Published in final edited form as:

Arterioscler Thromb Vasc Biol. 2021 June ; 41(6): 2019–2026. doi:10.1161/ATVBAHA.121.316293.

Janus Kinase 3 Deficiency Promotes Vascular Re-endothelialization

Yung-Chun Wang[1], **Dunpeng Cai**[1,2], **Xiao-Bing Cui**[1], **Ya-Hui Chuang**[1], **William P. Fay**[2,3,4], **Shi-You Chen**[1,2,*]

[1]Department of Surgery, University of Missouri School of Medicine, Columbia, MO 65212

[2]Medical Pharmacology & Physiology, University of Missouri School of Medicine, Columbia, MO 65212

[3]Medicine, University of Missouri School of Medicine, Columbia, MO 65212

[4]The Research Service, Harry S. Truman Memorial Veterans Hospital, Columbia, MO 65212

Abstract

Objective——The objective of this study is to determine the role of Janus kinase 3 (JAK3) in re-endothelialization after vascular injury.

Methods and Results——By using mouse carotid artery wire injury and rat balloon injury model, we found that JAK3 regulates re-endothelialization and EC proliferation after vascular injury. JAK3 and phospho-JAK3 levels were increased in neointimal smooth muscle cells (SMCs) in response to vascular injury in mice. JAK3 deficiency dramatically attenuated the injury-induced intimal hyperplasia in carotid arteries of both male and female mice. Importantly, JAK3 deficiency caused an increased rate of re-endothelialization following mechanical injury. Likewise, knockdown of JAK3 in medial SMCs elicited an accelerated re-endothelialization with reduced intimal hyperplasia following balloon injury in rat carotid arteries. Interestingly, knockdown of JAK3 restored the expression of SMC contractile protein smooth muscle α-actin in injury-induced intimal SMCs while increased the proliferating ECs in the intima area.

Conclusion——Our results demonstrate a novel role of JAK3 in the regeneration of endothelium after vascular injury, which may provide a new strategy to enhance re-endothelialization while suppressing neointimal formation for effective vascular repair from injury.

Keywords

Janus kinase 3; Endothelial proliferation; Re-endothelialization

Subject codes

115; 123; 162

[*]**Correspondence to** Shi-You Chen, PhD, Department of Surgery, University of Missouri School of Medicine, One Hospital Drive, Columbia, MO 65212 USA, scqvd@missouri.edu, Tel: 573-884-0371.

Disclosures: None

Introduction

Maintenance of an intact and functional endothelium is essential for the blood vessel health. Endothelial cells (ECs) play a central role in vascular hemostasis and control multiple critical processes, such as vascular tone, inflammation, thrombosis, and angiogenesis.[1, 2] Endovascular treatment of obstructive atherosclerotic lesions in coronary, carotid, and peripheral arteries with balloon-tipped catheters or metallic stents injures the vascular endothelium, which renders the injured vascular segment vulnerable to thrombosis and disrupts normal vascular function, such as vasodilation.[3] Proliferation of endothelial cells is required to restore an intact endothelial monolayer. Vascular sheer stress and inherent properties of endothelial cells control the pace of endothelial cell proliferation after denuding injury.[4] Vascular smooth muscle cells (SMCs), which subtend the endothelium, also control endothelial cell proliferation through the release of growth factors, including fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF).[5] However, other pathways by which SMCs control endothelial cell recovery after vascular injury remain poorly defined.

JAK3, a member of Janus kinase (JAK) family of non-receptor tyrosine kinases, transduces signals induced by cytokines via receptors containing common γ chain.[6] JAK3 has been shown to regulate cellular function of different cell types during physiological and pathological processes.[7] Absence of JAK3 deters the differentiation and maturation of lymphocytes, such as NK, T and B cells.[8-12] In addition, JAK3 regulates the proliferation of lung epithelial cells. Our previous studies show that JAK3 is also important for SMC proliferation and neointimal formation.[13, 14] Since JAK3 tunes myocardial vascular permeability via modulating the physiological function of ECs[15], we hypothesized that JAK3 may play a role in re-endothelialization following vascular injury.

In this study, we found that JAK3 deficiency promoted endothelium recovery after endothelial denudation in mouse and rat carotid arteries. Knockdown of JAK3 in medial SMCs of injured rat carotid arteries restored the expression of contractile proteins in intimal SMCs while enhancing EC proliferation in the neointima area. It appeared that JAK3 regulates EC proliferation via facilitating the interaction between SMCs and ECs.

Materials and Methods

The authors declare that all supporting data are available within the article (and its online supplementary files).

Animals

Male Sprague-Dawley rats weighing 450-500 g were purchased from Envigo. Both male and female JAK3+/− (B6;129S4-Jak3tm1Ljb/J; Stock#: 002852) and ApoE−/− (B6;129P2-Apoetm1Unc/J; Stock#: 002052) mice were purchased from The Jackson Laboratory. JAK3+/+, which serves as wild type (WT), and JAK3−/− mice were obtained through breeding JAK3+/− mice. JAK3+/+; ApoE−/− and JAK3+/−; ApoE−/− mice were obtained through breeding JAK3+/−; ApoE−/− mice. Mice were fed with Lab Diet 5053 (Lab Supply). Animals were housed in a state-of-the-art double barrier facility and received

humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals. Animal surgical procedures were approved by the Animal Care and Use Committee of the University of Missouri.

Cell Culture

Rat aortic smooth muscle cells (SMCs) were cultured by enzymatic digestion method from rat thoracic aorta as described previously.[14, 16] SMCs were maintained in Dulbecco's modification of Eagle's medium (DMEM, Corning, 17-207-CV) containing 10% fetal bovine serum (FBS, R&D, S11550H) and 5% L-glutamine (Hyclone, SH30034.01) at 37°C in a humidified atmosphere with 5% CO2. Phenotypes of primary cultured SMCs were confirmed by the expression of smooth muscle α-actin and SM22α. Male rat aortic endothelial cells (ECs, Cell Biologics, RN-6052) were maintained in complete rat endothelial cell medium (Cell Biologics, M1266) at 37°C in a humidified atmosphere with 5% CO2. SMC phenotypic modulation was induced by PDGF-BB (R&D, 220-BB).

Antibodies

Antibodies were obtained from the following sources: JAK3 (Cell Signaling, #8863), phospho-JAK3 (Santa Cruz, sc-16567), SMMHC (Abcam, ab53219), α-SMA (Abcam, ab5694), CNN1 (Cell signaling, 17819), SM22α (Abcam, ab14106), α-Tubulin (Sigma Aldrich, T9026), CD31 (Santa Cruz, sc-376764), VE-cadherin (Santa Cruz, sc-28644), PCNA (Santa Cruz, sc-56).

Construction of Adenovirus

Construction of adenovirus was described previously.[14, 17] Briefly, cDNA fragment encoding full length of human JAK3 was amplified from JAK3 plasmid (DNASU, HsCD00038537) by polymerase chain reaction (PCR), and then inserted into the pShuttle-IRES-hrGPF-1 vector (Agilent) through XhoI site. The resultant recombinant JAK3 plasmid was verified by sequencing. Rat JAK3 short hairpin RNA (shJAK3) was constructed into pRNAT-H1.1/Adeno vector (Genscript) through MluI and HindIII site. Adenoviral vector of JAK3 and shJAK3 was constructed using AdEasy system described previously.[18] Adenoviral control vectors are packaged using pShuttle-IRES-hrGPF-1 vector (for JAK3 expression) and pRNAT-H1.1/Adeno vector (for JAK3 shRNA). Adenovirus was purified by gradient density ultracentrifugation of cesium chloride followed by dialyzing in dialysis buffer (135 mmol/L NaCl, 1 mmol/L MgCl2, 10 mmol/L Tris-HCl, pH 7.5, 10% glycerol). JAK3 shRNA cDNA sequences were: 5' -CGC GTC TCT ACT TGC AGT CCA GAA TGC CAG CTT CAA GAG AGC TGG CAT TCT GGA CTG CAA GTA GAT TTT TTC CAA A −3' (top strand) and 5'- AGC TTT TGG AAA AAA TCT ACT TGC AGT CCA GAA TGC CAG CTC TCT TGA AGC TGG CAT TCT GGA CTG CAA GTA GAG A −3' (bottom strand).

Mouse Carotid Artery Wire-injury Model

Mice were anesthetized with 2% isoflurane inhalation. The left common carotid artery was exposed, and injury was made by insertion of a straight spring wire (0.38 mm in diameter;

Cook Medical) through the left external carotid artery and advanced toward the thoracic aorta into the common carotid artery. The wire was left in place for 1 minute to denude the artery. Wire-injured segments were collected at 5 days or 14 days after surgery, and the right common carotid arteries were collected as controls. The artery segments were perfused with saline, fixed with 4% paraformaldehyde, and embedded in paraffin for subsequent sectioning and morphometric analyses in a blinded manner.

Rat Carotid Artery Balloon Injury Model and Adenoviral Gene Transfer

Rat carotid artery balloon injury was performed using 2F Fogarty arterial embolectomy balloon catheter (Baxter Edwards Healthcare) as described previously.[14, 19] Adenovirus expressing control vector (Ad-Ctrl) or shJAK3 (Ad-shJAK3) was introduced into balloon-injured carotid artery by incubation of 100 μL adenovirus (5 x 10^9 pfu) for 20 minutes as described previously.[20] Balloon-injured artery segments were collected at 14 days after the surgery. The segments were perfused with saline, fixed with 4% paraformaldehyde, and embedded in paraffin for subsequent sectioning and morphometric analyses in a blinded manner.

Evans Blue Staining

To assess the extent of re-endothelialization, mouse and rats were given sterile solution of 0.5% Evans blue (Sigma Aldrich, E2129), 0.1 mL and 0.5 mL, respectively, by intravenous tail injection 30 minutes prior to the collection of injured artery segments.[21] Re-endothelialization was assessed by measuring the Evans blue-unstained area, and the re-endothelialization rate was calculated by the following formula: [(Mean value of Evans blue-unstained area) / (Mean value of total area)].

Blood Pressure Measurement

To measure the blood pressure of mice, tail-cuff system (CODA-MNTR, KENT) were performed as described previously.[22] Briefly, five mice of each group (WT, JAK3+/− and JAK3−/−) were trained by placing into holder for 15 minutes for 3 consecutive days prior to data collection. The temperature of mice was maintained at around 32 to 35 °C by using infrared warming pads. Occlusion cuff and volume pressure recording sensors were placed on the mouse tail sequentially, and mean value of systolic blood pressure was calculated from 20 cycles of measurements per mouse.

Histomorphometry Analysis, Immunohistochemistry (IHC) and Immunofluorescent Staining (IF)

Control and injured artery sections (5 μm) used for analyses among different groups were evenly distributed in the vessel segment collected.[19, 23] For morphology, the sections were stained with hematoxylin and eosin (H&E, American MasterTech, KTHNEPT) or Elastica van Gieson (VG, Sigma Aldrich, HT25A) reagents, and the cross-sectional images were captured using Eclipse 90i Nikon microscope. The circumference of lumen, internal elastic lamina, and external elastic lamina were measured by Image-pro Plus Software. For IHC or IFC, sections were rehydrated, blocked with 10% goat or donkey serum, permeabilized with 0.01% Triton X-100 in PBS, and incubated with JAK3, phospho-JAK3, CD31, VE-cadherin,

Ki-67 and α-SMA primary antibody at 4°C overnight followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody for IHC or with FITC- or TRITC-conjugated secondary antibodies for IF. The sections were counterstained with hematoxylin for IHC or DAPI for IF. Negative control was performed by incubating with host immunoglobulin G (IgG). Image J software was used to measure the intensity of IF positive staining by following the previous publication.[24] Mean value of the staining intensity for each group was acquired from 10 artery sections. To quantify the protein level, the mean value of IF-positive signal of each group less the background signal (negative control) was calibrated to the mean value of the staining intensity in uninjured vessels, in which the background signal was also subtracted. The protein level relative to the control group was shown as a fold increase of the signal intensity that was assessed by the following formula: [(Mean value of staining intensity – Mean value of negative control staining intensity) / (Mean value of staining intensity of uninjured vessels – Mean value of negative control staining intensity)].

Conditioned Smooth Muscle Cell Medium Preparation

Equal numbers (3×10^6) of SMCs seeded in 60 mm culture dishes were transduced with adenovirus expressing JAK3 or JAK3 shRNA, and then cultured in the complete medium (DMEM containing 10% FBS and 5% L-glutamine) for 48 hours. After SMC monolayer was formed, SMCs were starved in serum-free DMEM for another 24 hours, and then treated with PDGF-BB for additional 48 hours. Cells were washed with warm DPBS three times and then incubated with serum-free DMEM for 24 hours, followed by collection of conditioned SMC medium, which was filtered through a 0.22-μm filter.

Western Blot Analysis

Rat ECs were starved in serum-free DMEM for 24 hours, and then treated with conditioned SMC medium for 24 hours. Cells were washed with PBS twice, followed by protein extraction using RIPA buffer (50 mmol/liters Tris-HCI, pH 7.4, 1% Triton X-100, 0.25% w/v sodium deoxycholate, 150 mmol/ liter NaCl, 1 mmol/liter EGTA, protease inhibitors (Thermo Scientific), phosphatase inhibitors (Thermo Scientific), 0.1% SDS). Protein concentration was measured using BCA Protein Assay (Thermo Scientific). Equal amounts of proteins were resolved on SDS-PAGE gels and then transferred to PVDF (Bio-Rad) or nitrocellulose membranes (Bio-Rad). Nonspecific bindings were blocked with 5% BSA, and then incubated with primary antibodies in blocking buffer at 4°C for 16 hours, followed by incubation with HRP-conjugated secondary antibody at room temperature for 1 hour (Sigma). Detection was performed with enhanced chemiluminescence (Millipore). The experiments were repeated for three times with three replicates for each treatment.

Real-time Quantitative PCR (qPCR)

Total RNA of cultured cells was extracted using Trizol Reagent (Thermo Scientific, 10296028), and then reverse transcribed to cDNA using iScript cDNA Synthesis kit (Bio-Rad, 1708890) as described previously.[25, 26] Real-time qPCR was performed with Stratagene Mx3005P qPCR instrument using SYBR Green master mix (GeneCopoeia, QP001). Cyclophilin (CYP) was used as an internal control.[14] Primer sequences were provided in the major resources table.

Statistical Analysis

Data were analyzed using Prism 7 software (GraphPad Software) and are presented as mean ± S.D. To determine whether data were normally distributed with equal variance, Pearson chi-square normality test was performed. Comparison between two groups was evaluated with two-tailed independent Student's t-test. Comparison among more than two groups was evaluated by one-way ANOVA followed by Fisher's least significant difference (LSD) test. P value < 0.05 was considered as statistically significant.

Results

JAK3 deficiency Increases Re-endothelialization and Attenuates Injury-Induced Neointimal Formation.

Delayed proliferation of endothelium impairs the vascular repair following cardiovascular intervention, such as angioplasty and stent implantation.[27] Our previous study has shown that JAK3 is induced and activated in injury-induced neointimal SMCs in rat carotid artery.[14] To determine whether JAK3 plays a role in re-endothelialization, we performed wire injury in the carotid arteries of male WT and JAK3-deficient mice and then detected JAK3 expression and phosphorylation (pJAK3) in the injured arteries. Both JAK3 and pJAK3 protein levels were elevated in neointimal SMCs as early as 3 days after the injury (Online Figure I). Importantly, JAK3 deficiency suppressed the injury-induced neointimal formation in mouse carotid arteries (Figure 1A). The neointima area was reduced by nearly 60% in JAK3 heterozygous knockout (JAK3+/−) and 80% in JAK3 homozygous knockout (JAK3−/−) arteries compared to the WT (37.1%±5.2% and 15.2%±2.6% vs. 91.2%±16.3%, respectively. $P<0.05$, n=5, Figure 1B). Interestingly, JAK3 deficiency caused more endothelial coverage in mouse carotid arteries 5 days after the wire injury (Figure 1C). The percentage of endothelial coverage was increased by nearly 30% and 40% in JAK3+/− and JAK3−/− arteries, respectively, as compared to WT arteries (Figure 1D). This phenomenon was confirmed by immunofluorescent staining with endothelial cell marker, platelet endothelial cell adhesion molecule (PECAM-1, also known as CD31). Both JAK3+/− and JAK3−/− arteries showed more CD31-positive cells aligned at the lumen side of arteries (Figure 1E and Online Figure IIA). These effects were specific to JAK3 because JAK3+/− or JAK3−/− did not alter the expression of other JAK family members, i.e., JAK1, JAK2, and TYK2 (Online Figure IIIA), consistent with the results observed in rat balloon injury model.[14]

We also observed similar results in female mice. JAK3 deficiency suppressed the injury-induced neointimal formation while promoting endothelium regeneration (Online Figure IV). To better recapitulate the clinical condition such as intima-media thickness and in-stent restenosis, we performed wire injury in carotid arteries of apolipoprotein E knockout (ApoE−/−) and JAK3 +/−;ApoE−/− mice. As shown in Online Figure V, JAK3 deficiency caused less injury-induced neointima with more endothelial coverage as indicated by the staining of EC marker vascular endothelial cadherin (VE-cadherin) under hypercholesterolemia condition. These results suggested that JAK3 not only promotes neointimal formation, but also impairs re-endothelialization in injured arteries of clinically-relevant animal models.

Knockdown of JAK3 in SMCs Promotes Arterial Recovery from Injury.

It has been shown that injury-induced neointimal SMCs could inhibit EC proliferation, which delays vascular repair after injury.[28-30] Since knockdown of JAK3 in SMCs suppresses injury-induced neointima formation,[14] we sought to determine whether it promotes re-endothelialization through inhibiting the proliferation of SMCs. Thus, adenoviral vector expressing JAK3 short hairpin RNA (shRNA; Ad-shJAK3) was constructed, which effectively knocked down JAK3 (Online Figure VI, A-B) without affecting other JAK family members (Online Figure IIIB). Transduction of Ad-shJAK3 into endothelium-denuded arteries significantly increased the endothelium recovery or the re-endothelialization as assessed by Evans blue staining (Figure 2A). The percentage of endothelial coverage was increased by more than 40% with Ad-shJAK3 administration compared to the Ad-Ctrl transduction (88.2%±9.3% vs. 61.9%±8.7%; $P<0.05$, n=5, Figure 2B). To further confirm this phenomenon, the expression of CD31 and VE-cadherin was detected by immunostaining. Both CD31- and VE-cadherin- positive cells were increased in Ad-shJAK3-treated arteries compared to the Ad-Ctrl-treated arteries (Figure 2C and Online Figure IIB).

Previous studies have shown that EC proliferation is initiated rapidly in response to injury. However, EC proliferation is suppressed by the presence of neointimal SMCs before vascular healing is completed.[30] Indeed, no ECs were observed, while numerous proliferating SMCs were present in neointima of the arteries with injury and Ad-Ctrl administration for 14 days (Figure 2D). However, the injured arteries with Ad-shJAK3 transduction showed less neointimal SMCs with a higher level of expression of contractile protein α-SMA and SMMHC (Online Figure VI, C-D) and the presence of proliferating ECs at the lumen side of the neointima (Figure 2D and Online Figure IIC). Consistently, knockdown of JAK3 restored the SMC marker expression that was inhibited by PDGF-BB in vitro (Online Figure VI, A-B). On the other hand, overexpression of JAK3 suppressed SMC marker protein expression, similar to the effect of PDGF-BB (Online Figure VII). These data suggest that JAK3 regulates EC proliferation/re-endothelialization by modulating SMC phenotype.

To determine if JAK3 affects EC proliferation though SMCs, we overexpressed JAK3 in SMCs and used JAK3-conditioned SMC medium to treat ECs. As shown in Online Figure VIIIA, JAK3-conditioned SMC medium inhibited EC proliferation to the similar extent as with the culture medium of PDGF-BB-treated SMCs, a synthetic SMC phenotype. Moreover, JAK3-conditioned SMC culture medium inhibited the expression of proliferating cell nuclear antigen (PCNA) in ECs (Online Figure VIII, B-C). Conversely, conditioned medium of PDGF-BB-treated SMCs transduced with Ad-shJAK3 impeded the suppressive effect of PDGF-BB-conditioned SMC culture medium on EC proliferation and the protein expression of PCNA (Figure 2E-2G), suggesting that JAK3 was essential for synthetic SMCs to suppress EC proliferation. Collectively, these results indicated that JAK3 attenuated the arterial endothelial recovery through promoting synthetic SMC phenotype following vascular injury.

Discussion

Regeneration of endothelium achieved by local EC proliferation is critical for the vascular repair after injury.[28] However, EC proliferation rate is hindered by the inhibitory effect of neointimal SMCs.[29, 30] In this study, we have identified JAK3 as a novel regulator for the vascular endothelial cell recovery. JAK3 deficient mouse carotid arteries showed a relatively higher endothelium coverage after wire injury. These findings are also observed in atherosclerosis-prone hypercholesterolemic mice. Hypercholesterolemia is associated with primitive carotid intima-media thickness and in-stent restenosis.[31-33] Although we were unable to acquire results from JAK3−/−; ApoE−/− mice because of the severe inflammatory bowel disease (IBD)-like symptoms [34], their smaller size, and the increased mortality after wire injury, JAK3+/−; ApoE−/− mouse arteries displayed less neointima formation with better coverage of endothelium following the injury, suggesting a clinical relevance of our results. Moreover, knockdown of JAK3 in medial SMCs not only suppressed the injury-induced neointimal formation, but also enhanced the re-endothelialization, as shown by the increased number of proliferating ECs aligned at the lumen side of the arteries. Of interest, knockdown of JAK3 seemed to alter injury-induced neointimal SMCs from synthetic to contractile phenotype, which could allow an effective regeneration of endothelium after vascular injury.

Systolic blood pressure is associated with spontaneous carotid intima-media thickness in human.[35] Although the cause and effect between high blood pressure and incidence of restenosis remain to be determined,[36] blood pressure control is recommended to lower the morbidity and mortality after carotid endarterectomy.[37] JAK3−/− mice exhibited a slightly lower systolic blood pressure (Online Figure IX), consistent with a previous report.[38] Although a lower blood pressure could be beneficial to endothelial function, it is unclear if it can also contribute to the accelerated regeneration of endothelium, which requires extensive studies in the future.

In addition to ECs and SMCs, circulating cells such as endothelial progenitor cells (EPCs) and leukocytes, may also contribute to the increased re-endothelialization in our animal studies. Indeed, intravenous transfusion of EPCs in a mouse model of arterial injury has been shown to enhance re-endothelialization along with decreased neointimal formation.[39] It remains controversial whether EPCs are able to differentiate into mature ECs. However, it is more likely that EPCs induce the proliferation of resident ECs via a paracrine effect.[40, 41] Local inflammation at the site of endothelial denudation after angioplasty or stent implantation is initiated immediately and contributes to the subsequent SMC proliferation and thus neointimal formation.[42, 43] Initial inflammation response in the lesion site is incited due to leukocytes recruitment, including neutrophil and monocyte infiltrations.[44] Knockout of leukocyte integrin macrophage-1 antigen reduces injury-induced neointimal formation via diminished accumulation of leukocytes at lesion site.[45] Therefore, the enhanced re-endothelialization in injured arteries with JAK3 deficiency or administration of JAK3 shRNA may also be attributable to the pro-angiogenic effect of EPCs and the decreased accumulation of leukocytes, which can be studied in the future. Our findings have therapeutic implications, as they suggest that drug targeting of JAK3 could promote vascular healing after catheter-based vascular interventions.

Our study is the first to demonstrate the role of JAK3 in re-endothelialization after vascular injury. Both JAK3 deficiency and shRNA administration resulted in less neointimal formation along with an increased coverage of endothelium. Mechanically, it appears that JAK3 regulates EC proliferation via controlling the transition of SMC phenotype. Although it is beneficial to promote endothelial regeneration via downregulation of JAK3 in injured arteries, whether the new vascular endothelial lining has the physiological function of endothelium, such as vascular tone regulation, response to environmental stimuli (*i.e.* mechanical stress and cytokines), and vascular barrier, is worthy of future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding sources:

This work was supported by grants from National Institutes of Health (HL117247, HL119053, HL135854, and HL147313 to S.Y.C; HL136746 to W.P.F.).

Non-standard Abbreviations and Acronyms:

SMC	Smooth muscle cell
JAK3	Janus kinase 3
SMMHC	Smooth muscle myosin heavy chain
αSMA	Smooth muscle α-actin
CD31	Cluster of differentiation 31
VE-cadherin	Vascular endothelial cadherin
shRNA	Short hairpin RNA

Reference

1. Michiels C Endothelial cell functions. J Cell Physiol. 2003;196:430–43. [PubMed: 12891700]
2. Bazzoni G and Dejana E. Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. Physiol Rev. 2004;84:869–901. [PubMed: 15269339]
3. Barone GW, Conerly JM, Farley PC, Flanagan TL and Kron IL. Endothelial injury and vascular dysfunction associated with the Fogarty balloon catheter. J Vasc Surg. 1989;9:422–5. [PubMed: 2646459]
4. Van der Heiden K, Gijsen FJ, Narracott A, Hsiao S, Halliday I, Gunn J, Wentzel JJ and Evans PC. The effects of stenting on shear stress: relevance to endothelial injury and repair. Cardiovasc Res. 2013;99:269–75. [PubMed: 23592806]
5. Holderfield MT and Hughes CC. Crosstalk between vascular endothelial growth factor, notch, and transforming growth factor-beta in vascular morphogenesis. Circ Res. 2008;102:637–52. [PubMed: 18369162]
6. Yamaoka K, Saharinen P, Pesu M, Holt VE 3rd, Silvennoinen O and O'Shea JJ. The Janus kinases (Jaks). Genome Biol. 2004;5:253. [PubMed: 15575979]

7. Wu W and Sun XH. Janus kinase 3: the controller and the controlled. Acta Biochim Biophys Sin (Shanghai). 2012;44:187–96. [PubMed: 22130498]
8. Thomis DC, Gurniak CB, Tivol E, Sharpe AH and Berg LJ. Defects in B lymphocyte maturation and T lymphocyte activation in mice lacking Jak3. Science. 1995;270:794–7. [PubMed: 7481767]
9. Nie L, Perry SS, Zhao Y, Huang J, Kincade PW, Farrar MA and Sun XH. Regulation of lymphocyte development by cell-type-specific interpretation of Notch signals. Mol Cell Biol. 2008;28:2078–90. [PubMed: 18195039]
10. Wilson A, MacDonald HR and Radtke F. Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. J Exp Med. 2001;194:1003–12. [PubMed: 11581321]
11. Pui JC, Allman D, Xu L, DeRocco S, Karnell FG, Bakkour S, Lee JY, Kadesch T, Hardy RR, Aster JC and Pear WS. Notch1 expression in early lymphopoiesis influences B versus T lineage determination. Immunity. 1999;11:299–308. [PubMed: 10514008]
12. Guo Y, Arakawa F, Miyoshi H, Niino D, Kawano R and Ohshima K. Activated janus kinase 3 expression not by activating mutations identified in natural killer/T-cell lymphoma. Pathol Int. 2014;64:263–6. [PubMed: 24965108]
13. Liu J and Kern JA. Neuregulin-1 activates the JAK-STAT pathway and regulates lung epithelial cell proliferation. Am J Respir Cell Mol Biol. 2002;27:306–13. [PubMed: 12204892]
14. Wang YC, Cui XB, Chuang YH and Chen SY. Janus Kinase 3, a Novel Regulator for Smooth Muscle Proliferation and Vascular Remodeling. Arterioscler Thromb Vasc Biol. 2017;37:1352–1360. [PubMed: 28473442]
15. Lee JE, Lee AS, Kim DH, Jung YJ, Lee S, Park BH, Lee SH, Park SK, Kim W and Kang KP. Janex-1, a JAK3 inhibitor, ameliorates tumor necrosis factor-alpha-induced expression of cell adhesion molecules and improves myocardial vascular permeability in endotoxemic mice. Int J Mol Med. 2012;29:864–70. [PubMed: 22344597]
16. Golovina VA and Blaustein MP. Preparation of primary cultured mesenteric artery smooth muscle cells for fluorescent imaging and physiological studies. Nat Protoc. 2006;1:2681–7. [PubMed: 17406524]
17. Wang YC, Chuang YH, Shao Q, Chen JF and Chen SY. Brain cytoplasmic RNA 1 suppresses smooth muscle differentiation and vascular development in mice. J Biol Chem. 2018;293:5668–5678. [PubMed: 29467228]
18. Luo J, Deng ZL, Luo X, Tang N, Song WX, Chen J, Sharff KA, Luu HH, Haydon RC, Kinzler KW, Vogelstein B and He TC. A protocol for rapid generation of recombinant adenoviruses using the AdEasy system. Nat Protoc. 2007;2:1236–47. [PubMed: 17546019]
19. Tulis DA. Rat carotid artery balloon injury model. Methods Mol Med. 2007;139:1–30. [PubMed: 18287662]
20. Guzman RJ, Lemarchand P, Crystal RG, Epstein SE and Finkel T. Efficient and selective adenovirus-mediated gene transfer into vascular neointima. Circulation. 1993;88:2838–48. [PubMed: 8252697]
21. Radu M and Chernoff J. An in vivo assay to test blood vessel permeability. J Vis Exp. 2013:e50062. [PubMed: 23524912]
22. Daugherty A, Rateri D, Hong L and Balakrishnan A. Measuring blood pressure in mice using volume pressure recording, a tail-cuff method. J Vis Exp. 2009.
23. Tang R, Mei X, Wang YC, Cui XB, Zhang G, Li W and Chen SY. LncRNA GAS5 regulates vascular smooth muscle cell cycle arrest and apoptosis via p53 pathway. Biochim Biophys Acta Mol Basis Dis. 2019;1865:2516–2525. [PubMed: 31167125]
24. Jensen EC. Quantitative analysis of histological staining and fluorescence using ImageJ. Anat Rec (Hoboken). 2013;296:378–81. [PubMed: 23382140]
25. Tang R, Zhang G, Wang YC, Mei X and Chen SY. The long non-coding RNA GAS5 regulates transforming growth factor beta (TGF-beta)-induced smooth muscle cell differentiation via RNA Smad-binding elements. J Biol Chem. 2017;292:14270–14278. [PubMed: 28659340]
26. Tang R, Wang YC, Mei X, Shi N, Sun C, Ran R, Zhang G, Li W, Staveley-O'Carroll KF, Li G and Chen SY. LncRNA GAS5 attenuates fibroblast activation through inhibiting Smad3 signaling. Am J Physiol Cell Physiol. 2020;319:C105–C115. [PubMed: 32374674]

27. Stefanini GG and Holmes DR Jr. Drug-eluting coronary-artery stents. N Engl J Med. 2013;368:254–65. [PubMed: 23323902]
28. Van Belle E, Bauters C, Asahara T and Isner JM. Endothelial regrowth after arterial injury: from vascular repair to therapeutics. Cardiovasc Res. 1998;38:54–68. [PubMed: 9683907]
29. Schwartz SM and Benditt EP. Clustering of replicating cells in aortic endothelium. Proc Natl Acad Sci U S A. 1976;73:651–3. [PubMed: 1061164]
30. Reidy MA, Clowes AW and Schwartz SM. Endothelial regeneration. V. Inhibition of endothelial regrowth in arteries of rat and rabbit. Lab Invest. 1983;49:569–75. [PubMed: 6632774]
31. Rapp JH, Qvarfordt P, Krupski WC, Ehrenfeld WK and Stoney RJ. Hypercholesterolemia and early restenosis after carotid endarterectomy. Surgery. 1987;101:277–82. [PubMed: 3824155]
32. Tonstad S, Joakimsen O, Stensland-Bugge E, Leren TP, Ose L, Russell D and Bonaa KH. Risk factors related to carotid intima-media thickness and plaque in children with familial hypercholesterolemia and control subjects. Arterioscler Thromb Vasc Biol. 1996;16:984–91. [PubMed: 8696963]
33. Kusters DM, Wiegman A, Kastelein JJ and Hutten BA. Carotid intima-media thickness in children with familial hypercholesterolemia. Circ Res. 2014;114:307–10. [PubMed: 24192652]
34. Murata Y, Yamashita A, Saito T, Sugamura K and Hamuro J. The conversion of redox status of peritoneal macrophages during pathological progression of spontaneous inflammatory bowel disease in Janus family tyrosine kinase 3(−/−) and IL-2 receptor gamma(−/−) mice. Int Immunol. 2002;14:627–36. [PubMed: 12039914]
35. Ferreira JP, Girerd N, Bozec E, Machu JL, Boivin JM, London GM, Zannad F and Rossignol P. Intima-Media Thickness Is Linearly and Continuously Associated With Systolic Blood Pressure in a Population-Based Cohort (STANISLAS Cohort Study). J Am Heart Assoc. 2016; 5.
36. Hager A, Kanz S, Kaemmerer H, Schreiber C and Hess J. Coarctation Long-term Assessment (COALA): significance of arterial hypertension in a cohort of 404 patients up to 27 years after surgical repair of isolated coarctation of the aorta, even in the absence of restenosis and prosthetic material. J Thorac Cardiovasc Surg. 2007;134:738–45. [PubMed: 17723827]
37. Stoneham MD and Thompson JP. Arterial pressure management and carotid endarterectomy. Br J Anaesth. 2009;102:442–52. [PubMed: 19233880]
38. Umbach AT, Luo D, Bhavsar SK, Hosseinzadeh Z and Lang F. Intestinal Na+ loss and volume depletion in JAK3-deficient mice. Kidney Blood Press Res. 2013;37:514–20. [PubMed: 24281140]
39. Werner N, Junk S, Laufs U, Link A, Walenta K, Bohm M and Nickenig G. Intravenous transfusion of endothelial progenitor cells reduces neointima formation after vascular injury. Circ Res. 2003;93:e17–24. [PubMed: 12829619]
40. Hagensen MK, Raarup MK, Mortensen MB, Thim T, Nyengaard JR, Falk E and Bentzon JF. Circulating endothelial progenitor cells do not contribute to regeneration of endothelium after murine arterial injury. Cardiovasc Res. 2012;93:223–31. [PubMed: 22012957]
41. Rehman J, Li J, Orschell CM and March KL. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. Circulation. 2003;107:1164–9. [PubMed: 12615796]
42. Tanaka H, Sukhova GK, Swanson SJ, Clinton SK, Ganz P, Cybulsky MI and Libby P. Sustained activation of vascular cells and leukocytes in the rabbit aorta after balloon injury. Circulation. 1993;88:1788–803. [PubMed: 7691431]
43. Welt FG and Rogers C. Inflammation and restenosis in the stent era. Arterioscler Thromb Vasc Biol. 2002;22:1769–76. [PubMed: 12426203]
44. Welt FG, Edelman ER, Simon DI and Rogers C. Neutrophil, not macrophage, infiltration precedes neointimal thickening in balloon-injured arteries. Arterioscler Thromb Vasc Biol. 2000;20:2553–8. [PubMed: 11116052]
45. Simon DI, Dhen Z, Seifert P, Edelman ER, Ballantyne CM and Rogers C. Decreased neointimal formation in Mac-1(−/−) mice reveals a role for inflammation in vascular repair after angioplasty. J Clin Invest. 2000;105:293–300. [PubMed: 10675355]

Highlights

- JAK3 deficiency promotes endothelial recovery after vascular injury in mice.
- Knockdown of JAK3 alters smooth muscle cells (SMCs) from synthetic to contractile phenotype.
- Synthetic phenotype of SMCs suppressed proliferation of endothelial cells (ECs).

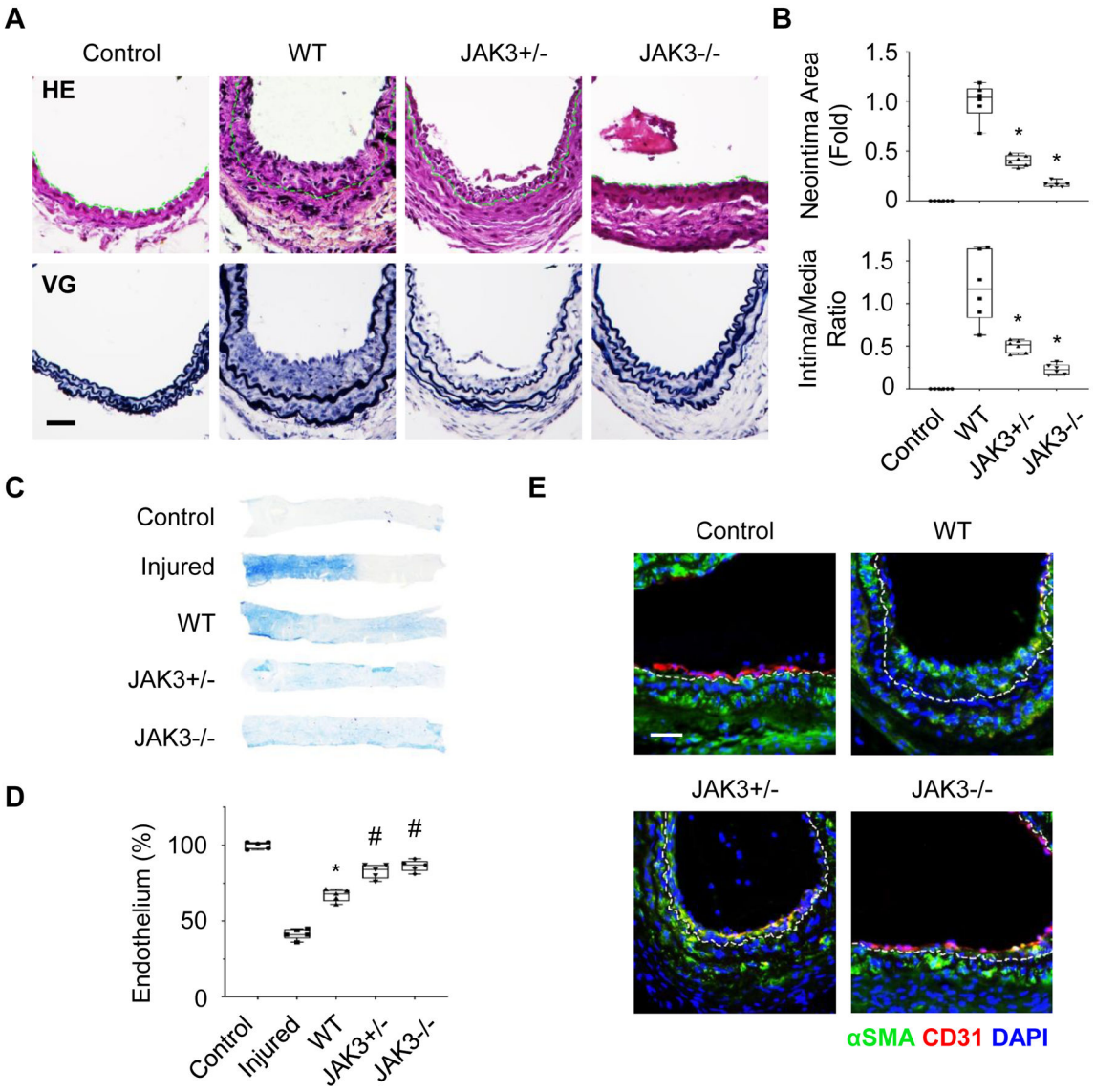

Figure 1. JAK3 deficiency suppresses neointima formation and accelerates re-endothelialization after carotid artery wire injury.

A, JAK3 heterozygous knockout (JAK3+/−) partially while its homozygous knockout (JAK3−/−) almost completely blocked neointima formation following mouse carotid artery wire injury as shown by hematoxylin and eosin (HE) and Elastica van Gieson (VG) staining. Bar: 50 μm. **B**, Quantification of the neointima area and intima/media ratio by measuring the size of intima and media. $*P < 0.05$ vs injured wild type arteries collected 5 days after the surgery (WT), n=6. **C**, JAK3 deficiency accelerated re-endothelialization after wire injury of mouse carotid arteries. The injured area without re-endothelialization was stained by Evans blue. **D**, Quantification of the extent of re-endothelialization in control or injured arteries by measuring the Evans blue–unstained area as shown in **C**. $*P < 0.05$ vs control injured arteries collected right after the surgery (Injured); $^{\#}P < 0.05$ vs injured wild type arteries collected 5 days after the surgery (WT), n=5. **E**, JAK3 deficiency promoted the lining of

CD31+ cells in the lumen of injured arteries as shown by immunostaining of CD31 (Red). The media layer was stained with αSMA (Green). Bar: 50 μm.

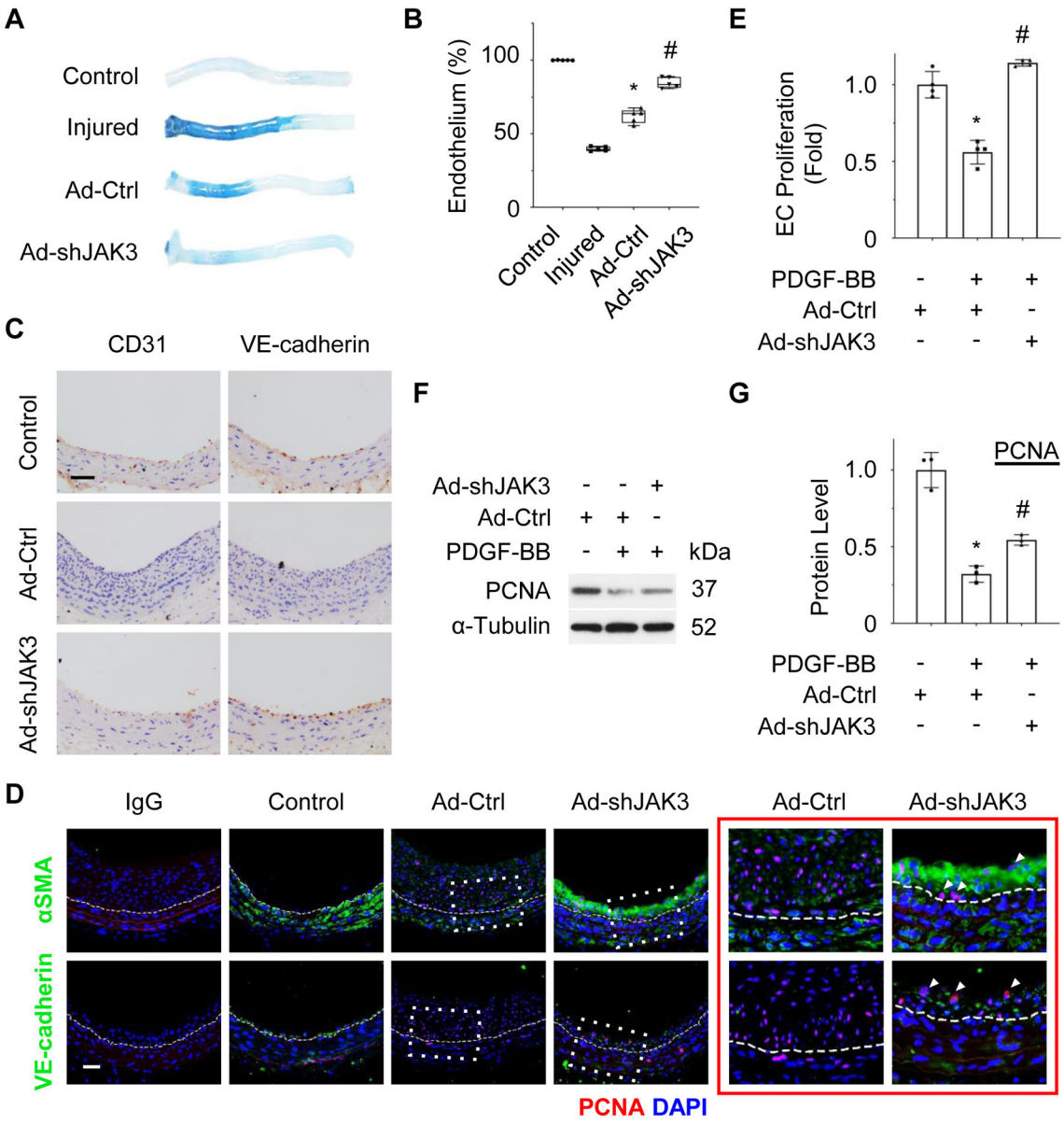

Figure 2. Blockade of JAK3 expression in neointimal SMCs promotes re-endothelialization of injured arteries via inducing EC proliferation.

A, Knockdown of JAK3 (Ad-shJAK3) accelerated reendothelialization following rat carotid artery balloon injury. The area without re-endothelialization was stained with Evans blue. **B,** Quantification of the re-endothelialization in control or injured arteries by measuring the Evans blue–unstained area as shown in **A**. $^{*}P < 0.05$ vs Control arteries with injury (Injured); $^{\#}P < 0.05$ vs control adenoviral vector-treated arteries collected at day 7 after surgery (Ad-Ctrl), n=5. **C,** CD31- and VE-cadherin-positive cells appeared in injured arteries with Ad-shJAK3 treatment as shown by immunostaining of CD31 or VE-Cadherin. Bar: 50 μm. **D,** Knockdown of JAK3 induced EC proliferation (white arrow) in injured arteries as detected by immunostaining of αSMA (Green), VE-cadherin (Green), and PCNA (Red). Bar: 50 μm. Images in the red rectangle are higher magnifications of the white dot

rectangles-denoted areas in the lower magnification images. **E,** Knockdown of JAK3 by Ad-shJAK3 in SMCs impeded the suppressive effect of PDGF-BB-conditioned SMC medium on EC proliferation as measured by EdU assay. $^{*}P < 0.05$ vs control adenoviral vector-transduced cells (Ad-Ctrl) with vehicle (−) treatment; $^{\#}P < 0.05$ vs Ad-Ctrl-transduced cells with PDGF-BB treatment; n=4. **F,** Knockdown of JAK3 in SMCs impeded the suppressive effect of PDGF-BB-conditioned SMC medium on PCNA protein expression in ECs. **G,** Quantification of PCNA protein level shown in F by normalizing to α-Tubulin. $^{*}P < 0.05$ vs control adenoviral vector-transduced cells (Ad-Ctrl) with vehicle (−) treatment; $^{\#}P < 0.05$ vs Ad-Ctrl-transduced cells with PDGF-BB treatment; n=3.