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Adipolin/CTRP12 protects against pathological vascular remodelling through suppression of smooth muscle cell growth and macrophage inflammatory response

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Aims Secreted factors produced by adipose tissue are involved in the pathogenesis of cardiovascular disease. We previously identified adipolin, also known as C1q/TNF-related protein 12, as an insulin-sensitizing adipokine. However, the role of adipolin in vascular disease remains unknown. Here, we investigated whether adipolin modulates pathological vascular remodelling.

Methods and results Adipolin-knockout (APL-KO) and wild-type (WT) mice were subjected to wire-induced injury of the femoral artery. APL-KO mice showed increased neointimal thickening after vascular injury compared with WT mice, which was accompanied by an enhanced inflammatory response and vascular cell proliferation in injured arteries. Adipolin deficiency also led to a reduction in transforming growth factor-β (TGF-β) 1 protein levels in injured arteries. Treatment of cultured macrophages with adipolin protein led to a reduction in lipopolysaccharide-stimulated expression of inflammatory mediators, including tumour necrosis factor (TNF)-α, interleukin (IL) 6, and monocyte chemotactic protein (MCP)-1. These effects were reversed by inhibition of TGF-β receptor II (TGF-βRII)/Smad2 signalling. Adipolin also reduced platelet-derived growth factor (PDGF)-BB-stimulated proliferation of vascular smooth muscle cells (VSMCs) through a TGF-βRII/Smad2-dependent pathway. Furthermore, adipolin treatment significantly increased TGF-β1 concentration in media from cultured VSMCs and macrophages.

Conclusion These data indicate that adipolin protects against the development of pathological vascular remodelling by attenuating macrophage inflammatory responses and VSMC proliferation.

Keywords Adipolin • Vascular remodelling • Vascular smooth muscle cell • Macrophage

1. Introduction

Cardiovascular diseases are the major causes of death worldwide.[1] Pathological vascular remodelling is a crucial feature during the development of various vascular diseases, including atherosclerosis and in-stent restenosis.[2–4] Cellular responses, including macrophage inflammation and vascular smooth muscle cell (VSMC) growth are involved in the pathogenesis of pathological remodelling of the arterial wall.[3,5–8] Thus, the identification of factors that can prevent macrophage inflammatory and VSMC proliferative responses could uncover novel therapeutic targets for vascular disorders, such as restenosis after vascular intervention.

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Obesity is causally linked to the cluster of Type 2 diabetes, dyslipidaemia, and hypertension, finally leading to the development of cardiovascular disease.[9,10] However, the molecular link between obesity and cardiovascular diseases is not completely understood. Adipose tissue produces a variety of bioactive secretory molecules, also known as adipokines or adipocytokines, which directly act on nearby or remote organs.[11,12] Most adipokines, including tumour necrosis factor (TNF)-α and interleukin (IL) 6, are pro-inflammatory and typically exacerbate obesity-related diseases. In contrast, a small number of adipokines, including adiponectin, are anti-inflammatory and can protect against the complications of obesity.[11] C1q/TNF-related proteins (CTRPs) are adiponectin paralogs and some CTRPs serve as adipokines that exert beneficial actions on metabolic and cardiovascular diseases.[13] We have previously reported that CTRP1 and CTRP9 protect the heart from ischaemia–reperfusion injury and attenuate neointimal formation after mechanical vascular injury.[14–18]

We previously identified adipolin, also known as CTRP12, as an adipokine that is abundantly expressed in adipose tissue.[19] Adipolin levels are down-regulated in adipose tissue and plasma in rodent models of obesity.[19] Mechanistically, adipose tissue inflammation, under conditions of obesity, reduces adipolin expression through suppression of Krüppel-like factor-15.[20] Systemic delivery of adipolin improves insulin sensitivity in obese mice, partly through suppression of adipose tissue inflammation.[19] However, nothing is known about the role of adipolin in the regulation of cardiovascular disease. Here, we used loss-of-function genetic manipulations to investigate whether adipolin modulates pathological vascular remodelling.

2. Methods

2.1 Materials

Antibodies against phosphorylated Smad2 (Ser-465/467), NF-κB p65 (Ser-536), c-Jun N-terminal kinase (JNK, Thr-183/Tyr-185), AMP-activated protein kinase (AMPK, Thr-172) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against NF-κB, Cyclin D1, JNK, AMPK, and α-tubulin were also purchased from Cell Signaling Technology. Antibodies against β-actin, αSMA, BrdU, and transforming growth factor-β (TGFβ) receptor II (TGFβRII) were purchased from Abcam (Cambridge, UK). Neutralization antibodies against TGFβRII (Anti-TGFβRII Ab), recombinant soluble TGFβRII Fc Chimera (sTGFβRII), and recombinant human TGF-β1 were purchased from R&D Systems (Minneapolis, MN, USA). The polyclonal antibody against mouse adipolin was generated as described previously.[21] Lipopolysaccharide (LPS) was purchased from Sigma (St Louis, MO, USA). SB431542 was purchased from Cayman (Ann Arbor, MI, USA). Recombinant mouse TNFα and recombinant human platelet-derived growth factor (PDGF)-BB were purchased from PeproTech (Rocky Hill, NJ, USA).

2.2 Mouse model of vascular injury

To generate adipolin-knockout (APL-KO: *Fam132a/CTRP12* Accession No. CDB1102K: http://www2.clst.riken.jp/arg/mutant%20mice%20list.html) mice, the adipolin gene was disrupted by homologous recombination between exon 1–4 with a loxP-flanked neomycin resistance gene cassette (Supplementary material online, *Figure S1A*). Genotyping primers for the adipolin (*Fam132a/CTRP12*) wild-type (WT) allele were as follows: 5′-GCCTGAATCCCCCACTAACT-3′ (P1) and 5′-TCTGGTAGCCCTGAGAATCG-3′ (P2). Primers for the adipolin (*Fam132a/CTRP12*) null allele were as follows: 5′-GGAAGTGCCCAATGAGTCC-3′ (P3) and 5′-GTGGATGTGGAATGTGTGC-3′ (P4). Homozygous APL-KO and WT littermate mice with a C57BL/6J background were used in the present study. At 12 weeks, heart rate and blood pressure were determined using a tail-cuff pressure analysis system (Softron, Tokyo, Japan). Plasma glucose levels were determined by a Glutest glucose metre (Sanwa Kagaku Kenkyusho Co., Ltd., Nagoya, Japan). Insulin concentrations were measured by an enzyme linked immuno-sorbent assay (ELISA) system (Morinaga, Tokyo, Japan). TGF-β1 protein levels of femoral arteries were evaluated by ELISA (R&D Systems), according to the manufacturer's instructions.

At 8–12 weeks of age, mice were subjected to a wire injury operation, as previously described.[18] In brief, after anaesthetization (medetomidine, midazolam, and butorphanol at doses of 0.15, 2.0, and 2.5 mg/kg, i.p., respectively), the adequacy of anaesthesia was confirmed by the lack of a toe-pinch withdrawal response during the surgical procedure. A straight spring wire (0.38 mm in diameter; No. C-SF-15-15; Cook, Bloomington, IN, USA) was then inserted into the left femoral artery to denude and dilate the artery. Buprenorphine, at 0.25 mg/kg, was administered for analgesia before surgery and every 8 h for 48 h after surgery. At the indicated time points after sham or vascular injury operation, mice were killed after anaesthesia with medetomidine, midazolam, and butorphanol at doses of 0.15, 2.0, and 2.5 mg/kg, respectively. Arteries were then excised, fixed in 4% paraformaldehyde (PFA), and embedded in paraffin. Serial sections were stained with haematoxylin–eosin (HE). In some experiments, 1.0×10^9 plaque formation units (PFU) of adenoviral vectors expressing full length adipolin or control β-galactosidase (β-gal) were intravenously administered to WT mice 3 days before wire injury.[19] The intima/media (I/M) area ratio was evaluated using Image J v1.48 software (National Institute of Health, Bethesda, MA, USA).

Study protocols were approved by the Institutional Animal Care and Use Committees at Nagoya University and RIKEN Kobe Branch. Our study conformed to the Guide for the Care and Use of Laboratory Animals, published by the United States National Institutes of Health (8th Edition, 2011).

2.3 Immunohistochemical analysis

After mice were sacrificed, the injured arteries were excised and embedded in paraffin. Sections from injured arteries were stained with anti-mouse CD68 antibodies (Abcam) to evaluate macrophage infiltration, as previously described.[15] To identify proliferating cells in the neointima of wire-injured arteries, *in vivo* detection of DNA synthesis by bromodeoxyuridine (BrdU) labelling was performed. In brief, BrdU (40 μg/g mouse) was injected intraperitoneally 24 h before preparation of the artery. Sections from injured arteries were stained with anti-α-smooth muscle actin (α-SMA) antibodies (Abcam) and anti-BrdU antibodies (Abcam) to detect proliferating smooth muscle cells.

2.4 Assessment of endothelial repair

Evans blue dye was intravenously injected into mice 10 days after vascular injury. After fixation with 4% PFA, arteries were excised and the stained and unstained areas were quantified using WinROOF2015 software (MITANI Corporation, Fukui-shi, Japan).

2.5 Preparation of recombinant mouse adipolin protein

A pCDNA3.1 vector expressing full-length mouse adipolin cDNA tagged with His at the C terminus, was transfected into HEK293F cells

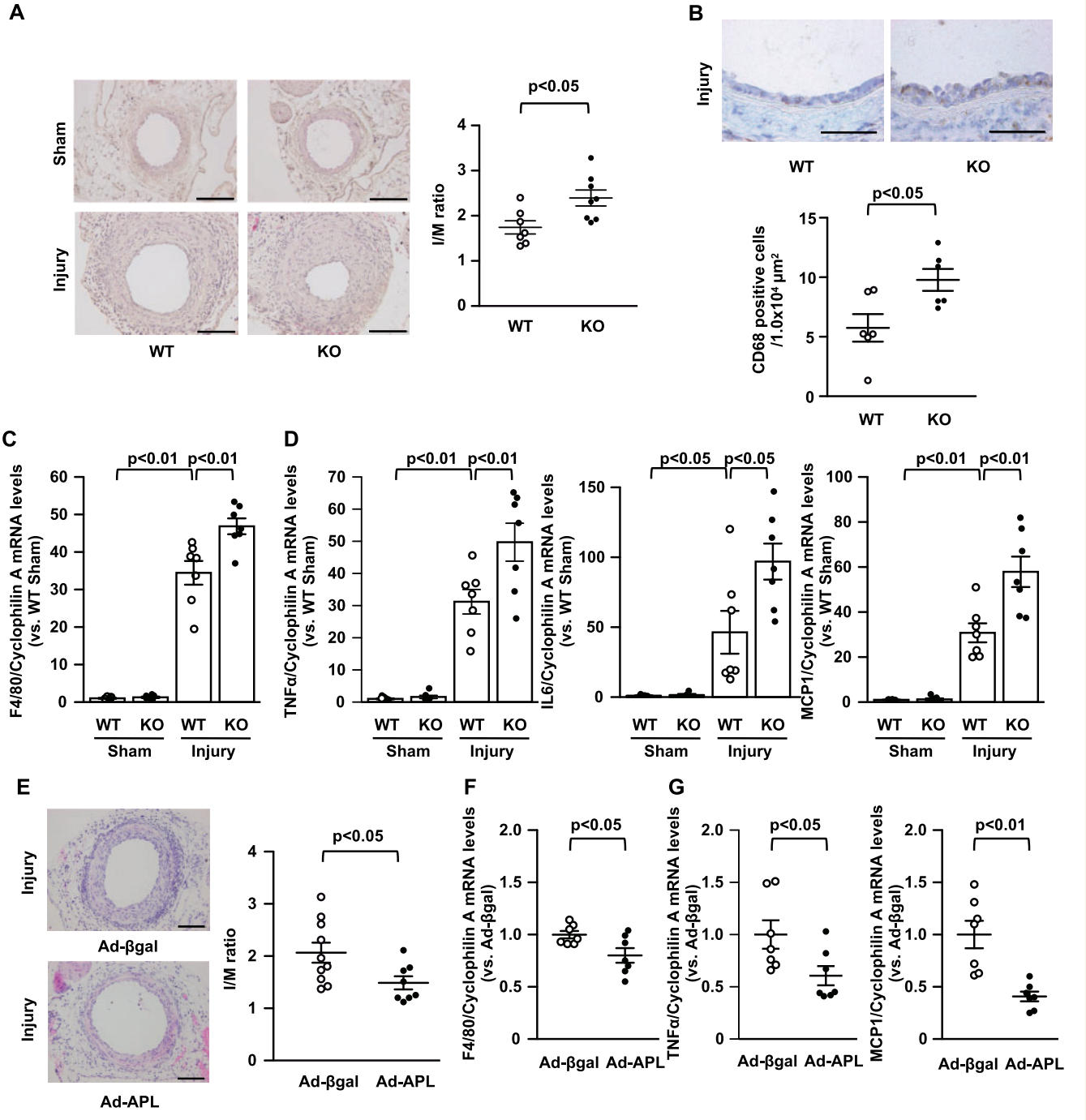

Figure 1 Adipolin deficiency promotes neointimal formation following arterial injury. (*A*) Analysis of neointimal thickening in injured arteries in wild-type (WT) and adipolin knockout (APL-KO) mice. Left panels show the representative photographs of haematoxylin–eosin-stained sections of femoral arteries from WT and APL-KO mice at 21 days after wire injury or sham operation. Scale bars, 100 μm. Right panel shows the quantitative analysis of I/M ratio (the ratio of intimal area/medial area). $N = 7$ in WT, $N = 8$ in APL-KO group. (*B*) Macrophage accumulation in injured artery in WT and APL-KO mice. Upper panels show the representative photographs of CD68-stained sections of femoral arteries from WT and APL-KO mice at Day 7 after vascular injury. Scale bars, 50 μm. Lower panel shows the quantitative analysis of CD68-positive cells. $N = 6$ in each group. (*C*) The mRNA expression of F4/80 in femoral arteries in WT and APL-KO mice at 10 days after wire injury or sham operation. $N = 7$ in each group. (*D*) The mRNA expression of TNFα, IL6, and MCP1 in femoral arteries in WT and APL-KO mice at 10 days after wire injury or sham operation. $N = 5$–7 in each group. (*E*) Analysis of neointimal thickening in injured arteries of WT mice treated with Ad-β-gal ($N = 10$) or Ad-APL ($N = 8$). Left panels show the representative photographs of injured femoral arteries at Day 21 after wire injury. Scale bars, 100 μm. Right panel shows the quantitative analyses of I/M ratio. (*F* and *G*) The mRNA expression of F4/80 (*F*), TNFα and MCP1 (*G*) in injured arteries of WT mice treated with Ad-β-gal ($N = 7$) or Ad-APL ($N = 7$) on the post-operative Day 10. Student's *t*-test [*A*, *B*, *E*, and *G* (TNFα)], Welch's *t*-test [*F* and *G* (MCP1)], and one-way ANOVA with Tukey's multiple comparisons test (*C* and *D*) were used to produce the *P*-values.

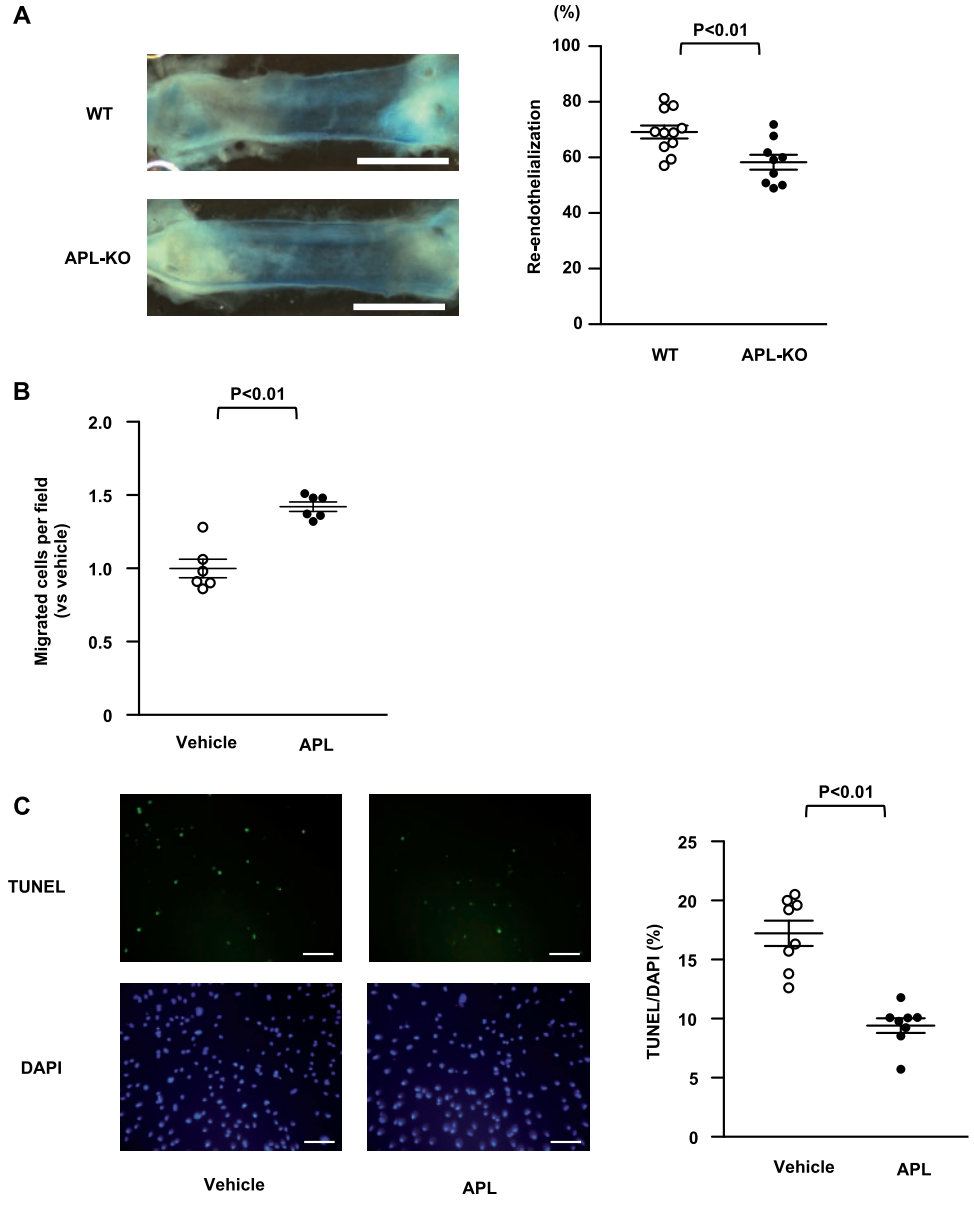

Figure 2 Adipolin deficiency contributes to impaired re-endothelialization after vascular injury. (*A*) Left panels show the representative photos stained by Evans blue dye at 10 days after vascular injury. White area shows the re-endothelialized area. Blue area shows impaired re-endothelialized area. Right panel shows the quantitative analysis of the ratio of white area per total area (white area + blue area) in WT ($N = 11$) and APL-KO mice ($N = 9$). Scale bars, 1 mm. (*B*) Effect of adipolin (300 ng/mL) on migratory activity of HUVECs. $N = 6$ in each group. (*C*) Effect of adipolin (300 ng/mL) on endothelial cell apoptosis induced by 48 h-serum deprivation. Left panels show the representative photos stained by TUNEL and DAPI. Right panel shows the quantitative analysis of the frequency of TUNEL-positive HUVECs. Scale bars, 100 μm. $N = 8$ in each group. Student's *t*-test (*A* and *B*) and Welch's *t*-test (*C*) were used to produce the *P*-values.

using the Targefect-293F reagent, according to the manufacturer's instructions (Targeting Systems, El Cajon, CA, USA). HEK293F cells were cultured in Freestyle293 expression medium (12338; Life Technologies, Carlsbad, CA, USA) under 8% carbon dioxide. Culture supernatants were collected and purified using His Ni-NTA resin. Adipolin protein was eluted by incubation with imidazole and dialyzed with phosphate-buffered saline (PBS). The anti-inflammatory and anti-proliferative effects of adipolin appear to be maximal at 50–300 ng/mL and 100–300 ng/mL, respectively (see *Figure 3A* and *4B*). We have chosen the dose of adipolin for most of the *in vitro* studies to induce maximal effects of adipolin, although the lower doses seem to be more physiologically relevant.

2.6 Cell culture and treatment

Macrophages were isolated from mouse peritoneal cavities 3 days after intraperitoneal thioglycolate injection and were cultured in RPMI1640 (Invitrogen, Carlsbad, CA, USA) with 10% foetal bovine serum (FBS).

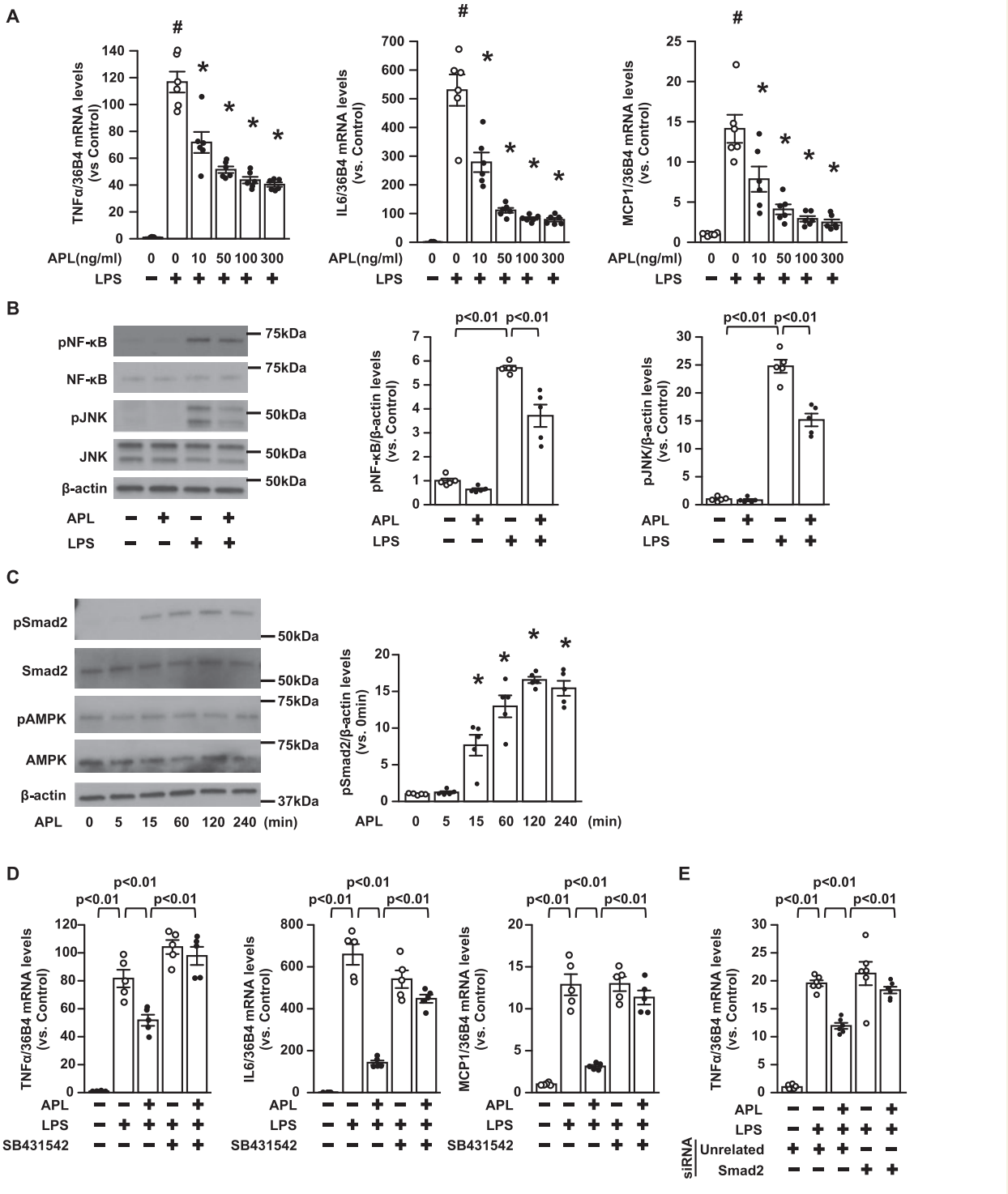

Figure 3 Adipolin attenuates inflammatory response of macrophages through Smad2 signalling. (*A*) Effect of adipolin on LPS-induced expression of TNFα, IL6, and MCP1 in peritoneal macrophages. Macrophages were treated with adipolin (10, 50, 100, and 300 ng/mL) or vehicle followed by stimulation with LPS (100 ng/mL) ($N = 6$ in each group) or vehicle for 6 h. $^{\#}P < 0.01$ vs. control. $^{*}P < 0.01$ vs. LPS treatment alone. (*B*) Effect of adipolin on phosphorylation of NF-κB and c-Jun N-terminal kinase (JNK) in RAW264.7 cells. Macrophages were treated with adipolin (300 ng/mL) or vehicle followed by stimulation with LPS (100 ng/mL) or vehicle for 30 min ($N = 5$ in each group). (*C*) Time dependent changes in phosphorylation of Smad2 and AMP-activated kinase (AMPK) in peritoneal macrophages after treatment with adipolin (300 ng/mL). Right panel shows the quantitative analysis of phosphorylation levels of Smad2 ($N = 5$ in each group). $^{*}P < 0.01$ vs. 0 min. (*D*) Effect of SB431542 on adipolin-mediated inhibition of macrophage inflammatory response. Peritoneal macrophages were pretreated with SB431542 (10 μM) or vehicle for 1 h and treated with adipolin (300 ng/mL) or vehicle followed by stimulation with LPS (100 ng/mL) or vehicle for 6 h. $N = 5$ in each group. (*E*) Contribution of Smad2 to anti-inflammatory effect of adipolin in RAW264.7 cells. Macrophages were transfected with siRNA targeting Smad2 or unrelated siRNA, and treated with adipolin (300 ng/mL) or vehicle followed by stimulation with LPS (100 ng/mL) or vehicle for 6 h ($N = 6$ in each group). One-way ANOVA with Tukey's multiple comparisons test was used to produce the *P*-values.

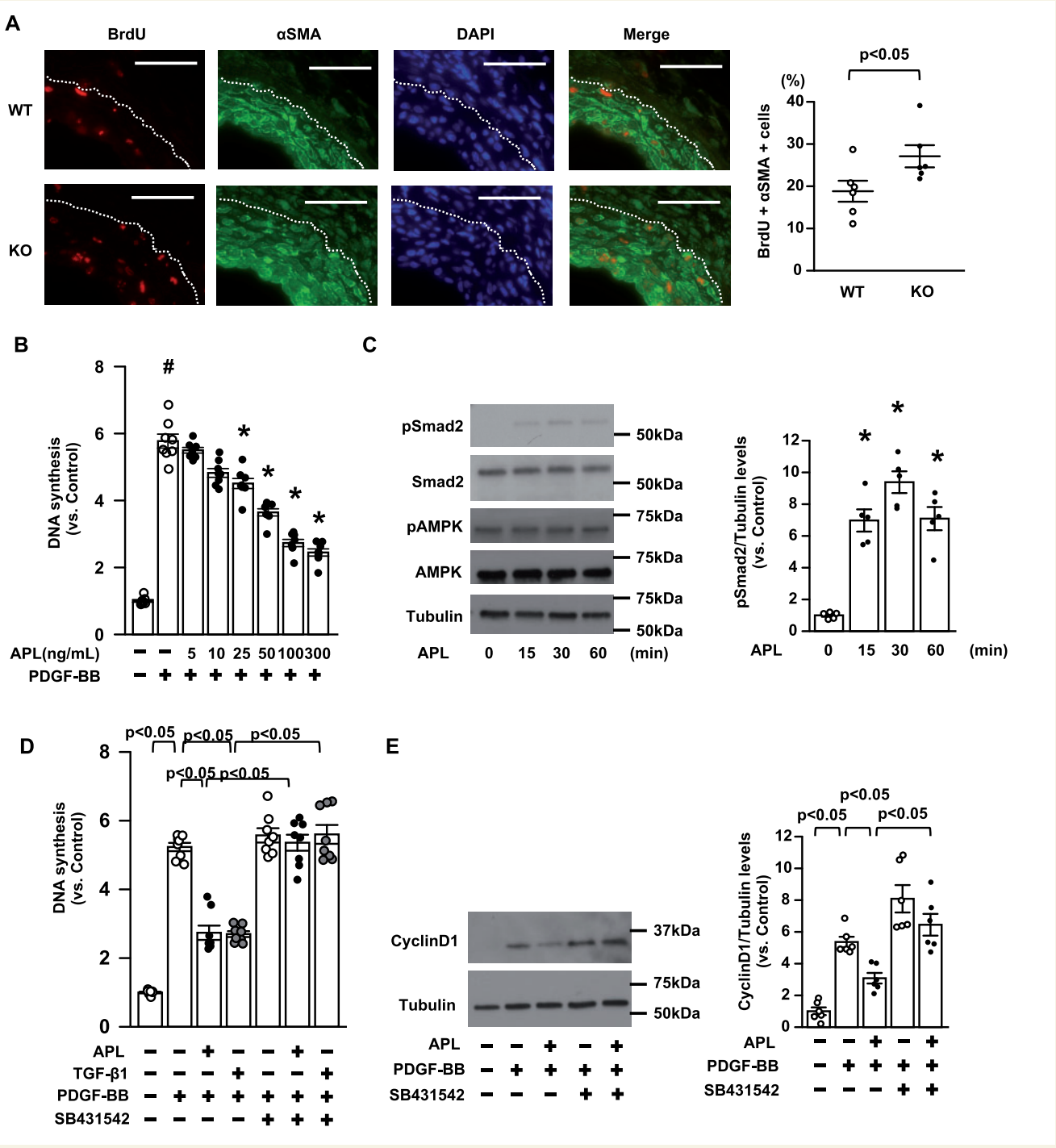

Figure 4 Adipolin reduces the proliferation of vascular smooth muscle cells via Smad2 signalling. (*A*) Assessment of smooth muscle cell proliferation in injured arteries of WT and APL-KO mice. Left panels show the representative photographs of BrdU/αSMA double-stained sections of femoral artery from WT and APL-KO mice at Day 14 after wire injury. Scale bars, 50 μm. Right panel shows the quantitative analysis of BrdU+/αSMA+ cells. The extent of BrdU+/αSMA+ cells in neointima was evaluated by dividing the number of BrdU/αSMA double-positive cells by the number of total DAPI ($N = 6$ in each group). (*B*) Effect of adipolin on platelet-derived growth factor (PDGF)-BB-induced DNA synthesis of vascular smooth muscle cells (VSMCs). VSMCs were treated with adipolin (5, 10, 25, 50, 100, and 300 ng/mL) or vehicle, followed by stimulation with PDGF-BB (50 ng/mL) or vehicle ($N = 8$ in each group) for 24 h. $^{\#}P < 0.05$ vs. control, $^{*}P < 0.05$ vs. PDGF-BB treatment alone. (*C*) Time dependent changes in phosphorylation of Smad2 and AMPK in VSMCs following adipolin treatment. Quantitative analysis of phosphorylated levels of Smad2 is shown in right panel ($N = 5$ in each group). $^{*}P < 0.01$ vs. 0 min. (*D* and *E*) Involvement of Smad2 signalling in anti-proliferative effects (*D*) and cyclin D1 suppression (*E*) of adipolin or TGF-β1 in VSMCs. VSMCs were pretreated with SB431542 (1 μM) or vehicle for 1 h, and treated with adipolin (300 ng/mL), TGF-β1 (10 ng/ml), or vehicle, followed by stimulation with PDGF-BB (50 ng/mL) for 24 h ($N = 8$ or $N = 6$ in each group, respectively). Student's *t*-test (*A*), Steel-Dwass test (*B*, *D*, and *E*), and one-way ANOVA with Tukey's multiple comparisons test (*C*) were used to produce the *P*-values.

Table 1 Primers used for quantitative RT-PCR

Mouse	
Adipolin	forward 5′-TACCACGTCCAACCGTGAG-3′
	reverse 5′-GTCATGTGGGCATCTGAGAG-3′
TNFα	forward 5′-CGGAGTCCGGGCAGGT-3′
	reverse 5′-GCTGGGTAGAGAATGGATGAACA-3′
IL6	forward 5′-GCTACCAAACTGGATATAATCAGGA-3′
	reverse 5′-CCAGGTAGCTATGGTACTCCAGAA-3′
MCP1	forward 5′-CCACTCACCTGCTGCTACTCAT-3′
	reverse 5′-TGGTGATCCTCTTGTAGCTCTCC-3′
F4/80	forward 5′-CTTTGGCTATGGGCTTCCAGTC-3′
	reverse 5′-GCAAGGAGGACAGAGTTTATCGTG-3′
Smad2	forward 5′-CCAGTCTTGCCTCCAGTCTTAG-3′
	reverse 5′-CTGTTGGTCACTTGTTTCTCCA-3′
TGF-β1	forward 5′-CACCGGAGAGCCCTGGATA-3′
	reverse 5′-TTCCAACCCAGGTCCTTCCT-3′
CD45	forward 5′-GGTTGTTCTGTGCCTTCTTC-3′
	reverse 5′-TAGATGCTGGCGATGATGTC-3
CD206	forward 5′-TCCCTGCTATGAACCTCCTC-3′
	reverse 5′-CCTGACCCCAACTTCTCGTA-3
Adiponectin	forward 5′-AGGTTGGATGGCAGGC-3′
	reverse 5′-GTCTCACCCTTAGGACCAAGAA-3
CTRP1	forward 5′-TGGAGGATTTCATTGCACAG-3′
	reverse 5′-CTTCCCACTCCTGTTGTTCC-3
CTRP9	forward 5′-TGGTGAACGTGGTGCCTACA-3′
	reverse 5′-TGCAGTCACATCCCACCCT-3
36B4	forward 5′-GCTCCAAGCAGATGCAGCA-3′
	reverse 5′-CCGGATGTGAGGCAGCAG-3′
Cyclophilin A	forward 5′-GTCTCCTTCGAGCTGTTTGC-3′
	reverse 5′-GATGCCAGGACCTGTATGCT-3′
Human	
TGF-β1	forward 5′-CCCTGGACACCAACTATTGC-3′
	reverse 5′-GTCCAGGCTCCAAATGTAGG-3′
36B4	forward 5′-TGCTCAACATCTCCCCCTTCTC-3′
	reverse 5′-ACCAAATCCCATATCCTCGTCC-3′

CTRP, C1q/TNF-related protein; IL6, interleukin 6; MCP1, monocyte chemoattractant protein-1; TGF-β1, transforming growth factor-β1; TNFα, tumour necrosis factor α.

Table 2 Characteristics of WT and APL-KO mice at the age of 12 weeks

	WT ($n = 8$)	APL-KO ($n = 8$)	*P*-value
BW (g)	24.2 ± 0.3	24.5 ± 0.4	0.488
Systolic BP (mmHg)	98.6 ± 1.3	101.8 ± 2.4	0.268
Diastolic BP (mmHg)	63.8 ± 2.5	63.9 ± 3.7	0.978
HR (b.p.m.)	600.1 ± 12.2	624.5 ± 14.0	0.211
White adipose Tissue (mg)	230.0 ± 1.7	235.7 ± 3.2	0.877
Gastrocnemius (mg)	316.1 ± 5.5	312.8 ± 6.9	0.711
Liver (mg)	1066.4 ± 36.4	1079.1 ± 39.0	0.815
Heart (mg)	109.5 ± 1.8	113.0 ± 2.5	0.282
Lung (mg)	134.7 ± 4.2	138.5 ± 1.9	0.431
FBS (mmol/L)	4.25 ± 0.34	4.39 ± 0.32	0.759

Data are presented as mean ± SE.
P-values were evaluated by Student's *t*-test.
APL-KO, adipolin knockout mice; BP, blood pressure; BW, body weight; FBS, fasting blood sugar; HR, heart rate; WT, wild-type mice.

Murine macrophage RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS. Macrophages were incubated in the presence of adipolin protein or vehicle [phosphate buffered saline (PBS)], followed by stimulation with LPS (100 ng/mL), TNFα (100 ng/mL), or vehicle (PBS) for the indicated times. In some experiments, macrophages were pretreated with SB431542 (10 μM) or vehicle [dimethyl sulfoxide (DMSO)] for 1 h, followed by treatment with adipolin protein or vehicle (PBS).

Human aortic smooth muscle cells (KS-4009; Kurabo, Osaka, Japan) were used as VSMCs in this study. VSMCs at passages 4 to 6 were used for all experiments. VSMCs were cultured in Humedia-SG2 medium (Kurabo). VSMCs were treated with adipolin protein (300 ng/mL), TGF-β1 (10 ng/mL), or vehicle (PBS) for the indicated times, followed by stimulation with PDGF-BB (50 ng/mL) or vehicle for 24 h.[15,22,23] In some experiments, VSMCs were pretreated with SB431542 (1 μM), anti-TGFβRII Ab (20 μg/mL), or sTGFβRII (1 μg/mL). Knockdown of Smad2 or TGFβRII was achieved by siRNA transduction at 100 nM (RAW264.7) or 1 nM (VSMCs), respectively, with lipofectamine RNAiMAX (Invitrogen) 48 h before experiments. Lipofectamine RNAiMAX and siRNAs were dissolved in Opti-MEM (Thermo Scientific, Waltham, MA, USA). ON-TARGETplus siRNA SMART pools targeting each gene were purchased from Thermo Scientific. Control cultures were transfected with unrelated scrambled siRNAs (ON-TARGETplus Control Non-Targeting Pool, Thermo Scientific). Serum deprivation (0.5% FBS) was performed for 16 h prior to starting all *in vitro* experiments.

TGF-β1 concentration in cultured media from macrophages and VSMCs was evaluated using by ELISA (R&D Systems), according to the manufacturer's instructions.

Human umbilical vein endothelial cells (HUVECS; C2519A; Lonza, Basel, Switzerland) were maintained in Endothelial Basal Media-2, supplemented with EGM-2 growth factor set (Lonza). HUVECs at passages 4 to 6 were used for all experiments. Apoptosis was induced by serum deprivation for 48 h. Apoptotic nuclei were stained using an *In Situ* Cell Death Detection Kit (Roche, Basel, Switzerland), according to the manufacturer's instructions. The number of apoptotic cells was counted in three random fields of each well and presented as the ratio of apoptotic cells to total cells. To assess endothelial cell migration, a transwell assay was performed as previously described.[24] In brief, upper chambers of the transwell (8.0μm pore size; Corning, Corning, NY, USA) were coated with fibronectin. Serum-deprived HUVECs were added to the upper chambers and allowed to migrate for 6 h. The number of migrated cells in three random fields was counted.

2.7 Proliferation assays

DNA synthesis was measured by a BrdU proliferation assay kit (Roche), according to the manufacturer's instructions.[15,18,22]

2.8 Quantification of mRNA levels

Gene expression levels were analysed by a quantitative real-time PCR method. In brief, total RNA was extracted from femoral arteries and cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was synthesized using a ReverTra Ace kit (TOYOBO, Osaka, Japan).

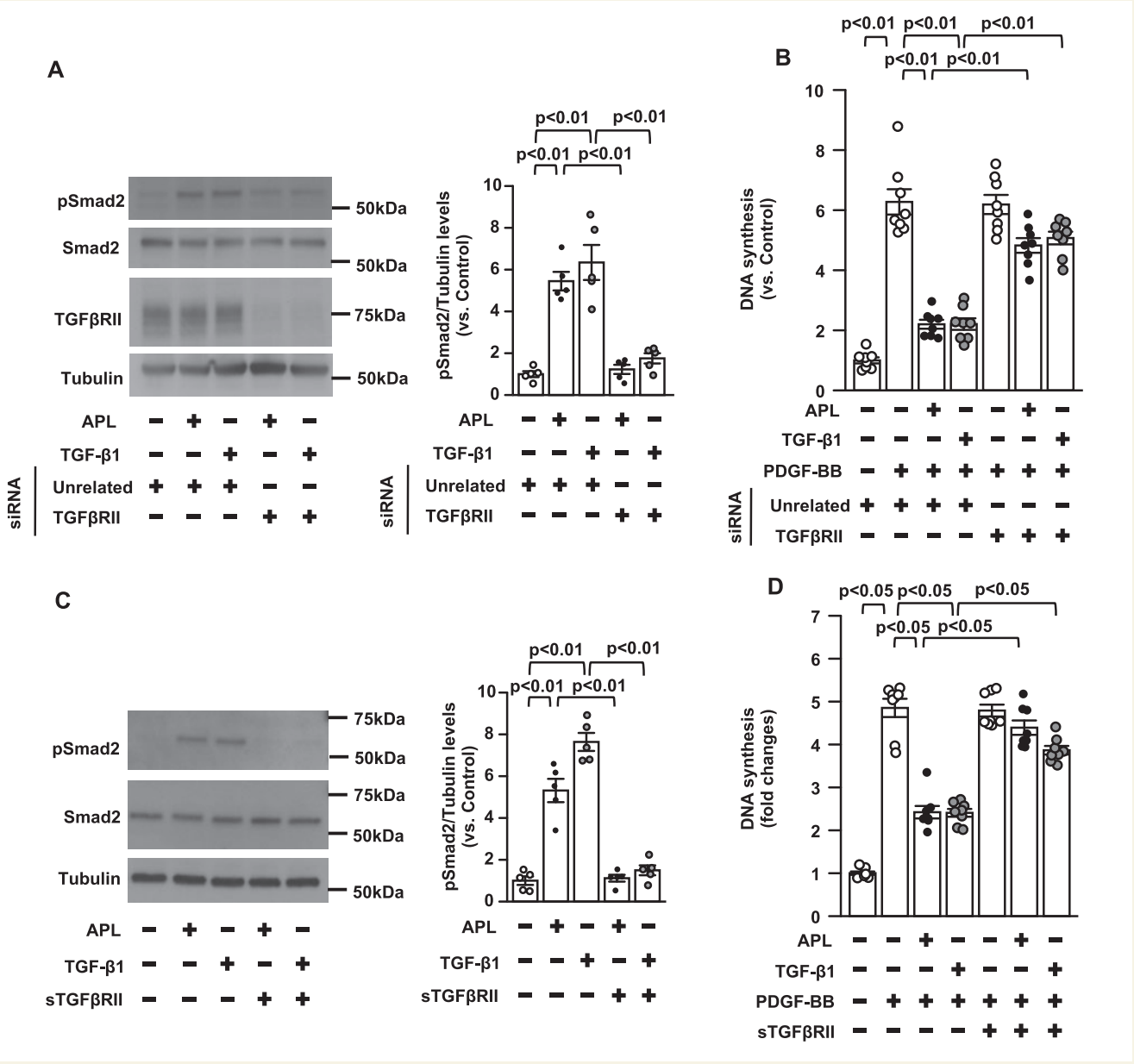

Figure 5 Adipolin inhibits VSMC growth through the TGFβ receptor II-dependent pathway. (*A*) Effect of TGF-β receptor II (TGFβRII) knockdown on Smad2 phosphorylation induced by adipolin or TGF-β1 in VSMCs ($N = 5$ in each group). VSMCs were transfected with siRNA targeting TGFβRII or unrelated siRNA, and treated with adipolin (300 ng/mL), TGF-β1 (10 ng/mL), or vehicle for 15 min. (*B*) Effect of TGFβRII ablation on anti-proliferative actions of adipolin or TGF-β1 in VSMCs ($N = 8$ in each group). VSMCs were transfected with siRNA targeting TGFβRII or unrelated siRNA, and treated with adipolin (300 ng/mL), TGF-β1 (10 ng/mL), or vehicle followed by stimulation with PDGF-BB (50 ng/mL) for 24 h. (*C*) Effect of soluble form of TGFβRII Fc chimera (sTGFβRII) on Smad2 phosphorylation in VSMCs ($N = 5$ in each group). VSMCs were cultured in the presence or absence of sTGFβRII (1 μg/mL) and treated with adipolin (300 ng/mL), TGF-β1 (10 ng/mL), or vehicle for 15 min. (*D*) Effect of sTGFβRII on anti-proliferative actions of adipolin or TGF-β1 in VSMCs ($N = 8$ in each group). VSMCs were pretreated with sTGFβRII (1 μg/mL) or vehicle for 15 min and treated with adipolin (300 ng/mL), TGF-β1 (10 ng/mL), or vehicle for 1 h followed by PDGF-BB stimulation for 24 h. One-way ANOVA with Tukey's multiple comparisons test (*A*, *B*, and *C*) and Steel-Dwass test (*D*) were used to produce the *P*-values.

Real-time PCR was performed on a Bio-Rad real-time PCR detection system (Bio-Rad, Hercules, CA, USA) using the THUNDERBIRD SYBR qPCR Mix (TOYOBO) as a double-stranded DNA-specific dye. The qPCR primers are listed in *Table 1*. Expression levels of examined transcripts were divided by the corresponding level of cyclophilin A or 36B4 expression and were expressed relative to the control group.

2.9 Western blotting analysis

Tissue or cell samples were homogenized in lysis buffer containing Tris-HCl (20 mM), NaCl (150 mM), Na2EDTA (1 mM), EGTA (1 mM), Triton (1%), sodium pyrophosphate (2.5 mM), sodium orthovanadate (1 mM), β-glycerophosphate (1 mM), PMSF (1 mM), leupeptin (1 μg/mL), and Complete Mini Proteinase Inhibitor Cocktail (Roche). Equal amounts of

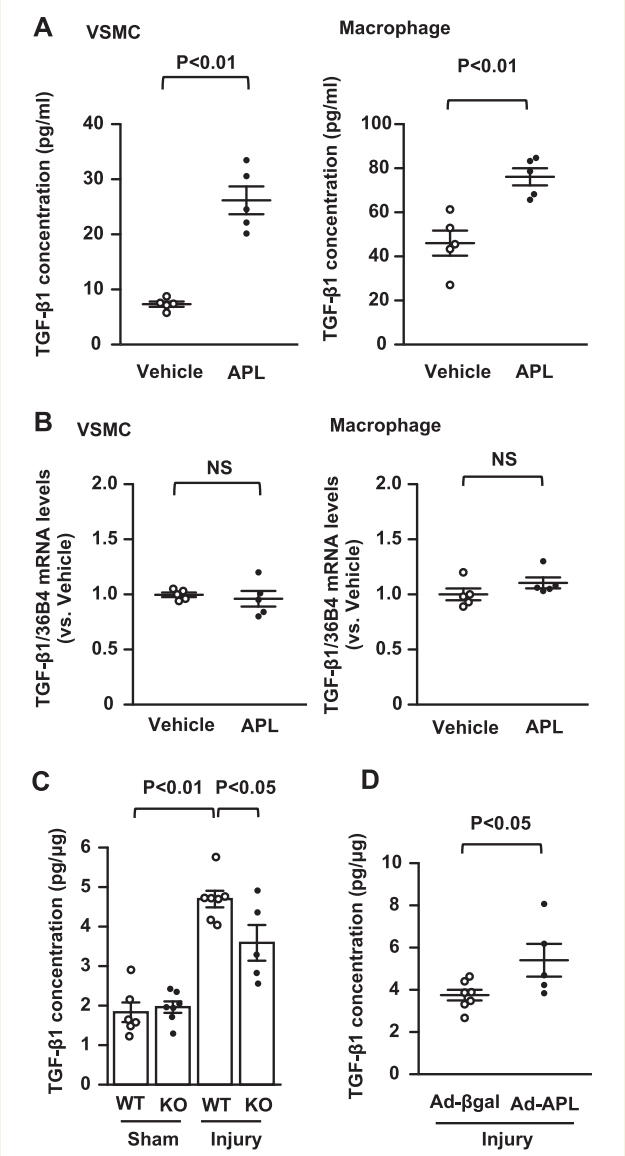

Figure 6 Effects of adipolin on TGF-β1 secretion and expression in VSMCs and macrophages. (*A*) Effect of adipolin on TGF-β1 levels in cultured media from VSMCs and peritoneal macrophages. (*B*) Effect of adipolin on TGF-β1 mRNA expression in VSMCs and peritoneal macrophages. VSMCs and macrophages were treated with adipolin (300 ng/mL) or vehicle for 15 min followed by incubation in serum free medium for 60 min. Concentration of TGF-β1 was measured by ELISA system. The mRNA levels of TGF-β1 were evaluated by quantitative real-time PCR methods. $N = 5$ in each group. (*C*) TGF-β1 protein levels of femoral arteries in WT and APL-KO mice after wire injury or sham operation. $N = 5$–7 in each group. (*D*) Analysis of TGF-β1 protein levels in injured arteries of WT mice treated with Ad-β-gal ($N = 7$) or Ad-APL ($N = 5$). TGF-β1 protein levels were normalized by total protein levels. Student's *t*-test [*A* (macrophage), *B* (VSMC), and *D* (Ad-βgal and Ad-APL)], Welch's *t*-test [*A* (VSMC)], Mann–Whitney *U* test (macrophage), and one-way ANOVA with Tukey's multiple comparisons test (*C*) were used to produce the *P*-values.

protein or serum were subjected to SDS-PAGE and transferred to PVDF membranes. Membranes were incubated with the indicated antibodies, followed by incubation with secondary antibodies conjugated with horseradish peroxidase. Protein signals were using an ECL Plus System (Thermo Scientific). Protein levels were evaluated by measuring band intensities using Image J software.

2.10 Statistical analysis

Data are presented as mean ± SE. The differences between two groups for variables with normal distributions were evaluated by unpaired Student's *t*-test (Welch's *t*-test was applied for variables with unequal variances). Differences between three or more groups were evaluated using one-way analysis of variance, with a *post hoc* Tukey's test. The differences between groups for variables with non-normal distribution were analysed by Mann–Whitney *U* test (for two groups) or Steel-Dwass test (for three or more groups). Data distributions were evaluated by Shapiro–Wilk test. A *P*-value <0.05 denoted the presence of a statistically significant difference. All statistical analyses were performed using JMP Pro 13 software (SAS, Marlow, UK).

3. Results

3.1 Adipolin deficiency promotes neointimal thickening after arterial injury

To elucidate the role of adipolin in vascular disease *in vivo*, we generated APL-KO mice. We confirmed the absence of adipolin mRNA in adipose tissue and adipolin protein levels in serum in homozygous APL-KO mice (Supplementary material online, *Figure S1B* and *C*). There were no significant differences in body weight; systolic or diastolic blood pressure; heart rate; tissue weight of white adipose tissue, gastrocnemius, liver, heart, and lung; or fasting blood sugar level between WT and APL-KO mice at the age of 12 weeks (*Table 2*). To investigate the effect of adipolin on vascular remodelling after vascular injury, APL-KO and WT mice were subjected to wire-induced injury of the femoral artery. *Figure 1A* shows the representative photos of HE-stained sections of femoral arteries from APL-KO and WT mice at Day 21 after sham operation or arterial injury. Quantitative analysis of neointimal thickening showed that the I/M area ratio was significantly increased in injured arteries of APL-KO mice compared with that of WT mice (*Figure 1A*). The intima and media were intact in arteries from APL-KO and WT mice after sham operation. There were no significant differences in plasma glucose or insulin levels after 12 h of fasting between WT and APL-KO mice, after sham operation or arterial injury (Supplementary material online, *Table S1*). In addition, there were no significant differences in the expression of other CTRP family members, including adiponectin, CTRP1, and CTRP9 in epididymal fat tissues between WT and APL-KO mice (Supplementary material online, *Figure S2*).

Because increased inflammatory responses promote injury-induced vascular remodelling,[25,26] we measured the number of macrophages and the expression of inflammatory mediators in injured femoral arteries. The number of cells expressing the macrophage marker, CD68, in injured arteries was significantly higher in APL-KO mice compared with WT mice (*Figure 1B*). Consistent with this result, the mRNA expression of another macrophage marker, F4/80, was also significantly higher in injured arteries of APL-KO mice than in those of WT mice (*Figure 1C*). In addition, mRNA levels of a leucocyte marker, CD45, were significantly higher in injured arteries of APL-KO mice than in those of WT mice (Supplementary material online, *Figure S3A*). Moreover, mRNA levels of TNFα, IL6, and monocyte chemotactic protein 1 (MCP1), which is also referred to as CCL2, were significantly higher in injured arteries of APL-KO mice than in those of WT mice (*Figure 1D*). In contrast, there were

no significant differences in the mRNA expression of F4/80, TNFα, IL6, or MCP1 in arteries of APL-KO and WT mice after sham operation. Furthermore, the mRNA levels of CD206, which is a marker of M2 macrophages, did not differ between the injured arteries of WT and APL-KO mice (Supplementary material online, *Figure S3B*).

The mRNA levels of adipolin were also evaluated in epididymal and perivascular fat tissues of WT mice 7 days after wire injury or sham operation. Adipolin mRNA levels did not differ between perivascular and epididymal fat tissues after sham operation (Supplementary material online, *Figure S4A*), suggesting that adipolin is secreted to the same extent from both local perivascular adipose tissue of femoral arteries and distant sites of adipose tissue. Of note, wire injury reduced adipolin mRNA expression in perivascular fat, but not in epididymal fat (Supplementary material online, *Figure S4A*), indicating that adipolin from local perivascular fat may play a role in regulating pathological vascular remodelling.

There were no significant differences in TNFα or MCP1 expression in perivascular fat tissue between WT and APL-KO mice after wire injury (Supplementary material online, *Figure S4B*).

To test whether increased levels of serum adipolin can affect neointimal formation after injury, we systemically administered adenoviral vectors expressing adipolin (Ad-APL) or β-gal (Ad-β-gal, control) to WT mice. Treatment of WT mice with Ad-APL led to a 1.8 ± 0.1-fold increase in serum adipolin levels compared with Ad-β-gal treatment at 6 days after injection, as assessed by western blotting analysis (Supplementary material online, *Figure S5*). This is in agreement with the results of our previous study.[19] Systemic delivery of Ad-APL, but not Ad-β-gal significantly attenuated the increase in I/M area ratio in injured arteries of WT mice (*Figure 1E*). Ad-APL treatment also significantly reduced the mRNA levels of F4/80 in injured arteries of WT mice (*Figure 1F*). Furthermore, Ad-APL administration significantly suppressed the mRNA levels of TNFα and MCP1 in injured arteries of WT mice (*Figure 1G*). No significant differences were observed in body weight or the weights of white adipose tissue, gastrocnemius muscle, liver, and heart between Ad-APL-treated and Ad-β-gal-treated mice (Supplementary material online, *Table S2*). In addition, there were no significant differences in plasma glucose or insulin levels between the two groups (Supplementary material online, *Table S3*). These data indicate that adipolin negatively regulates neointimal formation following arterial injury *in vivo*.

3.2 Adipolin deficiency reduces re-endothelialization after vascular injury

Accelerated re-endothelialization after vascular injury is reported to be associated with reduced neointimal thickening.[27] Therefore, we assessed the extent of endothelial repair in injured femoral arteries in APL-KO and WT mice by Evans blue staining. APL-KO mice exhibited impaired re-reendothelialization at Day 10 after vascular injury compared with WT mice (*Figure 2A*).

To investigate the effects of adipolin on endothelial cell function at a cellular level, HUVECs were treated with recombinant mouse adipolin protein, produced from 293F cells, or with vehicle. Treatment of HUVECs with adipolin promoted migratory activity (*Figure 2B*) and reduced the number of TUNEL-positive cells under conditions of serum deprivation (*Figure 2C*). These findings indicate that adipolin promotes endothelial cell migration and survival, thereby resulting in enhanced endothelial repair after vascular injury.

3.3 Adipolin reduces the inflammatory response of macrophages through the Smad2 signalling pathway

To investigate the effects of adipolin on the macrophage inflammatory response at a cellular level, mouse peritoneal macrophages were treated with adipolin protein or vehicle, followed by stimulation with LPS or TNFα. Treatment of macrophages with adipolin protein significantly suppressed LPS-stimulated expression of TNFα, IL6, and MCP1 in a dose-dependent manner (*Figure 3A*). Of note, treatment with adipolin protein at 10 ng/mL attenuated the LPS-induced inflammatory response in macrophages. Likewise, adipolin treatment reduced TNFα-stimulated expression of TNFα, IL6, and MCP1 in macrophages (Supplementary material online, *Figure S6*). Treatment with adipolin protein also attenuated the LPS-induced phosphorylation of NF-κB and JNK in murine macrophage RAW264.7 cells (*Figure 3B*). Thus, it is likely that adipolin exerts anti-inflammatory effects *in vitro*. A previous study reported that the concentration of circulating adipolin is 38.19 ± 32.02 ng/mL in patients with Type 2 diabetes.[28] In another study, serum adipolin concentration was reported to be significantly lower in patients with Type 2 diabetes (446.8 ± 59.6 pg/mL) than in healthy subjects (808.5 ± 83.9 pg/mL).[29] Thus, the data regarding circulating adipolin concentrations are inconsistent and future research will be needed to define its exact pathophysiological levels.

To dissect the mechanism by which adipolin attenuates inflammatory responses in macrophages, we assessed the phosphorylation levels of anti-inflammatory signalling molecules by western blotting analysis. Treatment of mouse peritoneal macrophages with adipolin protein time-dependently increased the phosphorylation of Smad2, whereas it had no effect on the phosphorylation levels of AMPK in macrophages (*Figure 3C*). Consistent with these *in vitro* data, APL-KO mice showed decreased phosphorylation of Smad2 and increased phosphorylation of NF-κB and JNK in injured vessels, when compared with WT mice (Supplementary material online, *Figure S7A* and *B*).

To test the possible contribution of Smad2 signalling to adipolin-induced suppression of inflammation *in vitro*, mouse peritoneal macrophages were pretreated with SB431542, a selective inhibitor of the TGFβ Type I receptor, activin receptor-like kinase (ALK)-5. Inhibition of Smad2 signalling with SB431542 reversed the suppressive effects of adipolin on LPS-induced expression of TNFα, IL6, and MCP1 in macrophages (*Figure 3D*). RAW264.7 macrophage cells were transduced with siRNAs against Smad2 or unrelated siRNAs. Transduction of RAW264.7 cells with siRNAs targeting Smad2 reduced the mRNA expression of Smad2 by 77 ± 1% (Supplementary material online, *Figure S8*). Knockdown of Smad2 reversed the inhibitory effect of adipolin on LPS-induced expression of TNFα in RAW264.7 cells (*Figure 3E*). Therefore, adipolin can attenuate the inflammatory response of macrophages through Smad2 signalling.

3.4 Adipolin reduces vascular smooth muscle cell proliferation through the Smad2 signalling pathway

To evaluate the effect of adipolin on the proliferative status of neointimal cells, double staining for BrdU and the smooth muscle cell marker, αSMA, was performed in sections of wire-injured femoral arteries from APL-KO and WT mice. The frequency of BrdU/αSMA double-positive cells in the neointima was significantly higher in APL-KO mice than in WT mice (*Figure 4A*).

To investigate the effect of adipolin on vascular cell proliferation at a cellular level, human VSMCs were treated with adipolin protein or vehicle, followed by stimulation with PDGF-BB or vehicle. Treatment of VSMCs with adipolin protein dose-dependently attenuated DNA synthesis stimulated by PDGF-BB (*Figure 4B*). In addition, adipolin treatment time-dependently enhanced Smad2 phosphorylation in VSMCs (*Figure 4C*). In contrast, adipolin treatment had no effects on the phosphorylation levels of AMPK in VSMCs. Furthermore, blockade of Smad signalling with SB431542 reversed the adipolin-mediated inhibition of PDGF-BB-stimulated DNA synthesis in VSMCs (*Figure 4D*). SB431542 pretreatment also cancelled the suppressive effect of TGF-β1 on VSMC growth, which is consistent with a previous report.[30] In addition, treatment of VSMCs with adipolin led to a reduction in PDGF-BB-stimulated expression of the cell cycle modulator, cyclin D1, and this was reversed by SB431542 pretreatment (*Figure 4E*). Thus, these data indicate that adipolin can reduce VSMC growth through modulation of Smad2 signalling pathways.

3.5 TGFβ receptor II is involved in the inhibitory effects of adipolin on VSMC growth and macrophage inflammatory response

To clarify the upstream molecule involved in adipolin-activated Smad2 signalling, we evaluated the possible involvement of TGFβ receptor II (TGFβRII), which transduces TGF-β signalling by forming a heteromeric complex with ALK5. Transduction of VSMCs with siRNAs against TGFβRII reduced the protein expression of TGFβRII by 87 ± 3% (Supplementary material online, *Figure S9*). Knockdown of TGFβRII attenuated adipolin- or TGF-β1-induced phosphorylation of Smad2 in VSMCs (*Figure 5A*). Ablation of TGFβRII also reversed the inhibitory effects of adipolin and TGF-β1 on PDGF-BB-stimulated DNA synthesis in VSMCs (*Figure 5B*). Moreover, treatment with a neutralizing antibody against TGFβ receptor II (anti-TGFβRII Ab) blocked adipolin-stimulated phosphorylation of Smad2 in VSMCs, compared with control IgG treatment (Supplementary material online, *Figure S10*).

To further test the involvement of TGFβRII in adipolin-induced activation of Smad2, VSMCs were pretreated with soluble forms of TGFβRII, encoding amino acids 24–184 of the extracellular domain, which can inhibit the binding of TGF-β to TGFβRII and the receptor's function.[31] Pretreatment with soluble forms of TGFβRII diminished the stimulatory effects of adipolin and TGF-β1 on Smad2 phosphorylation in VSMCs (*Figure 5C*). Moreover, the inhibitory effects of adipolin and TGF-β1 on PDGF-BB-induced DNA synthesis in VSMCs were cancelled by pretreatment with soluble forms of TGFβRII (*Figure 5D*). Similar to the results in VSMCs, pretreatment with soluble forms of TGFβRII also abolished adipolin-induced phosphorylation of Smad2 in mouse peritoneal macrophages (Supplementary material online, *Figure S11A*). Furthermore, soluble TGFβRII treatment also reversed the suppressive effect of adipolin on LPS-induced expression of TNFα in macrophages (Supplementary material online, *Figure S11B*). We also examined the effect of adipolin on the secretion and expression of TGF-β1 in cultured VSMCs and macrophages. Adipolin treatment significantly increased TGF-β1 levels in cultured media both from VSMCs and macrophages (*Figure 6A*). In contrast, adipolin treatment had no effects on mRNA levels of TGF-β1 in VSMCs or macrophages (*Figure 6B*). Wire injury increased TGF-β1 protein levels in arteries from both WT and APL-KO mice compared with those from sham-operated mice, but this induction of TGF-β1 expression in injured arteries was diminished in APL-KO mice (*Figure 6C*). Furthermore, Ad-APL administration to WT mice increased TGF-β1 protein levels in injured arteries (*Figure 6D*). These findings indicate that adipolin stimulates TGF-β1 secretion and promotes TGFβRII-dependent activation of Smad2, thereby leading to reduced VSMC proliferation and macrophage inflammatory responses (Supplementary material online, *Figure S12*).

4. Discussion

The present study provides the first evidence that endogenous adipolin exerts protective effects on the development of vascular disease in an established mouse model of vascular injury. Adipolin deficiency led to increased neointimal formation in response to injury in WT mice. Conversely, systemic administration of adipolin attenuated injury-induced neointimal formation in WT mice. Our *in vitro* findings demonstrated that treatment with adipolin protein led to the suppression of macrophage inflammatory responses to pathological stimuli and growth factor-induced proliferation of VSMCs, which are important features in various vascular disorders. These data show that adipolin negatively regulates adverse vascular remodelling, at least in part, by directly affecting macrophage and VSMC behaviours. Thus, these observations suggest that a reduction in circulating adipolin levels may contribute to the development of vascular diseases.

Inflammation is linked with the initiation and progression of atherosclerosis and vascular remodelling.[5,6] Our present observations showed that adipolin deficiency led to enhanced neointimal thickening in response to injury, which was associated with increases in macrophage recruitment, expression of inflammatory mediators, and activation of pro-inflammatory signalling molecules, including NF-κB and JNK in injured vessels. Furthermore, our *in vitro* data demonstrated that adipolin reduced the expression of pro-inflammatory genes and the activation of pro-inflammatory signalling molecules in macrophages. Thus, it is plausible that adipolin prevents adverse vascular remodelling, at least in part, through the suppression of inflammatory signal cascades in macrophages. Here, we demonstrated that the anti-inflammatory actions of adipolin in macrophages *in vitro* were dependent on its ability to promote TGFβRII/Smad2 signalling. It has been shown that TGFβ1/Smad signalling generally contributes to reduced inflammatory responses in many types of cells, including macrophages.[32–34] Moreover, Smad3-deficient mice exhibit more severe pathological vascular remodelling.[35] Our data also showed that adipolin promoted TGF-β1 secretion in cultured macrophages. Therefore, these findings suggest that adipolin reverses the macrophage inflammatory response, at least in part, through induction of the TGF-β1/Smad-dependent pathway, thereby contributing to vascular protection. On the other hand, TGF-β1/Smad signalling contributes to vascular fibrosis during the development of atherosclerosis.[36] Thus, we cannot exclude the possibility that adipolin promotes vascular disease by increasing arterial fibrosis, although this requires further investigation.

Enhanced VSMC proliferation contributes to the progression of neointimal formation and vessel stenosis during injury-induced vascular remodelling.[3,7,8] In the present study, adipolin attenuated growth factor-induced proliferative activity and cyclin D1 expression in VSMCs via Smad2 signalling. TGF-β1 has been reported to attenuate PDGF-BB-induced VSMC proliferation through down-regulation of cyclin D1.[30] Our data indicated that both adipolin and TGF-β1 can attenuate VSMC growth through a TGFβRII-dependent signalling mechanism. Moreover, treatment of cultured VSMCs with adipolin led to increased secretion of TGF-β1. Our *in vivo* data also indicated that adipolin disruption resulted

in the enhanced proliferation of α-SMA-positive SMCs in injured arteries. Furthermore, postnatal SMC-specific deletion of TGFβRII is reported to cause the rapid thickening of the thoracic aorta, which is accompanied by decreased Smad signalling.[37] Thus, these findings suggest that adipolin protects against pathological vascular remodelling by inducing TGFβRII/Smad2-dependent suppression of VSMC proliferation, at least in part, through the enhancement of TGF-β1 secretion.

We and others have shown that increased levels of plasma adipolin are linked to reduced insulin resistance in diet-induced obese mice.[19,38] Our findings demonstrated that, under normal chow feeding conditions, deficiency or overexpression of adipolin had no significant effect on circulating glucose or insulin levels in mice after vascular injury, suggesting that adipolin prevents the pathological remodelling of the vascular wall without affecting insulin sensitivity. Insulin resistance, under conditions of obesity, is associated with the progression of pathological vascular remodelling.[39] It has been shown that female mice with heterozygous adipolin deficiency exhibit mild insulin resistance with high fat diet-induced obesity.[40] Thus, although it is conceivable that a lack of adipolin may enhance insulin resistance in obese mice, leading to further exacerbation of neointimal formation after injury, this requires further investigation in a high-fat diet study.

The strategy of attenuating adverse vascular remodelling may be useful for the prevention and/or treatment of vascular diseases. Thus, it is likely that adipolin represents a therapeutic target molecule for preventing the progression of atherosclerosis and vascular restenosis after interventional therapy. A mouse model of wire-induced injury of the femoral artery may be widely used to study the mechanism of arterial restenosis, but this model produces localized vascular lesions, rather than systemic vascular damage. Therefore, to further understand the role of adipolin in cardiovascular and metabolic diseases, future investigations should be performed using more common experimental models of systemic vascular diseases, including atherosclerosis (e.g. atherosclerosis-prone apolipoprotein-E-deficient mice on a Western or high-fat diet).

Adipolin belongs to the CTRP family of adiponectin paralogs. It has been shown that adiponectin-KO mice have increased neointimal formation after arterial injury.[41–43] Adiponectin has been reported to suppress LPS-induced expression of TNFα in cultured macrophages and attenuate growth factor-induced proliferation of VSMCs.[44–46] Here, we have demonstrated that, like adiponectin, adipolin suppresses macrophage inflammatory reactions, and VSMC growth, thereby protecting against vascular disease. Mechanistically, adiponectin has been shown to attenuate VSMC proliferation and migration by directly interacting with growth factors and/or by activation of the AMPK signalling pathway.[42,46] Adiponectin also promotes phenotypic changes of macrophages to anti-inflammatory phenotypes, partly via activation of AMPK.[47] It has been shown that adiponectin-induced activation of AMPK is mediated by its interactions with the adiponectin receptors.[48] However, our data suggest that adipolin reduces inflammatory responses in macrophages and proliferative activity in VSMCs via TGFβRII/Smad2 signalling, independently of AMPK. Thus, adipolin appears to be functionally similar to adiponectin in vascular regulation, but there seems to be a difference in the receptor-mediated signalling pathways affected by adipolin and adiponectin.

In conclusion, our observations indicate that adipolin functions as an anti-inflammatory adipokine that plays a protective role in pathological vascular remodelling. In particular, elevation of plasma adipolin led to a reduction in vascular inflammation and remodelling in response to injury. Thus, therapeutic approaches aimed at increasing circulating adipolin levels may be useful for the prevention and treatment of vascular disorders.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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