

Local CXCR4 Upregulation in the Injured Arterial Wall Contributes to Intimal Hyperplasia

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ABSTRACT

CXCR4 is a stem/progenitor cell surface receptor specific for the cytokine stromal cell-derived factor-1 (SDF-1 α). There is evidence that bone marrow-derived CXCR4-expressing cells contribute to intimal hyperplasia (IH) by homing to the arterial subintima which is enriched with SDF-1 α . We have previously found that transforming growth factor- β (TGF β) and its signaling protein Smad3 are both upregulated following arterial injury and that TGF β /Smad3 enhances the expression of CXCR4 in vascular smooth muscle cells (SMCs). It remains unknown, however, whether locally induced CXCR4 expression in SM22 expressing vascular SMCs plays a role in neointima formation. Here, we investigated whether elevated TGF β /Smad3 signaling leads to the induction of CXCR4 expression locally in the injured arterial wall, thereby contributing to IH. We found prominent CXCR4 upregulation (mRNA, 60-fold; protein, 4-fold) in TGF β -treated, Smad3-expressing SMCs. Chromatin immunoprecipitation assays revealed a specific association of the transcription factor Smad3 with the CXCR4 promoter. TGF β /Smad3 treatment also markedly enhanced SDF-1 α -induced ERK1/2 phosphorylation as well as SMC migration in a CXCR4-dependent manner. Adenoviral expression of Smad3 in balloon-injured rat carotid arteries increased local CXCR4 levels and enhanced IH, whereas SMC-specific depletion of CXCR4 in the wire-injured mouse femoral arterial wall produced a 60% reduction in IH. Our results provide the first evidence that upregulation of TGF β /Smad3 in injured arteries induces local SMC CXCR4 expression and cell migration, and consequently IH. The Smad3/CXCR4 pathway may provide a potential target for therapeutic interventions to prevent restenosis. STEM CELLS 2016;34:2744–2757

SIGNIFICANCE STATEMENT

CXCR4 is a stem/progenitor cell surface receptor specific for the cytokine stromal cell-derived factor (SDF)-1 alpha. While previous studies show that blood-borne CXCR4-expressing progenitor cells contribute to intimal hyperplasia by homing to the arterial subintima which is enriched with SDF-1 alpha, it remains unclear whether CXCR4-expressing cells from other sources also play a role. Here our studies using tissue-specific CXCR4 knockout mice clarify that CXCR4 can be induced locally in the injured arterial wall and play a big part in the development of intimal hyperplasia by promoting smooth muscle cell migration to the SDF-1 alpha rich subintima space.

INTRODUCTION

Over one million vascular reconstructions are performed in the United States each year. However, the long-term success is plagued by the development of recurrent vascular disease either within the bypass or the treated artery. Intimal hyperplasia (IH) is a major component of this process which leads to vessel narrowing [1]. A hallmark of IH is the proliferation of vascular smooth muscle cells (SMCs) and their migration to the subintima of the injured blood vessel wall. The underlying mechanism

of IH is complex. One of the important and still unanswered questions is the origin of the cells that produce the neointimal lesion.

There is a large body of evidence revealing the importance of transforming growth factor- β (TGF β) in the development of IH. A number of studies have shown that exogenous TGF β enhances, and blocking TGF β inhibits IH [2]. TGF β plays an important role in a wide range of pathophysiological processes including cell proliferation, differentiation, and apoptosis [3]. TGF β signals via Smad-dependent canonical and Smad-independent noncanonical pathways.

Canonically, TGF β binds to its type II receptor leading to receptor phosphorylation and the subsequent activation of signaling proteins Smad2 and Smad3, which then form a heterotrimer with Smad4; this complex then translocates into the nucleus. Once inside the nucleus, Smad3 is able to regulate gene transcription by binding to consensus Smad-binding elements (SBEs) in the promoter region of multiple target genes. We have demonstrated that levels of TGF β and Smad3 are elevated after angioplasty both in animals as well as in humans [4, 5]. TGF β inhibits SMC proliferation and migration in vitro [6]. However, in the context of elevated Smad3, TGF β has the opposite effect [5, 7]. Furthermore, our group has shown that enhancing Smad3 expression following rat carotid balloon injury aggravates IH, whereas inhibition of Smad2/3 suppresses the formation of neointimal lesion [2].

CXCR4 is a receptor for chemo-attractant stromal cell-derived factor-1 (SDF-1 α). CXCR4 is expressed in stem/progenitor cells and lymphocytes but barely detectable in highly differentiated cells (such as SMCs). However, expression of CXCR4 is elevated under some pathological conditions. For example, during tumor metastasis CXCR4 plays an important role in directing cancer stem cell migration [8–10]. CXCR4 has been shown to play a role in IH. It has been demonstrated that after vascular injury SDF-1 α is released from apoptotic cells from the arterial wall. Local SDF-1 α then induces chemotaxis, attracting CXCR4-expressing progenitor cells to the site of injury [11]. Binding of SDF-1 α to CXCR4 expressing progenitor cells initiates a series of changes including enhanced motility and proliferation as well as differentiation leading to a significant contribution of these cells to the growth of neointimal lesion.

The origin of neointimal cells has been a hotly debated issue [12]. The current view is that medial SMCs migrate into the subintima and proliferate producing IH [2]. A large body of literature supports that medial SMCs are the primary source of neointimal cells [2]. However, there are also reports that myofibroblasts in the adventitia [13], or CXCR4-expressing progenitor cells derived from bone marrow, migrate to the subintima and participate in the formation of this highly cellular neointima layer [11]. The contribution of bone marrow-derived cells to IH remains controversial. It has been reported that CXCR4-expressing bone marrow-derived cells are attracted to the subintima by SDF-1 α following balloon or wire-induced arterial injury in mice [11]. However, in studies employing rat carotid artery transplants following balloon injury, it appears that majority of cells in the neointima are derived from residential SMCs [14].

We have made the interesting observation that SMCs, when activated by TGF β /Smad3 (which are both enhanced at the time of arterial injury), strongly express CXCR4. This finding raises the question as to whether SDF-1 α released at the time of injury might act as a chemoattractant of cells already within the arterial wall, that being CXCR4 expressing SMCs that have been activated by TGF β /Smad3. This phenomenon could occur rather than or in addition to the effect of SDF-1 α on progenitor cells. To test this question, we fully evaluated both in vitro and in vivo, the ability of SMCs to express CXCR4. In addition, we created a new mouse line with CXCR4 selectively depleted in SM22-expressing arterial wall SMCs. We found that IH was substantially reduced in wire-injured femoral arteries where SMCs were lacking the CXCR4

receptor. In sum, we have identified a novel mechanism whereby SDF-1 α produces chemotaxis of local SM22 expressing vascular SMCs which have been induced to express CXCR4 in response to TGF β /Smad3. These events in turn contribute to the development of injury-induced restenosis.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA, <http://www.criver.com/>). In order to create a SMC-specific CXCR4 knockout model, a mouse strain that expresses Cre (recombinase) under the control of SM22- α promoter (SM22-Cre) and another strain with loxP sites flanking the CXCR4 gene were acquired from Jackson Laboratories (Bar Harbor, ME, <https://www.jax.org/>). These two strains were crossed, and homozygous offspring without CXCR4 expression in the aorta were selected through RT-PCR genotyping (Supporting Information Fig. S1). The experiments involving animal use were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Wisconsin-Madison. Surgery was performed under isoflurane anesthesia (through inhaling, flow rate 2 ml/minute), and all efforts were made to minimize suffering. Animals were euthanized in a chamber gradually filled with CO₂.

Reagents

Recombinant TGF β 1 and SDF-1 α were purchased from R&D Systems (Minneapolis, MN, <https://www.rndsystems.com/>). The MAPK inhibitors (PD98059 and U0126) were from Selleck Chemicals Company (Houston, TX, <http://www.selleckchem.com/>). Dulbecco's modified Eagle's medium (DMEM) and cell culture reagents were from Invitrogen (Carlsbad, CA, <https://www.thermofisher.com/us/en/home.html>). Other reagents, if not specified, were purchased from Sigma-Aldrich (St. Louis, MO, <https://www.sigmaaldrich.com/united-states.html>).

SMC Culture and AdSmad3/TGF β Treatment

Rat SMCs were isolated from the thoracoabdominal aorta of male Sprague-Dawley rats. SMCs at passages 4–5 were used for all experiments and maintained in DMEM supplemented with 10% fetal bovine solution (FBS) at 37°C with 5% CO₂. Cell viability was >95% as assayed using the Trypan Blue exclusion method. Adenoviral vectors expressing Smad3 (AdSmad3) and green fluorescent protein (AdGFP control) were constructed as previously described [15]. SMCs were infected for 4 hour with AdSmad3 (or AdGFP) (3×10^4 particles/cell) in DMEM containing 2% FBS, and recovered for 20 hour with 10% FBS, and then starved with 0.5% FBS for 24 hour followed by treatment with human recombinant TGF β 1 (5 ng/ml, R&D Systems, Minneapolis, MN) or equivalent amount of solvent (final 4 μ M HCl and 1 μ g/ml BSA) for 6 or 24 hour.

Real-Time PCR Analysis

RNA was isolated from cultured cells using a Trizol reagent (Invitrogen, Carlsbad, CA) following manufacturer's protocol. Potential contaminating genomic DNA was removed by using

gDNA Eliminator columns provided in the kit. RNA (2 μ g) was used for the first-strand cDNA synthesis (Applied Biosystems, Carlsbad, CA, www.thermofisher.com/). Quantitative RT-PCR was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Each cDNA template was amplified in triplicate using SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) with gene specific primers.

Western Blotting Analysis

Cells were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40 and 0.1% sodium dodecyl sulfate) containing Protease Inhibitor Cocktail I (Millipore, Billerica, MA, www.emdmillipore.com/). Protein concentration was determined using a Bio-Rad DC Protein Assay kit (Hercules, CA). Thirty micrograms of proteins from each sample were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting was then performed to detect a protein specifically using the following antibodies: rabbit anti-CXCR4 (Santa Cruz Biotechnology, Dallas, TX, <http://www.scbt.com/>), rabbit anti-phospho-ERK, rabbit anti-ERK (Cell Signaling Technology, Boston, MA), mouse antibodies for α -smooth muscle actin (SMA) and β -actin (Sigma, St. Louis, MO). After incubation with an appropriate primary antibody and then a horseradish peroxidase (HRP)-conjugated secondary antibody, the specific protein band on the membrane was visualized by using enhanced chemiluminescence reagents (Pierce Biotechnology, Rockford, IL, www.thermofisher.com/). The signal was analyzed by using LAS 4000 mini (GE Healthcare Bio-Sciences, Pittsburgh, PA).

Immunocytochemistry

Immunostaining was performed to detect in situ the expression of CXCR4 on SMCs, following our published method [16]. Cells were fixed in 4% paraformaldehyde in PBS for 10 minutes, permeabilized with 0.1% triton x-100 (in PBS), and then blocked with 5% BSA/5% donkey serum for 1 hour. A rabbit anti-CXCR4 primary antibody (Abcam, Cambridge, MA, <http://www.abcam.com/>) was incubated with the cells overnight at 4°C, and then a secondary antibody was applied for 1 hour at room temperature followed by fluorescence microscopy.

Chromatin Immunoprecipitation

SMCs were crosslinked with 1% formaldehyde for 15 minute at room temperature, after which 125 mM glycine was added to quench formaldehyde. The cells were then lysed in 500 μ l lysis buffer supplemented with a protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland, <http://www.roche.com/index.htm>). Nuclei were pelleted at 3000 \times g and 4°C for 5 minute and resuspended in 400 μ l of nuclear lysis buffer. The samples were sonicated to yield DNA fragments between 200 and 700 bp, and lysates were cleared by centrifugation. The samples were then incubated with 2.5 μ g of a rabbit anti-Smad3 antibody (Abcam, Cambridge, MA) or control IgG (Upstate/Millipore, Billerica, MA) for 1 hour at 4°C. To reduce nonspecific association, 30 μ g of sonicated salmon sperm DNA and 50 μ g of BSA (Promega, Madison, WI, www.promega.com/) were included in each sample.

Immunoprecipitation was carried out using 50 μ l of 50% (v/v) Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology Inc., Dallas, TX) at 4°C overnight. The immunoprecipitated

complexes were washed sequentially with low-salt buffer, high-salt buffer, LiCl buffer, and TE buffer. The DNA-protein complexes were eluted with 200 μ l elution buffer (1.5% SDS in 50 mM NaHCO₃), and then incubated at 65°C overnight to reverse formaldehyde crosslink. DNA was first extracted with phenol-chloroform and then purified by ethanol precipitation and finally dissolved in 20 μ l of 10 mM Tris-HCl, pH 8.5 for quantitative RT-PCR analysis of the CXCR4 promoter. The DNA sequence of SBE was from references 17–19.

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay (EMSA) was performed using a Light Shift Chemiluminescent EMSA Kit (Pierce Biotechnology, Rockford, IL). A biotinylated DNA oligonucleotide containing the SBE in the CXCR4 promoter was synthesized in the University of Wisconsin Biotechnology Center (Madison, WI). Briefly, SMCs were infected with AdSmad3 and treated with TGF β 1 for 2 hour and then harvested for nuclei extraction. The extract (30 μ g of nuclear protein) was incubated with 5 ng of biotin-oligo at room temperature for 30 minute in 20 μ l total volume of binding buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol (v/v), and 0.5 μ g poly(deoxyinosinic-deoxycytidylic acid). The reaction was stopped by adding gel loading buffer and subjected to nondenaturing 4% polyacrylamide gel electrophoresis in 0.5 \times TBE buffer (Tris-borate-EDTA). The gel was transferred to a nylon membrane provided in the kit and DNA was UV crosslinked to the nylon membrane. After incubating the membrane with streptavidin-conjugated HRP, the biotinylated DNA bands were visualized using enhanced chemiluminescence reagents (Pierce Biotechnology, Rockford, IL).

SDF-1 α Binding Assay

SMCs infected with AdSmad3 and then treated with TGF β 1 (for 24 hour) were seeded in 96-well plates at a density of 3000 cells/well. Biotinylated SDF-1 α was then added and incubated with the cells for 1 h at 4°C. The cells were washed three times with HBSS media, incubated with streptavidin-conjugated HRP for 45 minute at 4°C, and then washed again three times with HBSS media. A colorimetric substrate, *o*-phenylenediamine dihydrochloride in citrate buffer was added to assess streptavidin-conjugated HRP bound with SDF-1 α /CXCR4 on the cell surface. Spectrophotometry was determined at 450 nm using a Flex Station 3 microplate reader (Molecular Devices, Sunnyvale, CA, www.moleculardevices.com/Microplates).

SMC Migration Assay

Migration assays were performed in Transwell plates (Corning Costar; Cambridge, MA) with a well diameter of 6.5 mm and filter pore size of 8 μ m. SMCs infected with AdSmad3 (or AdGFP) and then treated with TGF β 1 (or solvent) for 24 hour were added to the upper chamber (1.5×10^4 cells), and then 600 μ l of media with SDF-1 α (100 ng/ml) was added to the lower chamber. After 4 hour, the cells that migrated through the Transwell insert membrane were counted.

Rat Balloon Injury Model and In Vivo Gene Transfer

Male Sprague-Dawley rats (~350 g) underwent balloon injury of the left common carotid artery as described previously

[14]. Briefly, after induction of anesthesia with isoflurane, a 2-French balloon catheter (Edwards Lifesciences, Irvine, CA, www.edwards.com/) was inserted through the left external carotid artery into the common carotid artery and insufflated with 2 atm of pressure three times. After injury, animals received intraluminal infusion of adenoviral vectors (2.5×10^9 plaque-forming units) in 200 μ l of PBS over 20 minute. The external carotid artery was then ligated, and blood flow was resumed. Rats were sacrificed at 3, 7, or 14 days after injury. Half the artery was processed for mRNA analysis and the other half was fixed in 4% paraformaldehyde overnight and then cryopreserved in the Optimal Cutting Temperature reagent.

Mouse Femoral Wire Injury Model

Mouse femoral artery wire injury was performed as described previously [20]. Under anesthetization, a midline incision was made on the ventral left thigh to dissect the femoral artery. The distal and proximal ends of the femoral artery were looped with surgical suture for temporary control of blood flow. An arteriotomy was made on the femoral artery muscular branch, through which a 0.015" guide wire (REF#C-SF-15-15, Cook Medical, Bloomington, IN, https://www.cookmedical.com/products/di_tscf_webds/) was inserted and remained in the artery for 1 minute. After removal of the wire, the muscular branch was ligated and blood flow in the femoral artery was resumed. At 28 days after injury, femoral arteries were collected from anesthetized animals following perfusion fixation at a physiological pressure of 100 mmHg, the animals were then euthanized.

Morphometric Analysis of IH

Paraffin sections (5 μ m thick) were excised at equally spaced intervals and stained with hematoxylin-eosin (H&E) for morphometric analysis, as described in previous reports [21]. Planimetric parameters as follows were measured on the sections and calculated using ImageJ: the area inside external elastic lamina (EEL area) or internal elastic lamina (IEL area), lumen area, intima area (= IEL area – lumen area), and media area (= EEL area – IEL area). Measurements were performed by a student blinded to the experimental conditions. IH was quantified as a ratio of intima area versus media area. Six to eight sections from each animal were used. The data from all sections were pooled to generate the mean for each animal. The means from all the animals in each treatment group were then averaged, and standard error of the mean (SEM) was calculated.

Immunohistochemistry

Immunostaining was performed on carotid or mouse femoral artery sections following our published method [21]. Briefly, the sections were first incubated with each of the primary antibodies for 12 hour with dilution ratios as follows: rabbit anti-CXCR4 (Abcam, Cambridge, MA), 1:200; rabbit anti-SDF-1 α (Santa Cruz Biotechnology Inc., Dallas, TX), 1:200; mouse anti- α -SMA (Sigma-Aldrich, St. Louis, MO), 1:1000. Normal IgG was used for background control. The detected proteins were then visualized by fluorescence microscopy after incubating the cells with species-matched secondary antibodies conjugated with either Alexa Fluor 546 or Alexa Fluor 488 (Invitrogen, Carlsbad, CA).

For quantification, five immunostained sections from each animal were used. Positively stained cells were counted (based on fluorescence intensity) using ImageJ by a student blinded to experimental conditions. The numbers from all five sections were pooled to generate the mean for each animal. The means from all the animals in each treatment group were then averaged, and the SEM was calculated.

Statistical Analysis

All data are presented as mean \pm SEM. One-way or 2-way ANOVA was performed, as appropriate, followed by Tukey's HSD post hoc test. $p < .05$ was considered statistically significant.

RESULTS

TGF β /Smad3 Stimulates CXCR4 Expression in Rat Aortic SMCs In Vitro

We have shown that TGF β and its signaling protein Smad3 are upregulated in the arterial wall in response to injury and contribute to the development of IH [7, 21]. In an effort to identify the molecular determinants that mediate TGF β /Smad3-stimulated IH, we performed gene arrays which revealed a dramatic increase in CXCR4 gene expression in SMCs in the presence of elevated TGF β /Smad3 signaling [16]. In order to further explore the influence of CXCR4 upregulation on SMC behavior and IH, we first confirmed that TGF β /Smad3-induced SMC CXCR4 expression by quantitative RT-PCR. To mimic elevation of TGF β and Smad3 in SMCs following vascular injury, cultured rat aortic medial SMCs were infected with adenovirus expressing Smad3 (AdSmad3) followed by stimulation with TGF β . Cells infected with AdGFP served as a control. As shown in Figure 1A, compared to AdGFP control, TGF β alone increased CXCR4 gene expression by 12-fold, however, AdSmad3 infection increased CXCR4 expression by 18-fold and TGF β treatment (6 hour) following AdSmad3 infection further stimulated CXCR4 gene transcription by 60-fold. To test whether TGF β stimulated CXCR4 expression depends on Smad3, we incubated SMCs with Smad3 siRNA and then treated with TGF β for 6 hour. The result showed that preincubation with Smad3 siRNA significantly decreased level of TGF β -induced CXCR4 mRNA elevation (Fig. 1B). We then determined the effect of TGF β /Smad3 on CXCR4 protein levels by Western blotting at a later time point (24 hour TGF β treatment). Consistent with our findings with gene expression, CXCR4 protein levels were also increased by treatment of either TGF β or AdSmad3 alone, and were further elevated (4-fold) by the combination of Smad3 and TGF β (Fig. 1C). We then evaluated CXCR4 expression via immunocytochemistry which revealed intense fluorescence in SMCs stimulated by TGF β /Smad3 (Fig. 1D). These data convincingly demonstrate that TGF β /Smad3 signaling strongly enhances the expression of CXCR4 likely on the cell membrane of SMCs.

Smad3 Interacts with the CXCR4 Promoter in Rat Aortic SMCs

We next explored the molecular mechanism through which TGF β /Smad3 stimulates expression of CXCR4. Since the CXCR4 promoter contains consensus SBE, we hypothesized that Smad3 may regulate CXCR4 expression by directly interacting with the CXCR4 promoter. Using chromatin immunoprecipitation (ChIP),

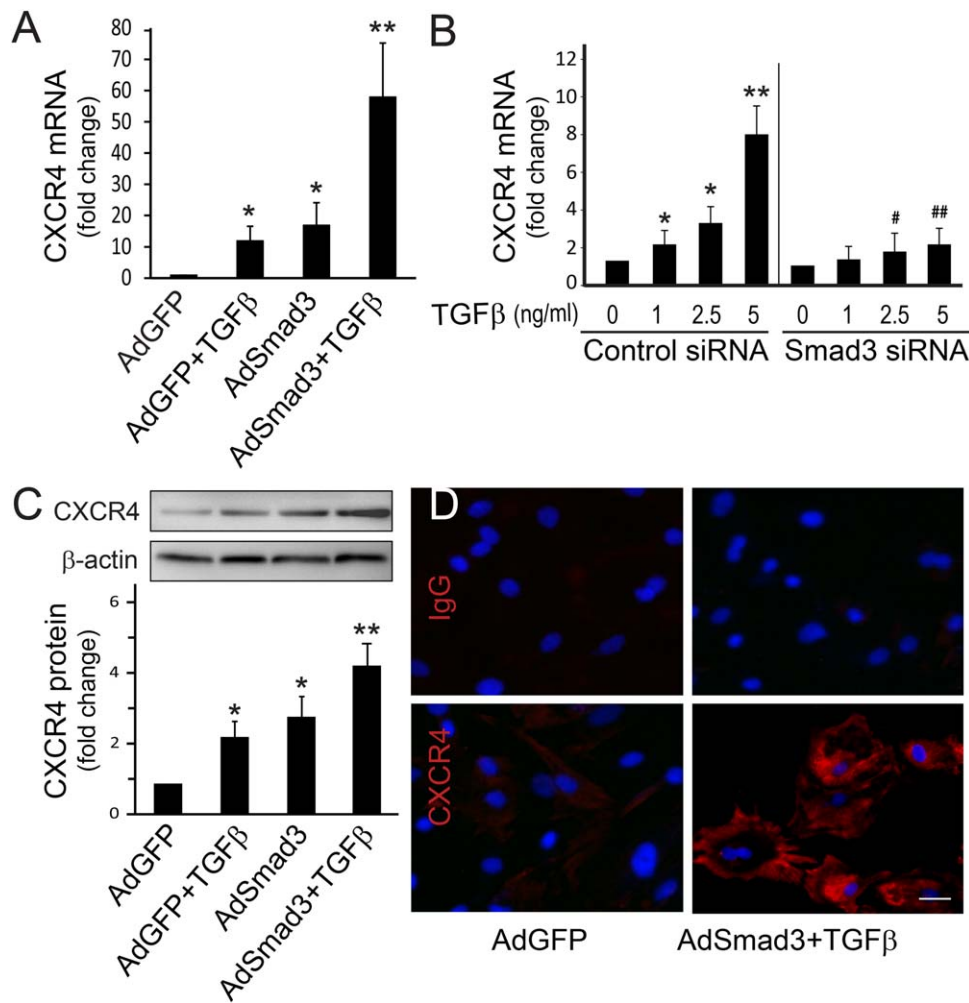


Figure 1. TGFβ/Smad3 stimulates CXCR4 expression in cultured rat aortic smooth muscle cells (SMCs). (A): Quantitative RT-PCR. mRNA levels of CXCR4 were determined from SMCs infected with AdGFP (control) or AdSmad3 followed by treatment with solvent or TGFβ1 for 6 hour. Error bar represents SEM. * $p < .05$ compared with AdGFP control; ** $p < .05$ compared with all other conditions; $n = 3$. (B): Quantitative RT-PCR. mRNA levels of CXCR4 were determined with SMCs transfected with scrambled siRNA (control) or Smad3 siRNA followed by treatment with TGFβ1 for 6 hour. * $p < .05$ compared with solvent (0 ng/ml) in control siRNA group; ** $p < .01$ compared with all other conditions in control siRNA group; # $p < .05$ compared with 2.5 ng/ml TGFβ1 treatment in control siRNA group; ## $p < .05$ compared with 5 ng/ml TGFβ1 treatment in control siRNA group; $n = 3$. (C): Western blotting. Protein levels of CXCR4 were assessed from SMCs infected with AdGFP or AdSmad3 followed by treatment with solvent or TGFβ1 (5 ng/ml) for 24 hour. * $p < .05$ compared with AdGFP control; ** $p < .05$ compared with all other conditions; $n = 3$. (D): Immunocytochemistry. CXCR4 expression on SMCs was visualized (red) using SMCs treated with AdGFP or AdSmad3 followed by treatment with TGFβ1 (5 ng/ml) for 24 hour. Nuclei were stained blue by 4',6-diamidino-2-phenylindole (DAPI). Scale bar = 20 μm. Image magnification: 200×. Abbreviation: TGFβ, transforming growth factor-β.

we found a greater immunoprecipitation of the CXCR4 promoter from AdSmad3-infected SMCs than from AdGFP control cells, which was further enhanced by stimulation with TGFβ (Fig. 2A). TGFβ alone also significantly increased immunoprecipitation of the CXCR4 promoter, likely through activation of endogenous Smad3. These results suggest that Smad3 can directly regulate CXCR4 through an interaction with its promoter. To further confirm the specific binding of Smad3 to the CXCR4 promoter, we incubated nuclear extracts from TGFβ/Smad3-treated SMCs, with a biotinylated DNA oligo that contains the SBE present in the CXCR4 promoter. The mixture was resolved on a nondenaturing polyacrylamide gel and then subjected to transfer and detection of the biotinylated oligo using streptavidin-conjugated HRP. Binding of Smad3 with the oligo was indicated by upshifting of the oligo band due to increased size of the

Smad3/oligo complex (Fig. 2B). Our data show that the nuclear extracts from TGFβ/Smad3-treated SMCs produced an upshift of the biotin-oligo band, which was abolished by adding the same oligo but without the biotin label (which competes for binding with Smad3) to the mixture of the biotin-oligo and nuclear extracts (Fig. 2C). A Smad3 antibody produced the oligo bands super shift while the biotinylated oligo precipitated Smad3 super proteins (Fig. 2D and 2E). These results together with the ChIP assay support that there is a specific interaction between Smad3 and the CXCR4 promoter.

Stimulation of SMCs with TGFβ/Smad3 Promotes CXCR4-Dependent SMC Migration Toward SDF-1α

A well-established cellular function of CXCR4 is its mediation of cell migration to SDF-1α. We thus hypothesized that since

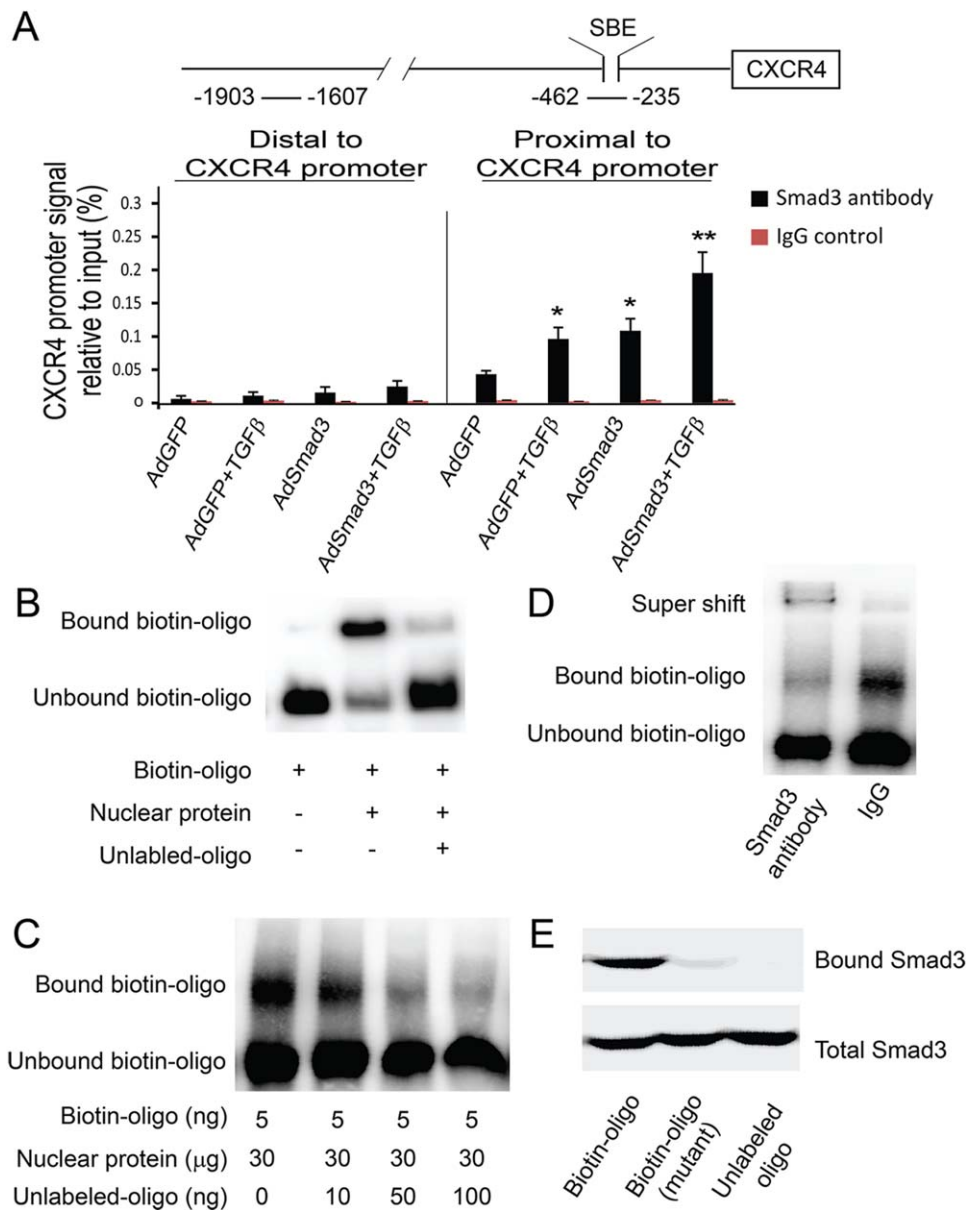


Figure 2. TGFβ/Smad3 treatment increases Smad3 binding to the CXCR4 promoter. (A): Schematic illustration of the rat CXCR4 promoter depicting the location of Smad binding element (SBE) and chromatin immunoprecipitation (ChIP) primer sets relative to the transcription start site. ChIP was performed using Smad3 antibody or normal IgG as described in the Methods section. Quantitative RT-PCR was performed with precipitated DNA using primer sets flanking SBE amplifying the CXCR4 promoter proximal region. A primer set amplifying the distal region of CXCR4 promoter was used as a control. **p* < .05, compared with AdGFP control; ***p* < .05, compared with AdGFP, AdGFP + TGFβ or AdSmad3; *n* = 3. (B): Electrophoretic mobility shift assay (EMSA) was performed as described in Methods. A synthetic biotinylated oligo containing the SBE from the CXCR4 promoter was used to react with nuclear protein extracts from AdSmad3-infected and then TGFβ-treated smooth muscle cells (SMCs). Shifted bands were detected with streptavidin-conjugated horseradish peroxidase. The same DNA oligo without biotin (unlabeled oligo) was used to compete with the biotinylated oligo for binding with Smad3. (C): EMSA was performed with increasing amounts of unlabeled oligo to compete with the biotinylated oligo for binding with Smad3. (D): EMSA in the presence of a Smad3 antibody showed super shift. (E): A synthetic biotinylated oligo containing the SBE of the CXCR4 promoter was incubated with nuclear protein extracts from AdSmad3 infected and TGFβ1-treated SMCs. Protein bound to biotinylated oligo was precipitated with avidin beads. Western blot was performed with a Smad3 antibody. A biotinylated oligo containing a mutated SBE or unlabeled SBE oligo were used as controls. Abbreviation: TGFβ, transforming growth factor-β.

TGFβ/Smad3 increase expression of CXCR4 this same stimulus through enhanced CXCR4 would promote SMC migration toward SDF-1α. We first evaluated whether there was increased binding of SDF-1α to SMCs treated with TGFβ/Smad3. SMCs were infected with AdGFP (control) or AdSmad3 followed by treatment with solvent or TGFβ1 for 24 hour, and

then incubated with increasing concentrations of SDF-1α conjugated with a biotin label. SDF-1α binding to SMCs was detected via streptavidin-conjugated HRP. As shown in Figure 3A, there was significantly more SDF-1α bound to TGFβ/Smad3-treated SMCs compared to the various controls at SDF-1α concentrations >40 ng/ml.

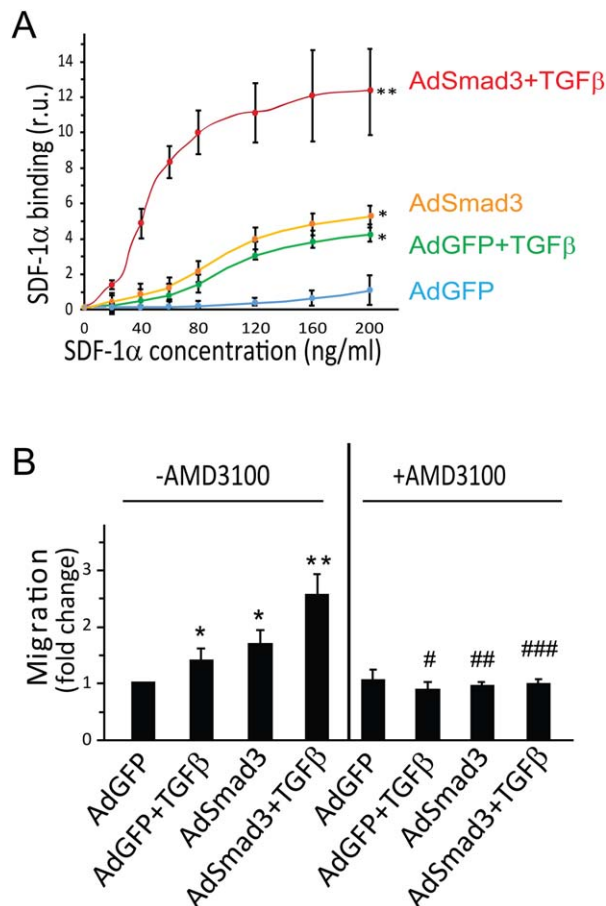


Figure 3. TGF β /Smad3 treatment stimulates CXCR4-dependent smooth muscle cell (SMC) migration toward SDF-1 α . **(A):** Binding of biotin-SDF-1 α to CXCR4 expressed on the unpermeabilized SMC surface was assayed *via* streptavidin-conjugated HRP, as described in Methods. Prior to the addition of biotin-SDF-1 α , SMCs were infected with AdGFP (control) or AdSmad3 and then treated with solvent or TGF β 1 for 24 hour. Error bar represents SEM; * p < .05 compared with AdGFP control; ** p < .05 compared with all other three conditions ($n = 3$). **(B):** Migration assay was performed as described in Methods. SMCs were infected with AdGFP (control) or AdSmad3 followed by treatment with solvent or TGF β 1 for 24 hour, and then transferred to a Boyden Transwell insert. AMD3100 (10 μ M) or solvent were added to the upper chamber. Sixty minutes later, SDF-1 α was added to the lower chamber (100 ng/ml). After 4 hour, the cells that migrated through the Transwell membrane were counted. * p < .05 compared with AdGFP control; ** p < .05 compared with all other conditions; # p < .05 compared with AdGFP+TGF β treated group without AMD3100; ### p < .05 compared with AdSmad3 treated group without AMD3100; #### p < .05 compared with AdSmad3+TGF β treated group without AMD3100; $n = 3$. Abbreviation: SDF-1, stromal cell-derived factor-1; TGF β , transforming growth factor- β .

In order to assess the functional effect of TGF β /Smad3-stimulated CXCR4 production on SDF-1 α induced SMC migration, we performed a chemotaxis assay using a Transwell with treated SMCs in the upper chamber and SDF-1 α in the lower chamber. While treatment of SMCs with TGF β or AdSmad3 alone slightly stimulated SMC migration in response to SDF-1 α , chemotaxis of SMCs stimulated with both TGF β and Smad3 was enhanced by >2.5-fold (Fig. 3B). The addition of a selective CXCR4 inhibitor (AMD3100, 10 μ M) abolished SMC

migration in response to SDF-1 α proving that the observed increase in chemotaxis was the consequence of CXCR4 expression. Finally, in separate assays, incubating SMCs with SDF-1 α did not alter CXCR4 mRNA levels (data not shown), ruling out the possibility that exposure to SDF-1 α during the chemotaxis assay induced SMC CXCR4 expression. Taken together, these results support the conclusion that TGF β /Smad3 stimulates CXCR4 expression on SMCs which in turn promotes chemotaxis to SDF-1 α .

SDF-1 α Stimulates ERK Phosphorylation in TGF β /Smad3-Treated SMCs in a CXCR4-Dependent Manner

In the forgoing studies, we have shown that TGF β /Smad3 enhances the expression of CXCR4 in SMCs, which then leads to their migration in response to SDF-1 α . In order to determine the mechanism through which SDF-1 α binding to CXCR4 enhances SMC migration, we explored the ERK pathway. We and others have shown that activation of ERK can lead to SMC migration [22, 23] and it has previously been shown in cell types other than SMCs that SDF-1 α can enhance phosphorylation of ERK [24]. We first determined the dose response of SDF-1 α -induced ERK activation by Western blotting. SMCs were infected with AdGFP (control) or AdSmad3 followed by treatment with solvent or TGF β 1 (5 ng/ml) for 24 hour before SDF-1 α stimulation. In AdGFP-treated control SMCs, SDF-1 α stimulation did not induce a detectable ERK MAPK activation (Fig. 4A). In AdGFP + TGF β -treated SMCs, stimulation with 100 ng/ml SDF-1 α led to a modest increase in ERK phosphorylation (Fig. 4B). Similarly, in AdSmad3-treated SMCs, stimulation with 100 ng/ml SDF-1 α also increased ERK phosphorylation (Fig. 4C). Then we tested in AdSmad3/TGF β 1 treated SMCs and found that the SDF-1 α induced ERK phosphorylation was markedly increased compared to cells treated with either AdGFP + TGF β 1 or AdSmad3 only (Fig. 4D). In the time course, study performing in AdSmad3-infected and TGF β 1-treated SMCs, we showed that SDF-1 α -induced ERK activation became visible at 5 minute, peaked at 10 minute, and then declined but remained above the baseline for up to 60 minute (Fig. 4E). These results indicate that SDF-1 α -stimulated ERK activation can be potentiated by TGF β /Smad3 signaling. In order to verify that the enhancement of SDF-1 α -stimulated ERK activation is the consequence of Smad3/TGF β induction of CXCR4, we employed AMD3100 to inhibit CXCR4. AMD3100-abolished ERK phosphorylation potentiated either by TGF β , Smad3, or both (Fig. 4F). Next, we isolated SMCs from aorta of wild type or CXCR4 conditional knockout mice and treated the cells with solvent (4 mM HCl) or 5 ng/ml TGF β 1 for 24 hour. SDF-1 α (10 minute; 100 ng/ml) failed to induce ERK phosphorylation in the absence of CXCR4 (Fig. 4G), confirming that SDF-1 α -induced ERK phosphorylation indeed through CXCR4. To prove the essential role of ERK phosphorylation in the SDF-1 α triggered SMC migration, we repeated the migration study in the presence or absence of the MEK inhibitor U0126 (5 μ M) or PD98058 (10 μ M). Both inhibitors abolished SMC migration toward SDF-1 α (Fig. 4H). These results, together with the foregoing, suggest a mechanism whereby TGF β /Smad3 upregulates CXCR4 in SMCs, which in turn mediates the activation of the ERK pathway and the subsequent SMC migration toward SDF-1 α .

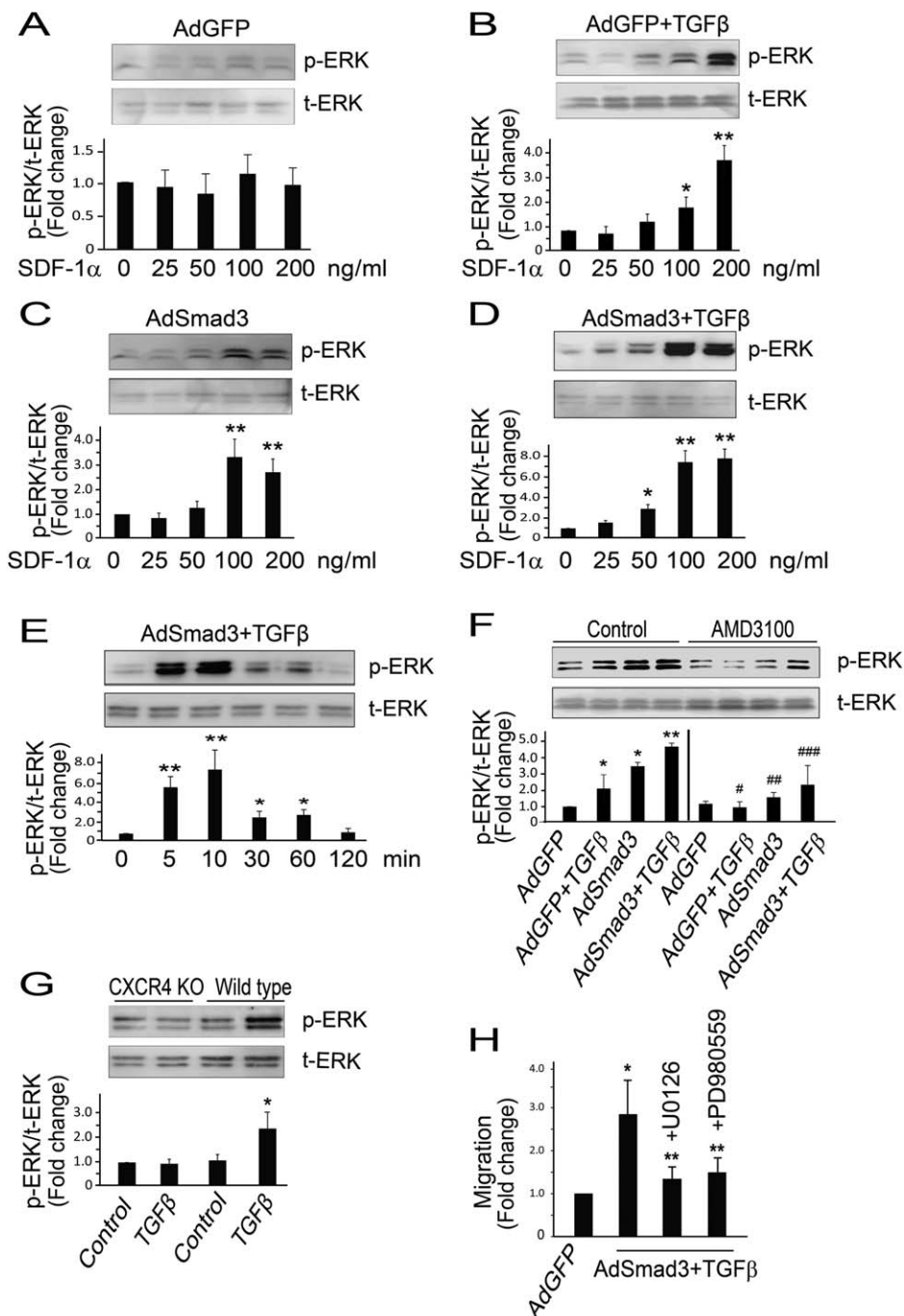


Figure 4. TGF β /Smad3 treatment stimulates SDF-1 α -induced, CXCR4-dependent ERK phosphorylation in smooth muscle cells (SMCs). AdSmad3 infected and TGF β 1 (5 ng/ml) treated SMCs were stimulated with SDF-1 α and ERK phosphorylation was detected by Western blotting. (A-D): Dose response of SDF-1 α induced (10 min) ERK phosphorylation in SMCs treated with AdGFP (A), AdGFP + TGF β 1 (B), AdSmad3 (C), and AdSmad3+TGF β 1 (D), respectively. * p < .05 compared with 0 ng/ml; ** p < .01 compared with 0 ng/ml; n = 3. (E): AdSmad3 infected and TGF β 1 (5 ng/ml) treated SMCs were stimulated with SDF-1 α (100 ng/ml) for indicated time and ERK phosphorylation was detected by Western blotting. * p < .05 compared with 0 min; ** p < .01 compared with 0 min; n = 3. (F): SDF-1 α -induced (10 min, 100 ng/ml) ERK phosphorylation in SMCs without or with AMD3100 (10 μ M). * p < .05 compared with AdGFP in control group; ** p < .05 compared with all other conditions in control group; # p < .05 compared with AdGFP+TGF β in control group; ## p < .05 compared with AdSmad3 in control group; ### p < .05 compared with AdSmad3+TGF β in control group; n = 3. (G): SMCs were isolated from aorta of wild type or CXCR4 conditional knockout mice and treated with control (solvent, 4 mM HCl) or TGF β 1 (5 ng/ml) for 24 hour. SDF-1 α -induced (10 min; 100 ng/ml) ERK phosphorylation was analyzed by Western blotting. * p < .05 compared with all other conditions, n = 3. (H): SMCs were infected with AdGFP (control) or AdSmad3 followed by treatment with TGF β 1 (5 ng/ml) for 24 hour, and then transferred to a Boyden Transwell insert. Cells were incubated with solvent or U0126 (5 μ M) or PD980559 (10 μ M) for 60 min, and then 100 ng/ml SDF-1 α was added to the lower chamber. The cells that migrated through the Transwell insert membrane after 4 hour were counted. * p < .05 compared with AdGFP; ** p < .05 compared with AdSmad3+TGF β without an inhibitor; n = 3. Abbreviations: SDF-1, stromal cell-derived factor-1; TGF β , transforming growth factor- β .

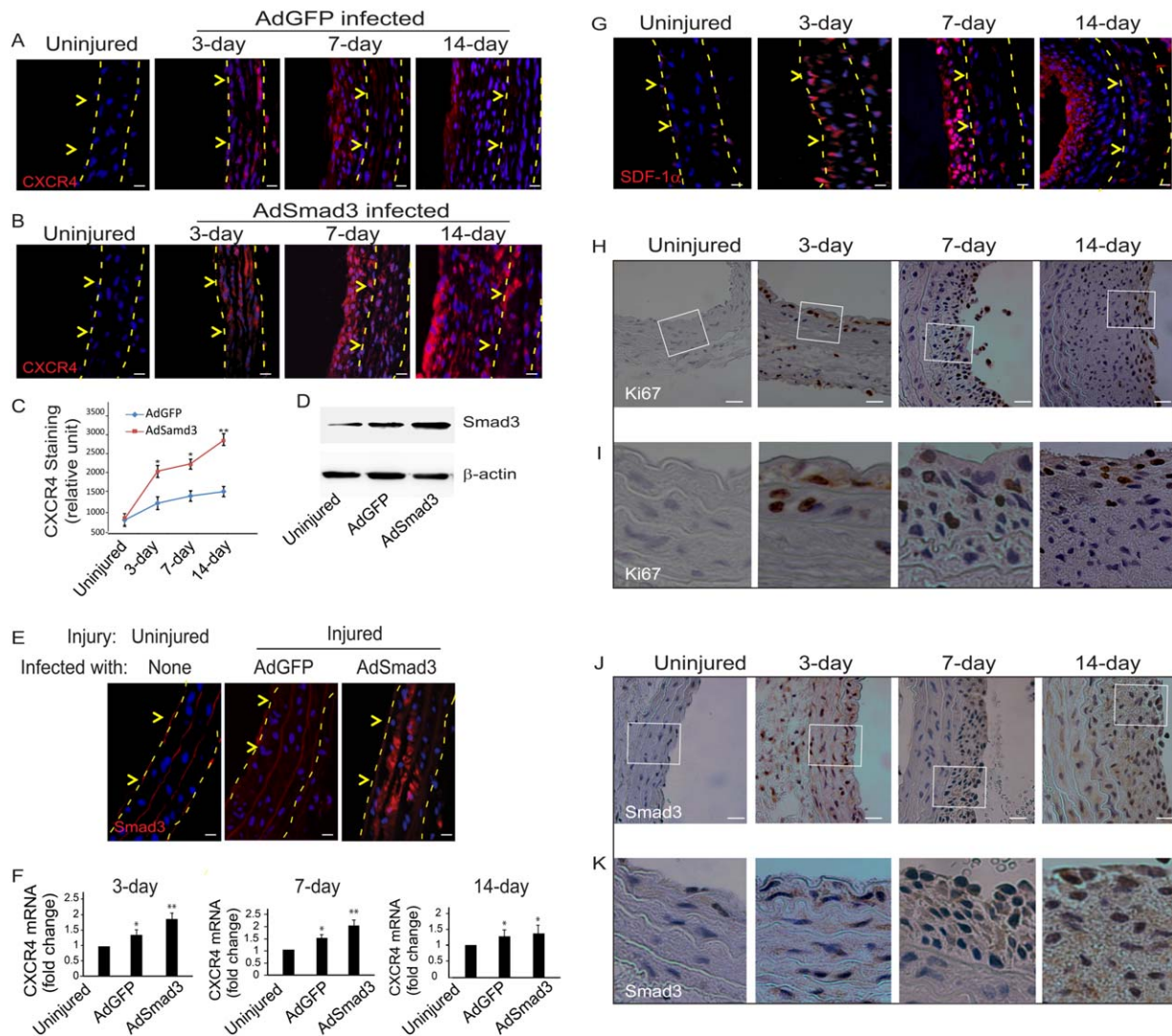


Figure 5. Enhanced Smad3 expression up-regulates CXCR4 production in balloon-injured rat carotid arteries. Balloon angioplasty was performed in rat carotid arteries followed by infusion of AdGFP or AdSmad3 (2.5×10^9 plaque-forming units) for 20 minutes. Uninjured carotid arteries were used as control. Carotid arteries were retrieved at the indicated time points (3, 7, or 14 days) for preparation of cross sections. (A and B): Immunostaining was performed to detect CXCR4 (red) and DAPI was used to stain nuclei (blue). Dashed lines define the media layer; arrowheads mark internal elastic lamina (IEL). (C): Relative fluorescent intensity was measured using ImageJ software. Each bar is a mean \pm SEM ($n = 3-5$); $*p < .05$ compared with AdGFP control; $**p < .05$ compared with all other conditions. (D): Western blot was performed on proteins extracted from uninjured or injured with AdGFP or AdSmad3 infused carotid arteries 3 days after angioplasty. (E): Immunostaining of Smad3 on sections from uninjured, or injured with AdGFP or AdSmad3 infused carotid artery. Dashed lines define the media layer; arrowheads mark IEL. (F): RT-PCR for CXCR4 levels after vascular injury. $*p < .05$ compared with uninjured; $**p < .05$ compared with uninjured or injured with AdGFP infused; $n = 3$. (G): Immunostaining of SDF-1 α on sections of carotid arteries on 3, 7, or 14 days after injury. Dashed lines define the media layer; arrowheads mark IEL. (H) and (J) are immunohistochemistry of Ki67 or Smad3 in injured sections of carotid arteries at the indicated time points (3, 7, or 14 days). (I) and (K) are magnified views of the boxed regions in H and J, respectively. Scale bar = 30 μ m.

Expression of CXCR4 Is Elevated in Balloon-Injured Rat Carotid Arteries and Further Enhanced following Increased Expression of Smad3

We have previously shown that TGF β and Smad3 are both upregulated following arterial injury [20]. Having demonstrated the novel upregulation of CXCR4 by TGF β /Smad3 in vascular SMCs, we next examined CXCR4 expression in vivo. We performed balloon angioplasty in male Sprague-Dawley rats, followed by luminal infusion of AdSmad3 or AdGFP control. Animals were sacrificed at 3, 7, or 14 days and carotid arteries were collected and sectioned followed by immunostaining. Compared to the uninjured controls, levels of CXCR4 in the

intima and media of the injured arteries were upregulated at all three time points. On day 14, the CXCR4 positive cells were primarily localized to the neointima. Moreover, CXCR4 expression was substantially increased in AdSmad3-treated arteries compared to AdGFP control (Fig. 5A, 5B, 5C, 5F). Western blotting and immunostaining showed higher endogenous Smad3 levels after angioplasty, while AdSmad3 infusion further increased Smad3 level compared to AdGFP infected controls (Fig. 5D and 5E). As reported previously, the accumulation of SDF-1 α was evident 3 days after injury and sustained up to day 14. The SDF-1 α positivity was concentrated in the intima (Fig. 5G). Similarly, the expression of Ki67 and Smad3

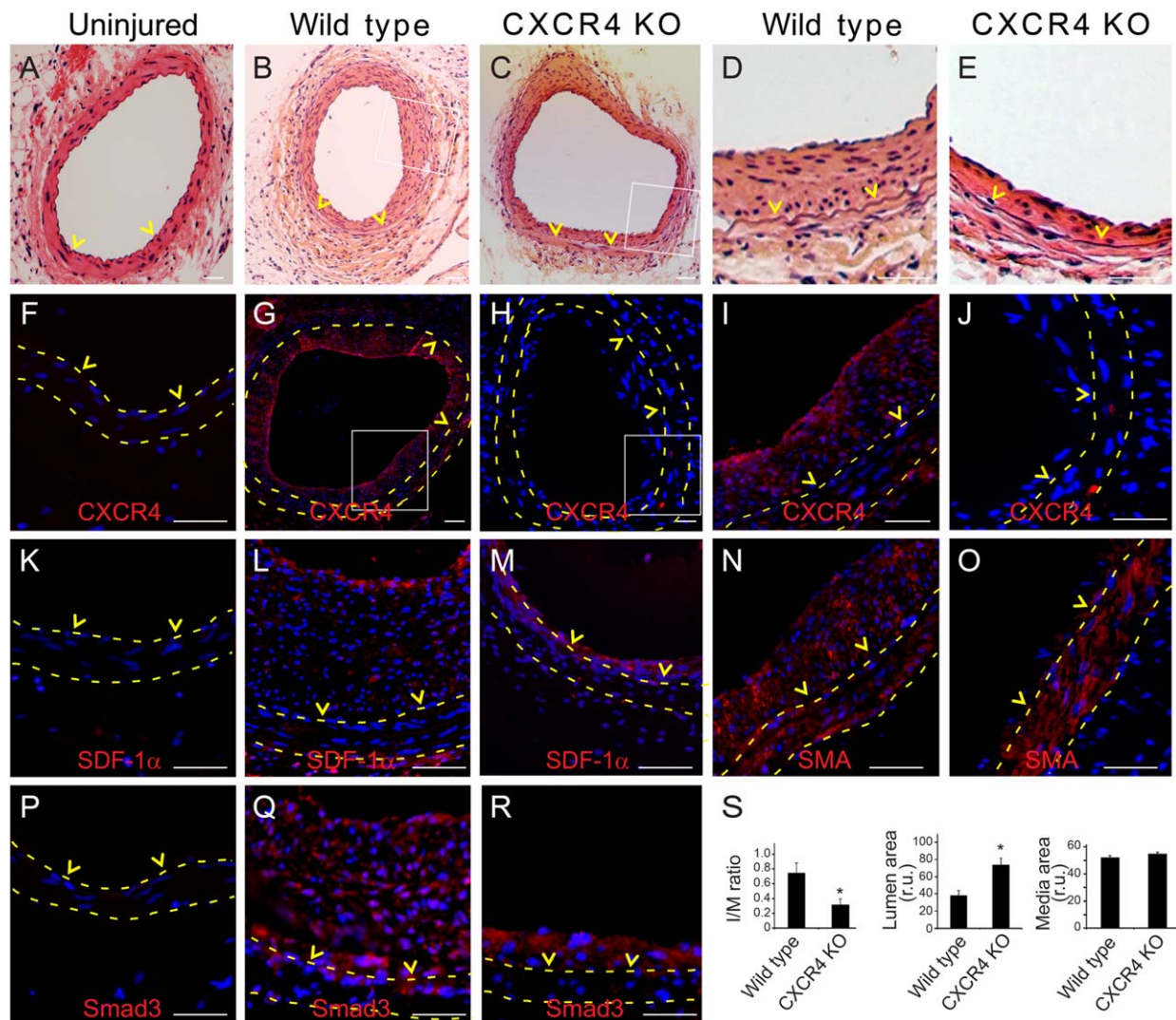


Figure 6. Selective knockout of CXCR4 in smooth muscle cells (SMCs) reduces intimal hyperplasia (IH) in wire-injured mouse femoral arteries. SM22-driven conditional CXCR4 knockout model was created and wire injury was performed in wild type and CXCR4 knockout mice, as described in Methods. (A–E): Representative H&E stained femoral arterial sections in uninjured (A), injured wild type (B) or injured knockout mice (C). (D) and (E) are enlarged views of the boxed areas in (B) and (C). (F–J): CXCR4 immunostaining in uninjured (F), injured wild type (G), or injured knockout mice (H). (I) and (J) are enlargement of the boxed regions from (G) and (H). (K–M): SDF-1 α immunostaining in uninjured (K), injured wild type (L), or injured knockout mice (M). (N–O): SMA immunostaining in injured wild type (N) or injured knockout mice (O). (P–R): Smad3 immunostaining in uninjured (P), injured wild type (Q), or injured knockout mice (R). (S): IH (intima/media area ratio), lumen area, and media area were quantified as described in Methods. * $p < .05$ compared to wild type; $n = 12$ – 13 . Scale bar = 50 μm . Abbreviation: SDF-1, stromal cell-derived factor-1.

also increased 3 days after injury and concentrated in the intimal area 7 and 14 days after angioplasty (Fig. 5H–5K; H&E and phospho-Smad3 IHC in Supporting Information Figure S4; Ki67 quantification in Supporting Information Figure S5).

Selective Knockout of CXCR4 in Vascular SMCs Inhibits Intimal Hyperplasia in Wire-Injured Mouse Femoral Arteries

Our hypothesis is that local expression of CXCR4 by vascular SMCs following arterial injury is enhanced by elevated TGF β /Smad3. This in turn leads to SMC migration and contributes to the development of IH. To generate SMC-specific CXCR4 knockout, we crossed a SM22-driven Cre mouse line with a CXCR4-floxed mouse strain to allow the CXCR4 gene to be

deleted during genomic DNA recombination. PCR genotyping using samples taken from the aortic SMCs of the recombined SM22-Cre/CXCR4-Flox crossover strain confirmed the absence of CXCR4 (Supporting Information Figure S1). In contrast, CXCR4 was present in other organs including liver and tail, indicating that the CXCR4 knockout was specific to the SMCs. The wire-injured femoral segments were retrieved 4 weeks after surgery. Representative H&E stained sections showed reduced neointima in CXCR4 conditional knockout mice compared to that in wild type. Immunostaining revealed abundant CXCR4 staining concentrated in the neointima of injured femoral artery in wild type mice (Fig. 6G and 6I). In contrast, the CXCR4 staining was essentially absent in the media and neointima of the conditional knockout mice (Fig. 6H and 6J). The SDF-1 α expression which is

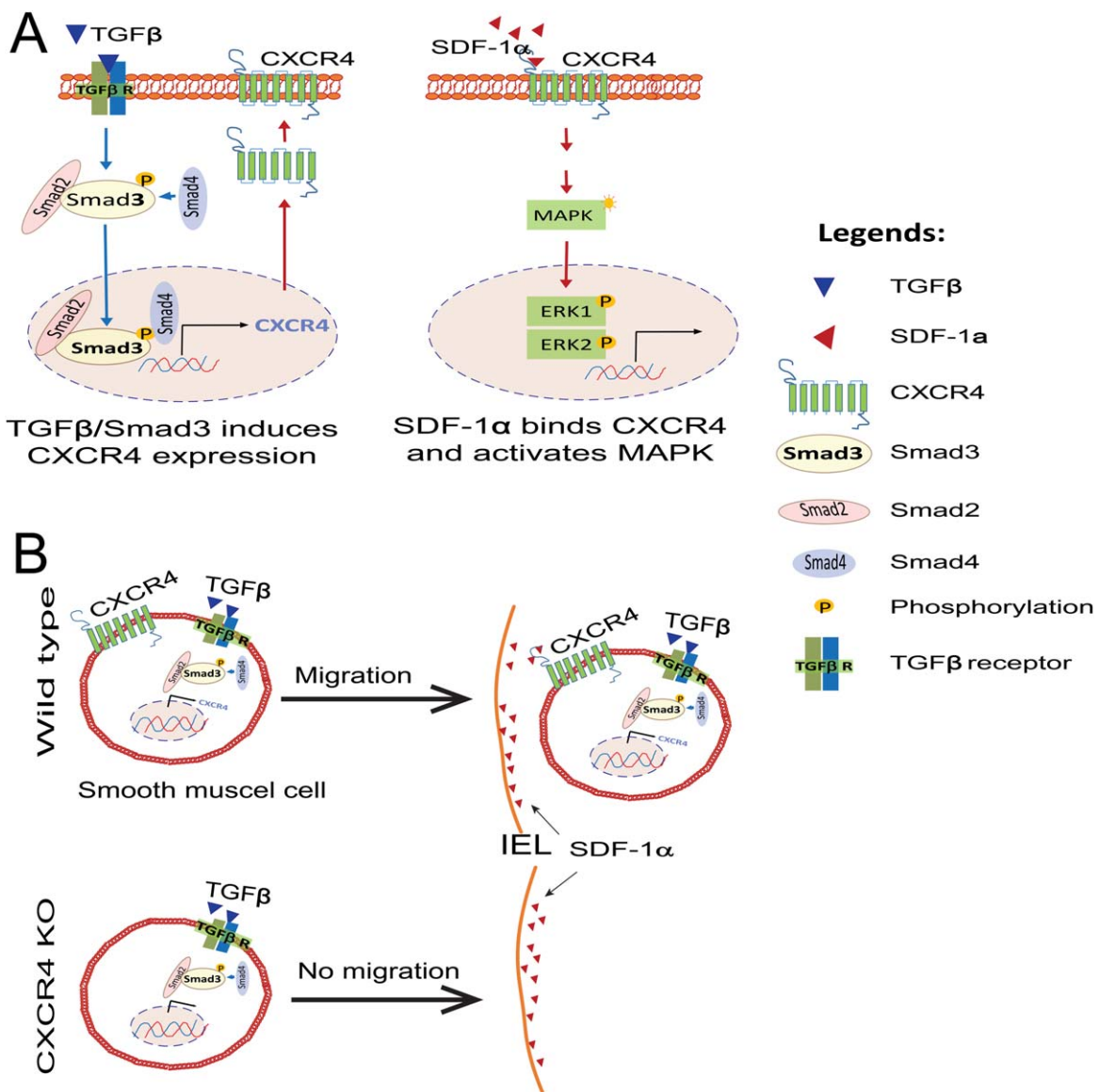


Figure 7. A schematic model showing the role of TGFβ/Smad3 regulated CXCR4 expression in SMC migration toward SDF-1α. (A): TGFβ/Smad3 stimulates CXCR4 expression while SDF-1α activates MAPK through CXCR4. (B): Elevated SDF-1α in the subintima of injured artery attracts SMCs from wildtype mice but not from CXCR4 knockout mice. Abbreviations: SDF-1, stromal cell-derived factor-1; SMCs, smooth muscle cells; TGFβ, transforming growth factor-β.

mainly localized to the luminal surface of the intima was not affected by the CXCR4 knockout (Fig. 6L and 6M). Similarly, SMA staining was comparable in both genotypes (Fig. 6N and 6O). Smad3 staining confirmed the elevation of this signaling protein in the intimal area and tunica media (Fig. 6Q and R; Ki67 and phospho-Smad3 in Supporting Information Figure S6). However, morphometric analyses revealed drastic differences in intimal formation between the wild type and SMC-specific CXCR4 knockout mice. At 4 weeks post injury, IH (measured as an I/M area ratio) was 60% less in knockout mice (0.29 ± 0.08) compared to their wild type littermates (0.74 ± 0.13). As the result, the lumen size increased by ~80% in the knockout compared to the wild type (Fig. 6S). These in vivo findings demonstrate that injury-induced local CXCR4 expression is an essential contributing factor to hyperplastic neointimal growth (Fig. 7).

DISCUSSION

Our studies have produced several interesting findings. We have discovered that elevated TGFβ/Smad3 signaling markedly upregulates CXCR4 in vascular SMCs in vitro as well as in balloon-injured rat carotid arteries. Moreover, enhanced CXCR4 expression in vitro results in the migration of SMCs toward SDF-1α. Using a mouse line designed to deplete the CXCR4 gene in a SM22-specific manner, we found that knocking out CXCR4 expression in vascular SMCs substantially reduced wire injury-induced IH. These results suggest that local (residential) CXCR4-expressing SMCs play an important role in the development of IH and suggest a novel mechanism of restenosis; i.e., in response to arterial injury, upregulated TGFβ/Smad3 activates the expression of CXCR4 in medial SMCs, which are then attracted to the

subintima in response to high levels of SDF-1 α , thereby enhancing IH.

There exists considerable evidence that CXCR4-expressing bone marrow-derived cells, namely progenitor or mesenchymal stem cells, are attracted to the subintima in response to SDF-1 α , thus contributing to IH [11, 25–27]. The findings of these studies have varied and are dependent upon the type of injury model used. M-CSF mobilized CXCR4⁺ stem cells enhance neointimal formation [26] while bone marrow transplantation from CXCR4 knockout to wild type mice reduced the IH by ~50% [11]. These studies have demonstrated the importance of CXCR4⁺ cells in IH. Our study is the first to demonstrate that locally induced expression of CXCR4 in residential SMCs plays an important role in the development of IH. The most compelling evidence is from our wire injury experiments, where we employed a mouse strain deprived of CXCR4 in local SMCs within the arterial wall. We were able to demonstrate a pronounced diminution of IH in wire-injured femoral arteries lacking CXCR4 in SM22-expressing cells. The fact that we do observe some positive staining for CXCR4 in the neointima of the knockout mice suggests that SDF-1 α acts as a chemoattractant of both internal CXCR4⁺ SMCs as well as external CXCR4⁺ bone marrow cells. Thus, it is plausible to propose that both residential and “foreign” CXCR4-expressing cells contribute to the development of neointima, although we were unable to compare their pathogenic capacity in the current study.

In accordance with an important role of local inducible CXCR4 expression in the development of IH, elevated CXCR4 has also been documented in other vascular pathological conditions. CXCR4 expression has been found to be upregulated in atherosclerotic lesions [28], and also in the tunica media of rat carotid arteries in a type I diabetes model [29], although the identity of SMCs as the source of CXCR4 was not confirmed in either of these settings. The only previous observation of vascular SMCs expressing CXCR4 was in vitro studies showing high glucose stimulated CXCR4 expression with the resultant promotion of proliferation and migration of rat SMCs [29]. Additionally, Schecter et al. [30] demonstrated that HIV gp120 activated the expression of tissue factor by SMCs through a mechanism that involved CXCR4, leading to thrombogenicity of atherosclerotic plaques. In spite of these prior studies, an important role for CXCR4 expression in local SMCs has not been previously demonstrated.

Our hypothesis is that locally induced SMC CXCR4 leads to SDF-1 α -mediated attraction of SMCs to the intima. This hypothesis is supported by the well-established role of SDF-1 α /CXCR4 in chemotaxis. The most pertinent evidence might be that SDF-1 α attracts circulating, CXCR4-expressing bone marrow-derived progenitor or mesenchymal stem cells to the subintimal layer [11]. These findings were confirmed by the reports that SDF-1 α neutralizing antibodies inhibit IH after femoral wire injury [31, 32]. Moreover, the chemoattractant role of SDF-1 α has been extensively studied in development, cancer metastasis, as well as several other pathological processes. For example, the SDF-1 α /CXCR4 axis has been found to be important in the maintenance of hematopoietic stem cell pool in bone marrow [33, 34]; this chemokine/receptor pair is also known to play an important role in directing cancer stem cell migration during tumor metastasis [8, 35]. In the current study, while our in vitro data confirmed migration of CXCR4-expressing SMCs toward SDF-1 α

(Fig. 3B), we also observed abundant SDF-1 α in the intima of injured mouse arteries (Fig. 6L and 6M). Thus, it is reasonable to propose that following arterial injury, residential SMCs bearing a high content of induced CXCR4 are attracted to the subintima by SDF-1 α , thereby enhancing neointimal formation. In addition to CXCR4, CXCR7 is another potential receptor for SDF-1 α [36, 37]. Since we did not observe significant effect of TGF β /Smad3 treatment on CXCR7 expression (Supporting Information Fig. S3), we focused the current study on CXCR4.

Aside from stimulating chemotaxis, SDF-1 α /CXCR4 has been shown to influence cell proliferation, albeit its role varies with different cell types and experimental contexts. In some settings, CXCR4 induces proliferation; examples include a glioblastoma cell line [38], pancreatic cancer cell lines [39], and several types of progenitor and stem cells [40, 41]. There is also a report suggesting that SDF-1 α can stimulate proliferation of vascular SMCs [29]. In contrast, other investigators have found that CXCR4 negatively regulates proliferation (e.g., keratinocytes in IL-23-Mediated Psoriasisiform Dermatitis) [42]. Since neointimal SMCs are characterized by a higher proliferative rate, we asked whether the interaction of CXCR4-expressing SMCs with SDF-1 α in the neointima would result in enhanced SMC proliferation. Despite the ability of SDF-1 α /CXCR4 to stimulate SMC chemotaxis, we did not demonstrate SDF-1 α -enhanced proliferation of CXCR4-expressing SMCs in vitro (Supporting Information Fig. S2). However, the lack of proliferation effects in vitro does not necessarily exclude the possibility that the SDF1/CXCR4 axis directly or indirectly contributes to the highly proliferative events taking place following vascular injury. Indeed, we noted a very similar temporal and spatial pattern of CXCR4⁺ and Ki67⁺ cells in the injured arteries. It is also possible that the high CXCR4 expression facilitates the phenotypic switch of SMCs, sensitizing them to the many mitogenic factors including PDGF-BB and bFGF to which SMCs are exposed following injury.

Our study also presents a novel finding that the TGF β /Smad3 axis is a potent stimulant of CXCR4, suggesting a new mechanism for the SDF-1 α /CXCR4 axis in the development of IH. Supporting evidence for the role of TGF β /Smad3 in expression of CXCR4 can be found from studies in other disease systems. For example, rat hepatoma cells normally express low levels of CXCR4, but when treated with TGF β , levels of CXCR4 are increased [43]. In response to TGF β 1, rheumatoid synovial T cells also express high levels of CXCR4 and become responsive to SDF-1 α [44]. In addition, Zhao and colleagues [45] showed that MCF-7 breast cancer cells significantly increased CXCR4 expression after TGF β 1 treatment. Our group has previously shown that TGF β and its signaling protein, Smad3, are upregulated at sites of arterial injury [20]. In the presence of enhanced Smad3 expression, TGF β appears to transform vascular SMCs in vitro from proapoptotic to proliferative. We believe that the effect of TGF β in promoting IH is related to coregulation of both TGF β and Smad3 following arterial injury, an observation we have made both in rats and in humans [5]. In the current study, we have demonstrated that TGF β /Smad3 stimulates CXCR4 expression in vascular SMCs, which ultimately leads to enhanced IH. While we believe that TGF β stimulates SMC CXCR4 expression primarily through canonical Smad3-dependent pathway, our work does not rule out a possible contribution of noncanonical pathways. It remains to be determined whether the inhibition of TGF β signaling attenuates postinjury CXCR4 upregulation. Of

note, other factors, such as HIF-1 α , have also been shown to contribute to the induction of CXCR4 expression in different cell types [46].

In search of a mechanism for the SDF-1 α -stimulated migration of TGF β /Smad3-treated cells, we identified MAPK ERK as the intermediate. MAPK ERK is a known critical mediator of cell migration in multiple cell types. We have shown that TGF β /Smad3 significantly enhances SDF-1 α -induced ERK activation. Moreover, SMC migration in response to SDF-1 α is dependent upon both CXCR4 as well as MAPK ERK (Figs. 3 and 4). Binding of SDF-1 α to the CXCR4 receptor activates a myriad of downstream signaling pathways that comprise Akt, ERK, and mTORC1 to regulate chemotaxis of mature dendritic cells [47]. The SDF-1 α /CXCR4 axis also regulates osteosarcoma cell migration through MEK1/2, ERK, and NF-KB pathways [48]. Regulation of SDF-1 α /CXCR4 for hematopoietic stem cell migration through PKC-zeta, Pyk-2 and ERK1/2 has also been documented [49]. Our results indicated that MAPK ERK is critical for SDF-1 α -induced SMC migration.

To explore the mechanism by which TGF β /Smad3 stimulates CXCR4 expression in SMCs, we focused on the observation that the CXCR4 promoter has consensus SBEs. Previous computational analysis predicted that Smad3 might directly interact with the CXCR4 promoter [50]. Our results from the ChIP assay reveal that Smad3 specifically binds to the CXCR4 promoter following TGF β stimulation (Fig. 2). Our study is the first to report a link between TGF β /Smad3 and CXCR4 in vascular SMCs. We also provide solid evidence supporting that Smad3 directly regulates CXCR4 expression by binding to SBEs in the promoter region of the CXCR4 gene.

In light of the fact that CXCR4 is a progenitor/stem cell marker, our observation of TGF β /Smad3-stimulated SMC CXCR4 expression is in accordance with recent reports suggesting that SMC de-differentiation is a critical component of the response to vascular injury [51]. In our recent work, we have shown that TGF β in the presence of elevated Smad3 enhances SMC de-differentiation by upregulating multiple stem or progenitor cell genes as well as by downregulating SMC markers or "differentiation" genes [16]. Our new findings with CXCR4 reinforce this hypothesis in that we have shown in the presence of TGF β /Smad3, that SMCs express CXCR4, a de-differentiation marker. Thus, it is conceivable that following arterial injury, CXCR4 may together with other de-

differentiation genes transform SMCs into a phenotype that promotes IH.

CONCLUSION

We have identified a new pathway through which TGF β stimulates the formation of IH. Following arterial injury, elevated levels of TGF β and Smad3 induce expression of CXCR4 on medial vascular SMCs. These cells in turn are attracted to the neointima by the cytokine SDF-1 α (Fig. 7). Moreover, we have demonstrated the mechanism by which TGF β /Smad3 controls the expression of CXCR4 as well as the mechanism used by CXCR4 to influence SMC migration. To confirm our hypothesis in vivo, we have created transgenic mice to selectively knock-out CXCR4 in residential SMCs. Local depletion of CXCR4 in vascular SMCs can effectively mitigate IH in injured arteries. These data suggest a novel mechanism for restenosis. Thus, modulation of TGF β /Smad3 function or local interruption of the CXCR4/SDF-1 α axis might lead to effective therapeutic strategies to prevent the development of the hyperplastic neointima that leads to restenosis.

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AUTHOR CONTRIBUTIONS

X.S., L.-W.G., S.S., B.L., and K.C.K.: Designed experiments; X.S., S.S., T.T., B.W., M.Z., M.A.C., and Y.S.: Performed experiments; X.S., L.-W.G., S.S., M.A.C., B.L., and K.C.K.: Analyzed data; X.S., L.-W.G., B.W., S.R.F., B.L., and K.C.K.: Wrote manuscript; X.S., L.-W.G., and S.S.: These authors contributed equally to this work.

DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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