Janus Kinase 3, a Novel Regulator for Smooth Muscle Proliferation and Vascular Remodeling

Yung-Chun Wang, Xiao-Bing Cui, Ya-Hui Chuang, Shi-You Chen

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 neointimal 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.

Visual Overview—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:1352-1360. DOI: 10.1161/ATVBAHA.116.308895.)

Key Words: apoptosis ■ interleukin ■ Janus kinase 3 ■ smooth muscle proliferation ■ vascular remodeling

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. Evidently, SMC proliferation contributes to numerous vascular diseases such as systemic hypertension, atherosclerosis, aortic aneurysm, postangioplasty restenosis, etc. 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.

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. In addition to lymphocytes, JAK3 is also expressed in certain nonhematopoietic cells, including vascular cells and carcinoma. JAK3 mediates mucosal homeostasis through regulating IL-2–induced intestinal epithelial cell migration, proliferation, and cell apoptosis. Moreover, JAK3 is involved in the progression of human colon cancer, renal fibrosis, vascular calcification, and myocardial ischemia and reperfusion injury. 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**

Materials and Methods are available in the online-only Data Supplement.

**Results**

**PDGF-BB Induced the Expression and Phosphorylation of JAK3 in SMCs**

JAK2 has been shown to play a role in angiotensin II–induced SMC proliferation and vascular remodeling. 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 SMCs 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 (Figure IA in the online-only Data Supplement). 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 1A through 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 1B and 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 1D and 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). 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 2A and 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.

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**Nonstandard Abbreviations and Acronyms**

| IL | interleukin |
| JAK3 | Janus kinase 3 |
| JNK | c-Jun N-terminal kinase |
| PCNA | proliferating cell nuclear antigen |
| PDGF-BB | platelet-derived growth factor-BB |
| SMC | smooth muscle cell |
| STAT3 | signal transducer and activator of transcription 3 |
| shRNA | short hairpin RNA |

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**Figure 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).
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 2C and 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 2E and 2F). Importantly, all the pathway inhibitors attenuated PDGF-BB–induced PCNA expression (Figure 2E and 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 3A through 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 3D). JAK3 expression also induced PCNA expression (Figure 3E and 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-1.15 As shown in Figure 3G and 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.16 As shown in Figure II in the online-only Data Supplement and Figure 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 (Figure II in the online-only Data Supplement; Figure 4A and 4B). JAK3 and phospho-JAK3 were mostly present in neointimal SMCs because they costained with smooth muscle α-actin (Figure III in the online-only Data Supplement). 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.
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injury and decreased gradually afterward (Figure 4B and 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 (Figure IB in the online-only Data Supplement), 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 (Figure IV in the online-only Data Supplement). Knockdown of JAK3 significantly inhibited injury-induced neointimal formation (Figure 4D and 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 4F). The intima/media area ratios were also significantly reduced (Figure 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 4H and 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 (Figure IIIC in the online-only Data Supplement). 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. 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 Figure V in the online-only Data Supplement, knockdown of JAK3 induced apoptosis in a small portion of

Figure 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.
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 (Figure VI in the online-only Data Supplement). 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.19,20 Inhibition of STAT3 and its signaling prevent SMC proliferation and injury-induced neointimal formation.16,19 JNK has also been shown to mediate PDGF-BB–induced SMC proliferation and injury-induced neointima formation.16,21 Therefore, we hypothesized that JAK3 may regulate PDGF-BB–mediated SMC proliferation by activating STAT3 and JNK signaling. As shown in Figure 5A through 5C, PDGF-BB, indeed, induced the expression and phosphorylation of both STAT3 and JNK (Figure VI in the online-only Data Supplement). Conversely, ectopic expression of JAK3 stimulated STAT3 and JNK phosphorylation (Figure VII in the online-only Data Supplement). Because
STAT3 and JNK regulate cell proliferation through inducing cyclin D1 expression\(^22\text{–}^{24}\) and protect cell from programmed death via altering Bcl-2/Bax expression.\(^25\text{,}^{26}\) We detected whether JAK3 affects the expression of cyclin D1 and apoptosis regulators. As shown in Figure VIII in the online-only Data Supplement, 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 (Figure IX in the online-only Data Supplement).

**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 biphasic 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 (Figure X in the online-only Data Supplement). STAT3 is known to stimulate SMC proliferation and survival, contributing to the injury-induced neointimal formation.\(^18\text{,}^{22}\) In addition to cell proliferation, STAT3 has also been shown to interact with myocardin to regulate SMC phenotypic modulation.\(^23\) 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 (Figure VI in the online-only Data Supplement). 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. Blockade of JNK activity by gene transfer of dominant-negative mutant suppresses balloon injury–induced neointimal formation via inhibiting SMC proliferation. 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 (Figure VIII in the online-only Data Supplement). 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. In fact, blockade of STAT3 or JNK activity and their signaling also causes pathological cell death by activating apoptotic pathways in SMCs. 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. In the early times after vascular injury, leukocyte recruitment has a strong correlation with the subsequent neointimal formation. Furthermore, inhibition of inflammatory cell accumulation in vascular lesion through blocking mononuclear leukocyte (lymphocyte and monocyte) trafficking reduces neointimal formation. JAK3 is involved in IL-6–induced M1 macrophage differentiation and IL-8–induced neutrophil chemotaxis. 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. Because STAT3 is a central regulator in vascular responses to the inflammatory cytokines, 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 neointima formation. Although JAK2 also regulates SMC proliferation, it mainly mediates angiotensin II–induced proliferation.

Figure 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.
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.

Sources of Funding

This work was supported by grants from National Institutes of Health (HL119053, HL123302, and HL135854).

Disclosures

None.

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### Highlights

- Janus kinase 3 is upregulated in platelet-derived growth factor-BB–induced smooth muscle cell proliferation.
- Knockdown of Janus kinase 3 suppresses smooth muscle cell proliferation.
- Janus kinase 3 contributes to injury-induced vascular remodeling and smooth muscle cell proliferation in vivo.
- Janus kinase 3 regulates smooth muscle cell proliferation by activating signal transducer and activator of transcription 3 and c-Jun N-terminal kinase signaling pathways.
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Arterioscler Thromb Vasc Biol. 2017;37:1352-1360; originally published online May 4, 2017;
doi: 10.1161/ATVBAHA.116.308895

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
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Detailed Materials and Methods

Janus Kinase 3, a Novel Regulator for Smooth Muscle Proliferation and Vascular Remodeling

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Detailed 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.⁴ 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-SAPK/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.⁵ 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 CGA TTA 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. JAK3 primer sequences were 5′- CCT GCC TGT TTA TCA TTC GCT -3′ (forward) and 5′- AAG ACT TGA GTG TCC TCC ACG TCC -3′ (reverse).

**Western Blot Analysis**

Rat SMCs were starved in DMEM containing no FBS and 5% L-glutamine for 24 hour, 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-HCl, 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 hour, followed by incubation with HRP-conjugated secondary antibody (Sigma) at Room temperature for 1 hour. The protein levels were detected with enhanced chemiluminescence (Millipore).  

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. 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 10^9 pfu) for 20 minutes as described previously. 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. 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. 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 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: 

$$\frac{(\text{Mean value of IHC staining intensity} - \text{Mean value of negative control staining intensity})}{(\text{Mean value of IHC staining intensity of uninjured vessels} - \text{Mean value of negative control staining intensity})}.$$ 

The subsequent statistical analyses 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.

**References:**


Janus Kinase 3, a Novel Regulator for Smooth Muscle Proliferation and Vascular Remodeling

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Supplementary Data
Supplementary Figure I. 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 II. 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 III: 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 IV. 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 V. 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 VI. 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 VII. 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 VIII. 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 IX.** 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 X. 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.