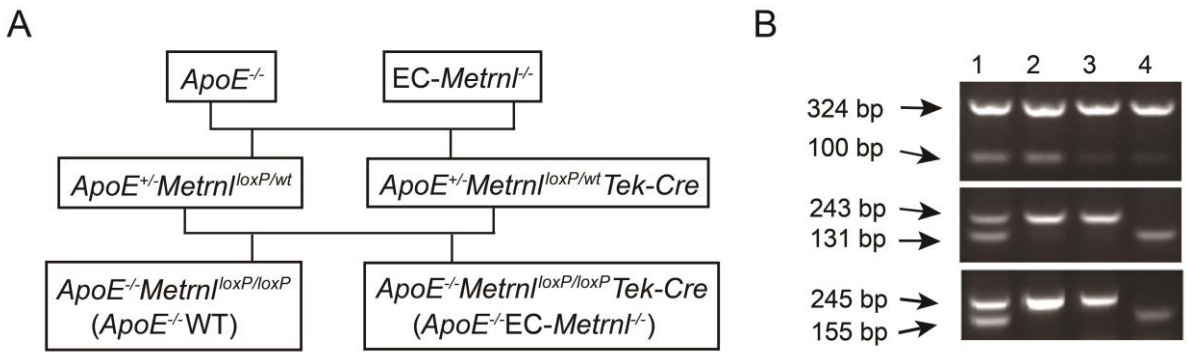

Figure S2 Generation and identification of *ApoE*$^{-/-}$EC-*Metrnl*$^{-/-}$ double knockout mice. (A) Breeding strategy for double knockout mice with deficiency of *ApoE* and endothelial cell-specific ablation of *Metrnl* (*ApoE*$^{-/-}$EC-*Metrnl*$^{-/-}$). (B) Genotyping the mice used in generation of *ApoE*$^{-/-}$EC-*Metrnl*$^{-/-}$ mice. 1, *ApoE*$^{+/-}$*Metrnl*$^{loxP/wt}$*Tek-Cre*; 2, *ApoE*$^{-/-}$*Metrnl*$^{loxP/loxP}$*Tek-Cre* (*ApoE*$^{-/-}$EC-*Metrnl*$^{-/-}$); 3, *ApoE*$^{-/-}$*Metrnl*$^{loxP/loxP}$ (*ApoE*$^{-/-}$WT); 4, *ApoE*$^{+/+}$*Metrnl*$^{wt/wt}$. Sizes of PCR products: *internal control*–324 bp, *Cre*–100 bp, *Metrnl-floxed*–243 bp, *Metrnl-wt*–131 bp, *ApoE null*–245 bp, *ApoE-wt*–155 bp.

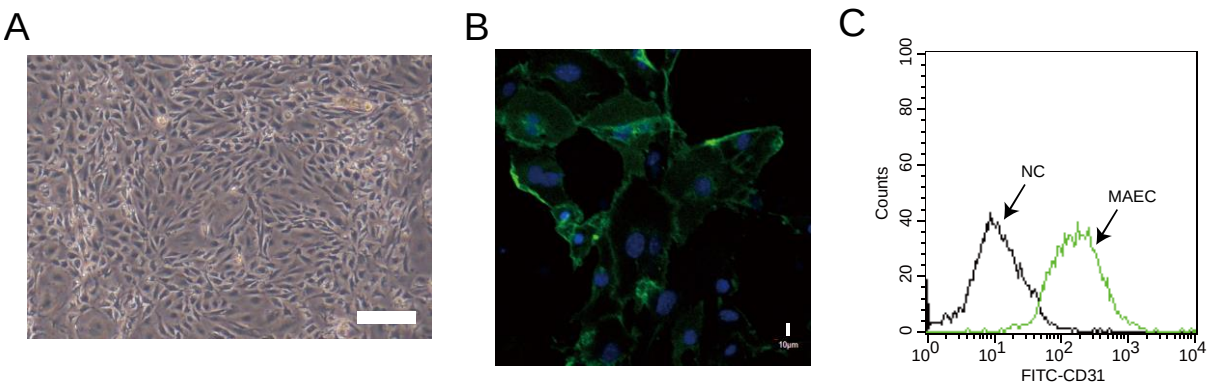

Figure S3 Identification of primary cultured mouse aortic endothelial cells (MAECs). (A) Cultured MAECs on Day 7 under optical microscopy. Bar = 100 μm. (B) CD31 (green) in cultured MAECs is detected by immunofluorescence. DAPI (blue) labels nuclei. Bar = 10 μm (C) Flow cytometry is used to identify the CD31 positive cells in cultured MAECs. NC: negative control. Three independent experiments performed.

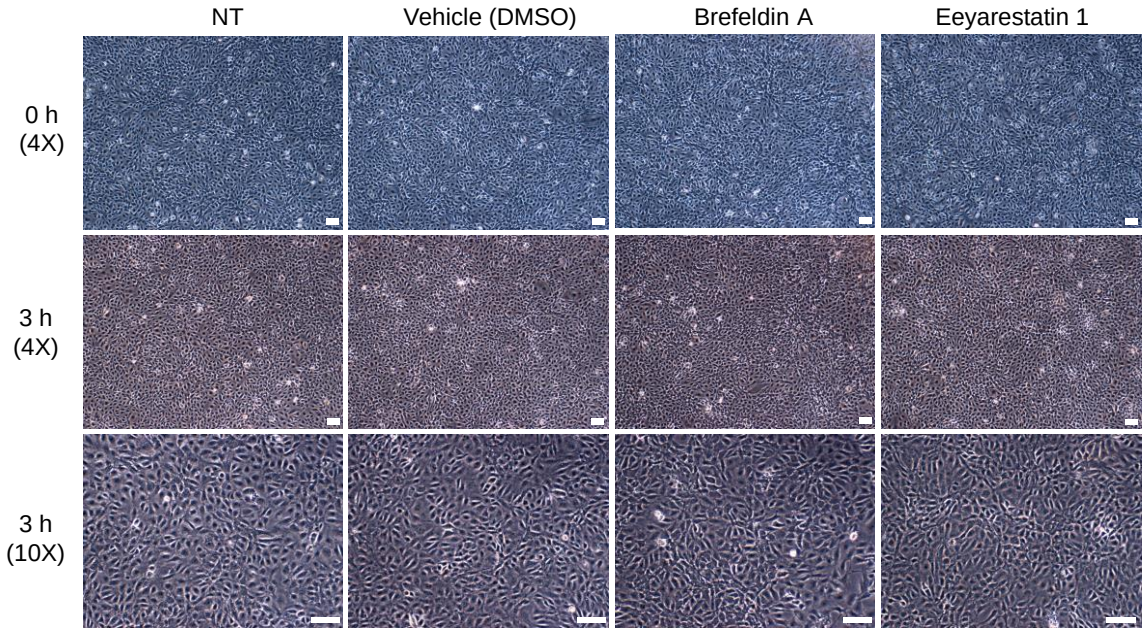

Figure S4 Human umbilical vein endothelial cells (HUVECs) under optical microscopy after 3 h of drug incubation. Brefeldin A (5 μg/mL), eeyarestatin 1 (5 μg/mL) or a solvent control (dimethyl sulfoxide, DMSO) was added to the serum-free culture medium 3 h before medium collection for METRNL measurement. NT: no treatment as blank control. Bar = 100 μm. Three independent experiments performed.

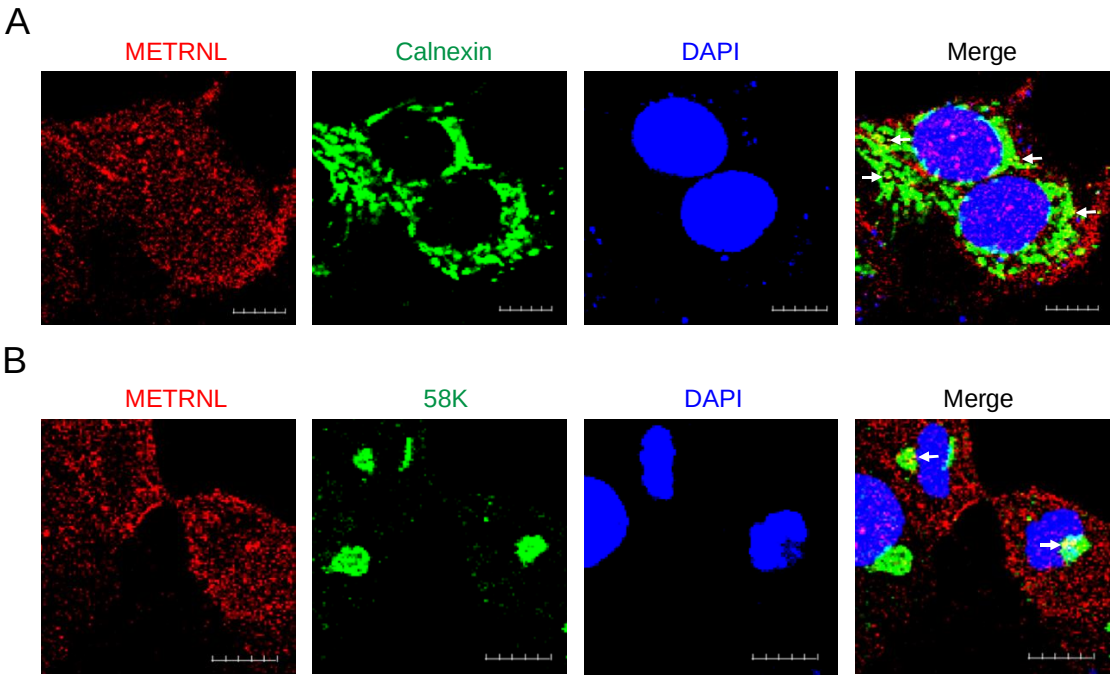

Figure S5 The intracellular location of METRNL in endothelial cells. (A) METRNL is present in endoplasmic reticulum (ER). Primary cultured HUVECs were immunostained for METRNL (red) and calnexin (green), an ER integral membrane chaperone (a marker of ER). The nuclei were stained with DAPI (blue). Merged image showed colocalization of METRNL with calnexin as indicated by white arrows. (B) METRNL is present in Golgi apparatus. Primary cultured HUVECs were immunostained for METRNL (red) and Golgi marker 58K (green). The nuclei were stained with DAPI (blue). Merged image shows colocalization of METRNL with 58K as indicated by white arrows. Bar = 10 μm.

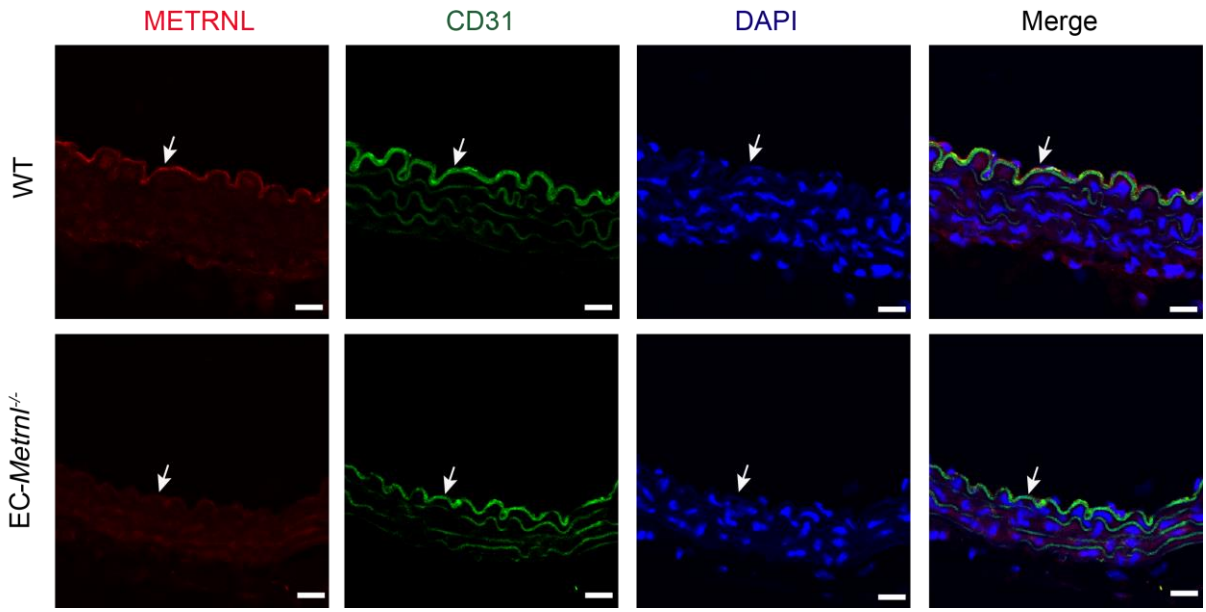

Figure S6 METRNL in the endothelium of aortae from EC-*Metrnl*$^{-/-}$ and wild-type (WT) mice. Aortae from EC-*Metrnl*$^{-/-}$ and WT mice were immunostained for METRNL (red) and vascular endothelial marker CD31 (green). The nuclei were stained with DAPI (blue). METRNL was abundantly expressed in aortic endothelium from WT mice, but absent in aortic endothelium from EC-*Metrnl*$^{-/-}$ mice. Scale bar = 20 μm.

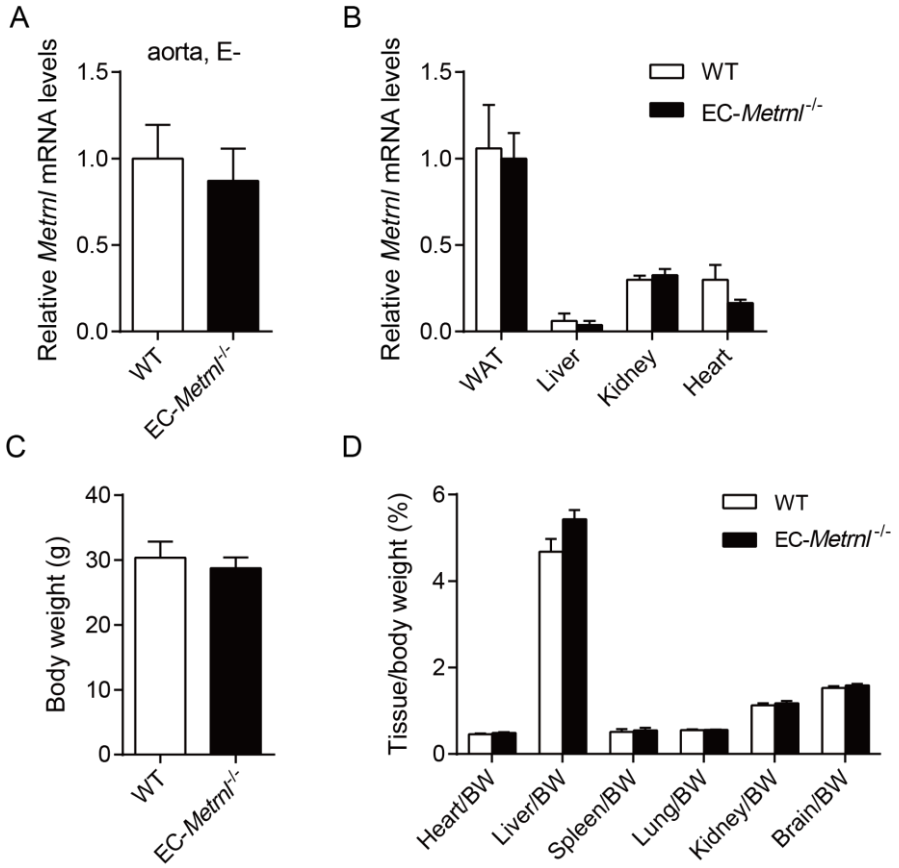

Figure S7 Characterization of EC-*Metrnl*$^{-/-}$ mice. (A) *Metrnl* mRNA expression in aortae with endothelium removal (E–) of EC-*Metrnl*$^{-/-}$ and wild type (WT) mice (n=9–10). (B) *Metrnl* mRNA expression in white adipose tissue (WAT), liver, kidney and heart of EC-*Metrnl*$^{-/-}$ and WT mice (n=4). (C, D) Body weight (C) and tissue weight (D) of EC-*Metrnl*$^{-/-}$ and WT mice (n=7–8). BW: body weight. Data are mean ± SEM.

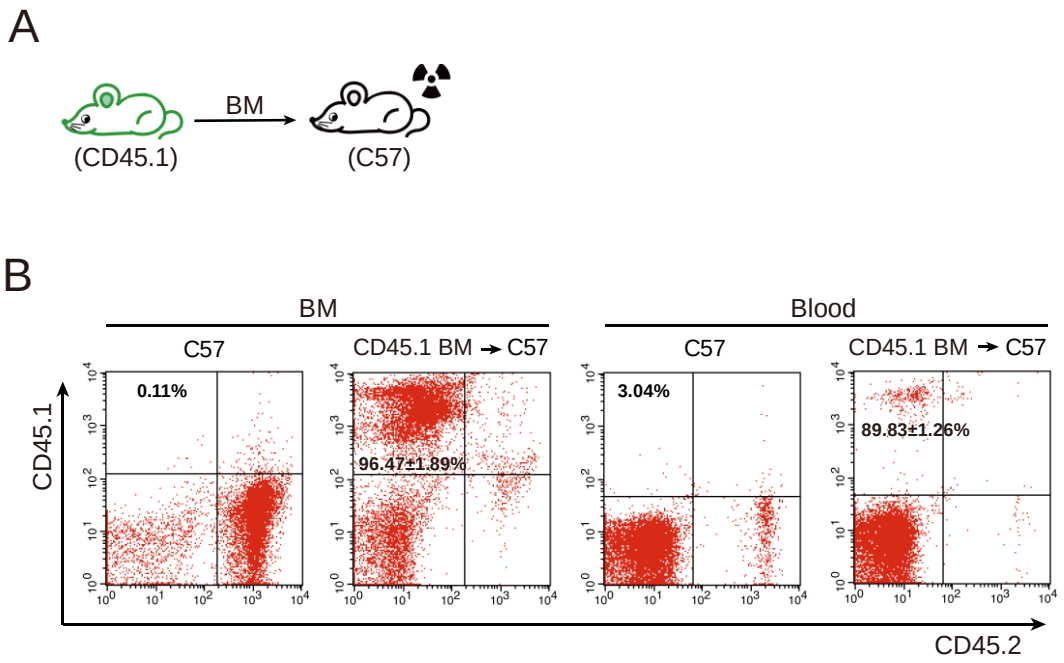

Figure S8 Transplantation of bone marrow (BM) and analysis of transplantation efficacy. (A) Experimental design of BM transplantation. The recipient C57BL/6 mice using irradiation to destroy their own BM and then receving donor BM cells from CD45.1 mice. (B) CD45.1 and CD45.2 positive cells from BM and peripheral blood, identified by flow cytometry. Transplantation efficacy analysis was performed in the recipient mice who received BM from CD45.1 mice. Cells from peripheral blood and BM were isolated and stained with anti-CD45.1 PE and anti-CD45.2 FITC conjugated antibodies (1:100, BD Biosciences, San Diego, CA, USA), and were then analyzed in a BD LSR II flow cytometer (BD Biosciences, NJ, USA) for the percentage of CD45.1 and CD45.2 positive cells. The transplantation efficacy was calculated by the formulas: CD45.1 positive cells/(CD45.1 positive cells+CD45.2 positive cells). The mouse mononuclear cells were isolated from anticoagulated venous blood using HISTOPAQUE® 1083 (#1083-1, Sigma–Aldrich). n = 1 in C57BL/6 control group, and n = 3 in BM transplantation (CD45.1 BM→C57) group.

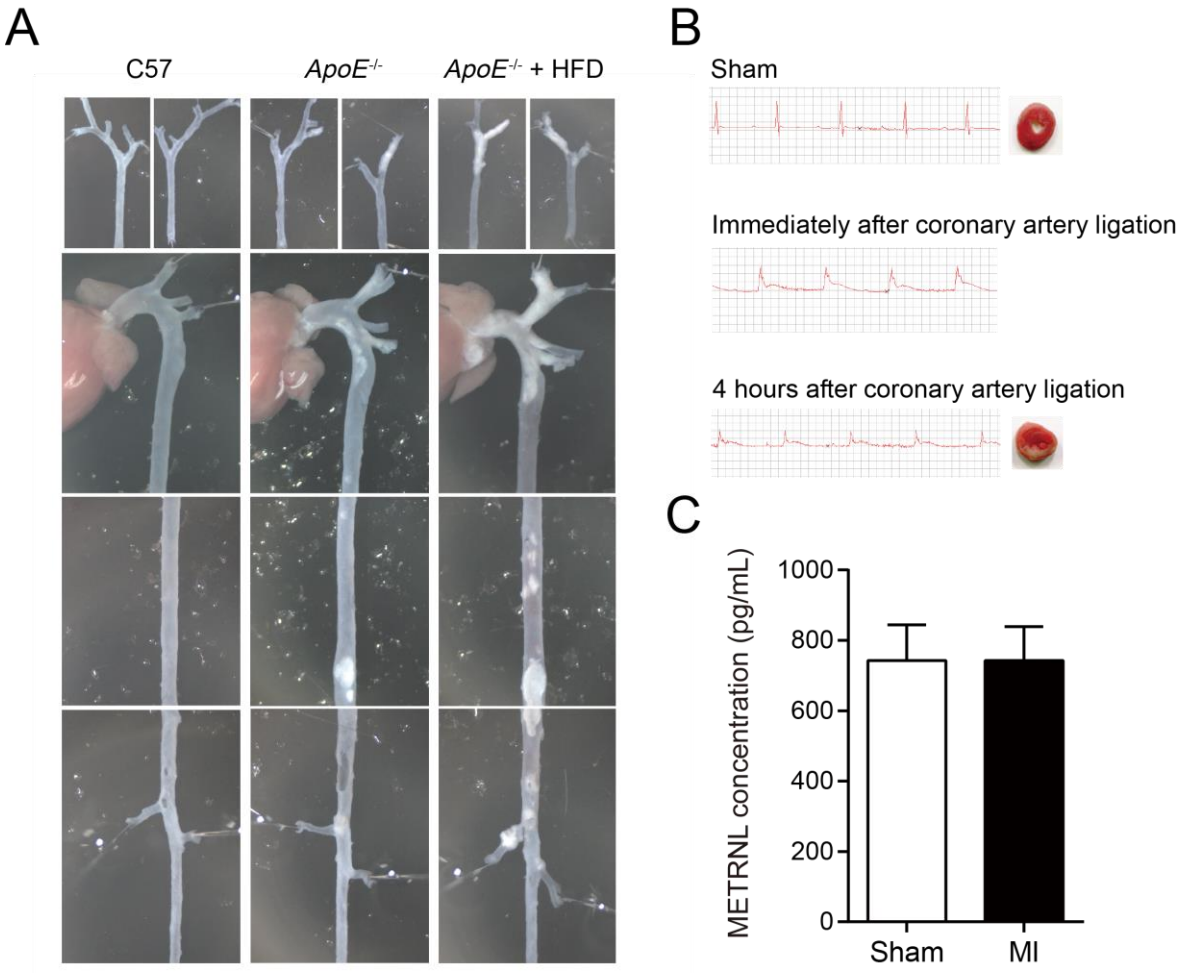

Figure S9 Atherosclerosis in *ApoE*$^{-/-}$ mice and serum METRNL levels in myocardial infarction mice. (A) Morphology of left and right carotid arteries (upper) as well as aortae from C57BL/6 mice, *ApoE*$^{-/-}$ mice (one-year-old) fed normal chow and *ApoE*$^{-/-}$ mice (one-year-old) fed high fat diet (HFD) during 22 weeks. Atherosclerotic lesions existed in multiple sites of the artery system in *ApoE*$^{-/-}$ mice, with more severity in all *ApoE*$^{-/-}$ mice fed HFD than normal chow. *n*=4–5 per group. (B) Representative electrocardiograms (ECG) of mice and representative photographs of 2,3,5-triphenyltetrazolium chloride-stained heart sections. Sham: sham operation. (C) Serum METRNL levels in mice 4 hours after myocardial infarction (MI) produced by coronary artery ligation surgery. *n*=8–10 per group. Sham: sham operation.

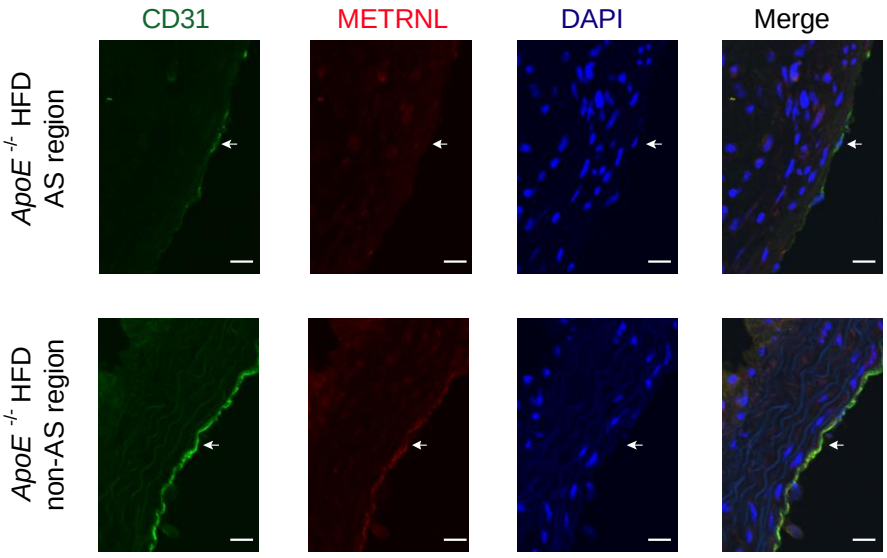

Figure S10 METRNL (red) and CD31 (green, a marker of endothelial cells) in the endothelium of aortae from *ApoE*$^{-/-}$ mice fed high fat diet (HFD). The nuclei were stained with DAPI (blue). Endothelial METRNL expression was decreased in *ApoE*$^{-/-}$ mice fed HFD, with obvious lack of endothelial METRNL in the region of atherosclerotic lesion (AS). Bar = 20 μm.

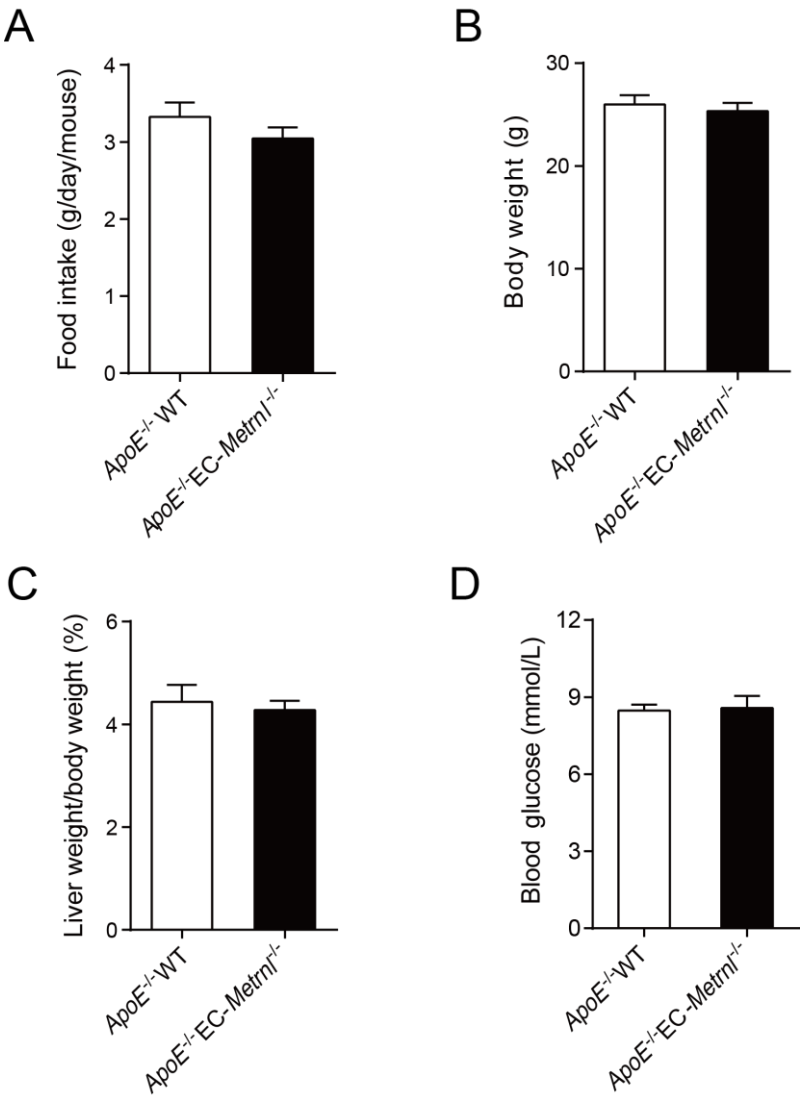

Figure S11 Food intake (A), body weight (B), liver weight (C) and blood glucose (D) in *ApoE*$^{-/-}$EC-*Metrnl*$^{-/-}$ and *ApoE*$^{-/-}$WT mice fed high fat diet for 10 weeks. *n*=4–5 per group.

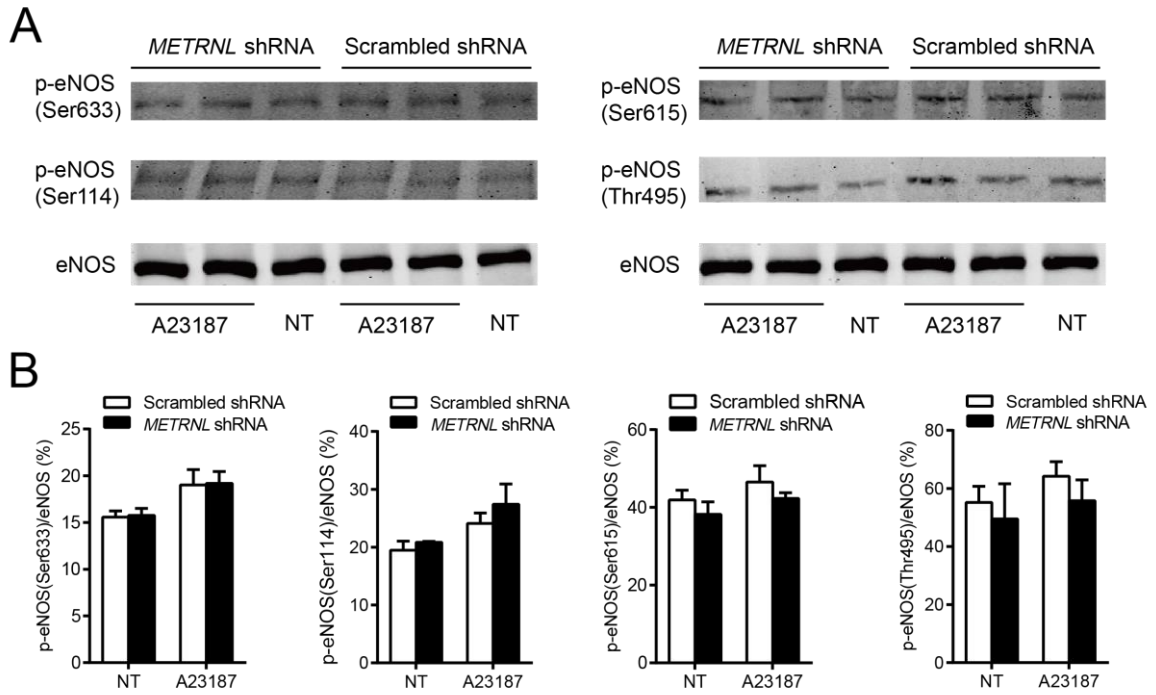

Figure S12 Effects of METRNL on the phosphorylations of eNOS at Ser633, Ser114, Ser615, and Thr495 sites. HUVECs transfected with lentivirus containing *METRNL* shRNA or scrambled RNA were treated, or not, with A23178 (1 μmol/L) during 10 min. Subsequently, the cells were harvested and Western blotting was performed with phosphorylation-specific antibodies. Total eNOS protein was used as internal reference. *n*=3–4 per group. Data are mean ± SEM. NT, no treatment with drug.

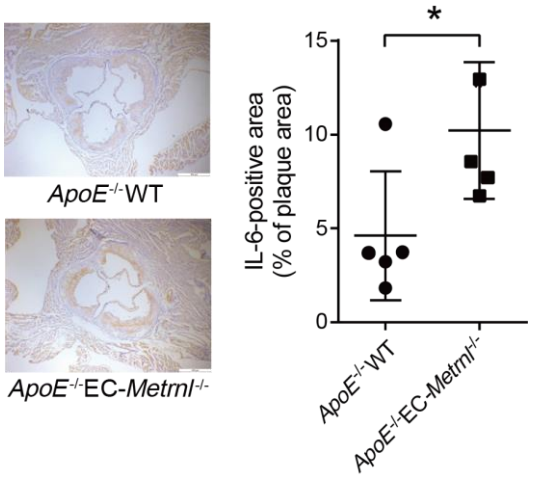

Figure S13 Immunohistochemical staining for IL-6 in aortic roots from *ApoE*$^{-/-}$EC-*Metrnl*$^{-/-}$ and *ApoE*$^{-/-}$WT mice after 22 weeks of high fat diet. Bar = 500 μm. *n* = 5 per group. Data are mean ± SEM. **P*<0.05 by two-tailed Student's *t* test.

Methods

1. Myocardial infarction model

Myocardial infarction (MI) was generated in mice as described in our previous study[1]. Briefly, 8 to 10 week-old male C57BL/6 mice were anesthetized by isoflurane inhalation. The left anterior descending (LAD) coronary artery was ligated to induce MI. Sham control mice were subjected to the same surgery without LAD ligation. The mice were then euthanized 4 or 24 h after the surgery, with the venous blood collected for METRNL measurement and the heart harvested for infarct assessment by 2% 2,3,5-triphenyltetrazolium chloride staining.

2. BM transplantation in atherosclerosis study

Recipient 10–11-week-old male *ApoE*$^{-/-}$ mice were irradiated with 12 Gy, and each mouse received 1×10^7 BM cells from *ApoE*$^{-/-}$EC-*Metrnl*$^{-/-}$ mice or *ApoE*$^{-/-}$WT littermates. Four weeks after BM transplantation, the mice were then subjected to a HFD for 10 weeks to study the occurrence of atherosclerosis, and the cells harvested from peripheral blood and BM were stained with anti-F4/80 APC and anti-CD11b FITC conjugated antibodies (1:100, Biolegend, San Diego, CA, USA) to analyze the percentage of marcophages by flow cytometer.

3. Lesion area analysis of atherosclerosis

The lesion area of the aorta or the aorta root was quantified as a percentage of the total area, measured using Image J software (Version 1. 46; National Institutes of Health, Bethesda, MD, USA) by an experienced researcher who was blinded to the mouse genotypes under examination. For the oil red O (ORO) staining of whole aortic lesions, the fixed aorta was placed into phosphate buffered saline, cut longitudinally and pinned flat with lumen side up. Next, phosphate buffered saline was replaced by ORO working solution prepared as described[2]. The aorta was immersed in ORO solution during 15 min, after which the ORO solution was replaced by 70% ethanol for another incubation of five to ten minutes. Finally, the aorta was washed with double distilled water. For the staining of the aortic roots, frozen sections were dried at 37 °C during one hour and then immersed in ORO solution during 15 min. Then, the section was transferred into 60% ethanol for one minute and washed in double distilled water during 50 s. Finally, excess water on the section was removed and glycerol

was used to cover it.

4. Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated and pooled from ten freshly delivered umbilical cords with established methods[3] and grown in endothelial cell medium (ECM, ScienCell, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS, Sciencell) and 1% endothelial cell growth supplement (ECGS, Sciencell). HUVECs at passage 3 to 5 were used for experiments. Mouse aortic endothelial cells (MAECs) were isolated and cultured as described by us and others[1,4]. MAECs were used at passage 3 after identification.

Mouse bone marrow-derived macrophages were isolated and cultured as described previously[5]. At day 7 of culture, when the macrophages were considered fully differentiated (also named M0 macrophages), cells were harvested and incubated with anti-F4/80 APC and anti-CD11b FITC conjugated antibodies to identify the percentage of macrophages by flow cytometer. M0 macrophages at day 8 were used for other experiments after identification.

5. Virus-mediated overexpression and knockdown of *METRNL*

Adeno-associated virus (AAV) for overexpression of full-length METRNL protein or METRNL without N-terminal signal peptide (METRNL-△SP) was constructed by Vigene Biosciences, Inc. (Shandong, China). HUVECs were transfected at a multiplicity of infection of 30.

Lentivirus for knockdown of human *METRNL* was constructed by Shanghai Bio-Link Company (Shanghai, China). Briefly, to construct human *METRNL* shRNA lentivirus, four targeting sequences were designed, including 5′- GCTTCCAGTACGAGCTGGTTA-3′, 5′-AGAACTGAGACTGCTGGTA-3′, 5′-GCCGATTGGAAATGCTGTAAA-3′, and 5′-CAGGTGCTCTCATCGTTAACC-3′. The last target displayed the highest knockdown efficiency and was used in the present study. HUVECs were transfected at a multiplicity of infection of 10.

6. Immunohistochemistry

Immunohistochemistry was performed as described in our previous studies[6,7]. Paraffin sections (4 μm) of tissues were dewaxed and frozen sections of aortic roots (8 μm) were dried. The slides were placed in a pressure cooker at high-temperature for antigen repair, and were then immersed in 3% H_2O_2 to block endogenous peroxidase activity. Goat serum (as blocking buffer) was added to the slides during 15 min at 37 °C. Then the sections were incubated with primary antibodies against METRNL (1:200, Abcam, Cambridge, MA, USA), F4/80 (1:200, Abcam) or IL-6 (1:200, Abcam) overnight at 4 °C. Slides were then incubated with biotinylated goat anti-rabbit or goat anti-mouse secondary antibodies (Boster, Wuhan, China) followed by an avidin-conjugated horseradish peroxidase, and were detected by diaminobutyric acid substrate. The sections were examined with a Leica DM LB2 microscope (Leica Microsystems, Wetzlar, Germany).

7. Immunofluorescence

Immunofluorescence was performed as described in our previous study[8]. As a pre-processing step, frozen sections (8 μm) of the descending thoracic aorta were dried and then citrate buffer heat repairing was performed. Cultured MAECs were harvested on Day 7 and fixed by 4% paraformaldehyde (PFA) during ten minutes. Goat serum (as blocking buffer) was added to the samples during one hour at room temperature. The samples were then incubated with primary antibodies against METRNL (1:200), CD-31 (1:200, Abcam), F4/80 (1:200) or IL-6 (1:200) overnight at 4 °C, followed by exposure appropriate fluorescence-labeled secondary antibodies (1:250, Molecular Probes, Life Technologies, Carlsbad, CA, USA), with cell nuclei counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Life Technologies). The samples were examined with a FLUOVIEW FV1000 Confocal laser scanning microscope (Olympus, Tokyo, Japan) by setting excitation wavelength at 488 nm for FITC-labeled secondary antibody (Thermo Scientific, Waltham, MA, USA) and setting excitation wavelength at 549 nm for Cy3-labeled secondary antibody (Thermo Scientific). DAPI was monitored with the excitation wavelength at 358 nm.

8. Detection of METRNL intracellular location with cell immunofluorescence

Primary HUVECs were seeded in 20 mm culture dish (NEST, Wuxi, China) at the density of

10^5 cells/dish for 24 h. Cells were fixed with 4% paraformaldehyde (Bio-light BIOTCH, Shanghai, China) for 10 min, permeabilized with 0.2% Triton X-100 for 10 min, and blocked with donkey serum for 2 h at room temperature. Then, the samples were incubated with METRNL antibody (1:300, R&D system, Minnesota, USA) mixed with calnexin antibody (1:500, Invitrogen, State of CA, USA) or 58K antibody (1:500, Abcam, Cambridge, UK) overnight at 4 ℃. Alex flow 488 or Alex flow 594 labeled secondary antibody (1:400, Jackson ImmunoResearch, PA, USA) was incubated for 90 min in the dark, and cell nuclei were counterstained with DAPI (Beyotime, Shanghai, China) for 15 min. Images were acquired with a FLUOVIEW FV1000 Confocal laser scanning microscope (Olympus, Tokyo, Japan).

9. Real time PCR

Total RNA from tissues or cells was extracted with Trizol Reagent (Invitrogen, Grand Island, NY, USA). Real time PCR was performed with a FastStart Universal SYBR green Master (Roche, Mannheim, Germany) and with an ABI7500 Real-time PCR system (Applied Biosystems, Grand Island, NY, USA) as described in our previous study[9]. The primers used are listed in Supporting Information Table S3. Data was normalized to GAPDH and analyzed with the $2^{-\Delta\Delta Ct}$ method[6]. Data are expressed as fold change.

10. Western blotting

Western blotting was performed as described in our previous study[6]. Briefly, the samples were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and then electrically transferred to Nitrocellulose Blotting membranes (ProTran, Schleicher and Schuell, Dasel, Germany). The membranes were blocked in 5% evaporated milk during 3 hours, probed with primary antibodies at 4 °C overnight. The primary antibodies were listed in Table S4. Then the membranes were incubated with Infrared-Dyes-conjugated secondary antibodies (Abcam) during 30 min. The images were captured with an Odyssey infrared imaging system (Li-Cor Bioscience, Lincoln, NE, USA).

11. ELISA measurements of METRNL

To detect circulating METRNL levels, venous blood was collected and kept at room temperature during two hours, followed by centrifugation at 3000×*g* during 15 min at 4 °C to obtain mouse serum samples. Each sample was aliquoted in time into sterile microtubes and stored at –20 °C until used. Every aliquot was used for the enzyme-linked immunosorbent assay (ELISA) only once.

To verify METRNL secretion from MAECs and HUVECs, serum-free medium was used to incubate the cells during several hours and then collected for METRNL detection. To investigate METRNL secretory pathway, HUVECs were incubated in serum-free medium with brefeldin A (5 μg/mL), eeyarestatin 1 (5 μg/mL) or a solvent control (DMSO) for 3 h before medium was collected for METRNL measurement. To determine if the inhibitory effect of brefeldin A is reversible, drug-treated cells were washed for three times and incubated in normal growth medium without brefeldin A for 16 h followed by another 3 h of serum-free medium incubation and METRNL detection of the culture supernatant. METRNL concentrations were determined with a Mouse or Human Meteorin-like DuoSet ELISA kit (DY6679/DY7867-05, R&D Systems, Minneapolis, MN, USA) as described in our previous studies[7,9,10].

12. Blood lipids assay

Mouse blood was collected by inferior vena cava puncture following serum separation. The serum samples packed with dry ice were then sent to Servicebio (Wuhan, Hubei, China) for serum lipid assays with an automatic biochemistry analyzer (Chemray 240, Rayto Life and Analytical Sciences Co., Ltd., Shenzhen, China).

13. Oil red O staining for lipid-laden macrophage

At the end of Day 7 of culture, M0 macrophages were incubated with oxidized low density lipoprotein (oxLDL, Yiyuan Biotechnologies, Guangzhou, China) at 50 μg/mL for 24 h. The cells were then washed with PBS twice and fixed in freshly prepared 4% PFA for 10 min in dark. After one wash, the cells were rinsed in PBS for 1 min, and then in 60% isopropanol for 15 s. The isopropanol was aspirated and the cells were stained with ORO at room temperature for 15 min. After that, ORO was aspirated and the cells were again rinsed in 60% isopropanol

for 15 s. Finally, the cells were washed with PBS for three times.

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