NOVEL MECHANISMS REGULATING SMOOTH MUSCLE PHENOTYPE AND VASCULAR REMODELING

by

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ABSTRACT

Smooth muscle cell (SMC) proliferation and differentiation contributes to several vascular diseases, such as atherosclerosis, post-transplant vasculopathy, restenosis after angioplasty, etc. Delayed re-endothelialization and vascular repair are common results of cardiovascular intervention, such as implantation of drug-eluting stents, due to the inhibitory effect of anti-proliferative drugs on endothelial cell proliferation. Therefore, identifying novel molecular mechanisms that differentially regulate the proliferation of SMCs and ECs is important for developing new therapeutics for proper vascular repair. Objective of this study is to determine the role and mechanism of Janus kinase 3 (JAK3) and brain cytoplasmic RNA 1 (BC1) in SMC proliferation and differentiation.

JAK3 induces SMC proliferation while suppresses EC proliferation. A relatively higher expression of JAK3 in ECs than SMCs at quiescent stage is observed. However, JAK3 is induced in proliferative SMCs but decreased in proliferative ECs. Mechanistically, JAK3 promotes SMC proliferation via activation of signal transducer and activator of transcription 3 and c-Jun N-terminal kinase. Conversely, JAK3 inhibits EC proliferation via two pathways. Firstly, JAK3 regulates EC proliferation by altering the expression of pro- and anti-angiogenic factors, vascular

endothelial growth factor A and thrombospondin 1, respectively. Secondly, JAK3 arrests cell cycle progression of EC at G0/G1 stage by diminishing the stability of G1-S transition regulator, Cyclin E. In vivo, knockdown of JAK3 reduces injury-induced neointimal formation along with acceleration of re-endothelialization. Our results demonstrate that JAK3 plays an opposite role in regulating SMC and EC proliferation in response to vascular injury.

SMC differentiation is an essential process not only in vascular development, but also the progression of vascular diseases. We found that long non-coding RNA BC1 is a critical regulator for this process. Mechanistically, BC1 binds to Smad3 via RNA Smad-binding elements (rSBEs) and thus impedes TGF-β-induced Smad3 nuclear translocation, which prevents Smad3 from binding to SBE in SMC marker gene promoters. In vivo, overexpression of BC1 in mouse embryo impairs vascular SMC differentiation, causing defective artery development such as random breaks of elastic lamina, inordinate stack of SMCs, and unorganized extra cellular matrix proteins in the media of neonatal aorta. Our results suggest that BC1 is a negative regulator for SMC differentiation during vascular development.

INDEX WORDS: Smooth muscle cell, endothelial cell, neointimal formation, re-endothelialization, drug eluting stent, Janus kinase 3, platelet-derived growth factor-BB, signal transducer and activator of transcription 3, c-Jun N-terminal kinase, cell cycle, proliferating cell nuclear antigen, cyclin D, cyclin E, vascular endothelial growth factor A, thrombospondin 1, long non-coding RNA, brain cytoplasmic RNA 1, transforming growth factor-β, Smad3, RNA Smad-binding elements, Vascular remodeling, Smooth muscle cell differentiation.

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“If we knew what it was we were doing, it would not be called research, would it?” said Albert Einstein. This high-minded aphorism always shed lights on me when I was confronted experimental dilemmas.

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CHAPTER 1

INTRODUCTION

Problem Statement

Percutaneous coronary intervention has been widely used for coronary artery revascularization. Initial intervention device, balloon angioblast, have showed its limited efficacy because of the high rates of clinical restenosis, which is caused by coronary dissections, arterial recoil, neointimal formation, and negative vascular remodeling. Although the introduction of coronary stents succeeds to reduce the rate of restenosis with stabilization of vascular dissections and reduction of arterial recoil, the neointimal formation caused by smooth muscle cell (SMC) proliferation in stent remains problematic. Drug-eluting stent (DES) is developed to suppress neointimal formation by releasing anti-proliferative agents at the site of stent placed. DES successfully improves the outcomes after percutaneous coronary intervention; however, the delayed re-endothelialization due to the universally anti-proliferative effect and late stent thrombosis has become a challenge. Therefore, elucidating mechanisms controlling SMC proliferation specifically or selectively is critical for identifying more effective therapeutic targets.

Janus kinase (JAK) family is a family of non-receptor tyrosine kinases that transduce signal from transmembrane receptor to nucleus and further modulate transcription of target genes to control cell differentiation, proliferation and apoptosis. In mammalian, JAK family has four members: JAK1, JAK2, JAK3 and Tyk2. Unlike other JAKs that are ubiquitously expressed and associated with diverse cytokine receptors, JAK3 is predominantly expressed in hematopoietic cells and is induced by cytokine receptors that contain a common γ chain, such as receptors for IL-

2, IL-5, IL-7, IL-9, IL-15, and IL-21. JAK3 plays a critical role in T lymphocyte development, proliferation and differentiation. Defect of JAK3 leads to severe combined immunodeficiency (SCID) with almost absence of T lymphocytes and functionally defective B lymphocytes. Although JAK3 is mainly expressed in hematopoietic cells, its expression has also been found in non-hematopoietic cells, such as vascular cells and carcinoma. Despites of function in immune system, JAK3 mediates mucosal homeostasis and involves in the progression of many diseases, such as human colon cancer, renal fibrosis, vascular calcification, and myocardial ischemia and reperfusion injury. However, it remains unknown if JAK3 plays a role in injury-induced neointimal formation and re-endothelialization.

Differentiation of smooth muscle cells (SMCs) is not only an essential process for cardiovascular system formation in embryo, but also a critical event for vascular diseases. Disruption of this process during embryonic development causes vascular abnormalities, such as thoracic aortic aneurysms and vascular anomalies, or leads to embryo lethality. De-differentiation of SMCs, characterized by loss of SMC contractile function with higher rate of proliferation, migration, and protein synthesis, results in neointimal formation. SMC differentiation is regulated elaborately at transcriptional and translational level. In addition to protein factors, non-coding RNAs (ncRNAs), such as microRNAs and long ncRNAs (lncRNAs), also play important roles in SMC differentiation. Investigating molecular mechanisms underlying lncRNA function in SMC differentiation will advance our understanding of vascular diseases caused by SMC proliferation.

Brain cytoplasmic RNA 1 (BC1) is a cytoplasmic lncRNA derived from $tRNA^{Ala}$ molecule and mainly presents in specific subset of neurons of the central and peripheral nervous system in rodents. BC200 RNA is the analog of BC1 in primates with similar function and expression pattern. BC1-deficient mice show reduced exploratory activity along with increased anxiety, and

increased seizure susceptibility although there is no observed anatomical or neurological abnormality. In human, BC200 plays a role in tumorigenesis and neurodegeneration. The elevated RNA expression level of BC200 has been detected in different cancer tissues. In breast cancer, BC200 contributes to the progression of tumorigenesis via regulating the survival of tumor cells. In addition to cancer, BC200 RNA expression is increased in brains with Alzheimer's disease (AD), a chronic neurodegenerative disease, and presents a correlation with AD progression. Since vascular and neural systems share similar anatomic localization, structural formation process, and signaling molecules for developmental regulation, and BC1 serves as an important regulator for neural plasticity, we sought to determine whether BC1 plays a role in vascular development.

Objective, Hypotheses and Specific Aims

Our goal is to identify a new therapeutic target, RNA or protein, for specifically inhibiting SMC proliferation, because the current drugs applied on stents inhibit proliferation of both SMCs and ECs, which causes delayed vascular recovery and late thrombosis, the major complications after angioplasty and stent implantation. Ideally, with inhibition of the target gene function or expression, SMC proliferation is suppressed while EC proliferation is not affected or is enhanced. Our studies suggest that Janus kinase 3 (JAK3), a non-receptor tyrosine kinase, plays differential role in regulating proliferation of SMCs and ECs. Therefore, we hypothesize that targeting JAK3 may inhibit injury-induced neointimal formation while enhance re-endothelialization.

Specific Aim 1 tests the hypothesis that JAK3 regulates SMC proliferation and injury-induced neointimal formation. We will determine if JAK3: 1) mediates PDGF-BB-induced SMC proliferation; 2) regulates SMC phenotypic modulation; 3) involves in balloon-

injury neointimal formation. We also identify the underlying mechanisms underlying JAK3 regulation of SMC proliferation.

Specific Aim 2 tests the hypothesis that knockdown of JAK3 promotes arterial repair via enhancing re-endothelialization. We will determine if JAK3: 1) mediates EC proliferation; 2) regulates EC migration; 3) is involved in re-endothelialization. We also identify the mechanisms underlying JAK3 regulation of EC proliferation and migration.

It has been shown that SMC has plasticity, characterized by de-differentiation during the progression of vascular diseases. In addition to protein factors, non-coding RNAs (ncRNAs), such as microRNAs and long ncRNAs (lncRNAs), also play important roles in regulation of SMC differentiation. Brain cytoplasmic RNA 1 (BC1) is a cytoplasmic lncRNA derived from $tRNA^{Ala}$ molecule and mainly presents in specific subset of neurons of the central and peripheral nervous system in rodents. BC1 serves as an important regulator for neural plasticity. Since vascular and neural systems share similar anatomic localization, structural formation process, and signaling molecules for developmental regulation, we sought to determine whether BC1 plays a role in SMC differentiation.

Specific Aim 3 tests the hypothesis that BC1 regulates SMC differentiation and vascular development. We will determine if BC1: 1) mediates TGF-β-induced SMC differentiation; 2) is involved in SMC fate determination in vascular system; 3) is involved in vascular development. We also identify the mechanisms underlying BC1 regulation of SMC differentiation.

CHAPTER 2

LITERATURE REVIEW

Abstract

Atherosclerosis-related disease has become the leading cause of death among all cardiovascular diseases. Although drugs with reduced side effects have been developed for local administration at the lesion site, the current obstacle for the atherosclerosis treatment in clinical field are in-stent restenosis (ISR) and very late thrombosis (VLT). Proliferation of smooth muscle cells (SMCs) is the major cause in ISR development. Besides, the delayed endothelium repair due to non-specific anti-proliferative drugs leads to late thrombosis formation. To prevent ISR and VLT, it is important to develop a drug specifically or selectively targets on smooth muscle cells (SMCs).

Evolution of Clinical Intervention

Occlusion of blood vessels tends to limit the blood supply and further leading to tissue death. Partial occlusion in coronary artery may have patients suffered from angina. With more percentage of occlusion in coronary artery, it may lead to myocardial infraction (MI) and further sudden cardiac death. Percutaneous transluminal coronary angioplasty (PTCA) is the primary medical procedure for coronary artery revascularization in modern day. In 1979, PTCA was firstly addressed by Grüntzig, and showed its efficacy on vascular restoration. With a mean follow-up time of nine months, thirty-one patients with successful medical procedure out of fifty total patients had improved their functional status (Class I-IV) by one to two grades.[1] However, few following studies indicated the process of restenosis begins early after successful angioplasty.[2, 3] There are

few mechanisms involve in causing restenosis after PTCA, including elastic recoil, reorganization of thrombus, neointimal formation, and negative vascular remodeling. Reorganization of thrombus is induced by platelet deposition at the site of endothelial denudation after angioplasty.[4] Neointimal formation is the result of platelet-derived growth factor (PDGF)-induced proliferation of medial smooth muscle cells.[5, 6] Thrombus formation is the result of platelet activation/ aggregation which was initiated by endothelial denudation and medial dissection after angioplasty.[7] Ischemic events, one of complications of PTCA, occur greatest immediately after surgery are mainly because of platelet-dependent thrombus formation.[7, 8] In IMPACT-II (Integrilin to Minimize Platelet Aggregation and Coronary Thrombosis-II) trial, there was 407 patients (10.01%) out of 4001 total patients suffered from ischemic events after angioplasty. Among of these ischemic patients, 66% of events occurred within 6 hours after angioplasty.[9] Although pretreatment with antiplatelet drug reduces ischemic events, the long-term oral administration of antiplatelet therapy after PTCA is necessary to lower the risk of atherothrombosis. PCI-CURE (Clopidogrel in Unstable angina to prevent Recurrent ischaemic Events) showed pretreatment with clopidogrel and aspirin followed by long-term oral administration of alopidogrel after PTCA reduced the rate of cardiovascular death, myocardial infraction, and any revascularization.[10] Despite the beneficial effects of long-term treatment with antiplatelet therapy after angioplasty, there remains adverse effects in cardiovascular events. Short term intravenous administration of platelet glycoprotein IIb/IIIa inhibitors is beneficial to reduce clinic outcomes;[11] however, long term oral application showed a toxic effect leading to increase of mortality.[12] Prolonged duration of dual antiplatelet therapy (DAPT), defined as use of both aspirin and a P2Y12 receptor inhibitor, was associated with increased risk of TIMI (Thrombolysis in Myocardial Infarction) major bleeding.[13, 14] Implanting coronary stent, bare-metal stent (BMS), after PTCA showed noninferiority of primary outcome

with lower percentage of medial dissection, elastic recoil and larger luminal diameter. At follow-up time of six months, angiography showed there is no dramatic difference to the percentage of luminal diameter; however, the incidence of restenosis (in-stent restenosis, ISR) was decreased 10% compared to the group without stent implantation (31.6% vs. 42.1%).[15] Four patterns of ISR was introduced based on angiography, they are focal ISR (≤10 mm in length), diffuse ISR (>10 mm in length but within the stent), proliferative ISR (>10 mm in length extending outside the stent) and total occlusion.[16] Prior studies showed that ISR is majorly caused by intimal hyperplasia without occurring of recoil or arterial constriction, and it tends to be distributed beyond the margin of stents.[17] Drug-eluting stents (DES) showed its efficacy on reducing incidence of restenosis and repeat revascularization through continuously local release of anti-proliferative agents.[18, 19] DES are composed of three components: a metallic stent platform, a polymer-based drug delivery platform, and a pharmacologic agent (majorly are immunosuppressant or anti-proliferative compound). Two common anti-proliferative compounds for first generation DES are sirolimus (also known as rapamycin), impeding cell cycle progression by blocking G1 to S phase transition, and paclitaxel, arresting cell division. Although sirolimus-eluting stent (SES) and paclitaxel-eluting stent (PES) showed a marked reduction in restenosis with lower rate of major adverse cardiac events (such as myocardial ischemia),[20, 21] they have been associated with an increased risk of late stent thrombosis (LST).[22-24] Stent thrombosis is classified by the Academic Research Consortium (ARC) as definite, probable, or possible and as early (occurring within 30 days), late (occurring between 1 month and 1 year), or very late (occurring beyond 1 year).[25] The precise cause of stent thrombosis remains unclear, but it might be due to a local pro-inflammatory reaction, induced by polymer coating on the DES, or the suppression of normal endothelial healing process because of the toxic effect of the stent drug. Evidently, first generation DES with higher rate of

LST failed to complete re-endothelialization even at 40 months although BMS showed a full re-endothelialization at 7 months.[22] Second generation DES was thus developed and improved with thinner stent strut, enhanced polymer biocompatibility, and drug elution profiles to optimize re-endothelialization. Zotarolimus and everolimus are two major anti-proliferative compounds in second generation DES. In Cassese' study, angiography among 10,004 patients with 6-8 months' follow-up showed second generation DES improved the rate of restenosis.[26] ENDEAVOR III and IV trials showed Zotarolimus-eluting stent (ZES) meet goal of non-inferiority in 12-month outcome compared to first generation DES.[27, 28] With following study, 5-year outcome showed ZES had improved incidence of cardiac death, myocardial infraction, and target lesion revascularization (TLR); however, the overall rates of definite/probable stent thrombosis are similar between ZES and first generation DES although ZES has lower rate of very late thrombosis compared to PES.[29, 30] In Ra¨ber's study, with up to 4 years' follow-up time window, Everolimus -eluting stent (EES) declined the incidence of early, late, and very late stent thrombosis compared to first generation DES.[31] Overall, both ZES and EES provide a similar efficacy of improving primary outcome compared with SES, but are more effective than PES; however, only EES significantly reduced the stent thrombosis events and therefore serve as the safest DES nowadays.[32] In recent year, drug-coated balloon (DCB) have become an alternative therapeutic strategy for vascular reconstruction in femoral artery diseases, coronary de novo lesions, and in-stent restenosis.[33, 34] Paclitaxel coated balloon showed its efficacy in restenosis inhibition after coronary angioplasty in porcine model.[33] In 2006, first application of DCB in human showed a significantly reduction of restenosis incidence among 52 patients with ISR. Follow-up angiography at 6 months, it showed less late luminal loss (LLL) in paclitaxel-coated balloon (PCB) group (0.03 ± 0.48 mm vs 0.74 ± 0.86 in uncoated-balloon group). At 12 months, the rate of major

adverse cardiac events (MACE) was reduced in PCB group (4% vs 31% in uncoated-balloon group).[35] Following clinical study showed PCB persistently reduces repeat revascularization in treating patients with drug-eluting stents in-stent restenosis (DES-ISR) up to 2 years. PEPCAD-DES trial demonstrated the efficacy of PCB in patients with DES-ISR at 3 years. At third year, the rates of TLR (19.4% vs 36.8% in uncoated-balloon group) and MACE (20.8% vs 52.6% in uncoated-balloon group) were significantly reduced in PCB group.[36] Although the increasing evidences to show the benefits of PCB in treating ISR, its therapeutic role in *de novo* coronary lesions remains uncertain. The first meta-analysis for assessing the efficacy of PCB to treat *de novo* coronary lesions in clinical showed PCB were not superior to current widely used interventional therapy, DES and BMS.[37] Besides, in PICCOLETO study, PCB failed to show noninferiority to DES in treating small coronary vessels with higher diameter stenosis percentage (43.6% vs 24.3%) and rate of restenosis (32.1% vs 10.3%).[38] Despite the state of the art interventional therapy improves the surgical outcome, the post-surgical rate of restenosis incidence, myocardial ischemia, and the need for repeat revascularization still remains space to be progressed.

Animal Model Used in Study

Animal models have long been used to observe diseases development morphologically and physiologically. Nowadays, animal models are mostly developed to evaluate therapeutic target in molecular level which can be more specific or selective in treating cardiovascular diseases, such as atherosclerosis, systemic hypertension, aortic aneurysm and in-stent restenosis. There are many developed animal models available for coronary artery restenosis studies. In general, animal models like rats or mice are popular options due to their shorter life span, less cost and easier

operation; rabbits, dogs, pigs or nonhuman primates are also crucial models regardless of its longer study window and comparatively difficult to maintain. In studying restenosis and testing the efficacies of candidate drugs, every animal model is chosen for its own characteristics of arterial morphology and physiological responses to injury. Although rats are less susceptible to atherosclerosis and morphologically different from human artery,[39, 40] they are still considered the most studied animal model in arterial wall because they are relatively inexpensive to maintain, easily genetic manipulated, most inbred strains, predictable and rapid response to injury, and also can be seen disease development in a shorter period of time. In comparison, rabbits have higher susceptibility to develop intimal lesion under certain conditions,[41] and fewer genetic modifications. A well-known strain is Watanabe hereditary hypercholesterolemic (WHHL) rabbit with a defect in the LDLR, and this model is generally conducted in studying atherosclerosis development. Recent studies show that larger animal models are more suitable for preclinical trials due to their similarity to humans in cellular and metabolic levels.[42-44] This trait has enhanced a better chance in testing disease prevention therapies. Among larger animal models, pigs have shed lights on human disease development for a long time. Pigs are expensive to maintain and have no genetic modifications so far, but they possess human-like lipoprotein and similar injury response to human. Specifically, pig coronary model was developed to evaluate stent progression,[45] while rat carotid artery balloon injury model is mostly done for balloon denudation. Compared to all other larger animal models, nonhuman primates have longest life span, longer onset of fertility with much higher maintenance expense and limited genetic information, but their humanoid lipoprotein metabolism makes them a good choice for observation of coronary lesions development, especially coronary diseases have interacted with other chronic disease.[46] Other considerations like growth stages or inbred strains are also taken into account in age-related or diet-related diseases.[47-49]

Overall, animal models have played an important role in translating laboratories findings into clinical applications. Therefore, based on various purposes of experiments, fully understanding the limitation and advantages of every animal models will facilitate therapies advancement.

PDGF-BB Signaling in Regulating Restenosis

Artery stenosis after angioplasty had been shown to be associated with the local proliferation of smooth muscle cells (SMCs) in human.[5] Smooth muscle cells within adult animals are not terminally differentiated and remain its plasticity to undergo reversibly phenotypic change in response to environmental stimuli, such as vascular injury, reactive oxygen species, and mechanical stretch.[50] Normal adult vascular SMCs are in the quiescent stage (G_0) of cell cycle with low rate of proliferation, non-migratory status, and low rate of extraocular matrix synthesis. However, quiescent SMCs have high expression of contractile proteins, ion channels, calcium regulatory proteins, and proteins necessary for its contractile abilities. Upon vascular injury, quiescent SMCs undergoes phenotypic switching to highly synthetic phenotype for playing a role in vascular repair.[51] Culture arterial SMCs was first found can proliferate in whole blood serum; however, the serum derived from platelet-poor plasma failed to induce SMCs proliferation.[6] In later study, by administration of anti-platelets antibody into rat after vascular injury it showed the deceased intimal lesion formation in 7-day post injury. However, the proliferative rate of medial SMCs has no difference in absence of platelets.[52] These results indicated platelet-derived growth factor (PDGF) is essential for SMCs migration but sufficient for proliferation. Another possibility is the compensatory effect of other cells, such as local SMCs. Indeed, SMCs can express PDGF in response to vascular injury.[53, 54] Platelet-derived growth factor (PDGF) is formed as a disulfide-linked dimer, either homo- or hetero-, from different polypeptide chains (A, B, C, and D). PDGF

is not expressed in vascular endothelial cells (ECs) and SMCs at normal; however, its expression is induced in activated ECs and SMCs during atherosclerotic lesion development and artery injury. Activated macrophage also express PDGF in advanced atherosclerotic lesions.[55] PDGF receptor (PDGFR) are cell membrane tyrosine kinase receptor, and has two forms- alpha (PDGFR-α) and beta (PDGFR-β). Dimerization of PDGFR is the critical event for signal activation.[56] In vivo, PDGF-AA and PDGF-BB is recognized by PDGFR-α, while PDGF-BB is recognized by PDGFR-β. PDGF-BB is the major growth factor responsible for injury-induced SMCs migration and proliferation resulting in neointimal formation.[57, 58] Besides, integrins can also interact with PDGFR to enhance cell proliferation and migration.[59] PDGF intracellular signal transduction has been well established. PDGF signaling begins at dimerization of PDGFR and auto-phosphorylation of tyrosine residue inside the kinase domain (Tyr-849 in α-receptor; Tyr-857 in β-receptor). The activated kinase domain creates a docking site for signal transduction molecules containing Src homology 2 (SH2) or phosphotyrosine binding (PTB) domain. These signal transduction molecules include phosphatidylinositol 3'-kinase (PI3K), Src family of tyrosine kinases, tyrosine phosphatase SHP-2, and GTPase activating protein (GAP) for Ras, and growth factor receptor-bound protein 2 (Grb2). Activated PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to form phosphatidylinositol 3,4,5-trisphosphate (PIP3), which serve as secondary messenger to activate Ser/Thr kinases PDK1 and Akt. Activation of Akt leads to inhibition of FoxO1/3, which has inhibitory effect on cell cycle progression, for further stimulation of cell proliferation. Grb2, an adaptor molecule, binds to a guanine nucleotide exchange factors, son of sevenless (SOS), for activating the Ras/Raf/MEK/ERK signaling pathway. Activated ERK is responsible for regulation of multiple transcription factors related to cell proliferation and differentiation. SAPK/JNK and p38 MAP kinase signaling also serve a key role in cell proliferation

regulated by PDGF-BB signaling.[60, 61] De-differentiation of SMCs by PDGF-BB with a decrease of contractile-related protein (SMMHC, calponin, SM22α, and αSMA) expression is also important to vascular diseases, such as in-stent restenosis, aortic aneurysm, and atherosclerosis.[62, 63] Serum response factor (SRF) regulates SMC contractile-related protein expression through binding to $CC(A/T)_6GG$ (CArG) elements upstream of their promoters. Coactivator, myocardin and myocardin-related transcription factors A and B (MRTF-A and -B), are essential for SRF-dependent gene regulation. PDGF-BB induces Ras/Raf/MEK/ERK signaling leading to phosphorylation of ETS domain-containing protein-1 (Elk-1). The phosphorylated Elk-1 disrupts the interaction between myocardin and SRF to further repress the gene expression. Moreover, Kruppel-like factor-4 (KLF4), a transcription factor, also shows its effect on regulating SRF-mediated gene expression in SMCs. In normal adult SMCs, there is no detectible expression of KLF4; however, PDGF-BB induces expression of KLF4 in SMCs. Induced KLF4 suppress the SRF-mediated gene expression via multiple ways, including disruption of SRF-myocardin complex, dissociation of SRF from CArG elements, and downregulation of myocardin. SMC proliferation in response to injury after angioplasty is the key events leading subsequent restenosis. Current prevalent anti-proliferative drugs, paclitaxel and sirolimus, used in DES showed their effect on reducing incidence of restenosis. However, the increasing rate of very late thrombosis due to impaired re-endothelialization has become a major problem in modern clinical applications.[64] The reason causes impaired re-endothelialization is believed because of the universal anti-proliferative effect of current drugs. To improve outcome of current therapeutic intervention, specific or selective anti-proliferative drug on SMC is required. In 1995, Asahara group showed the potential therapeutic role of vascular endothelial growth factor (VEGF). It showed with local delivery of VEGF suppressed the neointimal formation via enhancing re-

endothelialization.[65] However, later study on rabbit iliac artery injury model showed there was no beneficial effect with local delivery of VEGF by DES on re-endothelialization, nor on neointimal formation.[66] Walter group showed an acceleration of re-endothelialization with lower neointimal formation in rabbit iliac artery injury model via gene-eluting stents carrying VEGF expression vector.[67] Kuopio Angiogenesis Trial (KAT) trial showed a promising effect of VEGF gene transfer in prevention of restenosis and improvement of myocardial perfusion.[68] Delivery of endothelial nitric oxide synthase (eNOS) by viral or non-viral means in rabbit iliac artery injury model showed an acceleration of re-endothlialization with inhibition of restenosis.[69, 70] Recent study showed delivery of eNOS via liposome-based system failed to reduce restenosis in a rabbit hypercholesterolemic iliac artery injury model although it enhanced the re-endothelialization.[71] There are also other molecules showing their potential effect on enhancing re-endothelialization while suppressing SMC proliferation was mentioned in our previous review article.[72]

Conclusion

Second generation DES with everolimus have shown its efficacy in reducing rate of ISR and VLT, and DCB is also a good alternative way for those smaller vessels which cannot be performed with DES. The translational issue from laboratories to clinical may not be solved in a short time, but it can be improved by an appropriated animal model study. Identification of more specific or selective anti-proliferative drugs have shown its importance in reduction of ISR and VLT in this field.

Reference

1. Gruntzig AR, Senning A and Siegenthaler WE. Nonoperative dilatation of coronary-artery stenosis: percutaneous transluminal coronary angioplasty. *N Engl J Med*. 1979;301:61-8.
2. Serruys PW, Luijten HE, Beatt KJ, Geuskens R, de Feyter PJ, van den Brand M, Reiber JH, ten Katen HJ, van Es GA and Hugenholtz PG. Incidence of restenosis after successful coronary angioplasty: a time-related phenomenon. A quantitative angiographic study in 342 consecutive patients at 1, 2, 3, and 4 months. *Circulation*. 1988;77:361-71.
3. Wijns W, Serruys PW, Reiber JH, de Feyter PJ, van den Brand M, Simoons ML and Hugenholtz PG. Early detection of restenosis after successful percutaneous transluminal coronary angioplasty by exercise-redistribution thallium scintigraphy. *Am J Cardiol*. 1985;55:357-61.
4. Wilentz JR, Sanborn TA, Haudenschild CC, Valeri CR, Ryan TJ and Faxon DP. Platelet accumulation in experimental angioplasty: time course and relation to vascular injury. *Circulation*. 1987;75:636-42.
5. Austin GE, Ratliff NB, Hollman J, Tabei S and Phillips DF. Intimal proliferation of smooth muscle cells as an explanation for recurrent coronary artery stenosis after percutaneous transluminal coronary angioplasty. *J Am Coll Cardiol*. 1985;6:369-75.
6. Rutherford RB and Ross R. Platelet factors stimulate fibroblasts and smooth muscle cells quiescent in plasma serum to proliferate. *J Cell Biol*. 1976;69:196-203.
7. Furie B and Furie BC. Mechanisms of thrombus formation. *N Engl J Med*. 2008;359:938-49.
8. Harker LA. Role of platelets and thrombosis in mechanisms of acute occlusion and restenosis after angioplasty. *Am J Cardiol*. 1987;60:20B-28B.

9. Thel MC, Califf RM, Tardiff BE, Gardner LH, Sigmon KN, Lincoff AM, Topol EJ, Kitt MM, Blankenship JC and Tcheng JE. Timing of and risk factors for myocardial ischemic events after percutaneous coronary intervention (IMPACT-II). Integrilin to Minimize Platelet Aggregation and Coronary Thrombosis. *Am J Cardiol*. 2000;85:427-34.
10. Mehta SR, Yusuf S, Peters RJ, Bertrand ME, Lewis BS, Natarajan MK, Malmberg K, Rupprecht H, Zhao F, Chrolavicius S, Copland I, Fox KA and Clopidogrel in Unstable angina to prevent Recurrent Events trial I. Effects of pretreatment with clopidogrel and aspirin followed by long-term therapy in patients undergoing percutaneous coronary intervention: the PCI-CURE study. *Lancet*. 2001;358:527-33.
11. Kong DF, Califf RM, Miller DP, Moliterno DJ, White HD, Harrington RA, Tcheng JE, Lincoff AM, Hasselblad V and Topol EJ. Clinical outcomes of therapeutic agents that block the platelet glycoprotein IIb/IIIa integrin in ischemic heart disease. *Circulation*. 1998;98:2829-35.
12. Chew DP, Bhatt DL, Sapp S and Topol EJ. Increased mortality with oral platelet glycoprotein IIb/IIIa antagonists: a meta-analysis of phase III multicenter randomized trials. *Circulation*. 2001;103:201-6.
13. Zhang L, Li Y, Jing QM, Wang XZ, Ma YY, Wang G, Xu B, Gao RL, Han YL and Investigators C. Dual antiplatelet therapy over 6 months increases the risk of bleeding after biodegradable polymer-coated sirolimus eluting stents implantation: insights from the CREATE study. *J Interv Cardiol*. 2014;27:119-26.
14. Pandit A, Giri S, Hakim FA and Fortuin FD. Shorter (</=6 months) versus longer (>/=12 months) duration dual antiplatelet therapy after drug eluting stents: a meta-analysis of randomized clinical trials. *Catheter Cardiovasc Interv*. 2015;85:34-40.

15. Fischman DL, Leon MB, Baim DS, Schatz RA, Savage MP, Penn I, Detre K, Veltri L, Ricci D, Nobuyoshi M and et al. A randomized comparison of coronary-stent placement and balloon angioplasty in the treatment of coronary artery disease. Stent Restenosis Study Investigators. *N Engl J Med*. 1994;331:496-501.
16. Mehran R, Dangas G, Abizaid AS, Mintz GS, Lansky AJ, Satler LF, Pichard AD, Kent KM, Stone GW and Leon MB. Angiographic patterns of in-stent restenosis: classification and implications for long-term outcome. *Circulation*. 1999;100:1872-8.
17. Hoffmann R, Mintz GS, Dussaillant GR, Popma JJ, Pichard AD, Satler LF, Kent KM, Griffin J and Leon MB. Patterns and mechanisms of in-stent restenosis. A serial intravascular ultrasound study. *Circulation*. 1996;94:1247-54.
18. Sousa JE, Costa MA, Abizaid AC, Rensing BJ, Abizaid AS, Tanajura LF, Kozuma K, Van Langenhove G, Sousa AG, Falotico R, Jaeger J, Popma JJ and Serruys PW. Sustained suppression of neointimal proliferation by sirolimus-eluting stents: one-year angiographic and intravascular ultrasound follow-up. *Circulation*. 2001;104:2007-11.
19. Moses JW, Leon MB, Popma JJ, Fitzgerald PJ, Holmes DR, O'Shaughnessy C, Caputo RP, Kereiakes DJ, Williams DO, Teirstein PS, Jaeger JL, Kuntz RE and Investigators S. Sirolimus-eluting stents versus standard stents in patients with stenosis in a native coronary artery. *N Engl J Med*. 2003;349:1315-23.
20. Grube E, Silber S, Hauptmann KE, Mueller R, Buellesfeld L, Gerckens U and Russell ME. TAXUS I: six- and twelve-month results from a randomized, double-blind trial on a slow-release paclitaxel-eluting stent for de novo coronary lesions. *Circulation*. 2003;107:38-42.
21. Schofer J, Schluter M, Gershlick AH, Wijns W, Garcia E, Schampaert E, Breithardt G and Investigators ES. Sirolimus-eluting stents for treatment of patients with long atherosclerotic

lesions in small coronary arteries: double-blind, randomised controlled trial (E-SIRIUS). *Lancet*. 2003;362:1093-9.

22. Joner M, Finn AV, Farb A, Mont EK, Kolodgie FD, Ladich E, Kutys R, Skorija K, Gold HK and Virmani R. Pathology of drug-eluting stents in humans: delayed healing and late thrombotic risk. *J Am Coll Cardiol*. 2006;48:193-202.
23. Stone GW, Moses JW, Ellis SG, Schofer J, Dawkins KD, Morice MC, Colombo A, Schampaert E, Grube E, Kirtane AJ, Cutlip DE, Fahy M, Pocock SJ, Mehran R and Leon MB. Safety and efficacy of sirolimus- and paclitaxel-eluting coronary stents. *N Engl J Med*. 2007;356:998-1008.
24. Takano M, Yamamoto M, Xie Y, Murakami D, Inami S, Okamatsu K, Seimiya K, Ohba T, Seino Y and Mizuno K. Serial long-term evaluation of neointimal stent coverage and thrombus after sirolimus-eluting stent implantation by use of coronary angioscopy. *Heart*. 2007;93:1533-6.
25. Holmes DR, Jr., Kereiakes DJ, Garg S, Serruys PW, Dehmer GJ, Ellis SG, Williams DO, Kimura T and Moliterno DJ. Stent thrombosis. *J Am Coll Cardiol*. 2010;56:1357-65.
26. Cassese S, Byrne RA, Tada T, Pinieck S, Joner M, Ibrahim T, King LA, Fusaro M, Laugwitz KL and Kastrati A. Incidence and predictors of restenosis after coronary stenting in 10 004 patients with surveillance angiography. *Heart*. 2014;100:153-9.
27. Kandzari DE, Leon MB, Popma JJ, Fitzgerald PJ, O'Shaughnessy C, Ball MW, Turco M, Applegate RJ, Gurbel PA, Midei MG, Badre SS, Mauri L, Thompson KP, LeNarz LA, Kuntz RE and Investigators EI. Comparison of zotarolimus-eluting and sirolimus-eluting stents in patients with native coronary artery disease: a randomized controlled trial. *J Am Coll Cardiol*. 2006;48:2440-7.

28. Leon MB, Mauri L, Popma JJ, Cutlip DE, Nikolsky E, O'Shaughnessy C, Overlie PA, McLaurin BT, Solomon SL, Douglas JS, Jr., Ball MW, Caputo RP, Jain A, Tolleson TR, Reen BM, 3rd, Kirtane AJ, Fitzgerald PJ, Thompson K, Kandzari DE and Investigators EI. A randomized comparison of the Endeavor zotarolimus-eluting stent versus the TAXUS paclitaxel-eluting stent in de novo native coronary lesions 12-month outcomes from the ENDEAVOR IV trial. *J Am Coll Cardiol*. 2010;55:543-54.
29. Kandzari DE, Mauri L, Popma JJ, Turco MA, Gurbel PA, Fitzgerald PJ and Leon MB. Late-term clinical outcomes with zotarolimus- and sirolimus-eluting stents. 5-year follow-up of the ENDEAVOR III (A Randomized Controlled Trial of the Medtronic Endeavor Drug [ABT-578] Eluting Coronary Stent System Versus the Cypher Sirolimus-Eluting Coronary Stent System in De Novo Native Coronary Artery Lesions). *JACC Cardiovasc Interv*. 2011;4:543-50.
30. Kirtane AJ, Leon MB, Ball MW, Bajwa HS, Sketch MH, Jr., Coleman PS, Stoler RC, Papadakos S, Cutlip DE, Mauri L, Kandzari DE and Investigators EI. The "final" 5-year follow-up from the ENDEAVOR IV trial comparing a zotarolimus-eluting stent with a paclitaxel-eluting stent. *JACC Cardiovasc Interv*. 2013;6:325-33.
31. Raber L, Magro M, Stefanini GG, Kalesan B, van Domburg RT, Onuma Y, Wenaweser P, Daemen J, Meier B, Juni P, Serruys PW and Windecker S. Very late coronary stent thrombosis of a newer-generation everolimus-eluting stent compared with early-generation drug-eluting stents: a prospective cohort study. *Circulation*. 2012;125:1110-21.
32. Navarese EP, Kowalewski M, Kandzari D, Lansky A, Gorny B, Koltowski L, Waksman R, Berti S, Musumeci G, Limbruno U, van der Schaaf RJ, Kelm M, Kubica J and Suryapranata H. First-generation versus second-generation drug-eluting stents in current clinical practice:

updated evidence from a comprehensive meta-analysis of randomised clinical trials comprising 31 379 patients. *Open Heart*. 2014;1:e000064.

33. Scheller B, Speck U, Abramjuk C, Bernhardt U, Bohm M and Nickenig G. Paclitaxel balloon coating, a novel method for prevention and therapy of restenosis. *Circulation*. 2004;110:810-4.
34. Tepe G, Zeller T, Albrecht T, Heller S, Schwarzwalder U, Beregi JP, Claussen CD, Oldenburg A, Scheller B and Speck U. Local delivery of paclitaxel to inhibit restenosis during angioplasty of the leg. *N Engl J Med*. 2008;358:689-99.
35. Scheller B, Hehrlein C, Bocksch W, Rutsch W, Haghi D, Dietz U, Bohm M and Speck U. Treatment of coronary in-stent restenosis with a paclitaxel-coated balloon catheter. *N Engl J Med*. 2006;355:2113-24.
36. Scheller B, Hehrlein C, Bocksch W, Rutsch W, Haghi D, Dietz U, Bohm M and Speck U. Two year follow-up after treatment of coronary in-stent restenosis with a paclitaxel-coated balloon catheter. *Clin Res Cardiol*. 2008;97:773-81.
37. Frohlich GM, Lansky AJ, Ko DT, Archangelidi O, De Palma R, Timmis A and Meier P. Drug eluting balloons for de novo coronary lesions - a systematic review and meta-analysis. *BMC Med*. 2013;11:123.
38. Cortese B, Micheli A, Picchi A, Coppolaro A, Bandinelli L, Severi S and Limbruno U. Paclitaxel-coated balloon versus drug-eluting stent during PCI of small coronary vessels, a prospective randomised clinical trial. The PICCOLETO study. *Heart*. 2010;96:1291-6.
39. Sims FH. A comparison of structural features of the walls of coronary arteries from 10 different species. *Pathology*. 1989;21:115-24.

40. Paigen B, Morrow A, Brandon C, Mitchell D and Holmes P. Variation in susceptibility to atherosclerosis among inbred strains of mice. *Atherosclerosis*. 1985;57:65-73.
41. Lacko AG, Rutenberg HL and Soloff LA. Serum cholesterol esterification in species resistant and susceptible to atherosclerosis. *Atherosclerosis*. 1974;19:297-305.
42. Kastellorizios M, Papadimitrakopoulos F and Burgess DJ. Prevention of foreign body reaction in a pre-clinical large animal model. *J Control Release*. 2015;202:101-7.
43. Shim J, Al-Mashhadi RH, Sorensen CB and Bentzon JF. Large animal models of atherosclerosis--new tools for persistent problems in cardiovascular medicine. *J Pathol*. 2016;238:257-66.
44. Tsang HG, Rashdan NA, Whitelaw CB, Corcoran BM, Summers KM and MacRae VE. Large animal models of cardiovascular disease. *Cell Biochem Funct*. 2016;34:113-32.
45. Suzuki Y, Yeung AC and Ikeno F. The representative porcine model for human cardiovascular disease. *J Biomed Biotechnol*. 2011;2011:195483.
46. Getz GS and Reardon CA. Animal models of atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2012;32:1104-15.
47. Drechsler S, Lynch MA, Novella S, Gonzalez-Navarro H, Hecimovic S, Barini E, Tucci V, Castro RE, Vandenbroucke RE, Osuchowski M and Potter PK. With mouse age comes wisdom: A review and suggestions of relevant mouse models for age-related conditions. *Mech Ageing Dev*. 2016;160:54-68.
48. Mirzaei H, Di Biase S and Longo VD. Dietary Interventions, Cardiovascular Aging, and Disease: Animal Models and Human Studies. *Circ Res*. 2016;118:1612-25.

49. Tsihlis ND, Rodriguez MP, Jiang Q, Schwartz A, Flynn ME, Vercammen JM and Kibbe MR. Nitric oxide differentially affects proteasome activator 28 after arterial injury in type 1 and type 2 diabetic rats. *J Surg Res*. 2016;202:413-21.
50. Owens GK. Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev*. 1995;75:487-517.
51. Owens GK, Kumar MS and Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev*. 2004;84:767-801.
52. Fingerle J, Johnson R, Clowes AW, Majesky MW and Reidy MA. Role of platelets in smooth muscle cell proliferation and migration after vascular injury in rat carotid artery. *Proc Natl Acad Sci U S A*. 1989;86:8412-6.
53. Libby P, Warner SJ, Salomon RN and Birinyi LK. Production of platelet-derived growth factor-like mitogen by smooth-muscle cells from human atheroma. *N Engl J Med*. 1988;318:1493-8.
54. Majesky MW, Reidy MA, Bowen-Pope DF, Hart CE, Wilcox JN and Schwartz SM. PDGF ligand and receptor gene expression during repair of arterial injury. *J Cell Biol*. 1990;111:2149-58.
55. Heldin CH and Westermark B. Mechanism of action and in vivo role of platelet-derived growth factor. *Physiol Rev*. 1999;79:1283-316.
56. Kelly JD, Haldeman BA, Grant FJ, Murray MJ, Seifert RA, Bowen-Pope DF, Cooper JA and Kazlauskas A. Platelet-derived growth factor (PDGF) stimulates PDGF receptor subunit dimerization and intersubunit trans-phosphorylation. *J Biol Chem*. 1991;266:8987-92.

57. Jawien A, Bowen-Pope DF, Lindner V, Schwartz SM and Clowes AW. Platelet-derived growth factor promotes smooth muscle migration and intimal thickening in a rat model of balloon angioplasty. *J Clin Invest*. 1992;89:507-11.
58. Millette E, Rauch BH, Defawe O, Kenagy RD, Daum G and Clowes AW. Platelet-derived growth factor-BB-induced human smooth muscle cell proliferation depends on basic FGF release and FGFR-1 activation. *Circ Res*. 2005;96:172-9.
59. Assoian RK. Anchorage-dependent cell cycle progression. *J Cell Biol*. 1997;136:1-4.
60. Zhan Y, Kim S, Yasumoto H, Namba M, Miyazaki H and Iwao H. Effects of dominant-negative c-Jun on platelet-derived growth factor-induced vascular smooth muscle cell proliferation. *Arterioscler Thromb Vasc Biol*. 2002;22:82-8.
61. Zhan Y, Kim S, Izumi Y, Izumiya Y, Nakao T, Miyazaki H and Iwao H. Role of JNK, p38, and ERK in platelet-derived growth factor-induced vascular proliferation, migration, and gene expression. *Arterioscler Thromb Vasc Biol*. 2003;23:795-801.
62. Holycross BJ, Blank RS, Thompson MM, Peach MJ and Owens GK. Platelet-derived growth factor-BB-induced suppression of smooth muscle cell differentiation. *Circ Res*. 1992;71:1525-32.
63. Vukovic I, Arsenijevic N, Lackovic V and Todorovic V. The origin and differentiation potential of smooth muscle cells in coronary atherosclerosis. *Exp Clin Cardiol*. 2006;11:123-8.
64. Stefanini GG and Holmes DR, Jr. Drug-eluting coronary-artery stents. *N Engl J Med*. 2013;368:254-65.
65. Asahara T, Bauters C, Pastore C, Kearney M, Rossow S, Bunting S, Ferrara N, Symes JF and Isner JM. Local delivery of vascular endothelial growth factor accelerates reendothelialization

and attenuates intimal hyperplasia in balloon-injured rat carotid artery. *Circulation*. 1995;91:2793-801.

66. Swanson N, Hogrefe K, Javed Q, Malik N and Gershlick AH. Vascular endothelial growth factor (VEGF)-eluting stents: in vivo effects on thrombosis, endothelialization and intimal hyperplasia. *J Invasive Cardiol*. 2003;15:688-92.
67. Walter DH, Cejna M, Diaz-Sandoval L, Willis S, Kirkwood L, Stratford PW, Tietz AB, Kirchmair R, Silver M, Curry C, Wecker A, Yoon YS, Heidenreich R, Hanley A, Kearney M, Tio FO, Kuenzler P, Isner JM and Losordo DW. Local gene transfer of phVEGF-2 plasmid by gene-eluting stents: an alternative strategy for inhibition of restenosis. *Circulation*. 2004;110:36-45.
68. Hedman M, Hartikainen J, Syvanne M, Stjernvall J, Hedman A, Kivela A, Vanninen E, Mussalo H, Kauppila E, Simula S, Narvanen O, Rantala A, Peuhkurinen K, Nieminen MS, Laakso M and Yla-Herttuala S. Safety and feasibility of catheter-based local intracoronary vascular endothelial growth factor gene transfer in the prevention of postangioplasty and in-stent restenosis and in the treatment of chronic myocardial ischemia: phase II results of the Kuopio Angiogenesis Trial (KAT). *Circulation*. 2003;107:2677-83.
69. Sharif F, Hynes SO, Cooney R, Howard L, McMahon J, Daly K, Crowley J, Barry F and O'Brien T. Gene-eluting stents: adenovirus-mediated delivery of eNOS to the blood vessel wall accelerates re-endothelialization and inhibits restenosis. *Mol Ther*. 2008;16:1674-80.
70. Brito LA, Chandrasekhar S, Little SR and Amiji MM. Non-viral eNOS gene delivery and transfection with stents for the treatment of restenosis. *Biomed Eng Online*. 2010;9:56.
71. Sharif F, Hynes SO, McCullagh KJ, Ganley S, Greiser U, McHugh P, Crowley J, Barry F and O'Brien T. Gene-eluting stents: non-viral, liposome-based gene delivery of eNOS to the blood

vessel wall in vivo results in enhanced endothelialization but does not reduce restenosis in a hypercholesterolemic model. *Gene therapy*. 2012;19:321-8.

72. Tang R and Chen SY. Smooth muscle-specific drug targets for next-generation drug-eluting stent. *Expert Rev Cardiovasc Ther*. 2014;12:21-3.

CHAPTER 3

JANUS KINASE 3, A NOVEL REGULATOR FOR SMOOTH MUSCLE PROLIFERATION AND VASCULAR REMODELING[1]

[1] 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. http://atvb.ahajournals.org/content/37/7/1352. Reprinted here with permission of the publisher.

Abstract

Objective—Vascular remodeling because of smooth muscle cell (SMC) proliferation is a common process occurring in several vascular diseases, such as atherosclerosis, aortic aneurysm, post-transplant vasculopathy, restenosis after angioplasty, etc. The molecular mechanism underlying SMC proliferation, however, is not completely understood. The objective of this study is to determine the role and mechanism of Janus kinase 3 (JAK3) in vascular remodeling and SMC proliferation.

Approach and Results—Platelet-derived growth factor-BB, an SMC mitogen, induces JAK3 expression and phosphorylation while stimulating SMC proliferation. Janex-1, a specific inhibitor of JAK3, or knockdown of JAK3 by short hairpin RNA, inhibits the SMC proliferation. Conversely, ectopic expression of JAK3 promotes SMC proliferation. Mechanistically, JAK3 promotes the phosphorylation of signal transducer and activator of transcription 3 and c-Jun N-terminal kinase in SMC, 2 signaling pathways known to be critical for SMC proliferation and vascular remodeling. Blockade of these 2 signaling pathways by their inhibitors impeded the JAK3-mediated SMC proliferation. In vivo, knockdown of JAK3 attenuates injury-induced neointima formation with attenuated neointimal SMC proliferation. Knockdown of JAK3 also induces neointimal SMC apoptosis in rat carotid artery balloon injury model.

Conclusions—Our results demonstrate that JAK3 mediates SMC proliferation and survival during injury-induced vascular remodeling, which provides a potential therapeutic target for preventing neointimal hyperplasia in proliferative vascular diseases.

Non-standard Abbreviations and Acronyms:

SMC: Smooth muscle cell

STAT3: Signal transducer and activator of transcription 3

JNK: c-Jun N-terminal kinase

JAK3: Janus kinase 3

IL: Interleukin

PDGF-BB: Platelet-derived growth factor-BB

PCNA: Proliferating cell nuclear antigen

shRNA: Short hairpin RNA

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

Introduction

Smooth muscle cells (SMCs) within adult blood vessel are in the quiescent stage of cell cycle characterized by the low proliferative rate, low mobility, and low synthetic activity. In response to vascular injury, including mechanical stretch, medial dissection, and endothelial denudation, SMCs change to a synthetic phenotype with an increased rate of proliferation, migration, and synthetic activity.[1] Evidently, SMC proliferation contributes to numerous vascular diseases such as systemic hypertension, atherosclerosis, aortic aneurysm, postangioplasty restenosis, etc.[2] Therefore, investigating molecular mechanisms underlying SMC proliferation is important for advancing our understanding of the development of proliferative vascular diseases.

Janus kinase (JAK) is a family of nonreceptor tyrosine kinases that transduce signal from transmembrane receptor to nucleus and further modulate transcription of target genes to control cell differentiation, proliferation, and apoptosis via JAK/signal transducers and activators of transcription (JAK/ STAT) pathway. In mammalian, JAK family has 4 members: JAK1, JAK2, JAK3 and Tyk2. Unlike other JAKs that are ubiquitously expressed and associated with diverse cytokine receptors, JAK3 is predominantly expressed in hematopoietic cells and is induced by cytokine receptors that contain a common γ chain, such as receptors for interleukin-2 (IL-2), IL-4, IL-7, IL-9, IL-15, and IL-21.[3]

JAK3 has been shown to play an essential role in T-lymphocytes development, proliferation, and differentiation. JAK3 deficiency causes defective T-lymphocyte immunity, leading to a severe combined immunodeficiency.[4] In addition to lymphocytes, JAK3 is also expressed in certain nonhematopoietic cells, including vascular cells and carcinoma.[5] JAK3 mediates mucosal homeostasis through regulating IL-2–induced intestinal epithelial cell migration, proliferation, and cell apoptosis.[6] Moreover, JAK3 is involved in the progression of human colon cancer, renal

fibrosis, vascular calcification, and myocardial ischemia and reperfusion injury.[7–10] However, it remains unknown if JAK3 plays a role in SMC proliferation and related vascular remodeling.

In this study, we found that JAK3 expression was induced in SMCs by platelet-derived growth factor-BB (PDGF-BB) in vitro and neointimal SMCs after vascular injury in vivo. Knockdown of JAK3 or blockade of its activity suppressed SMC proliferation, whereas overexpression of JAK3 induced SMC proliferation. Importantly, knockdown of JAK3 attenuated injury-induced neointimal formation along with the suppression of SMC proliferation and induction of SMC apoptosis. JAK3 appeared to promote SMC proliferation via activating STAT3 and c-Jun N-terminal kinase (JNK).

Materials and Methods

Reagents and Cell Culture

Rat aortic smooth muscle cells (SMCs) were cultured by enzymatic digestion method from rat thoracic aorta as described previously.[11, 12] SMCs were maintained in Dulbecco's modification of Eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS, Hyclone) and 5% L-glutamine (Corning) at 37°C in a humidified atmosphere with 5% CO2. Phenotype of primary cultured SMCs were confirmed by the expression of smooth muscle α-actin and SM22α. Chemicals were obtained from the following sources: rhPDGF-BB (R&D Systems, 220-BB), Janex-1 (Santa Cruz, sc-205354), Sp600125 (Sigma Aldrich, S5567), S3I-201 (Sigma Aldrich, SML0330), SB203580 (Sigma Aldrich, S8307), LY294002 (EMD Millipore, 440202), U0126 (Sigma Aldrich, U120). Antibodies were obtained from the following sources: JAK3 (Cell Signaling, #8863), phospho-JAK3 (Santa Cruz, sc-16567), STAT3 (Cell Signaling, #9139), phospho-STAT3 (Cell Signaling, #9145), SAPK/JNK (Cell Signaling, #9252), phospho-

SPAK/JNK (Cell Signaling, #9251), PCNA (Santa Cruz, sc-56), α-SMA (Abcam, ab5694), Cyclin D1 (Santa Cruz, sc-8396), Bcl-2 (Santa Cruz, sc-492), Bax (Cell Signaling, #2772), cleaved Caspase 3 (Cell Signaling, #9661), α-Tubulin (Cell Signaling, #2125).

Construction of Adenovirus

cDNA fragment encoding the full length of human JAK3 was amplified from JAX3 plasmid (DNASU, HsCD00038537) by 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.[13] 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). Control scramble shRNA (shScr) sequences were: 5'-CGC GTC GAT CGA TGA TTC GCC CGG CGT CTT CAT AAT TCA AGA GAT TAT GAA GAC GCC GGG CGA ATC ATC GAT CTT TTT TCC AAA-3' (top strand) and 5'-AGC TTT TGG AAA AAA GAT CGA TGA TTC GCC CGG CGT CTT CAT AAT CTC TTG AAT TAT GAA GAC GCC GGG CGA ATC ATC GAT CGA-3' (bottom strand).

EdU Cell Proliferation Assay

Equal numbers (5 x 104) of SMCs were seeded into 12-well cell culture plates. Cells were starved (DMEM containing no FBS and 5% L-glutamine) for 24 hours, and then treated with PDGF-BB or other factors as indicated for 48 hours. Cells were then incubated with 5-Ethynyl-deoxyuridine (5-EdU) by following the manufacturer's recommendation (EMD Millipore). EdU-positive cells were counted from 10 different microscopic fields (10x). Proliferation rate was assessed by the following formula: [Cell numbers at 48 hours / Cell numbers at 0 hour]. 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 (Invitrogen), and then reverse transcribed to cDNA using iScript cDNA Synthesis kit (Bio-Rad). Real time qPCR was performed with Stratagene Mx3005P qPCR instrument using SYBR Green master mix (Agilent Technologies). Each sample was amplified in triplicate.[14] JAK3 primer sequences were 5′-CCT GCC TGT TTA TCA TTC GCT -3′ (forward) and 5′-AAG ACT TGA GTG TCC ACG TCC -3′ (reverse).

Western Blot Analysis

Rat SMCs were starved in DMEM containing no FBS and 5% L-glutamine for 24 hours, and then treated with PDGF-BB or other factors as indicated 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), and 0.1% SDS). Protein concentration was measured using BCA Protein Assay Reagent (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 (Sigma) at Room temperature for 1 hour. The protein levels were detected with enhanced chemiluminescence (Millipore).[15]

Rat Carotid Artery 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.[11, 16] Adenovirus expressing green fluorescent protein (Ad-GFP) or shJAK3 (Ad-JAK3) was introduced into balloon-injured carotid artery by incubation of 100 μl adenovirus (5 x 109 pfu) for 20 minutes as described previously.[17] Balloon-injured artery segment was collected at 3, 7, and/or 14 days after the surgery. The segments were perfused with saline, fixed with 4% paraformaldehyde, and then embedded in paraffin for further sectioning and subsequent morphometric analyses in a double-blinded manner.

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

Balloon-injured artery sections (5 μm) used for analyses among different groups were evenly distributed in the vessel segment collected.[18] The sections were stained with modified hematoxylin and eosin (HE) or Elastica van Gieson (VG) 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. Sections for IHC and IF were rehydrated, blocked with 10% goat serum or donkey serum, permeabilized with 0.01% Triton X-100 in PBS, and then incubated with JAK3, phospho-JAK3, PCNA, 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 of IHC and IF was performed by incubating with Immunoglobulin G (IgG) antibody. Image J software was used to measure the intensity of IHC positive staining by following the previous publication.[19] Mean value of the staining intensity for each group was acquired from 10 artery sections. To quantify the protein level, the mean value of IHC postive signal of each group less the background (negative control) signal was caliberated to the mean vavlue of the staininig intensity in uninjured vessels, in which the background signal was also substracted. 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 IHC staining intensity – Mean value of negative control staining intensity) / (Mean value of IHC staining intensity of uninjured vessels – Mean value of negative control staining intensity)]. The subsequent statistical anaylses were performed as described below.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

The artery samples were prepared from the serial sectioning of balloon-injured artery, and the in vivo cell apoptosis was evaluated by detecting DNA fragmentation using a TUNEL assay kit (R&D System) by following the manufacturer's instruction.

Statistical Analysis

Results were presented as mean ± S.D. 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

Phosphorylation of JAK3 in SMCs

JAK2 has been shown to play a role in angiotensin II–induced SMC proliferation and vascular remodeling.[20, 21] However, the role of JAK3 in SMC proliferation remains unknown. To determine whether JAK3 is involved in SMC proliferation, we treated primary cultured rat aortic SMC with PDGF-BB and detected the expression of JAK family members. PDGF-BB did not induce the mRNA expression of JAK1 and Tyk2 while modestly induced JAK2 expression (Supplementary Figure S3.1). However, PDGF-BB dramatically and dose-dependently induced the mRNA and protein expression of JAK3 along with the expression of proliferating cell nuclear antigen (PCNA) (Figure 3.1A through 3.1C), suggesting that JAK3, but not other JAK family members, may be involved in PDGF-BB–induced SMC proliferation. Importantly, PDGF-BB also induced the phosphorylation of JAK3, indicating an activation of JAK3 signaling (Figure 3.1B and 3.1C). Because 20 ng/mL of PDGF-BB treatment resulted in the highest level of induction of JAK3 expression and its phosphorylation, we used 20 ng/mL of PDGF-BB for all subsequent experiments. Time-dependent studies showed that PDGF-BB induced a steady increase of JAK3 expression up to 48 hours, whereas its phosphorylation reached the highest level at 24 hours, correlating with the expression of PCNA (Figure 3.1D and 3.1E). These data suggest that JAK3 expression and activation may play a role in PDGF-BB–induced SMC proliferation.

PDGF-BB–Induced JAK3 Expression/Activation via p38 Mitogen-Activated Protein Kinase, Extracellular Signal-Regulated Kinase, and Phosphoinositide 3-Kinase/Akt Signaling Pathways

PDGF-BB stimulates the activation of multiple signaling pathways, such as phosphoinositide 3-kinase (PI3K)/Akt, extracellular signal-regulated kinase (ERK), and p38 mitogen activated protein

kinase (p38 MAPK).[22, 23] Thus, we sought to determine whether PDGF-BB–induced JAK3 phosphorylation through these pathways. Because most of these kinases activate downstream signaling rapidly, we tested how early JAK3 can be activated by PDGF-BB. As shown in Figure 3.2A and 3.2B, JAK3 phosphorylation was detected as early as 10 minutes after the PDGF-BB induction, and it was further increased after 60 minutes of the treatment. The 10-minute activation is likely because of the direct effect of PDGF receptors, whereas the later JAK3 activation may be regulated by PDGF-BB downstream signaling pathways. Thus, we blocked individual pathways with their pathway-specific inhibitors in SMCs followed by PDGF-BB treatment for 60 minutes. As shown in Figure 3.2C and 3.2D, blockade of ERK and PI3K/Akt signaling, but not the p38 MAPK, significantly attenuated PDGF-BB–induced JAK3 phosphorylation, suggesting that ERK and PI3K/Akt mediated the JAK3 activation. However, p38 MAPK, but not ERK or PI3K/Akt signaling, seemed to be important for JAK3 expression because only p38 MAPK inhibitor blocked JAK3 expression when the cells were treated with PDGF-BB for 24 hours (Figure 3.2E and 3.2F). Importantly, all the pathway inhibitors attenuated PDGF-BB–induced PCNA expression (Figure 3.2E and 3.2G), consistent with the roles of these signaling pathways in PDGF-BB–induced SMC proliferation.

JAK3 Regulated SMC Proliferation In Vitro

To test whether JAK3 is important for SMC proliferation, we used adenoviral vector to express JAK3 short hairpin RNA (shRNA; Ad-shJAK3) or its cDNA (Ad-JAK3) to manipulate JAK3 expression in SMCs. As shown in Figure 3.3A through 3.3C, knockdown of JAK3 suppressed PDGF-BB–induced SMC proliferation and PCNA expression. Conversely, ectopic expression of JAK3 stimulated SMC proliferation similar to the effect of PDGF-BB (Figure 3.3D). JAK3 expression also induced PCNA expression (Figure 3.3E and 3.3F). To determine whether the

activation of JAK3 is essential for regulating PDGF-BB–induced SMC proliferation, we blocked JAK3 activity by a selective JAK3 inhibitor Janex-1.[24] As shown in Figure 3.3G and 3.3I, Janex-1 significantly suppressed PDGF-BB–induced SMC proliferation and PCNA expression. These results indicated that PDGF-BB–induced SMC proliferation is mediated by JAK3 expression and activation.

JAK3 Was Induced and Activated in SMCs In Vivo During Injury-Induced Vascular Remodeling

Because SMC proliferation is an important process in vascular remodeling, we sought to determine whether JAK3 is involved in the injury-induced neointima formation. We first detected whether JAK3 is induced or activated in neointimal SMCs in balloon-injured rat carotid artery.[25] As shown in Supplementary Figure S3.2 and Figure 3.4A, only low levels of JAK3 and phospho-JAK3 were present in normal artery media. Balloon injury, however, dramatically induced JAK3 expression in neointimal SMCs. Phospho-JAK3 was also dramatically increased at 7 days although decreased at 14 days after the injury (Supplementary Figure S3.2; Figure 3.4A and 3.4B). JAK3 and phospho-JAK3 were mostly present in neointima SMCs because they co-stained with smooth muscle α-actin (Supplementary Figure S3.3). To confirm the upregulation of JAK3 in injured arteries, JAK3 expression and activation were quantified by Western blot. As shown in Figure 4B and 4C, JAK3 was induced as early as 3 days and progressively increased and remained at a high level until 14 days after the injury. Phospho-JAK3 reached the highest level 3 days after injury and decreased gradually afterward (Figure 3.4B and 3.4C), similar to the activation of many other kinases. JAK1 and JAK2 were only slightly induced with no change in TYK2 expression in the injured carotid arteries (Supplementary Figure S3.1B), consistent with the expression of other JAK family

members in cultured SMCs. These results indicated that JAK3 may play a major role in the artery response to vascular injury.

Knockdown of JAK3 Attenuated Injury-Induced Neointimal Formation

To determine whether JAK3 plays a role in injury-induced neointimal formation in vivo, Ad-shJAK3 was transduced into endothelium-denuded arteries. Immunohistochemistry staining showed that Ad-shJAK3 successfully reduced JAK3 expression by 64% in the neointimal SMCs compared with the control adenovirus expressing green fluorescent protein (Ad-GFP) transduction (Supplementary Figure S3.4). Knockdown of JAK3 significantly inhibited injury-induced neointimal formation (Figure 3.4D and 3.4E). The neointima area was reduced by 63% with Ad-shJAK3 incubation compared with the control group (0.021±0.001 versus 0.057±0.002 mm2; $P<0.05$, n=5, Figure 3.4F). The intima/media area ratios were also significantly reduced (Figure 3.4G). To determine whether JAK3 regulates neointimal SMC proliferation in vivo, we detected the PCNA expression in injured arteries. Immunohistochemistry staining showed that knockdown of JAK3 reduced the PCNA expression by 64% in neointima when compared with Ad-GFP–treated arteries (Figure 3.4H and 3.4I). The majority of proliferating cells in the neointima were SMCs because most PCNA-positive cells also expressed SMC marker smooth muscle myosin heavy chain (Supplementary Figure S3.3C). These data indicated that JAK3 regulates injury-induced neointimal formation, at least in part, by promoting SMC proliferation in vivo. Previous studies have shown that increased SMC survival is also involved in neointima formation.[25, 26] Therefore, we tested whether JAK3 regulates SMC survival by performing the Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling assay in balloon-injured arteries. As shown in Supplementary Figure S3.5, knockdown of JAK3 induced apoptosis in a small portion of neointimal cells. These apoptotic cells seemed to be SMCs because the apoptotic marker cleaved

caspase 3 was mainly presented in smooth muscle α-actin–positive cells (Supplementary Figure S3.6). Taken together, our results indicated that JAK3 mediates injury-induced neointimal formation by promoting both SMC proliferation and survival.

JAK3-Mediated PDGF-BB–Induced Activation of STAT3 and JNK Signaling in SMCs

JAK3/STAT3 signaling is known to promote survival and cell cycle progression of different cancer cells.[7,27,28] Inhibition of STAT3 and its signaling prevent SMC proliferation and injury-induced neointimal formation.[16,27] JNK has also been shown to mediate PDGF-BB–induced SMC proliferation and injury-induced neointima formation.[16,29] Therefore, we hypothesized that JAK3 may regulate PDGF-BB–mediated SMC proliferation by activating STAT3 and JNK signaling. As shown in Figure 3.5A through 3.5C, PDGF-BB, indeed, induced the expression and phosphorylation of both STAT3 and JNK in SMCs. However, blockade of JAK3 activity by Janex-1 or knockdown of JAK3 expression by JAK3 shRNA significantly decreased the phosphorylation of both STAT3 and JNK (Figure 3.5A through 3.5F). Conversely, ectopic expression of JAK3 stimulated STAT3 and JNK phosphorylation (Supplementary Figure S3.7). Because STAT3 and JNK regulate cell proliferation through inducing cyclin D1 expression[30–32] and protect cell from programmed death via altering Bcl-2/Bax expression[33,34], we detected whether JAK3 affects the expression of cyclin D1 and apoptosis regulators. As shown in Supplementary Figure S3.8, knockdown of JAK3 suppressed PDGF-BB–induced cyclin D1 expression while restored PDGF-BB–decreased cleaved caspase 3 level, which was likely because of the reduction of Bcl2 and the increase in Bax expression by JAK3 shRNA. These results indicated that JAK3 mediates PDGF-BB–induced SMC proliferation and survival via the activation of STAT3 and JNK signaling. In fact, STAT3 and JNK were also activated in balloon-injured carotid arteries with neointimal hyperplasia (Supplementary Figure S3.9).

STAT3 and JNK-Mediated JAK3 Activity in SMC Proliferation

To determine whether STAT3 or JNK signaling plays a role in JAK3-mediated SMC proliferation, we used a selective STAT3 inhibitor S3I-201 to block STAT3 phosphorylation (Figure 3.6A and 3.6B)[35] and a JNK selective inhibitor SP600125 to block JNK phosphorylation (Figure 3.6C and 3.6D).[36] Both S3I-201 and SP600125 significantly attenuated the JAK3-induced SMC proliferation and PCNA expression (Figure 3.6E through 3.6G), suggesting that both STAT3 and JNK mediated JAK3-mediated SMC proliferation. Importantly, combined treatment of S3I-201 and SP600125 further inhibited SMC proliferation and completely blocked JAK3-mediated PCNA expression when compared with the individual inhibitor (Figure 3.6E through 3.6G), indicating that STAT3 and JNK signaling pathways may synergistically or cooperatively mediate JAK3-induced SMC proliferation.

Discussion

In this study, we demonstrate that JAK3 is a novel regulator for SMC proliferation. Although JAK3 has a low level of expression in normal vascular SMCs, its expression and activity are dramatically induced by PDGF-BB in vitro and by balloon injury in vivo. Interestingly, there is a biphase regulation for JAK3 phosphorylation by PDGF-BB, that is, 10 to 30 minutes phase and 60 minutes forward phase. The immediate activation of JAK3 after 10 minutes of PDGF-BB treatment is likely mediated by PDGF-BB receptor. The second phase of activation is mediated by ERK and PI3K/Akt signaling because blockade of these signaling pathways attenuates the JAK3 phosphorylation. JAK3 expression seems to be regulated differently from its activation because the signaling pathways regulating JAK3 phosphorylation do not affect its expression and vice versa. Nevertheless, as blockade of any of these signaling pathways inhibits PCNA expression

along with a reduction in either JAK3 expression or activation, JAK3 is likely to mediate, at least in part, the function of these signaling pathways.

JAK3 seems to play a critical role in SMC proliferation both in vitro and in vivo. Blockade of JAK3 expression or activity attenuates PDGF-BB–induced proliferation of the cultured SMCs. Moreover, knockdown of JAK3 inhibits the injury-induced intimal hyperplasia and the expression of proliferating cell marker in neointimal SMCs. Mechanistically, JAK3 regulates SMC proliferation through activation of both STAT3 and JNK (Supplementary Figure S3.10). STAT3 is known to stimulate SMC proliferation and survival, contributing to the injury-induced neointimal formation.[26, 30] In addition to cell proliferation, STAT3 has also been shown to interact with myocardin to regulate SMC phenotypic modulation.[31] Hence, JAK3 could potentially regulate SMC phenotype through STAT3 signaling. Indeed, a higher level of smooth muscle α-actin expression is presented in neointimal SMCs with JAK3 knockdown (Supplementary Figure S3.6). However, this concept cannot be firmly established without extensive future studies. JNK also serves as a central signaling molecule for growth factors, cytokines, and stress stimuli in regulating cell growth, differentiation, apoptosis, and inflammation response.[37] Blockade of JNK activity by gene transfer of dominant-negative mutant suppresses balloon injury–induced neointimal formation via inhibiting SMC proliferation.[28] Our results show that JAK3 activates both STAT3 and JNK signaling to mediate PDGF-BB–induced SMC proliferation and consequently vascular remodeling.

In addition to SMC proliferation, JAK3 also affects SMC survival during vascular remodeling. JAK3 seems to inhibit Bax while enhancing Bcl-2 expression, which blocks the cleavage of caspase 3, and thus hinders programmed cell death and promotes SMC survival. Indeed, knockdown of JAK3 increases cleaved caspase 3 level and alters Bcl-2/Bax expression ratio

(Supplementary Figure S3.8). Activation of STAT3 and JNK may be essential for JAK3-mediated SMC survival because transient activation of STAT3 or JNK has been shown to stimulate cancer cell survival.[38,39] In fact, blockade of STAT3 or JNK activity and their signaling also causes pathological cell death by activating apoptotic pathways in SMCs.[30, 40] Therefore, the increased neointimal SMC apoptosis in injured arteries with JAK3 knockdown is likely because of the inhibition of STAT3 or JNK activity.

Inflammation also contributes to the vascular remodeling in injured vessels.[34] In the early times after vascular injury, leukocyte recruitment has a strong correlation with the subsequent neointimal formation.33 Furthermore, inhibition of inflammatory cell accumulation in vascular lesion through blocking mononuclear leukocyte (lymphocyte and monocyte) trafficking reduces neointimal formation.[42] JAK3 is involved in IL-6–induced M1 macrophage differentiation and IL-8–induced neutrophil chemotaxis.[43, 44] In our animal studies, JAK3 shRNA in the injured arteries may also affect leukocytes or remnant endothelial cells. Therefore, the reduction of neointimal formation in injured arteries with JAK3 knockdown may also attributable to a decreased leukocyte activation or trafficking. Indeed, several cytokines produced by inflammatory cells regulate SMC phenotype and proliferation, and thus contribute to the vascular remodeling. These cytokines include tumor necrosis factor-α, interferon-γ, IL-6, etc.[45–48] Because STAT3 is a central regulator in vascular responses to the inflammatory cytokines,[49] and JAK3 can regulate STAT3 activation, it is likely that JAK3 also mediate the function of these cytokines in vascular remodeling, which can be studied in the future.

Our study is the first time to demonstrate the role of JAK3 in SMC proliferation and injury-induced neointimal formation. Although JAK2 also regulates SMC proliferation, it mainly mediates angiotensin II–induced proliferation.[20,21] Because JAK2 is only marginally induced in

SMC by PDGF-BB or vascular injury, its function in PDGF-BB–induced SMC proliferation is likely to be less significant. Moreover, as JAK2 participates in a variety of cytokine receptor signaling and is expressed ubiquitously while JAK3 only interacts with cytokine receptors containing common γ chain and expresses restrictedly in certain cell types,3 blocking JAK3 signaling may have less off-target effect than blocking JAK2. Therefore, targeting JAK3 activity may be a more effective approach to treat proliferative vascular disorders comparing to JAK2.

Reference

1. Bennett MR. In-stent stenosis: pathology and implications for the development of drug eluting stents. *Heart*. 2003;89:218-24.

2. Owens GK, Kumar MS and Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev*. 2004;84:767-801.

3. Yamaoka K, Saharinen P, Pesu M, Holt VE, 3rd, Silvennoinen O and O'Shea JJ. The Janus kinases (Jaks). *Genome Biol*. 2004;5:253.

4. Wu W and Sun XH. Janus kinase 3: the controller and the controlled. *Acta Biochim Biophys Sin (Shanghai)*. 2012;44:187-96.

5. Verbsky JW, Bach EA, Fang YF, Yang L, Randolph DA and Fields LE. Expression of Janus kinase 3 in human endothelial and other non-lymphoid and non-myeloid cells. *J Biol Chem*. 1996;271:13976-80.

6. Mishra J, Waters CM and Kumar N. Molecular mechanism of interleukin-2-induced mucosal homeostasis. *Am J Physiol Cell Physiol*. 2012;302:C735-47.

7. Lin Q, Lai R, Chirieac LR, Li C, Thomazy VA, Grammatikakis I, Rassidakis GZ, Zhang W, Fujio Y, Kunisada K, Hamilton SR and Amin HM. Constitutive activation of JAK3/STAT3 in colon carcinoma tumors and cell lines: inhibition of JAK3/STAT3 signaling induces apoptosis and cell cycle arrest of colon carcinoma cells. *Am J Pathol*. 2005;167:969-80.

8. Yan J, Zhang Z, Yang J, Mitch WE and Wang Y. JAK3/STAT6 Stimulates Bone Marrow-Derived Fibroblast Activation in Renal Fibrosis. *J Am Soc Nephrol*. 2015;26:3060-71.

9. Kakutani Y, Shioi A, Shoji T, Okazaki H, Koyama H, Emoto M and Inaba M. Oncostatin M Promotes Osteoblastic Differentiation of Human Vascular Smooth Muscle Cells Through JAK3-STAT3 Pathway. *J Cell Biochem*. 2015;116:1325-33.

10. Oh YB, Ahn M, Lee SM, Koh HW, Lee SH, Kim SH and Park BH. Inhibition of Janus activated kinase-3 protects against myocardial ischemia and reperfusion injury in mice. *Exp Mol Med*. 2013;45:e23.

11. Wang JN, Shi N, Xie WB, Guo X and Chen SY. Response gene to complement 32 promotes vascular lesion formation through stimulation of smooth muscle cell proliferation and migration. *Arterioscler Thromb Vasc Biol*. 2011;31:e19-26.

12. Guo X, Shi N, Cui XB, Wang JN, Fukui Y and Chen SY. Dedicator of cytokinesis 2, a novel regulator for smooth muscle phenotypic modulation and vascular remodeling. *Circ Res*. 2015;116:e71-80.

13. 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.

14. Shi N, Xie WB and Chen SY. Cell division cycle 7 is a novel regulator of transforming growth factor-beta-induced smooth muscle cell differentiation. *J Biol Chem*. 2012;287:6860-7.

15. Tang R, Cui XB, Wang JN and Chen SY. CTP synthase 1, a smooth muscle-sensitive therapeutic target for effective vascular repair. *Arterioscler Thromb Vasc Biol*. 2013;33:2336-44.

16. Tulis DA. Rat carotid artery balloon injury model. *Methods Mol Med*. 2007;139:1-30.

17. 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.

18. Tulis DA. Histological and morphometric analyses for rat carotid balloon injury model. *Methods Mol Med*. 2007;139:31-66.

19. Jensen EC. Quantitative analysis of histological staining and fluorescence using ImageJ. Anat Rec (Hoboken). 2013;296:378-81.

20. Seki Y, Kai H, Shibata R, Nagata T, Yasukawa H, Yoshimura A and Imaizumi T. Role of the JAK/STAT pathway in rat carotid artery remodeling after vascular injury. *Circ Res*. 2000;87:12-8.

21. Kirabo A, Oh SP, Kasahara H, Wagner KU and Sayeski PP. Vascular smooth muscle Jak2 deletion prevents angiotensin II-mediated neointima formation following injury in mice. *J Mol Cell Cardiol*. 2011;50:1026-34.

22. Kaplan-Albuquerque N, Garat C, Desseva C, Jones PL and Nemenoff RA. Platelet-derived growth factor-BB-mediated activation of Akt suppresses smooth muscle-specific gene expression through inhibition of mitogen-activated protein kinase and redistribution of serum response factor. J Biol Chem. 2003;278:39830-8.

23. Zhan Y, Kim S, Izumi Y, Izumiya Y, Nakao T, Miyazaki H and Iwao H. Role of JNK, p38, and ERK in platelet-derived growth factor-induced vascular proliferation, migration, and gene expression. *Arterioscler Thromb Vasc Biol*. 2003;23:795-801.

24. Sudbeck EA, Liu XP, Narla RK, Mahajan S, Ghosh S, Mao C and Uckun FM. Structure-based design of specific inhibitors of Janus kinase 3 as apoptosis-inducing antileukemic agents. *Clin Cancer Res*. 1999;5:1569-82.

25. Shibata R, Kai H, Seki Y, Kato S, Morimatsu M, Kaibuchi K and Imaizumi T. Role of Rho-associated kinase in neointima formation after vascular injury. *Circulation*. 2001;103:284-9.

26. Daniel JM, Dutzmann J, Bielenberg W, Widmer-Teske R, Gunduz D, Hamm CW and Sedding DG. Inhibition of STAT3 signaling prevents vascular smooth muscle cell proliferation and neointima formation. *Basic Res Cardiol*. 2012;107:261.

27. Dien Bard J, Gelebart P, Anand M, Zak Z, Hegazy SA, Amin HM and Lai R. IL-21 contributes to JAK3/STAT3 activation and promotes cell growth in ALK-positive anaplastic large cell lymphoma. *Am J Pathol*. 2009;175:825-34.

28. Krejsgaard T, Ralfkiaer U, Clasen-Linde E, Eriksen KW, Kopp KL, Bonefeld CM, Geisler C, Dabelsteen S, Wasik MA, Ralfkiaer E, Woetmann A and Odum N. Malignant cutaneous T-cell lymphoma cells express IL-17 utilizing the Jak3/Stat3 signaling pathway. *J Invest Dermatol*. 2011;131:1331-8.

29. Shibata R, Kai H, Seki Y, Kato S, Wada Y, Hanakawa Y, Hashimoto K, Yoshimura A and Imaizumi T. Inhibition of STAT3 prevents neointima formation by inhibiting proliferation and promoting apoptosis of neointimal smooth muscle cells. *Hum Gene Ther*. 2003;14:601-10.

30. Izumi Y, Kim S, Namba M, Yasumoto H, Miyazaki H, Hoshiga M, Kaneda Y, Morishita R, Zhan Y and Iwao H. Gene transfer of dominant-negative mutants of extracellular signal-regulated kinase and c-Jun NH2-terminal kinase prevents neointimal formation in balloon-injured rat artery. *Circ Res*. 2001;88:1120-6.

31. Liao XH, Wang N, Zhao DW, Zheng DL, Zheng L, Xing WJ, Ma WJ, Bao LY, Dong J and Zhang TC. STAT3 Protein Regulates Vascular Smooth Muscle Cell Phenotypic Switch by Interaction with Myocardin. J Biol Chem. 2015;290:19641-52.

32. Schwabe RF, Bradham CA, Uehara T, Hatano E, Bennett BL, Schoonhoven R and Brenner DA. c-Jun-N-terminal kinase drives cyclin D1 expression and proliferation during liver regeneration. Hepatology. 2003;37:824-32.

33. Nielsen M, Kaestel CG, Eriksen KW, Woetmann A, Stokkedal T, Kaltoft K, Geisler C, Ropke C and Odum N. Inhibition of constitutively activated Stat3 correlates with altered Bcl-2/Bax expression and induction of apoptosis in mycosis fungoides tumor cells. Leukemia. 1999;13:735-8.

34. Dhanasekaran DN and Reddy EP. JNK signaling in apoptosis. *Oncogene*. 2008;27:6245-51.

35. Pang M, Ma L, Gong R, Tolbert E, Mao H, Ponnusamy M, Chin YE, Yan H, Dworkin LD and Zhuang S. A novel STAT3 inhibitor, S3I-201, attenuates renal interstitial fibroblast activation and interstitial fibrosis in obstructive nephropathy. *Kidney Int*. 2010;78:257-68.

36. Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM and Anderson DW. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc Natl Acad Sci U S A*. 2001;98:13681-6.

37. Weston CR and Davis RJ. The JNK signal transduction pathway. *Curr Opin Genet Dev*. 2002;12:14-21.

38. Al Zaid Siddiquee K and Turkson J. STAT3 as a target for inducing apoptosis in solid and hematological tumors. *Cell Res*. 2008;18:254-67.

39. Mingo-Sion AM, Marietta PM, Koller E, Wolf DM and Van Den Berg CL. Inhibition of JNK reduces G2/M transit independent of p53, leading to endoreduplication, decreased proliferation, and apoptosis in breast cancer cells. *Oncogene*. 2004;23:596-604.

40. Kornowski R, Hong MK, Tio FO, Bramwell O, Wu H and Leon MB. In-stent restenosis: contributions of inflammatory responses and arterial injury to neointimal hyperplasia. *J Am Coll Cardiol*. 1998;31:224-30.

41. Welt FG and Rogers C. Inflammation and restenosis in the stent era. *Arterioscler Thromb Vasc Biol*. 2002;22:1769-76.

42. Shah PK. Inflammation, neointimal hyperplasia, and restenosis: as the leukocytes roll, the arteries thicken. *Circulation*. 2003;107:2175-7.

43. Mangan JK, Rane SG, Kang AD, Amanullah A, Wong BC and Reddy EP. Mechanisms associated with IL-6-induced up-regulation of Jak3 and its role in monocytic differentiation. *Blood*. 2004;103:4093-101.

44. Henkels KM, Frondorf K, Gonzalez-Mejia ME, Doseff AL and Gomez-Cambronero J. IL-8-induced neutrophil chemotaxis is mediated by Janus kinase 3 (JAK3). *FEBS Lett*. 2011;585:159-66.

45. Wang Y, Bai Y, Qin L, Zhang P, Yi T, Teesdale SA, Zhao L, Pober JS and Tellides G. Interferon-gamma induces human vascular smooth muscle cell proliferation and intimal

expansion by phosphatidylinositol 3-kinase dependent mammalian target of rapamycin raptor complex 1 activation. Circ Res. 2007;101:560-9.

46. Jovinge S, Hultgardh-Nilsson A, Regnstrom J and Nilsson J. Tumor necrosis factor-alpha activates smooth muscle cell migration in culture and is expressed in the balloon-injured rat aorta. Arterioscler Thromb Vasc Biol. 1997;17:490-7.

47. Ikeda U, Ikeda M, Oohara T, Oguchi A, Kamitani T, Tsuruya Y and Kano S. Interleukin 6 stimulates growth of vascular smooth muscle cells in a PDGF-dependent manner. Am J Physiol. 1991;260:H1713-7.

48. Niida T, Isoda K, Kitagaki M, Ishigami N, Adachi T, Matsubara O, Takeda K, Kishimoto T and Ohsuzu F. IkappaBNS regulates interleukin-6 production and inhibits neointimal formation after vascular injury in mice. Cardiovasc Res. 2012;93:371-9.

49. Shuai K and Liu B. Regulation of JAK-STAT signalling in the immune system. Nat Rev Immunol. 2003;3:900-11.

Figure Legends

Figure 3.1. Janus kinase 3 (JAK3) was upregulated and activated by platelet-derived growth factor (PDGF)-BB in smooth muscle cells (SMCs). A, JAK3 mRNA expression was dose dependently induced by PDGF-BB. Rat aortic SMCs were treated with PDGF-BB (20 ng/mL) for 8 hours. JAK3 mRNA levels were detected by quantitative real-time polymerase chain reaction. **B**, JAK3, phospho-JAK3 (pJAK3), and proliferating cell nuclear antigen (PCNA) protein was dose dependently induced by PDGF-BB. SMCs were treated with PDGF-BB (20 ng/mL) for 24 hours. JAK3, pJAK3, and PCNA protein were detected by Western blot. **C**, Quantification of the protein levels shown in **B** by normalizing to α-tubulin. **D**, JAK3, pJAK3, and PCNA proteins were time dependently induced by PDGF-BB (20 ng/mL). **E**, Quantification of JAK3, pJAK3, and PCNA protein levels shown in **D** by normalizing to α-tubulin. **P*<0.05 vs vehicle-treated cells (Ctrl), n=3.

Figure 3.2. p38 mitogen-activated protein kinase (p38 MAPK), extracellular signal-regulated kinase 1/2 (ERk1/2), and PI3K/Akt signaling regulated platelet-derived growth factor (PDGF)-BB–induced Janus kinase 3 (JAK3) expression or activation. A, PDGF-BB (20 ng/mL) time dependently induced JAK3 activation during the initial treatment. **B**, Quantification of pJAK3 level shown in **A** by normalizing to α-tubulin. **C**, Blockade of either PI3K/Akt or ERk1/2 signaling by their pathway-specific inhibitors attenuated PDGF-BB–induced JAK3 activation. Rat aortic smooth muscle cells (SMCs) were pretreated with pathway-specific inhibitor SB203580 (p38 MAPK), LY294002 (PI3K/Akt), or U0126 (ERk1/2) for an hour followed by PDGF-BB induction for another hour. JAK3 phosphorylation was detected by Western blot. **D**, Quantification of pJAK3 levels shown in **C** by normalizing to α-tubulin. **E**, The effect of pathway inhibitors on PDGF-BB–induced JAK3 and proliferating cell nuclear antigen (PCNA) expression. SMCs were treated with pathway inhibitors the same as in **C** followed by 24 hours of PDGF-BB treatment.

JAK3 and PCNA expression was detected by Western blot. Quantification of the JAK3 (**F**) and PCNA (**G**) levels shown in **E** by normalizing to α-tubulin. **P*<0.05 vs vehicle-treated cells (Ctrl or −); #*P*<0.05 vs PDGF-BB–treated cells without inhibitors (−), n=3.

Figure 3.3. Janus kinase 3 (JAK3) was essential for smooth muscle cell (SMC) proliferation in vitro. Cell proliferation was measured by EdU assay as described in Materials and Methods of this article. **A**, Knockdown of JAK3 by adenovirus-expressed short hairpin RNA (shRNA; Ad-shJAK3) blocked platelet-derived growth factor (PDGF)-BB–induced SMC proliferation. **B**, Knockdown of JAK3 decreased PDGF-BB–induced proliferating cell nuclear antigen (PCNA) protein expression. **C**, Quantification of JAK3 and PCNA protein expression shown in **B** by normalizing to α-tubulin level. **P*<0.05 vs scramble shRNA (Ad-shScr)–transduced cells; #*P*<0.05 vs Ad-shScr–transduced cells with PDGF-BB treatment (n=3). **D**, Forced expression of JAK3 by adenoviral vector (Ad-JAK3) stimulated SMC proliferation. **E**, Forced expression of JAK3 induced PCNA protein expression. **F**, Quantification of JAK3 and PCNA protein expression shown in **E** by normalizing to α-tubulin level. **P*<0.05 vs control group (Ad-GFP) (n=3). **G**, JAK3 selective inhibitor, Janex-1, blocked PDGF-BB–induced SMC proliferation. **H**, Janex-1 decreased PDGF-BB–induced PCNA protein expression. **I**, Quantification of the PCNA protein expression shown in **H** by normalizing to α-tubulin level. **P*<0.05 vs vehicle-treated cells (−); #*P*<0.05 vs PDGF-BB–treated cells without Janex-1 (−), n=3.

Figure 3.4. Blockade of Janus kinase 3 (JAK3) expression suppressed injury-induced neointima formation and attenuated smooth muscle cell (SMC) proliferation in vivo. A, JAK3 and pJAK3 levels in neointimal SMCs of balloon-injured rat carotid arteries as measured by immunohistochemistry staining (**Supplementary Figure S3.2**) and quantified by calibrating their staining intensity to the mean signal in uninjured vessels (Ctrl, set as 1). **B**, JAK3, pJAK3, and

proliferating cell nuclear antigen (PCNA) protein expression in the injured arteries was detected by Western blot. **C**, Quantification of JAK3, pJAK3, and PCNA protein expression shown in **B** by normalizing to α-tubulin level. **P*<0.05 vs uninjured arteries (Ctrl), n=5. **D** and **E,** Knockdown of JAK3 by short hairpin RNA (Ad-shJAK3) blocked neointima formation after balloon injury, as shown by hematoxylin and eosin (HE) and Elastica van Gieson (VG) staining, respectively. Quantification of the neointima area (**F**) and intima/media ratio (**G**). **P*<0.05 vs control adenovirus (Ad-GFP)-treated arteries, n=5. **H**, Knockdown of JAK3 attenuated the PCNA expression in balloon-injured arteries. **I**, Quantification of the PCNA level shown in **H** by calibrating its staining intensity to the mean signal in uninjured arteries (Ctrl, set as 1). **P*<0.05 vs uninjured arteries (Ctrl); $^{\#}P$<0.05 vs control adenovirus (Ad-GFP)-treated arteries, n=5.

Figure 3.5. Janus kinase 3 (JAK3)–mediated platelet-derived growth factor (PDGF)-BB function in activating signal transducer and activator of transcription 3 (STAT3) and c-Jun N-terminal kinase (JNK). A, Janex-1 attenuated PDGF-BB–induced phosphorylation of STAT3 and JNK. **B** and **C**, Quantification of pSTAT3 and pJNK protein expression shown in **A** by normalizing to α-tubulin level. **P*<0.05 vs vehicle-treated cells (−); $^{\#}P$<0.05 vs PDGF-BB–treated groups for each individual protein, respectively, n=3. **D**, Knockdown of JAK3 by short hairpin RNA (shRNA; Ad-shJAK3) attenuated PDGF-BB–induced STAT3 and JNK phosphorylation. **E** and **F**, Quantification of pJAK3, pSTAT3, and pJNK levels shown in **D** by normalizing to α-tubulin level, respectively. **P*<0.05 vs scramble shRNA (Ad-shScr)–transduced cells; $^{\#}P$<0.05 vs Ad-shScr–transduced cells with PDGF-BB, for each individual protein, respectively, n=3.

Figure 3.6. Blockade of signal transducer and activator of transcription 3 (STAT3) or c-Jun N-terminal kinase (JNK) activity attenuated Janus kinase 3 (JAK3)–induced smooth muscle cell (SMC) proliferation. A, STAT3 inhibitor S3I-201 blocked JAK3-induced phosphorylation

of STAT3. **B**, Quantification of the pSTAT3 level shown in **A** by normalizing to the α-tubulin level. **C**, JNK inhibitor SP600125 blocked JAK3-induced JNK phosphorylation. **D**, Quantification of the pJNK level in **C** by normalizing to the α-tubulin level. **E**, Blockade of STAT3 or JNK signaling by their inhibitors inhibited JAK3-induced SMC proliferation as measured by EdU assay. **F**, Blockade of STAT3 or JNK signaling by their inhibitors inhibited JAK3-induced proliferating cell nuclear antigen (PCNA) expression. **G**, Quantification of PCNA expression shown in **F** by normalizing to the α-tubulin level. Combined use of the 2 inhibitors achieved a greater inhibition of cell proliferation (**E**) and PCNA expression (**F**) than the individual inhibitor. *P<0.05 vs Ad-GFP group within each panel; $^{\#}P$<0.05 vs Ad-JAK3–treated group within each panel; $^{\$}P$<0.05 vs individual inhibitor-treated cells in **E** and **G**; n=3.

Supplementary Figure S3.1. The expression of JAK family members in PDGF-BB-induced SMCs and neointimal SMCs of balloon-injured arteries. mRNA expression was measured by qRT-PCR. **A**, The mRNA expression of JAK3, but not other family members, was dramatically induced by PDGF-BB treatment for 8 hours. **B**, The mRNA expression of JAK3, but not other family members, was strikingly induced in the injured-arteries. *P < 0.05 vs vehicle (Ctrl)-treated SMCs (A) or uninjured (Ctrl) arteries (B); n=3.

Supplementary Figure S3.2. JAK3 was up-regulated and activated in neointimal SMC during injury-induced vascular remodeling. JAK3 expression (**A)** and phosphorylation (pJAK3, **B**) were detected in rat carotid arteries without (Ctrl) or with balloon-injury for the times indicated by immunohistochemistry staining with their respective antibody. Both JAK3 and pJAK3 levels were elevated in neointimal SMCs following balloon injury.

Supplementary Figure S3.3. Co-expression of JAK3, pJAK3, and PCNA with SMC markers in neointimal SMCs in injured rat carotid arteries. Co-immunofluorescent staining in normal

or balloon-injured artery sections was performed using antibodies against individual proteins as indicated. DAPI stains nuclei. **A**, JAK3 was expressed in most smooth muscle α-actin (α-SMA)-positive neointimal SMCs. **B**, Most pJAK3-positive cells also expressed α-SMA in the neointima. **C**, Most PCNA-expressing cells were smooth muscle myosin heavy chain (SMMHC)-positive.

Supplementary Figure S3.4. JAK3 expression was effectively blocked by adenoviral vector-expressed JAK3 shRNA (Ad-shJAK3). A, shRNA (Ad-shJAK3) significantly attenuated injury-induced JAK3 expression in neointimal SMC (Ad-GFP) as shown by immunohistochemistry staining. JAK3 was undetectable in uninjured arteries (Ctrl). **B**, Quantification of the relative JAK3 levels shown in **A** by calibrating the JAK3 staining intensity to the mean signal in injured arteries (Ctrl, set as 1). $^{*}P < 0.05$ vs uninjured arteries (Ctrl); $^{\#}P < 0.05$ vs Ad-GFP-transduced arteries; n=5.

Supplementary Figure S3.5. Knockdown of JAK3 expression caused cell apoptosis in neointima of the injured rat carotid artery. A, Knockdown of JAK3 by shRNA (Ad-shJAK3) resulted in cell apoptosis in injured arteries as shown by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Both negative control (NC) and positive control (PC) were prepared from artery sections treated with Ad-shJAK3. NC was not treated with Terminal deoxynucleotidyl transferase, and PC had additional treatment with DNase I. Arrows point to the positively stained apoptotic cells. **B**, The apoptotic cell population in the neointima shown in **A** were quantified by normalizing the positively-stained cells to total cell numbers (%) in neointimal areas of the control (Ad-GFP) and Ad-shJAK3-treated arteries, respectively. $^{*}P < 0.05$ vs other groups, n=5.

Supplementary Figure S3.6. Knockdown of JAK3 caused neointimal SMC apoptosis in balloon-injured arteries. The injured arteries were transduced with control (Ad-GFP) or JAK3

shRNA-expressing adenoviral vector (Ad-shJAK3). The uninjured (Ctrl) and injured arterial sections were double-stained with α-SMA and apoptotic cell marker cleaved caspase 3 antibodies. Cleaved caspase 3-positive cells also expressed α-SMA in Ad-shJAK3-treated artery sections (white arrows), indicative of apoptotic SMCs.

Supplementary Figure S3.7. Forced expression of JAK3 activated signal transducer and activator of transcription 3 (STAT3) and c-Jun N-terminal kinase (JNK). A, Forced expression of JAK3 by adenoviral vector transduction (Ad-JAK3) stimulated the phosphorylation of STAT3 and JNK in SMCs. **B-C**, Quantification of pJAK3 (B), pSTAT3 and pJNK (C) protein levels shown in **A** by normalizing to their corresponding total protein that was normalized to the α-Tubulin level, respectively. $*P < 0.05$ vs control adenoviral vector (Ad-GFP)-transduced cells, n=3. JAK3-mediated STAT3 and JNK phosphorylation was comparable to the levels induced by PDGF-BB.

Supplementary Figure S3.8. JAK3 mediated PDGF-BB-induced cyclin D1 expression and caspase 3 activation. A, Knockdown of JAK3 by shRNA (Ad-shJAK3) attenuated the expression of cyclin D1 and Bcl-2 while increased Bax and cleaved caspase 3 levels. **B**, Quantification of cyclin D1 level shown in **A** by normalizing to α-Tubulin. **C**, Quantification of Bcl-2 and Bax expressions shown in **A** by normalizing to α-Tubulin. **D**, Quantification of cleaved caspase 3 level shown in **A** by normalizing to α-Tubulin. $*P < 0.05$ vs scramble shRNA (Ad-shScr)-transduced cells without PDGF-BB; $^{\#}P < 0.05$ vs Ad-shScr-transduced cells treated with PDGF-BB, for each individual protein, respectively, n=3.

Supplementary Figure S3.9. STAT3 and JNK were activated in injured arteries. A, Balloon injury stimulated the phosphorylation of STAT3 and JNK in rat carotid arteries. **B** and **C**,

Quantification of pSTAT3 and pJNK protein levels shown in **A** by normalizing to the α-Tubulin level. *$P < 0.05$ vs uninjured arteries (Ctrl), n=3.

Supplementary Figure S3.10. A schematic mechanism by which JAK3 regulated SMC proliferation/survival. Upon PDGF-BB stimulation, JAK3 is phosphorylated by PDGF-BB receptor, ERK, and PI3K/Akt signaling, which then activates STAT3 and JNK. Meanwhile, p38-MAPK signaling is activated to enhance JAK3 expression. Activated STAT3 and JNK drive cyclin D1 and PCNA expression to promote cell cycle progression, resulting in cell proliferation. On the other hand, activated STAT3 and JNK can block caspase 3 activation via regulating Bcl-2/ Bax expression, promoting SMC survival. Both the increased SMC proliferation and survival contribute to the neointima formation in arteries in response to the mechanical injury.

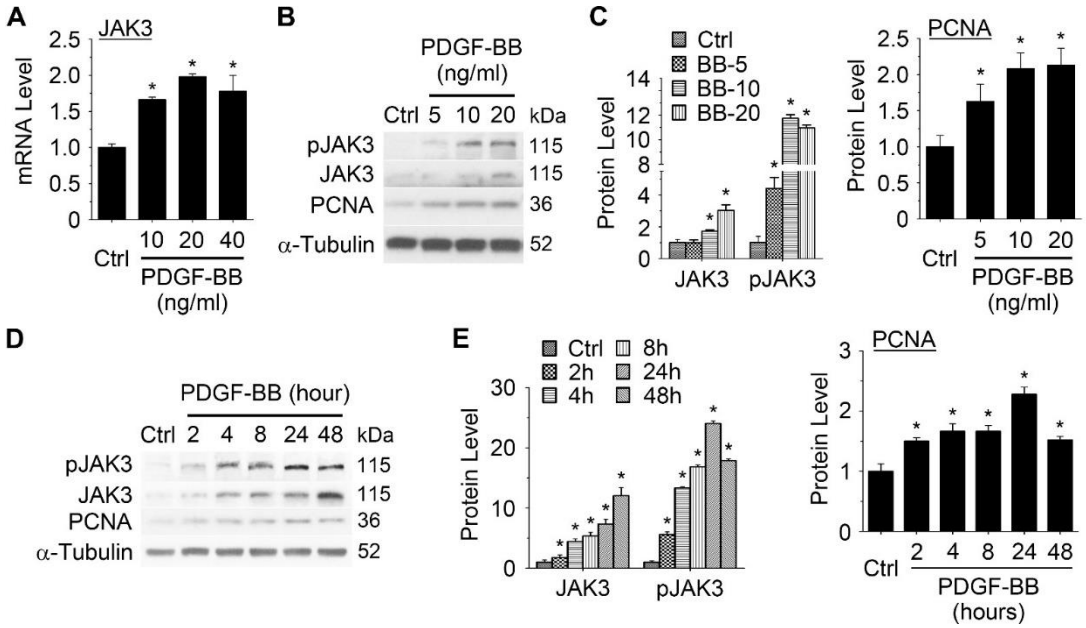


Figure 3.1. JAK3 was upregulated and activated by PDGF-BB in SMCs.

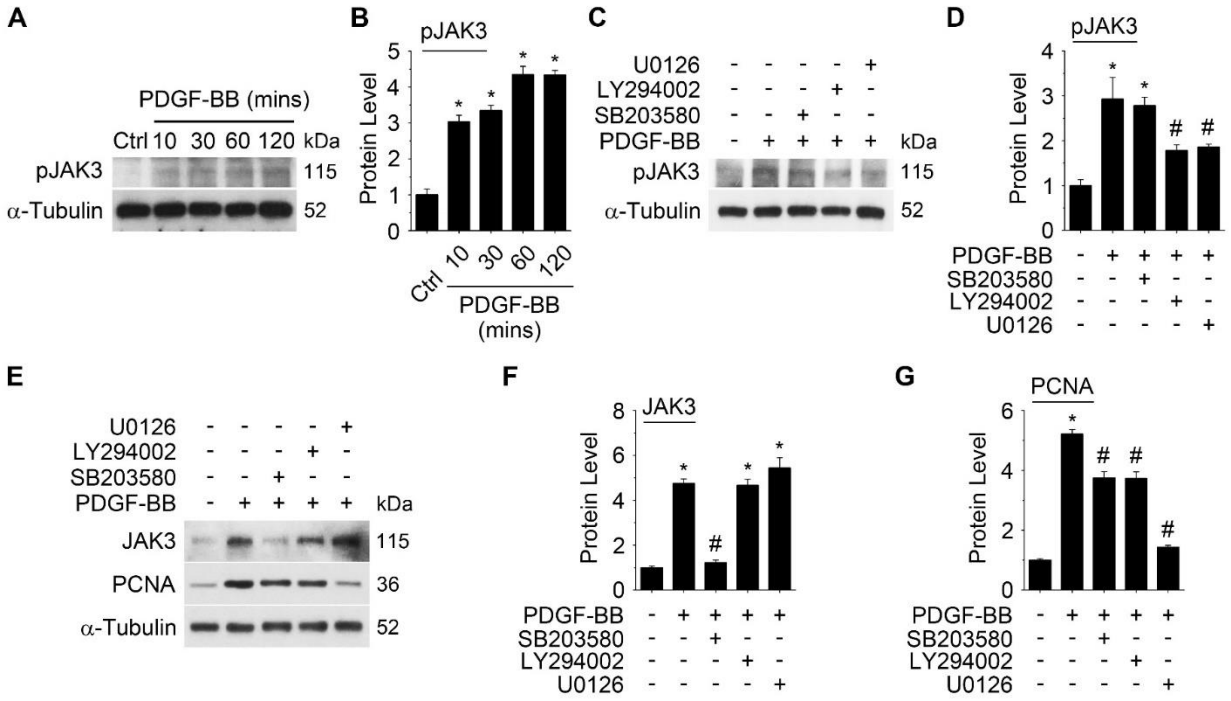


Figure 3.2. p38 MAPK, ERk1/2, and PI3K/Akt signaling regulated PDGF-BB–induced JAK3 expression or activation.

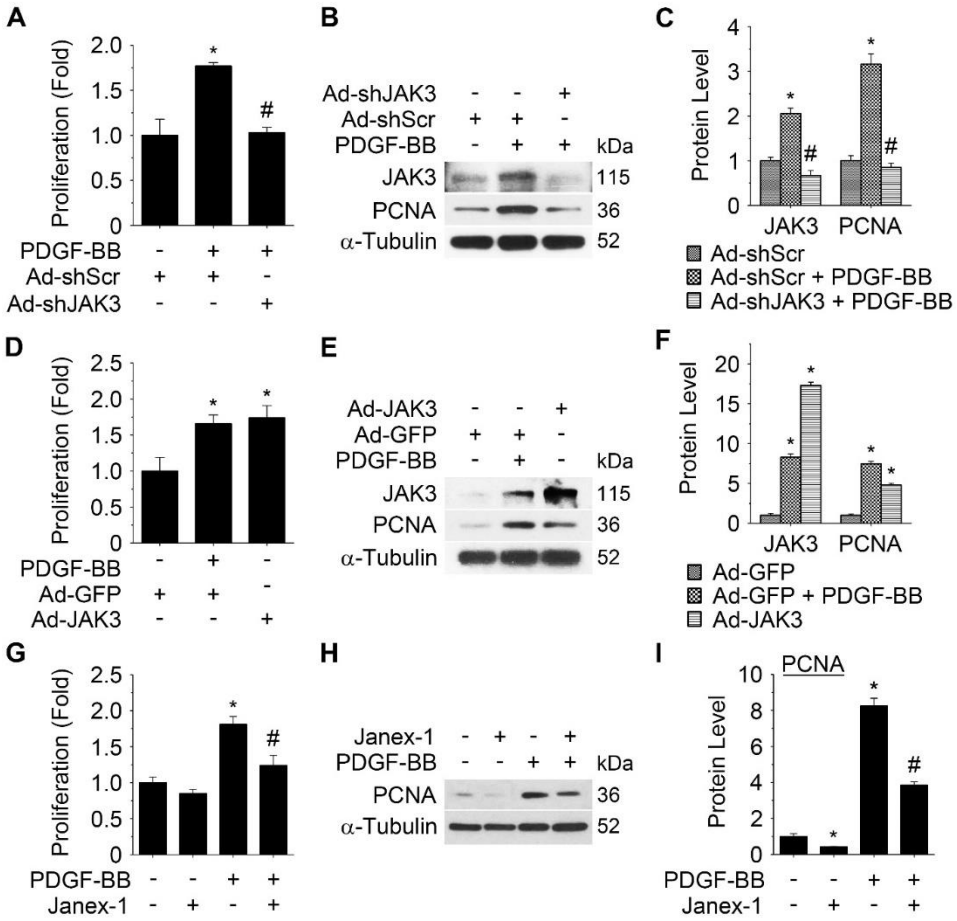


Figure 3.3. JAK3 was essential for SMC proliferation in vitro.

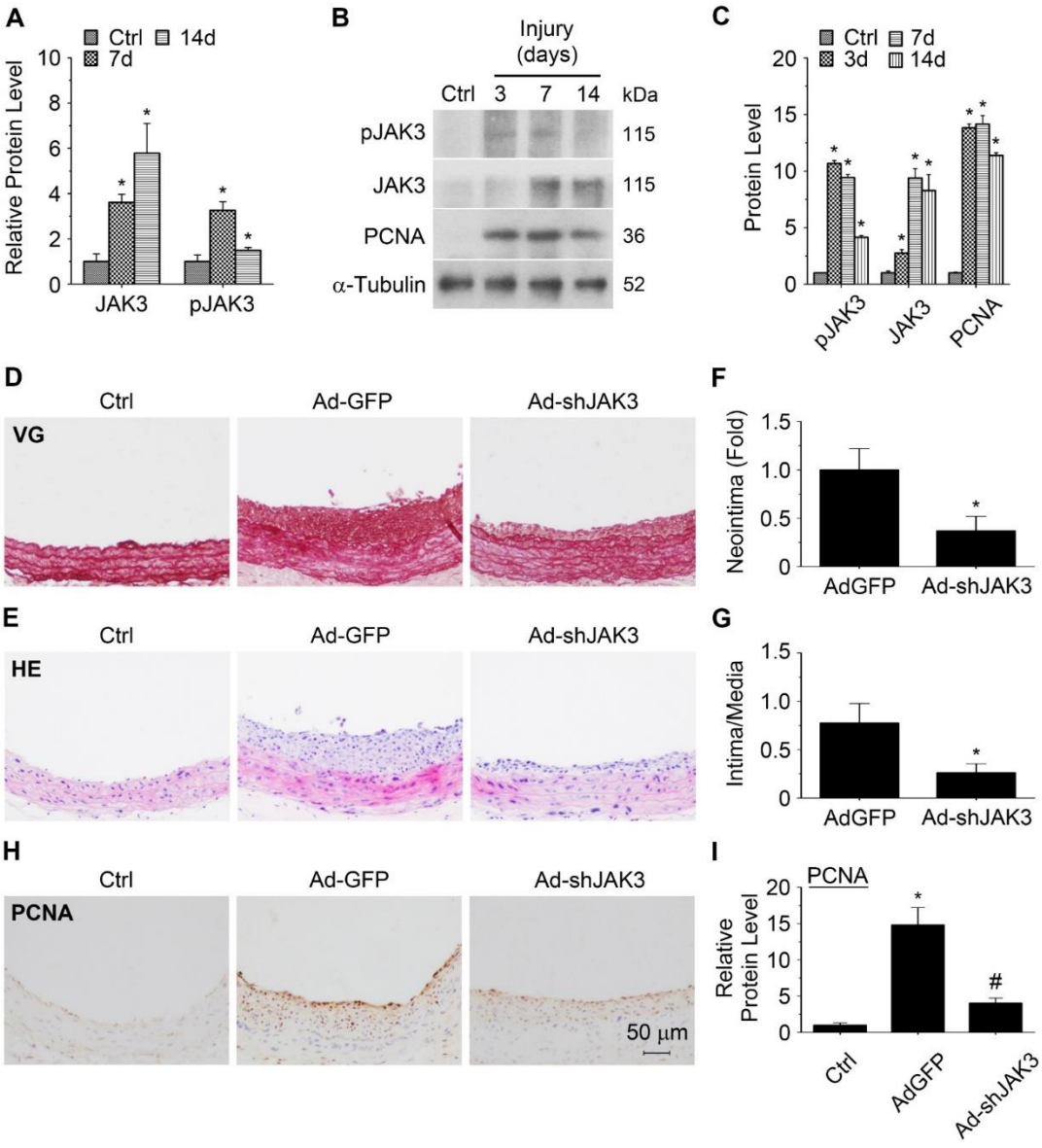


Figure 3.4. Blockade of Janus kinase 3 (JAK3) expression suppressed injury-induced neointima formation and attenuated smooth muscle cell (SMC) proliferation in vivo.

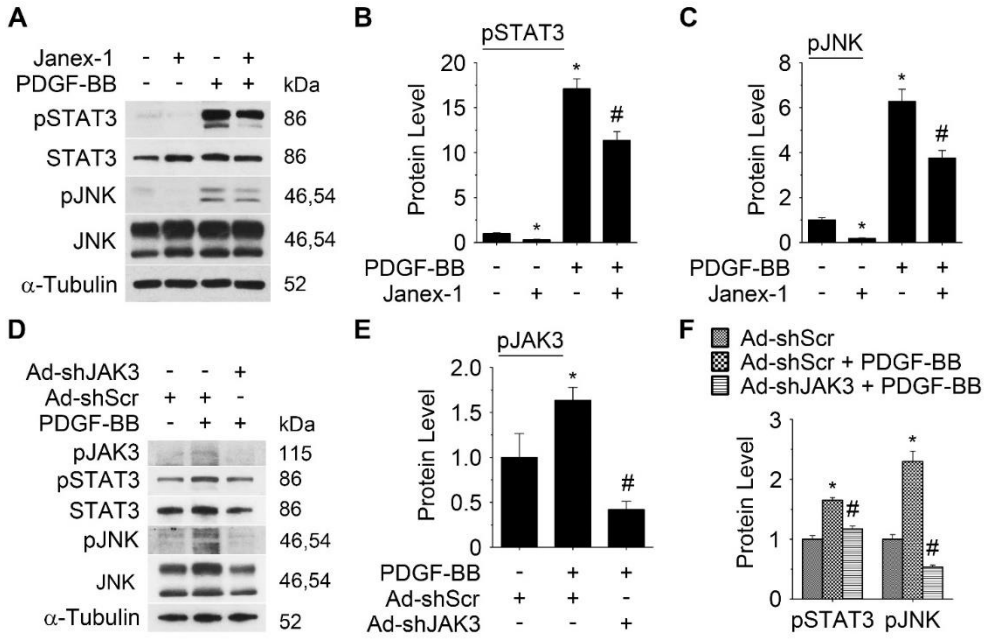


Figure 3.5. JAK3–mediated PDGF-BB function in activating STAT3 and JNK.

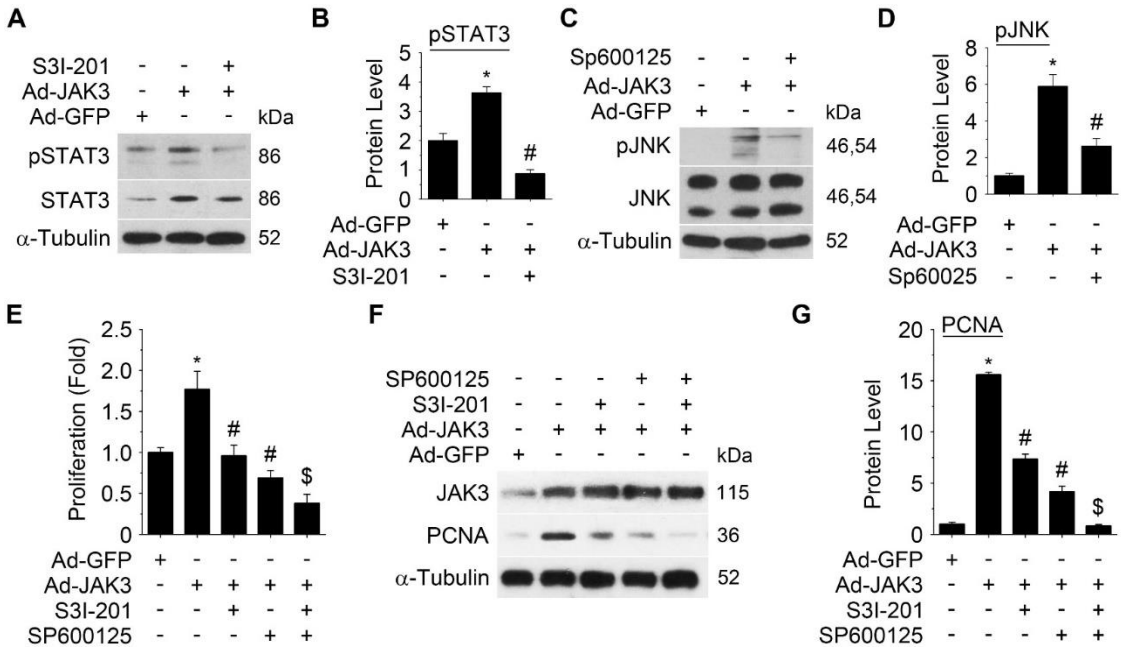
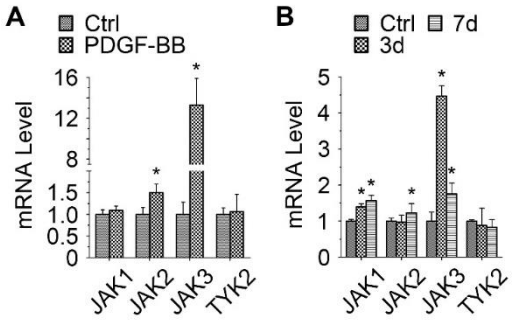
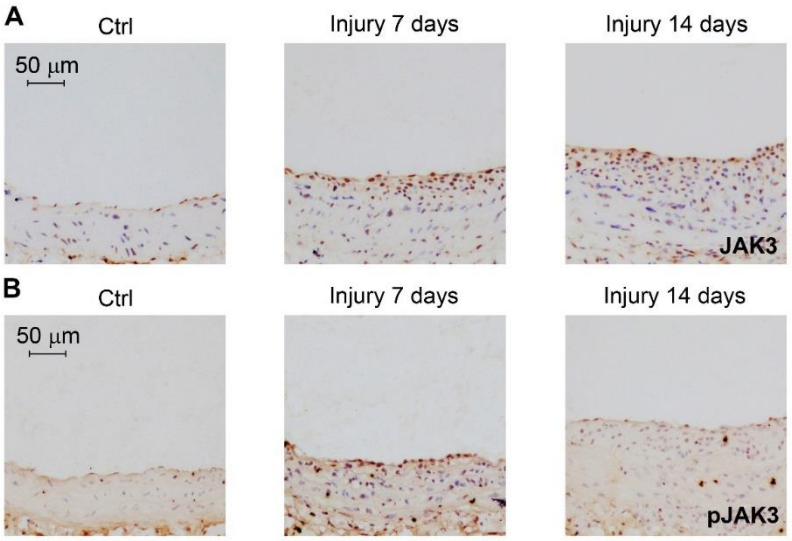


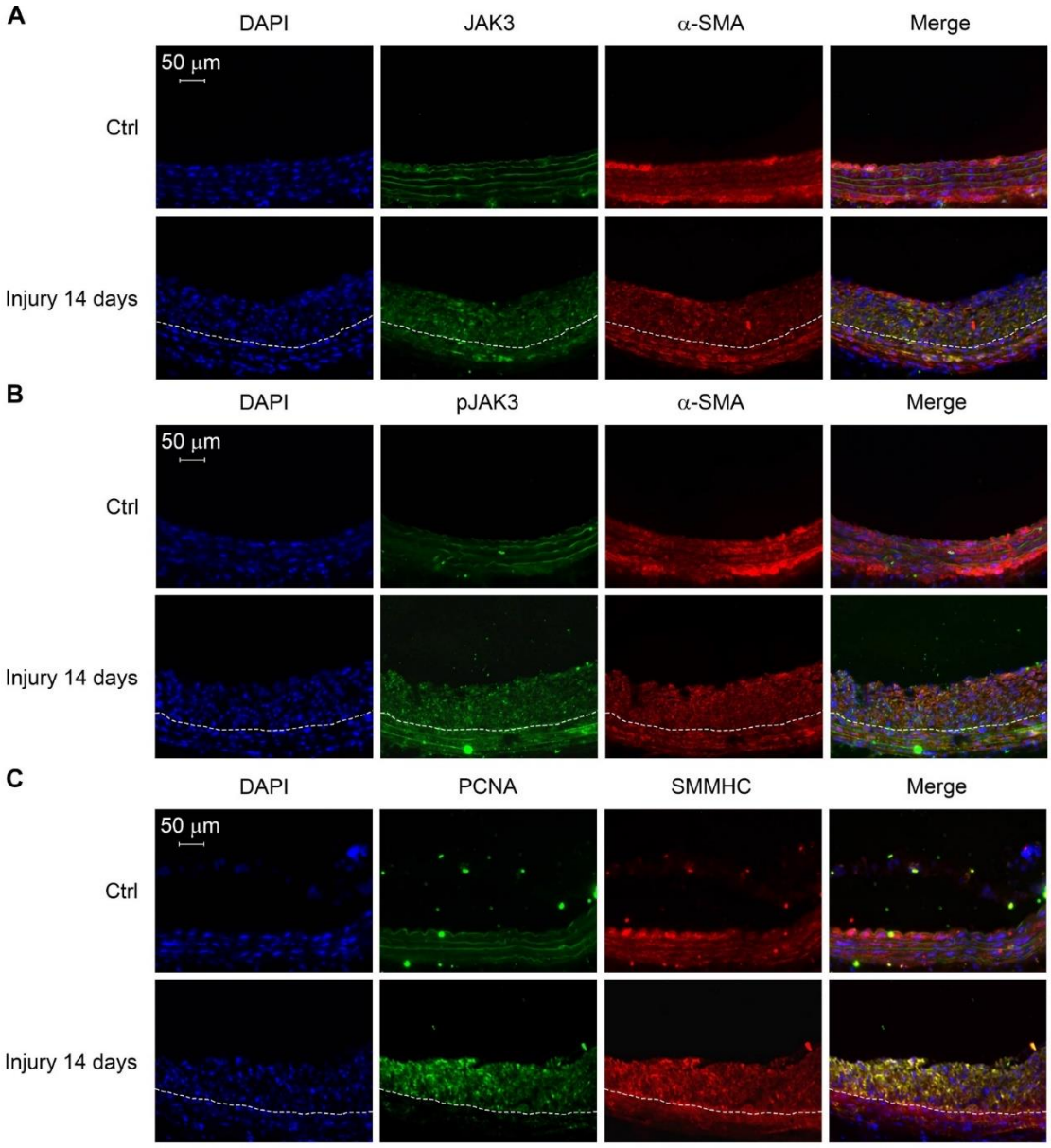
Figure 3.6. Blockade of STAT3 or JNK activity attenuated JAK3–induced SMC proliferation.



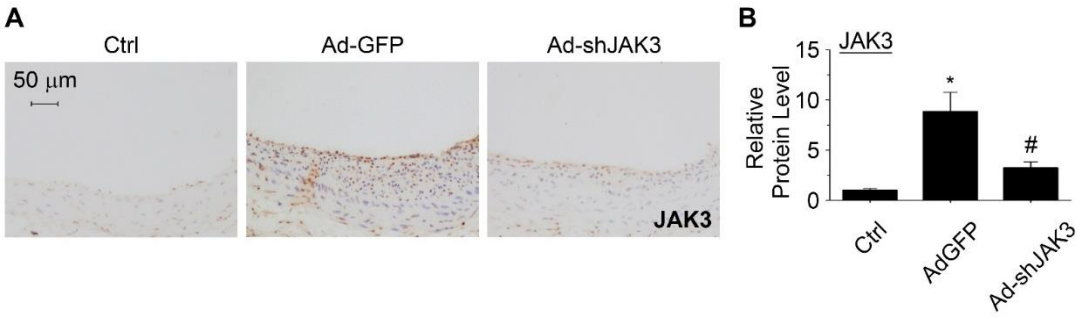
Supplementary Figure S3.1. The expression of JAK family members in PDGF-BB-induced SMCs and neointimal SMCs of balloon-injured arteries.



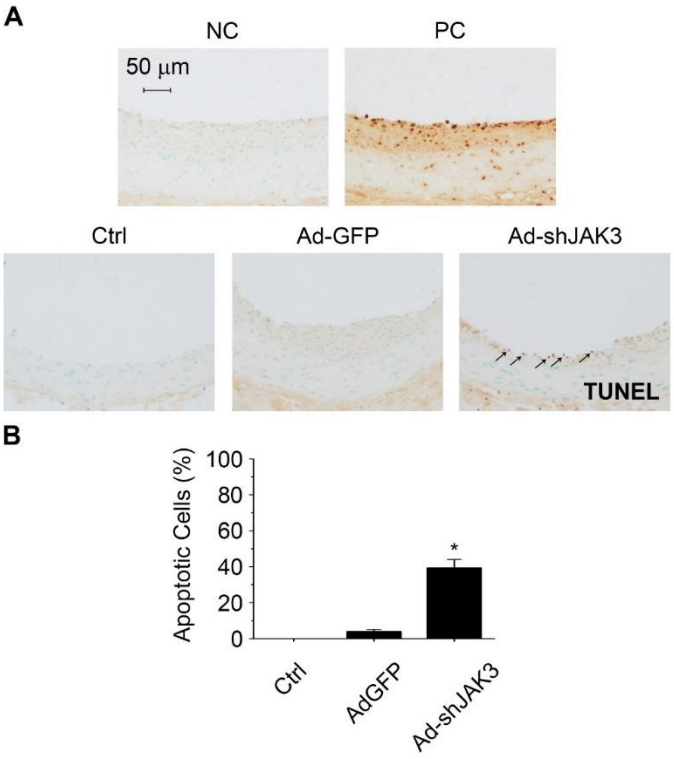
Supplementary Figure S3.2. JAK3 was up-regulated and activated in neointimal SMC during injury-induced vascular remodeling.



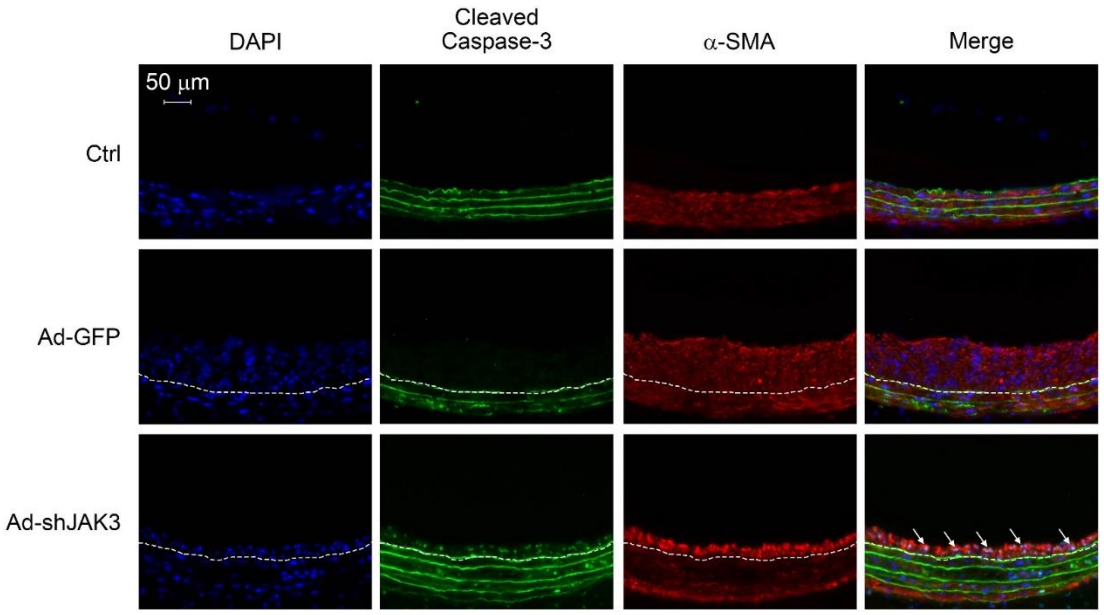
Supplementary Figure S3.3. Co-expression of JAK3, pJAK3, and PCNA with SMC markers in neointimal SMCs in injured rat carotid arteries.



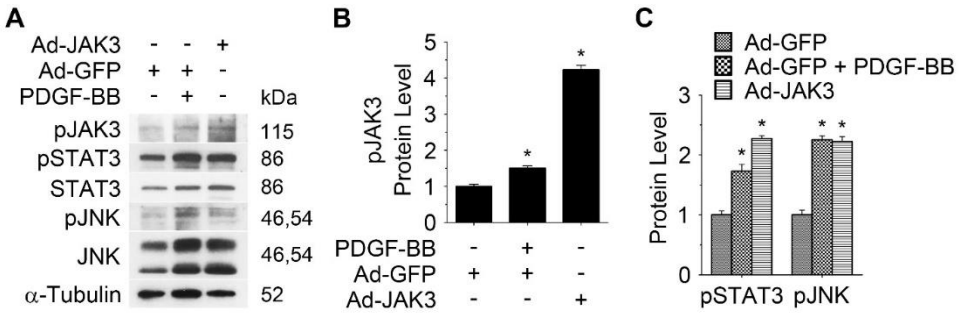
Supplementary Figure S3.4. JAK3 expression was effectively blocked by adenoviral vector-expressed JAK3 shRNA (Ad-shJAK3).



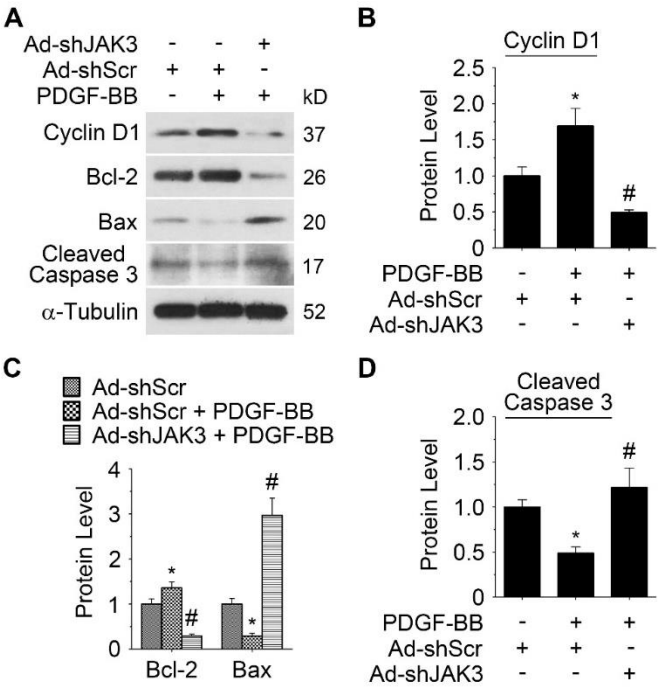
Supplementary Figure S3.5. Knockdown of JAK3 expression caused cell apoptosis in neointima of the injured rat carotid artery.



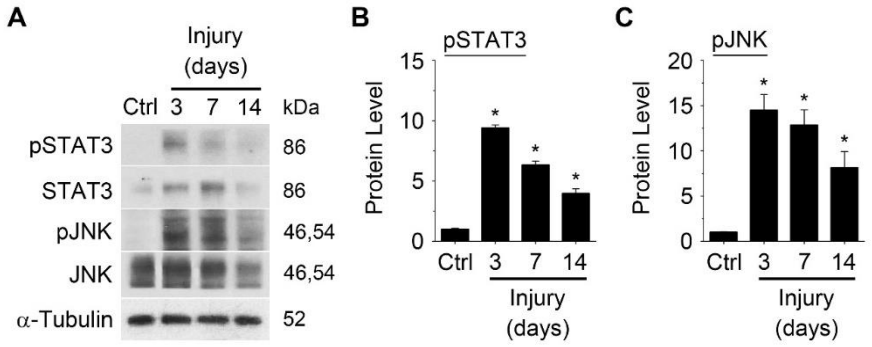
Supplementary Figure S3.6. Knockdown of JAK3 caused neointimal SMC apoptosis in balloon-injured arteries.



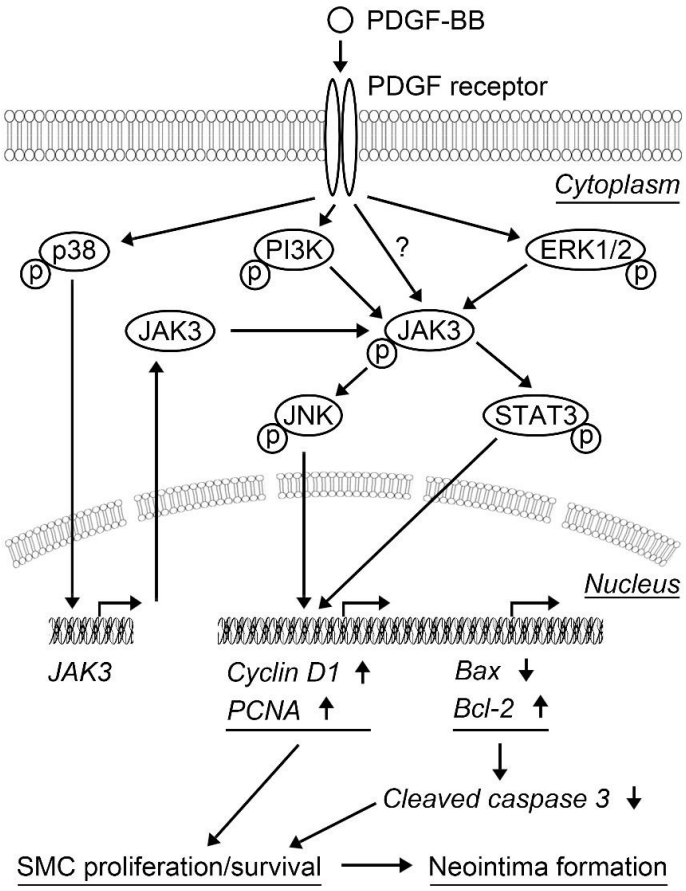
Supplementary Figure S3.7. Forced expression of JAK3 activated STAT3 and JNK.



Supplementary Figure S3.8. JAK3 mediated PDGF-BB-induced cyclin D1 expression and caspase 3 activation.



Supplementary Figure S3.9. STAT3 and JNK were activated in injured arteries.



Supplementary Figure S3.10. A schematic mechanism by which JAK3 regulated SMC proliferation/survival.

Supplementary Table S3.1: Primers used in qRT-PCR.

Primer	Sequence
rno-JAK3-F	GCCTCCATCTCTGGAGTTTTCT
rno-JAK3-R	AGAGGCTACAAGAGCGCTGA
rno-JAK1-F	AGTGCAGTATCTCTCCTCTCTG
rno-JAK1-R	GATTCGGTTCGGAGCGTACC
rno-JAK2-F	CTTGTGGTATTACGCCTGTGT
rno-JAK2-R	TGCCTGGTTGACTCGTCTATG
rno-TYK2-F	AGAGCGCATACCTGTGTGTC
rno-TYK2-R	GTCACCAGTACCTCGTGGGT

CHAPTER 4

JANUS KINASE 3 DEFICIENCY PROMOTES VASCULAR RE-ENDOTHELIALIZATION[2]

[2] Wang YC, Cui XB, Chuang YH, Cuneo C and Chen SY. Janus Kinase 3 Deficiency Promotes Vascular Re-endothelialization. To be submitted to Arterioscler Thromb Vasc Biol.

Abstract

***Objective*—**Delayed regeneration of endothelium following drug-eluting stent implantation is due to the universal effect of anti-proliferative drugs on stents. Identification of new therapeutic targets that differentially regulate the proliferation of smooth muscle cells (SMCs) and endothelial cells (ECs) is important for developing therapeutics for the proper recovery from vascular injury. The objective of this study is to determine the role and mechanism of Janus kinase 3 (JAK3) in EC proliferation and vascular re-endothelialization after injury.

***Methods and Results*—**Ectopic expression of JAK3 in SMCs suppresses the proliferation and migration of ECs. Conversely, knockdown of JAK3 in SMCs promotes proliferation and migration of ECs. Mechanistically, JAK3 modulates the expression levels of pro- and anti-angiogenic factor in SMC, *i.e.*, vascular endothelial growth factor A and thrombospondin 1, respectively. Moreover, although ECs express a relatively higher level of JAK3 at the basal state as compared to SMCs, JAK3 is up-regulated in proliferative SMCs but down-regulated in proliferative ECs. JAK3 appears to arrest cell cycle progression of EC at G0/G1 phase by attenuating the stability of G1-S transition regulator, Cyclin E. In vivo, knockdown of JAK3 promotes re-endothelialization with accelerating EC proliferation.

***Conclusion*—**Our results demonstrate that JAK3 oppositely regulates SMC and EC proliferation, which provides a novel strategy via inhibiting JAK3 activity to suppress neointima formation while enhancing re-endothelialization for effective vascular repair following injury.

Non-standard Abbreviations and Acronyms:

SMC: Smooth muscle cell

JAK3: Janus kinase 3

TSP1: Thrombospondin 1

VEGFA: Vascular endothelial growth factor A

PDGF-BB: Platelet-derived growth factor-BB

PCNA: Proliferating cell nuclear antigen

shRNA: Short hairpin RNA

Introduction

Endothelial cells (ECs) are the major regulator of vascular hemostasis, vascular tone, angiogenesis, vascular barrier, inflammation, etc.[1, 2] Endothelial dysfunction characterized by loss of vasodilator, particularly nitric oxide, has been shown to be associated with atherosclerosis, hypertension, coronary artery disease, diabetes, and injury-induced restenosis.[3-6] Endothelial denudation after angioplasty or stent implantation induces proliferation of smooth muscle cells (SMCs) resulting in restenosis.[7-9] Besides, a discontinuation of endothelial monolayer because of EC apoptosis caused by cardiovascular risk factors contributes to numerous cardiovascular diseases.[10, 11] How to replace the loss of endothelium via inducing resident EC proliferation or recruiting circulating endothelial progenitor cells is a major challenge in the field of cardiovascular intervention.[12] Therefore, investigating molecular mechanisms underlying EC proliferation and re-endothelialization is important for improving the treatment of these cardiovascular diseases.

ECs maintain SMCs in a quiescent state by the growth arrest effect of nitric oxide.[13] When confluent monolayer of ECs is formed, ECs cease replication due to the inhibitory effect of cell contact.[14] Although ECs rapidly replicate in response to vascular injury, the endothelial regeneration stops before wound heals completely due to the suppressive effect of injury-induced proliferative SMCs.[15] Communication between ECs and SMCs is an essential process for the maintenance of normal vascular physiology and the progression of cardiovascular diseases.[16-18] ECs promotes SMC differentiation via phosphoinositide 3-kinase/Akt signaling pathway.[19] In contrast, SMCs suppress endothelial cell growth factor-induced EC growth.[20] In a co-culture system, the expression of growth factors in ECs and SMCs is regulated by their cell-cell contact.[21] Although it has been shown that proliferative SMCs inhibit ECs proliferation and migration via

altering the gene expression profile of pro- and anti-angiogenic factors[22], the molecular mechanisms underlying this phenomenon remains largely unknown.

JAK3, a member of Janus kinase (JAK3) family of non-receptor tyrosine kinases, transduces signals induced by cytokines via receptors containing common γ chain.[23] JAK3 has been shown to regulate cellular function of different cell types during physiological and pathological processes.[24] Absence of JAK3 deters common lymphoid progenitors from differentiating into T- and B-lymphocyte.[25] The location of T- or B- lymphocytes in body, thymus or bone marrow respectively, is determined by the presence of JAK3 and its signaling.[26-28] Moreover, mutation of JAK3 has been observed in extranodal natural killer/T cell lymphomas (ENKTL) of patients.[29] In addition to the immune cells, JAK3 regulates lung epithelial cell proliferation induced by neuregulin-1 via signal transducer and activator of transcription (STAT) signaling.[30] Besides, JAK3 involves in tumor necrosis factor-α-induced cell adhesion molecules in vascular ECs via STAT3 and nuclear factor-κB leading to the increase of myocardial vascular permeability.[31] Our previous study have shown that JAK3 induces SMC proliferation via STAT3 and c-Jun N-terminal kinase resulting in injury-induced neointimal formation.[32] However, it is unknown whether JAK3 plays a role in EC proliferation and re-endothelialization following vascular injury.

In this study, we found knockdown of JAK3 accelerated endothelium recovery after endothelial denudation via stimulating EC proliferation. Conditioned medium of SMCs with JAK3 overexpression suppresses EC proliferation and migration via anti-angiogenic factor, thrombospondin 1 (TSP1) because this phenomenon is inhibited by blocking TSP1 activity through its neutralizing antibody. Conversely, Conditioned medium of SMCs with JAK3 knockdown restored PDGF-BB-suppressed EC proliferation via pro-angiogenic factor vascular

endothelial growth factor A (VEGFA) because this effect is impeded by VEGFA neutralizing antibody. Of interest, JAK3 is highly expressed in quiescent ECs but was down-regulated in proliferative ECs. In contrast, JAK3 expression was significantly induced in SMCs undergoing proliferation, suggesting that JAK3 may differentially regulate EC and SMC proliferation. Indeed, although JAK3 promotes SMC proliferation, it inhibits EC proliferation by arresting its cell cycle at G0/G1 phase through reducing Cyclin E protein stability.

Materials and Methods

Reagents and Cell Culture

Rat aortic smooth muscle cells (SMCs) were cultured by enzymatic digestion method from rat thoracic aorta as described previously.[32, 33] SMCs were maintained in Dulbecco's modification of Eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS, Hyclone) and 5% L-glutamine (Corning) at 37°C in a humidified atmosphere with 5% CO2. Phenotype of primary cultured SMCs were confirmed by the expression of smooth muscle α-actin and SM22α. Rat aortic endothelial cells (ECs) were purchased from Cell Biologics (RN-6052), and were maintained in complete rat endothelial cell medium (Cell Biologics, M1266) at 37°C in a humidified atmosphere with 5% CO2. Chemicals were obtained from the following sources: rhPDGF-BB (R&D, 220-BB), Mitomycin C (Sigma Aldrich, M4287), Evans Blue (Sigma Aldrich, E2129), Cycloheximide (Sigma Aldrich, 01810), Calcein AM (Sigma Aldrich, C1359), Vybrant CM-Dil (Fisher Scientific, V22888). Antibodies were obtained from the following sources: JAK3 (Cell Signaling, #8863), PCNA (Santa Cruz, sc-56), SMMHC (Biomedical Technologies, BT-562), α-SMA (Abcam, ab5694), Calponin (Abcam, ab46794), SM22α (Abcam, ab10135), CD31 (Santa Cruz, sc-376764), VE-cadherin (Santa Cruz, sc-28644), TSP1 (Santa Cruz, sc-393504), TSP1 for neutralizing

(Thermo Scientific, MA5-13377), VEGFA (Santa Cruz, sc-7269), VEGFA (Thermo Scientific, PA5-47026), Cyclin E (Santa Cruz, sc-377100), β-actin (cell signaling, 3700S), α-Tubulin (Cell Signaling, #2125).

Construction of Adenovirus

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.[34] 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). Adenovirus expression control vector (Ad-Ctrl) was used as a control.

Rat Carotid Artery 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.[32, 35] 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.[36] Balloon-injured artery segment was collected at 14 days after the surgery. The segments were perfused with

saline, fixed with 4% paraformaldehyde, and then embedded in paraffin for further sectioning and subsequent morphometric analyses in a double-blinded manner. Protein of injured arteries was collected at 3, 7, and 14 days after the surgery, and was analyzed by Western Blot Analysis. To assess the extent of re-endothelialization, rats were given 0.5 ml sterile solution of Evans blue (0.5 %) by intravenous tail injection 30 minutes prior to injured artery segment collection.[37] Re-endothelialization was assessed by measuring the Evans blue-unstained area, and the rate was calculated by the following formula : [(Mean value of Evans blue-unstained area) / (Mean value of total area)]. The subsequent statistical analyses were performed as described below.

Immunohistochemistry (IHC), and Immunofluorescent Staining (IF)

Balloon-injured artery sections (5 μm) used for analyses among different groups were evenly distributed in the vessel segment collected.[35] Sections were rehydrated, blocked with 10% goat serum or donkey serum, permeabilized with 0.01% Triton X-100 in PBS, and then incubated with JAK3, phospho-JAK3, PCNA, 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 of IHC and IF was performed by incubating with Immunoglobulin G (IgG) antibody. Image J software was used to measure the intensity of IHC positive staining by following the previous publication.[38] Mean value of the staining intensity for each group was acquired from 10 artery sections. To quantify the protein level, the mean value of IHC positive signal of each group less the background (negative control) signal 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 IHC staining

intensity – Mean value of negative control staining intensity) / (Mean value of IHC staining intensity of uninjured vessels – Mean value of negative control staining intensity)]. The subsequent statistical analyses were performed as described below.

Conditioned Smooth Muscle Cell Medium

SMCs were transduced with adenovirus as indicated, and then cultured in complete medium (DMEM containing 10% FBS and 5% L-glutamine) for 48 hours. Equal numbers (3×10^6) of SMCs were seeded into 60 mm culture dishes. 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. The conditioned SMC medium were then stored at -80°C. To block TSP1 or VEGFA activity, conditioned SMC medium was incubated with neutralizing antibody (1 μg/ml) at 4°C for at least 1 hour prior to treat the ECs. For treating the cell, conditioned SMC medium was mixed with complete rat endothelial cell medium (1:1).

EdU Cell Proliferation Assay

Equal numbers (5×10^4 cells) of ECs were seeded into 12-well cell culture plates. Cells were starved in serum-free DMEM for 24 hours, and then treated with conditioned SMC medium for 48 hours. Cells were then incubated with 5-Ethynyl-deoxyuridine (5-EdU) by following the manufacturer's recommendation (EMD Millipore). EdU-positive cells were counted from 10 different microscopic fields (10x). Proliferation rate was assessed by the following formula: [Cell numbers at 48 hours / Cell numbers at 0 hour]. The experiments were repeated for three times with three replicates for each treatment.

Western Blot Analysis

Rat ECs and SMCs were transduced with either Ad-Ctrl, Ad-JAK3, or Ad-shJAK3 for 48 hours. Then, the infected-cells were re-seeded into 6-well cell culture plate. Next day, cells were starved in serum-free DMEM for 24 hours, and then treated with conditioned SMC medium for 48 hours or 20% FBS DMEM 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 Reagent (Thermo Scientific). Equal amount of protein was 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 for 16 hours at 4°C, followed by incubation with HRP-conjugated secondary antibody for 1 hour (Sigma) at Room temperature. Detection was performed with enhanced chemiluminescence (Millipore). The experiments were repeated for three times with three replicates for each treatment.

Wound-healing assay

Wound healing assay was performed with modification using CytoSelect Wound Healing Assay Kit (Cell Biolabs). Wound healing inserts were put into 24-well cell culture plates coated with fibronectin. 250 μl Cell suspension (1×10^4 cells/ml) was added to either side of the insert and incubated overnight to form a monolayer. The inserts were then removed to allow the cells to migrate. Cells were starved in serum-free DMEM containing mitomycin C (10 μg/ml) for 2 hours. Then, treated with conditioned SMC medium for additional 48 hours. Images were captured using Eclipse 90i Nikon microscope. Migration rate was assessed by the following formula: [Cell

migration distance at 48 hours / Cell migration distance at 0 hour]. The experiments were repeated for three times with three replicates for each treatment.

Endothelial Tube Formation Assay

Tube formation assay was performed using Matrigel Basement Membrane Matrix (Corning) and the procedure was performed with modification from previous study.[39] Rat ECs and SMCs were transduced with either vehicle, Ad-Ctrl, Ad-JAK3, or Ad-shJAK3 for 48 hours. Then, the infected-cells were stained with either Calcein AM (5 μM, Green) or Vybrant CM-Dil (5 μM, Red) for 30 minutes prior to re-seeding. The cells (5 x 10^4 cells) were then re-seeded into 24-well cell culture plate pre-coated with Matrigel and then incubated for 10 hours. Images were captured using Eclipse 90i Nikon microscope. Image J software was used to measure and calculate different parameters (total length, number of branch point, and number of sprouts) to indicate the extent of angiogenesis.[39] Mean value of each parameter for each group was acquired from 10 images. The experiments were repeated for three times with three replicates for each treatment.

Cell Cycle Flow Cytometry Analysis

Rat ECs were transduced with either Ad-Ctrl, Ad-JAK3, or Ad-shJAK3 for 48 hours. Then, the infected-cells were re-seeded into 6-well cell culture plate for overnight. Next day, cells were starved in serum-free DMEM for 24 hours, and then treated with 20% FBS DMEM for 24 hours. 1×10^6 cells were resuspended in 500 μl of PBS, and then fixed in ice-cold 70% ethanol for 30 minutes at 4°C. Cells were washed with PBS twice, and resuspended in 500 μl of reaction buffer containing 10 μl of Nuclear-IDTM Red dye (Enzo Life Sciences, ENZ-52406) and 2.5 μl RNase A/T1 (Thermo Scientific, EN0551) for overnight at 4°C. Cell cycle analysis was performed on a CyAn ADP (Beckman Coulter, Hialeah, Florida), and analyzed by the FlowJo software (Treestar,

Inc., Ashland, Oregon). The experiments were repeated for three times with three replicates for each treatment.

Protein stability assay

Rat ECs were transduced with either Ad-Ctrl or Ad-shJAK3 for 48 hours. Then, the infected-cells were re-seeded into 6-well cell culture plate for overnight. Next day, cells were starved in serum-free DMEM for 24 hours, and then treated with 20% FBS DMEM for 16 hours. Cells were then treated with 10 μg/ml of cycloheximide (CHX) to block protein synthesis. Cellular proteins were collected at 0, 1, 2, 4, 8, and 24 hours after CHX treatment. Cyclin E protein level was detected by Western Blot Analysis. The experiments were repeated for three times with three replicates for each treatment.

Statistical Analysis

Results were presented as mean ± S.D. 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

Knockdown of JAK3 Promoted Endothelium Recovery from Injury.

Although SMC proliferation in response to injury causes vascular stenosis, impaired re-endothelialization is the critical factor leading to the failure of vascular repair.[40] Our previous study has shown that knockdown of JAK3 attenuates injury-induced stenosis in artery along with suppression of SMC proliferation.[32] However, it remains unknown if JAK3 affects vascular endothelium. To determine if JAK3 plays a role in re-endothelialization, adenoviral vector

expressing JAK3 short hairpin RNA (shRNA; Ad-shJAK3) was transduced into endothelium-denuded arteries, and the degree of re-endothelialization was assessed by Evans blue staining. As shown in Figure 4.1A, knockdown of JAK3 significantly improved the endothelial recovery from vascular injury compared to control adenoviral vector (Ad-Ctrl)-treated arteries. The percentage of endothelium was increased by 50% with Ad-shJAK3 administration compared to Ad-Ctrl (37.1%±11.9% versus 92.3%±5.5%; $P<0.05$, n=5, Figure 4.1B). To further confirm this phenomenon, endothelial marker expression was detected by immunohistochemistry staining. Two standard markers for ECs, platelet endothelial cell adhesion molecule (PECAM-1, also known as CD31) and vascular endothelial cadherin (VE-cadherin), was increased in Ad-shJAK3-treated arteries compared to Ad-Ctrl-treated arteries (Figure 4.1C).

EC proliferation is initiated rapidly in response to injury at early time point. However, its proliferation rate is suppressed by the presence of neointimal SMCs before wound healing is completed.[15] As expected, there was no ECs observed in arteries with injury for 14-days when abundant proliferative SMCs were present in the neointima (Figure 4.1D). However, injured arteries transduced with Ad-shJAK3 displayed a small number of proliferative ECs in neointima, as indicated by white arrows (Figure 4.1D). These results suggested that JAK3 knockdown not only suppressed SMC proliferation/neointimal formation but also increased EC proliferation/re-endothelialization.

Knockdown of JAK3 in SMCs Promoted EC Proliferation and Migration.

SMCs has been shown to affect EC growth in a co-culture system.[20, 41] Besides, our previous study has shown that proliferative SMCs treated by platelet-derived growth factor-BB (PDGF-BB) inhibits EC proliferation and migration.[22] Because JAK3 regulates PDGF-BB-induced SMC proliferation[32], we sought to determine if the increased re-endothelialization in injured arteries

treated with Ad-shJAK3 was due to the effect of SMCs. As shown in Figure 4.2A through 4.2C, conditioned medium of SMCs transduced with adenoviral vector expressing JAK3 cDNA (Ad-JAK3) suppressed EC proliferation as well as the expression of proliferating cell nuclear antigen (PCNA). Conversely, conditioned medium of PDGF-BB-treated SMCs with JAK3 knockdown impeded the suppression of EC proliferation and PCNA expression (Figure 4.2D through 4.2F). EC migration is also important for re-endothelialization after arterial injury.[42] Ad-JAK3-conditioned SMC culture medium suppressed EC migration while Ad-shJAK3-conditioned SMC culture medium inhibited the suppression of EC migration by PDGF-BB-conditioned SMC culture medium (Figure 4.2G and 4.2H). To further confirm the JAK3 regulation of EC proliferation and migration through the effect of SMCs, we performed in vitro tube formation assay via co-culturing ECs with SMCs transduced either with Ad-Ctrl, Ad-JAK3, or Ad-shJAK3 in Matrigel Matrix. As shown in Supplementary Figure S4.1, ectopic expression of JAK3 in SMCs suppressed while knockdown of JAK3 enhanced the EC tube formation. These results indicated that SMCs with JAK3 knockdown may have pro-angiogenic property.

JAK3 Regulated TSP1 and VEGFA Expression in SMCs.

SMCs have been shown to express angiogenic and anti-angiogenic factors to control EC physiological function.[21, 22, 43] Matrix protein thrombospondin-1 (TSP1) and vascular endothelial growth factor (VEGF) are two major factors to regulate EC angiogenesis.[44] Blockade of TSP1 via neutralizing antibody promotes re-endothelialization along with decrease of neointimal formation in balloon-injured rat carotid artery.[45] Besides, local delivery of VEGFA enhances re-endothelialization and suppresses neointimal formation in balloon-injured rat carotid artery.[46] Therefore, we hypothesized that JAK3 may promote SMC to express these two factors to regulate EC proliferation and migration. As shown in Figure 4.3A and 4.3B, ectopic expression of JAK3

induced TSP1 expression and suppressed VEGFA expression in SMCs. In contrast, knockdown of JAK3 suppressed PDGF-BB-induced TSP1 expression and induced VEGFA expression (Figure 4.3C and 4.3D). To determine if TSP1 and VEGFA are also regulated by JAK3 in neointimal SMCs, we performed immunofluorescent staining of TSP1/VEGFA and co-stained with smooth muscle myosin heavy chain (SMMHC) on injured arteries. As expected, knockdown of JAK3 suppressed injure-induced TSP1 expression but enhanced VEGFA expression in injured arteries (Figure 4.3E). Protein expression of TSP1 and VEGFA in injured artery was confirmed by Western blot. As shown in Supplementary Figure S4.2, both TSP1 and VEGFA protein expression was induced by injury at early time points. However, VEGFA expression decreased to background level while TSP1 expression sustained at 14 days after injury, consistent with the finding of previous study.[15]

To determine if TSP1 and VEGFA play a role in JAK3-regulated communication between SMCs and ECs, we introduced neutralizing antibodies of TSP1 and VEGFA to block their function in SMC conditioned medium. As shown in Figure 4.4A through 4.4C, blockade of TSP1 via neutralizing antibody impeded the suppressive effect of Ad-JAK3-conditioned SMC medium on EC proliferation and migration. Conversely, blockade of VEGFA via neutralizing antibody demolished the enhanced effect of Ad-shJAK3-conditioned SMC culture medium on EC proliferation and migration (Figure 4.4D through 4.4F). These data indicated that JAK3 regulates EC functional properties by controlling TSP1 and VEGFA expression in SMCs. Our previous study has shown that the alteration of SMC pro- or anti- angiogenic property is accompanied by the phenotypic switch of SMCs.[22] Indeed, ectopic expression of JAK3 inhibited SMC marker protein expression while knockdown of JAK3 induced the expression of SMC markers that were suppressed by PDGF-BB (Supplemental Figure S4.3).

JAK3 Expression was Differentially Regulated in Proliferative SMCs and ECs.

Although JAK3 is predominantly expressed in hematopoietic cells, it is also observed in human ECs.[47] To determine if JAK3 is directly involved in EC function, we firstly detected its expression in rat ECs (Figure 4.5A). Interestingly, the basal expression level of JAK3 was much higher in ECs than SMCs (Figure 4.5B). Moreover, immunohistochemistry staining showed that JAK3 was mainly expressed in endothelium, but not in the media of rat arteries, as indicated by black arrows (Figure 4.5C), suggesting that JAK3 may have different roles in these two cell types. Fetal bovine serum (FBS) is known to stimulate proliferation of different types of cell.[48-50] To determine how JAK3 is regulated in proliferative SMCs and ECs, we used 20% FBS to induce cell proliferation. As shown in Figure 4.1D and 4.1E, JAK3 expression was up-regulated in proliferative SMCs, as indicated by the increased PCNA expression. However, JAK3 was suppressed time-dependently in proliferative ECs (Figure 4.5F and 4.5G), suggesting that JAK3 may regulate SMC and EC proliferation distinctly.

JAK3 Mitigated Cell Cycle Progression in ECs via Decreasing Cyclin E Stability

Our previous study shows that JAK3 promotes SMC proliferation via regulating the expression of cyclin D, a critical regulator for cell cycle.[32] We thus hypothesized that JAK3 may regulate EC proliferation via controlling cell cycle progression. Indeed, ectopic expression of JAK3 arrested FBS-induced cell cycle progression at G0/G1 stage (Figure 4.6A and Supplementary Figure S4.4). In contrast, knockdown of JAK3 promotes cell cycle progression of EC with higher ratio of S and G2/M stage (Figure 4.6A and Supplementary Figure S4.4). Because Cyclin E is a central regulator for G1-S transition of cell cycle[51], we sought to determine if JAK3 regulate cell cycle progression of ECs via Cyclin E. Ectopic expression of JAK3 suppressed Cyclin E protein expression that was

induced by FBS (Figure 4.6B and 4.6C), while knockdown of JAK3 enhanced FBS-induced Cyclin E expression (Figure 4.6D and 4.6E). Since the time window for the presence of Cyclin E is short during cell cycle progression due to the activity of ubiquitin proteasome system,[52] we hypothesized that JAK3 may regulate the stability of Cyclin E. To test this, we performed the protein stability assay by blocking protein synthesis in ECs with translation inhibitor cycloheximide (CHX) and then measured the Cyclin E protein level by Western blot. As expected, protein level of Cyclin E dramatically decreased 1 hour after the CHX treatment. However, knockdown of JAK3 prolonged the lifespan of Cyclin E protein.

To further confirm JAK3 activity in EC proliferation, we detected if JAK3 affects EC tube formation. As shown in Supplementary Figure S4.5, ectopic expression of JAK3 in ECs suppressed the EC tube formation although knockdown of JAK3 had no effect. Collectively, our data demonstrated that JAK3 inhibited EC proliferation via controlling cell cycle progression through decreasing cyclin E protein stability.

Discussion

Regeneration of endothelium achieved by resident EC proliferation is critical for the vascular repair after injury.[42] However, the rate of EC proliferation is limited due to the inhibitory effect of neointimal smooth muscle cells (SMCs).[14, 15] It has been shown that proliferative SMCs inhibit EC proliferation and migration[20], due to the alteration of SMCs in producing pro-/anti-angiogenic factors.[22] In this study, we have identified that JAK3 is a novel regulator for promoting endothelium regeneration via inducing proliferation and migration of ECs. JAK3 modulates the pro- and anti-angiogenic property of SMCs (Figure 4.3) accompanied with the phenotypic alteration, characterized by the alteration in contractile protein expression (Supplementary Figure

S4.3). Although JAK3 regulates both the pro-/anti-angiogenic property and phenotypic change of SMCs, further study is required to determine whether these two phenomena have a cause-effect relationship.

Anti-angiogenic property of thrombospondin 1 (TSP1) and angiogenic-property of vascular endothelial growth factor A (VEGFA) is well established.[53, 54] In the case of vascular injury, local delivery of VEGF promotes endothelium regeneration with inhibition of neointimal thickness[46], while blockade of TSP1 activity accelerates re-endothelialization with attenuated neointimal formation.[45] However, the effect of VEGFA delivery on endothelium regeneration after vascular injury remains controversial,[55, 56] likely due to the production of the anti-angiogenic factors, such as TSP1, after endothelial denudation.[57] TSP1 has been shown to inhibit angiogenic effect of VEGFA via interfering VEGFA-mediated Akt signaling and disrupting the association of VEGF receptor-2 with CD47, a critical event for initiating VEGFA signaling.[58, 59] Moreover, injure-induced TSP1 expression promotes SMC proliferation which further suppresses EC proliferation.[57] JAK3 regulates EC properties also by manipulating the expression of pro- and anti-angiogenic factors, VEGFA and TSP1 respectively, in SMCs (Figure 4.3 and 4.4). Knockdown of JAK3 inhibits the injury-induced TSP1 expression while inducing VEGFA expression, resulting in enhanced re-endothelialization. Therefore, it is beneficial to target JAK3 during pathological vascular remodeling because of its multiple effects including inhibition of SMC proliferation, acceleration of EC proliferation, and alteration of SMC pro-/anti-angiogenic property.

Suppressed re-endothelialization is the major obstacle hindering the effectiveness of angioplasty or stent implantation caused by the anti-proliferative agents targeting both SMCs and ECs.[40] Therefore, a selective or SMC-specific anti-proliferative agent is necessary. Our current results indicate that JAK3 plays a differential role in regulating the proliferation of SMCs and ECs.

JAK3 promoted SMC proliferation via inducing proliferating cell nuclear antigen and cyclin D through signal transducer and activator of transcription 3 and c-Jun N-terminal kinase signaling.[60] In contrast, JAK3 inhibited EC proliferation via decreasing Cyclin E protein stability (Figure 4.6). These data further indicate that JAK3 is an excellent target for suppressing injury-induced neointimal formation and accelerating re-endothelialization. Although the signaling involves in the regulation of JAK3 in EC proliferation is still unknown, it has been shown that JAK3 mediates interleukin-2 (IL-2)-induced wound healing of the intestinal epithelial cells via β-catenin signaling.[60, 61] Activation of β-catenin signaling has been shown to induce EC proliferation, likely due to the remodeling of adherences junction.[62, 63] Moreover, IL-2 receptor is presented in ECs[64], and the level of IL-2 in plasma is increased after vascular injury.[65] On the other hand, IL-2 causes injury of ECs via the increase of activated T-lymphocytes in vascular leak syndrome.[66, 67] However, the circumstance of the vascular injury in endothelial denudation and vascular leak syndrome is different. Therefore, it is likely that JAK3 may regulate EC proliferation through β-catenin signaling, which need extensive future studies.

In addition to ECs and SMCs, circulating cells such as endothelial progenitor cells (EPCs) and leukocytes, may contribute to the increases re-endothelialization in our animal studies. Indeed, intravenous transfusion of EPCs in a mouse model of arterial injury enhances re-endothelialization along with decreased neointimal formation.[68] Although whether EPCs are able to differentiate into mature ECs remains controversial, it can induce the proliferation of resident ECs via a paracrine effect.[69, 70] 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.[71, 72] The initial inflammation response in the lesion site is induced by the recruited leukocytes such as platelets, neutrophils, and monocytes.[73] Knock out of leukocyte

integrin, Macrophage-1 antigen, reduces injury-induced neointimal formation via diminished accumulation of leukocytes at lesion site.[74] Therefore, there is a possibility that the enhanced re-endothelialization with administration of JAK3 shRNA is partially due to the pro-angiogenic effect of EPCs and the decreased leukocyte accumulation, which can be studied in the future.

Our study is the first to demonstrate the role of JAK3 in re-endothelialization after vascular injury. JAK3 regulates the pro-/anti-angiogenic property of SMCs, characterized by the expression of VEGFA or TSP1, to affect the cellular function of ECs. In addition, JAK3 plays an opposite role in regulating cell cycle progression of ECs and SMCs. Mechanistically, JAK3 induces cyclin D expression level to promote SMC proliferation[60] while decreases cyclin E level to cause cell cycle arrest at G0/G1 stage in ECs. Although it is beneficial to promote endothelial regeneration via downregulation of JAK3 in injured arteries, whether the new vascular endothelial lining has proper physiological function of endothelium, such as vascular tone regulation, response to environmental stimuli (*i.e.*, mechanical stress and cytokines), and vascular barrier, is an important subject for future investigation.

Reference

1. Michiels C. Endothelial cell functions. *J Cell Physiol*. 2003;196:430-43.
2. Bazzoni G and Dejana E. Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. *Physiol Rev*. 2004;84:869-901.
3. Davignon J and Ganz P. Role of endothelial dysfunction in atherosclerosis. *Circulation*. 2004;109:III27-32.
4. Hadi HA, Carr CS and Al Suwaidi J. Endothelial dysfunction: cardiovascular risk factors, therapy, and outcome. *Vasc Health Risk Manag*. 2005;1:183-98.
5. Lerman A and Zeiher AM. Endothelial function: cardiac events. *Circulation*. 2005;111:363-8.

6. Eto M, Toba K, Akishita M, Kozaki K, Watanabe T, Kim S, Hashimoto M, Sudoh N, Yoshizumi M and Ouchi Y. Reduced endothelial vasomotor function and enhanced neointimal formation after vascular injury in a rat model of blood pressure lability. *Hypertens Res*. 2003;26:991-8.
7. Serruys PW, Luijten HE, Beatt KJ, Geuskens R, de Feyter PJ, van den Brand M, Reiber JH, ten Katen HJ, van Es GA and Hugenholtz PG. Incidence of restenosis after successful coronary angioplasty: a time-related phenomenon. A quantitative angiographic study in 342 consecutive patients at 1, 2, 3, and 4 months. *Circulation*. 1988;77:361-71.
8. Fischman DL, Leon MB, Baim DS, Schatz RA, Savage MP, Penn I, Detre K, Veltri L, Ricci D, Nobuyoshi M and et al. A randomized comparison of coronary-stent placement and balloon angioplasty in the treatment of coronary artery disease. Stent Restenosis Study Investigators. *N Engl J Med*. 1994;331:496-501.
9. Kipshidze N, Dangas G, Tsapenko M, Moses J, Leon MB, Kutryk M and Serruys P. Role of the endothelium in modulating neointimal formation: vasculoprotective approaches to attenuate restenosis after percutaneous coronary interventions. *J Am Coll Cardiol*. 2004;44:733-9.
10. Choy JC, Granville DJ, Hunt DW and McManus BM. Endothelial cell apoptosis: biochemical characteristics and potential implications for atherosclerosis. *J Mol Cell Cardiol*. 2001;33:1673-90.
11. Dimmeler S, Haendeler J and Zeiher AM. Regulation of endothelial cell apoptosis in atherothrombosis. *Curr Opin Lipidol*. 2002;13:531-6.
12. Versari D, Lerman LO and Lerman A. The importance of reendothelialization after arterial injury. *Curr Pharm Des*. 2007;13:1811-24.

13. Garg UC and Hassid A. Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J Clin Invest*. 1989;83:1774-7.
14. Schwartz SM and Benditt EP. Clustering of replicating cells in aortic endothelium. *Proc Natl Acad Sci U S A*. 1976;73:651-3.
15. 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.
16. Lusis AJ. Atherosclerosis. *Nature*. 2000;407:233-41.
17. Curcio A, Torella D and Indolfi C. Mechanisms of smooth muscle cell proliferation and endothelial regeneration after vascular injury and stenting: approach to therapy. *Circ J*. 2011;75:1287-96.
18. Gao Y, Chen T and Raj JU. Endothelial and Smooth Muscle Cell Interactions in the Pathobiology of Pulmonary Hypertension. *Am J Respir Cell Mol Biol*. 2016;54:451-60.
19. Brown DJ, Rzucidlo EM, Merenick BL, Wagner RJ, Martin KA and Powell RJ. Endothelial cell activation of the smooth muscle cell phosphoinositide 3-kinase/Akt pathway promotes differentiation. *J Vasc Surg*. 2005;41:509-16.
20. Hoover GA, McCormick S and Kalant N. Effects of porcine aortic smooth muscle cell conditioned medium on endothelial cell replication. *Arteriosclerosis*. 1989;9:76-83.
21. Heydarkhan-Hagvall S, Helenius G, Johansson BR, Li JY, Mattsson E and Risberg B. Co-culture of endothelial cells and smooth muscle cells affects gene expression of angiogenic factors. *J Cell Biochem*. 2003;89:1250-9.

22. Tang R, Zhang G and Chen SY. Smooth Muscle Cell Proangiogenic Phenotype Induced by Cyclopentenyl Cytosine Promotes Endothelial Cell Proliferation and Migration. *J Biol Chem*. 2016;291:26913-26921.
23. Yamaoka K, Saharinen P, Pesu M, Holt VE, 3rd, Silvennoinen O and O'Shea JJ. The Janus kinases (Jaks). *Genome Biol*. 2004;5:253.
24. Wu W and Sun XH. Janus kinase 3: the controller and the controlled. *Acta Biochim Biophys Sin (Shanghai)*. 2012;44:187-96.
25. 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.
26. 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.
27. 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.
28. 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.
29. 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.
30. 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.

31. 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.
32. 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.
33. 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.
34. 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.
35. Tulis DA. Rat carotid artery balloon injury model. Methods Mol Med. 2007;139:1-30.
36. 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.
37. Radu M and Chernoff J. An in vivo assay to test blood vessel permeability. J Vis Exp. 2013:e50062.
38. Jensen EC. Quantitative analysis of histological staining and fluorescence using ImageJ. Anat Rec (Hoboken). 2013;296:378-81.
39. DeCicco-Skinner KL, Henry GH, Cataisson C, Tabib T, Gwilliam JC, Watson NJ, Bullwinkle EM, Falkenburg L, O'Neill RC, Morin A and Wiest JS. Endothelial cell tube formation assay for the in vitro study of angiogenesis. J Vis Exp. 2014:e51312.

40. Stefanini GG and Holmes DR, Jr. Drug-eluting coronary-artery stents. *N Engl J Med*. 2013;368:254-65.
41. Orlidge A and D'Amore PA. Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. *J Cell Biol*. 1987;105:1455-62.
42. 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.
43. Ucuzian AA, Gassman AA, East AT and Greisler HP. Molecular mediators of angiogenesis. *J Burn Care Res*. 2010;31:158-75.
44. Lawler PR and Lawler J. Molecular basis for the regulation of angiogenesis by thrombospondin-1 and -2. *Cold Spring Harb Perspect Med*. 2012;2:a006627.
45. Chen D, Asahara T, Krasinski K, Witzenbichler B, Yang J, Magner M, Kearney M, Frazier WA, Isner JM and Andres V. Antibody blockade of thrombospondin accelerates reendothelialization and reduces neointima formation in balloon-injured rat carotid artery. *Circulation*. 1999;100:849-54.
46. Asahara T, Bauters C, Pastore C, Kearney M, Rossow S, Bunting S, Ferrara N, Symes JF and Isner JM. Local delivery of vascular endothelial growth factor accelerates reendothelialization and attenuates intimal hyperplasia in balloon-injured rat carotid artery. *Circulation*. 1995;91:2793-801.
47. Verbsky JW, Bach EA, Fang YF, Yang L, Randolph DA and Fields LE. Expression of Janus kinase 3 in human endothelial and other non-lymphoid and non-myeloid cells. *J Biol Chem*. 1996;271:13976-80.

48. Rolf CG, Fu BS, Pau A, Wang W and Chan B. Increased cell proliferation and associated expression of PDGFRbeta causing hypercellularity in patellar tendinosis. *Rheumatology (Oxford)*. 2001;40:256-61.
49. Ryan JM. Effect of different fetal bovine serum concentrations on the replicative life span of cultured chick cells. *In Vitro*. 1979;15:895-9.
50. Zheng X, Baker H, Hancock WS, Fawaz F, McCaman M and Pungor E, Jr. Proteomic analysis for the assessment of different lots of fetal bovine serum as a raw material for cell culture. Part IV. Application of proteomics to the manufacture of biological drugs. *Biotechnol Prog*. 2006;22:1294-300.
51. Bertoli C, Skotheim JM and de Bruin RA. Control of cell cycle transcription during G1 and S phases. *Nat Rev Mol Cell Biol*. 2013;14:518-28.
52. Lin DI, Barbash O, Kumar KG, Weber JD, Harper JW, Klein-Szanto AJ, Rustgi A, Fuchs SY and Diehl JA. Phosphorylation-dependent ubiquitination of cyclin D1 by the SCF(FBX4-alphaB crystallin) complex. *Mol Cell*. 2006;24:355-66.
53. Lawler J. Thrombospondin-1 as an endogenous inhibitor of angiogenesis and tumor growth. *J Cell Mol Med*. 2002;6:1-12.
54. Simons M, Gordon E and Claesson-Welsh L. Mechanisms and regulation of endothelial VEGF receptor signalling. *Nat Rev Mol Cell Biol*. 2016;17:611-25.
55. Swanson N, Hogrefe K, Javed Q, Malik N and Gershlick AH. Vascular endothelial growth factor (VEGF)-eluting stents: in vivo effects on thrombosis, endothelialization and intimal hyperplasia. *J Invasive Cardiol*. 2003;15:688-92.
56. Walter DH, Cejna M, Diaz-Sandoval L, Willis S, Kirkwood L, Stratford PW, Tietz AB, Kirchmair R, Silver M, Curry C, Wecker A, Yoon YS, Heidenreich R, Hanley A, Kearney M,

Tio FO, Kuenzler P, Isner JM and Losordo DW. Local gene transfer of phVEGF-2 plasmid by gene-eluting stents: an alternative strategy for inhibition of restenosis. *Circulation*. 2004;110:36-45.

57. Bonnefoy A, Moura R and Hoylaerts MF. The evolving role of thrombospondin-1 in hemostasis and vascular biology. *Cell Mol Life Sci*. 2008;65:713-27.
58. Sun J, Hopkins BD, Tsujikawa K, Perruzzi C, Adini I, Swerlick R, Bornstein P, Lawler J and Benjamin LE. Thrombospondin-1 modulates VEGF-A-mediated Akt signaling and capillary survival in the developing retina. *Am J Physiol Heart Circ Physiol*. 2009;296:H1344-51.
59. Kaur S, Martin-Manso G, Pendrak ML, Garfield SH, Isenberg JS and Roberts DD. Thrombospondin-1 inhibits VEGF receptor-2 signaling by disrupting its association with CD47. *J Biol Chem*. 2010;285:38923-32.
60. Mishra J, Das JK and Kumar N. Janus kinase 3 regulates adherens junctions and epithelial mesenchymal transition through beta-catenin. *J Biol Chem*. 2017;292:16406-16419.
61. Kumar N, Mishra J, Narang VS and Waters CM. Janus kinase 3 regulates interleukin 2-induced mucosal wound repair through tyrosine phosphorylation of villin. *J Biol Chem*. 2007;282:30341-5.
62. Masckauchan TN, Shawber CJ, Funahashi Y, Li CM and Kitajewski J. Wnt/beta-catenin signaling induces proliferation, survival and interleukin-8 in human endothelial cells. *Angiogenesis*. 2005;8:43-51.
63. Sawant DA, Tharakan B, Hunter FA, Smythe WR and Childs EW. Role of beta-catenin in regulating microvascular endothelial cell hyperpermeability. *J Trauma*. 2011;70:481-7; discussion 487-8.

64. Hicks C, Cooley MA and Penny R. Investigation of interleukin 2 receptors on human endothelial cells. *Growth Factors*. 1991;5:201-8.

65. Miller JD, Clabaugh SE, Smith DR, Stevens RB and Wrenshall LE. Interleukin-2 is present in human blood vessels and released in biologically active form by heparanase. *Immunol Cell Biol*. 2012;90:159-67.

66. Wylezinski LS and Hawiger J. Interleukin 2 Activates Brain Microvascular Endothelial Cells Resulting in Destabilization of Adherens Junctions. *J Biol Chem*. 2016;291:22913-22923.

67. Rafi-Janajreh AQ, Chen D, Schmits R, Mak TW, Grayson RL, Sponenberg DP, Nagarkatti M and Nagarkatti PS. Evidence for the involvement of CD44 in endothelial cell injury and induction of vascular leak syndrome by IL-2. *J Immunol*. 1999;163:1619-27.

68. 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.

69. 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.

70. 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.

71. 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.

72. Welt FG and Rogers C. Inflammation and restenosis in the stent era. *Arterioscler Thromb Vasc Biol*. 2002;22:1769-76.

73. 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.

74. 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.

Figure Legends

Figure 4.1. Blockade of JAK3 expression promoted re-endothelialization of injured arteries via inducing Endothelial cell proliferation. A, Reendothelialization in injured artery was accelerated by knockdown of JAK3. The area without re-endothelialization was stained by Evans blue. **B,** Quantification of the extent of re-endothelialization in control or injured arteries by measuring the Evans blue–unstained area as shown in **A**. **P* < 0.05 vs uninjured arteries (Control), n=5. **C,** CD31 and VE-cadherin expression was increased in injured arteries with Ad-shJAK3 treatment as measured by immunohistochemistry staining. Bar: 50 μm. **D,** Knockdown of JAK3 induced proliferation of ECs (white arrow) in injured arteries as measured by immunofluorescent staining for αSMA (Green), VE-cadherin (Green), and Ki 67 (Red). Bar: 50 μm.

Figure 4.2. JAK3 was essential for SMCs to suppress EC proliferation and migration. A, Ad-JAK3-conditioned SMC medium suppressed EC proliferation as measured by EdU assay. **B,** Ad-JAK3-conditioned SMC medium decreased PCNA protein expression in ECs. **C,** Quantification of PCNA protein levels shown in **B** by normalizing to α-Tubulin level. **P* < 0.05 vs control adenoviral vector-transduced cells (Ad-Ctrl), n=3. **D,** Knockdown of JAK3 by Ad-shJAK3 in SMCs impeded the suppression of PDGF-BB-conditioned SMC medium on EC proliferation as measured by EdU assay. **E,** Knockdown of JAK3 in SMC impeded the suppressive effect of PDGF-BB-conditioned SMC medium on PCNA protein levels in ECs. **F,** Quantification of PCNA protein expression shown in **E** by normalizing to α-tubulin level. **P* < 0.05 vs control adenoviral vector-transduced cells (Ad-Ctrl), n=3. **G,** JAK3 regulated the effect of SMC on EC migration as measure by wound healing assay. **H,** Quantification of EC migration shown in **G** by normalizing their migratory distance to the Ad-Ctrl-conditioned SMC

medium treated ECs (Ad-Ctrl). *P < 0.05 vs ECs treated with Ad-Ctrl-conditioned SMC medium (Ad-Ctrl); $^{\#}P$ < 0.05 vs ECs treated with Ad-Ctrl-PDGF-BB-conditioned SMC medium treated (Ad-Ctrl + PDGF-BB), n=3.

Figure 4.3. JAK3 regulated TSP1 and VEGFA expression in SMCs. A, Forced expression of JAK3 by Ad-JAK3 induced TSP1 expression and reduced VEGFA expression in SMCs. **B,** Quantification of JAK3, TSP1, and VEGFA protein levels shown in **A** by normalizing α-tubulin level. *P < 0.05 vs control adenoviral vector-transduced cells (Ad-Ctrl), n=3. **C,** Knockdown of JAK3 by Ad-shJAK3 reduced TSP1 expression and increased VEGFA expression in SMCs. **D,** Quantification of JAK3 TSP1, and VEGFA protein levels shown in **C** by normalizing α-tubulin level. *P < 0.05 vs control adenoviral vector-transduced cells (Ad-Ctrl); $^{\#}P$ < 0.05 vs PDGF-BB treated control cells (Ad-Ctrl + PDGF-BB), n=3. **E,** Knockdown of JAK3 suppressed TSP1 expression and induced VEGFA expression (white arrow) in neointimal SMCs in the injured arteries as measured by immunofluorescent staining for TSP1 (Green), VEGFA (Green), and SMMHC (Red). Bar: 50 μm.

Figure 4.4. JAK3 regulated pro- or anti- angiogenic effect of SMC via TPS1 and VEGFA. A and **B,** TSP1-neutralizing antibody (TSP1 Ab, 1 μg/ml) restored EC proliferation **(A)** and migration **(B)** suppressed by Ad-JAK3-conditioned SMC medium. IgG-neutralizing antibody (IgG Ab, 1 μg/ml) was used as control. **C,** Quantification of EC migration shown in **B** by normalizing their migratory distance to the Ad-Ctrl-conditioned SMC medium treated ECs with IgG-neutralizing antibody incubation (Ad-Ctrl + IgG Ab). *P < 0.05 vs ECs treated with Ad-Ctrl-conditioned SMC medium containing IgG-neutralizing antibody (Ad-Ctrl + IgG Ab); $^{\#}P$ < 0.05 vs ECs treated with Ad-JAK3-conditioned SMC medium containing IgG-neutralizing antibody (Ad-Ctrl + IgG Ab) n=3. **D** and **E,** VEGFA-neutralizing antibody

(VEGFA Ab, 1 μg/ml) suppressed EC proliferation **(D)** and migration **(E)** that was induced by Ad-shJAK3-conditioned SMC medium. IgG-neutralizing antibody (IgG Ab, 1 μg/ml) was used as control. **F,** Quantification of EC migration shown in **E** by normalizing their migratory distance to the Ad-Ctrl-conditioned SMC medium treated ECs with IgG-neutralizing antibody incubation (Ad-Ctrl + IgG Ab). *P < 0.05 vs ECs treated with Ad-Ctrl-conditioned SMC medium containing IgG-neutralizing antibody (Ad-Ctrl + IgG Ab); $^{\#}P$ < 0.05 vs ECs treated with Ad-shJAK3-PDGF-BB-conditioned SMC medium containing IgG-neutralizing antibody (Ad-shJAK3 + PDGF-BB + IgG Ab), n=3.

Figure 4.5. JAK3 differentially regulated SMC and EC proliferation. A, JAK3 expression was higher in ECs than SMCs. **B,** Quantification of JAK3 protein level shown in **A** by normalizing to α-Tubulin level. *P < 0.05 vs SMC, n=3. **C,** JAK3 was expressed highly in arterial ECs as measured by immunohistochemistry staining. Bar: 50 μm. **D,** JAK3 and PCNA proteins were time dependently induced by 20% FBS in SMCs. **E,** Quantification of JAK3 and PCNA protein levels shown in **D** by normalizing to α-Tubulin. *P < 0.05 vs vehicle-treated cells (Ctrl), n=3. **F,** JAK3 and PCNA proteins were time dependently reduced by 20% FBS in ECs. **G,** Quantification of JAK3 and PCNA levels shown in **F** by normalizing to α-Tubulin. *P < 0.05 vs vehicle-treated cells (0h), n=3.

Figure 4.6. JAK3 impaired cell cycle progression via regulating Cyclin E stability. A, Forced expression of JAK3 by Ad-JAK3 caused SMC cell cycle arrest at G0/G1 stage in ECs. *P < 0.05 vs control adenoviral vector-treated cells (Ad-Ctrl); $^{\#}P$ < 0.05 vs 20% FBS-treated control cells (Ad-Ctrl + 20% FBS), n=3. **B,** Forced expression of JAK3 by Ad-JAK3 reduced Cyclin E protein expression. **C,** Quantification of Cyclin E protein level shown in **B** by normalizing to β-Actin. *P < 0.05 vs control adenoviral vector-treated cells (Ad-Ctrl); $^{\#}P$ <

0.05 vs 20% FBS-treated control cells (Ad-Ctrl + 20% FBS), n=3. **D,** Knockdown of JAK3 by Ad-shJAK3 increased Cyclin E protein level. **E,** Quantification of Cyclin E protein level shown in **D** by normalizing to β-Actin. *P < 0.05 vs control adenoviral vector-treated cells (Ad-Ctrl); #P < 0.05 vs 20% FBS-treated control cells (Ad-Ctrl + 20% FBS), n=3. **F,** Knockdown of JAK3 by Ad-shJAK3 increased the stability of Cyclin E. Cycloheximide (CHX, 10 μg/ml) was used to inhibit protein synthesis. **G,** Quantification of Cyclin E protein levels shown in **D** by normalizing to β-Actin. *P < 0.05 vs vehicle-treated cells (0h), n=3.

Supplementary Figure S4.1. JAK3 is essential for SMC to regulate EC tube formation in vitro. A, JAK3 regulated the effect of SMC on EC tube formation as measure by Matrigel angiogenesis assay. SMC was treated with either Ad-Ctrl, Ad-JAK3, or Ad-shJAK3. SMC was stained with Calcein AM (Green) and EC was stained with Vybrant (Red). **B,** Quantification of the extent of angiogenesis by measuring the total length, number of branch point, and number of sprouts as shown in **A**. *P < 0.05 vs control group with Ad-Ctrl-treated SMCs; n=3.

Supplementary Figure S4.2. TSP1 and VEGFA expression in the injured arteries. A, Immunohistochemistry staining of TSP1 and VEGFA expression in injured arteries. **B,** Quantification of TSP1 and VEGFA protein levels shown in **A** by normalizing to α-tubulin level. *P < 0.05 vs uninjured arteries (Ctrl), n=5.

Supplementary Figure S4.3. JAK3 induced phenotypic switch of SMCs. A, Forced expression of JAK3 by Ad-JAK3 decreased contractile protein expression in SMCs. **B,** Quantification of SMMHC, αSMA, CNN1, and SM22α protein levels shown in **A** by normalizing to α-tubulin level. *P < 0.05 vs control adenoviral vector-treated cells (Ad-Ctrl); #P < 0.05 vs PDGF-BB treated control cells (Ad-Ctrl + PDGF-BB), n=3. **C**, Knockdown of JAK3 by Ad-shJAK3 induced contractile protein expression in PDGF-BB-treated SMCs. **D,**

Quantification of SMMHC, αSMA, CNN1, and SM22α protein levels shown in **C** by normalizing to α-tubulin level. *$P < 0.05$ vs control adenoviral vector-treated cells (Ad-Ctrl); #$P < 0.05$ vs PDGF-BB treated control cells (Ad-Ctrl + PDGF-BB), n=3.

Supplementary Figure S4.4. JAK3 induced EC cell cycle arrest at G0/G1 stage. Forced expression of JAK3 by Ad-JAK3 caused SMC cell cycle arrest at G0/G1 stage in ECs.

Supplementary Figure S4.5. JAK3 suppressed EC tube formation. A, JAK3 inhibited EC tube formation as measure by in vitro Matrigel angiogenesis assay. EC was treated with either Ad-Ctrl, Ad-JAK3, or Ad-shJAK3. EC was stained with Vybrant (Red). **B,** Quantification of EC tube formation by measuring the total length, number of branch point, and number of sprouts as shown in **A**. *$P < 0.05$ vs control group with Ad-Ctrl-treated ECs; n=3.

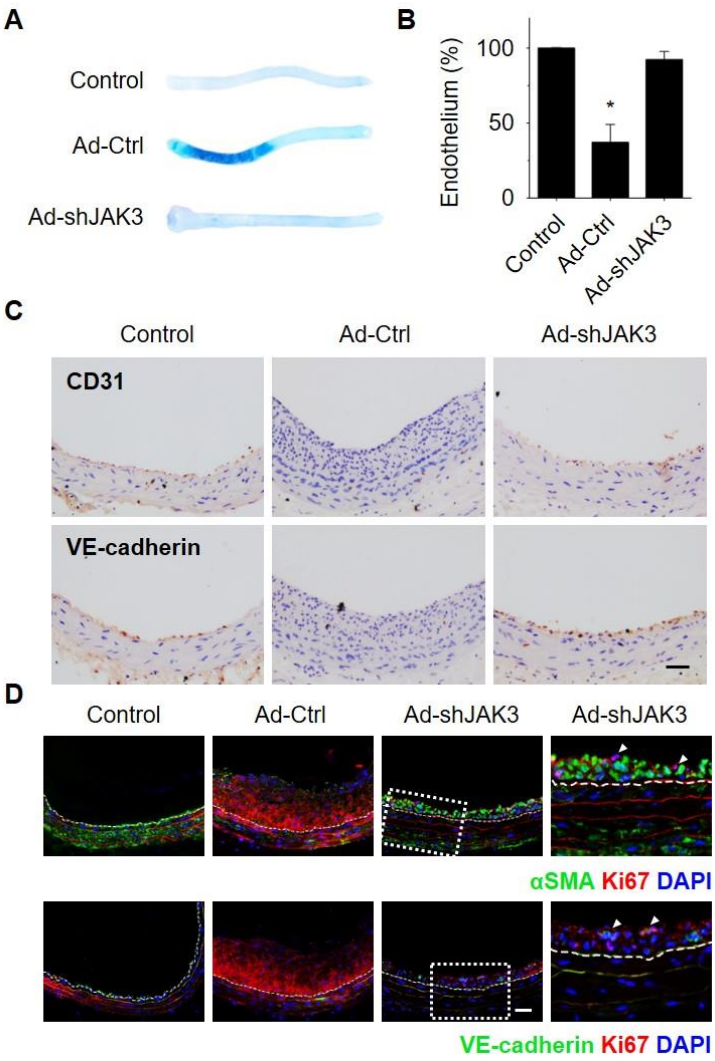


Figure 4.1. Blockade of JAK3 expression promoted re-endothelialization of injured arteries via inducing Endothelial cell proliferation.

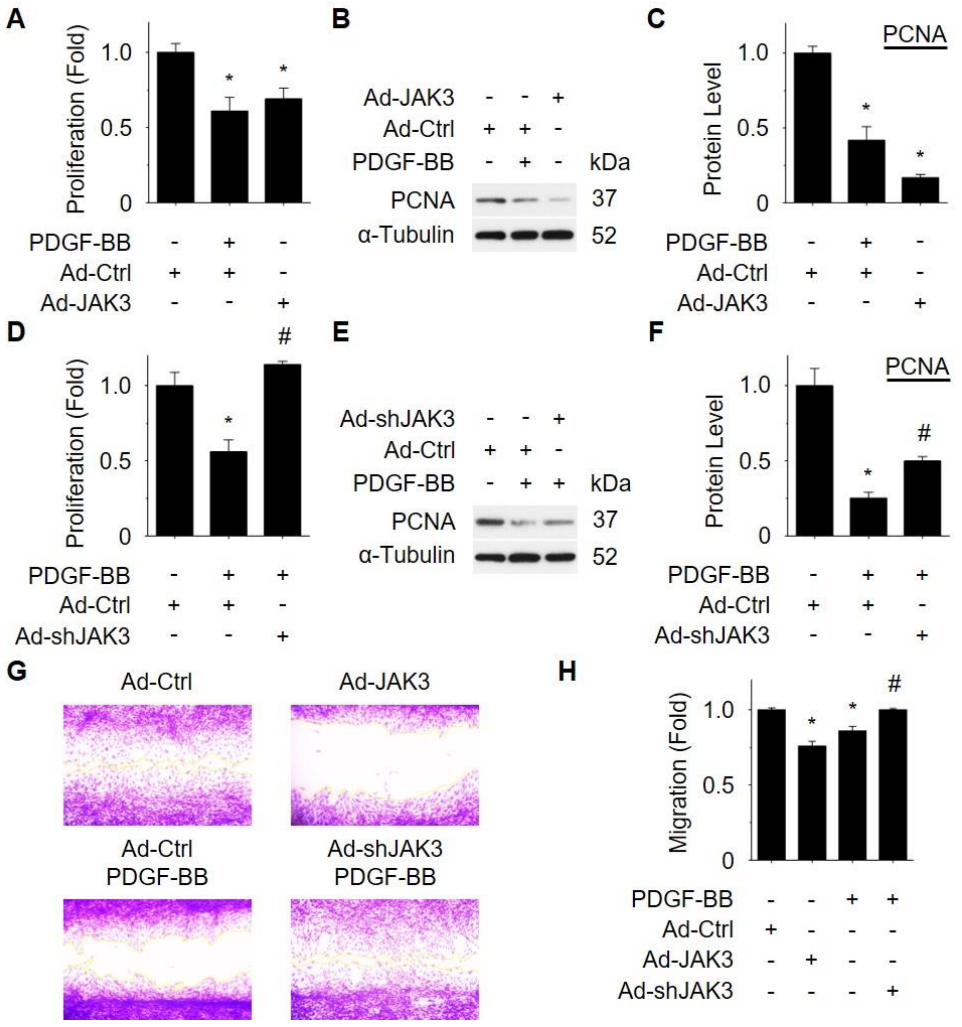


Figure 4.2. JAK3 was essential for SMC to suppress EC proliferation and migration.

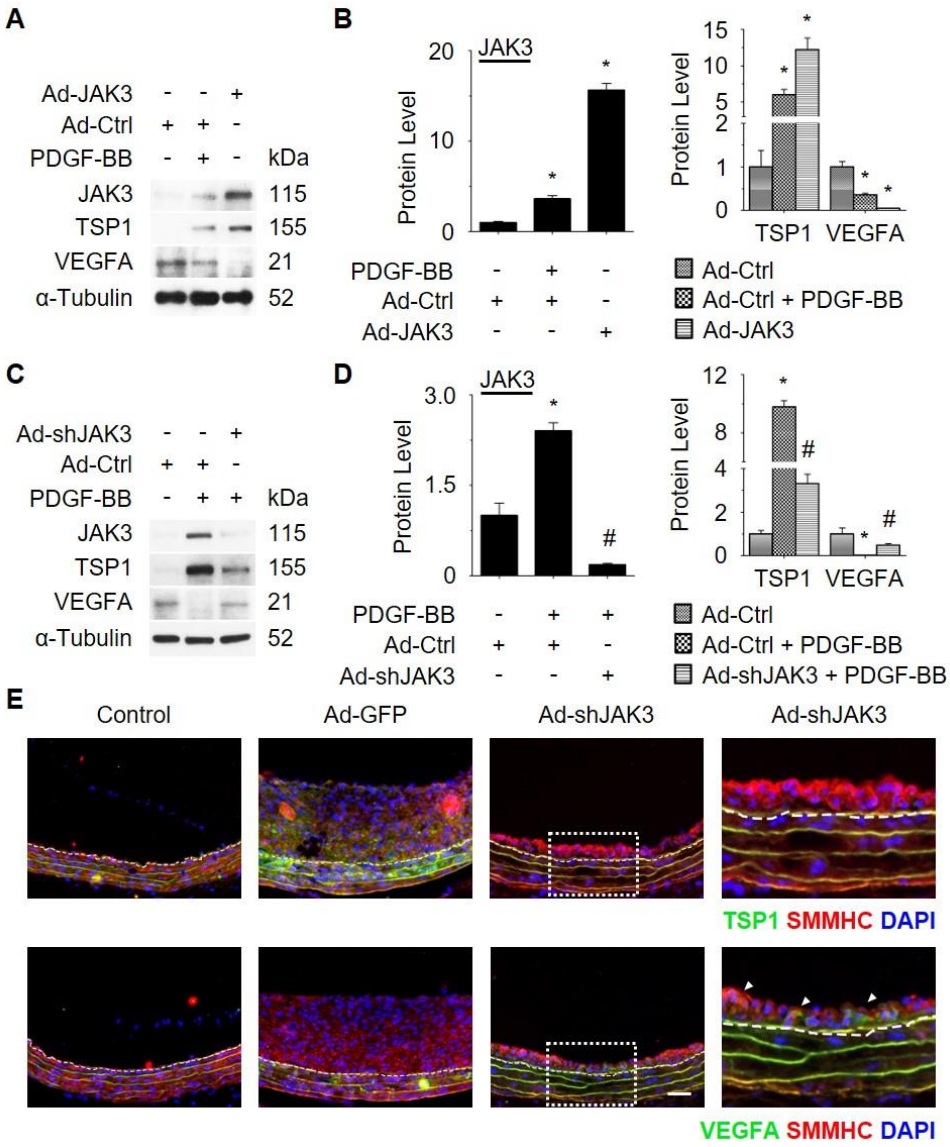


Figure 4.3. JAK3 regulated the expression of TSP1 and VEGFA in SMC.

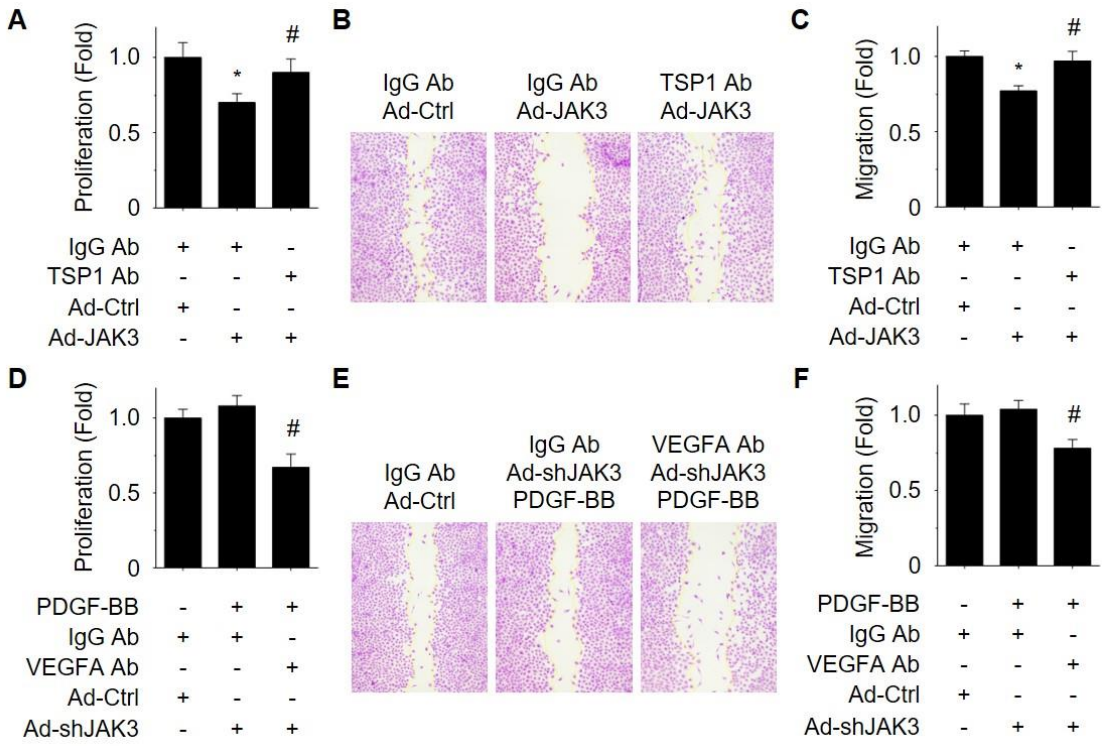


Figure 4.4. JAK3 regulated pro- or anti- angiogenic effect of SMC via TPS1 and VEGFA.

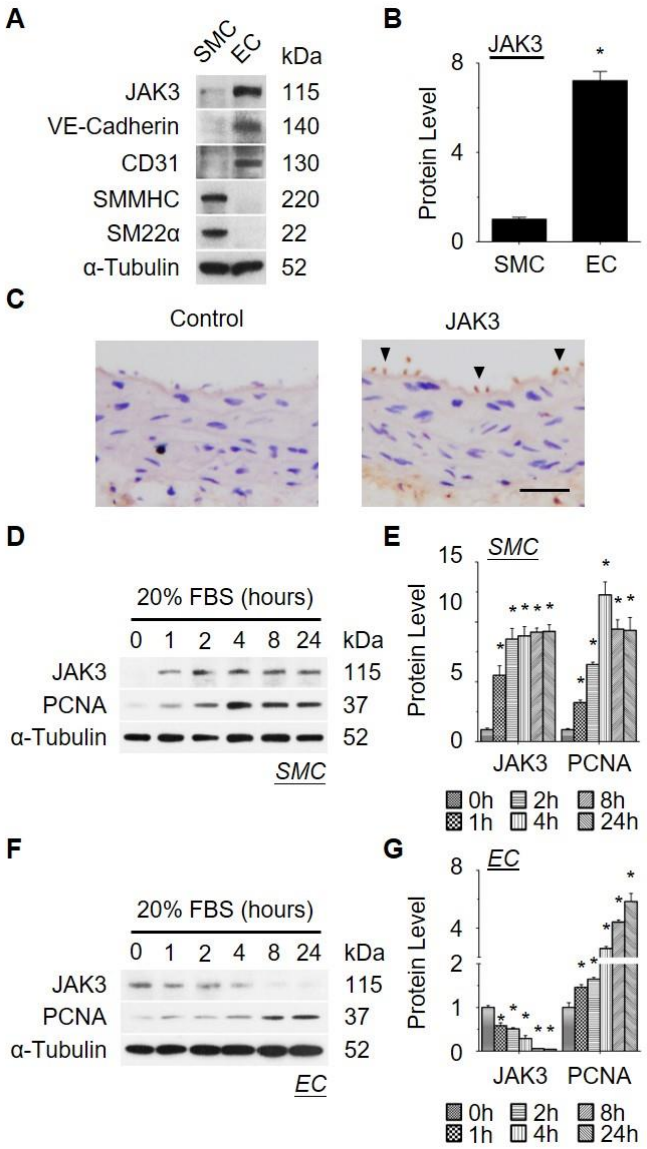


Figure 4.5. JAK3 regulated proliferation of SMC and EC oppositely.

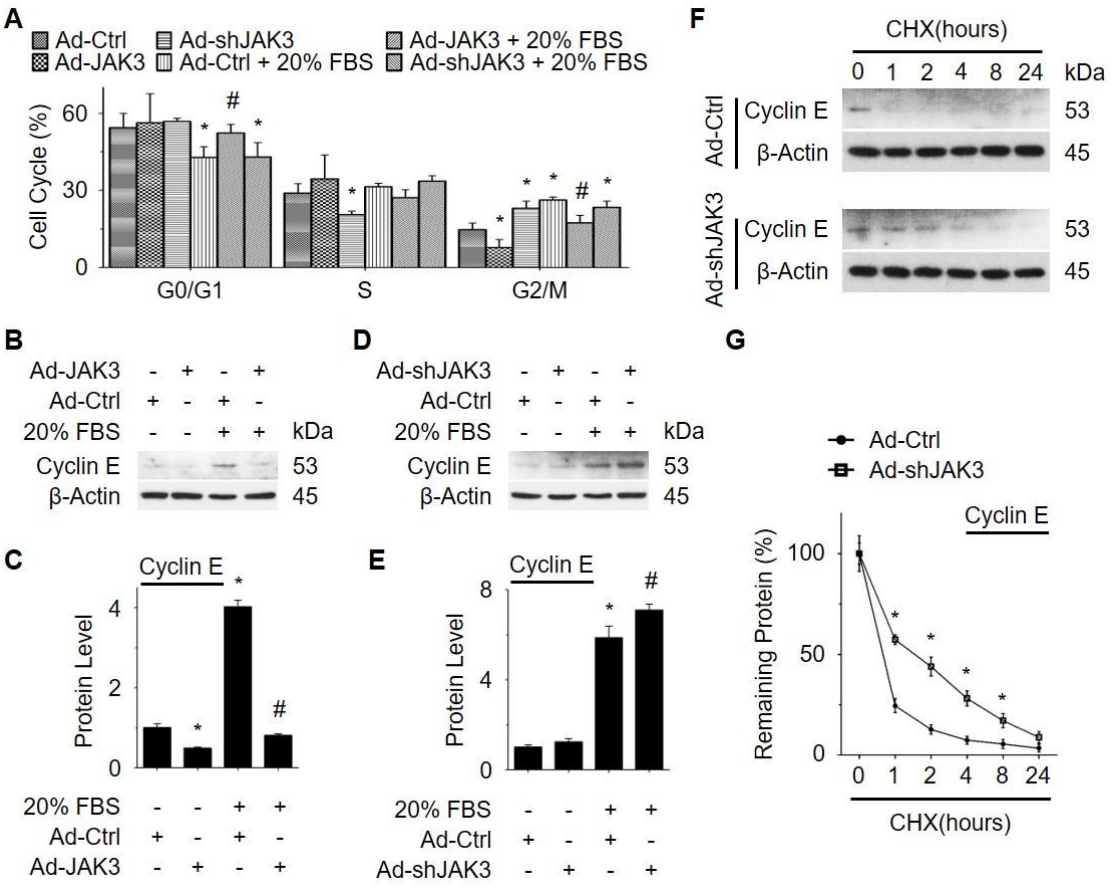
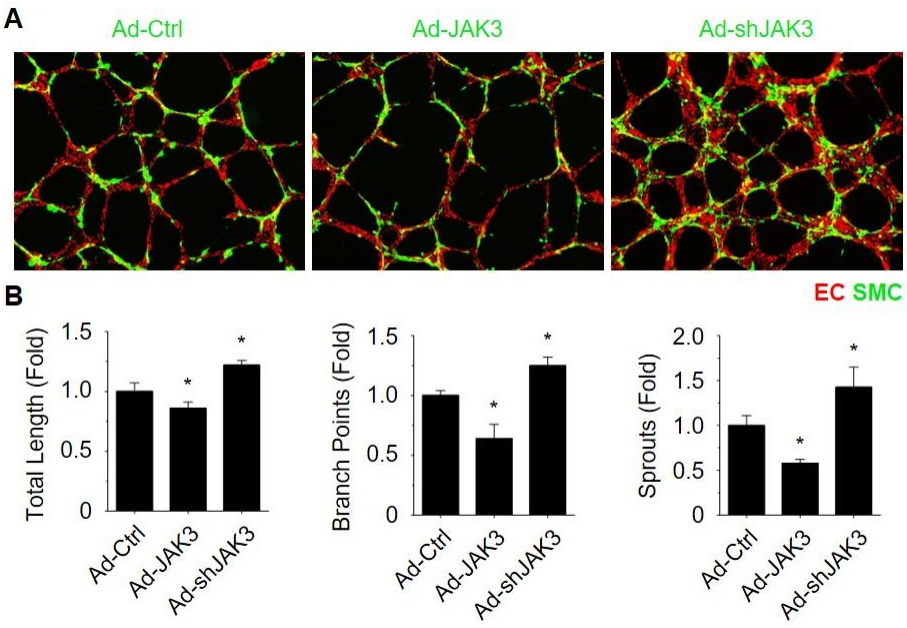
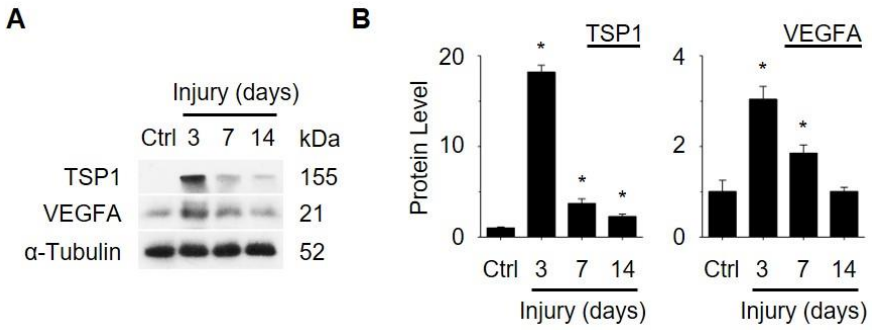


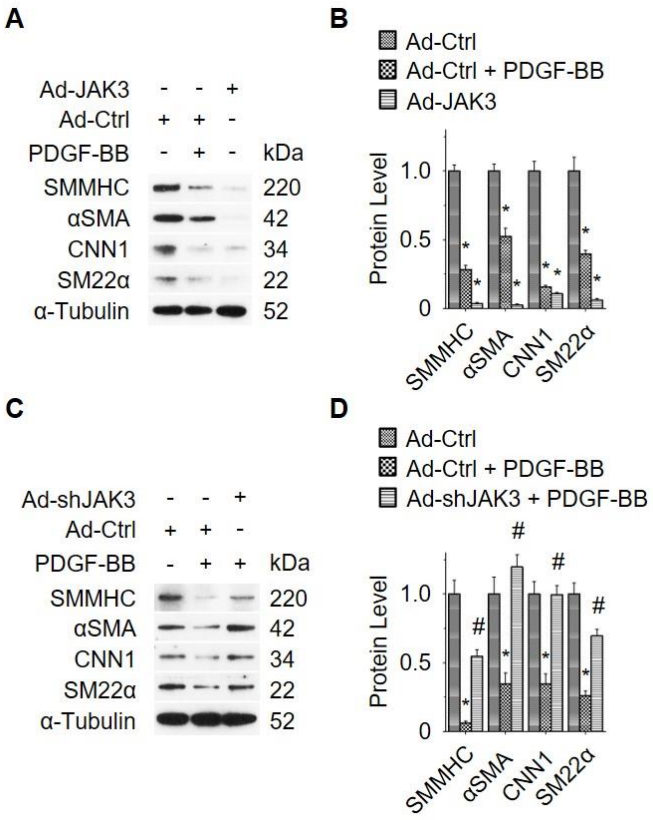
Figure 4.6. JAK3 impaired cell cycle progression via regulating Cyclin E existence.



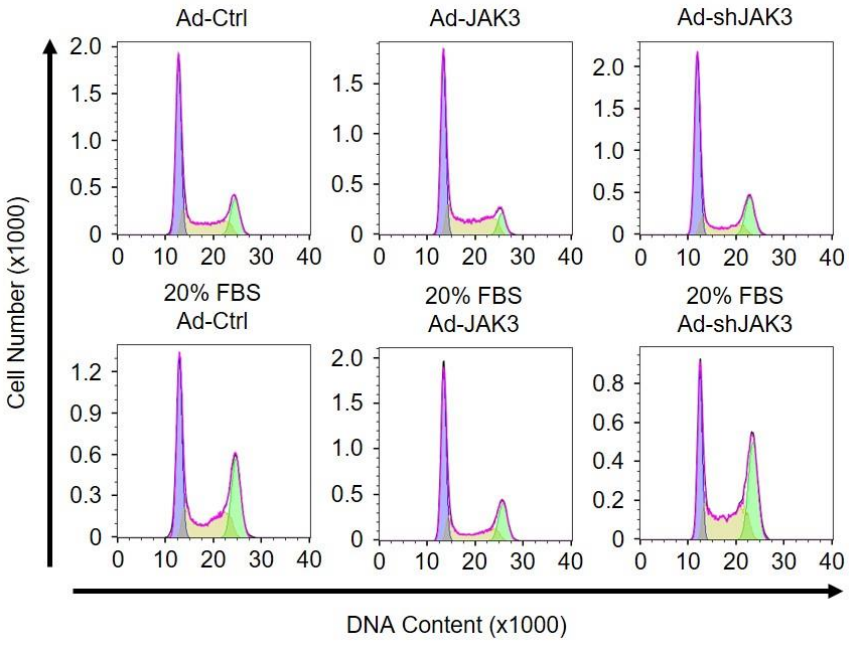
Supplementary Figure S4.1. JAK3 is essential for SMC to regulate EC angiogenesis in vitro.



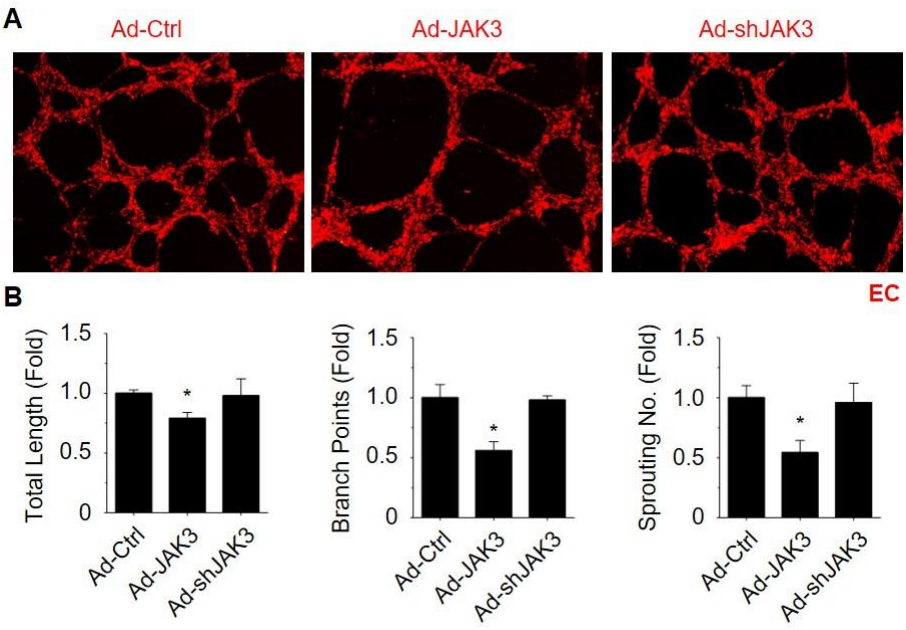
Supplementary Figure S4.2. TSP1 and VEGFA expression in the injured arteries.



Supplementary Figure S4.3. JAK3 induced phenotypic switch of SMC.



Supplementary Figure S4.4. JAK3 induced cell cycle arrest at G0/G1 stage.



Supplementary Figure S4.5. JAK3 suppressed angiogenesis of EC in vitro.

CHAPTER 6

BRAIN CYTOPLASMIC RNA 1 SUPPRESSES SMOOTH MUSCLE DIFFERENTIATION AND VASCULAR DEVELOPMENT IN MICE [3]

[3] 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(15):5668-5678. Reprinted here with permission of the publisher.

Abstract

Smooth muscle cell (SMC) differentiation is an essential process during vascular development. Transforming growth factor (TGF)-β/Smad3 signaling plays a pivotal role in regulating SMC differentiation. Long non-coding RNAs (lncRNAs) regulate various cellular events, such as proliferation, differentiation, apoptosis, etc. However, mechanisms underlying lncRNA regulation of SMC differentiation remain largely unknown. Our present study indicates brain cytoplasmic RNA 1 (BC1) is an important regulator for the SMC differentiation. Forced expression of BC1 suppresses while knockdown of BC1 promotes TGF-β-induced SMC differentiation as shown by altered cell morphology and expression of multiple SMC markers including αSMA, calponin, and SM22α. BC1 appears to block Smad3 activity and further inhibit SMC marker gene transcription. Mechanistically, BC1 binds to Smad3 via RNA Smad-binding elements (rSBEs) and thus impedes TGF-β-induced Smad3 nuclear translocation, which prevents Smad3 from binding to SBE in SMC marker gene promoters, an essential event for SMC marker transcription. In vivo, overexpression of BC1 in mouse embryo impairs vascular SMC differentiation, leading to artery development defects such as random breaks of elastic lamina, inordinate stack of SMCs, and unorganized extra cellular matrix proteins in the media of neonatal aorta. Our results suggest BC1 is a negative regulator for SMC differentiation during vascular development.

Non-standard Abbreviations and Acronyms:

SMC: Smooth muscle cell

LncRNA: Long non-coding RNA

BC1: Brain cytoplasmic RNA 1

αSMA: Alpha smooth muscle actin

CNN1: Calponin 1

SM22α: Smooth muscle 22 alpha

TGF-β: Transforming growth factor-beta

SBE: Smad binding element

shRNA: Short hairpin RNA

FISH: Fluorescence in situ hybridization

Introduction

Cardiovascular system is developed in the early stage of embryogenesis. Differentiation of smooth muscle cells (SMCs) is an essential process for cardiovascular system formation in embryo.[1] Embryonic vascular development begins with primitive vascular plexus formation by incorporation of endothelial cells (ECs) and becomes a complex vascular network.[2] SMC progenitors are recruited and then differentiated to SMCs to ensheath the endothelial vasculature.[3] Disruption of this process during embryonic development causes vascular abnormalities, such as thoracic aortic aneurysms and vascular anomalies, or leads to embryo lethality.[4-6] SMC differentiation is regulated elaborately in embryo at transcriptional and translational level.[7] In addition to protein factors, non-coding RNAs (ncRNAs), such as microRNAs and long ncRNAs (lncRNAs), also play important roles in SMC differentiation.[8-12] Investigating molecular mechanisms underlying lncRNA function in SMC differentiation will advance our understanding of vascular development.

During embryogenesis, SMCs are derived from multiple origins including neural crest, mesoangioblasts, secondary heart field, and proepicardium, etc.[13] Thus, various in vitro cell models such as C3H/10T1/2 (10T1/2) cells, Monc-1 cells, and JoMa1 cells, have been developed to study SMC differentiation.[14] Transforming growth factor (TGF)-β is one of essential growth factors inducing SMC differentiation during vascular development.[15] Besides, Smad proteins serve as core intracellular mediators for transducing TGF-β signaling from transmembrane receptors to nucleus and further modulating the expression of targeted genes via binding to Smad-binding elements (SBEs) in gene promoters to initiate the SMC differentiation.[16] Although a few lncRNAs are regulated by TGF-β and involved in TGF-β-induced gene expression in disease states[17-19], the role of lncRNAs in TGF-β-induced SMC differentiation remains largely unknown.

Brain cytoplasmic RNA 1 (BC1) is a cytoplasmic lncRNA derived from $tRNA^{Ala}$ molecule and mainly presents in specific subset of neurons of the central and peripheral nervous system in rodents.[20] BC200 RNA is the analog of BC1 in primates with similar function and expression pattern.[21] Both BC1 and BC200 regulates protein biosynthesis in dendrites of neurons via interacting with eukaryotic initiation factor 4A (eIF4A), poly(A)-binding protein (PABP), and fragile X mental retardation protein (FMRP).[22-24] BC1-deficient mice show reduced exploratory activity along with increased anxiety, and increased seizure susceptibility although there is no observed anatomical or neurological abnormality.[25-27] In human, BC200 plays a role in tumorigenesis and neurodegeneration.[28] The elevated RNA expression level of BC200 has been detected in different cancer tissues.[29] In breast cancer, BC200 contributes to the progression of tumorigenesis via regulating the survival of tumor cells.[30] In addition to cancer, BC200 RNA expression is increased in brains with Alzheimer's disease (AD), a chronic neurodegenerative disease, and presents a correlation with AD progression.[31] Since vascular and neural systems share similar anatomic localization, structural formation process, and signaling molecules for developmental regulation,[32, 33] and BC1 serves as an important regulator for neural plasticity[34], we sought to determine whether BC1 plays a role in vascular development.

In this study, we found BC1 negatively regulates TGF-β-induced SMC differentiation and vascular development in mouse embryo. Ectopic expression of BC1 suppressed TGF-β-induced SMC differentiation via impeding TGF-β-induced Smad3 nuclear translocation in 10T1/2 cells. Mechanistically, BC1 binds to Smad3 via its RNA SBE (rSBE), which inhibits Smad3 nuclear translocation and subsequent activation of SMC genes. Importantly, ectopic expression of BC1 in mouse embryo resulted in abnormalities of vasculature due to the impaired SMC differentiation.

Materials and Methods

Reagents and Cell Culture

Human aortic smooth muscle cells (SMCs) and endothelial cells (ECS) were purchased from Lonza. C3H10T1/2 (10T1/2) cells were purchased from American Type Culture Collection (ATCC). Mouse aortic SMCs and ECs were cultured from abdominal artery of C57BL/6J mice with following the protocol from previous publication.[35] Cells were then maintained in Dulbecco's modification of Eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS, Hyclone) and 5% L-glutamine (Corning) at 37°C in a humidified atmosphere with 5% CO2. Chemicals were obtained from the following sources: TGF-β1 (R&D System, 240-B), Smad3 inhibitor SIS3 (Sigma-Aldrich, S0447), recombinant human Smad3 protein (Sigma-Aldrich, SRP5132). Antibodies were purchased from various vendors: α-SMA (Abcam, ab5694), Calponin (Abcam, ab46794), SM22α (Abcam, ab10135), α-Tubulin (Cell Signaling, #2125), Smad3 (Cell Signaling, #9523S), phospho-Smad3 (Cell Signaling, #9520S), GAPDH (Sigma-Aldrich, G8795), PCNA (Santa Cruz, sc-56).

Construction of Adenovirus

cDNA fragment encoding the full length of mouse BC1 was amplified from 10T1/2 cells by polymerase chain reaction (PCR), and then inserted into pRNAT-H1.1/Adeno vector (Genscript) between the Mlu I and Hind III site. Primer used to amplify mouse BC1 were as follows. Forward primer: 5'- CCC AAG CTT GGG GTT GGG GAT TTA GCT CAG T. Reverse primer: 5'- CGG AAT TCA AAG GTT GTG TGT GCC AGT TAC. Mouse BC1 short hairpin RNA (shBC1) was inserted into pRNAT-H1.1/Adeno vector (Genscript) between Mlu I and Hind III sites. BC1 shRNA cDNA sequences were: 5'- CCC AAG CTT GGG TAG CTC AGT GGT AGA GCG CTT GCT TCA AGA GAG CAA GCG CTC TAC CAC TGA GCT ATT TTT TCC AAC GG -3' (top

strand) and 5’- CGG AAT TCC GTT GGA AAA AAT AGC TCA GTG GTA GAG CGC TTG CTC TCT TGA AGC AAG CGC TCT ACC ACT GAG CTA CCC A -3’ (bottom strand). Adenoviral vector of BC1 and shBC1 was constructed using AdEasy system described previously.[36] 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).

Real-time Quantitative PCR (RT-qPCR)

Total RNA of cultured cells was extracted from 10T1/2 cells using Trizol Reagent (Invitrogen), and then reverse transcribed to cDNA using iScript™ cDNA Synthesis kit (Bio-Rad). Real time qPCR was performed on a Stratagene Mx3005P qPCR instrument using SYBR Green master mix (Agilent Technologies). Each sample including no-template control was amplified in triplicate. mRNA expression was normalized to cyclophilin (CYP), and LncRNA BC1 expression was normalized to U6. Primer sequences used in this study were listed below. BC1: 5’- ACA CTC CAG CTG GGT TCG GTC CTC AGC TCT GGA A (forward) and 5’- CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG TGT GCC AGT TAC CTT GTT -3’ (reverse); U6: 5’- CTC GCT TCG GCA GCA CA -3’ (forward) and 5’- AAC GCT TCA CGA ATT TGC GT -3’ (reverse); αSMA: 5’- AAT GGC TCT GGG CTC TGT AAG -3’ (forward) and 5’- CAC GAT GGA TGG GAA AAC AGC -3’ (reverse); CNN1: 5’- AAT ACG ACC ATC AGC GGG AG -3’ (forward) and 5’- ATG AAG TTG TTC CCG ATG CG -3’ (reverse); SM22α: 5’- GGT CCA TCC TAC GGC ATG AG -3’ (forward) and 5’- CCT ACA TCA GGG CCA CAC TG -3’ (reverse); CYP: 5’- GAG CTG TTT GCA GAC AAA GTT C -3’ (forward) and 5’- CCC TGG CAC ATG AAT CCT GG -3’ (reverse).

Western Blot Analysis

10T1/2 cells were starved in DMEM containing 5% L-glutamine without FBS for 24 hours, and then treated with TGF-β (5 ng/ml) or vehicle as indicated for 1 hour to detect phospho-Smad3 or 24 hours to detect Smad3, αSMA, CNN1, and SM22α. Cells were infected with adenovirus or transfected with plasmid as indicated before starvation and further treatment. 10T1/2 cells were then washed with PBS twice, followed by protein extraction using RIPA buffer (50 mmol/L 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), and 0.1% SDS). Protein concentration was measured using BCA Protein Assay Reagent (Thermo Scientific). Equal amounts of proteins were resolved on Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to Polyvinylidene fluoride (PVDF, Bio-Rad) or nitrocellulose membranes (Bio-Rad). Nonspecific bindings were blocked with 5% Bovine serum albumin (BSA) in Tris-buffered saline (TBS) plus 0.1% Tween-20 (TBST) at 4°C for 1 hour, and then incubated with primary antibodies in blocking buffer at 4°C for 16 hours. After washing for three times with TBST, membrane was incubated with HRP-conjugated secondary antibody (Sigma) at room temperature for 1 hour. Following washing with TBST for three times, the protein levels were detected with enhanced chemiluminescence (Millipore).

Cell Morphology Observation

10T1/2 cells were seeded on gelatin-coated cover glasses using gelatin-based coating solution (Cell Biologics, 6950). Cells were transduced with either Ad-Ctrl, Ad-BC1, or Ad-shBC1 for two days. The culture medium was then replaced with starving DMEM (no FBS, 5% L-glutamine) when cell density reached 50% confluence. After starvation for 24 hours, cells were treated with vehicle or TGF-β (5 ng/ml) for additional 48 hours. Cells were then fixed with 1% formaldehyde

and stained with crystal violet (Thermo Fisher Scientific, 23-750-025). Cell morphology was observed using a Eclipse 90i Nikon microscope.

Cell Fractionation

Cell fractionation was performed using Nuclear Extraction Kit (EMD Millipore, 2900) by following the procedure recommended by the manufacture. Briefly, 10T1/2 cells were washed with cold PBS and then scraped off. Cell pellets were collected through centrifuging at 250 x g at 4°C for 5 minutes. The cell pellets were re-suspended with ice-cold cytoplasmic lysis buffer on ice for 15 minutes. Cells lysates were passing through a syringe with 27-gauge needle for 10 times followed by centrifugation at 8,000 x g at 4°C for 20 minutes to separate cytoplasmic fraction (supernatant) and nuclear fraction (pellet). The pellet containing nuclear fraction was then lysed with RIPA buffer containing protease and phosphatase inhibitors. Protein concentrations of both cytoplasmic and nuclear fractions were measured using BCA Protein Assay Reagent (Thermo Scientific). Protein partition of each fraction was measured via Western Blot Analysis.

Immunofluorescent Staining (IF)

10T1/2 cells or artery cryo-sections (10 μm) were fixed with methanol/acetone (1:1) at -20°C for 15 minutes. Fixed cells or sections were then washed with PBS for three times, followed by blocking with 10% goat serum at room temperature for 30 minutes. After blocking, cells or sections were incubated with primary antibody (Smad3, αSMA, or PCNA) at 4°C for overnight. Next day, cells or sections were washed with PBST for three times followed by secondary antibody incubation at 37°C for 30 minutes. Then, sections were washed with PBST for three times followed by counterstaining with DAPI. The cell and cross-sectional images were captured using Eclipse 90i Nikon microscope.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assay was performed with modification using a ChIP assay kit (EMD Millipore, 17-295). 10T1/2 cells were fixed with 1% formaldehyde at room temperature (RT) for 15 minutes, then incubated with 0.2 M glycine at RT for additional 5 minutes. Cells were scraped off, and then lysed in SDS Lysis Buffer containing protease and phosphatase inhibitors at 4°C for 10 minutes. Cell lysates were sonicated for 5 rounds of 50% power to shear the nucleic acids, followed by collecting the supernatant. Cell supernatants were diluted with ChIP dilution buffer containing protease and phosphatase inhibitors. After pre-clearing cell supernatants with 50 µl protein A/G agarose beads (Santa Cruz, sc-2003) at 4°C for 4 hours, cell supernatants were incubated with rabbit IgG (negative control), or Smad3 antibodies at 4°C for overnight. Input sample (10%) was collected after the pre-clear step. Next day, the DNA-protein complex was captured by 50 µl protein A/G agarose beads at 4°C for 2 hours, followed by serial wash steps with Low Salt Immune Complex Wash Buffer, High Salt Immune Complex Wash Buffer, LiCl Immune Complex Wash Buffer, and TBE Buffer. Each wash step was repeated for three times. After the washing, A/G agarose beads were incubated with freshly prepared Elution Buffer (1% SDS, 0.1M $NaHCO_3$) at 37°C for 15 minutes to elute DNA-protein complex. Elutes containing DNA-protein complex were added 5M NaCl and incubated at 65°C for 4 hours. Then, elutes were added EDTA, 1M Tris-HCl (pH 6.5), and Proteinase K and incubated at 45°C for 1 hour. DNA was recovered via phenol/chloroform extraction and ethanol precipitation. Purified DNA was then examined via PCR to measure the enrichment of αSMA and SM22α gene promoter region containing Smad Binding Element (SBE). Primer sequences used in this experiment were as follows: αSMA-SBE: 5'- GTT CTG AGG GCT TAG GAT GT -3' (forward) and 5'- CCA GTA AAT CAA GCG TTG TT -3'

(reverse); SM22α-SBE: 5'- GGT GTT GAG CCA AGC AGA C -3' (forward) and 5'- CGA GTT GCA TTA GCC CTG G -3' (reverse).

Luciferase Reporter Assay

10T1/2 cells were transduced with either Ad-Ctrl, Ad-BC1, or Ad-shBC1 for two days. Then, the infected-10T1/2 cells were re-seeded into 12-well cell culture plate. Next day, cells were transfected with plasmids containing firefly luciferase reporter driven by either α-SMA, SM22α, or SBE (SBE-Luc) promoter via Lipofectamine LTX (Thermo Fisher Scientific, 15338100) for 6 hours. Then, 10T1/2 cells were starved overnight followed by TGF-β (5ng/ml) treatment for additional 16 hours. Luciferase activity was measured by Dual-Luciferase Reporter Assay System (Promega, E1910).

In Vitro Transcription

DNA template of BC1 full length (FL), BC1 fragment 1 (F1), and F1 fragment with mutated rSBE (mt1, mt2, and mt12) driven by T7 (sense strand) or T3 (anti-sense strand) promoter were amplified by PCR. In vitro transcription was performed using the T7 or T3 RNA polymerase kit (Roche) by following the protocol provided by the manufacture. Briefly, 1 μg DNA template was used in the reaction containing 40 U/ml RNase inhibitor (RNasin) (Sigma-Aldrich, R1158). Reaction was then incubated at 37°C for 1 hour followed by DNase I treatment. Synthesized RNA was purified using a RNeasy Mini Kit (Qiagen, 74104). BC1 synthesized from the in vitro transcription was labeled with biotin (Thermo Fisher Scientific, AM8452) for pull-down assay or Fluorescein (Thermo Fisher Scientific, R0101) for Fluorescence in Situ Hybridization (FISH) assay. Primer sequences used in this experiment were as follows: BC1-T7-FL: 5'- GAG TAA TAC GAC TCA CTA TAG GGA AGG GGT TGG GGA TTT AGC T -3' (forward); BC1-T3-FL: 5'- GAG ATT AAC CCT CAC TAA AGG GAT TAA AGG TTG TGT GTG CCA G -3' (reverse);

BC1-T3-F1: 5'- GAG ATT AAC CCT CAC TAA AGG GAT TAG AGC TGA GGA CCG AAC C -3' (reverse); BC1-T7-F1-mt1: 5'- GAG TAA TAC GAC TCA CTA TAG GGA AGG TTG GGG ATT TAG CTT GGT GGT A -3' (forward); BC1-T3-F1-mt2: 5'- GAG ATT AAC CCT CAC TAA AGG GAT TAG AAC TAA GGA CCG AAC C -3' (reverse). Different DNA templates were amplified by using different combination of primer sets: BC1-FL template: BC1-T7-FL and BC1-T3-FL; BC1-F1 template: BC1-T7-FL and BC1-T3-F1; BC1-F1-mt1 template: BC1-T7-F1-mt1 and BC1-T3-F1; BC1-F1-mt2 template: BC1-T7-FL and BC1-T3-F1-mt2; BC1-F1-mt12 template: BC1-T7-F1-mt1 and BC1-T3-F1-mt2.

Fluorescence in Situ Hybridization (FISH)

FISH was performed as described previously.[37] 10T1/2 cells or artery cryo-sections (10 μm) were fixed with methanol/acetone (1:1) at -20°C for 15 minutes. Fixed cells or sections were then washed with PBST containing 40 U/ml RNasin for four times followed by washing with 1:1 PBST/Hybridization Buffer (50% formamide, 5X SSC, 100 μg/mL fragmented salmon testes DNA, 50 μg/mL heparin, 0.1% Tween 20, 40 U/ml RNasin) for 10 minutes. Then, cells or sections were incubated with hybridization buffer in serial steps at 50°C for 5, 30, and 30 minutes. BC1 RNA probe in hybridization buffer was prepared at a final concentration of 1 μg/ml. BC1 RNA probe labeled with fluorescein was synthesized via in vitro transcription as described above. BC1 RNA probe was denatured at 80°C for 2 minutes, and then placed on ice before using. Fixed cells and sections were incubated with denatured BC1 RNA probe in a dark and humid environment at 50°C overnight. Negative control was performed with same procedure without BC1 RNA probe in hybridization buffer. Next day, cells or sections were incubated with hybridization buffer in serial steps at 50°C for 5, 30, and 30 minutes, followed by washing with 1:1 PBST/hybridization buffer for additional 10 minutes. Cells or sections were then washed with PBST containing 40

U/ml RNasin for four times. To perform the co-staining procedure, cells or sections were blocked with 10% goat serum at room temperature for 30 minutes. After blocking, cells or sections were incubated with Smad3 primary antibody containing 40 U/ml RNasin at 4°C overnight. Next day, cells or sections were washed with PBST containing 40 U/ml RNasin for three times followed by incubation with secondary antibody containing 40 U/ml RNasin at 37°C for 30 minutes. Then, cells or sections were washed with PBST containing 40 U/ml RNasin for three times followed by counterstaining with DAPI. The cell and cross-sectional images were captured using Eclipse 90i Nikon microscope.

RNA Immunoprecipitation (RIP) Assay

RIP assay was performed with modification as described previously.[18, 38] Briefly, 10T1/2 cells were fixed with 1% formaldehyde at room temperature (RT) for 15 minutes, then incubated with 0.2 M glycine at RT for additional 5 minutes. Cells were scraped off, and then lysed in FA lysis buffer (50 mM HEPES, 140 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.1% (w/v) sodium deoxycholate) containing 40 U/ml RNase inhibitor (RNasin) (Sigma-Aldrich, R1158) and 1x Halt™ Protease inhibitor cocktail (Thermo Scientific). After incubation at 4°C for 1 hour, cell lysates were passing through a syringe with 27-gauge needle for 10-times. Cell lysates were sonicated for 5 rounds of 50% power to shear the nucleic acids, followed by collecting the supernatant. Then, cell supernatants were treated with DNase I. After pre-clearing cell supernatants with 50 µl protein A/G agarose beads (Santa Cruz, sc-2003) at 4°C for 4 hours, cell supernatants were incubated with rabbit IgG (negative control), or Smad3 antibodies at 4°C for overnight. Input sample (10%) was collected after the pre-clear step. Next day, the RNA-protein complex was captured by 50 µl protein A/G agarose beads at 4°C for 2 hours, followed by serial washing with FA lysis buffer, FA500 (50 mM HEPES, 500 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100,

0.1% (w/v) sodium deoxycholate), LiCL washing buffer (10 mM Tris-Cl, 250 mM LiCl, 0.5% (v/v) NP-40, 0.1% (w/v) sodium deoxycholate, 1 mM EDTA), and TE/100 mM NaCl (10 mM Tris·Cl, 1 mM EDTA, 100 mM NaCl). Each washing buffer was added 40 U/ml RNasin before use, and each wash step was repeated for three times. After washings, A/G agarose beads were incubated with RIP elution buffer (100 mM Tris-Cl, 10 mM EDTA, 1% (w/v) SDS) at 37°C for 10 minutes to elute RNA-protein complex from beads. RNA-protein complex was incubated with 5M NaCl together with Protease K at 42°C for 1 hour, and then at 65°C for additional 1 hr. The immunoprecipitated RNA was purified using a RNeasy Mini Kit (Qiagen, 74104). Purified RNA was analyzed by qRT-PCR for measuring the lncRNA enrichment.

Biotin-Avidin Pull Down Assay

The procedure was performed as previous study.[18] Biotin labeled full length BC1 (FL), Fragment 1 (F1), or F1 with mutation at either one (mt1, mt2) or both rSBE sites (mt12) were synthesized via the in vitro transcription as described above. For in vitro binding assay, different amount of synthesized RNA (0, 100, 200, 400 ng) was incubated with 400 ng of recombinant human Smad3 (rhSmad3) in 50 μl of Wash/Binding Buffer (PBS with 0.1% SDS, 1% NP-40, 40 U/ml RNase inhibitor (RNasin)) at 4°C overnight followed by the pull-down procedure. For in cell binding assay, 10T1/2 cells were transfected with 1 μg of synthesized RNA via Lipofectamine LTX for overnight followed by fixation with 1% formaldehyde. Negative control for the binding between nucleotides and Smad3 was performed with same procedure using biotin-labeled random nucleotide sequence that was not overlapped with mouse genome as examined by the Basic Local Alignment Search Tool provided by National Center for Biotechnology Information. The random nucleotide fragment sequence is 5'-Bio-ATC GTT TCC GCT TAA CGG CG-Bio -3'. Cells were then lysed with FA lysis buffer containing protease inhibitor and 40 U/ml RNasin, followed by

DNase I treatment, and then the pull-down procedure. For pull-down procedure, BC1-Smad3 mixture and cell lysates were pre-cleared with Pierce™ Control Agarose Resin (Thermo Fisher Scientific, 26150) at 4°C for 2 hours followed by incubating with Pierce™ Streptavidin Agarose (Thermo Fisher Scientific, 20347) for BC1 pull down or Pierce™ Control Agarose Resin for negative control at 4°C for additional 2 hours. Input sample (10%) was collected after the pre-clear step. After washing with washing/binding Buffer for four times and RIPA buffer containing 40 U/ml RNasin for additional two times, the beads-RNA-protein complex was collected and lysed in RIPA buffer containing protease inhibitors. Protein concentration was measured using BCA Protein Assay Reagent (Thermo Scientific) and the abundance of Smad3 was measured via Western Blot Analysis.

Viral Inoculation of Mouse Embryo

C57BL/6J mice were housed under conventional conditions in the animal care facilities and received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals. Animal surgical procedures were approved by the Institutional Animal Care and Use Committee of the University of Georgia. Two months old C57BL/6J mice were mated to produce embryos for viral inoculation. Females were checked to see whether there is vaginal plug every morning. The day that plug was found was consider as E0.5. Pregnant C57BL/6J mice with E12.5 embryos were anesthetized with inhalation of isoflurane. Adenovirus administration to mouse embryos were performed with modification from previous study.[39] Glass micropipette needle (diameter: 50-100 μm) was used to inject adenovirus to embryo. Labyrinth zone of embryo placenta was injected with 5 μl of Ad-Ctrl or Ad-BC1 (1×10^9 pfu). To prevent abortion unnaturally, we avoided the viral inoculation to the embryos next to ovaries and upper vagina.

Pups were euthanized at the day of birth by CO_2 inhalation (~2.0 L/min). Thoracic aorta of pups were then perfused with saline, fixed with 4% formaldehyde, dehydrated with 15% and 30% Sucrose prepared in PBS, and embedded in OCT for further sectioning and subsequent morphometric analyses.

Histomorphometric analysis

Newborn mouse aorta cryo-sections (10 μm) were fixed with 1% formaldehyde, and hydrated with PBS. The sections were then stained with either modified hematoxylin and eosin (H&E) (American MasterTech, KTHNEPT), Elastica van Gieson (VG) (Electron Microscopy sciences, 26350), or Masson's trichrome staining reagents (American MasterTech, KTMTR2). The procedure was performed by following protocols provided in each kit. The cross-sectional images were captured using Eclipse 90i Nikon microscope.

Predicted secondary structure of BC1

Mus musculus BC1 secondary structure was predicted based on minimum free energy (MFE) structure.[40] Schematic secondary structure was then illustrated and modified via RnaViz2 program.[41]

Statistical Analysis

Results were presented as mean ± S.D. 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

BC1 inhibited TGF-β-induced SMC differentiation.

Transforming growth factor (TGF)-β is a central regulator for smooth muscle cell (SMC) fate determination during vascular development.[15] To determine whether BC1 is involved in SMC differentiation, we treated C3H10T1/2 (10T1/2) cells with TGF-β to induce SMC differentiation in vitro.[14, 15] TGF-β induced the expression of SMC markers αSMA, CNN1, and SM22α (Figure 5.1A through 5.1C), indicative of a SMC differentiation. Interestingly, BC1 expression was time-dependently decreased along with the increase of SMC markers, suggesting that BC1 may negatively regulate TGF-β-induced SMC differentiation.

To determine whether BC1 regulates TGF-β-induced SMC differentiation, we used adenoviral vector to express BC1 cDNA (Ad-BC1) or its short hairpin RNA (shRNA; Ad-shBC1) to alter BC1 expression in 10T1/2 cells (Supplementary Figure S5.1). As shown in Figure 5.1D and 5.1E, ectopic expression of BC1 suppressed TGF-β-induced expression of SMC marker protein αSMA, CNN1, and SM22α. Conversely, knockdown of BC1 enhanced the expression of SMC markers (Figure 5.1F and 5.1G). Morphological change from a polygonal to a spindled-shaped phenotype with TGF-β induction have been observed in 10T1/2 cells.[14] Consistent with the marker gene expression, Ectopic expression of BC1 suppressed TGF-β-induced morphological change, while knockdown of BC1 induced a spindled-shaped morphology even without TGF-β stimulation (Figure 5.1H). These results indicated that BC1 was a negative regulator for TGF-β-induced SMC differentiation.

BC1 function in SMC differentiation is associated with Smad3 activity.

Smad3 is essential for transducing the TGF-β signaling[42] and is important in TGF-β-induced SMC differentiation[43]. Thus, we sought to determine whether BC1 affects Smad3 activity. Ectopic

expression of BC1 suppressed while knockdown of BC1 enhanced Smad3 phosphorylation/expression along with the alteration of SMC marker expression (Figure 5.2A through 5.2D). However, ectopic expression of Smad3 (pcDNA-Smad3) in BC1-transfected 10T1/2 cells rescued the expression of SMC marker genes that was suppressed by BC1 (Figure 5.2A-5.2B and Supplemental Figure S5.2A). Conversely, knockdown of Smad3 via its shRNA (Ad-shSmad3) or blockade of Smad3 activity via its selective inhibitor SIS3 impeded the expression of SMC markers that was enhanced due to the knockdown of BC1 (Figure 5.2C through 5.2F, and Supplemental Figure S5.2, B-C). These data suggested that BC1 inhibited TGF-β-induced SMC differentiation through suppressing Smad3 signaling.

BC1 inhibited Smad3 transcription activity via suppressing its nuclear translocation.

Since Smad3 regulates SMC marker gene transcription, we tested whether BC1 regulates SMC marker gene promoter activity by luciferase reporter assay. As shown in Figure 5.3A and Supplemental Figure S5.3, ectopic expression of BC1 suppressed while knockdown of BC1 enhanced TGF-β-induced promoter activity of αSMA and SM22α genes. Since TGF-β-induced gene transcription is mediated by Smad3 binding to Smad binding element (SBE) in the promoter of targeted genes,[44] we tested whether BC1 regulates the promoter mainly composed of SBE elements (SBE-Luc) and assessed whether BC1 affects Smad3 binding to the SBEs in αSMA and SM22α promoter in a chromatin setting by performing chromatin immunoprecipitation (ChIP) assay. As shown in Figure 5.3A and Supplemental Figure S5.3, ectopic expression of BC1 suppressed while knockdown of BC1 enhanced SBE promoter activity. ChIP assay showed that TGF-β induction for 2 hours caused strong Smad3 binding to αSMA and SM22α promoter. However, BC1 impeded the Smad3 binding to both promoters (Figure 5.3B).

Smad3 nuclear translocation is required for its transcriptional regulation.[45, 46] Therefore, we sought to determine whether BC1 is involved in TGF-β-induced Smad3 nuclear translocation. As shown in Figure 5.3C, BC1 expression in 10T1/2 cells caused more Smad3 presented in cytoplasm within 1 hour of TGF-β induction as compared to the cells without BC1 expression. These results indicated that Smad3 nuclear translocation was inhibited by BC1. To further confirm this phenomenon, cytoplasmic and nuclear fraction of BC1 and Smad3 was quantified by real-time quantitative PCR (for BC1) and Western blot (for Smad3), respectively. As shown in Figure 5.3D through 5.3F, ectopic expression of BC1 blocked Smad3 nuclear translocation by nearly 40% at 1 hour of TGF-β treatment. These results demonstrated that BC1 suppressed Smad3 transcriptional activity via inhibiting its nuclear translocation.

BC1 physically interacted with Smad3.

Since BC1 physically interacts with eIF4A, PABP, and FMRP, [22-24] BC1 may affect Smad3 nuclear translocation through a physical interaction. We therefore first detected whether BC1 co-localize with Smad3 in 10T1/2 cells. As shown in Figure 5.4A, fluorescence in situ hybridization (FISH) for BC1 and immunofluorescent staining (IF) for Smad3 showed that they were co-localized in the cytoplasm of 10T1/2 cells prior to TGF-β induction. Interestingly, TGF-β induced nuclear translocation of Smad3, but not BC1, indicating that Smad3 nuclear translocation may require the dissociation of Smad3 from BC1. To determine whether BC1 specifically binds to Smad3, we performed RNA immunoprecipitation (RIP) assay to pull down BC1-Smad3 complex using Smad3 antibody and detected the BC1 RNA enrichment in 10T1/2 cells. As shown in Figure 5.4B, BC1 was present in Smad3 complex, and TGF-β significantly reduced the BC1 binding to Smad3. To further confirm the interaction between BC1 and Smad3, we transfected biotin-labeled BC1 into 10T1/2 cells and then detected the presence of Smad3 in avidin-precipitated complex. Indeed,

Smad3 was presented in BC1 complex pulled down with avidin beads in vehicle-treated cells (Figure 5.4C and 5.4D). However, presence of Smad3 in avidin-precipitated complex was decreased with TGF-β induction (Figure 5.4C and 5.4D). To further test whether BC1 directly binds to Smad3, we performed in vitro binding assays by incubating biotin-labeled BC1 with recombinant Smad3. Smad3 was detected in the BC1-Smad3 complex pulled down with avidin beads but not in control complex, indicating that BC1 directly and specifically bound to Smad3 (Figure 5.4E and 5.4F). These data indicated that BC1 regulated Smad3 nuclear translocation/activity via physical binding to its protein.

BC1 interacted with Smad3 and regulated TGF-β/Smad3 signaling via its rSBE.

Previous studies have shown that Smad3 binds to microRNA and LncRNA via a conserved RNA Smad binding elements (rSBE).[11, 47] Therefore, we sought to determine whether BC1 interacted with Smad3 via its rSBE. As shown in Supplementary Figure S5.4, two tentative rSBEs were observed in the first stem structure of BC1 RNA. To test whether BC1 interacted with Smad3 via rSBE, we mutated these two tentative rSBE and then performed biotin-avidin pull down assay in 10T1/2 cells. As shown in Figure 5.5A through 5.5D, BC1 fragment 1 (F1) with first stem structure containing two rSBEs bound Smad3 with an abundance similar to the result of full length BC1 group. However, the F1 fragment with mutations in either rSBE bound significantly less amount of Smad3. Importantly, mutations at both rSBEs almost abolished Smad3-BC1 interaction. These results demonstrated that the full interaction between BC1 and Smad3 required both rSBEs in the first stem of BC1 RNA.

To determine whether BC1 regulates SMC differentiation via the rSBEs, we tested whether BC1 fragment with mutant rSBEs affects the function of BC1 on SMC gene expression. As shown in Figure 5.6A through 5.6C, both full length (FL) and wild type F1 fragment of BC1 inhibited the

TGF-β-induced αSMA, SM22α, and SBE promoter activity. However, BC1 fragments with mutations at either one or both rSBE sites abolished the BC1 activity in suppressing the promoter activity. Moreover, rSBE mutations also restored the protein expression of SMC markers (αSMA, CNN1, and SM22α) that was attenuated by BC1 (Figure 5.6B and 5.6C). These data indicated that the rSBEs were essential for BC1 to inhibit TGF-β-induced SMC differentiation of 10T1/2 cells.

BC1 altered SMC phenotype during vascular development.

Impaired differentiation of smooth muscle cells (SMCs) during embryonic development leads to abnormal formation of vasculature. [48] To determine whether BC1 affects SMC differentiation in vivo, we delivered adenoviral vector expressing BC1 into mouse embryos via intra-placental injection on the embryonic day 12.5 (E12.5).[39] Thoracic aorta of pups were then collected on the postnatal day 0 (P0) to analyze the vascular development. As shown in Figure 5.7A, BC1 was expressed nearly 2.3-fold more in the aorta with BC1 adenoviral vector, which reduced the mRNA expression of SMC marker genes, αSMA, CNN1, and SM22α. To verify whether BC1 suppressed SMC marker gene expression in media of the mouse aorta, we performed immunostaining of αSMA and fluorescence in situ hybridization (FISH) of BC1 on the aorta sections. As shown in Figure 5.7B and 5.7C, BC1 was expressed in a portion of SMCs where a lower level of αSMA was observed, indicating that BC1 inversely correlated with the αSMA expression. In addition to αSMA, CNN1 expression was also suppressed by BC1 (Supplemental Figure S5.5A). These results showed that BC1 suppressed SMC marker gene expression in vivo.

SMCs exhibits a high rate of proliferation during embryonic development although in mature vessels SMCs have extremely low rate of proliferation.[7] By quantifying the SMC numbers in the media, we found that BC1 overexpression increased the medial SMC numbers in the artery (Figure 5.7D). In addition, artery media expressing BC1 showed more proliferating cell nuclear antigen

(PCNA)-positive cells (Figure 5.7E). To confirm the proliferative cells were SMCs, we co-stained PCNA and αSMA. Indeed, the PCNA-positive cells also expressed αSMA (Supplemental Figure S5.5B), indicating that the medial SMCs expressing BC1 exhibited less contractile but more proliferative property.

Impaired SMC differentiation and maturation often disrupt vascular integrity, such as defective elastic lamina, abnormal SMC investment, or disorganized extracellular matrix (ECM).[49-51] Indeed, artery with BC1 expression exhibited random breakage and irregular distribution of elastic lamina (Figure 5.7G). There were also an inordinate stack of SMCs, disorganized ECM, and excessive collagen accumulated around SMCs in the artery media (Supplemental Figure S5.6A) although the collagen deposition on the elastic lamina was reduced (Supplemental Figure S5.6B). These data indicated that BC1 adversely impacted the normal development of vascular system.

Discussion

We have identified BC1 as a novel regulator for TGF-β-induced SMC differentiation. BC1 suppresses SMC marker gene expression via binding to Smad3, which prevents Smad3 binding to SMC marker gene promoters and thus inhibiting SMC gene transcription, leading to decreased expression of SMC contractile genes. Importantly, BC1 expression in mouse embryo via adenoviral delivery impairs SMC marker gene expression in neonatal aorta while increasing SMC proliferation, indicating that BC1 suppresses SMC differentiation and alters SMC to proliferative phenotype. The impaired SMC differentiation appears to have detrimental effect to the artery structure. Mechanistically, BC1 inhibits Smad3 nuclear function by attenuating its phosphorylation and nuclear translocation.

BC1 may inhibit Smad3 nuclear translocation through different mechanisms. Smad3 phosphorylation is required for Smad3 nuclear translocation.[52] However, ectopic expression of BC1 suppresses the phosphorylation of Smad3 (Figure 5.2A), indicating that the blockage of Smad3 nuclear translocation is due to, at least in part, the reduction of Smad3 phosphorylation by BC1. Since Smad3 is recruited to TGF-β receptor for phosphorylation by Smad Anchor for Receptor Activation (SARA),[53] BC1 may indirectly interfere Smad3 phosphorylation by interrupting the Smad3 association with SARA. In addition to this indirect effect, BC1 may also directly affect Smad3 nuclear translocation. Smad3 nuclear translocation is facilitated by nuclear transport protein, importin β.[54] It has been shown that a lncRNA named noncoding repressor of NFAT (NRON) physically interacts with importin β to suppress importin β nuclear trafficking, leading to cytoplasmic retention of nuclear factor of activated T cells (NFAT).[55] BC1 may also interact with importin β to interrupt the interaction between Smad3 and importin β and thus blocks the Smad3 nuclear translocation. On the other hand, BC1 may accelerate the process of Smad3 nuclear export. Nuclear export of Smad3 is mediated by exportin 4 in a Ran-dependent manner.[56] It has been shown that RNA binding to the third double-stranded RNA binding domains (dsRBDs) of double-stranded RNA-specific adenosine deaminase (ADAR) promotes a member of exportin binding to ADAR, leading to ADAR nuclear export.[57] Therefore, the aberrant Smad3 nuclear localization caused by BC1 may be due to the binding between exportin 4 and Smad3 mediated by BC1. Extensive future studies are required to fully understand how BC1 affects the Smad3 phosphorylation and nuclear translocation. Our current data support that BC1 binds to Smad3 in cytoplasm at the basal state to prevent Smad3 shuttling into nuclei (Figure 5.4A, upper panel and Figure 5.4B). However, Smad3 is released from BC1 by TGF-β induction in order to translocate into nuclei to activate downstream genes (Figure 5.4A, lower panel and Figure 5.4B).

BC1 binds to Smad3 via its rSBEs. It is known that nuclear Smad3 binds to the DNA SBE in specific gene promoters to initiate their transcription.[44] RNA molecules appear to interact with Smad3 through the same manner. rSBEs have been identified in microRNA and lncRNA and have been reported to regulate TGF-β signaling via binding to Smad3.[11, 47] Our previous studies have shown that lncRNA GAS5 binds to Smad3 via rSBE to regulate TGF-β/Smad3 signaling. However, GAS5 does not affect Smad3 phosphorylation.[11] Therefore, BC1 function represents a new mechanism by which lncRNA regulates TGF-β/Smad3 signaling, *i.e.*, modulating Smad3 phosphorylation and nuclear translocation. Although BC1 binds Smad3 mainly through the two rSBEs in the first stem structure, it may also bind to Smad3 via other unknown sequences because mutation of both rSBE does not completely abrogate the Smad3 binding to BC1 (Figure 5.5C and 5.5D). Further studies are required to test this possibility or identify these sequences.

In addition to Smad3 phosphorylation and nuclear translocation, BC1 appears to also regulate Smad3 expression because less Smad3 is observed in BC1-expressed cells (Figure 5.2A and 5.2B). Conversely, more Smad3 is present in cells with BC1 knockdown (Figure 5.2C through 5.2F). BC1 is reported to serves as a translational repressor to block the assembly of 48S preinitiation complex via binding to eIF4A, PABP, and FMRP. [23, 58] Therefore, it is possible that BC1 suppresses Smad3 expression by serving as translational repressor through a similar mechanism. Likewise, the decreased SMC marker expression may also partially due to BC1-mediated translational suppression. Moreover, BC1 may affects the Smad3 or SMC marker protein stability. Indeed, TGF-β signaling is regulated and terminated by ubiquitin-proteasome system.[59] LncRNAs have also been shown to either enhance or suppress targeted protein degradation via interacting with certain E3 ligase.[60, 61] These potential interesting mechanisms are excellent subjects for future

studies. Nevertheless, our data indicate that BC1 not only regulates the nuclear trafficking of Smad3 but also affects Smad3 gene expression.

One limitation of this study is that the function of BC1 in endothelial cells is unknown. Although the adenoviral delivery of BC1 to mouse embryo also targets endothelium, BC1 appears not to alter the endothelial structure in aorta as shown by the intact VEcadherin-labeled endothelial layer (Figure 5.7F), which is in sharp contrast to the structural defects observed in media layer. Since BC1 expression in endothelial cells is much lower than vascular SMCs as measured by real-time quantitative PCR (Supplemental Figure S5.7), BC1 may not play a role in endothelial cells. However, extensive future studies are required to exclude this possibility.

Collectively, this is the first study to demonstrate the role of BC1 in SMC differentiation and vascular development. BC1 binds directly to Smad3 via rSBE in its 5′ stem structure, and thus impedes the TGF-β/Smad3 signaling required for SMC differentiation. The blockage of SMC differentiation by BC1 in vascular development leads to the abnormalities of vasculature characterized by random breaks of elastic lamina, inordinate stack of SMCs, and disorganized ECM in artery media. Our data are also consistent with the absence of vascular abnormality in BC1-deficient mice [25] because the normal vascular development requires the absence of BC1 in vascular SMCs.

Reference

1. Rzucidlo EM, Martin KA and Powell RJ. Regulation of vascular smooth muscle cell differentiation. *J Vasc Surg*. 2007;45 Suppl A:A25-32.

2. Conway EM, Collen D and Carmeliet P. Molecular mechanisms of blood vessel growth. *Cardiovasc Res*. 2001;49:507-21.

3. Gittenberger-de Groot AC, DeRuiter MC, Bergwerff M and Poelmann RE. Smooth muscle cell origin and its relation to heterogeneity in development and disease. *Arterioscler Thromb Vasc Biol*. 1999;19:1589-94.

4. Brouillard P and Vikkula M. Vascular malformations: localized defects in vascular morphogenesis. *Clin Genet*. 2003;63:340-51.

5. Wang Q, Zhao N, Kennard S and Lilly B. Notch2 and Notch3 function together to regulate vascular smooth muscle development. *PLoS One*. 2012;7:e37365.

6. Jiao J, Xiong W, Wang L, Yang J, Qiu P, Hirai H, Shao L, Milewicz D, Chen YE and Yang B. Differentiation defect in neural crest-derived smooth muscle cells in patients with aortopathy associated with bicuspid aortic valves. *EBioMedicine*. 2016;10:282-90.

7. Owens GK, Kumar MS and Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev*. 2004;84:767-801.

8. Song Z and Li G. Role of specific microRNAs in regulation of vascular smooth muscle cell differentiation and the response to injury. *J Cardiovasc Transl Res*. 2010;3:246-50.

9. Jakob P and Landmesser U. Role of microRNAs in stem/progenitor cells and cardiovascular repair. *Cardiovasc Res*. 2012;93:614-22.

10. Bell RD, Long X, Lin M, Bergmann JH, Nanda V, Cowan SL, Zhou Q, Han Y, Spector DL, Zheng D and Miano JM. Identification and initial functional characterization of a human vascular cell-enriched long noncoding RNA. *Arterioscler Thromb Vasc Biol*. 2014;34:1249-59.

11. 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.

12. Zhao J, Zhang W, Lin M, Wu W, Jiang P, Tou E, Xue M, Richards A, Jourd'heuil D, Asif A, Zheng D, Singer HA, Miano JM and Long X. MYOSLID Is a Novel Serum Response Factor-Dependent Long Noncoding RNA That Amplifies the Vascular Smooth Muscle Differentiation Program. *Arterioscler Thromb Vasc Biol*. 2016;36:2088-99.

13. Majesky MW. Developmental basis of vascular smooth muscle diversity. *Arterioscler Thromb Vasc Biol*. 2007;27:1248-58.

14. Xie C, Ritchie RP, Huang H, Zhang J and Chen YE. Smooth muscle cell differentiation in vitro: models and underlying molecular mechanisms. *Arterioscler Thromb Vasc Biol*. 2011;31:1485-94.

15. Hirschi KK, Rohovsky SA and D'Amore PA. PDGF, TGF-beta, and heterotypic cell-cell interactions mediate endothelial cell-induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate. *J Cell Biol*. 1998;141:805-14.

16. Guo X and Chen SY. Transforming growth factor-beta and smooth muscle differentiation. *World J Biol Chem*. 2012;3:41-52.

17. Mondal T, Subhash S, Vaid R, Enroth S, Uday S, Reinius B, Mitra S, Mohammed A, James AR, Hoberg E, Moustakas A, Gyllensten U, Jones SJ, Gustafsson CM, Sims AH, Westerlund F, Gorab E and Kanduri C. MEG3 long noncoding RNA regulates the TGF-beta pathway genes through formation of RNA-DNA triplex structures. *Nature communications*. 2015;6:7743.

18. Tang R, Zhang G, Wang YC, Mei X and Chen SY. The long non-coding RNA GAS5 regulates TGF-beta-induced smooth muscle cell differentiation via RNA-Smad binding element. *J Biol Chem*. 2017.

19. Richards EJ, Zhang G, Li ZP, Permuth-Wey J, Challa S, Li Y, Kong W, Dan S, Bui MM, Coppola D, Mao WM, Sellers TA and Cheng JQ. Long non-coding RNAs (LncRNA) regulated by transforming growth factor (TGF) beta: LncRNA-hit-mediated TGFbeta-induced epithelial to mesenchymal transition in mammary epithelia. *J Biol Chem*. 2015;290:6857-67.

20. Martignetti JA and Brosius J. BC1 RNA: transcriptional analysis of a neural cell-specific RNA polymerase III transcript. *Mol Cell Biol*. 1995;15:1642-50.

21. Martignetti JA and Brosius J. BC200 RNA: a neural RNA polymerase III product encoded by a monomeric Alu element. *Proc Natl Acad Sci U S A*. 1993;90:11563-7.

22. Lin D, Pestova TV, Hellen CU and Tiedge H. Translational control by a small RNA: dendritic BC1 RNA targets the eukaryotic initiation factor 4A helicase mechanism. *Mol Cell Biol*. 2008;28:3008-19.

23. Kondrashov AV, Kiefmann M, Ebnet K, Khanam T, Muddashetty RS and Brosius J. Inhibitory effect of naked neural BC1 RNA or BC200 RNA on eukaryotic in vitro translation systems is reversed by poly(A)-binding protein (PABP). *J Mol Biol*. 2005;353:88-103.

24. Zalfa F, Adinolfi S, Napoli I, Kuhn-Holsken E, Urlaub H, Achsel T, Pastore A and Bagni C. Fragile X mental retardation protein (FMRP) binds specifically to the brain cytoplasmic RNAs BC1/BC200 via a novel RNA-binding motif. *J Biol Chem*. 2005;280:33403-10.

25. Skryabin BV, Sukonina V, Jordan U, Lewejohann L, Sachser N, Muslimov I, Tiedge H and Brosius J. Neuronal untranslated BC1 RNA: targeted gene elimination in mice. *Mol Cell Biol*. 2003;23:6435-41.

26. Lewejohann L, Skryabin BV, Sachser N, Prehn C, Heiduschka P, Thanos S, Jordan U, Dell'Omo G, Vyssotski AL, Pleskacheva MG, Lipp HP, Tiedge H, Brosius J and Prior H. Role of a neuronal small non-messenger RNA: behavioural alterations in BC1 RNA-deleted mice. *Behav Brain Res*. 2004;154:273-89.

27. Gitai DL, Fachin AL, Mello SS, Elias CF, Bittencourt JC, Leite JP, Passos GA, Garcia-Cairasco N and Paco-Larson ML. The non-coding RNA BC1 is down-regulated in the hippocampus of Wistar Audiogenic Rat (WAR) strain after audiogenic kindling. *Brain research*. 2011;1367:114-21.

28. Sosinska P, Mikula-Pietrasik J and Ksiazek K. The double-edged sword of long non-coding RNA: The role of human brain-specific BC200 RNA in translational control, neurodegenerative diseases, and cancer. *Mutat Res Rev Mutat Res*. 2015;766:58-67.

29. Chen W, Bocker W, Brosius J and Tiedge H. Expression of neural BC200 RNA in human tumours. *J Pathol*. 1997;183:345-51.

30. Singh R, Gupta SC, Peng WX, Zhou N, Pochampally R, Atfi A, Watabe K, Lu Z and Mo YY. Regulation of alternative splicing of Bcl-x by BC200 contributes to breast cancer pathogenesis. *Cell Death Dis*. 2016;7:e2262.

31. Mus E, Hof PR and Tiedge H. Dendritic BC200 RNA in aging and in Alzheimer's disease. *Proc Natl Acad Sci U S A*. 2007;104:10679-84.

32. Eichmann A and Thomas JL. Molecular parallels between neural and vascular development. *Cold Spring Harb Perspect Med*. 2013;3:a006551.

33. Larrivee B, Freitas C, Suchting S, Brunet I and Eichmann A. Guidance of vascular development: lessons from the nervous system. *Circ Res*. 2009;104:428-41.

34. Wang H, Iacoangeli A, Lin D, Williams K, Denman RB, Hellen CU and Tiedge H. Dendritic BC1 RNA in translational control mechanisms. *J Cell Biol*. 2005;171:811-21.

35. Kobayashi M, Inoue K, Warabi E, Minami T and Kodama T. A simple method of isolating mouse aortic endothelial cells. *J Atheroscler Thromb*. 2005;12:138-42.

36. 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.

37. Eckersley-Maslin MA, Thybert D, Bergmann JH, Marioni JC, Flicek P and Spector DL. Random monoallelic gene expression increases upon embryonic stem cell differentiation. *Dev Cell*. 2014;28:351-65.

38. Gilbert C and Svejstrup JQ. RNA immunoprecipitation for determining RNA-protein associations in vivo. *Curr Protoc Mol Biol*. 2006;Chapter 27:Unit 27 4.

39. Mitchell M, Jerebtsova M, Batshaw ML, Newman K and Ye X. Long-term gene transfer to mouse fetuses with recombinant adenovirus and adeno-associated virus (AAV) vectors. *Gene Ther*. 2000;7:1986-92.

40. Hofacker IL. Vienna RNA secondary structure server. *Nucleic Acids Res*. 2003;31:3429-31.

41. De Rijk P, Wuyts J and De Wachter R. RnaViz 2: an improved representation of RNA secondary structure. *Bioinformatics*. 2003;19:299-300.

42. Datto MB, Frederick JP, Pan L, Borton AJ, Zhuang Y and Wang XF. Targeted disruption of Smad3 reveals an essential role in transforming growth factor beta-mediated signal transduction. *Mol Cell Biol*. 1999;19:2495-504.

43. Chen S, Kulik M and Lechleider RJ. Smad proteins regulate transcriptional induction of the SM22alpha gene by TGF-beta. *Nucleic Acids Res*. 2003;31:1302-10.

44. Zawel L, Dai JL, Buckhaults P, Zhou S, Kinzler KW, Vogelstein B and Kern SE. Human Smad3 and Smad4 are sequence-specific transcription activators. *Mol Cell*. 1998;1:611-7.

45. Kurisaki A, Kose S, Yoneda Y, Heldin CH and Moustakas A. Transforming growth factor-beta induces nuclear import of Smad3 in an importin-beta1 and Ran-dependent manner. *Mol Biol Cell*. 2001;12:1079-91.

46. Attisano L and Wrana JL. Smads as transcriptional co-modulators. *Curr Opin Cell Biol*. 2000;12:235-43.

47. Davis BN, Hilyard AC, Nguyen PH, Lagna G and Hata A. Smad proteins bind a conserved RNA sequence to promote microRNA maturation by Drosha. *Mol Cell*. 2010;39:373-84.

48. Goumans MJ, Liu Z and ten Dijke P. TGF-beta signaling in vascular biology and dysfunction. *Cell Res*. 2009;19:116-27.

49. Domenga V, Fardoux P, Lacombe P, Monet M, Maciazek J, Krebs LT, Klonjkowski B, Berrou E, Mericskay M, Li Z, Tournier-Lasserve E, Gridley T and Joutel A. Notch3 is required for arterial identity and maturation of vascular smooth muscle cells. *Genes Dev*. 2004;18:2730-5.

50. Liu D, Wang J, Kinzel B, Mueller M, Mao X, Valdez R, Liu Y and Li E. Dosage-dependent requirement of BMP type II receptor for maintenance of vascular integrity. *Blood*. 2007;110:1502-10.

51. Muratoglu SC, Belgrave S, Hampton B, Migliorini M, Coksaygan T, Chen L, Mikhailenko I and Strickland DK. LRP1 protects the vasculature by regulating levels of connective tissue growth factor and HtrA1. *Arterioscler Thromb Vasc Biol*. 2013;33:2137-46.

52. Souchelnytskyi S, Ronnstrand L, Heldin CH and ten Dijke P. Phosphorylation of Smad signaling proteins by receptor serine/threonine kinases. *Methods Mol Biol*. 2001;124:107-20.

53. Qin BY, Lam SS, Correia JJ and Lin K. Smad3 allostery links TGF-beta receptor kinase activation to transcriptional control. *Genes Dev*. 2002;16:1950-63.

54. Xiao Z, Liu X and Lodish HF. Importin beta mediates nuclear translocation of Smad 3. *J Biol Chem*. 2000;275:23425-8.

55. Willingham AT, Orth AP, Batalov S, Peters EC, Wen BG, Aza-Blanc P, Hogenesch JB and Schultz PG. A strategy for probing the function of noncoding RNAs finds a repressor of NFAT. *Science*. 2005;309:1570-3.

56. Kurisaki A, Kurisaki K, Kowanetz M, Sugino H, Yoneda Y, Heldin CH and Moustakas A. The mechanism of nuclear export of Smad3 involves exportin 4 and Ran. *Mol Cell Biol*. 2006;26:1318-32.

57. Fritz J, Strehblow A, Taschner A, Schopoff S, Pasierbek P and Jantsch MF. RNA-regulated interaction of transportin-1 and exportin-5 with the double-stranded RNA-binding domain regulates nucleocytoplasmic shuttling of ADAR1. *Mol Cell Biol*. 2009;29:1487-97.

58. Wang H, Iacoangeli A, Popp S, Muslimov IA, Imataka H, Sonenberg N, Lomakin IB and Tiedge H. Dendritic BC1 RNA: functional role in regulation of translation initiation. *J Neurosci*. 2002;22:10232-41.

59. Inoue Y and Imamura T. Regulation of TGF-beta family signaling by E3 ubiquitin ligases. *Cancer Sci*. 2008;99:2107-12.

60. Yoon JH, Abdelmohsen K, Kim J, Yang X, Martindale JL, Tominaga-Yamanaka K, White EJ, Orjalo AV, Rinn JL, Kreft SG, Wilson GM and Gorospe M. Scaffold function of long non-coding RNA HOTAIR in protein ubiquitination. *Nature communications*. 2013;4:2939.

61. Taniue K, Kurimoto A, Sugimasa H, Nasu E, Takeda Y, Iwasaki K, Nagashima T, Okada-Hatakeyama M, Oyama M, Kozuka-Hata H, Hiyoshi M, Kitayama J, Negishi L, Kawasaki Y and Akiyama T. Long noncoding RNA UPAT promotes colon tumorigenesis by inhibiting degradation of UHRF1. *Proc Natl Acad Sci U S A*. 2016;113:1273-8.

Figure Legends

Figure 5.1. BC1 blocked TGF-β-induced smooth muscle differentiation of 10T1/2 cells. A, BC1 was downregulated while SMC marker were upregulated by TGF-β. BC1 RNA and SMC marker mRNAs were detected by semi-quantitative PCR. **B,** BC1 expression was quantified by quantitative qRT-PCR. **P* < 0.05 vs vehicle-treated cells (0 h), n=3. **C,** alpha smooth muscle actin (αSMA), Calponin 1 (CNN1), smooth muscle 22 alpha (SM22α) mRNA expression were measured by qRT-PCR and normalized to cyclophilin (CYP). **P* < 0.05 vs vehicle-treated cells (0h), n=3. **D,** Forced expression of BC1 (Ad-BC1) suppressed TGF-β-induced protein expression of αSMA, CNN1, and SM22α. **E,** Quantification of αSMA, CNN1, and SM22α protein expression

shown in **D** by normalizing to α-tubulin level. *P < 0.05 vs control adenoviral vector-transduced cells (Ad-Ctrl), n=3. **F,** Knockdown of BC1 (Ad-shBC1) increased TGF-β-induced protein expression of αSMA, CNN1, and SM22α. **G,** Quantification of αSMA, CNN1, and SM22α protein expression shown in **F** by normalizing to α-tubulin level. *P < 0.05 vs control adenoviral vector-transduced cells (Ad-Ctrl), n=3. **H,** BC1 altered TGF-β-induced morphological changes of 10T1/2 cells. Bar: 50 μm.

Figure 5.2. BC1 inhibited TGF-β-induced smooth muscle differentiation through suppressing Smad3 activation and expression. A, The suppressive effect of BC1 on TGF-β-induced protein expression of αSMA, CNN1, and SM22α was reversed by Smad3 overexpression. **B,** Quantification of αSMA, CNN1, and SM22α protein expression shown in **A** by normalizing to α-tubulin level. *P < 0.05 vs vehicle-treated cells (Ctrl); $^{\#}P$ < 0.05 vs TGF-β treated control cells (Ctrl+TGF-β); $^{\$}P$ < 0.05 vs TGF-β treated cells with BC1 expression (BC1+TGF-β), n=3. **C,** The enhanced expression of αSMA, CNN1, and SM22α due to BC1 knockdown was impeded by Smad3 knockdown (Ad-shSmad3). **D,** Quantification of αSMA, CNN1, and SM22α protein expression shown in **C** by normalizing to α-tubulin level. *P < 0.05 vs control adenoviral vector-treated cells (Ad-Ctrl); $^{\#}P$ < 0.05 vs TGF-β treated control cells (Ad-Ctrl + TGF-β); $^{\$}P$ < 0.05 vs TGF-β treated cells with knockdown of BC1 (Ad-shBC1+TGF-β), n=3. **E,** The enhanced SMC marker expression due to BC1 knockdown was impeded by Smad3-selective inhibitor SIS3. **F,** Quantification of protein expression shown in **E** by normalizing to α-tubulin level. *P < 0.05 vs control adenoviral vector-treated cells (Ad-Ctrl); $^{\#}P$ < 0.05 vs TGF-β treated control cells (Ad-Ctrl + TGF-β); $^{\$}P$ < 0.05 vs TGF-β treated cells with knockdown of BC1 (Ad-shBC1 + TGF-β), n=3.

Figure 5.3. BC1 inhibited TGF-β-induced Smad3 nuclear translocation and its transcription activity in 10T1/2 cells. A, Forced expression of BC1 (Ad-BC1) suppressed TGF-β-induced promoter activity of αSMA, SM22α, and SBE-Luc as measured by Luciferase assay. $^{*}P < 0.05$ vs control adenoviral vector-treated cells (Ad-Ctrl); $^{\#}P < 0.05$ vs TGF-β treated control cells (Ad-Ctrl+TGF-β). **B,** Forced expression of BC1 (Ad-BC1) suppressed TGF-β-induced Smad3 binding to SBE in αSMA and SM22α promoter in a chromatin setting as measured by ChIP assay. **C,** Forced expression of BC1 (Ad-BC1) suppressed TGF-β-induced Smad3 nuclear translocation as shown by immunofluorescent staining of Smad3. Bar: 20 μm. **D,** Forced expression of BC1 (Ad-BC1) increased BC1 expression level in both cytoplasm and nuclei of TGF-β-treated 10T1/2 cells as shown by qRT-PCR. $^{*}P < 0.05$ vs control adenoviral vector-treated cells with TGF-β (Ad-Ctrl+TGF-β) in cytoplasm or nuclei, n=3. **E,** Forced expression of BC1 (Ad-BC1) suppressed TGF-β-induced Smad3 nuclear translocation. Cytoplasmic and nuclear portions of Smad3 were detected by Western blot. **F,** Quantification of pSmad3 and Smad3 protein level shown in **E** by normalizing to α-tubulin (cytoplasmic portion) or lamin B (nuclear portion). $^{*}P < 0.05$ vs control adenoviral vector-treated cells with TGF-β (Ad-Ctrl+TGF-β) in either cytoplasmic or nuclear portion, n=3.

Figure 5.4. BC1 physically associated with Smad3 protein. A, Distribution pattern of BC1 (Green) and Smad3 (Red) in 10T1/2 cells as shown by fluorescence in situ hybridization for BC1 and immunofluorescence staining for Smad3. BC1 co-localized with Smad3 in 10T1/2 cells (white arrow). Bar: 20 μm. **B,** TGF-β suppressed endogenous Smad3 binding to BC1 in 10T1/2 cells as measured by RIP assay. Cells were treated with vehicle or TGF-β for 2 hours. BC1-Smad3 complex was pulled down using anti-Smad3 antibody, and BC1 were detected by qRT-PCR. $^{*}P < 0.05$ vs vehicle-treated cells (Ctrl), n=3. **C,** TGF-β suppressed Smad3 binding to biotin-labeled

BC1 in 10T1/2 cells. 10T1/2 cells were transfected with biotin-labeled BC1 or biotin-labeled random nucleotide fragments (as control, Ctrl), and then treated with TGF-β for 1, 3, and 6 hours. BC1-Smad3 complex were pulled down using avidin-beads. The presence of Smad3 in the complex was detected by western blot. **D,** Quantification of Smad3 in the complex shown in **C** by normalizing to GAPDH level. $^{*}P < 0.05$ vs vehicle-treated cells (0h), n=3. **E,** Recombinant human Smad3 physically associated with biotin-labeled BC1 in vitro. Biotin-labeled random nucleotide fragments were served as control (Ctrl). **F,** Quantification of Smad3 shown in **E** by normalizing to input Smad3 level. $^{*}P < 0.05$ vs Ctrl, n=3.

Figure 5.5. BC1 interacted with Smad3 protein via rSBE elements. A and C, Mutations at either one (BC1-F1-mt1 or BC1-F1-mt2) or both rSBEs (BC1-F1-mt12) in the first stem structure of BC1 suppressed the interaction of BC1 with endogenous Smad3 in 10T1/2 cells **(A)** and recombinant Smad3 in vitro **(C)**. **B and D,** Quantification of Smad3 in BC1-Smad3 complex shown in **A** and **B** by normalizing to GAPDH **(A)** and Input Smad3 **(C)** level, respectively. $^{*}P < 0.05$ vs vehicle-treated cells (Ctrl); $^{\#}P < 0.05$ vs full length BC1 (BC1-FL) transfected cells (FL), n=3.

Figure 5.6. BC1 attenuated TGF-β-induced SMC marker expression via rSBE element. A, Mutations at either one (mt1 or mt2) or both rSBEs (mt12) in the first stem structure of BC1 reversed the inhibitory effect of BC1 on TGF-β-induced activities of SMC marker promoters and the promoter mainly containing SBE (SBE-Luc) as measured by Luciferase assay. $^{*}P < 0.05$ vs vehicle-treated cells (Ctrl); $^{\#}P < 0.05$ vs TGF-β treated cells (TGF-β); $^{\$}P < 0.05$ vs TGF-β treated cells with full length BC1 (FL+TGF-β), n=3. **B,** Mutations at either one (BC1-F1-mt1 or BC1-F1-mt2) or both rSBEs (BC1-F1-mt12) in the first stem structure of BC1 reversed the inhibitory effect of BC1 on TGF-β-induced protein expression of SMC markers. **C,** Quantification of αSMA, CNN1,

and SM22α protein expression shown in **B** by normalizing to α-tubulin level. *$P < 0.05$ vs vehicle-treated cells (Ctrl); $^{\#}P < 0.05$ vs TGF-β treated cells (TGF-β); $^{\$}P < 0.05$ vs TGF-β treated cells with full length BC1 (FL + TGF-β), n=3.

Figure 5.7. BC1 impaired SMC differentiation in vivo. A, Adenoviral delivery of BC1 increased BC1 expression and suppressed SMC marker mRNA expression in neonatal mouse aorta. Labyrinth of mouse placenta was injected with control (Ad-Ctrl) or BC1 adenoviral vector (Ad-BC1) on the embryonic day 12.5 (E12.5). Thoracic aorta of postnatal day 1 pups was collected and lysed for RNA extraction. The BC1 level and mRNA expression of SMC markers were detected by qPCR. *$P < 0.05$ vs Ad-Ctrl-treated group (Ad-Ctrl), n=5. **B,** BC1 suppressed αSMA expression (white arrow) in aorta media SMCs as measured by fluorescence in situ hybridization for BC1 (green) and immunofluorescent staining for αSMA (Red). In BC1-expressed artery, the media area lack of BC1 expression showed a high level of of αSMA expression (light blue arrow). Bar: 50 μm. **C,** Quantification of αSMA expression shown in **B** by calibrating its staining intensity relative to the mean signal in control arteries (Ad-Ctrl, set as 1). *$P < 0.05$ vs Ad-Ctrl, n=6. **D,** Forced expression of BC1 (Ad-BC1) increased aorta media cell number as measured by calculating DAPI-positive staining cell from arterial sections. *$P < 0.05$ vs Ad-Ctrl, n=8. **E,** BC1 (Ad-BC1) caused SMC proliferation. The proliferative SMCs were detected by the expression of proliferating cell nuclear antigen (PCNA) (white arrow). A small portion of BC1 (green)-positive cells expressed PCNA (Red) as shown by FISH for BC1 and IF staining for PCNA. Bar: 50 μm. **F,** BC1 did not alter the integrity of aorta endothelium as shown by the IF staining of vascular endothelial cadherin (VE-cad). Bar: 50 μm. **G,** BC1 caused abnormal elastic fiber structure (black arrow) as shown by Elastica van Gieson (VG) staining. Bar: 50 μm.

Supplementary Figure S5.1. BC1 was upregulated by Ad-BC1 and downregulated by Ad-shBC1 in 10T1/2 cells. A and B, Transduction of BC1 adenoviral vector (Ad-BC1) elevated BC1 expression in 10T1/2 cells as measured by semi-quantitative RT-PCR (A) and qRT-PCR (B). *P < 0.05 vs control adenoviral vector-treated cells (Ad-Ctrl), n=3. **C and D,** Adenovirus-expressed BC1 shRNA (Ad-shBC1) suppressed BC1 expression in 10T1/2 cells as measured by semi-quantitative RT-PCR **(C)** and qRT-PCR **(D)**. *P < 0.05 vs control adenoviral vector-treated cells (Ad-Ctrl), n=3.

Supplementary Figure S5.2. Quantification of pSmad3 and Smad3 levels shown in Figure 3. A, Quantification of pSmad3 and Smad3 levels in **Figure 3A** by normalizing to α-tubulin level. *P < 0.05 vs vehicle-treated cells (Ctrl); $^{\#}P$ < 0.05 vs TGF-β treated control cells (Ctrl + TGF-β); $^{\$}P$ < 0.05 vs TGF-β treated cells with BC1 expression (BC1 + TGF-β), n=3. **B,** Quantification of pSmad3 and Smad3 levels shown in **Figure 3C** by normalizing to α-tubulin level. *P < 0.05 vs control adenoviral vector-treated cells (Ad-Ctrl); $^{\#}P$ < 0.05 vs TGF-β treated control cells (Ad-Ctrl+TGF-β); $^{\$}P$ < 0.05 vs TGF-β treated cells with BC1 knockdown (Ad-shBC1 + TGF-β), n=3. **C,** Quantification of pSmad3 and Smad3 levels shown in **Figure 3E** by normalizing to α-tubulin level. *P < 0.05 vs control adenoviral vector-treated cells (Ad-Ctrl); $^{\#}P$ < 0.05 vs TGF-β treated control cells (Ad-Ctrl + TGF-β); $^{\$}P$ < 0.05 vs TGF-β treated cells with BC1 knockdown (Ad-shBC1 + TGF-β), n=3.

Supplementary Figure S5.3. Knockdown of BC1 increased TGF-β-induced promoter activity in 10T1/2 cells. A and B, Knockdown of BC1 (Ad-shBC1) increased TGF-β-induced αSMA, SM22α, and SBE promoter activity as measured by Luciferase assay. *P < 0.05 vs control adenoviral vector-treated cells (Ad-Ctrl); $^{\#}P$ < 0.05 vs TGF-β treated control cells (Ad-Ctrl + TGF-β).

Supplementary Figure S5.4. Schematic secondary structure of Mus musculus (mmu) BC1 and BC1 fragment with mutation at rSBE sites. A, Predicted secondary structure of mmu-BC1. Fragment 1 (F1) with a size of 74 base pair (bp) was indicated. Two rSBE sites was indicated in red (site 1) and green (site 2) color. **B,** BC1-F1 sequence was shown on the upper panel, and the sequence with mutant rSBE was shown on the lower panel. **C,** Predicted secondary structure of BC1-F1, BC1-F1-mt1, BC1-F1-mt2, and BC1-F1-mt12. Two rSBE elements were indicated in red (site 1) and green (site 2) color. The mutated rSBE sequences were indicated as lower-case letters with gray color.

Supplementary Figure S5.5. BC1 suppressed CNN1 expression while induced PCNA expression in SMCs. A, Adenoviral delivery of BC1 (Ad-BC1) suppressed CNN1 expression (white arrow) in thoracic aorta of newborn mice as measured by fluorescence in situ hybridization for BC1 (green) and immunofluorescent (IF) staining for CNN1 (red). In the aorta expressing BC1, the media area lack of BC1 showed a high level of CNN1 expression (light blue arrow). Bar: 50 μm. **B,** BC1 (Ad-BC1) induced SMCs to express proliferating cell nuclear antigen (PCNA). PCNA (red) co-localized with αSMA (green) as shown by IF staining. Bar: 50 μm.

Supplementary Figure S5.6. BC1 altered the structural integrity and collagen deposition in medial layer of aorta. A, BC1-expressed aorta exhibited a distorted structure in the media layer (black arrow) as shown by hematoxylin and eosin (HE) staining. Bar: 50 μm. **B,** Forced expression of BC1 induced excessive collagen (dark blue) accumulation in medial SMCs of aorta (white arrow) while reduced the collagen attachment on elastic fibers (green arrow) as shown by Masson's trichrome staining. Bar: 50 μm.

Supplementary Figure S5.7. BC1 expression in smooth muscle cell (SMC) and endothelial cell (EC). A, Difference of BC1 expression in SMC and EC of human and mouse as shown by quantitative real-time polymerase chain reaction (qRT-PCR). *$P < 0.05$ vs EC, n=3.

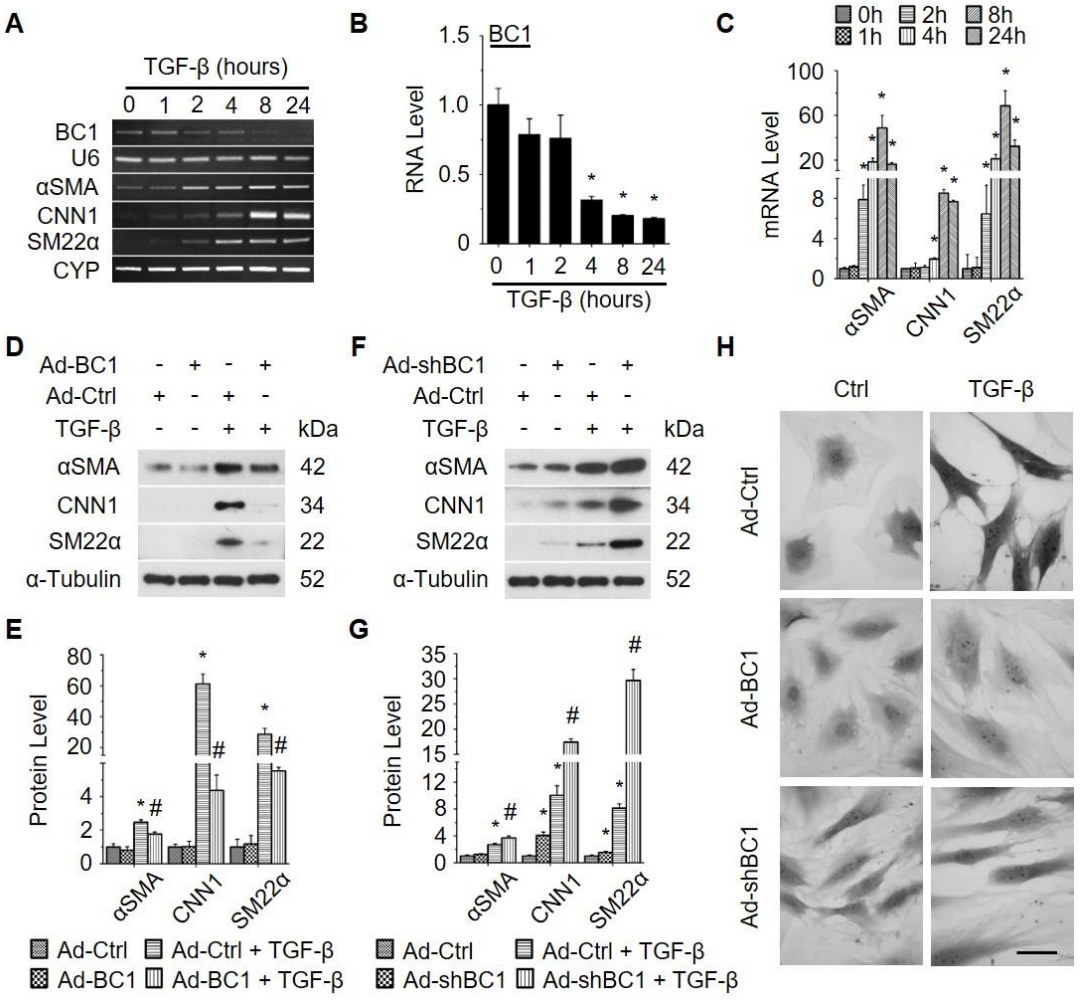


Figure 5.1. BC1 blocked TGF-β-induced smooth muscle differentiation of 10T1/2 cells.

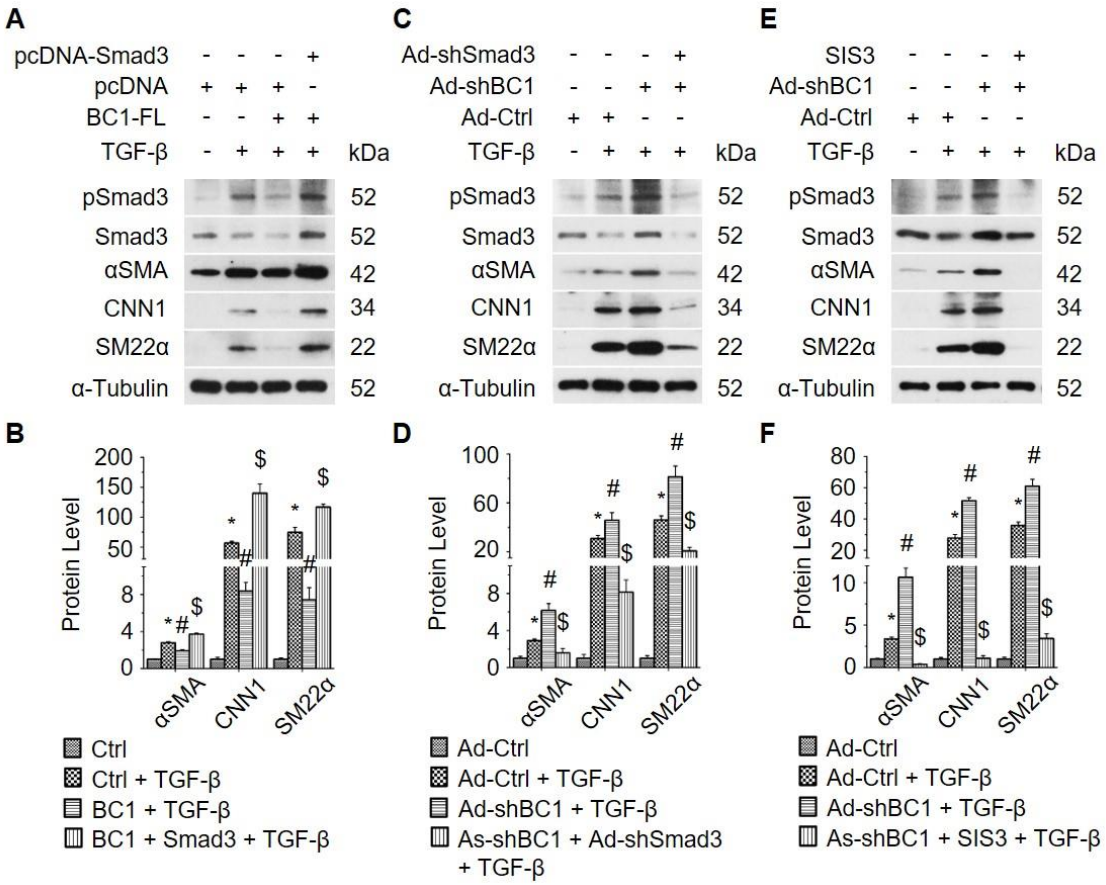


Figure 5.2. BC1 inhibited TGF-β-induced smooth muscle differentiation through suppressing Smad3 activation and expression.

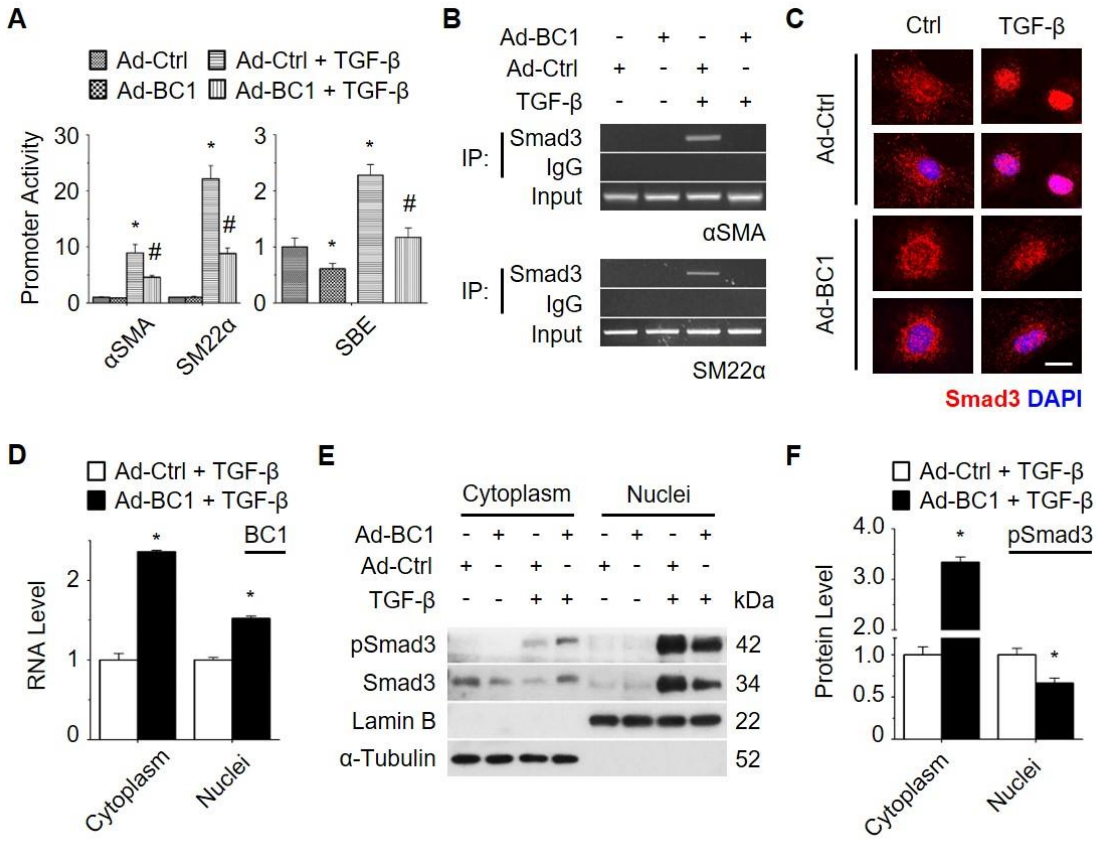


Figure 5.3. BC1 inhibited TGF-β-induced Smad3 nuclear translocation and its transcription activity in 10T1/2 cells.

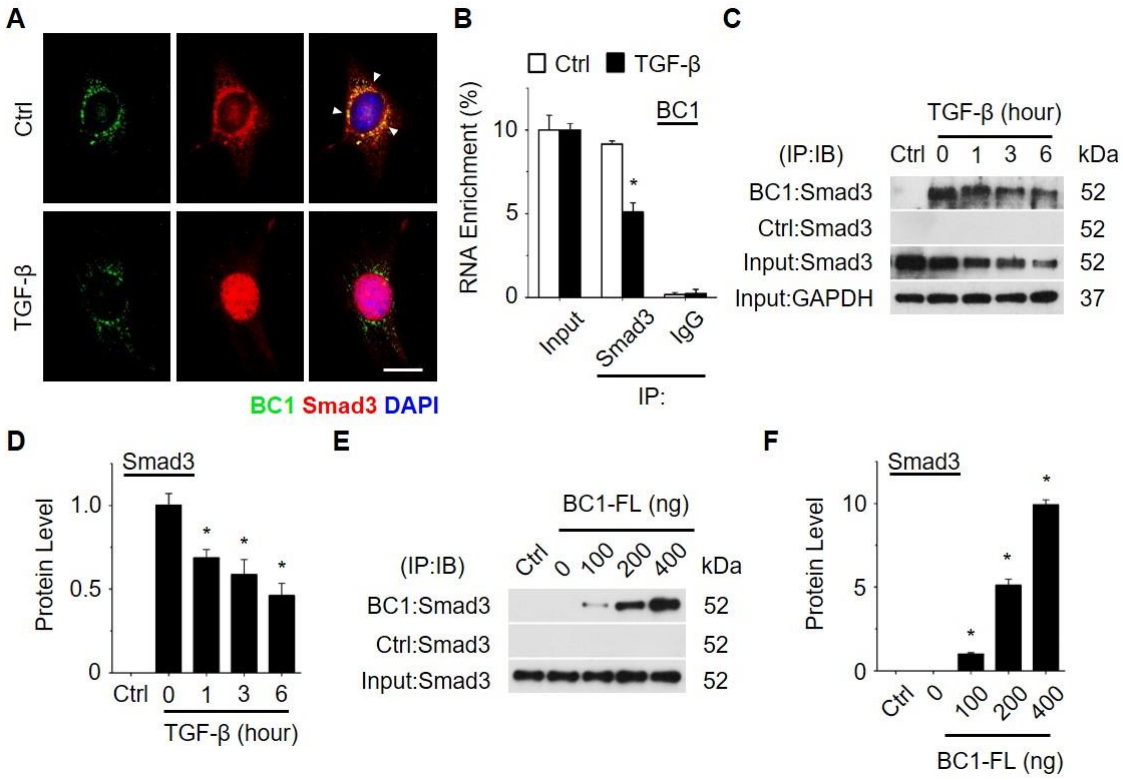


Figure 5.4. BC1 physically associated with Smad3 protein.

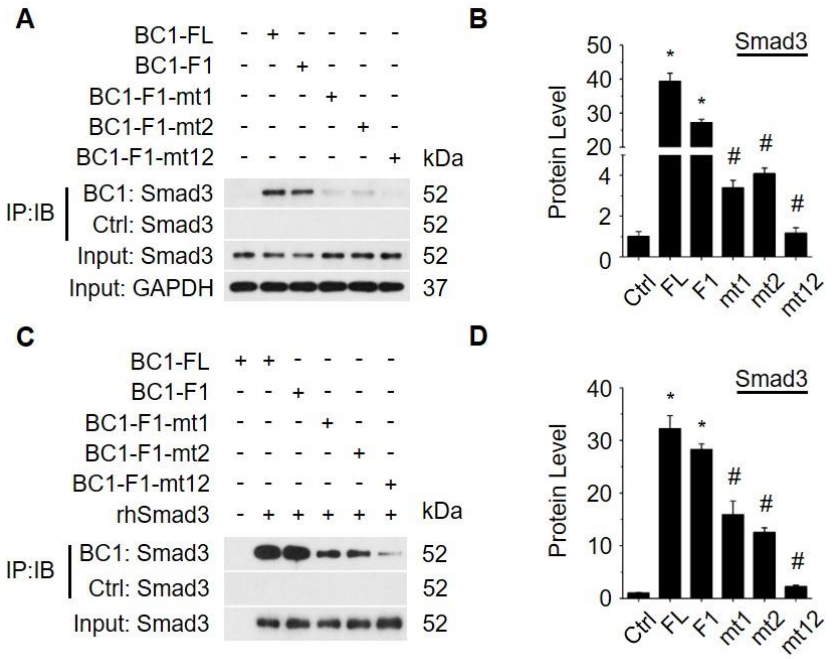


Figure 5.5. BC1 interacted with Smad3 protein via rSBE elements.

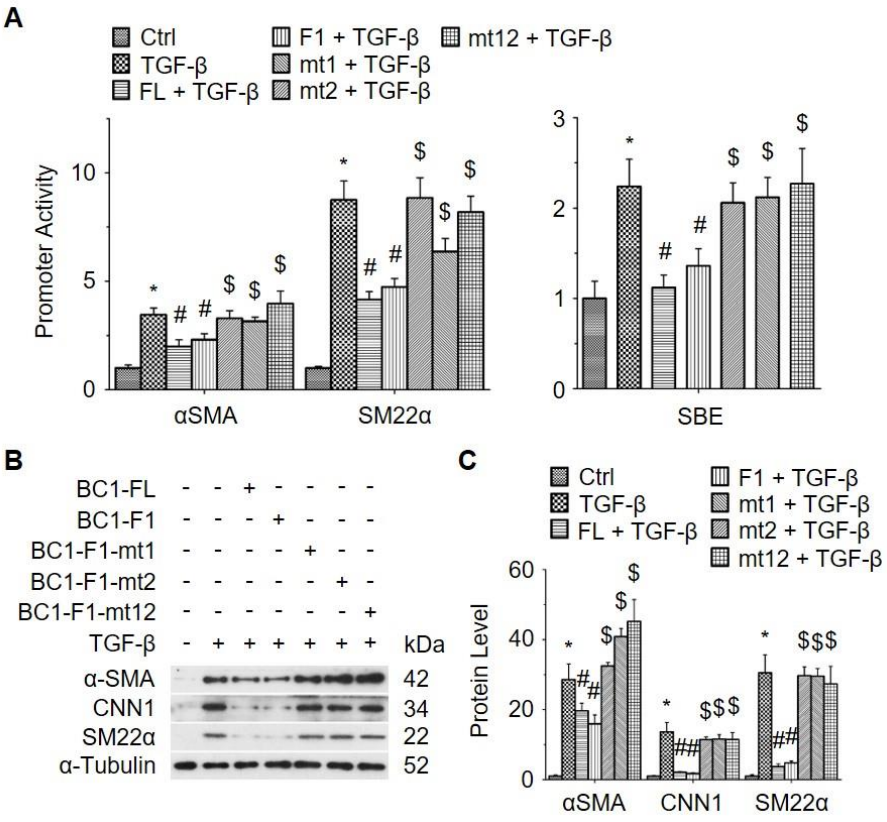


Figure 5.6. BC1 attenuated TGF-β-induced SMC marker expression via rSBE element.

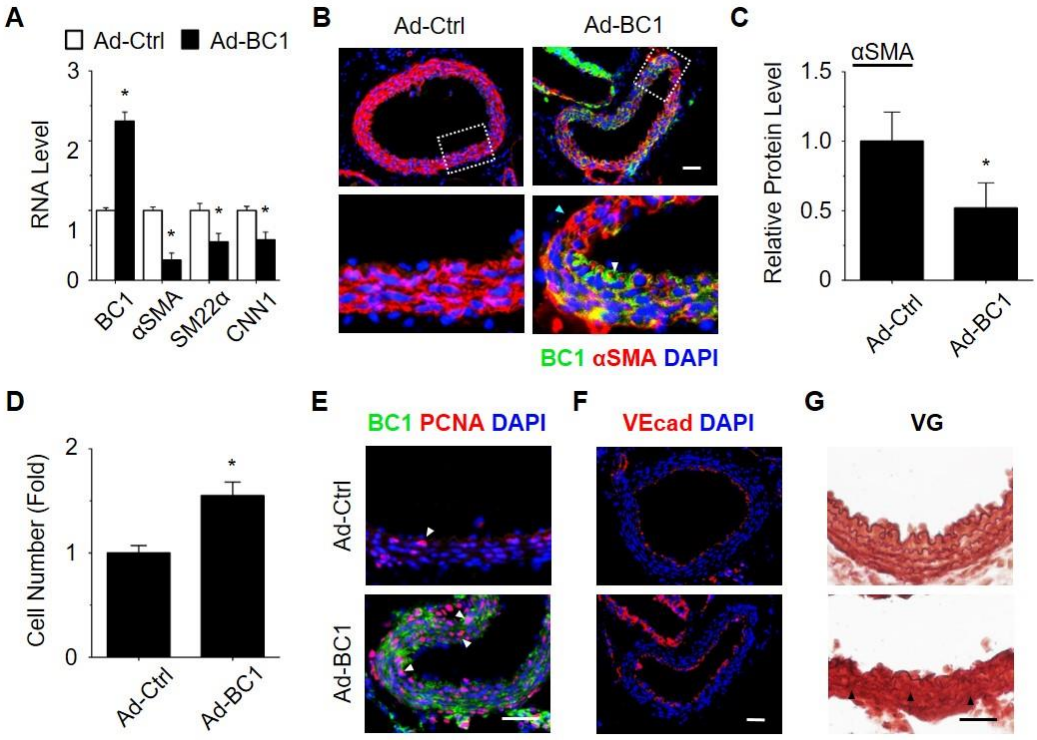
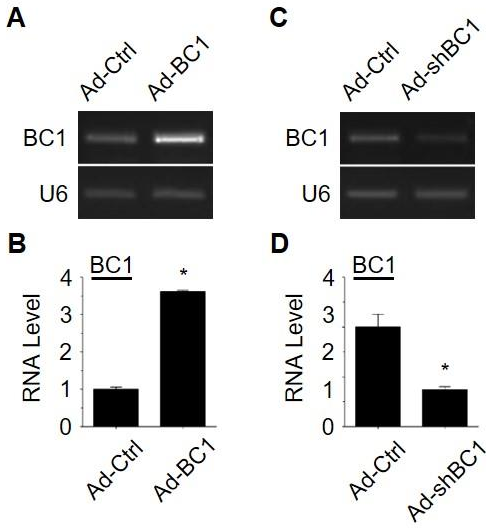
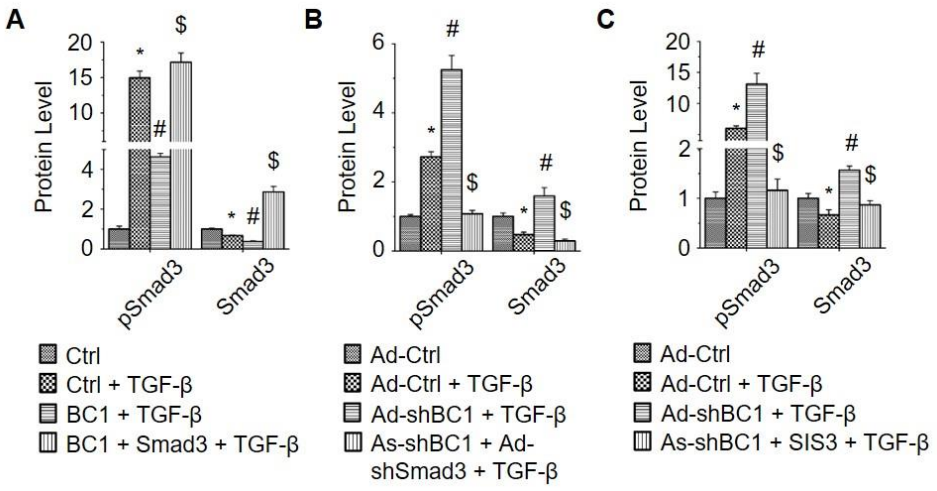


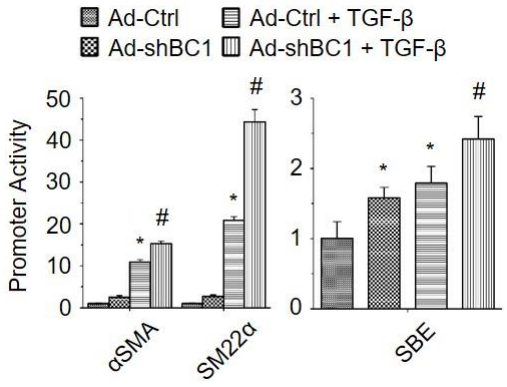
Figure 5.7. BC1 impaired SMC differentiation in vivo.



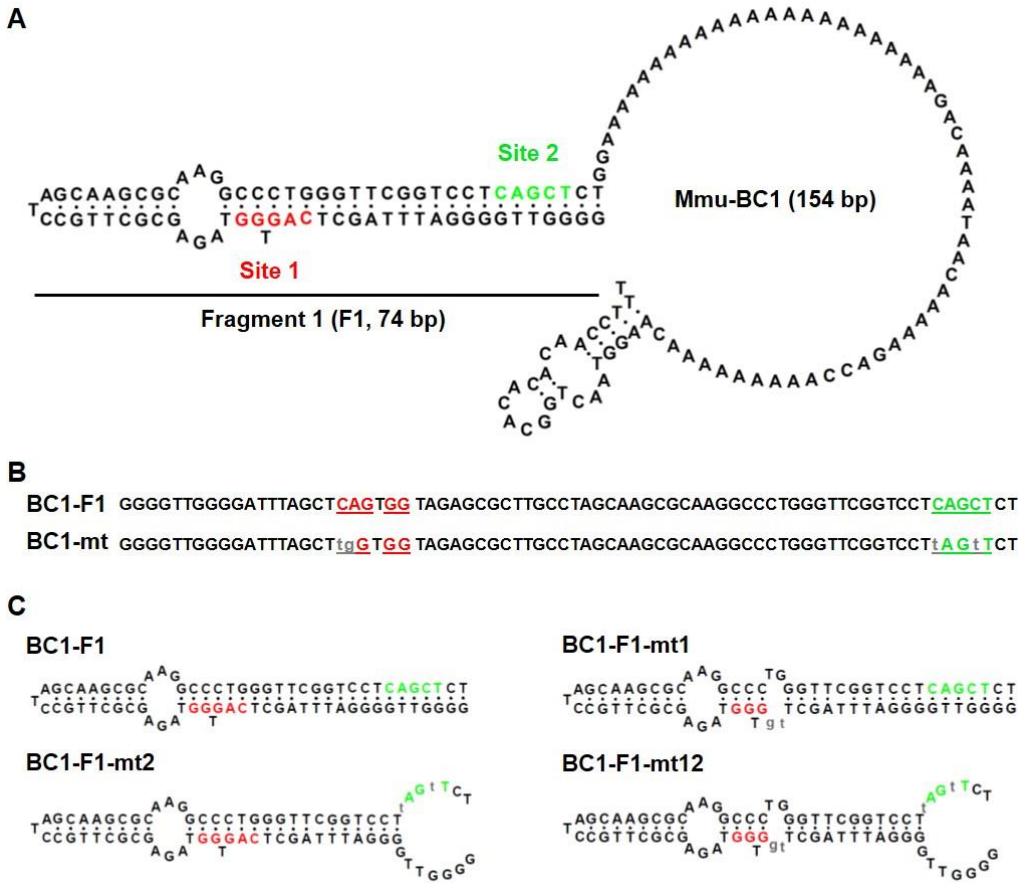
Supplementary Figure S5.1. BC1 was upregulated by Ad-BC1 and downregulated by Ad-shBC1 in 10T1/2 cells.



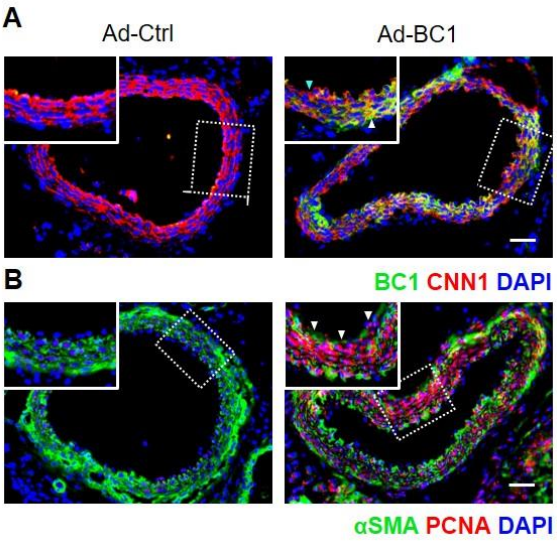
Supplementary Figure S5.2. Quantification of pSmad3 and Smad3 levels shown in Supplementary Figure S5.3.



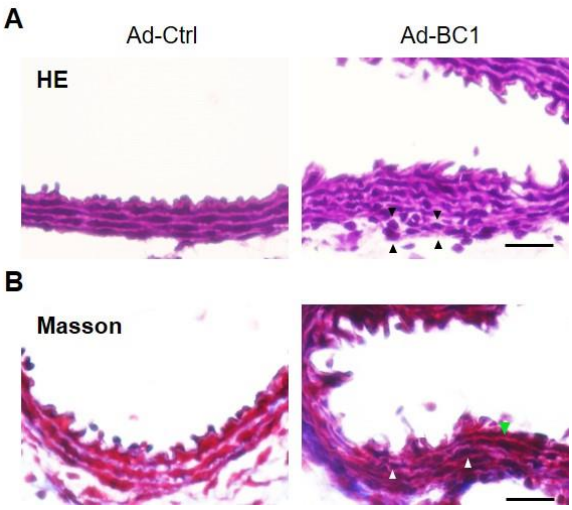
Supplementary Figure S5.3. Knockdown of BC1 increased TGF-β-induced promoter activity in 10T1/2 cells.



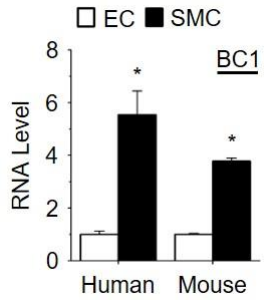
Supplementary Figure S5.4. Schematic secondary structure of Mus musculus (mmu) BC1 and BC1 fragment with mutation at rSBE sites.



Supplementary Figure S5.5. BC1 suppressed CNN1 expression while induced PCNA expression in SMCs.



Supplementary Figure S5.6. BC1 altered the structural integrity and collagen deposition in medial layer of aorta.



Supplementary Figure S5.7. BC1 expression in smooth muscle cell (SMC) and endothelial cell (EC).

Supplementary Table S5.1: Primers used in this study.

Primer	Sequence
mmu-BC1-F	ACACTCCAGCTGGGTTCGGTCCTCAGC TCTGGAA
mmu-BC1-R	CTCAACTGGTGTCGTGGAGTCGGCAAT TCAGTTGAGTGTGCCAGTTACCTTGTT
mmu-αSMA-F	AATGGCTCTGGGCTCTGTAAG
mmu-αSMA-R	CACGATGGATGGGAAAACAGC
mmu-CNN1-F	AATACGACCATCAGCGGGAG
mmu-CNN1-R	ATGAAGTTGTTCCCGATGCG
mmu-SM22α-F	GGTCCATCCTACGGCATGAG
mmu-SM22α-R	CCTACATCAGGGCCACACTG
mmu-CYP-F	GAGCTGTTTGCAGACAAAGTTC
mmu-CYP-R	CCCTGGCACATGAATCCTGG
mmu-U6-F	CTCGCTTCGGCAGCACA
mmu-U6-R	AACGCTTCACGAATTTGCGT
mmu-αSMA-SBE-F	GTTCTGAGGGCTTAGGATGT
mmu-αSMA-SBE-R	CCAGTAAATCAAGCGTTGTT
mmu-SM22α-SBE-F	GGTGTTGAGCCAAGCAGAC
mmu-SM22α-SBE-R	CGAGTTGCATTAGCCCTGG
BC1-T7-FL-F	GAGTAATACGACTCACTATAGGGAAG GGGTTGGGGA TTTAGCT

BC1-T3-FL-R	GAGATTAACCCTCACTAAAGGGATTAA AGGTTGTGTGTG CCAG
BC1-T3-F1-R	GAGATTAACCCTCACTAAAGGGATTAG AGCTGAGGACCGAACC
BC1-T7-F1-mt1-F	GAGTAATACGACTCACTATAGGGAAG GTTGGGGATTTAGCTTGGTGGTA
BC1-T3-F1-mt2-R	GAGATTAACCCTCACTAAAGGGATTAG AACTAAGGACCGAACC

CHAPTER 6

CONCLUSION

Although multiple anti-proliferative agents have been used and successfully inhibited SMC proliferation, its universal effect on endothelial cell may cause serious complication, called late thrombosis, due to the delayed vascular repair. Therefore, a more selective or specific therapeutic target for inhibiting SMC proliferation is needed.

To discover new therapeutic target, our study focused on the differential mechanisms underlying the proliferation of SMCs and ECs. We performed the microarray to profile gene expression in proliferative SMCs and ECs. JAK3 showed differential expressions in proliferative SMCs and ECs from our microarray results. We thus hypothesized that JAK3 plays differential roles in regulating SMC and EC proliferation, and thus JAK3 may be a better target to specifically block SMC proliferation. Indeed, our results indicated that JAK3 promotes SMC proliferation and restenosis after vascular intervention (Chapter 3). Conversely, JAK3 negatively regulates the proliferation and migration of ECs resulting in delayed vascular repair (Chapter 4). In addition to SMCs and ECs, prolonged inflammation at the lesion site of vessel also limits the vascular repair after clinical intervention. JAK3 has been shown to regulate cellular function (proliferation, migration, and differentiation) of different types of immune cells, such as neutrophil, macrophages, and T-lymphocyte. Local inflammation begins with adherence of neutrophil to the lesion site, and the progression of inflammation requires the recruitment of macrophages and lymphocytes. Although we haven't studied the role of JAK3 in the injury-induced local inflammation, it is likely that knockdown of JAK3 or block JAK3 signaling would suppress the

local inflammation via inhibiting the differentiation of macrophages and thus blocking the innate immune system. In summary, targeting JAK3 during the progress of restenosis may not only inhibit SMC proliferation but also suppress local inflammation, resulting in accelerated vascular repair due to the improved proliferation and migration of ECs.

Since vascular SMCs are not terminally differentiated in adult arteries, the initial step for SMC proliferation is undergoing de-differentiation, which is opposite to SMC differentiation during embryonic development. Therefore, studying mechanisms underlying SMC differentiation is important to understand how vascular SMCs undergo proliferation induced by injury. Although many protein factors regulating SMC differentiation have been identified, the role of noncoding RNAs, especially long noncoding RNAs (lncRNA) remain largely unknown. Our microarray analyses showed that Brain cytoplasmic RNA 1 (BC1) was expressed very differently in SMCs from ECs. We thus hypothesized that BC1 may be involved in the regulation of SMC differentiation during vascular development. Indeed, suppression of BC1 is necessary for SMC differentiation and the development of vasculature (Chapter 5). We observed the increased number of vascular SMCs in the artery with BC1 overexpression, suggesting that BC1 may regulate SMC proliferation as well. Since BC1 expression level in ECs is very low, it may not affect EC cellular function. In order to fully establish BC1 is an ideal therapeutic target for injury-induced vascular repair, additional future study is required.

This dissertation work was dedicated to identifying novel therapeutic strategies for an efficient vascular recovery from SMC-targeted treatment in cardiovascular intervention. We identified the role of JAK3 in injury-induced neointimal formation caused by SMC proliferation (Chapter 3), endothelial regeneration due to EC proliferation (Chapter 4), and the role of lncRNA BC1 in SMC differentiation (Chapter 5).

Several conclusions can be drawn from this dissertation:

1. **The activation and expression of JAK3 is induced by PDGF-BB.**

   JAK3 is phosphorylated as early as10 minutes after the PDGF-BB induction. Activation of JAK3 by PDGF-BB appears to be mediated by PI3K/Akt and Erk signaling. Moreover, PDGF-BB induces the expression of JAK3 via p38 MAPK signaling.

2. **JAK3 regulates SMC proliferation via STAT3 and JNK signaling.**

   Ectopic expression of JAK3 induces SMC proliferation while knockdown of JAK3 inhibits PDGF-BB-induced SMC proliferation. Selective inhibitor of STAT3, S3I-201, and JNK, Sp600125, impedes JAK3-induced SMC proliferation.

3. **JAK3 mediates injury-induced SMC proliferation, which results in neointimal formation.**

   Knockdown of JAK3 in injured-arteries shows a less neointima compared to vehicle-treated arteries with injury, due to the reduction of proliferative SMCs in neointima.

4. **JAK3 regulates EC physiological function by altering the pro-/anti angiogenic property of SMCs.**

   JAK3 promotes the expression of anti-angiogenic factor, TSP1, while decreases the expression of pro-angiogenic factor, VEGFA, in SMCs, resulting in an inhibition of EC proliferation, migration, and angiogenesis. On the other hand, knockdown of JAK3 suppresses the expression of TSP1 while induces the expression of VEGFA in SMCs causing the enhanced EC proliferation, migration, and angiogenesis.

5. **JAK3 arrests cell cycle progression of ECs via destabilization of G1-S transition regulator, Cyclin E.**

JAK3 expression is decreased during EC proliferation. The absence of JAK3 induces the protein stability of Cyclin E, a central regulator of G1-S transition, to promote the cell cycle progression.

6. **JAK3 mediates re-endothelialization by increasing EC proliferation.**

   Knockdown of JAK3 in injured arteries shows an enhanced re-endothelialization. The increased EC proliferation is observed in injured arteries with knockdown of JAK3.

7. **BC1 regulates TGF-β-induced SMC differentiation.**

   Ectopic expression of BC1 suppresses TGF-β-induced expression of SMC markers (αSMA, CNN1, and SM22α). In contrast, knockdown of JAK3 stimulates the expression of SMC marker genes. Besides, morphological change from a polygonal to a spindled-shaped phenotype induced by TGF-β is suppressed by the ectopic expression of BC1.

8. **BC1 regulates TGF-β/Smad3 signaling via its RNA Smad-binding elements (rSBEs).**

   BC1 physically binds to Smad3 via two rSBE located in the 5' stem structure of BC1. Mutation of these two rSBEs blocks the binding between BC1 and Smad3. Besides, BC1 with mutated rSBEs fails to suppress TGF-β-induced SMC marker gene expression.

9. **BC1 regulates vascular development.**

   With administration of adenoviral vector expressing BC1 into mouse embryos via intra-placental injection, SMC marker gene expression in thoracic artery is decreased compared to the vehicle-treated embryos. Meanwhile, SMC numbers in arteries of BC1-treated embryos are increased due to an increased proliferation rate. Of importance, vessels from BC1-treated embryos show an abnormality in vasculature, characterized by inordinate stack of SMCs, disorganized ECM, and excessive collagen accumulated around SMCs in the artery media.

There are several limitations in this dissertation, which could be addressed in the future studies:

1. **The reduced neointimal formation and accelerated re-endothelialization in our animal models may also be due to the decreased inflammation or infiltration of immune cells, such as macrophages, neutrophils, and lymphocytes.**

   It is well-known that inflammation contributes to the injury-induced neointimal formation. In our animal model, JAK3 shRNA in the injured arteries may also affect leukocyte function, and thus regulates SMC and/or EC proliferation. Indeed, several cytokines produced by leukocytes regulate SMC proliferation, contributing to the injury-induced neointimal formation. Therefore, it is important to study whether JAK3 plays a role in regulating physiological and pathological function of leukocyte in our animal model.

2. **It is unknown if the new vascular endothelial lining induced by knockdown of JAK3 have the same physiological function as the mature endothelium.**

   Endothelium is important for the different aspects of vascular biology, such as vascular hemostasis, vascular tone, angiogenesis, vascular barrier, inflammation, etc. It is important to determine whether the newly formed endothelium has normal EC function. If it behaves differently, the new EC lining may cause other complications.

3. **It remains to be determined if BC1 plays a role in ECs.**

   Although BC1 appears not to alter endothelial structure in aorta as shown by the intact VEcadherin staining, it cannot exclude the possibilities that BC1 may affect the physiological function of ECs. Therefore, it is important to determine whether BC1 plays a role in EC property or function.