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Semaphorin-3A protects against neointimal hyperplasia after vascular injury

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ABSTRACT

Background: Neointimal hyperplasia is a prominent pathological event during in-stent restenosis. Phenotype switching of vascular smooth muscle cells (VSMCs) from a differentiated/contractile to a dedifferentiated/synthetic phenotype, accompanied by migration and proliferation of VSMCs play an important role in neointimal hyperplasia. However, the molecular mechanisms underlying phenotype switching of VSMCs have yet to be fully understood.

Methods: The mouse carotid artery ligation model was established to evaluate Sema3A expression and its role during neointimal hyperplasia in vivo. Bioinformatics analysis, chromatin immunoprecipitation (ChIP) assays and promoter-luciferase reporter assays were used to examine regulatory mechanism of Sema3A expression. SiRNA transfection and lentivirus infection were performed to regulate Sema3A expression. EdU assays, Wound-healing scratch experiments and Transwell migration assays were used to assess VSMC proliferation and migration.

Findings: In this study, we found that semaphorin-3A (Sema3A) was significantly downregulated in VSMCs during neointimal hyperplasia after vascular injury in mice and in human atherosclerotic plaques. Meanwhile, Sema3A was transcriptionally downregulated by PDGF-BB via p53 in VSMCs. Furthermore, we found that overexpression of Sema3A inhibited VSMC proliferation and migration, as well as increasing differentiated gene expression. Mechanistically, Sema3A increased the NRP1-plexin-A1 complex and decreased the NRP1-PDGFRβ complex, thus inhibiting phosphorylation of PDGFRβ. Moreover, we found that overexpression of Sema3A suppressed neointimal hyperplasia after vascular injury in vivo.

Interpretation: These results suggest that local delivery of Sema3A may act as a novel therapeutic option to prevent in-stent restenosis.

1. Introduction

Atherosclerosis and related cardio-cerebrovascular incidents are leading causes of death in every part of the world [1,2]. Atherosclerotic plaque formation potentially results in interruption of blood flow to the brain and heart, and the consequent clinical symptoms. Surgical procedures, including balloon angioplasty and stent implantation, are important treatments for patients with life-threatening symptoms. However, in-stent restenosis after these surgical procedures is a prominent pathological issue greatly limiting the benefits of these interventions [3]. Although drug-eluting stents (DES), such as sirolimus, paclitaxel, zotarolimus and arsenic trioxide coated stents, were developed to prevent in-stent restenosis [4,5], the in-stent restenosis still occurred in as high as 10% of patients and remained a significant clinical problem to be solved [6]. The major cause of in-stent restenosis is neointimal hyperplasia resulting from phenotype switching of vascular smooth muscle cells (VSMCs) [7,8]. VSMC phenotype switching indicates a switch of VSMCs from a differentiated/contractile state to a dedifferentiated/synthetic phenotype accompanied by migration and proliferation of VSMCs [9]. However, the molecular mechanisms underlying VSMC phenotype switching have yet to be fully understood. Considering that phenotype switching of VSMCs also contributes to the expansion of atherosclerotic lesions [10], research on this issue may provide benefits with regard to both in-stent restenosis and atherosclerosis.

Although semaphorins were originally recognized as axon growth cones in the developing nervous system [11], accumulating evidence

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Research in context

Evidence before this study

Neointimal hyperplasia is a prominent pathological event during in-stent restenosis. Phenotype switching of vascular smooth muscle cells (VSMCs) from a contractile to a synthetic phenotype, accompanied by migration and proliferation of VSMCs play an important role in neointimal hyperplasia. However, the molecular mechanisms underlying phenotype switching of VSMCs have yet to be fully understood. Using custom mRNA arrays, a previous study indicated that semaphorin-3A (Sema3A) was downregulated in the atheroprone flow region and Sema3A signalling could inhibit leucocyte migration to maintain the endothelial barrier under normal conditions. However, little is known about the role of Sema3A on VSMC functions during neointimal hyperplasia.

Added value of this study

We found that Sema3A was significantly downregulated in VSMCs during neointimal hyperplasia after vascular injury in mice and in human atherosclerotic plaques. Meanwhile, Sema3A was transcriptionally downregulated by PDGF-BB via p53 in VSMCs. Furthermore, we found that overexpression of Sema3A inhibited VSMC proliferation and migration, as well as increasing contractile gene expression. Mechanistically, Sema3A increased the NRP1-plexin-A1 complex and decreased the NRP1-PDGFRβ complex, thus inhibiting phosphorylation of PDGFRβ. Moreover, we found that overexpression of Sema3A suppressed neointimal hyperplasia after vascular injury in vivo.

Implications of all the available evidence

Our findings that Sema3A plays an important role in VSMC phenotypic modulation and neointima hyperplasia may offer a possible basis for a site-specific delivery of Sema3A with coated stents to prevent in-stent restenosis.

indicates that semaphorins play a critical role in vascular homeostasis under physiological and pathological conditions [12–15]. Sema3A, one of the class 3 semaphorins, binds to a receptor complex of neuropilin-1 (NRP1) and one of the class A plexins (plexin-A1, plexin-A2, plexin-A3 or plexin-A4) to trigger its downstream signalling pathway [15]. Using custom mRNA arrays, a previous study indicated that semaphorin-3A (Sema3A) was downregulated in the atheroprone flow region and Sema3A /NRP1 signalling could inhibit leucocyte migration to maintain the endothelial barrier under normal conditions [16]. Furthermore, another study revealed an unanticipated role of Sema3A /NRP1 signalling in inhibiting the migration of perivascular pericytes during lymphatic vascular development [17]. However, little is known about the role of Sema3A in VSMC functions during neointimal hyperplasia.

In this study, Sema3A was found to be significantly downregulated in VSMCs during neointimal hyperplasia after vascular injury in mice and in human atherosclerotic plaques. PDGF-BB decreased the binding of p53 to Sema3A promoter, thus transcriptionally downregulating Sema3A in VSMCs. Meanwhile, our results indicated that Sema3A inhibited proliferation of VSMCs and their migration, as well as increasing differentiated gene expression. Mechanistically, Sema3A increased the NRP1-plexin-A1 complex and decreased the NRP1-PDGFRβ complex, thus inhibiting phosphorylation of PDGFRβ. Moreover, we found that overexpression of Sema3A suppressed neointimal hyperplasia after vascular injury in vivo. These results suggested that replenishment of Sema3A may act as a novel therapeutic option for prevention of neointimal hyperplasia and associated diseases.

2. Materials and methods

2.1. Cell Culture

Mouse VSMCs were separated from the thoracic arteries of 8-week-old male C57BL/6 mice using collagenase, as previously described [14]. In brief, the thoracic arteries were isolated after mice were euthanized with an intraperitoneal injection of pentobarbital sodium (100 mg/kg). The adventitia of the arteries was gently removed and the intima was gently erased after being incubated in Type II collagenase for 30 min (min.). After maintaining in 10% foetal bovine serum (FBS) overnight, the vessels were minced into small pieces and digested with elastase, DNase and collagenase. The cells were collected and cultured in growth medium SmGM-2 (Lonza) containing 5% FBS. VSMCs were identified by staining with an anti-α-SMA antibody (ab5694; Abcam) (Supplemental Fig. 1). MOVAS cells were cultured in 10% FBS and incubated at 37 °C in a humidified 5% CO_2 incubator.

2.2. Lentiviral and siRNA transfection

Lentivirus containing full-length cDNA of Sema3A (LV-Sema3A) and negative control viruses expressing GFP (LV-GFP) were purchased from GeneChem (Shanghai, China). The lentiviruses were transfected into VSMCs with MOI 50, according to the manufacturer's protocol. The efficiency of lentivirus was verified by PCR and western blot analysis after 72 h.

The Sema3A siRNA, plexin-A1 siRNA and scrambled siRNA were constructed by RiboBio (Guangzhou, China). The siRNAs were transfected into VSMCs with Lipofectamine 2000 (Life Technologies), in accordance with the instructions. The efficiency of siRNA transfection was verified by PCR and western blot analysis after 24 h. The sequences were:

Scrambled siRNA: 5′-UUCUCCGAACGUGUCACGU-3′;
Sema3A siRNA-1: 5′-GGAGCAGCAACAAGTGGAA-3′;
Sema3A siRNA-2: 5′-CATGCAAGAGAGCAAGAAA-3′;
Plexin-A1 siRNA: 5′-GCTTCTACTGCTGGACTAT-3′.

2.3. Western blotting and immunoprecipitation

Tissue and cell samples were lysed in RIPA buffer (Beyotime Biotechnology, China). An equal amount (50 μg) of protein in each group was loaded and separated on 8–12% SDS-PAGE, then transferred onto 0.4 mm PVDF membranes (EMD Millipore, Billerica, MA, USA). After blocking in 5% skim milk for 1 h (h), membranes were incubated at 4 °C overnight with primary antibodies against Sema3A (ab199475, Abcam), Sema3A (ab23393, Abcam) neuropilin-1 (ab81321, Abcam), plexin-A1 (ab92346, Abcam), cyclinD1 (ab16663, Abcam), PCNA (ab29, Abcam, RRID: AB_303394), PDGFRβ (ab32570, Abcam, RRID: AB_777165), p-PDGFRβ (3173, Cell Signalling, RRID: AB_2252179), p53 (2524, Cell Signalling, RRID: AB_331743) and β-actin (A01010, Abbkine, RRID: AB_2737288). Then, membranes were incubated with secondary antibodies for 1 h at room temperature. All protein bands were visualized using ECL solution on a BioSpectrum Imaging System (UVP, Upland, CA, USA). The intensities of the bands were analysed using the ImageJ software package (National Institutes of Health, Bethesda, MD, USA). For immunoprecipitation, an equal amount (500 μg) of protein in each group was incubated with specific antibodies at 4 °C overnight with gentle rotation. The complexes were precipitated with Protein A/G agarose beads and analysed by western blotting.

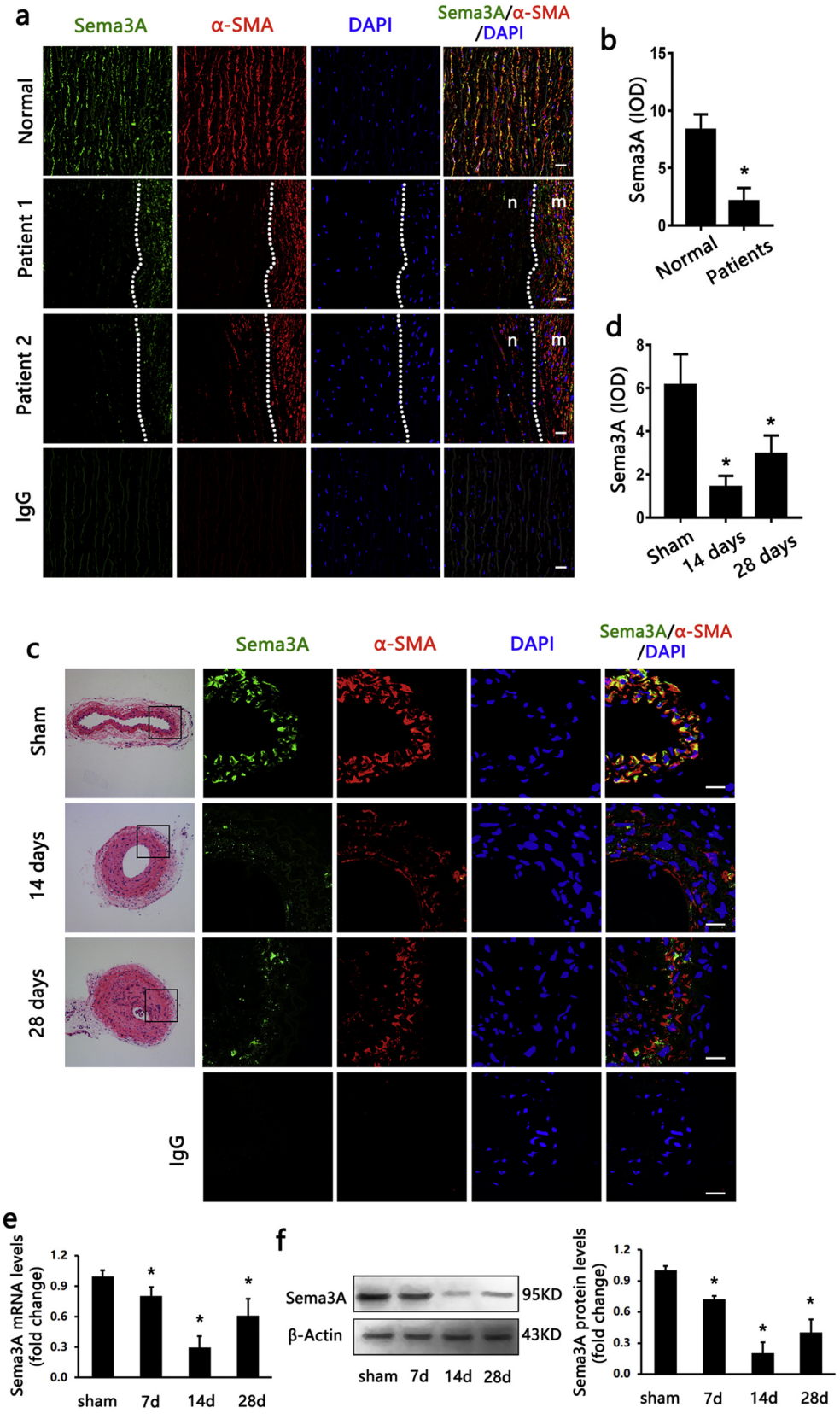


Fig. 1. Expression of Sema3A is decreased during neointimal hyperplasia. a, b Immunofluorescence staining showed that Sema3A (green) expression and localization in normal arteries and atherosclerotic plaques. The arterial smooth muscle cells are indicated by α-SMA (red). The dotted lines show the boundary of media and neointima. n: neointima; m: media. Bars = 20 μm (*P < .05 compared with Normal group, n = 6). c, d H&E-staining was used to identify the structure of arteries. Immunofluorescence staining showed that Sema3A was also evidently down-regulated in VSMCs after carotid artery ligation. Bars = 20 μm (*P < .05 compared with Sham group, n = 6). e, f qRT-PCR and Western blot analysis showed that the mRNA and protein levels of Sema3A were decreased at 7, 14 and 28 days after injury (*P < .05 compared with Sham group, n = 5–6).

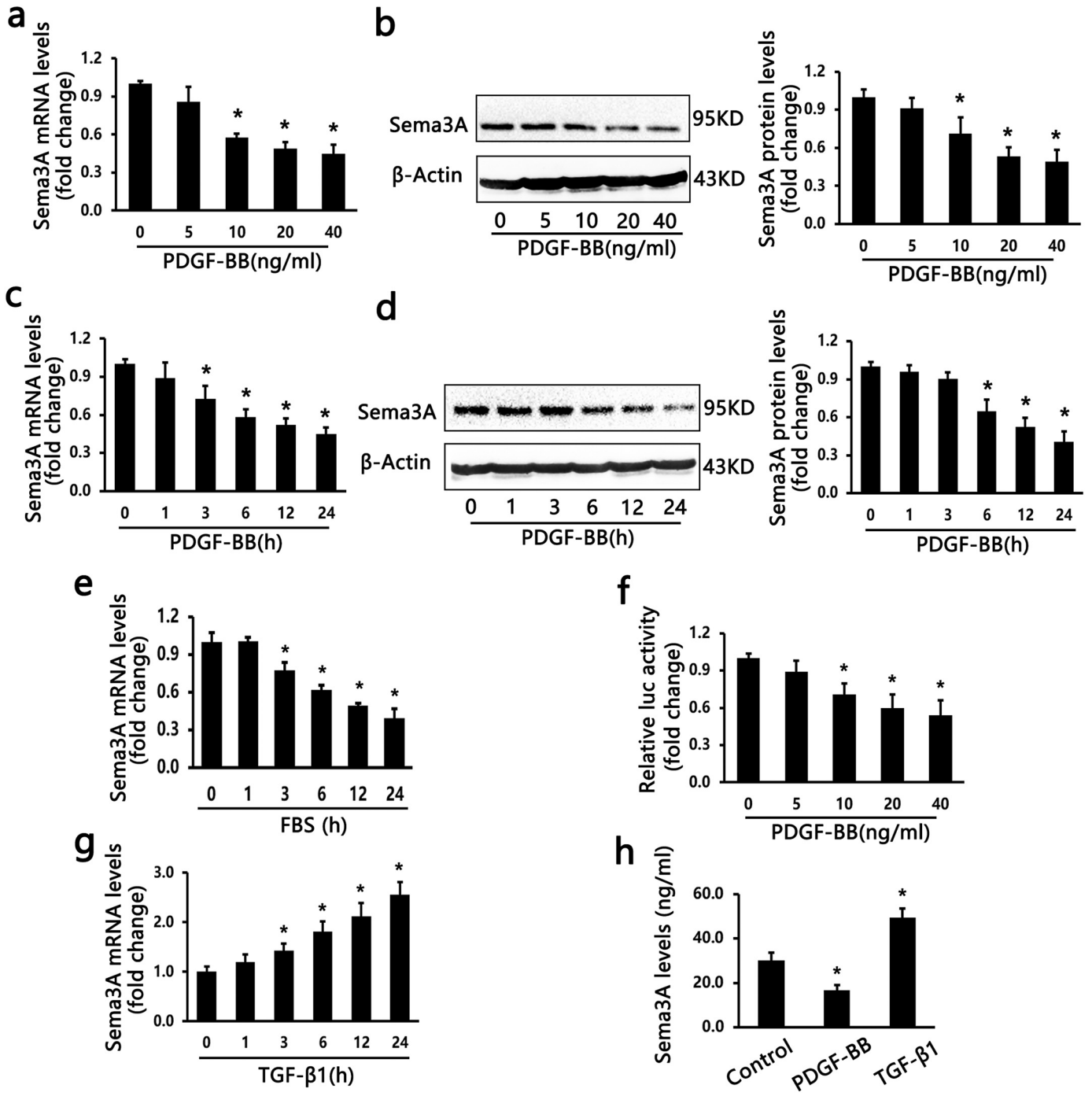
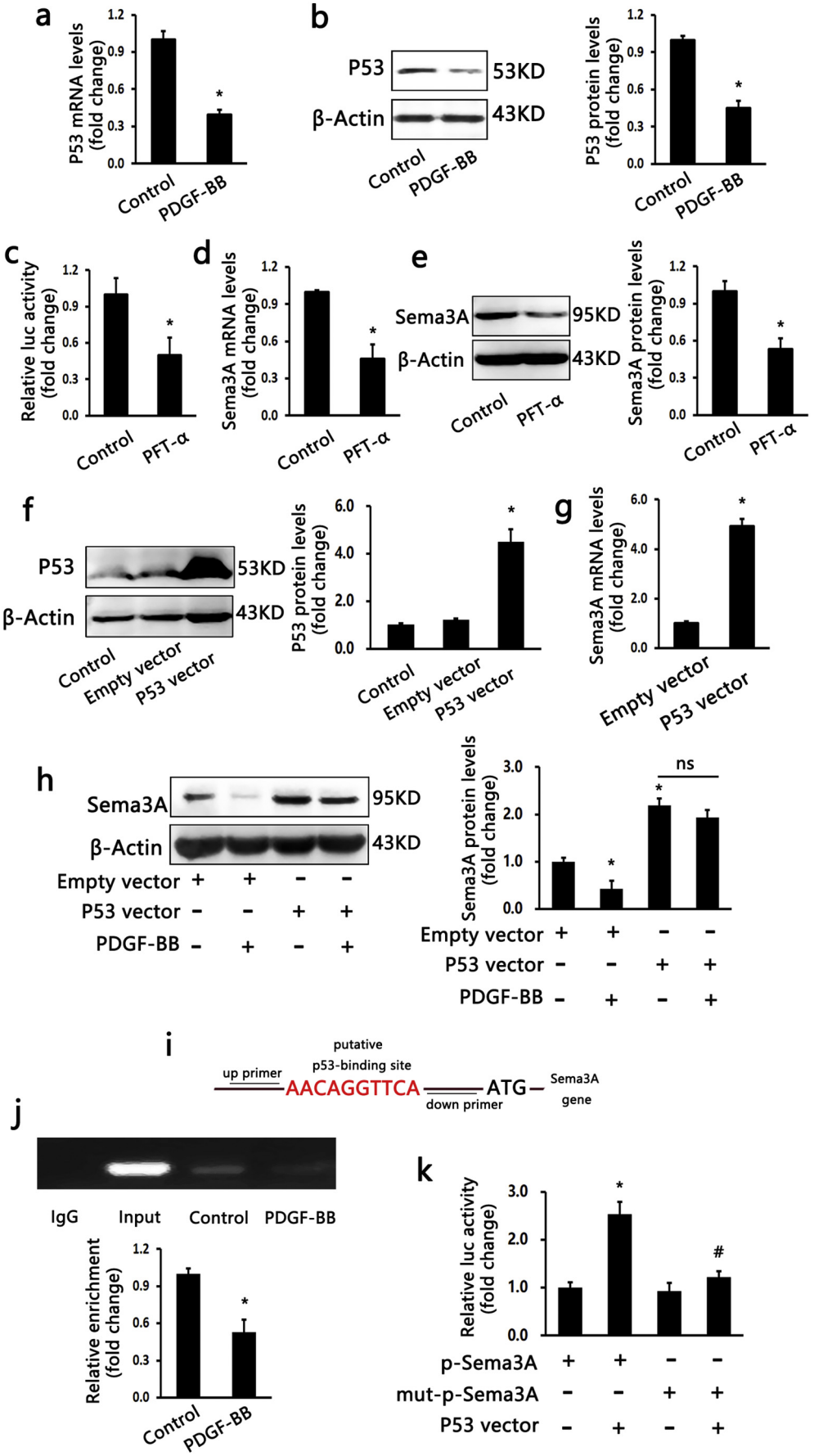


Fig. 2. PDGF-BB downregulates Sema3A in a time- and dose-dependent manner in VSMCs. a, b PDGF-BB down-regulated mRNA and protein levels of Sema3A in VSMCs in a dose-dependent manner after 24 h treatment, as determined by qRT-PCR and Western blot analysis (*P < .05 compared with no PDGF-BB group, n = 5–6). c, d PDGF-BB (20 ng/ml) caused a time-dependent decrease in Sema3A expression in VSMCs, as determined by qRT-PCR and Western blot analysis (*P < .05 compared with 0 h group, n = 5–6). e Foetal Bovine Serum (FBS, 5%) induced a time-dependent decrease of Sema3A mRNA levels in VSMCs, as demonstrated by qRT-PCR analysis (*P < .05 compared with no FBS group, n = 6). f 24 h after transfection with empty vector or Sema3A promoter plasmid, the cells were starved overnight and treated with PDGF-BB (20 ng/ml) for another 24 h before measuring the luciferase activity. PDGF-BB caused a dose-dependent decrease in Sema3A-promoter luciferase activity in MOVAS cells. (*P < .05 compared with no PDGF-BB group, n = 6). g qRT-PCR analysis indicated that TGF-β1 (20 ng/ml) induced mRNA levels of Sema3A in VSMCs in a time dependent manner (*P < .05 compared with 0 h group, n = 6). h ELISA assays of Sema3A protein levels in conditional medium of VSMCs after treated with PDGF-BB (20 ng/ml) or TGF-β1 (20 ng/ml) for 48 h (*P < .05 compared with control group, n = 6).

2.4. Quantitative real-time PCR

Total RNA was extracted from cells or tissues using an RNAiso Plus Kit (Takara, Kyoto, Japan). Then the RNA was reverse-transcribed with the cDNA Synthesis Kit (Takara, Kyoto, Japan), according to the manufacturer's protocol. SYBR Premix Ex Taq™ Kit (Takara, Kyoto, Japan) was used to amplify the cDNA using the Applied Biosystems StepOne Real-Time PCR System. Primers were as follows:

Sema3A forward primer: 5′-GGCTGGTTCACTGGGATTG-3′.
Sema3A reverse primer: 5′-CCGTTTGCATAGTTTGCTCTGG-3′.
p53 forward primer: 5′-CTCTCCCCCGCAAAAGAAAAA-3′.


a
P53 mRNA levels (fold change)
Control
PDGF-BB
b
P53
53KD
β-Actin
43KD
Control
PDGF-BB
P53 protein levels (fold change)
c
Relative luc activity (fold change)
Control
PFT-α
d
Sema3A mRNA levels (fold change)
e
Sema3A
95KD
β-Actin
43KD
Sema3A protein levels (fold change)
f
P53
53KD
β-Actin
43KD
Control
Empty vector
P53 vector
P53 protein levels (fold change)
g
Sema3A mRNA levels (fold change)
h
Sema3A
95KD
β-Actin
43KD
Empty vector
P53 vector
PDGF-BB
Sema3A protein levels (fold change)
ns
i
putative
p53-binding site
up primer
AACAGGTTCA
ATG
down primer
Sema3A
gene
j
IgG
Input
Control
PDGF-BB
Relative enrichment (fold change)
k
Relative luc activity (fold change)
p-Sema3A
mut-p-Sema3A
P53 vector

p53 reverse primer: 5′-CGGAACATCTCGAAGCGTTTA-3′.
β-actin forward primer: 5′-GGCTGTATTCCCCTCCATCG-3′.
β-actin reverse primer: 5′-CCAGTTGGTAACAATGCCATGT-3′.

Data were analysed using the $2^{-\Delta\Delta Ct}$ method against β-actin. Results were expressed as fold changes compared to the control.

2.5. Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed in accordance with a previous study [14], with minor modifications. VSMCs were treated with PDGF-BB for 24 h and fixed in formaldehyde for 15 min. at room temperature. Then the cells were lysed and sonicated into DNA fragments and the DNA fragment solution was diluted and incubated overnight with a p53 antibody (Cell Signalling) or an IgG antibody (negative control) at 4 °C. Immunocomplexes were precipitated with salmon sperm DNA/protein A agarose, and DNA fragments bound to p53 antibody were eluted from agarose beads with SDS elution buffer. After washing, the chromatin DNA was eluted, purified and amplified using real-time PCR with the following primers: forward 5′-ATTCTATCAGTGTACTGGATT-3′ and reverse 5′-GTTCTTGTAGTTGATAGCAG-3′.

2.6. DNA constructs and luciferase reporter assays

An overexpression vector containing full-length cDNA of p53 was prepared by PCR and cloned into the control vector. For reporter vectors, a sample of approximately 2000 bp of the Sema3A promoter (−2000 to −1) was prepared by PCR and cloned into the pGL3-basic vector (Promega). A mutant vector of Sema3A promoter was also cloned into luciferase reporter with deletion of the putative p53 binding site. MOVAS cells were co-transfected with luciferase reporter constructs with an empty vector or p53 overexpression vector. Following different levels of stimulation, cells were lysed, and the luciferase activity was assayed with a microplate reader, according to the manufacturer's instructions.

2.7. Immunofluorescence

Immunofluorescence was performed as previously described [18]. The paraffin-embedded sections were deparaffinized with xylene and treated with heat-mediated antigen unmasking solution. Then, the sections were permeabilized in 0.25% Triton X-100 for 15 min. After being blocked with 20% donkey serum for 30 min., the sections were incubated with the primary antibodies anti-Sema3A antibody (1:100, ab199475, Abcam), anti-CD31 antibody (1:50, AF3628, R&D Systems, RRID: AB_2161028), anti-α-SMA antibody (1:100, ab5694, Abcam, RRID: AB_2223021) and anti-α-SMA antibody (1:100, ab21027, Abcam, RRID: AB_1951138) at 4 °C overnight, then incubated with the corresponding fluorescein-conjugated secondary antibodies (1:100 dilution; Life Technologies). Nuclei were stained with DAPI for 10 min. All sections were observed under a confocal microscope (Nikon, Tokyo, Japan).

2.8. Immunohistochemistry

Paraffin-embedded vessel samples were cut into 4-μm thick sections and stained with haematoxylin and eosin (HE). The sections were deparaffinized, rehydrated and boiled in citrate buffer (pH 6.0) at 95 °C for 20 min. to retrieve antigens. The sections were treated with H_2O_2 to quench endogenous peroxidase activity and blocked with 10% goat serum before being incubated with primary antibody anti-Ki67 (Abcam, ab16667, RRID: AB_302459) overnight at 4 °C. The sections were sequentially incubated with biotinylated secondary antibodies at room temperature for 60 min. The slides were treated with EnVision™ Detection Systems Peroxidase/DAB (DAKO) following the manuals, then counterstained with haematoxylin. Slides were examined under an Olympus white-light microscope (Olympus, Japan).

2.9. Migration assays

Migration of VSMCs was assessed by wound-healing scratch and transwell assays. For wound-healing scratch assays, VSMCs were seeded into six-well plates to confluence and maintained with DMEM containing 5 μg/ml mitomycin-C for 24 h. Then, a sterile 200-μl pipette tip was used to produce a uniform linear scratch in the centre of the wells. Cells were exposed to different stimulations for an additional 24 h. The images of cell migration across the wound were captured at 0 h and 24 h using a microscope with a digital camera. Transwell assays were performed in transwell chambers (Corning, USA) with 24-well tissue culture plates composed of 8-μm pore polycarbonate filters. VSMCs were serum-starved in DMEM containing 0.5% FBS overnight. The cells were dissociated by trypsin and then suspended in DMEM. VSMCs (5.0×10^4 cells/well in 200 μl DMEM) were seeded into the upper chambers. The lower chambers were filled with 500 μl DMEM with or without recombinant PDGF-BB (20 ng/ml). After incubation at 37 °C for 6 h in a 5% CO_2 incubator, the cells on the filter surface were fixed with 4% paraformaldehyde (PFA) and stained with crystal violet for 10 min. Then, the cells in the upper chambers were erased using a cotton bud, and cells on the undersides of the filters were randomly counted under a light microscope (Olympus, Tokyo, Japan).

2.10. Cell proliferation assays

Proliferation of VSMCs was assessed by EdU (5-ethynyl-20-deoxyuridine) assay using Cell-Light EdU DNA Cell Proliferation Kit (RiboBio, Guangzhou, China) according to the manufacturer's protocol. VSMCs were seeded in six-well plates overnight. After different stimulation treatments, the media were replaced with DMEM containing 50 μM EdU and incubated for another 2 h. Cells were then trypsinized and fixed in 4% PFA for 30 min. Subsequently, cells were treated with 2 mg/ml glycine for neutralization and 0.5% Triton X-100 at room temperature for 10 min. After sequentially staining with Apollo dye solution, cells were analysed on an FACSCalibur flow cytometer.

2.11. Cell apoptosis assays

Cell apoptosis was assessed using a commercial assay kit (BD Biosciences, USA) as previously described [19]. Briefly, VSMCs after indicated

Fig. 3. PDGF-BB decreases Sema3A by inhibiting p53 expression in VSMCs. a, b qRT-PCR and Western blotting indicated that PDGF-BB reduced mRNA and protein levels of p53 at 24 h in VSMCs (*P < .05 compared with control group, n = 5–6). c 24 h after transfection with empty vector or Sema3A promoter plasmid, the cells were starved overnight and treated with a p53 inhibitor, pifithrin-α (PFT-α), for another 24 h before measuring the luciferase activity. PFT-αattenuated the Sema3A-promoter luciferase activity in MOVAS cells (*P < .05 compared with control group, n = 6). d, e PFT-α decreased the mRNA and protein levels of Sema3A at 24 h in VSMCs (*P < .05 compared with control group, n = 5–6). f MOVAS cells were transfected with a P53 vector or an empty vector. g qRT-PCR analysis showed that overexpression of P53 increased the mRNA levels of Sema3A in MOVAS cells 48 h after vector transfection (*P < .05 compared with empty vector, n = 6). h 24 h after transfection with empty vector or p53 vector, the cells were starved overnight and treated with PDGF-BB (20 ng/ml) for another 24 h before Western blotting. Overexpression of p53 abolished the inhibition of Sema3A induced by PDGF-BB (ns means no statistical significance, *P < .05 compared with empty vector, n = 5). i The candidate P53-binding site in the promoter region of Sema3A. j Chromatin immunoprecipitation assays indicated that PDGF-BB stimulation decreased the binding of p53 to the Sema3A promoter at 24 h (*P < .05 compared with control group, n = 5). k Luciferase reporters of Sema3A promoter with native (p-Sema3A) or mutated P53 binding site (mut-p-Sema3A) were cloned and co-transfected with P53 overexpressing vector in MOVAS cells for 48 h (*P < .05 compared with p-Sema3A group, # P < .05 compared with p-Sema3A + P53 vector group, n = 6).

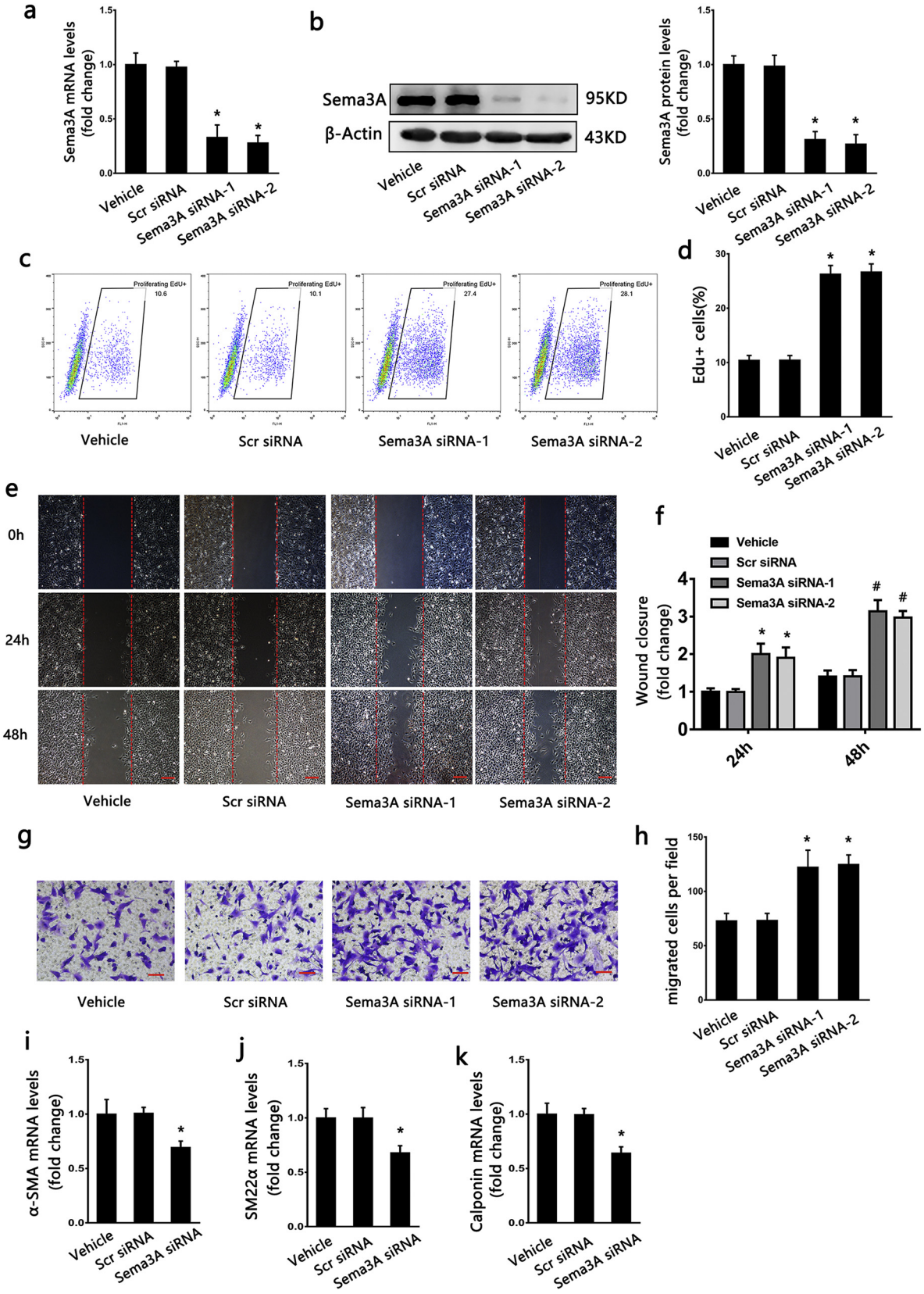


Fig. 4. Knockdown of Sema3A promotes VSMC proliferation and migration in vitro. a, b VSMCs were transfected with Sema3A specific siRNAs or scramble siRNA (Scr siRNA) (n = 3–6). c–k VSMCs were transfected with Sema3A specific siRNAs or scramble siRNA for 24 h, then the cells were starved overnight before experiments. c, d Edu assays showed that knockdown of Sema3A increased the percentage of proliferated VSMCs (n = 6). e–h Wound-healing scratch experiments and Transwell migration assays indicated that silencing Sema3A promoted VSMC migration. Bars indicate 200 μm for wound-healing images. Bars indicate 80 μm for transwell images (n = 6). i–k Silencing Sema3A down-regulated the mRNA levels of α-SMA, SM22α and calponin in VSMCs (n = 6). *P < .05 compared with Scr siRNA group.

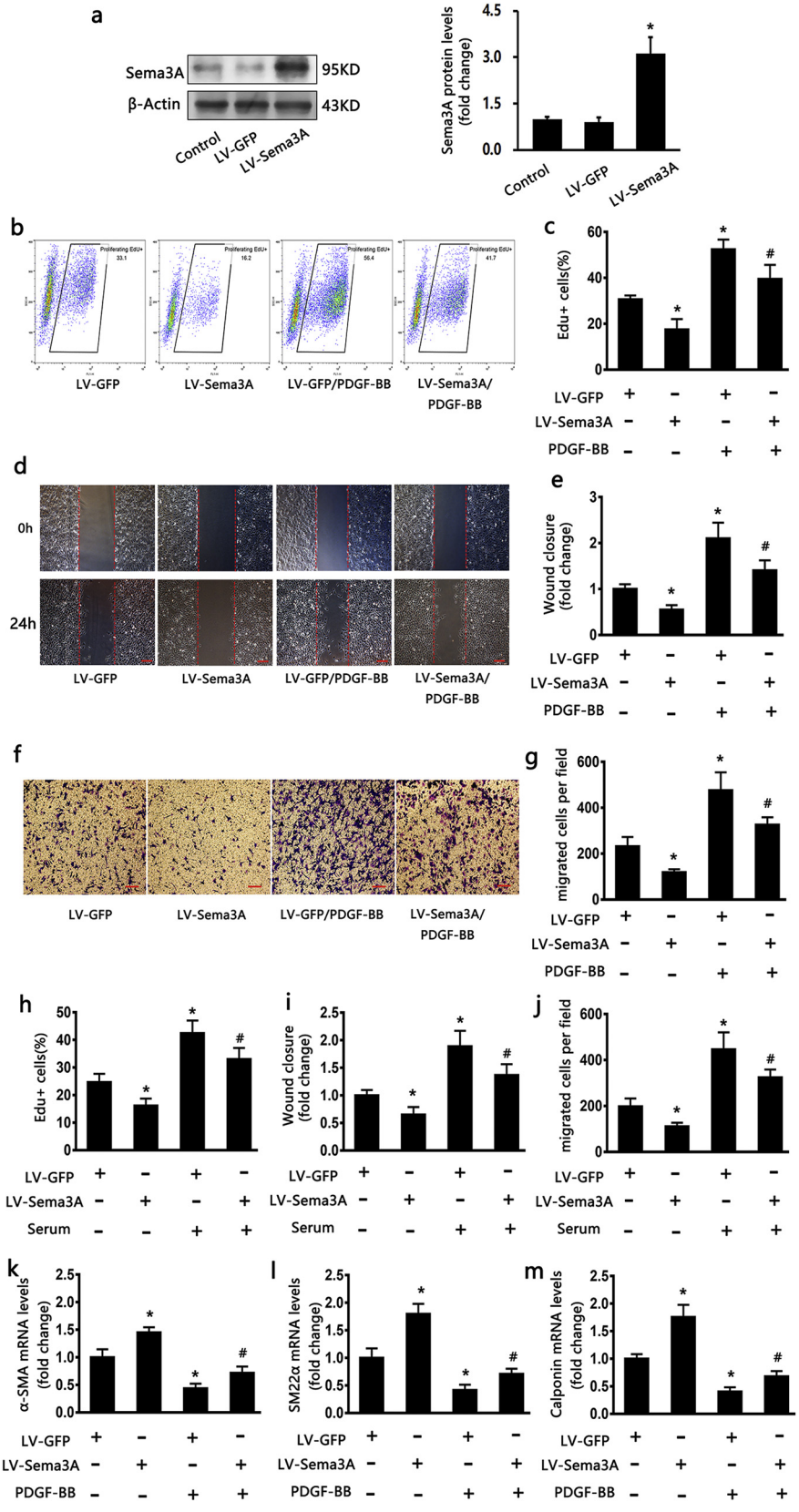
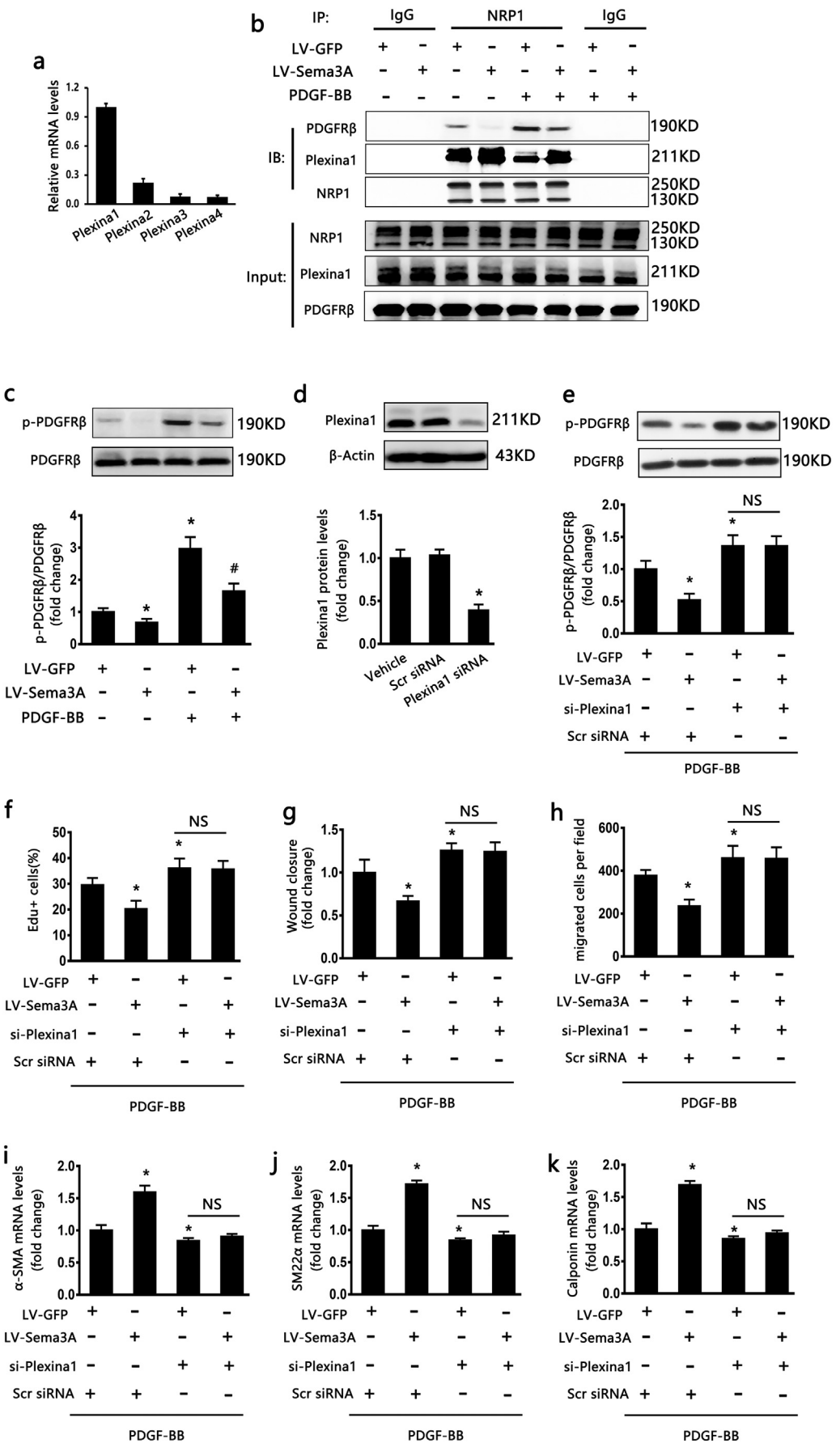


Fig. 5. Overexpression of Sema3A suppresses VSMC proliferation and migration in vitro. a Western blotting showed that Sema3A was overexpressed by LV-Sema3A compared to LV-GFP in VSMCs (n = 3). Lentivirus infected VSMCs were starved overnight for the following experiments. b, c Edu assays showed that overexpression of Sema3A decreased the percentage of proliferated VSMCs in the absence or presence of 20 ng/ml PDGF-BB for 24 h (n = 6). d–g Wound-healing scratch experiments (24 h) and Transwell migration (6 h) assays indicated that Sema3A overexpression inhibited VSMC migration in the absence or presence of PDGF-BB. Bars indicate 200 μm for wound-healing images. Bars indicate 40 μm for transwell images (n = 6). h–j Overexpression of Sema3A inhibited Serum-induced VSMC proliferation and migration (n = 6). k–m LV-Sema3A increased the mRNA levels of α-SMA, SM22α and calponin in VSMCs (n = 6). *P < .05 compared with LV-GFP group, # P < .05 compared with LV-GFP + PDGF-BB group.


a
Relative mRNA levels
1.2
0.9
0.6
0.3
0.0
Plexina1
Plexina2
Plexina3
Plexina4
b
IP:
IgG
NRP1
IgG
LV-GFP
LV-Sema3A
PDGF-BB
IB:
PDGFRβ
Plexina1
NRP1
190KD
211KD
250KD
130KD
Input:
NRP1
Plexina1
PDGFRβ
250KD
130KD
211KD
190KD
c
p-PDGFRβ
PDGFRβ
190KD
190KD
p-PDGFRβ/PDGFRβ (fold change)
LV-GFP
LV-Sema3A
PDGF-BB
d
Plexina1
β-Actin
211KD
43KD
Plexina1 protein levels (fold change)
Vehicle
Scr siRNA
Plexina1 siRNA
e
p-PDGFRβ
PDGFRβ
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PDGF-BB
f
NS
Edu+ cells(%)
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LV-Sema3A
si-Plexina1
Scr siRNA
PDGF-BB
g
NS
Wound closure (fold change)
LV-GFP
LV-Sema3A
si-Plexina1
Scr siRNA
PDGF-BB
h
NS
migrated cells per field
LV-GFP
LV-Sema3A
si-Plexina1
Scr siRNA
PDGF-BB
i
NS
α-SMA mRNA levels (fold change)
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LV-Sema3A
si-Plexina1
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Scr siRNA
PDGF-BB
k
NS
Calponin mRNA levels (fold change)
LV-GFP
LV-Sema3A
si-Plexina1
Scr siRNA
PDGF-BB

treatments were lysed by 0.25% trypsin without EDTA. The cells were centrifugated at 2000 rpm for 5 min and washed in ice-cold PBS. Then the cells were resuspended with 200 μl binding buffer and incubated with FITC-conjugated annexin V for 15 mins. After incubated with propidium iodide (PI), cells were analysed using an FACSCalibur flow cytometer.

2.12. Mouse carotid artery ligation injury model

The procedures were performed according to our previous study [14]. Briefly, male C57BL/6 mice (20–25 g) were anaesthetized with an intraperitoneal injection of pentobarbital sodium. The left common carotid artery was ligated with a 6–0 silk suture to disrupt the blood flow. The carotid arteries were collected at seven, 14 and 28 days after ligation. For perivascular delivery of lentivirus, the common carotid artery was dissected free of the surrounding connective tissue. LV-Sema3A or LV-GFP was suspended in 50 μl Pluronic F127 gel (BASF, 25% wt/vol) and the gel was applied around the carotid artery immediately after ligation. Carotid arteries were harvested at indicated timepoints after ligation. The carotid arteries were dissected, fixed with 4% paraformaldehyde and embedded in paraffin for sections. All sections in the figures were obtained at 200 μm proximal to the ligature site on the common carotid according to previous studies [20–22]. All animal experiments were approved by the institutional animal care and use committee at Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication, 8th edition, 2011).

2.13. Human tissue specimens

Human atherosclerotic artery specimens were collected from patients who received carotid endarterectomy. Normal artery specimens were obtained from patients with organ transplant donors. All procedures were carried out following the Declaration of Helsinki and with the approval of the ethics committee of Wuhan Union Hospital. In addition, written informed consent was acquired from each individual patient. Clinical samples were fixed in 4% PFA and embedded in paraffin for sections.

2.14. Statistical analysis

All data were expressed as mean ± SD. The Shapiro-Wilk test was used for testing the normality distribution of the data. For data with a normal distribution, two-tailed Student's *t*-tests were used between two groups and a one-way ANOVA was used among multiple groups followed by a Tukey's post hoc test for multiple comparisons. For data with non-normal distribution, the Mann-Whitney test was used between two groups and the Kruskal-Wallis test was used for multiple groups followed by a Dunn's test for multiple comparisons when necessary.

3. Results

3.1. Expression of Sema3A is decreased during neointimal hyperplasia

Neointimal hyperplasia resulting from phenotype switching of VSMCs is an important pathophysiological process during in-stent restenosis and atherosclerosis [23]. Because human in-stent restenosis samples are hard to obtain, the vast majority of studies have used human atherosclerotic samples with neointimal hyperplasia to partly represent the neointimal hyperplasia process [24–27]. We first explored the expression pattern of Sema3A in phenotype switching VSMCs during neointimal hyperplasia in human carotid arteries. We found that Sema3A was markedly co-localized with VSMCs in normal arteries and was evidently decreased in phenotype switching VSMCs of atherosclerotic neointima (Fig. 1a, b).

We then used a carotid artery ligation model to induce neointimal hyperplasia in mice. Consistently with human results, we found that Sema3A staining was also evidently downregulated in VSMCs after carotid artery ligation (Fig. 1c, d, Supplemental Fig. 2). The qRT-PCR results showed that the mRNA levels of Sema3A were decreased at seven, 14 and 28 days after injury. Western blotting revealed a similar tendency of decreased Sema3A protein after carotid artery ligation (Fig. 1e, f).

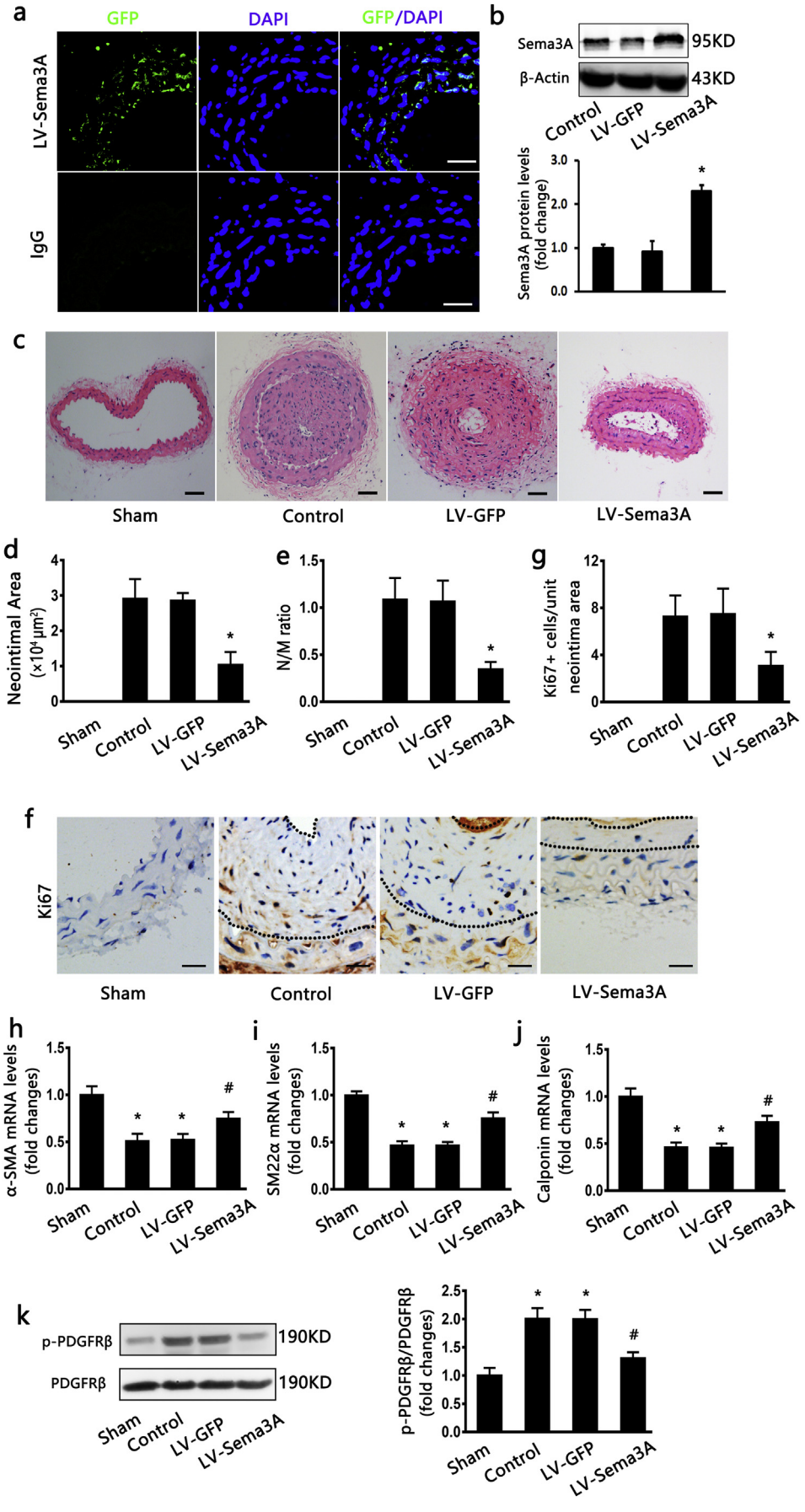
3.2. PDGF-BB downregulates Sema3A in a time- and dose-dependent manner in VSMCs

PDGF-BB is a potent stimulator of phenotypic switching of VSMCs and plays an important role during neointimal hyperplasia after vascular injury [28]. The qRT-PCR analysis revealed that PDGF-BB downregulated mRNA levels of Sema3A in VSMCs in a dose-dependent manner (Fig. 2a). Western blot analysis showed a similar inhibitory effect of PDGF-BB on Sema3A protein levels and the effect was significant at 20 ng/ml (Fig. 2b). Meanwhile, the mRNA and protein levels of Sema3A were also reduced by PDGF-BB over time (Fig. 2c, d). Furthermore, foetal bovine serum (FBS), another potent stimulator of VSMC phenotypic switching, also triggered an inhibitory effect on the mRNA levels of Sema3A (Fig. 2e). Moreover, we confirmed the transcriptional regulation of PDGF-BB on Sema3A by using the Sema3A promoter luciferase reporter (Fig. 2f). In contrast, we found that TGF-β1 induced mRNA levels of Sema3A in VSMCs over time (Fig. 2g). In addition, we found that protein levels of Sema3A in conditional medium of VSMCs were increased by TGF-β1 treatment and decreased by PDGF-BB treatment (Fig. 2h).

3.3. PDGF-BB decreases Sema3A by inhibiting p53 expression in VSMCs

We further investigated the molecular mechanisms underlying Sema3A downregulation induced by PDGF-BB in VSMCs. Previous studies showed that SEMA3B, SEMA3E and SEMA3F could be directly regulated by p53 transcription factor during tumour progression [14,29,30]; therefore, we considered whether p53 could mediate the inhibitory effect of PDGF-BB on Sema3A. The qRT-PCR and western blot analyses indicated that PDGF-BB decreased mRNA and protein levels of p53 in VSMCs (Fig. 3a, b). Inhibiting p53 activity via pifithrin-α (PFT-α), a specific p53 inhibitor, decreased the promoter activity and expression of Sema3A in VSMCs (Fig. 3c–e). Furthermore, overexpression of p53 evidently increased mRNA levels of Sema3A and abolished the inhibition of Sema3A induced by PDGF-BB (Fig. 3f–h). To determine whether Sema3A is a direct target gene of p53, we found using bioinformatic software that Sema3A's upstream promoter contains a candidate p53 binding site (http://jaspar.genereg.net) (Fig. 3i). Chromatin immunoprecipitation assays confirmed that p53 interacted with the promoter region of Sema3A and that PDGF-BB decreased the binding of p53 to the

Fig. 6. Mechanisms of effect of Sema3A on VSMC proliferation and migration. a The mRNA levels of class A plexins (PlexinA1, PlexinA2, PlexinA3, PlexinA4) in VSMCs (n = 6). b and c, Lentivirus infected VSMCs were starved overnight and then treated with PDGF-BB (20 ng/ml) for 30 mins. b VSMCs were immunoprecipitated with NRP1 or control IgG antibodies and then immunoblotted with the indicated antibodies (n = 4). c Western blotting revealed that Sema3A overexpression remarkably suppressed PDGF-BB-induced phosphorylation of PDGFRβ (*P < .05 compared with LV-GFP group, # P < .05 compared with LV-GFP + PDGF-BB group, n = 5). d Knockdown efficiency of PlexinA1 siRNA in VSMCs (n = 5). e–k Lentivirus infected VSMCs were transfected with indicated siRNA. 24 h after transfection, the cells were starved overnight and then treated with PDGF-BB (20 ng/ml) for different time (30 min for e, 24 h for f, g, i–k, 6 h for h). e Silencing PlexinA1 blocked the inhibitory effect of LV-Sema3A on PDGFRβ phosphorylation (n = 5). f–h The inhibitory effect of LV-Sema3A on VSMC proliferation and migration were both abolished by PlexinA1 knockdown (n = 6). i–k qRT-PCR analysis revealed that knockdown of PlexinA1 blocked the effect of LV-Sema3A on VSMC differentiated markers (n = 6). NS means no statistical significance, *P < .05 compared with LV-GFP group + Src siRNA group.


a
GFP
DAPI
GFP/DAPI
LV-Sema3A
IgG
b
Sema3A
95KD
β-Actin
43KD
Control
LV-GFP
LV-Sema3A
Sema3A protein levels (fold change)
c
Sham
Control
LV-GFP
LV-Sema3A
d
Neointimal Area (×10⁴ μm²)
e
N/M ratio
g
Ki67+ cells/unit neointima area
f
Ki67
h
α-SMA mRNA levels (fold changes)
i
SM22α mRNA levels (fold changes)
j
Calponin mRNA levels (fold changes)
k
p-PDGFRβ
190KD
PDGFRβ
190KD
p-PDGFRβ/PDGFRβ (fold changes)

Sema3A promoter (Fig. 3j). Moreover, we found that overexpression of p53 induced Sema3A promoter activity and that site-directed mutagenesis of the p53 binding site blunted this effect (Fig. 3k).

3.4. Knockdown of Sema3A promotes VSMC proliferation and migration in vitro

Because VSMC proliferation and migration from the media to the intima are essential events in the progression of neointimal hyperplasia [9,10], we further investigated the effect of Sema3A on VSMC proliferation and migration. We first used specific siRNAs to knock down Sema3A expression in VSMCs. The qRT-PCR and western blot analyses showed that mRNA and protein levels of Sema3A were significantly decreased by Sema3A-specific siRNAs (Fig. 4a, b). EdU assays showed that knockdown of Sema3A increased the percentage of proliferated VSMCs (Fig. 4c, d). Wound-healing scratch experiments indicated that silencing Sema3A promoted VSMC migration (Fig. 4e, f). We further performed transwell migration assays to validate our results. Consistently, transwell migration assays also indicated the same stimulated impact of Sema3A siRNA on VSMC migration (Fig. 4g, h). Furthermore, we found that silencing Sema3A downregulated three canonical differentiated markers (α-SMA, SM22α and calponin) of VSMCs and increased cell proliferative markers (cyclinD1, PCNA) (Fig. 4i–k, Supplemental Fig. 3a).

3.5. Overexpression of Sema3A suppresses VSMC proliferation and migration in vitro

We induced Sema3A expression in VSMCs via lentivirus-mediated Sema3A overexpression (LV- Sema3A). Western blotting showed that Sema3A was evidently overexpressed by LV- Sema3A compared to LV-GFP in VSMCs (Fig. 5a). EdU assays showed that overexpression of Sema3A decreased the percentage of proliferated VSMCs in the absence or presence of PDGF-BB (Fig. 5b, c). Wound-healing scratch experiments and transwell assays both revealed that Sema3A overexpression significantly inhibited VSMC migration in the absence or presence of PDGF-BB (Fig. 5d–g). Meanwhile, overexpression of Sema3A also inhibited serum-induced VSMC proliferation and migration (Fig. 5h–j). Moreover, LV- Sema3A increased the mRNA levels of α-SMA, SM22α and calponin in VSMCs and decreased the protein levels of cyclinD1, PCNA (Fig. 5k–m, Supplemental Fig. 4a). However, we found that Sema3A showed no significant effect on VSMC apoptosis (Supplemental Fig. 3b, 4b). In addition, we also found that recombinant Sema3A inhibited VSMC proliferation and migration in a dose-dependent manner (Supplemental Fig. 5).

3.6. Mechanisms underlying effect of Sema3A on VSMC proliferation and migration

We further explored the molecular mechanisms underlying the effect of Sema3A on VSMC proliferation and migration. Sema3A binds to a receptor complex of neuropilin-1 (NRP1) and one of the class A plexins (plexin-A1, plexin-A2, plexin-A3, plexin-A4) to trigger its downstream signalling pathway [15]. First, we examined the mRNA levels of class A plexins in VSMCs. The qRT-PCR assays showed that plexin-A1 was highly expressed in VSMCs, and therefore we chose plexin-A1 for further study (Fig. 6a).

Previous studies indicated that NRP1 could directly interact with PDGFR to promoted PDGF-induced phosphorylation of PDGFR in different cell types [31–33]. However, NRP1 was constitutively associated with plexin-A and maintained the plexin-A-NRP1 receptor complex [15,34]. Our immunoprecipitation assays revealed that overexpression of Sema3A increased the NRP1-plexin-A1 complex and decreased the NRP1-PDGFRβ complex (Fig. 6b). We next examined the phosphorylation levels of PDGFRβ in VSMCs. Western blotting revealed that overexpression of Sema3A suppressed phosphorylation of PDGFRβ to a remarkable degree (Fig. 6c). To verify whether plexin-A1 mediates the effects observed, we used a specific siRNA to silence its expression (Fig. 6d). We found that silencing plexin-A1 almost blocked the inhibitory effect of LV-Sema3A on PDGFRβ phosphorylation (Fig. 6e). Furthermore, the inhibiting effects of LV-Sema3A on VSMC migration and proliferation were abolished by plexin-A1 knockdown (Fig. 6f–h). Moreover, knockdown of plexin-A1 blocked the effect of LV-Sema3A on VSMC differentiated markers (Fig. 6i–k).

3.7. Sema3A inhibits neointimal hyperplasia after vascular injury in vivo

In order to further investigate the effect of Sema3A on neointimal hyperplasia after vascular injury in vivo, we conducted lentivirus-mediated Sema3A overexpression (LV-Sema3A) around the ligated arteries. Immunofluorescent staining showed that the lentiviruses, which could express GFP, were successfully transfected into the vascular cells (Fig. 7a). Western blotting confirmed the overexpression effect of LV-Sema3A in carotid arteries 28 days after transfection (Fig. 7b). H&E staining showed that LV-Sema3A significantly inhibited both the neointimal area and intima/media ratio, compared with LV-GFP, at 28 days after vascular injury (Fig. 7c–e). Meanwhile, Ki67 immunohistochemistry staining with injured arteries indicated that overexpression of Sema3A markedly decreased Ki67-positive cells in neointima (Fig. 7f, g). Furthermore, qRT-PCR assays indicated that replenishment of Sema3A partly reversed the decreased mRNA levels of α-SMA, SM22α and calponin after vascular injury (Fig. 7h–j). Moreover, LV-Sema3A also suppressed phosphorylation of PDGFRβ during vascular injury to a remarkable extent (Fig. 7k).

4. Discussion

The major findings of the present study were that: (1) the Sema3A levels of VSMCs were significantly decreased during neointimal hyperplasia after vascular injury in mice and in human atherosclerotic plaques, (2) Sema3A was transcriptionally downregulated by PDGF-BB via p53 in VSMCs, (3) Sema3A inhibited VSMC proliferation and migration, as well as increasing differentiated gene expression, (4) mechanistically, Sema3A increased the NRP1-plexin-A1 complex and decreased the NRP1-PDGFRβ complex, thus inhibiting phosphorylation of PDGFRβ and (5) replenishment of Sema3A suppressed neointimal hyperplasia after vascular injury in vivo. Our study is the first to demonstrate that Sema3A acts as a modulator for neointimal formation, and we further provided molecular mechanisms for how Sema3A inhibits VSMC proliferation and migration.

Although semaphorins were initially recognized as axon growth cones in developing nervous systems [11], accumulating evidence indicates that semaphorins are expressed in vessel walls and play a critical role in the balance of vascular homeostasis under physiological and pathological conditions [16,35]. Atheroprone flow patterns are important initial causes of neointimal formation, vascular remodelling and atherosclerosis [16,36]. Using custom mRNA arrays, Van Gils et al. [16] compared the neuronal guidance molecules gene in the atheroprone

Fig. 7. Sema3A inhibits neointimal hyperplasia after vascular injury in vivo. a Immunofluorescence showed that the lentiviruses, which could express GFP, were successfully transfected into the vascular cells (n = 6). b Western blotting confirmed the overexpressing effect of LV-Sema3A in carotid arteries 28 days after transfection (*P < .05 compared with LV-GFP group, n = 5). c–e H&E-staining showed that LV-Sema3A significantly inhibited both neointimal area and intima/media ratio as compared with LV-GFP at 28 days after vascular injury (*P < .05 compared with LV-GFP group, n = 10). f, g Ki67 immunohistochemistry staining with injured arteries indicated that overexpression of Sema3A remarkably decreased Ki67-positive cells in neointima. The black lines show the boundaries of neointima and media (*P < .05 compared with LV-GFP group, n = 10). h–j qRT-PCR assays indicated that replenishment of Sema3A partly reversed the decreased mRNA levels of α-SMA, SM22α, Calponin after vascular injury (*P < .05 compared with Sham group, # P < .05 compared with LV-GFP group, n = 6). k LV-Sema3A also remarkably suppressed phosphorylation of PDGFRβ during vascular injury (*P < .05 compared with Sham group, # P < .05 compared with LV-GFP group, n = 5).

flow region with the atheroprotective flow region of the aortic arch. In their study, four families of neuronal guidance molecules were covered, and 10 of 31 differentially expressed genes, including Sema3A, were significantly downregulated during turbulent flow-induced disruption of vascular homeostasis [16]. In agreement with their study, our study demonstrated that Sema3A was markedly decreased during neointimal hyperplasia. We further found that replenishment of Sema3A significantly suppressed neointimal formation, implying that Sema3A is a critical modulator of vascular homeostasis and a novel inhibitor of neointimal hyperplasia.

In vitro, our study revealed that PDGF-BB, which is of great potency for VSMC migration and proliferation and is substantially produced under vascular injury and atherosclerosis [28,37], significantly decreased Sema3A expression in VSMCs at both mRNA and protein levels. More importantly, our study demonstrated that PDGF-BB decreased the direct binding of p53 to the promoter region of Sema3A, thus inhibiting Sema3A transcription in VSMCs. Previously, several studies have confirmed that p53 plays an important role in VSMC proliferation and neointimal hyperplasia under vascular injury [38–40]. Our results suggest that Sema3A may account for the p53-induced suppressive effect on neointimal formation.

Previous studies demonstrated that Sema3A is involved in tumour progression [15], multiple sclerosis lesions [41], lymphatic perivascular cell coverage [42] and asthma [43], via regulation of cell proliferation and migration. It is known that VSMC migration and proliferation from the media to the intima are essential events in the progression of neointimal hyperplasia during in-stent restenosis [10]. In our study, we found that Sema3A significantly inhibited VSMC proliferation and migration. In vivo, we also confirmed that replenishment of Sema3A decreased the VSMC proliferation in neointima, with Ki67 immunostaining. Our findings indicate that local delivery of Sema3A may serve as a potential treatment to prevent in-stent restenosis.

It is known that that PDGF-BB-induced phosphorylation of PDGFRβ activates underlying signalling pathways of PDGF-BB and accelerates neointimal hyperplasia [44,45]. Previous studies indicated that NRP1 could directly interact with PDGFR to promote PDGF-induced phosphorylation of PDGFR in different cell types [31–33]. However, NRP1 is constitutively associated with plexin-A and maintains the plexin-A-NRP1 receptor complex [15,34]. Interestingly, in our study, we found that Sema3A increased the formation of the plexin-A1-NRP1 receptor complex, thereby suppressing the interaction of NRP1 with PDGFRβ and inhibiting phosphorylation of PDGFRβ and signal transduction of PDGF-BB. Similar competitive binding mechanisms mediated by Sema3A were reported in osteoclast differentiation and neurovascular patterning [34,46]. In osteoclast differentiation, Sema3A maintained the plexin-A1-NRP1 complex, thereby inhibiting RANKL-induced formation of the plexin-A1-TREM2-DAP12 complex [34]. In vascular development, Sema3A inhibited NRP1-mediated VEGF/VEGFR2 signalling by competing with VEGF for binding to the NRP1-plexin-A1 complex [46]. In addition, we examined the relative expression of plexin-A1, plexin-A2, plexin-A3 and plexin-A4 in VSMCs and found that the plexin-A1 mRNA level was highest. Furthermore, silencing plexin-A1 could block the inhibitory effect of Sema3A on VSMC proliferation and migration. However, whether other receptor components are involved in the effect of Sema3A on VSMCs should be explored in the future.

Our study has some limitations. First, although the most relevant translational effect of our study is the local delivery of Sema3A may serve as a potential treatment to prevent in-stent restenosis, the localized perivascular overexpression of Sema3A was inconsistent with the potential delivery approaches applied in patients. Recently, gene-eluting stents (GES), such as viruses, plasmid DNA, siRNA and miRNA coated stents [47–50], are being researched in full swing. Further investigation using GES with viral-mediated gene transfer of Sema3A should be tested in a stent model to increase the translational potential of our current findings. Second, previous studies [28,51,52] indicated that disordered blood flow after vascular injury could induce the expression of PDGFB in endothelial cells thus promoting phenotype switching of VSMCs and neointimal hyperplasia. Our group and others found that two other semaphorins (Sema4D, Sema3E) could regulate the expression of PDGFB in endothelial cells during tumour progression and ischemia stroke [12,53]. Future studies are warranted to explore whether Sema3A also regulates PDGFB in endothelial cells during neointimal hyperplasia.

Collectively, our study demonstrates that Sema3A suppresses VSMC migration and proliferation, thus inhibiting neointimal hyperplasia. Local delivery of Sema3A may act as a novel therapeutic option to prevent in-stent restenosis.

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Declaration of interests

The authors declare that there is no conflict of interest.

Author contributions

J.H.W., Y.F.Z. and C.D.H. performed major experiments. J.H.W., B.H. and Y.N.L. conceived the study, analysed the data, and wrote the manuscript. A.Q.C. and M.H. performed some immunostaining experiments. Y.L., L.M., Y.P.X., Q.W.H. and H.J.J. analysed data and edited the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.12.023.

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