

Nuclear Focal Adhesion Kinase Controls Vascular Smooth Muscle Cell Proliferation and Neointimal Hyperplasia Through GATA4-Mediated Cyclin D1 Transcription

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Rationale: Neointimal hyperplasia is characterized by excessive accumulation of vascular smooth muscle cells (SMCs) leading to occlusive disorders, such as atherosclerosis and stenosis. Blood vessel injury increases growth factor secretion and matrix synthesis, which promotes SMC proliferation and neointimal hyperplasia via FAK (focal adhesion kinase).

Objective: To understand the mechanism of FAK action in SMC proliferation and neointimal hyperplasia.

Methods and Results: Using combined pharmacological FAK catalytic inhibition (VS-4718) and SMC-specific FAK kinase-dead (Myh11-Cre-ER^{T2}) mouse models, we report that FAK regulates SMC proliferation and neointimal hyperplasia in part by governing GATA4- (GATA-binding protein 4) cyclin D1 signaling. Inhibition of FAK catalytic activity facilitates FAK nuclear localization, which is required for proteasome-mediated GATA4 degradation in the cytoplasm. Chromatin immunoprecipitation identified GATA4 binding to the mouse cyclin D1 promoter, and loss of GATA4-mediated cyclin D1 transcription diminished SMC proliferation. Stimulation with platelet-derived growth factor or serum activated FAK and redistributed FAK from the nucleus to cytoplasm, leading to concomitant increase in GATA4 protein and cyclin D1 expression. In a femoral artery wire injury model, increased neointimal hyperplasia was observed in parallel with elevated FAK activity, GATA4 and cyclin D1 expression following injury in control mice, but not in VS-4718-treated and SMC-specific FAK kinase-dead mice. Finally, lentiviral shGATA4 knockdown in the wire injury significantly reduced cyclin D1 expression, SMC proliferation, and neointimal hyperplasia compared with control mice.

Conclusions: Nuclear enrichment of FAK by inhibition of FAK catalytic activity during vessel injury blocks SMC proliferation and neointimal hyperplasia through regulation of GATA4-mediated cyclin D1 transcription. (*Circ Res.* 2019;125:152-166. DOI: 10.1161/CIRCRESAHA.118.314344.)

Key Words: atherosclerosis ■ cyclin D1 ■ FAK ■ GATA4 ■ hyperplasia ■ smooth muscle cell ■ vascular remodeling

Excessive proliferation and migration of vascular smooth muscle cells (SMCs) contribute to occlusive cardiovascular diseases such as atherosclerosis.¹ Although SMCs in mature vessels have low proliferative and migratory activity, physical and inflammatory stimuli after vessel injury increases growth factor secretion and matrix synthesis, which promotes SMC proliferation and migration into the intima leading to arterial stenosis.²⁻⁴ Current treatment strategies for stenotic atherosclerotic lesions include procedures, such as balloon angioplasty, stenting, or bypass vein graft surgery that restore blood flow through the atherosclerotic vessel.^{5,6}

Although these procedures are effective, they damage the artery and can lead to restenosis or reocclusion due to induction of SMC proliferation and migration. Drug-eluting stents and drug-coated balloons have been used to reduce restenosis,^{7,8} but better treatment options are needed as size of the affected artery is still a major problem for intervention and prevention of restenosis.⁹⁻¹¹

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Novelty and Significance

What Is Known?

- Vessel injury induces proliferation of vascular SMCs (smooth muscle cells) into the intima, leading to narrowing of the blood vessel.
- Expression of FAK (focal adhesion kinase), a protein tyrosine kinase, is important in promoting SMC proliferation.

What New Information Does This Article Contribute?

- FAK in healthy arteries is predominantly localized within nuclei of SMCs, but is activated and redistributes to the cytoplasm following injury.
- In injured arteries, FAK cytoplasmic localization increases GATA4 (GATA-binding protein 4) protein stability in the nucleus, which increases GATA4-mediated cyclin D1 transcription to promote SMC proliferation and hyperplasia.
- Catalytic inhibition of FAK results in FAK retention in the nucleus and reduction in GATA4 protein expression in injured arteries, thus blocking SMC proliferation and hyperplasia.

Current interventions to inhibit SMC proliferation, such as drug-coated balloons and drug-eluting stents, are limited to vessel size and location. Therefore, a better understanding of SMC proliferation during vessel injury could lead to new therapeutic treatments. We are the first to report the molecular mechanism of how FAK controls SMC proliferation and hyperplasia. We observed that FAK is predominantly located within the nucleus of healthy arteries, keeping GATA4 protein levels low by promoting proteasome-mediated degradation. However, vessel injury induces FAK activation resulting in FAK cytoplasmic localization, which increases GATA4 protein, but not mRNA. This is also the first study to show that GATA4 plays a key role in SMC proliferation and that knockdown of GATA4 prevents injury-induced SMC hyperplasia. During vessel injury, forced nuclear localization of FAK through pharmacological or genetic inhibition of FAK catalytic activity reduced GATA4 protein and SMC proliferation. These findings highlight the importance of FAK subcellular localization in regulating SMC proliferation and suggest that targeting GATA4 protein stability through forced nuclear localization of FAK could be used as a treatment to inhibit vessel narrowing.

Nonstandard Abbreviations and Acronyms

FAK	focal adhesion kinase
GATA	GATA-binding protein
KD	kinase-dead
Myh11	myosin heavy chain 11
NLM	nonnuclear localizing mutant
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
pY397	autophosphorylation at tyrosine 397
shGATA4	GATA4 shRNA
shRNA	short hairpin RNA
SMC	smooth muscle cell
SRF	serum response factor
TGF-β	transforming growth factor β
WT	wild-type

FAK (focal adhesion kinase) is a protein tyrosine kinase that mediates integrin and growth factor signaling pathways associated with cell proliferation and migration.^{12,13} Increased growth factor synthesis and matrix deposition are hallmarks of vessel injury,^{14,15} which lead to aberrant FAK activation.^{16,17} PDGF (platelet-derived growth factor), a key factor in vascular remodeling, promotes FAK-mediated SMC migration and proliferation.^{17–21} Increased matrix stiffness also activates integrin downstream signaling through FAK, resulting in expression of cyclin D1.^{20,22} FAK activity promotes SMC proliferation through increased protein stability of S-phase kinase-associated protein 2, a ubiquitin ligase subunit that promotes degradation of cell cycle inhibitors p27 and p21.^{23–25} SMC-specific FAK knockout studies revealed that FAK expression is important in promoting SMC hyperplasia upon vascular injury.²¹ FAK can function as either a kinase or a kinase-independent scaffold, but it is unknown how FAK

regulates these cell cycle proteins and whether FAK-mediated SMC proliferation is dependent on its kinase activity or not.

FAK consists of N-terminal FERM (four-one, ezrin, radixin, and moesin homology), central kinase, and C-terminal focal adhesion targeting domains.¹² FAK localizes to focal adhesions via the focal adhesion targeting domain, where integrin and growth factor signaling lead to FAK tyrosine 397 (Y397) autophosphorylation, an indicator of FAK activation.²⁶ FAK also shuttles between the cytoplasm and nucleus via nuclear localization and export signal within the FERM and kinase domains, respectively.^{27,28} Nuclear FAK interacts with nuclear factors, such as tumor suppressor p53, GATA4, MBD2 (methyl-CpG-binding domain protein 2), TAF9 (TATA-box-binding protein associated factor 9), MEF2 (myocyte enhancer factor 2), Runx1 (runt-related transcription factor 1), NANOG, and RNA polymerase II.^{27,29–35} These findings suggested a new paradigm for FAK in which nuclear FAK regulation of transcription factors may alter gene transcription.

Vessel injury alters expression of key transcription factors, such as SRF (serum response factor), myocardin, and KLF-4 (Krüppel-like factor 4), which regulate expression of SMC contractile genes and SMC phenotype.^{36,37} The GATA (GATA-binding protein) family of transcription factors, including GATA4, GATA5, and GATA6, are expressed in differentiated cardiac cells and SMCs.³⁸ GATA6, the most commonly studied GATA transcription factor in SMCs, promotes SMC differentiation through cell cycle suppression and induction of SMC-specific genes through association with SRF and myocardin.^{39,40} Rapamycin, used on drug-eluting stents,⁴¹ promotes SMC differentiation via increased GATA6 expression.⁴² Although there are some reports supporting GATA4 involvement in SMC-specific gene expression,^{43,44} GATA4 expression levels are low in differentiated SMCs,³⁸ and it is unknown how vascular injury regulates GATA factors in SMCs.

In this study, we observed a new link between FAK and GATA4 expression in SMC proliferation using *in vitro* and *in*

vivo models. We report that (1) FAK catalytic inhibition-induced nuclear FAK reduces GATA4 protein levels via a proteasome-mediated pathway in SMCs; (2) mitogen stimuli, such as PDGF and serum, increase GATA4 protein stability through decreased nuclear FAK and increased FAK localization in the cytoplasm; (3) GATA4 promotes SMC proliferation through upregulation of cyclin D1 transcription; and (4) upregulation of GATA4 upon vascular injury is important for neointimal hyperplasia. These findings suggest that nuclear FAK-mediated GATA4 expression contributes to SMC proliferation and neointimal hyperplasia via regulation of cyclin D1 expression.

Methods

The authors declare that all supporting data are available within the article and its [Online Data Supplement](#). Detailed descriptions of experimental materials and methods used in this study are provided in the [Online Data Supplement](#).

Generation of SMC-Specific FAK–Wild-Type and –Kinase-Dead Mice

SMC-specific Cre mice (Myh11 [myosin heavy chain 11]-Cre-ER^{T2}) were purchased from Jackson Laboratory. As Myh11-Cre-ER^{T2} was inserted into Y chromosome, only male mice were used.⁴⁵ By crossing male Myh11-Cre-ER^{T2} mice with female FAK flox/flox (FAK^{FL/FL}) mice, we generated FAK^{FL/FL};Myh11-Cre-ER^{T2} mice. Male FAK^{FL/FL};Myh11-Cre-ER^{T2} mice were crossed with female FAK wild-type (WT)/kinase-dead (KD; FAK^{WT/KD}) mice to generate FAK^{FL/WT};Myh11-Cre-ER^{T2} and FAK^{FL/KD};Myh11-Cre-ER^{T2} mice (Online Figure I). The flox FAK allele was excised by treating male mice (6 weeks old) with tamoxifen (75 mg/kg, intraperitoneal, every other day) for 2 weeks, generating FAK^{-WT};Myh11-Cre-ER^{T2} (FAK-WT) and FAK^{-KD};Myh11-Cre-ER^{T2} (FAK-KD) mice.⁴⁶

Femoral Arterial Injury

All animal procedures were performed under protocols approved by the Institutional Animal Care and Use Committee at the University of South Alabama. We performed femoral artery wire injury as described previously with minor modification.⁴⁷ For FAK inhibitor experiments, mice were treated twice daily with vehicle (10 mmol/L citrate, pH 6.0) or the FAK inhibitor VS-4718 (50 mg/kg) by oral gavage. For SMC-specific FAK-WT and FAK-KD mice experiments, wire injury was performed after 2 weeks of tamoxifen injections. For GATA4 short hairpin RNA (shRNA) lentiviral infection of the femoral artery, concentrated lentivirus (10⁷ transducing units) was mixed with nonionic surfactant (Pluronic F-127, Sigma-Aldrich) in PBS and applied to artery as described previously.⁴⁸

Cell Culture

Primary mouse aortic SMCs were isolated from C57BL/6, FAK^{FL/FL}, FAK^{FL/WT}, and FAK^{FL/KD} mice as previously reported with some modifications.⁴⁹ Isolated FAK^{FL/WT} and FAK^{FL/KD} SMCs were treated with Cre adenovirus (50 multiplicity of infection) to generate FAK-WT and FAK-KD SMCs. Isolated FAK^{FL/FL} cells were transduced with FLAG-FAK-WT or nonnuclear localizing mutant (FLAG-FAK-NLM; R177A/R178A) lentivirus (20 multiplicity of infection) and treated with Cre adenovirus to generate FLAG-FAK-WT and FLAG-FAK-NLM SMCs. Successful deletion of FAK flox allele was confirmed by immunoblotting.

Immunostaining

Paraffin-embedded sections were deparaffinized with Histo-Clear (National Diagnostics). Paraffin-embedded and frozen sections were fixed with cold acetone for 15 minutes. Cells grown on glass coverslips were fixed with 4% paraformaldehyde for 10 minutes. Samples were permeabilized with 0.1% Triton X-100 for 10 minutes, washed with PBS, and incubated with blocking solution containing 3% BSA and 1% goat serum for 1 hour at room temperature. Samples were incubated with indicated primary antibodies overnight at 4°C.

Samples were washed with PBS and incubated with conjugated goat anti-rabbit or anti-mouse secondary antibodies (1:1000; Alexa Fluor 594 or 488, Thermo Fisher) for 1 hour at room temperature. Species-specific IgG or secondary antibodies were used as negative control. Nuclei were stained with DAPI (Sigma). Slides were mounted (Fluoromount-G, SouthernBiotech), and images were acquired with a confocal microscope at 60-fold magnification (Nikon A1R, Nikon). Images were processed using Photoshop CS5. Quantification of all images was analyzed with ImageJ. At least 3 section images were acquired from each sample.

Statistical Analysis

Data sets underwent Shapiro-Wilk test for normality, and statistical significance between experimental groups was determined with student *t* test or 2-way ANOVA with Sidak multiple comparisons test (Prism software, v7.0d; GraphPad Software). Power analyses were performed to determine sample size for 2-way ANOVA. Blinding procedures were not employed in this study due to the following reasons: high reproducibility, noticeable differences between specimen, and data acquisition by quantification.

Results

Inhibition of FAK Catalytic Activity Reduces SMC Neointimal Hyperplasia and Proliferation

We evaluated the effect of FAK catalytic inhibition on SMC hyperplasia using femoral arterial wire injury model.⁴⁷ Four weeks postinjury, arteries were analyzed by hematoxylin & eosin staining to measure neointimal hyperplasia (Figure 1A). While injured arteries of vehicle-treated mice showed a significant increase in the intima/media ratio, mice treated with FAK inhibitor VS-4718 had a lower intima/media ratio similar to sham controls (Figure 1A). To further assess SMC-specific FAK catalytic activity in vivo, we used a Cre/loxP strategy to create new conditional SMC-specific FAK kinase-dead (FAK-KD) mice. As FAK^{KD/KD} mouse is embryonic lethal,^{29,50} we crossed FAK^{FL/FL};Myh11-Cre-ER^{T2} and FAK^{Wild-Type (WT)/KD} mice to generate tamoxifen-inducible FAK^{FL/WT};Myh11-Cre-ER^{T2} and FAK^{FL/KD};Myh11-Cre-ER^{T2} mice⁴⁵ (Online Figure I). Tamoxifen treatment led to deletion of FAK flox allele in SMCs, producing FAK^{-WT};Myh11-Cre-ER^{T2} (FAK-WT) and FAK^{-KD};Myh11-Cre-ER^{T2} (FAK-KD) SMCs in vivo. Four weeks postinjury, arteries were collected, and neointimal hyperplasia was determined via hematoxylin & eosin staining (Figure 1B). FAK-KD had a lower intima/media ratio compared with FAK-WT mice (Figure 1B). Notably, FAK-WT mice showed less neointimal formation compared with vehicle-treated mice (Figures 1A and 1B), suggesting that single copy of FAK is less robust at promoting injury-induced neointimal formation. The results indicated that both pharmacological and genetic SMC-specific FAK catalytic inhibition significantly reduced SMC neointimal hyperplasia.

Since SMC proliferation is critical in promoting neointimal hyperplasia, we tested the relation between FAK catalytic activity and SMC proliferation in vitro. A single treatment with VS-4718 reduced pY397 (autophosphorylation at tyrosine 397) FAK (an active marker) in mouse aortic SMCs for 4 days (Online Figure IIA) and significantly reduced SMC proliferation (Figure 1C). VS-4718 also lowered expression of PCNA (proliferating cell nuclear antigen), further supporting that FAK catalytic inhibition blocks cellular proliferation (Online Figure IIA).

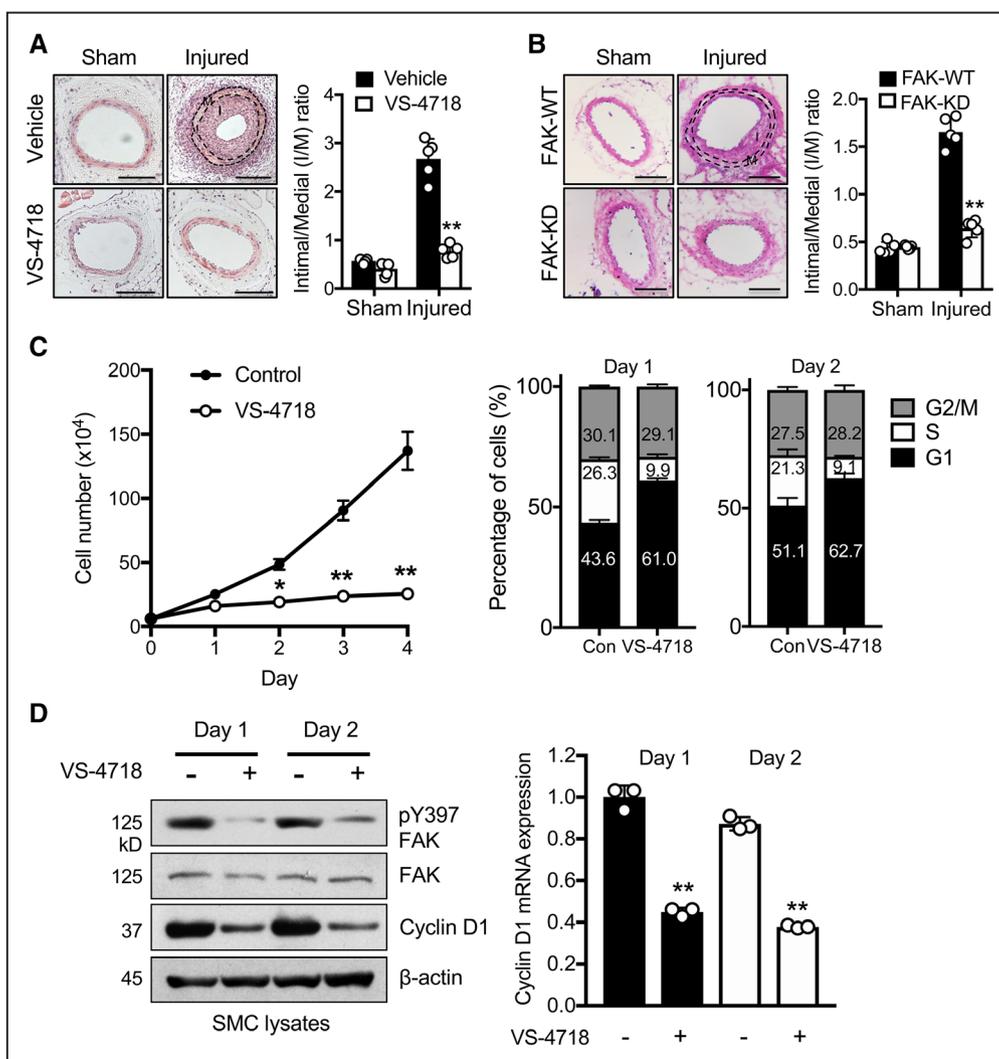


Figure 1. FAK (focal adhesion kinase) catalytic inhibition attenuates smooth muscle cell (SMC) hyperplasia and proliferation through G1 cell cycle arrest. **A** and **B**, Representative hematoxylin & eosin staining of femoral artery cross-sections 4 wk after wire injury for **(A)** vehicle or VS-4718 (50 mg/kg, twice daily) treated, and **(B)** genetic FAK-wild-type (WT) or FAK-kinase-dead (KD) mice. Scale bar, 50 μ m. Intima/media ratios were quantified (\pm SD; **A**, $n=4$; **B**, $n=5$; $**P<0.005$). **C**, SMC proliferation assay and percentages of SMCs in each phase of the cell cycle were determined with or without VS-4718 (2.5 μ mol/L) in the presence of 10% serum (\pm SD; $n=3$; $*P<0.01$, $**P<0.005$). **D**, Immunoblots of SMCs treated with or without VS-4718 (2.5 μ mol/L) in the presence of 10% serum for pY397 FAK, FAK, cyclin D1, and β -actin as loading control. Relative cyclin D1 mRNA levels were measured using real-time quantitative polymerized chain reaction (\pm SEM; $n=3$; $**P<0.005$).

FAK Catalytic Inhibition Causes SMC Cell Cycle Arrest at G1 in Part by Reducing Cyclin D1 Expression

Annexin V staining showed that 24-hour FAK catalytic inhibition did not induce SMC apoptosis (Online Figure IIB), and that reduction in cell number was due to decreased cell proliferation (Figure 1C). We hypothesized that SMC cell cycling may be impaired upon FAK inhibition. Cell cycle analyses revealed that VS-4718 increased G1 and decreased S-phase cell populations compared to control (Figure 1C), suggesting that FAK catalytic inhibition results in G1 arrest. It is known that cyclin D1 plays a critical role in G1/S phase transition by forming complexes with cyclin-dependent kinases 4/6 to phosphorylate retinoblastoma protein, resulting in cell cycle progression by phosphorylating E2F transcription factor 1.⁵¹ Therefore, we tested whether FAK activity could regulate cyclin D1 expression. VS-4718 treatment for 2 days reduced

cyclin D1 protein expression (Figure 1D). As cyclin D1 expression is tightly regulated at a transcriptional level, we further tested whether VS-4718 reduced cyclin D1 transcription by using real-time quantitative polymerized chain reaction. SMCs treated with VS-4718 had significantly less cyclin D1 mRNA when compared to control SMCs (Figure 1D). To ensure that these observations were not limited to mouse SMCs, we tested FAK catalytic inhibition in human coronary artery SMCs. VS-4718 treatment reduced human coronary artery SMC proliferation, caused G1 arrest, and reduced cyclin D1 protein and mRNA expression (Online Figure IIIA through IIID). Using a human cyclin D1 promoter luciferase construct, we also observed that VS-4718 impaired cyclin D1 promoter activity in human coronary artery SMCs (Online Figure IIIE). Therefore, FAK catalytic inhibition prevented G1 to S cell cycle transition by potentially downregulating cyclin D1 transcription in mouse and human SMCs.

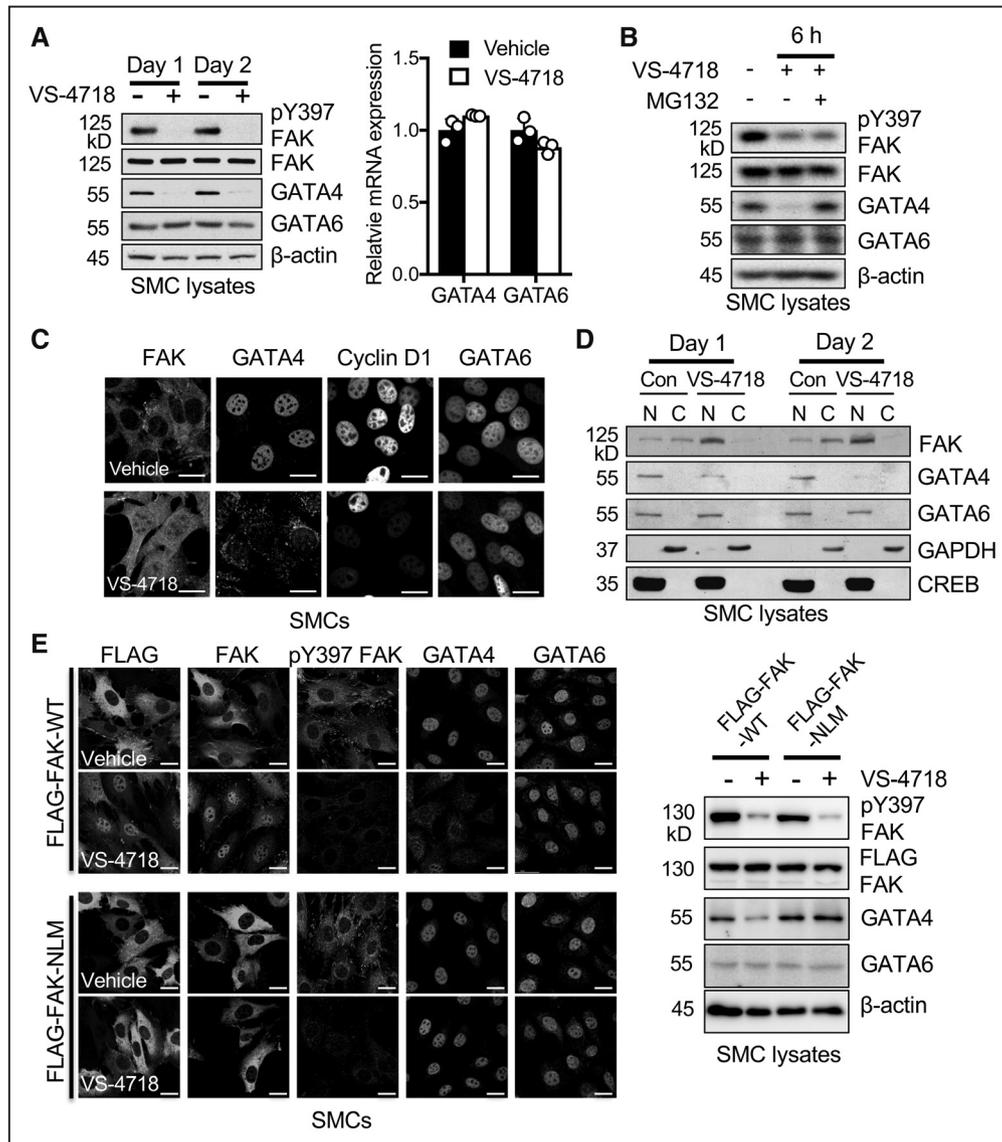


Figure 2. Pharmacological FAK (focal adhesion kinase) catalytic inhibition decreases GATA4 (GATA-binding protein 4) and cyclin D1 protein expression in smooth muscle cells (SMCs). All experiments were performed in the presence of 10% serum. **A**, Immunoblots of SMCs treated with or without VS-4718 (2.5 $\mu\text{mol/L}$) for pY397 FAK, FAK, GATA4, GATA6, and β -actin as loading control. Relative GATA4 and GATA6 mRNA levels were measured using real-time quantitative polymerized chain reaction after treatment with VS-4718 (2.5 $\mu\text{mol/L}$) for 2 days (\pm SEM; $n=3$). **B**, Immunoblots of SMCs treated with VS-4718 (2.5 $\mu\text{mol/L}$) only or VS-4718 together with MG132 (20 $\mu\text{mol/L}$) for pY397 FAK, FAK, GATA4, GATA6, and β -actin as loading control. **C**, Treatment with VS-4718 (2.5 $\mu\text{mol/L}$) for 12 h increased nuclear FAK and reduced GATA4 and cyclin D1 expression as visualized by immunofluorescence staining of FAK, GATA4, cyclin D1, and GATA6 in SMCs. Scale bar, 20 μm . **D**, Immunoblots of SMC nuclear (N) and cytosolic (C) fractionated lysates with or without VS-4718 (2.5 $\mu\text{mol/L}$) for FAK, GATA4, and GATA6. CREB and GAPDH as nuclear and cytosolic markers, respectively. **E**, Comparison of FLAG-FAK-wild-type (WT) or FLAG-FAK-NLM (nonnuclear localizing mutant) nuclear localization and GATA4 levels in FAK^{-/-} SMCs after treatment with VS-4718 (2.5 $\mu\text{mol/L}$) for 12 h. Immunofluorescence staining of FLAG, FAK, pY397 FAK, GATA4, and GATA6 in SMCs. Scale bar, 20 μm . Immunoblots of SMCs for pY397 FAK, FAK, GATA4, GATA6, and β -actin as loading control.

Nuclear FAK Regulation of GATA4 and Cyclin D1 Expression in SMCs

Next, we investigated how FAK regulates cyclin D1 expression at a transcriptional level. Since our previous study demonstrated that stability of GATA4 transcription factor is regulated by nuclear FAK induced via inhibition of FAK catalytic activity,²⁹ and others found that several cell cycle genes including cyclin D1 were downregulated in GATA4 knockout hearts,⁵² we chose GATA4 as a strong candidate for regulating FAK-mediated cyclin D1 transcription. Although GATA4 levels are lower in SMCs compared with GATA6,³⁸ it is known that

GATA4 also regulates SMC-specific gene expression through SRF and myocardin.⁴⁴ First, we tested whether FAK catalytic inhibition altered GATA4 expression in SMCs. VS-4718 treatment significantly reduced GATA4 protein but had no effect on GATA6 in both mouse and human SMCs (Figure 2A and Online Figure IIIC). However, there was no change in GATA4 or GATA6 mRNA levels, suggesting that FAK catalytic inhibition does not regulate GATA4 expression at a transcriptional level (Figure 2A). As it is known that nuclear FAK regulates GATA4 protein stability in mouse fibroblasts,²⁹ we used a proteasome inhibitor, MG132, to evaluate GATA4 stability

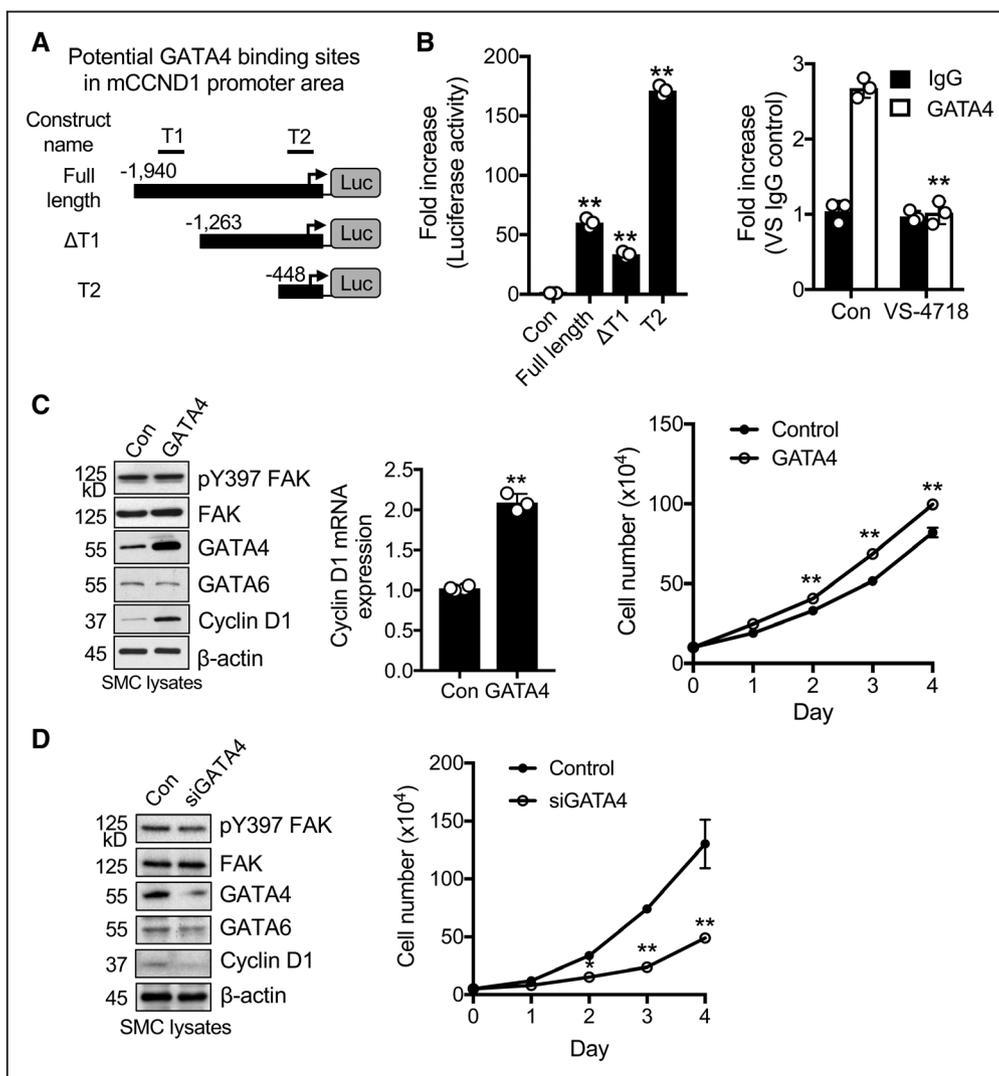


Figure 3. GATA4 (GATA-binding protein 4) is a direct transcription factor for cyclin D1 and promotes smooth muscle cell (SMC) proliferation. All experiments were performed in the presence of 10% serum. **A**, Schematic of mouse cyclin D1 (mCCND1) promoter luciferase constructs with 2 putative GATA4-binding sites (T1 and T2) indicated. Arrow represents transcription start site at +1. Luciferase activity in SMCs was measured 24 h post-transfection (\pm SEM; n=3; ** P <0.005). **B**, Chromatin immunoprecipitation analysis of GATA4 binding to the mCCND1 promoter in SMCs with or without VS-4718 (2.5 μ mol/L). Enrichment of potential site T2 over IgG control was quantified by real-time quantitative polymerized chain reaction (RT-qPCR; \pm SEM; n=3; ** P <0.005). **C**, GATA4 overexpression increases SMC proliferation and cyclin D1 expression. Immunoblots of SMCs overexpressing GATA4 for pY397 FAK, FAK, GATA4, GATA6, cyclin D1, and β -actin as loading control. Relative cyclin D1 mRNA levels were measured using RT-qPCR (\pm SEM; n=3). Proliferation assay of GATA4-overexpressing SMCs (\pm SD; n=3; ** P <0.005). **D**, GATA4 knockdown decreases SMC proliferation and cyclin D1 expression. Immunoblots of SMCs transfected with either control or GATA4 short interference RNA (siRNA; 200 pmole for 48 h) for pY397 FAK, FAK, GATA4, GATA6, cyclin D1, and β -actin as loading control. Proliferation assay of SMCs following GATA4 siRNA knockdown (\pm SD; n=3; * P <0.05, ** P <0.005).

in mouse SMCs. VS-4718 treatment significantly reduced GATA4 levels at 6 hours, but MG132 restored GATA4 protein suggesting that FAK regulates GATA4 stability (Figure 2B). To determine if GATA4 degradation occurred in the cytoplasm or nucleus, SMCs were treated with Leptomycin B, a nuclear export blocker. Leptomycin B treatment blocked GATA4 degradation upon FAK catalytic inhibition (Online Figure IV), suggesting GATA4 degradation takes place in the cytoplasm. VS-4718 treatment for 12 hours in both mouse and human SMCs increased nuclear FAK, which was correlated with reduced GATA4 and cyclin D1 staining (Figure 2C and Online Figure IIIF). Biochemical separation of SMCs into cytosolic and nuclear fractions also revealed that elevated levels of nuclear FAK upon VS-4718 treatment was associated with decreased GATA4 levels (Figure 2D). To verify if nuclear

FAK is required for reducing GATA4 stability, we generated FLAG-tagged FAK-WT (FLAG-FAK-WT) and FAK-NLM (FLAG-FAK-NLM) in FAK^{-/-} SMCs (Figure 2E). VS-4718 increased FLAG-FAK-WT nuclear localization and reduced GATA4 expression. We confirmed that FLAG-FAK-NLM still remained active in cytoplasm, similar to FLAG-FAK-WT in control. Although VS-4718 reduced FAK catalytic activity in both FLAG-FAK-WT and FLAG-FAK-NLM SMCs as monitored by FAK pY397 staining (Figure 2E), VS-4718 did not induce FLAG-FAK-NLM nuclear localization or loss of GATA4 (Figure 2E). Biochemical analyses also supported that FLAG-FAK-NLM did not reduce GATA4 levels in the nucleus (Online Figure V). Therefore, nuclear FAK regulates GATA4 stability, but not GATA6, in SMCs.

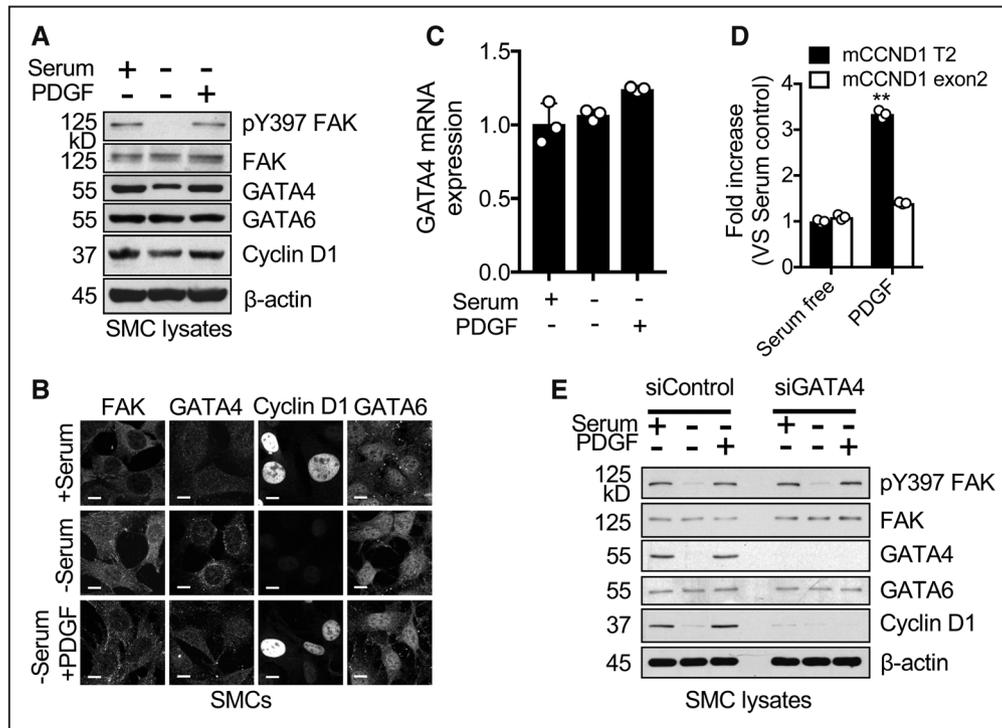


Figure 4. Mitogen-induced cyclin D1 expression is dependent on GATA4 (GATA-binding protein 4) in smooth muscle cells (SMCs). SMCs were serum-starved overnight, then treated with either serum (10% FBS) or PDGF (platelet-derived growth factor; 20 ng/ml) for 12 h. **A**, Correlation between pY397 FAK (focal adhesion kinase), GATA4, and cyclin D1 levels after serum or PDGF stimulation of serum-starved SMCs as observed from immunoblots for pY397 FAK, FAK, GATA4, GATA6, cyclin D1, and β -actin as loading control. **B**, Serum and PDGF redistribute FAK to cytoplasm and increase GATA4 and cyclin D1 as visualized by immunofluorescence staining of SMCs for FAK, GATA4, GATA6, and cyclin D1. Scale bar, 10 μ m. **C**, Relative GATA4 mRNA levels were measured using real-time quantitative polymerized chain reaction (\pm SEM; n=3). **D**, PDGF stimulation increased GATA4 binding to T2 site of mouse cyclin D1 (mCCND1) promoter as seen by chromatin immunoprecipitation analysis (\pm SEM; n=3). mCCND1 Exon 2 primers used as negative control. ** P <0.005. **E**, Immunoblots of SMCs transfected with either siControl or siGATA4 (200 pmole for 48 h) for pY397 FAK, FAK, GATA4, GATA6, cyclin D1, and β -actin as loading control.

GATA4 Regulation of Cyclin D1 Transcription in SMCs

As GATA4 regulation of cyclin D1 is not known, we looked into published GATA4 chromatin immunoprecipitation database using an interactive genome viewer to find potential GATA4 binding domains in the mouse cyclin D1 promoter (Online Figure VI). We identified 2 potential GATA4 binding regions overlapping with active histone marks such as H3K4me3, named T1 (−1621 to −1400) and T2 (−238 to −178). Using these predicted sites, we generated mouse cyclin D1 promoter luciferase constructs containing either full-length, T1 deleted (Δ T1), or T2 only regions (Figure 3A). The T2 construct, containing the region closest to the transcription start site, showed the highest levels of luciferase activity (Figure 3B). As both the full-length and Δ T1 constructs had lower luciferase activity than T2, the presence of suppressive elements was implicated upstream of the T2 GATA4 binding site (Figure 3B). To determine whether GATA4 binds to the cyclin D1 promoter, we performed GATA4 chromatin immunoprecipitation in mouse SMCs. GATA4 pulldown enriched the T2 region compared with control IgG, suggesting that GATA4 directly mediates cyclin D1 transcription via the T2 site (Figure 3B). To further test whether GATA4 levels affect cyclin D1 expression, we performed a gain-of-function study by overexpressing GATA4 in SMCs. Stable GATA4 overexpression in SMCs increased cyclin D1 mRNA and protein expression, and increased SMC proliferation compared to

control cells (Figure 3C). Additionally, GATA4 overexpression increased prespliced cyclin D1 mRNA in the nucleus (Online Figure VII), further supporting that GATA4 directly promotes cyclin D1 transcription. In contrast, GATA4 knockdown by using short interference RNA (siGATA4) reduced cyclin D1 protein expression in both mouse and human SMCs (Figure 3D and Online Figure VIII). siGATA4 also decreased SMC proliferation compared with scramble control (Figure 3D). In addition, cyclin D1 knockdown in control and GATA4-overexpressing SMCs reduced cell proliferation, indicating that cyclin D1 expression is indeed required for SMC proliferation (Online Figure IX). These results suggest that GATA4 promotes SMC proliferation through direct upregulation of cyclin D1 transcription.

PDGF-Induced Cyclin D1 Is Dependent on GATA4 Expression

As GATA4 protein levels are low in SMCs *in vivo*,³⁸ it is possible that proliferative SMCs located within the neointima or SMCs exposed to serum *in vitro* could have increased expression of GATA4. It is also well known that PDGF is increased in the neointima and is a strong promoter for SMC proliferation.^{53,54} We tested the effect of serum or PDGF stimulation in mouse SMCs on GATA4 expression. Overnight serum-starved mouse SMCs exhibited decreased pY397 FAK levels (Figure 4A), but increased levels of nuclear FAK (Figure 4B). This was associated with decreased

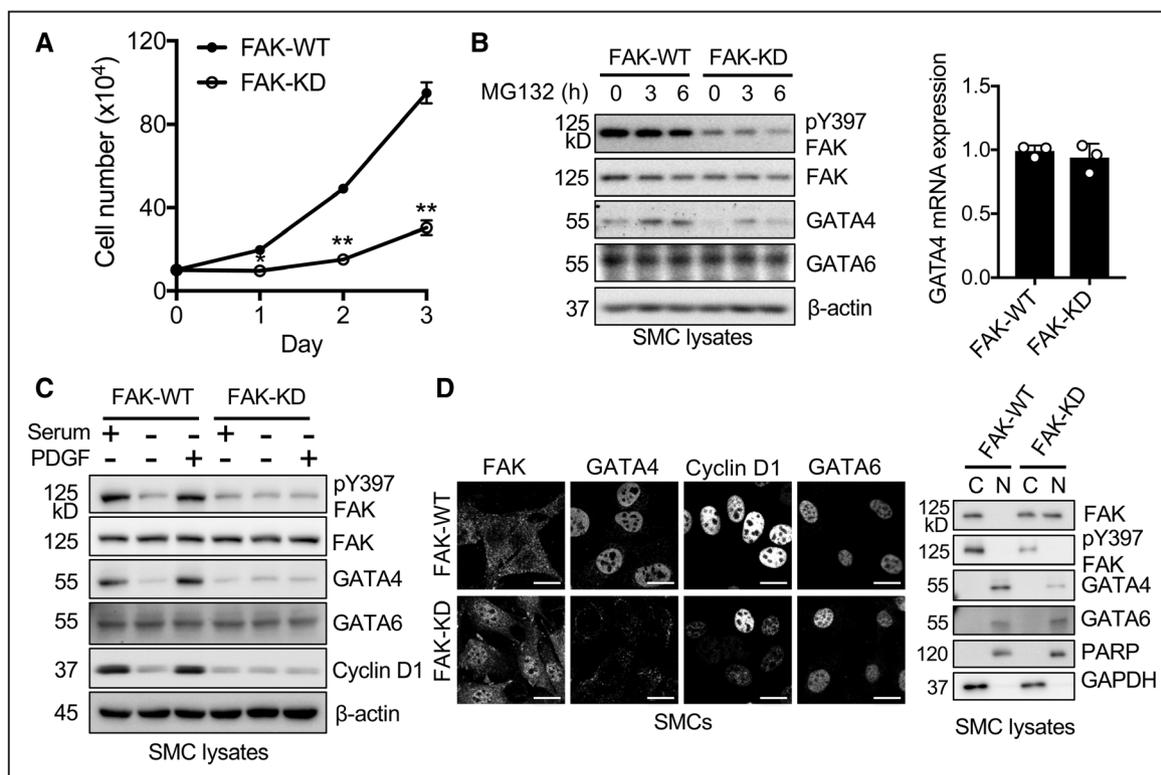


Figure 5. FAK (focal adhesion kinase)-kinase-dead (KD) smooth muscle cells (SMCs) exhibit prominent nuclear FAK localization, lower GATA4 (GATA-binding protein 4) expression, and slower proliferation. Experiments were performed in the presence of 10% serum, unless indicated otherwise. **A**, Proliferation assay was performed with FAK-wild-type (WT) and FAK-KD SMCs (\pm SD; n=3; * P <0.05, ** P <0.005). **B**, GATA4 turned over faster in FAK-KD SMCs as seen by immunoblots of MG132 (20 μ mol/L, 3 or 6 h) treated FAK-WT and FAK-KD SMCs for pY397 FAK, FAK, GATA4, GATA6, and β -actin as loading control. Relative level of GATA4 mRNA in FAK-WT and FAK-KD SMCs were measured using real-time quantitative polymerized chain reaction. (\pm SEM; n=3). **C**, SMCs were serum-starved overnight and treated with either serum (10% FBS) or PDGF (20 ng/ml) for 12 h. Immunoblots of FAK-WT and FAK-KD SMCs for pY397 FAK, FAK, GATA4, GATA6, cyclin D1, and β -actin as loading control. **D**, FAK-KD is enriched in the nucleus compared to FAK-WT. Representative immunofluorescence staining of FAK-WT and FAK-KD SMCs for FAK, GATA4, cyclin D1, and GATA6. Scale bars, 20 μ m. Immunoblots of FAK-WT and FAK-KD SMC nuclear (N) and cytosolic (C) lysate for FAK, pY397 FAK, GATA4, and GATA6. PARP (Poly ADP-ribose polymerase) and glyceraldehyde-3-phosphate dehydrogenase as nuclear and cytosolic markers, respectively.

levels of GATA4 and cyclin D1 (Figure 4A and 4B). In addition to increased FAK cytoplasmic localization and pY397 FAK levels, stimulation with serum or PDGF also increased GATA4 and cyclin D1 expression (Figure 4A and 4B). Under all conditions, GATA6 levels did not change (Figure 4A and 4B). Neither serum nor PDGF stimulation changed GATA4 mRNA levels (Figure 4C), further supporting the idea that nuclear FAK regulates GATA4 protein stability independent of GATA4 transcription in mouse SMCs. In addition to regulating GATA4 stability, we observed FAK activity is also important for PDGF-mediated phosphorylation of GATA4 at serine 105, which promotes GATA4 transcriptional activity⁵⁵ (Online Figure X). GATA4 chromatin immunoprecipitation assay revealed that PDGF stimulation increased GATA4 binding to the T2 region of the mouse cyclin D1 promoter, likely due to increased levels of GATA4 (Figure 4D). siGATA4 blocked serum- and PDGF-induced cyclin D1 expression in mouse SMCs (Figure 4E), further demonstrating that GATA4 expression is critical to promote mitogen-induced cyclin D1 expression in mouse SMCs. To test whether GATA4 expression is increased in proliferating SMCs, we used multipotent 10T1/2 cells that can be induced to exhibit a contractile SMC phenotype upon TGF- β (transforming growth factor β) or proliferative SMC phenotype upon PDGF treatment.⁵⁶ While

serum starvation and TGF- β treatment decreased pY397 FAK levels and increased FAK nuclear localization, serum and PDGF activated FAK and promoted FAK cytoplasmic redistribution (Online Figure XI). Serum or PDGF increased GATA4 and cyclin D1 (Online Figure XI). In contrast, TGF- β stimulation decreased both GATA4 and cyclin D1 expression in 10T1/2 cells (Online Figure XI). These data further support that FAK nuclear accumulation upon inhibition of FAK catalytic activity controls GATA4 protein stability and cyclin D1 expression in SMCs.

Kinase-Dead FAK Decreases GATA4 Stability

Although VS-4718 is a selective FAK inhibitor that is being tested for clinical use,⁵⁷ it was necessary to verify the specific effect of FAK catalytic activity in SMC proliferation. We isolated FAK-WT and FAK-KD aortic SMCs from FAK^{FL/FL} and FAK^{WT/KD} mouse crossings (Online Figure I). FAK-KD SMCs proliferated at a slower rate compared to FAK-WT SMCs (Figure 5A). FAK-KD SMCs exhibited reduced pY397 FAK phosphorylation and significantly reduced GATA4 and cyclin D1 levels compared with FAK-WT SMCs (Figure 5B). GATA4 mRNA levels were not significantly different between FAK-WT and FAK-KD SMCs (Figure 5B). MG132 treatment restored GATA4

expression in FAK-WT and to a lesser extent in FAK-KD SMCs (Figure 5B). Serum or PDGF stimulation failed to increase GATA4 and cyclin D1 levels in FAK-KD, but not FAK-WT, SMCs (Figure 5C). Immunostaining revealed that FAK-KD SMCs showed much higher nuclear FAK and reduced GATA4 and cyclin D1 levels compared with FAK-WT SMCs (Figure 5D). Biochemical fractionation also confirmed that nuclear FAK was abundant, and GATA4 levels were lower in FAK-KD SMCs (Figure 5D). There was no difference in GATA6 expression between FAK-KD and FAK-WT SMCs in all conditions (Figure 5). Next, we tested if cyclin D1 expression by itself is sufficient to promote cell proliferation in FAK-KD SMCs. However, cyclin D1 overexpression did not rescue proliferation defects of FAK-KD SMCs (Online Figure XII). Additionally, cyclin D1 overexpression had no effect on cell cycle inhibitors, such as p27 and p21, suggesting that cyclin D1 is necessary but not sufficient for SMC cell cyclin progression. These data further support the concept that nuclear FAK reduces GATA4 and cyclin D1 expression in SMCs.

Pharmacological FAK Catalytic Inhibition Blocks FAK Activation and GATA4 and Cyclin D1 Expression Induced by Wire Injury

To gain further insights into the function of FAK in vascular remodeling *in vivo*, we evaluated the effect of FAK catalytic inhibition on SMCs in the femoral arterial wire injury model.⁴⁷ As changes in gene and protein expression may occur rapidly after injury,⁵⁸ we evaluated whether changes occurred in FAK, GATA4, GATA6, cyclin D1, and PCNA expression soon after vascular injury. Immunoblotting with artery lysates showed that wire injury increased the levels of pY397 FAK, GATA4, and cyclin D1 in femoral arteries within 3 days after injury compared with uninjured control (Figure 6A). However, VS-4718 treatment reduced wire injury-induced pY397 FAK, GATA4, cyclin D1, and PCNA levels compared with the vehicle-treated group (Figure 6A). Although GATA4 protein was increased in control after vascular injury and reduced upon FAK catalytic inhibition, there was no change in GATA4 mRNA expression in artery samples compared with uninjured control (Figure 6B). Immunostaining also showed increased pY397 FAK levels in the injured artery, and that GATA4 and cyclin D1 were also increased in the neointima 4 weeks after injury (Figure 6C and Online Figure XIII A). These increases were blocked in mice treated with VS-4718 (Figure 6C and Online Figure XIII B). We also observed increased nuclear FAK accumulation in VS-4718 treated artery samples (Figure 6C and Online Figure XIII B). Relative fluorescence signals for nuclear FAK were significantly increased in vehicle- compared with VS-4718 treated mice (Figure 6C). Interestingly, we observed abundant nuclear FAK in uninjured control, suggesting that FAK in a healthy artery may remain less active. There were no differences in GATA6 levels between vehicle- and VS-4718-treated mice (Figure 6 and Online Figure XIII). These results indicate that VS-4718-induced loss of FAK activity and increased nuclear FAK blocks neointimal hyperplasia potentially through reduced expression of GATA4 and cyclin D1.

SMC-Specific FAK Catalytic Inhibition Prevents SMC Hyperplasia in Part via FAK-GATA4-Cyclin D1 Signaling

To evaluate SMC-specific FAK activity in wire injury model, we used our new FAK-KD model (Online Figure I). As SMC-specific FAK-KD mice showed significantly reduced SMC hyperplasia (Figure 1B), we further investigated changes in FAK activity, nuclear FAK, and expression of GATA4 and cyclin D1 after injury. Wire injury increased expression of GATA4 and cyclin D1 in FAK-WT mice but not in FAK-KD mice (Figure 7 and Online Figure XIV). FAK activity (pY397 FAK) was significantly decreased in FAK-KD mice, verifying deletion of FAK flox allele after tamoxifen treatment compared to FAK-WT (Figure 7). While wire injury redistributed FAK from the nucleus to cytoplasm in FAK-WT mice, FAK-KD mice showed strong FAK nuclear localization in control and injured arteries (Figure 7). Relative fluorescence signals for nuclear FAK were also significantly increased in FAK-KD compared to FAK-WT mice (Figure 7). These results demonstrate that SMC-specific FAK activity contributes to SMC proliferation via upregulation of GATA4 and cyclin D1 in the neointimal regions (Figure 7).

shRNA Knockdown of GATA4 Abolished Wire Injury-Induced Neointimal Hyperplasia

As wire injury-induced GATA4 upregulation was reduced by FAK catalytic inhibition (Figures 6 and 7), we evaluated whether GATA4 expression, *per se*, was important for neointimal formation after wire injury. We used either scramble or GATA4 shRNA (shGATA4) lentivirus coexpressing mCherry. Knockdown efficiency of 2 different GATA4 shRNAs was tested in mouse SMCs, and reduction of GATA4 and SMC proliferation were verified (Online Figure XV). One shGATA4 lentivirus was applied to the outside of the femoral artery after injury. shGATA4 completely abolished neointimal formation in injured arteries compared with the control lentivirus group (Figure 8A). Lentiviral infection was verified by monitoring mCherry expression, and we observed that shGATA4 lentivirus reduced wire injury-induced expression of GATA4 and cyclin D1 (Figure 8B and Online Figure XVI). Wire injury increased pY397 FAK levels in both scramble and shGATA4 arteries (Figure 8B and Online Figure XVI). Injured arteries of scramble and shGATA4 showed a significant decrease in FAK nuclear localization (Figure 8C and Online Figure XVII), suggesting that decreasing GATA4 levels may be enough to reduce SMC hyperplasia. Taken together, these data reveal that injury-induced upregulation of GATA4 contributes in neointimal hyperplasia.

Discussion

In this study, we discovered that inhibition of FAK catalytic activity blocked SMC proliferation by inducing G1 arrest in mouse and human SMCs (Figure 1 and Online Figure III). G1 arrest was a result of FAK catalytic inhibition-induced FAK nuclear localization, resulting in decreased stability of GATA4 protein (Figure 2) and reduced expression of cyclin D1 (Figure 1). Serum starvation of SMCs led to decreased FAK activity and increased FAK nuclear localization (Figure 4), and stimulation with serum or PDGF led to increased FAK

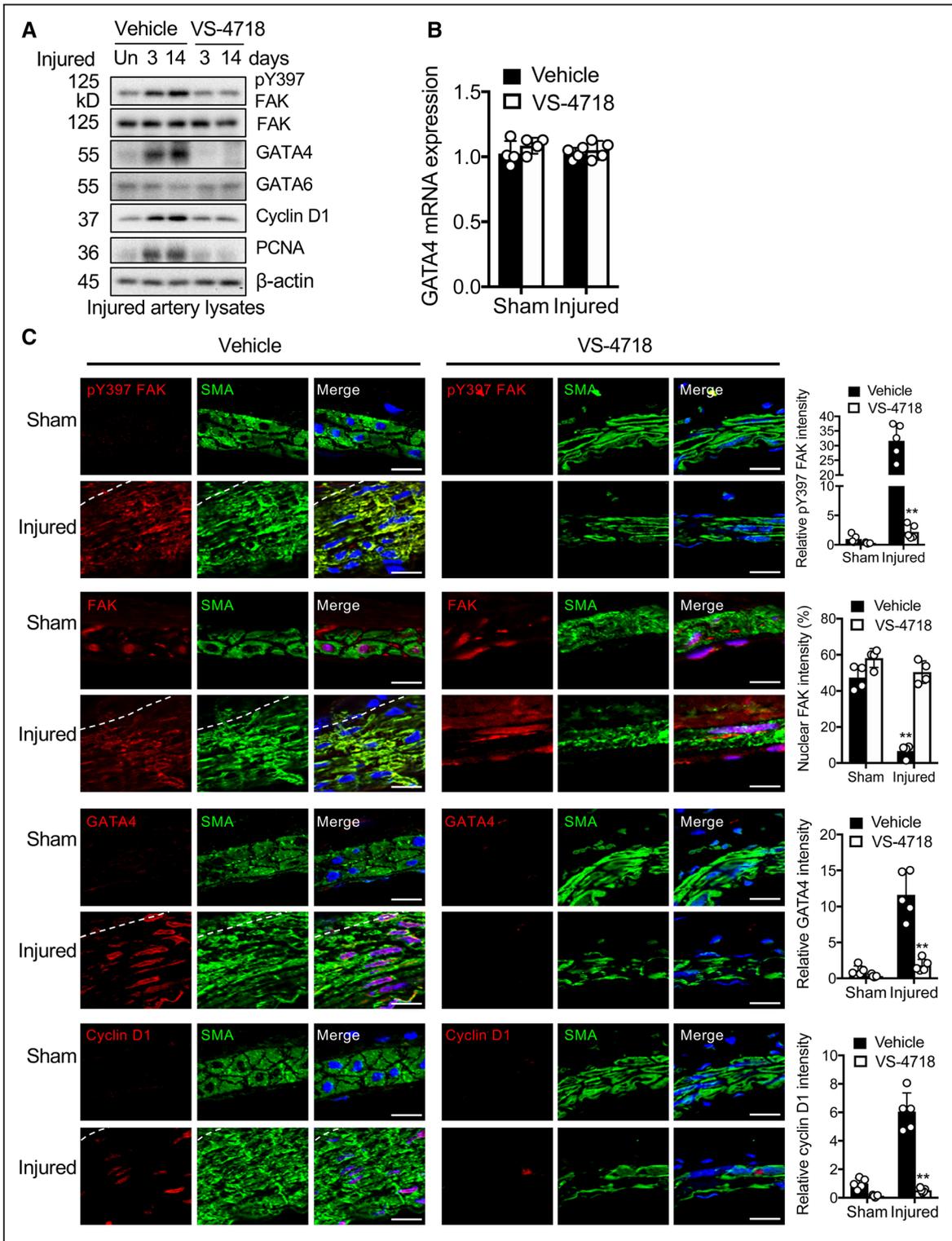


Figure 6. GATA4 (GATA-binding protein 4) is increased after wire injury, and pharmacological FAK (focal adhesion kinase) catalytic inhibition significantly blocks GATA4, cyclin D1, and neointimal hyperplasia. Mice were treated with vehicle or VS-4718 (50 mg/kg) twice daily following wire injury. **A**, Immunoblots of femoral arteries 3 and 14 days postinjury for pY397 FAK, FAK, GATA4, GATA6, cyclin D1, PCNA (proliferating cell nuclear antigen), and β -actin as loading control. Un: uninjured artery. **B**, Relative GATA4 mRNA expression in femoral arteries harvested 3 days postinjury were measured by real-time quantitative polymerized chain reaction (\pm SEM; n=4 biological samples). **C**, Immunofluorescence staining of femoral arteries 4 wk postinjury for pY397 FAK, FAK, GATA4, cyclin D1, and α -SMA (α -smooth muscle actin). Red, green (α -SMA), and blue (DAPI) were merged. Relative fluorescence intensity of nuclear FAK and indicated targets in α -SMA positive cells were quantified (\pm SD; n=5). Scale bar, 10 μ m. Dashed lines, boundary between media and intima. ** P <0.005.

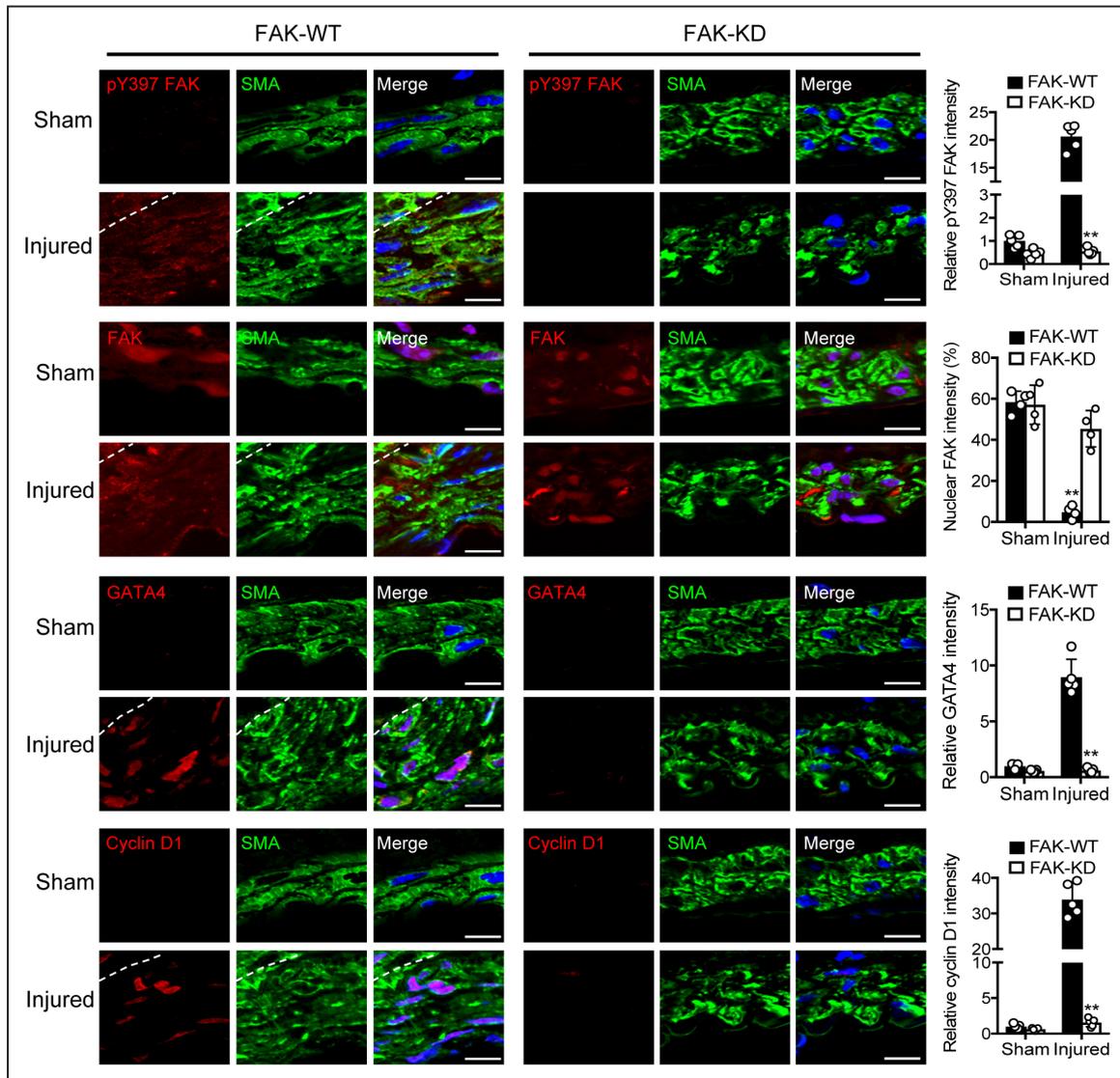


Figure 7. Smooth muscle cells (SMC)-specific FAK (focal adhesion kinase)-kinase-dead (KD) mice develop reduced hyperplasia and exhibit less GATA4 (GATA-binding protein 4) and cyclin D1 expression after wire injury. Immunofluorescence staining of FAK-wild-type (WT) and FAK-KD femoral arteries 4 wk postinjury for pY397 FAK, FAK, GATA4, cyclin D1, and α -SMA (α -smooth muscle actin). Red, green (SMA), and blue (DAPI) were merged. Relative fluorescence intensity of nuclear FAK and indicated targets in α -SMA positive cells were quantified (\pm SD; n=5). Scale bars, 10 μ m. Dashed lines, boundary between media and intima. ** P <0.005.

activity and cytoplasmic localization (Figure 4). In the multipotent 10T1/2 cells, stimulation with TGF- β , which promotes a nonproliferative SMC phenotype, increased FAK nuclear localization and GATA4 protein levels (Online Figure XI); however, treatment with PDGF increased FAK cytoplasmic localization, GATA4, and cyclin D1 expression (Online Figure XI). As FAK cytoplasmic localization was positively correlated with GATA4 and cyclin D1 expression, we concluded that a FAK-GATA4-cyclin D1 axis may be a general mechanism to regulate SMC proliferation in mouse and human SMCs. In the wire injury model, we showed that injury-induced FAK activation and cytoplasmic redistribution could be reversed by pharmacological FAK catalytic inhibition, resulting in significantly decreased neointimal formation in part via nuclear FAK-GATA4-cyclin D1 axis (Figure 6).

Homozygous FAK KD (FAK^{KD/KD}) knock-in mouse is embryonic lethal⁵⁰ and FAK knockout models induce

compensatory Pyk2 expression hindering the analyses of FAK-specific function.⁴⁶ To study the role of FAK catalytic activity in SMCs, we developed a new tamoxifen-inducible SMC-specific (Myh11-Cre-ERT²) FAK-KD mouse (Online Figure I). SMCs isolated from FAK-KD mice proliferated slower and showed increased FAK nuclear localization compared with FAK-WT SMCs (Figure 5). FAK-KD SMCs also had decreased GATA4 and cyclin D1 expression (Figure 5). In the femoral artery wire injury model, FAK-KD mice showed a significant reduction in SMC intimal thickening compared with FAK-WT mice (Figure 1B). FAK-KD mice also showed increased nuclear FAK and decreased GATA4 and cyclin D1 after wire injury compared with FAK-WT mice (Figure 7). The SMC-specific FAK-KD mouse model verified FAK-mediated GATA4-cyclin D1 connection in vitro and in vivo.

Our current animal models using pharmacological and genetic FAK catalytic inhibition reduced FAK cytoplasmic

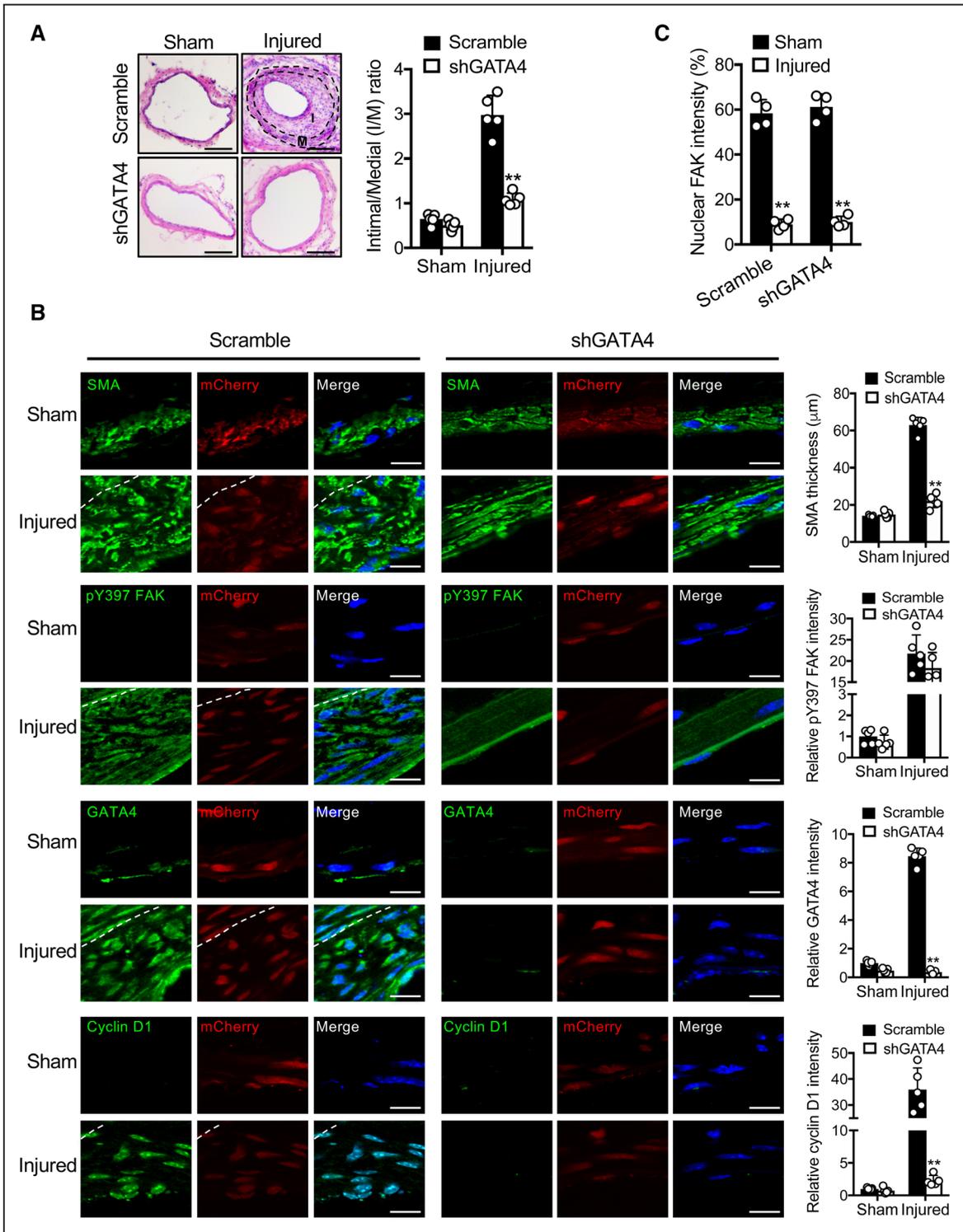


Figure 8. Knockdown of GATA4 (GATA-binding protein 4) ameliorates wire injury-induced neointimal hyperplasia. Femoral arteries were coated with either scramble or shGATA4 (GATA4 shRNA) lentivirus immediately following wire injury. **A**, Representative hematoxylin and eosin staining of femoral artery cross-sections 4 wk after wire injury. Intima/media ratios were quantified (\pm SD; $n=5$; $**P<0.005$). Scale bar, 50 μm . **B**, Immunofluorescence staining 4 wk postinjury for pY397 FAK, GATA4, cyclin D1, and α -SMA (α -smooth muscle actin). Green and blue (DAPI) were merged. mCherry was used to verify successful viral infection. Relative fluorescence intensity of indicated targets in α -SMA positive cells and α -SMA thickness were quantified (\pm SD; $n=5$). Scale bars, 10 μm . Dashed lines, boundary between media and intima. $**P<0.005$. **C**, Fluorescence intensity of FAK in nucleus was quantified by ImageJ (\pm SD; $n=4$; $**P<0.005$).

signaling and increased FAK nuclear localization, thus making it difficult to separate cytoplasmic and nuclear functions of FAK in the regulation of SMC proliferation. To help

address this, we generated a nonnuclear localizing FAK mutant (FLAG-FAK-NLM) in FAK^{-/-} mouse SMCs. Although FLAG-FAK-NLM normally localizes to adhesions and shows

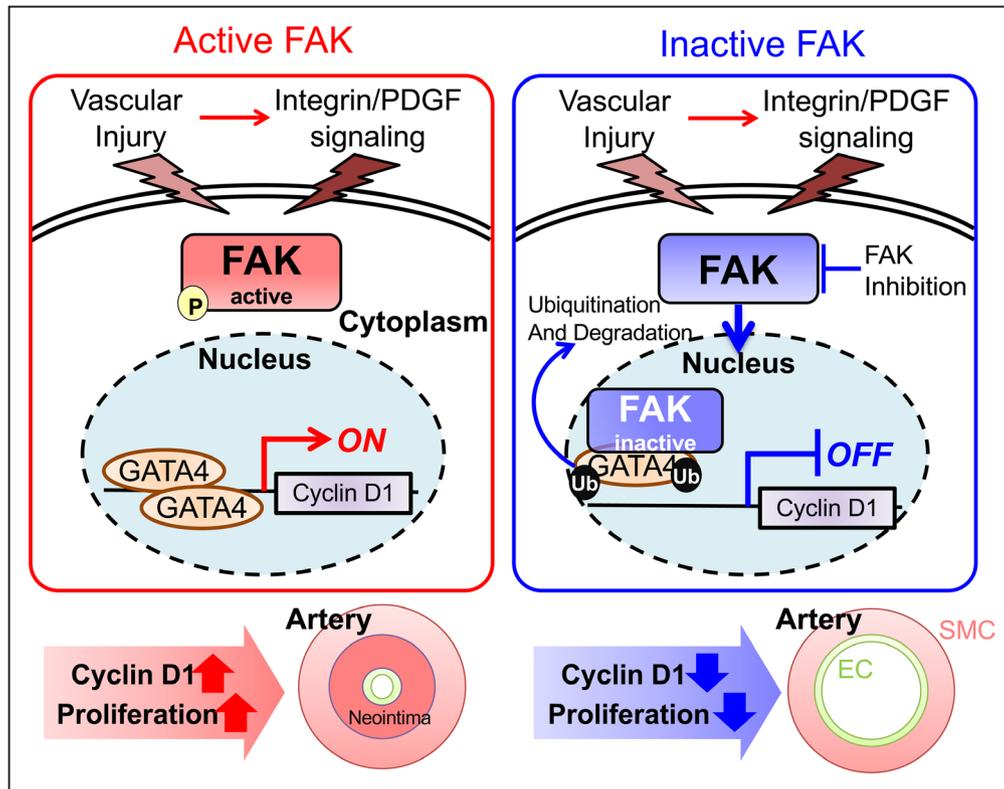


Figure 9. Proposed model of FAK (focal adhesion kinase)-GATA4 (GATA-binding protein 4)-cyclin D1 regulation in smooth muscle cell (SMC) neointimal hyperplasia. Upon vessel injury, growth factor secretion augments FAK activity and increases GATA4 stability and cyclin D1 expression leading to SMC proliferation and neointimal hyperplasia. Pharmacological or genetic FAK catalytic inhibition reduces SMC proliferation through increased nuclear FAK, which promotes GATA4 proteasomal degradation. Reduced GATA4 levels decreases cyclin D1 transcription and SMC proliferation. EC indicates endothelial cells; P, pY397 FAK; PDGF, platelet-derived growth factor; and Ub, ubiquitin.

pY397 autophosphorylation, VS-4718 treatment failed to induce FLAG-FAK-NLM nuclear localization and loss of GATA4 expression (Figure 2E and Online Figure V), suggesting that FAK nuclear localization is important for FAK-mediated GATA4 degradation. However, new animal models to better evaluate cytoplasmic versus nuclear FAK in neointimal formation are needed.

It is unknown what causes kinase-inhibited FAK to preferentially localize to the nucleus. This may be due to a conformational change that hides the nuclear export sequence and prevents it from leaving after it shuttles to the nucleus. Notably, we observed that nuclear FAK is abundant in healthy arteries and vessel injury redistributed FAK from the nucleus to cytoplasm, suggesting that FAK in a healthy condition is less active compared with injured artery (Figures 6 through 8). It is also unknown what keeps FAK inside the nucleus or triggers its cytoplasmic localization following stimulation with serum or PDGF. After vascular injury, SMCs express an endogenous inhibitor of FAK, termed FRNK (FAK-related nonkinase), that inhibits SMC proliferation to some extent.^{18,59} Currently small molecule FAK inhibitors are mostly used as a cancer therapy,⁵⁷ but several studies have investigated the efficacy of these inhibitors in the treatment of other diseases such as lung and liver fibrosis.^{60–62} In the present study, we observed that the FAK inhibitor VS-4718, currently in clinical development as a cancer therapy,⁶³ significantly reduced SMC hyperplasia, suggesting that anticancer FAK inhibitors

could also be evaluated as a potential therapy for vessel wall narrowing diseases.

GATA4 levels may be low in SMCs of mature vessels,³⁸ but we observed that GATA4 mRNA was constantly expressed in SMCs in vitro and in vivo (Figure 2 and 6) and that wire injury increased GATA4 protein expression in vivo (Figure 6). Knocking down GATA4 using shRNA significantly prevented wire injury-induced neointimal formation (Figure 8), suggesting that GATA4 upregulation after wire injury is important for SMC proliferation and migration in vivo. Although GATA4 shRNA reduced cyclin D1 levels, it did not reduce pY397 FAK staining after wire injury, suggesting that targeting GATA4 protein in neointimal hyperplasia may be beneficial (Figure 8). As there currently are no GATA4-specific inhibitors available, further investigation into the regulation of GATA4 in SMCs in vivo is required.

In summary, our new observations from this study are that healthy vessels have low FAK activity and abundant nuclear FAK (Figure 9). However, vascular injury promoted FAK activation and redistribution from the nucleus to cytoplasm (Figures 6 through 8 and Online Figures XIII, XIV, and XVI). We have demonstrated that regulation of FAK activity and cytoplasmic localization in SMCs is critical to promoting SMC proliferation in part via increased GATA4 stability and cyclin D1 expression. Targeting FAK catalytic activity or GATA4 levels may have potential in the treatment of vessel narrowing diseases.

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Disclosures

None.

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