

Original article

Vascular smooth muscle Jak2 deletion prevents angiotensin II-mediated neointima formation following injury in mice

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ABSTRACT

The *in vitro* treatment of vascular smooth muscle cells (VSMC) with angiotensin II (Ang II) causes Janus kinase 2 (Jak2) to interact with the Ang II type 1 receptor (AT₁-R) resulting in enhanced cell growth. However, the role that Jak2 plays in AT₁-R-mediated vascular cell growth and remodeling *in vivo* is less clear. We hypothesized that *in vivo*, Jak2 plays a rate-limiting role in Ang II-mediated neointima formation following vascular injury. Using the Cre-loxP system, we conditionally ablated Jak2 from the VSMC of mice. We found that these mice are protected from Ang II-mediated neointima formation following iron chloride-induced vascular injury. In addition, the VSMC Jak2 null mice were protected from injury-induced vascular fibrosis and the pathological loss of the contractile marker, smooth muscle α -actin. Finally, when compared to controls, the VSMC Jak2 null mice exhibited significantly less Ang II-induced VSMC proliferation and migration *in vitro* and *in vivo* and more apoptosis. These results suggest that Jak2 plays a central role in the causation of Ang II-induced neointima formation following vascular injury and may provide a novel target for the prevention of neointima formation.

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1. Introduction

Damage to the vascular endothelial cell (EC) layer often caused by stenting, angioplasty, or bypass surgery is one of the initial key events in the pathogenesis of various vascular diseases including neointima formation, atherosclerosis, restenosis, and hypertension. It causes phenotypic switching of vascular smooth muscle cells (VSMC) from a contractile to a synthetic phenotype characterized by increased cell proliferation, migration, and production of extracellular matrix [1,2]. Neointima formation resulting in vascular EC injury is also characterized by a general loss of critical VSMC contractile markers including smooth muscle α -actin (SMA), myosin heavy chain (SM-MHC), SM22 α and calponin (CNN) [2,3]. However, the mechanistic processes that trigger these phenotypic alterations are still not fully understood.

Janus kinase 2 (Jak2) is well known for its role in hematopoiesis and cytokine signaling. Mice that are completely Jak2 null die at embryonic day 12.5 due to impaired hematopoiesis and profound anemia [4,5]. Conversely, mutations that lead to hyper-kinetic Jak2 kinase activity result in various hematological diseases characterized increased cell proliferation and hematopoiesis [6]. As a consequence, numerous Jak2 inhibitors are currently under pre-clinical and clinical

investigation for their potential in the treatment of Jak2 mediated hematological diseases, but to date, none have been approved.

It is well accepted that the Jak2 signaling pathway couples to the angiotensin II type 1 receptor (AT₁-R) [7]. However, the physiological and/or pathological importance of this coupling is poorly understood in the *in vivo* setting. Angiotensin II (Ang II) has been shown to exhibit growth promoting effects and migration in cultured VSMCs [8,9]. In addition, chronic infusion of Ang II induces VSMC proliferation in normal and injured vessels *in vivo* [10,11]. Unfortunately, there have been conflicting reports as to which kinase signaling pathway(s) mediate(s) Ang II-induced cell proliferation and migration. For instance, some studies have shown that Ang II mediates its growth promoting effects via the Mitogen-Activated Protein (MAP) kinase pathway *in vitro* [12]. On the other hand, studies have reported a correlative involvement of the Jak/STAT signaling pathway in the growth factor-like signaling properties of Ang II and subsequent vascular neointima formation [13,14]. Previous attempts to determine the specific kinase pathway involved in Ang II-induced neointima formation have been limited by the lack of specific kinase inhibitors and the lack of conditional knockout animal models. For example, AG490 has been used in these types of studies [17], but in addition to inhibiting Jak2, it also inhibits Jak3 and MAP kinase [15]. Thus, it is still unclear which kinase signaling pathway acts downstream of the AT₁-R to mediate VSMC proliferation, migration, and neointima formation.

Previous studies have long shown that Ang II binding causes physical interaction of Jak2 with the AT₁-R resulting in the subsequent activation of the Signal Transducers and Activators of Transcription

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(STAT) proteins [16]. Activated STATs in turn form homo- and heterodimers which translocate into the nucleus and bind *cis*-inducible elements resulting in the activation of growth promoting genes [17,18]. However, there is no evidence indicating that the pathological AT₁-R-mediated growth effects occur exclusively through Jak2. The current study was aimed at determining whether Jak2 plays a central role in the pathogenesis of Ang II-mediated neointima formation following injury. Using an approach whereby we conditionally deleted Jak2 from the VSMC of mice, our data indicate for the first time that Jak2 plays a rate-limiting role in the causation of Ang II-induced neointima formation following vascular injury *in vivo*.

2. Materials and methods

2.1. Animals

Male mice generated on an FVB background strain were used in these experiments. All procedures using laboratory animals were approved by the Institutional Animal Care and Use Committee at the University of Florida. Animals were maintained in accordance with NIH standards established in the *Guidelines for the Care and Use of Experimental Animals*.

2.2. Generation of knockout mice

VSMC deletion of Jak2 was achieved by crossing mice carrying loxP sites around the first coding exon of the Jak2 gene [19] with mice expressing Cre recombinase under the control of the SM22 α promoter [20]. Specifically, male mice of genotype SM22 α Cre-Jak2fl/+ were crossed with Jak2fl/fl females, which resulted in mice whose VSMCs are devoid of Jak2 (SM22 α Cre(+)Jak2fl/fl). Genotyping was done by PCR using primers 5'-GCTAAACATGCTTCATCGTCGGTC and 5'-CAGATTACGTATATCCTGGCAGCG in the Cre coding region, 5'-ATTCTGAGATTCAGGTCGAGC and 5'-CTCACAACCATCTGTATCTCAC in the Jak2 coding region, and 5'-GTCTATACACCACCTCCTG and 5'-GAGCTGGAAGATAGGTCAGC to identify the null Jak2 allele. Expression of VSMC SM22 α Cre was confirmed by crossing mice expressing SM22 α Cre(+)Jak2fl/fl with Rosa26 β -galactosidase reporter mice, and X-Gal (5-bromo-4-chloro-3-indolyl β -D-galactoside) staining was analyzed as previously described [21].

2.3. Vascular injury model

Iron chloride-induced vascular injury was carried out as previously described with a few modifications [22]. Briefly, mice at 3 months of age were anesthetized using isoflurane. An incision was made directly on top of the right common carotid artery and vascular injury was induced by applying a sterile Q-tip saturated with 10% ferric chloride (Sigma, St. Louis, MO, USA) for 3 min. The left common carotid artery was exposed by blunt dissection, but not injured and thus served as a contralateral control. At the same time, the animals received an Alzet Model 1004 osmotic minipump (Alzet Corp) for subcutaneous infusion of 1000 ng/kg/min of Ang II [23]. The incision was closed and the animals were allowed to recover. To prevent thrombosis, animals were injected subcutaneously with 20 units of heparin prior to surgery. Animals were euthanized at 7 days ($n=6$ for each genotype) or 14 days ($n=6$ for each genotype) following vascular injury. The carotid arteries were fixed using 10% formalin and processed for histological determination of neointima formation.

2.4. Histology

Tissue samples were prepared for histology as previously described [24]. Briefly, tissues were fixed overnight at 4 °C in 10% buffered formalin (Fisher Scientific, Pittsburgh, PA). The tissues were subsequently dehydrated through a graded ethanol series, paraffin embedded

and sectioned. Five-micrometer sections were stained with hematoxylin and eosin (H&E) for morphological analysis. Transverse sections of the carotid arteries were subjected to morphometry for assessing the intima/media ratio (I/M ratio). Other tissues were stained with Masson's trichrome using a kit (87019 Richard-Allan Scientific) for analysis of fibrosis.

2.5. Immunohistochemistry

Five-micrometer sections mounted on gelatin-coated slides were dewaxed in ethanol, rehydrated, then blocked in 3% H₂O₂ followed by 5% normal goat serum. Sections were exposed to the primary antibody overnight at 4 °C, washed, and then treated with the biotinylated secondary antibody. After secondary antibody incubation, the samples were washed, exposed to the avidin-peroxidase reagent (Vectastain Elite, Vector Laboratories, Burlingame, CA), and reacted with diaminobenzidine to produce a brown reaction product. The sections were dehydrated in ethanol, mounted with Permount, and observed by light microscopy. Immunohistological detection of anti-smooth muscle α -actin (CM001B) was carried out using the Rat on Mouse AP-Polymer Kit (Biocare Medical) according to the manufacturer's instructions. Anti-Jak2 (ab39636 Abcam), anti-phospho-Jak2 (Ab32101 Abcam), anti-phospho-STAT5 (Ab32364 Abcam), and Ki-67 (M7249 DAKO) immunohistochemical detection was performed as previously described [24]. Apoptosis detection was carried out using a TUNEL staining kit (S7100 Chemicon) according to the manufacturer's instructions.

2.6. Immunoblotting

Protein sample was extracted from the homogenates of primary VSMC cultures and immunoprecipitated using STAT3 antibody (SC-482, Santa Cruz), or STAT5 antibody (SC-28685, Santa Cruz), followed by western-blotting using phospho-STAT3 antibody (SC-8059, Santa Cruz), or phospho-STAT5 antibody (716900, Invitrogen) respectively, as previously described [25].

2.7. Cell proliferation

VSMCs from each genotype were isolated and cultured as described [26]. Cell proliferation was assessed on the basis of mitochondrial dehydrogenase activity of the cells using the MTT dye-reduction assay [27]. In brief, after the addition of ligand or vehicle, the cells were incubated with the MTT labeling solution (0.5 mg/ml) at 37 °C for 4 h. The cells were then solubilized in 100 μ l of 0.1 N HCl and isopropyl alcohol, and shaken for 30 s on a plate rotator. Absorbance at 540 nm was measured with the use of a microtiter plate reader with a reference wavelength of 690 nm. Direct cell counts were also carried out to determine the number of viable cells using trypan blue exclusion.

2.8. Cell migration

The migration of VSMCs was determined using a QCM™ 24-well colorimetric cell migration assay kit (Millipore) according to the manufacturer's instructions. Briefly, the cells were suspended in serum free media (DMEM) at a concentration of 1×10^6 cells/ml. Aliquots (0.3 ml) of the cell suspension were added to the top chambers of the transwell membranes with 8- μ m pores. The lower transwell compartments contained 0.5 ml of DMEM containing various migration factors including Ang II (10^{-7} M), FBS (10%) or platelet-derived growth factor (PDGF-BB) (20 ng/ml). Incubation was continued for 24 h at 37 °C in a CO₂ incubator. The adherent cells were then stained, washed, and allowed to air dry. The dye was extracted from the cells, and optic density was measured at 560 nm.

2.9. Apoptosis

Annexin V/propidium iodide staining was employed to determine early apoptosis in the control and the Jak2 null VSMCs. VSMCs were serum-starved overnight and then treated with either vehicle, 10% FBS, 20 ng/ml PDGF or 10^{-7} M Ang II for 24 h. Cells (10^5) were re-suspended in 100 μ l of $1\times$ binding buffer and apoptotic levels were determined via the FITC Annexin V Apoptosis Detection Kit (BD Pharmingen) following the manufacturer's instructions and analyzed on a FACSCalibur flow cytometer (Becton Dickinson). Apoptotic cells were also identified using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method which specifically labels the 3'-hydroxyl termini of DNA strand breaks. The TUNEL assay was carried out using the ApopTag Fluorescein *In Situ* Apoptosis Detection Kit (Millipore) according to the manufacturer's instructions.

2.10. Statistical analysis

All results were expressed as means \pm SEM. Statistical comparison of the different genotypes were performed by unpaired Student's *t*-test. *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. Deletion of VSMC Jak2 prevents Ang II-mediated neointima formation and narrowing of the vascular lumen following injury

Vascular remodeling is a pathologic response to vascular injury characterized by VSMC proliferation, migration, neointima formation, and a narrowing of the vascular lumen [28]. We wanted to determine whether VSMC Jak2-null mice are protected from neointima formation and narrowing of the vascular lumen following vascular injury. For this, the right carotid arteries of control (SM22 α Cre(–);Jak2fl/fl) and

VSMC Jak2-null (SM22 α Cre(+);Jak2fl/fl) mice were subjected to iron chloride-induced vascular injury with simultaneous Ang II infusion. Left carotid arteries were exposed via blunt dissection, but not subjected to iron chloride-induced injury and thus served as contralateral controls. Iron chloride-induced vascular injury is a well-established vascular injury model which has been mostly used in thrombosis studies [22,29,30]. It causes de-endothelialization of the artery resulting in neointima formation due to erythrocyte hemolysis and hemoglobin oxidation [31]. Furthermore, iron chloride-induced arterial injury increases oxidative stress in the vessel which is an important component of all the risk factors leading to neointima formation including smoking, hypertension and vascular surgery.

Mice were euthanized 7 and 14 days after injury. H&E representative sections in Fig. 1A show a clear increase in thickness of the neointima in the injured arteries of the control mice at days 7 and 14, but neither in the VSMC Jak2-null mice nor any of the uninjured contralateral arteries. Using computer-assisted quantitative morphometric analysis, we found that compared to the non-injured contralateral carotid arteries, vascular injury induced significant increases in the intima/media ratio in the control mice at days 7 and 14, which was lacking in the VSMC Jak2 null mice (Fig. 1B). As a consequence, there was a significant narrowing on the carotid artery lumen in the injured arteries from the control mice while the VSMC Jak2-null mice were protected from this deleterious effect (Fig. 1C). These collective results suggest that deletion of VSMC Jak2 prevents neointima formation and the subsequent narrowing of the vessel following vascular injury.

3.2. Deletion of VSMC Jak2 prevents Ang II-mediated vascular fibrosis following injury

Vascular fibrosis is an important component of vascular injury response [32]. Thus, we quantified extracellular matrix (ECM) deposition using trichrome-blue staining of sections from iron chloride-injured

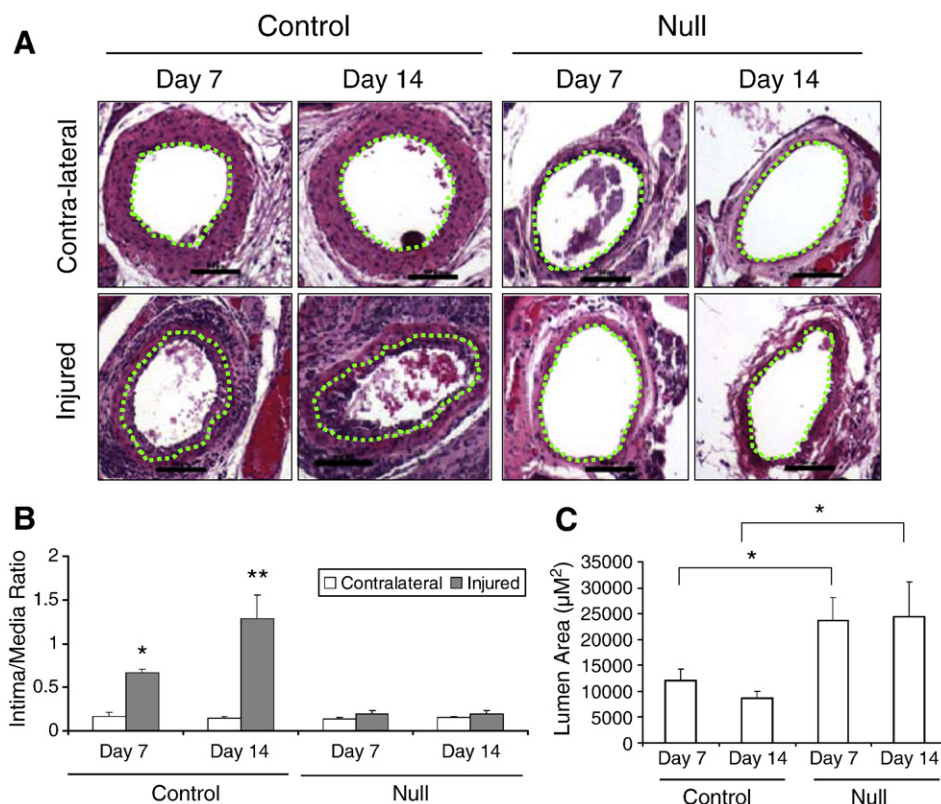


Fig. 1. Deletion of VSMC Jak2 prevents Ang II-mediated neointima formation and narrowing of the vascular lumen following injury. A, H&E stained sections showing neointima formation in control mice (the scale bar is 50 μ m long). The green dotted lines separate the intima from media. B, Intima/media ratio. C, Lumen area. **p* < 0.05 vs. contralateral control, ***p* < 0.01 vs. contralateral control.

control and Jak2 null mouse carotid arteries 7 and 14 days after injury. Representative sections indicated that the density of trichrome blue staining was significantly reduced in the Jak2 null mice at both time points when compared to the control group (Fig. 2A) and quantitative analysis of all sections found this difference to be significant (Fig. 2B). Overall, these results suggest that deletion of VSMC Jak2 reduces Ang II-induced ECM deposition following vascular injury.

3.3. Deletion of VSMC Jak2 prevents the loss of smooth muscle α -actin in response to Ang II-mediated vascular injury

Vascular injury is known to reduce the expression of contractile markers within VSMC [1,2]. We hypothesized that the Jak2-null mice would be protected from the injury-induced loss of smooth muscle α -actin (SMA). Immunohistochemistry of injured carotid sections showed that there was a significant loss of SMA immunoreactivity (red stain) at both day 7 and day 14 in the control mice when compared to the contralateral carotid artery (Fig. 2C). However, SMA staining was preserved in the injured carotid arteries in the Jak2 null mice, and was comparable to that observed in their contralateral arteries. Quantitative analysis of all sections again found this difference between the two genotypes to be significant (Fig. 2D). As such, these results suggest that VSMC Jak2-null mice are protected from the loss of the contractile phenotype that occurs following vascular injury.

3.4. Deletion of VSMC Jak2 prevents neointima formation by inhibiting cell proliferation and inducing apoptosis

We hypothesized that a mechanism whereby deletion of VSMC Jak2 prevents neointima formation is via reduced cellular proliferation. To determine this, the levels of the proliferative marker, Ki-67,

were measured in sections from each condition. Representative sections indicated an increase in the number of Ki-67 positive nuclei (brown stain) in the injured arteries of the control mice at days 7 and 14, but not in the VSMC Jak2 null-mice (Fig. 3A). Quantification of Ki-67 positive nuclei across all sections found that when compared to the non-injured contralateral arteries, injury caused significant increases in the number of Ki-67 positive cells in the control mice at days 7 and 14, but this was lacking in the Jak2-null mice (Fig. 3B). These results suggest that deletion of VSMC Jak2 prevents neointima formation by inhibiting cell proliferation.

Previous studies have shown that induction of apoptosis is a possible mechanism for the prevention of neointima formation [14,33]. Therefore, we wanted to determine whether the reduced neointima formation observed in the VSMC Jak2-null mice correlates with increased apoptosis. Shown are representative TUNEL stained sections (Fig. 3C; brown stained cells indicated by red arrows) and the average number of TUNEL positive cells plotted as a function of treatment group (Fig. 3D). There was no significant difference in the number of TUNEL positive cells observed in injured carotid arteries taken from the control group when compared to the non-injured, contralateral arteries. In contrast, there was a significant increase in the number of apoptotic cells 7 days after injury in the Jak2-null mice and this returned to near baseline levels by day 14. Altogether, the results in Fig. 3 suggest that VSMC Jak2 deletion prevents neointima formation by inhibiting cell proliferation and inducing apoptosis in VSMCs.

3.5. VSMC Jak2 induces neointima formation by increasing phosphorylation of Jak2 and STAT5

Immunohistochemistry was carried out in order to determine the relative levels of Jak2, phospho-Jak2, and phospho-STAT5 (a downstream

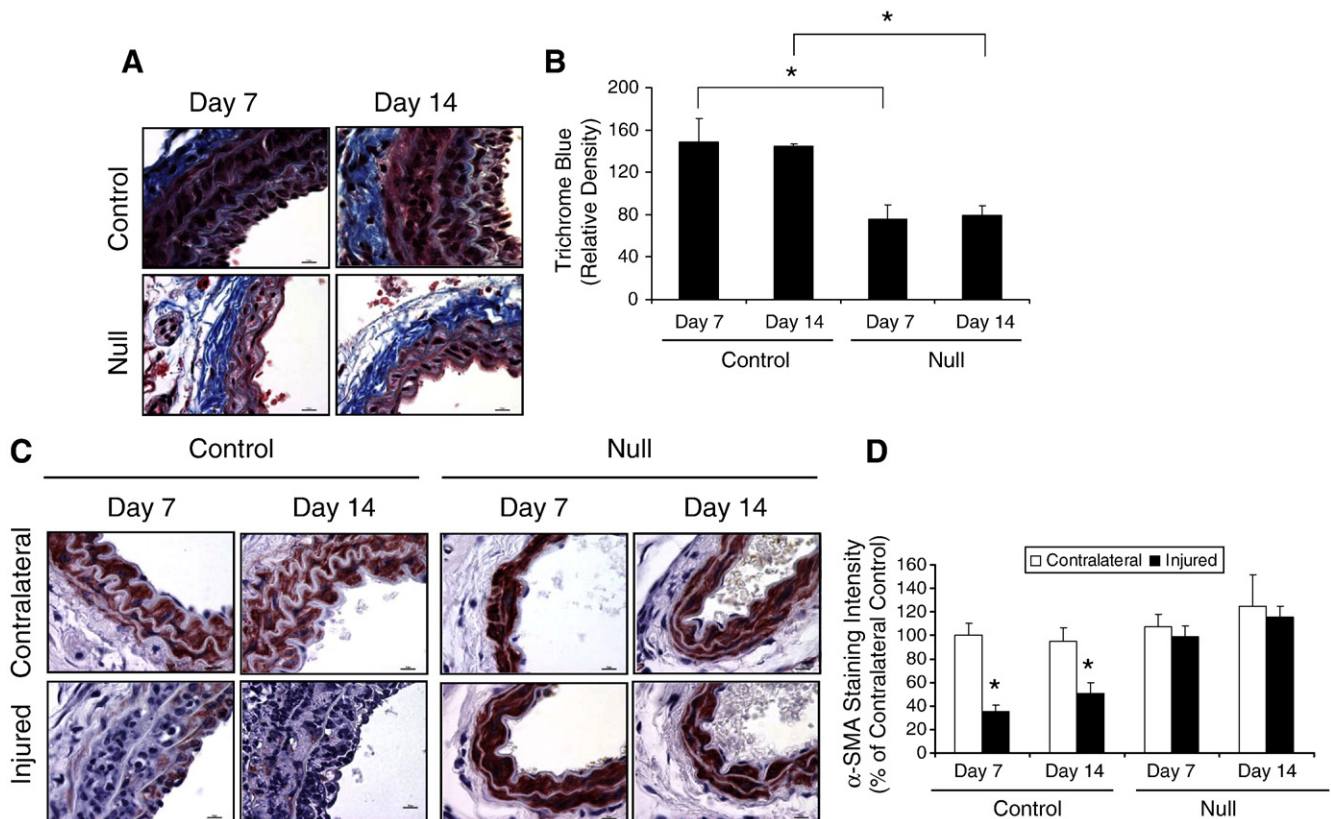


Fig. 2. Deletion of VSMC Jak2 prevents Ang II-mediated fibrosis and loss of SMA following injury. A, Trichrome-blue stained sections showing increased collagen density in control mice (the scale bar is 10 μ m long). B, Quantification of trichrome-blue staining. C, Immunohistochemistry of SMA (the scale bar is 10 μ m long). D, Quantification of SMA staining. * p <0.05 vs. contralateral non-injured artery.

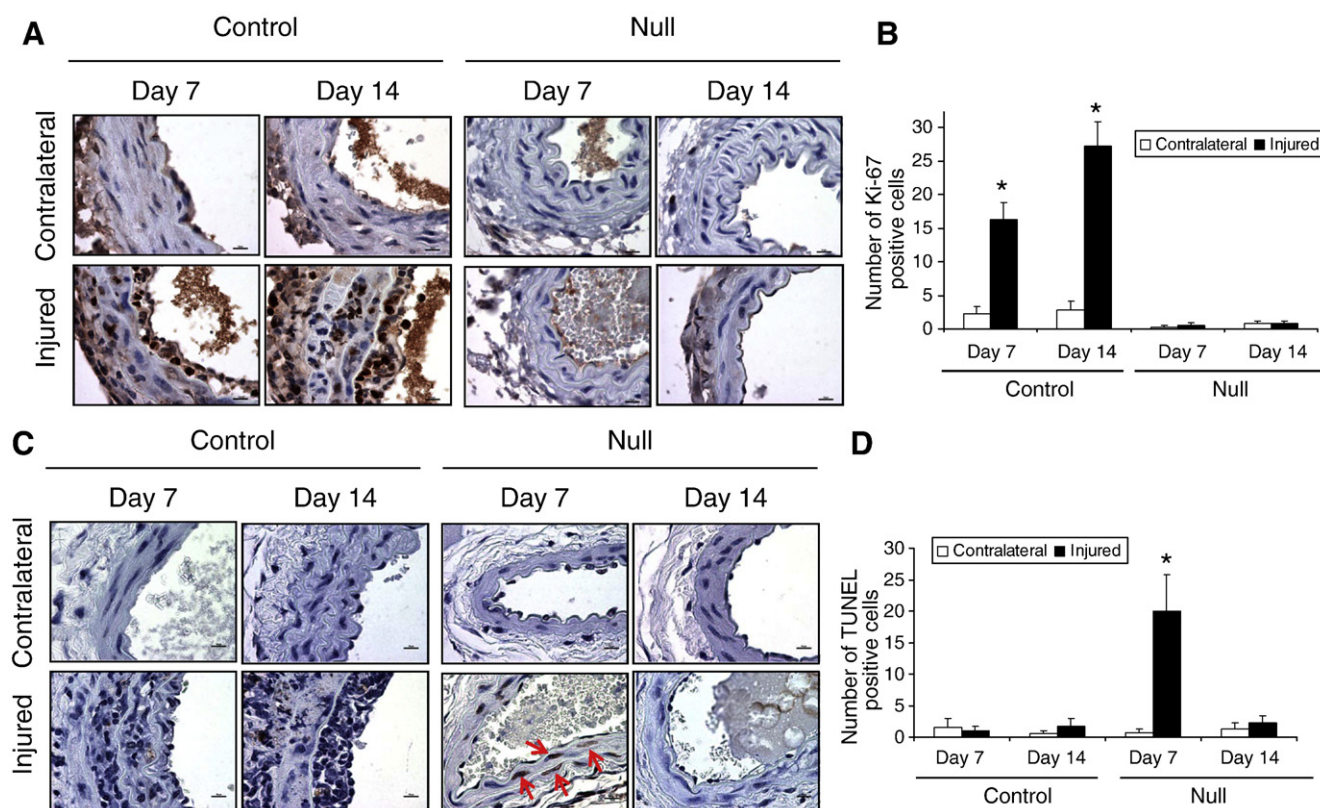


Fig. 3. Deletion of VSMC Jak2 inhibits cell proliferation and induces apoptosis. A, Representative sections showing immunohistochemistry of Ki-67 (the scale bar is 10 μ m long). B, Quantification of the number of Ki-67-positive nuclei. C, Representative sections showing immunohistochemistry of TUNEL (the scale bar is 10 μ m long). D, Quantification of the number of TUNEL positive cells. * $p < 0.05$ vs. contralateral non-injured control.

target of Jak2) in the injured and non-injured carotid arteries of both the control and VSMC Jak2-null mice (Fig. 4). With respect to the control mice, we found that there were readily detectable levels (brown stain) of Jak2, phospho-Jak2 and phospho-STAT5 in the contralateral arteries and vascular injury increased this staining pattern both in the media and neointima. With respect to the VSMC Jak2-null mice, there was little to no staining for Jak2, phospho-Jak2, and phospho-STAT5 both in the contralateral control and the injured arteries. These results demonstrate that the reduced neointima formation observed in the VSMC Jak2-null

mice correlates with reduced phosphorylation of Jak2 and STAT5 in VSMC.

3.6. VSMC Jak2 mediates Ang II-induced cell proliferation and migration

In order to determine whether the Jak2-dependent effects observed *in vivo* are specific for Ang II *per se*, we isolated VSMC from control and VSMC Jak2-null mice and cultured them *ex vivo*. The cells were subsequently treated with either vehicle, 10% FBS, platelet derived

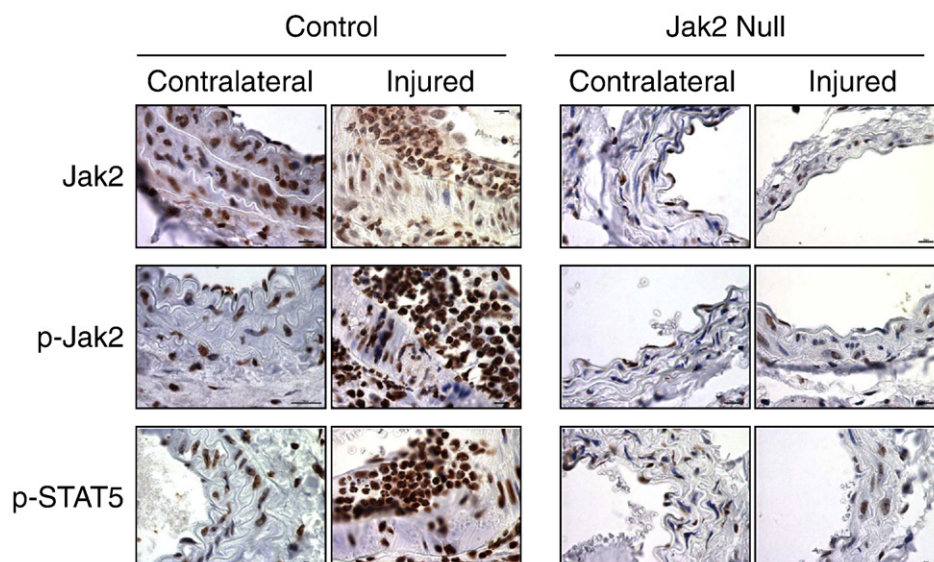


Fig. 4. VSMC Jak2 induces neointima formation by increasing phosphorylation of Jak2 and STAT5. Immunohistochemistry of representative sections showing relative expression of Jak2, phospho-Jak2 and phospho-STAT5 in injured and non-injured carotid arteries of the control and the Jak2-null mice (the scale bar is 10 μ m long).

growth factor (PDGF), or Ang II, and then evaluated for cell proliferation and cell migration properties. For the control cells, FBS, PDGF, and Ang II all induced robust cell proliferation as measured by MTT (Fig. 5A). For the VSMC Jak2 null cells, FBS and PDGF induced robust cell proliferation while Ang II did not (Fig. 5B). To confirm these results using a different approach, we repeated the study, but instead determined cell proliferation via a direct counting of cells. We similarly observed that FBS, PDGF, and Ang II all induced robust proliferation of the control cells (Fig. 5C), but only FBS and PDGF induced proliferation of the VSMC Jak2-null cells (Fig. 5D). Together, these results suggest that Jak2 is rate-limiting in Ang II-induced VSMC proliferation.

Next, we wanted to determine the role of Jak2 in VSMC migration in response to these same stimuli. We found that there was no significant difference in cell migration between the genotypes when the cells were treated with FBS or PDGF (Fig. 5E). However, when the cells were treated with Ang II, cell migration in the Jak2-null cells was significantly attenuated when compared to the control cells (Fig. 5E), suggesting that Jak2 mediates Ang II-induced cell migration. Collectively, the data in Fig. 5 correlate the specific loss of Jak2 with significantly impaired Ang II-mediated VSMC proliferation and migration.

3.7. VSMC Jak2 is required for Ang II-mediated cell survival

The *in vivo* immunohistochemistry data indicate that Jak2 promotes cell survival as cells that lack Jak2 are more prone to apoptosis after injury (Fig. 4C and D). Here, we wanted to determine whether this Jak2-dependent event was specific for Ang II. For this,

primary cultures of VSMC from control and VSMC Jak2-null mice were serum starved overnight. The following day, the cells were treated with the indicated ligands and early apoptosis was measured 24 h later. We found that in the control cells, ~10% of all cells treated with vehicle control solution were in early apoptosis (Fig. 6A). Treatment with FBS, PDGF, and Ang II promoted cell survival as indicated by the lower levels of apoptosis in these cells. When the same experiment was performed in the VSMC Jak2-null cells, the ability of Ang II to decrease the levels of apoptosis was lost, thereby indicating a critical role for Jak2 in Ang II-mediated cell survival. To demonstrate this using an alternate approach, the cells were treated in the same way, but this time apoptosis levels were measured via TUNEL stain, an indicator of both early and late apoptosis (Fig. 6B). We similarly found an essential requirement for Jak2 in promoting Ang II-mediated cell survival as evidenced by the inability of the null cells to decrease the levels of apoptosis in response to Ang II. As such, these data correlate the reduced neointimal formation observed in the VSMC Jak2-null genotype with increased apoptosis and this is Ang II specific.

3.8. VSMC Jak2 deletion is associated with reduced Ang II-mediated activation of STAT3 and STAT5

We next wanted to determine whether the activation of the Jak2/STAT signaling pathway is also specific for Ang II. For this, we analyzed the relative abilities of FBS, PDGF and Ang II to induce phosphorylation of STAT3 and STAT5; both of which are mitogenic proteins and are direct downstream signaling targets of Jak2. With respect to STAT3,

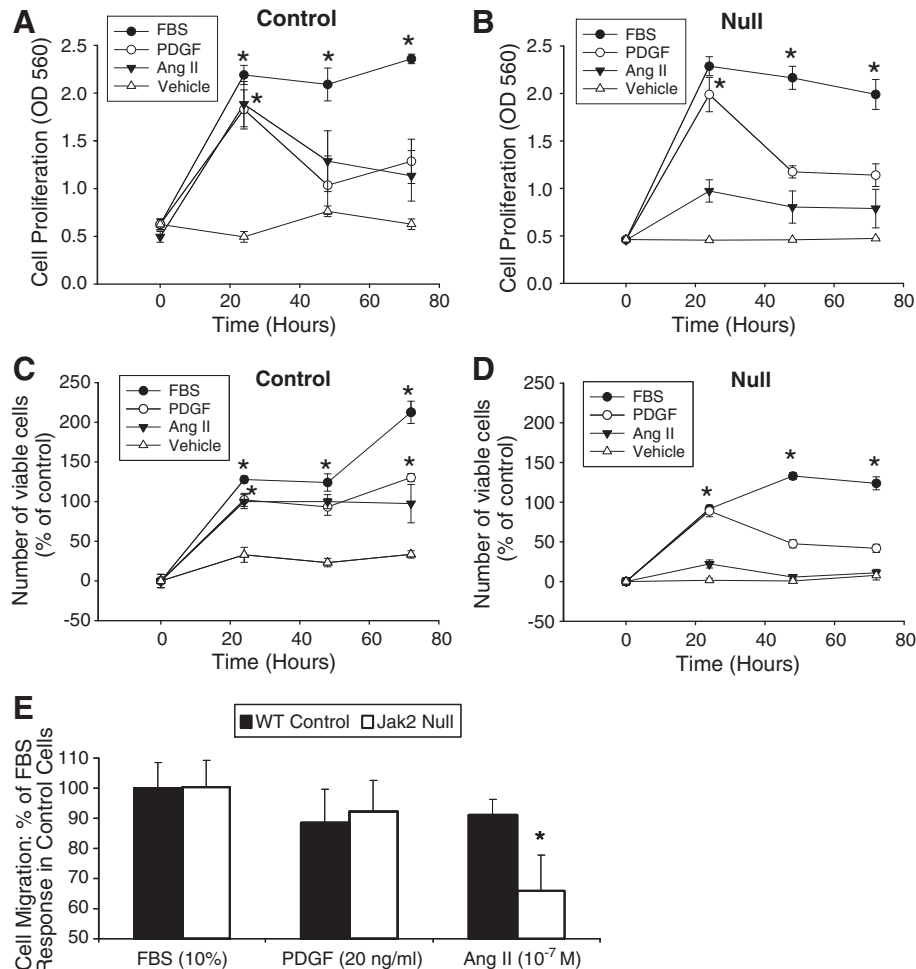


Fig. 5. VSMC Jak2 mediates Ang II-induced cell proliferation and migration. VSMC were treated with 10% FBS, 20 ng/ml PDGF or 10⁻⁷ M Ang II as indicated. Resulting cell proliferation of the control cells (A) and VSMC Jak2 null cells (B) is shown using the MTT cell proliferation assay. Cell proliferation using trypan blue exclusion for control (C) and VSMC Jak2 null (D) cells. E, Cell migration in these same cells. **p*<0.05 vs. control. Shown are mean ± SEM for three independent experiments, each run in triplicate.

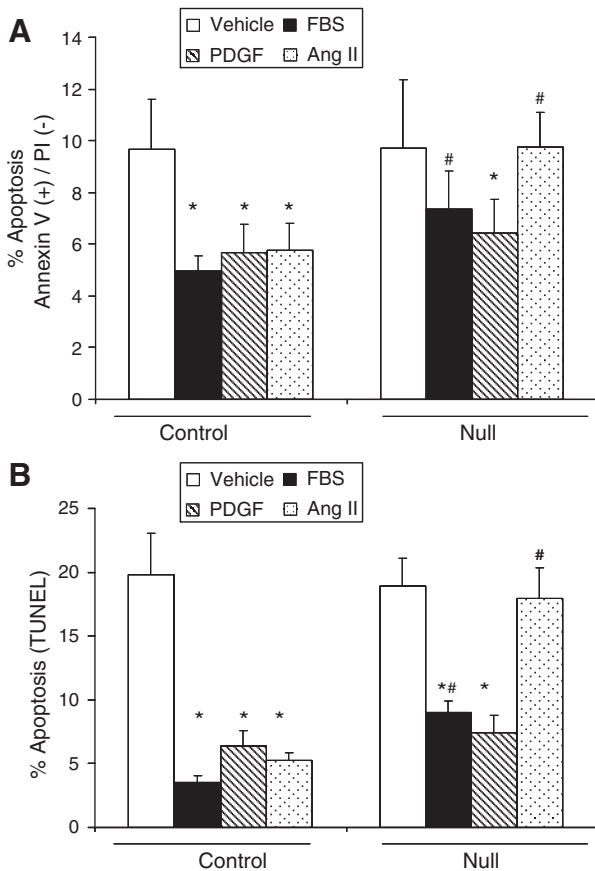


Fig. 6. Jak2 is required for Ang II-mediated VSMC survival. After overnight serum starvation, VSMC were treated with vehicle control solution, 10% FBS, 20 ng/ml PDGF or 10^{-7} M Ang II as indicated. Twenty-four hours later, apoptosis levels were measured via annexin V/propidium iodide selection (A) or via TUNEL staining (B). * $p \leq 0.05$ versus vehicle treated, # $p \leq 0.05$ versus control genotype. Shown are mean \pm SEM for three independent experiments, each run in triplicate.

ligand-induced phosphorylation of STAT3 was significantly reduced in the null cells when compared to the control cells for all conditions (Fig. 7A and B). These results suggest that activation of STAT3 is primarily mediated by Jak2 since the absence of Jak2 renders the cells incapable of phosphorylating STAT3 under any condition. In addition, we found that within the control cells, the Ang II-induced phosphorylation of STAT3 was significantly increased, when compared to the other stimulating ligands. With respect to STAT5, the phospho-STAT5 levels were not significantly different in the FBS- and PDGF-treated control cells when compared to vehicle-treated cells, but were significantly different in the Ang II-treated cells (Fig. 6C and D). Furthermore, there was no significant difference in ligand-induced phosphorylation of STAT5 amongst the Jak2-null cells (Fig. 7C and D). As such, these results suggest that Jak2 is the primary mediator of Ang II-induced phosphorylation of STAT5, and that activation of the Jak2/STAT5 pathway is specific for Ang II.

4. Discussion

Neointima formation is a pathologic consequence of vascular endothelial damage which often results from various risk factors including percutaneous vascular surgery procedures. We have investigated the impact of VSMC deletion of Jak2 on the prevention of Ang II-mediated neointima formation following vascular injury in mice. This study identifies Jak2 as a critical mediator of the pathological processes involved in Ang II-mediated neointima formation. To our knowledge, this is the first report to establish a causal relationship between Jak2 and Ang II-mediated neointima formation following vascular injury.

Following endothelial damage, the process of neointima formation starts with an initiation of VSMC proliferation and migration into the intima, followed by a sustained proliferation of VSMC in the neointima [27,34]. This compromises the vascular lumen, and limits blood supply to distal areas of the vessel. Ang II plays a key role in mediating the processes involved in the pathogenesis of neointima formation following vascular injury [35]. However, the specific mechanisms involved in Ang II-mediated neointima formation remain largely

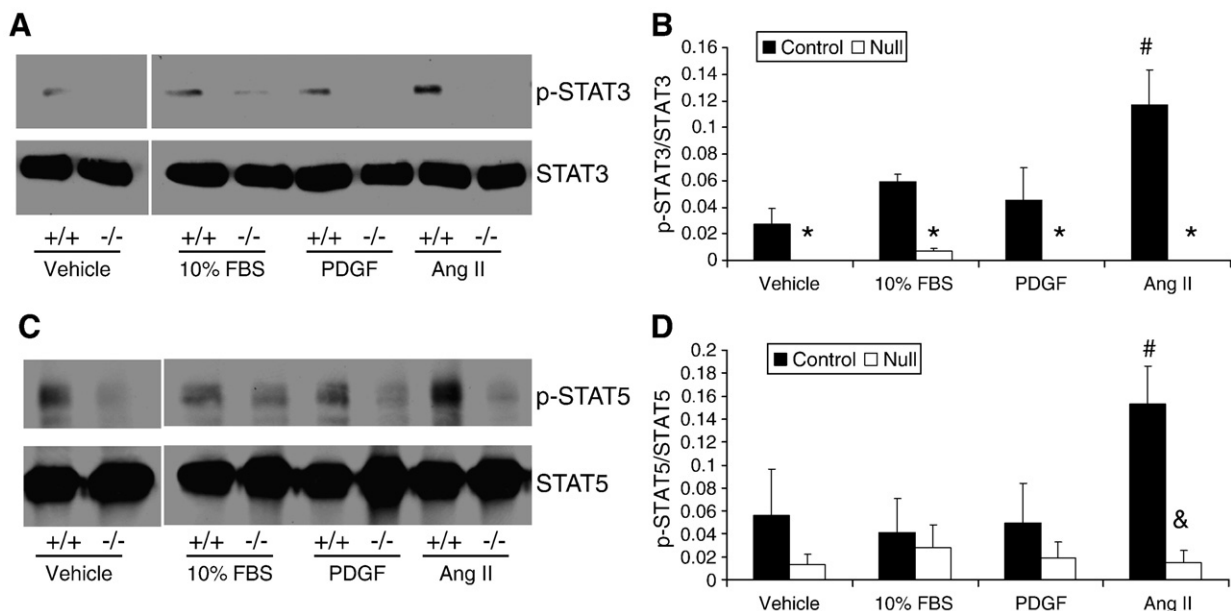


Fig. 7. VSMC Jak2 deletion reduces Ang II-mediated activation of STAT3 and STAT5. A, Representative blots of phospho-STAT3 and STAT3. B, Phospho-STAT3 quantification. C, Representative blots of phospho-STAT5 and STAT5. D, Phospho-STAT5 quantification. * $p < 0.05$ vs. ligand-treated control cells, # $p < 0.05$ vs. vehicle-treated control cells, & $p < 0.05$ vs. Ang II-treated control cells. Shown are mean \pm SEM for three independent experiments.

unidentified. Ang II induces tyrosine-phosphorylation and activation of Jak2 in rat VSMC resulting in activation of its downstream signaling substrates, the STATs [16]. Subsequent studies showed that the Ang II-induced activation of Jak2 results in proliferation of cultured VSMC [36]. We found that not only did VSMC Jak2 deletion prevent Ang II-induced neointima formation, but it also prevented the pathological phenotypic alterations induced by vascular injury including vascular fibrosis and loss of the contractile marker, SMA. The mechanisms by which VSMC Jak2 deletion prevents neointima formation include inhibition of cell proliferation and migration. Furthermore, we found that Ang II-induced activation of the proliferative proteins, STAT3 and STAT5, is impaired in the VSMC Jak2-null cells. The Jak2-mediated growth promoting and migratory effects are specific for Ang II since other ligands such as FBS and PDGF did not lead to differential effects on cell proliferation and migration in the control and the Jak2-null cells. From these data, we conclude that Jak2 plays a rate-limiting role in the causation of Ang II-induced neointima formation following vascular injury *in vivo*.

It is well established that Ang II plays a key role in mediating neointima formation following vascular injury [28]. This provided us with an excellent model of examining the role of VSMC Jak2 deletion in an environment in which neointima formation following injury is exasperated by chronic Ang II infusion. Although studies have shown that the Jak2 signaling pathway couples to the AT₁-R resulting in VSMC proliferation *in vitro* [7], the only study to date examining the relationship between Jak2 and neointima formation *in vivo* reported that Jak2 is only transiently expressed during the process of neointima formation [13]. The same study also reported that treatment of vascular strips with Ang II *ex vivo* enhances phosphorylation of Jak2 [13]. Therefore, until now, the pathophysiological link between Jak2 and Ang II-mediated neointima formation has been only correlative and no study has implicated Jak2 in playing a causative role in Ang II-mediated vascular remodeling *in vivo*.

Placing the Cre recombinase cDNA under the control of tissue specific promoters has allowed for the conditional deletion of genes such as Jak2. However, previous work has clearly demonstrated that the pattern of Cre expression in the targeted tissue is a mosaicism [37,38]. In other words, most cells will express Cre, but some will not. The reason(s) for this is not fully understood, but it is thought that it may be due to modifying events such as epigenetic silencing of the transgene within a given cell [39,40]. Hence, the gene targeted for deletion, in this case Jak2, is not removed from the cell. Quantitative examinations of our anti-Jak2 immunohistochemistry (Fig. 4) as well as Rosa26/LacZ expression patterns (data not shown) indicate that Jak2 is deleted from ~95% of all VSMC in the carotid arteries of these mice. This degree of deletion is clearly enough to impair Jak2 function as determined by the significantly reduced VSMC migration, VSMC proliferation, neointimal formation, and preservation of contractile markers, when compared to controls. Given that Jak2 has been implicated in a number of cardiovascular diseases including hypertension, heart failure, and diabetes [41–44], we now have an excellent model for the future examination of the role of VSMC derived Jak2 in these and other disorders.

In conclusion, the present study demonstrated for the first time that Jak2 plays a central role in the causation of Ang II-induced neointima formation following vascular injury *in vivo*. Therefore, inhibition of Jak2 may provide a potential prophylactic therapeutic strategy for prevention of neointima formation and progression following angioplasty and/or vascular stenting.

Disclosures

None.

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