Novel Paracrine Functions of Smooth Muscle Cells in Supporting Endothelial Regeneration Following Arterial Injury

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Rationale: **Regeneration of denuded or injured endothelium is an important component of vascular injury response. Cell-cell communication between endothelial cells and smooth muscle cells (SMCs) plays a critical role not only in vascular homeostasis but also in disease. We have previously demonstrated that PKCδ (protein kinase C-delta) regulates multiple components of vascular injury response including apoptosis of SMCs and production of chemokines, thus is an attractive candidate for a role in SMC-endothelial cells communication.**

Objective: **To test whether PKCδ-mediated paracrine functions of SMCs influence reendothelialization in rodent models of arterial injury.**

Methods and Results: **Femoral artery wire injury was performed in SMC-conditional *Prkcd* knockout mice, and carotid angioplasty was conducted in rats receiving transient *Prkcd* knockdown or overexpression. SMC-specific knockout of *Prkcd* impaired reendothelialization, reflected by a smaller Evans blue-excluding area in the knockout compared with the wild-type controls. A similar impediment to reendothelialization was observed in rats with SMC-specific knockdown of *Prkcd*. In contrast, SMC-specific gene transfer of *Prkcd* accelerated reendothelialization. In vitro, medium conditioned by AdPKCδ-infected SMCs increased endothelial wound closure without affecting their proliferation. A polymerase chain reaction-based array analysis identified *Cxcl1* and *Cxcl7* among others as PKCδ-mediated chemokines produced by SMCs. Mechanistically, we postulated that PKCδ regulates *Cxcl7* expression through STAT3 (signal transducer and activator of transcription 3) as knockdown of STAT3 abolished *Cxcl7* expression. The role of CXCL7 in SMC-endothelial cells communication was demonstrated by blocking CXCL7 or its receptor CXCR2, both significantly inhibited endothelial wound closure. Furthermore, insertion of a *Cxcl7* cDNA in the lentiviral vector that carries a *Prkcd* shRNA overcame the adverse effects of *Prkcd* knockdown on reendothelialization.**

Conclusions: **SMCs promote reendothelialization in a PKCδ-dependent paracrine mechanism, likely through CXCL7-mediated recruitment of endothelial cells from uninjured endothelium. (*Circ Res*. 2019;124:1253-1265. DOI: 10.1161/CIRCRESAHA.118.314567.)**

Key Words: chemokines, CXC ■ endothelial cells ■ muscle, smooth ■ protein kinase C-delta ■ regeneration ■ STAT3 transcription factor

A healthy endothelium is necessary for vascular homeostasis and blood flow. By the production of bioactive substances, endothelial cells (ECs) regulate vascular tone, proliferative state of the underlying vascular smooth muscle cells (SMCs) and maintain a nonthrombogenic blood-tissue interface that has a limited permeability.[1] Damaged or dysfunctional endothelium drives vascular inflammation through expression of adhesion molecules and enhanced permeability to leukocytes.[2] In addition, endothelial injury diminishes the production of nitric oxide, a potent inhibitor of SMC proliferation, and induces intimal hyperplasia (IH).[3] Thus, endothelial dysfunction plays a key role in various vascular diseases, such as atherosclerotic cardiovascular disease, the leading cause of death worldwide.[4,5] Angioplasty followed by stenting

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Novelty and Significance

What Is Known?

- Timely endothelial regeneration or reendothelialization after vascular injury prevents thrombotic events and restrains the development of intimal hyperplasia. Intimal hyperplasia contributes to the renarrowing of treated vessels and therefore hampers the long-term success of vascular interventions.
- Smooth muscle cells (SMCs) respond to injury with functional changes including proliferation, migration, and secretion of chemokines and cytokines.
- PKCδ (protein kinase C-δ) is a crucial stress-response gene that is up-regulated and activated after injury. PKCδ plays a critical role in the regulation of SMC apoptosis and chemokine production.

What New Information Does This Article Contribute?

- SMCs can regulate reendothelialization through a paracrine mechanism that requires PKCδ.
- Mice with SMC-specific *Prkcd* deficiency display impaired reendothelialization and develop greater intimal hyperplasia after femoral artery wire injury. Conversely, SMC-specific gene delivery of PKCδ accelerates reendothelialization.
- Paracrine signals from high PKCδ expressing SMCs promote endothelial wound closure but have minimal effect on endothelial cell proliferation.
- PKCδ activation in SMCs increases the expression of CXCR2 (chemokine [C-X-C motif] receptor 2) ligands CXCL1 (chemokine [C-X-C motif] ligand 1) and CXCL7 (chemokine [C-X-C motif] ligand 7) through STAT3 (signal transducer and activator of transcription 3), likely by increasing STAT3 phosphorylation at Serine 727.
- In the context of SMC-specific *Prkcd* deficiency, restoration of *Cxcl7* expression rescues the defective reendothelialization.

Injury to the endothelium occurs during vascular interventions, including balloon angioplasty and stenting. Current therapies to prevent restenosis primarily focus on inhibiting SMCs. It is generally believed that SMCs undergo a phenotypic change in response to injury associated with vascular surgical procedures. Great advances have been made in identifying the molecular mechanisms involved in modulating the SMC phenotypic switch. Less is known about the crosstalk between SMCs and other types of cells. In this article, we report that injured SMCs promote reendothelialization by attracting endothelial cells to the site of injury through a PKCδ-dependent paracrine mechanism. Mechanistically, activation of PKCδ upregulates the expression of the CXCR2 ligands CXCL1 and CXCL7 in SMCs. Regulation of *Cxcl7* by PKCδ in SMCs depends on STAT3, but not STAT1, likely through phosphorylation of STAT3 at Serine 727. The delayed reendothelialization in *Prkcd* deficient arteries can be rescued by restoration of CXCL7. These results, along with our previous findings, demonstrate the ability of SMCs to communicate with other cell types in the vascular wall as a part of a coordinated vascular injury response. In-depth understanding of cell-cell crosstalk expands our knowledge of the vascular injury response and promotes therapeutic development.

Nonstandard Abbreviations and Acronyms

ADAMTS7	a disintegrin and metalloproteinase with thrombospondin motifs 7
AdPKCδ	adenoviral vector expressing *Prkcd* gene
CXCL1	chemokine (C-X-C motif) ligand 1
CXCL7	chemokine (C-X-C motif) ligand 7
CXCR2	chemokine (C-X-C Motif) receptor 2
EC	endothelial cell
EGFP	enhanced green fluorescent protein
IH	intimal hyperplasia
MCP-1	monocyte chemoattractant protein-1
PKCδ	protein kinase C-δ
SMC	smooth muscle cell
SM-MHC	smooth muscle-myosin heavy chain
STAT	signal transducer and activator of transcription
TNFα	tumor necrosis factor-α
vWF	von Willebrand factor

effectively restores blood flow by compressing atherosclerotic plaques against the artery wall. However, this procedure causes regional damage to endothelium as well as SMCs and triggers the development of restenosis, which hampers the long-term success of vascular interventions.[6,7] Endothelial regeneration or reendothelialization correlates inversely with the growth of intimal lesion.[8] Furthermore, improved reendothelialization prevents thrombotic events and IH triggered by vascular interventions.[9,10] The rate of reendothelialization is thus critical in vascular repair.

Reendothelialization after arterial injury involves multiple types of resident vascular cells and circulating cells in a coordinated fashion.[11] Denuded endothelium is immediately covered by a layer of platelets and leukocytes.[12] In the hours after arterial injury, resident ECs surrounding damaged arterial endothelium rapidly enter the replication cycle to restore endothelial continuity.[12] Arrays of arterial injury studies demonstrate that the damaged endothelium is repaired by proliferation and migration of ECs in regions bordering the denuded area.[13–16] Experimental manipulations that inhibit endothelial proliferation such as gene deletion of endothelial microRNA-126 impair reendothelialization after denudation.[17] Conversely, administration of microRNA-126-5p or deletion of genes that negatively regulate EC proliferation/migration, such as ADAMTS7 (a disintegrin and metalloproteinase with thrombospondin motifs 7), promote endothelial repair and limit the growth of atherosclerotic plaques.[17,18]

As a major component of the arterial wall, SMCs are critical in orchestrating vascular injury response. Vascular injury induces a phenotypic switch of SMCs toward the synthetic phenotype characterized by increased rate of proliferation, migration, and synthesis of extracellular matrix components.[6] This phenotypic switch is generally believed to underlie the development of IH.[7] Proliferation and migration of synthetic SMCs are further powered by the loss of endothelial inhibition until endothelium is functionally recovered. Numerous studies have shown that SMCs are an important source of cytokines and chemokines in the vessel wall.[19–22] Recent data from our laboratory demonstrated that injured medial SMCs

signal to adventitial fibroblasts via the PKCδ (protein kinase C-δ)- mediated release of MCP-1 (monocyte chemoattractant protein-1).[23] Whether and how injured SMCs may influence endothelial repair remains largely unknown.

PKCδ is a member of the PKC family of serine-threonine kinases. Expressed by all major vascular cell types, PKCδ plays an essential role in the regulation of multiple cellular functions such as apoptosis and chemokine expression.[22,24] We have previously reported that levels of PKCδ are elevated in human restenotic lesions as well as balloon-injured rat carotid arteries.[25] Mice deficient in *Prkcd* develop exacerbated injury- and vein graft-related IH, which is associated with diminished medial SMC apoptosis.[25,26] Conversely, gene transfer of *Prkcd* to balloon-injured carotid arteries inhibits IH, which is associated with a profound upregulation of apoptotic activity within medial SMCs.[25] In cultured SMCs, PKCδ promotes cytokine expression and apoptosis but suppresses proliferation and migration.[27,28] In ECs, PKCδ is necessary for proliferation and migration.[29] Bai et al[29] showed the exaggerated neointimal lesions found in *Prkcd* gene-deficient mice are in part caused by compromised EC proliferation and migration during the reendothelialization process after arterial injury. In this current study, we report that SMC-specific upregulation of PKCδ promotes reendothelialization. Specifically, SMCs facilitate EC recovery in a PKCδ dependent manner, likely in part involving the release of CXCR2 (chemokine [C-X-C Motif] receptor 2) ligands which promotes EC migration.

Methods

The authors declare that all supporting data are available within the article and its Online Data Supplement. Detailed Methods are available in the Online Data Supplement.

Animal Models

All animal procedures were performed under protocols approved by the Institute Animal Care and Use Committee at the University of Wisconsin-Madison (No. M02285/M005894) and conformed to the Guide for the Care and Use of Laboratory Animals. The inducible SMC-specific *Prkcd* knockout mice were generated by breeding *Prkcd*$^{fl/fl}$ mice with Myh11-CreER^{T2} mice in which Myh11 (myosin heavy chain 11) promoter drives expression of a fusion protein that consists of Cre recombinase (Cre) fused to a mutated ligand-binding domain of the human ER (estrogen receptor). The congenic *Prkcd*$^{fl/fl}$ mice on a predominantly C57BL/6 background were generated by Bezy et al[30] (a kind gift from Dr C. Ronald Kahn at Harvard Medical School). The Myh11-CreER^{T2} mice were obtained from The Jackson Laboratory (Stock No. 019079) in which the expression of Cre-ER^{T2} recombinase is driven by smooth muscle-myosin heavy chain promoter and is activated by tamoxifen. Eight to 12 weeks male Myh11-CreER^{T2}-*Prkcd*$^{wt/wt}$, Myh11-CreER^{T2}-*Prkcd*$^{fl/fl}$, and *Prkcd*$^{fl/fl}$ mice received tamoxifen (75 mg/kg per day, i.p.) for 5 consecutive days. Three weeks after tamoxifen injections, mouse femoral artery wire injury was performed blindly as described previously.[31] Evans blue staining was performed on 7 days post wire-injury and arteries were harvested and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). Arterial denudation was performed in male Sprague-Dawley rats (8–12 weeks) obtained from Charles River Laboratories through carotid balloon angioplasty as described before.[25,32] Then, rats were randomly assigned to different groups. Gene transfer to medial SMCs was achieved by intraluminal perfusion with adenoviral vectors or lentiviral vectors within the injured segment after balloon-injury as described previously.[25] A sham group underwent surgery without balloon angioplasty/viral infection. Evans blue staining was performed on 14 days or 21 days post wire-injury and arteries were harvested and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). Evans blue staining was performed on 14 days or 21 days postinjury and arteries were harvested and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). Reendothelialization was determined by the percentage of Evans blue negative area over the total denuded areas.

Cell Culture

Primary SMCs were isolated from rat carotid arteries according to a method described previously[33] and maintained in DMEM supplemented with 10% fetal bovine serum, and penicillin-streptomycin. Mouse aortic ECs were isolated from C57BL/6J immorto mice as described previously using antiCD31 conjugated magnetic beads.[34] ECs were grown on gelatin-coated dishes in DMEM containing 10% fetal bovine serum, 2 mmol/L L-glutamine, 2 mmol/L sodium pyruvate, 20 mmol/L HEPES, 1% nonessential amino acids, 100 μg/mL streptomycin, 100 U/mL penicillin, freshly added heparin at 55 U/mL (Sigma, St. Louis, MO), endothelial growth supplement 100 μg/mL (Sigma), and the murine recombinant interferon-γ (R&D, Minneapolis, MN) at 44 U/mL. The endothelial identity was confirmed by fluorescence-activated cell sorting analysis for CD31, VE-cadherin (vascular endothelial cadherin), and B4-lectin.

Immunostaining

Immunostaining was performed on cryosections of 4% paraformaldehyde fixed arteries with the indicated antibodies. Quantification of immunostaining was performed using ImageJ Software (National Institutes of Health, Bethesda, MD).

Statistical Analysis

Results are presented as mean±SEM. Data were assessed for normality using the Shapiro-Wilk normality test. Data not exhibiting a normal distribution were log2-transformed and retested for normality. Two-tailed Student *t* test for normally distributed data and Mann–Whitney nonparametric test for skewed data that remained deviate from normality after transformation were used to compare between 2 conditions. One-way ANOVA with Tukey post hoc test for normally distributed data and Kruskal–Wallis nonparametric test for skewed data after transformation were used to compare ≥3 means. Statistical analyses were performed with GraphPad Prism 7.0 (GraphPad Software, Inc, San Diego, CA). Experiments were repeated as indicated. Differences with $P<0.05$ were considered statistically significant.

Results

Inhibition of PKCδ in SMCs Impairs Endothelial Regeneration and Increases IH

We have previously reported that levels of PKCδ protein are upregulated in the arterial wall after injury.[25] To delineate the function of PKCδ specifically in SMCs, we bred Myh11-CreER^{T2} mice with *Prkcd*$^{fl/fl}$ mice to generate a tamoxifen-inducible *Prkcd* gene deficiency in vascular SMCs. Five-day continuous injection of tamoxifen (75 mg/kg per day, IP) was administered to all mice, which produced a ≈70% reduction of arterial PKCδ levels in Myh11-CreER^{T2}-*Prkcd*$^{fl/fl}$ mice as compared with *Prkcd*$^{fl/fl}$ or Myh11-CreER^{T2}-*Prkcd*$^{wt/wt}$ mice (Figure 1A). Three weeks after tamoxifen injection, we performed wire injury in mice of the 3 genotypes (Figure 1B). The procedure denudes the endothelium and triggers a regeneration process that reaches completion at least 7 days after wire injury.[35,36] We, therefore, euthanized the mice 7 days after injury to evaluate endothelial regeneration using en face staining with Evans blue dye. Because Evans blue is excluded by uninjured or regenerated functional endothelium,[37] the dye-free area of denuded arterial segments reflects the degree of endothelial repair. Deletion of PKCδ in SMCs (Myh11-CreER^{T2}-*Prkcd*$^{fl/fl}$ mice) showed 41.90±2.36%

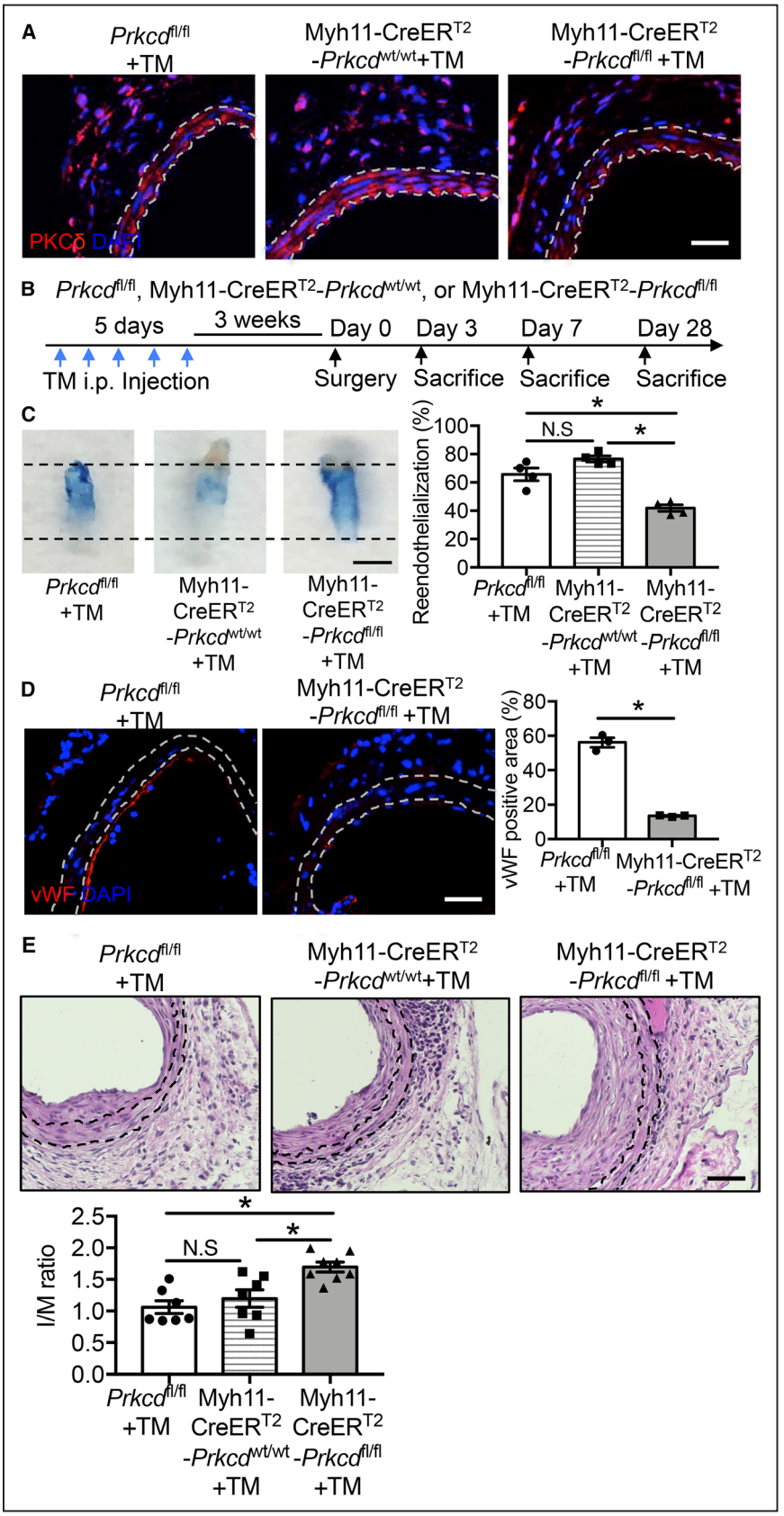

Figure 1. PKCδ (protein kinase C-δ) signaling in smooth muscle cells (SMCs) regulates reendothelialization. **A**, Representative images of uninjured femoral arteries harvested from *Prkcd*$^{fl/fl}$ mice, Myh11-CreER^{T2}-*Prkcd*$^{wt/wt}$, and Myh11-CreER^{T2}-*Prkcd*$^{fl/fl}$ mice 3 wk after tamoxifen (TM) injection. Sections were immunostained for PKCδ (red). Nuclei were stained with DAPI (blue). The locations of the internal and external elastic lamina defining the boundaries of the media are shown as white dashed lines. Scale bar=50 μm. **B**, Schematic design of wire injury study. **C**, Representative Evans blue dye-stained femoral arteries harvested 7 days after wire-injury to *Prkcd*$^{fl/fl}$+TM, Myh11-CreER^{T2}-*Prkcd*$^{wt/wt}$+TM, and Myh11-CreER^{T2}-*Prkcd*$^{fl/fl}$+TM mice. Boundaries of injured areas are indicated by dashed lines. Reendothelialization was determined by the percentage of Evans blue negative area over the total injured area using ImageJ software. Scale bar=1 mm. Results are expressed as mean±SEM. n=4, **P*<0.05, 1-way ANOVA. **D**, Representative images and quantifications of wire-injured femoral arteries harvested from *Prkcd*$^{fl/fl}$ mice and Myh11-CreER^{T2}-*Prkcd*$^{fl/fl}$ mice 3 days postinjury. Sections were immunostained for vWF (red). Nuclei were stained with DAPI (blue). The locations of the internal and external elastic lamina defining boundaries of the media are shown as white dashed lines. Scale bar=50 μm. Results are expressed as mean±SEM. n=3, **P*<0.05, 2-tailed Student *t* test. **E**, Representative images and quantifications of wire-injured femoral arteries harvested from *Prkcd*$^{fl/fl}$, Myh11-CreER^{T2}-*Prkcd*$^{wt/wt}$, and Myh11-CreER^{T2}-*Prkcd*$^{fl/fl}$ mice 28 days postinjury. Sections were immunostained for Haemotoxylin and Eosin (H&E). The locations of the internal and external elastic lamina defining boundaries of the media are shown as black dashed lines. Scale bar=50 μm. The intima area to media area ratio (I/M ratio) was measured as described in the Methods. Results are expressed as mean±SEM. n=7–8, **P*<0.05, 1-way ANOVA. Myh11-CreERT2 indicates Myh11 (myosin heavy chain 11) promoter drives expression of a fusion protein that consists of Cre recombinase (Cre) fused to a mutated ligand-binding domain of the human ER (estrogen receptor).

reendothelialization, which was significantly less compared with 65.66±4.46% and 76.56±2.18% in *Prkcd*$^{fl/fl}$ and Myh11-CreER^{T2}-*Prkcd*$^{wt/wt}$ mice, respectively (Figure 1C). Consistently, the injured segment of Myh11-CreER^{T2}-*Prkcd*$^{fl/fl}$ mice showed significantly diminished vWF (von Willebrand factor) positivity compared with *Prkcd*$^{fl/fl}$ mice (positive area:

13.48±0.29% versus 56.14±2.68%, respectively; Figure 1D). Together, these data suggest that SMC-specific loss of PKCδ impedes endothelial regeneration. In response to wire injury, mice of all 3 genotypes developed IH. However, the delayed reendothelialization in Myh11-CreER^{T2}-*Prkcd*$^{fl/fl}$ mice was associated with significantly larger IH. The intima/media ratio measured 28 days after injury was 59.72% and 41.76% higher in Myh11-CreER^{T2}-*Prkcd*$^{fl/fl}$ mice than *Prkcd*$^{fl/fl}$ and Myh11-CreER^{T2}-*Prkcd*$^{wt/wt}$ mice, respectively (Figure 1E).

To investigate whether PKCδ plays a similar role in rat endothelial regeneration, we employed the carotid balloon injury model, in which reendothelialization typically requires 28 days to complete.[37,38] We constructed a lentiviral vector to express a *Prkcd* short hairpin RNA (shRNA) driven by a chimeric promoter (enSM22αgc) that contains an SM-MHC (smooth muscle-myosin heavy chain) enhancer and a modified SM22α (smooth muscle protein 22-α) promoter whose G/C-rich *cis*-element is deleted (Lenti-enSM22αgc-ZsGreen-IRES-shPKCδ) (Figure 2A). The chimeric promoter has been reported in the literature to direct SMC-specific gene expression.[39] The G/C-rich *cis*-element mediates injury-induced downregulation of SM22α promoter, therefore the removal of this repressive element renders higher promoter activity in dedifferentiated SMCs.[40] The specificity of the lentivirus was tested in cultured SMCs, ECs, and adventitial cells. Contrasting to the ubiquitous activity of cytomegalovirus-promoter, enSM22αgc promoter selectively drove gene expression in SMCs (Figure 2B through 2D). Furthermore, the enSM22αgc promoter produced a comparable PKCδ knockdown as the cytomegalovirus promoter (Figure 2E).

The lentiviruses were delivered to intact or balloon-injured carotid arteries through intraluminal perfusion (Online Figure IA). It is believed that the intact ECs, as well as base membrane, prevent viruses from penetrating deep into the vascular wall.[41–43] Therefore, viral vectors delivered intraluminally primarily transduced intima and consequently produced weak transgene expression in medial and adventitia region. As expected, cytomegalovirus-promoter drove

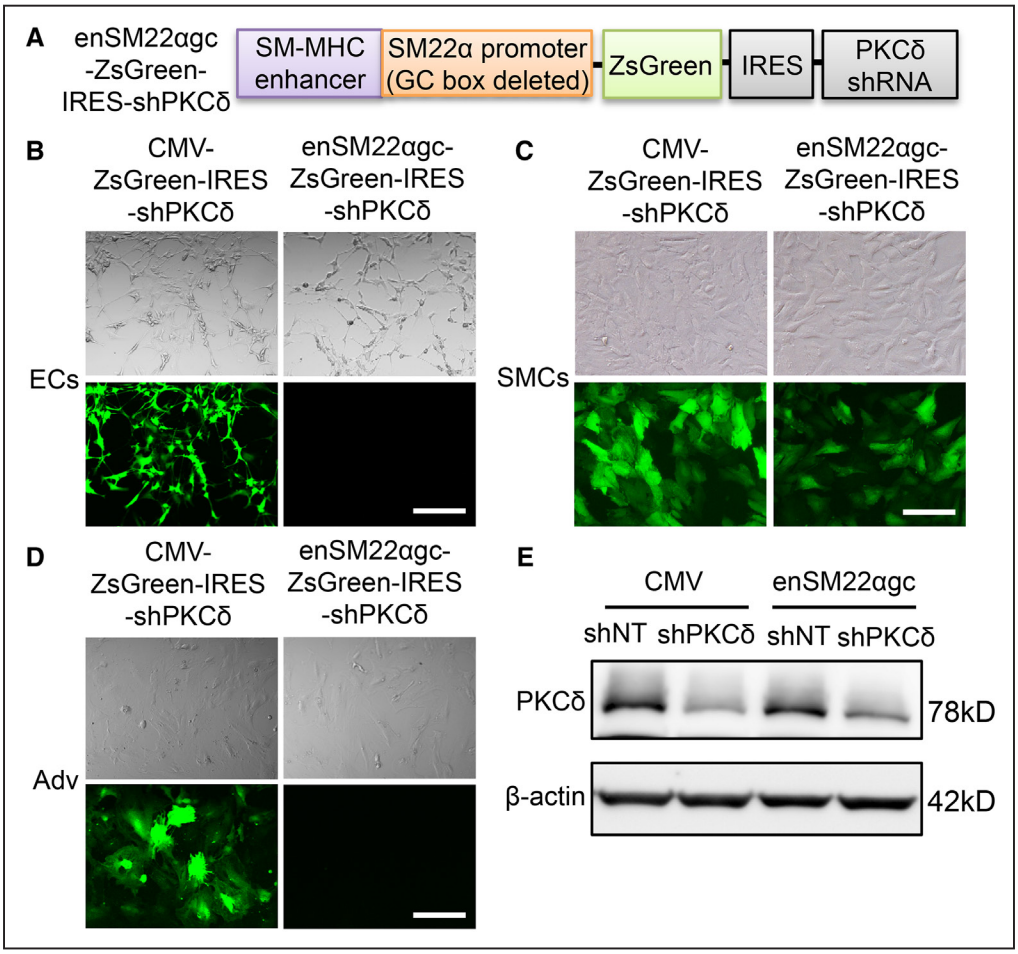

Figure 2. enSM22αgc promoter mediates smooth muscle cell (SMC)-specific gene expression. **A**, Schematic design of SMC-specific PKCδ (protein kinase C-δ) shRNA expressing vector. ZsGreen cDNA and PKCδ shRNA are driven by a chimeric promoter (enSM22αgc) that contains an SM-MHC (smooth muscle myosin heavy chain) enhancer and a modified SM22α (smooth muscle protein 22-α) promoter whose G/C-rich *cis*-element is deleted. **B–D**, enSM22αgc mediates gene expression specifically in SMCs (**C**), but not endothelial cells (ECs; **B**) and adventitial fibroblasts (Adv; **D**). Cells were infected with either the Lenti- cytomegalovirus (CMV)-ZsGreen-IRES-shPKCδ or the Lenti-enSM22αgc-ZsGreen-IRES-shPKCδ lentiviral particles. Bright-field (**upper**) and fluorescence (**bottom**) images of cells were taken 72 h after transduction. Scale bar=50 μm. **E**, SMCs were transduced with lentivirus expressing nontargeting shRNA (shNT) or PKCδ shRNA (shPKCδ) under the indicated promoter. After 72 h transduction, cells were harvested and the whole-cell lysates were subjected to immunoblot analysis. CMV indicates cytomegalovirus; enSM22αgc, a chimeric promoter that contains an SM-MHC enhancer and a modified SM22α (smooth muscle protein 22-α) promoter whose G/C-rich cis-element is deleted; IRES, internal ribosome entry site; shNT, nontargeting shRNA; and shPKCδ, shRNA against PKCδ.

transgene expression in ECs, SMCs, and adventitial cells (Figure 3A). In contrast, the enSM22αgc promoter was active in SMCs but not in ECs or adventitial cells (Figure 3A). Furthermore, enSM22αgc-driven shPKCδ produced downregulation of PKCδ in tunica media of injured arteries (Figure 3B). When evaluated 21 days postinjury, a late stage of reendothelialization after angioplasty in rats,[44] arteries treated with *Prkcd* shRNA had a significantly smaller dye-free area than the nontargeting shRNA treated arteries (41.31±6.54% versus 70.31±5.97%; Figure 3C), indicating an impairment in endothelial recovery.

Gene Transfer of PKCδ to SMCs Promotes Endothelial Regeneration

Using a cytomegalovirus-driven adenovirus we constructed previously,[25] we replicated the previous finding that *Prkcd* gene transfer to a balloon-injured rat carotid artery significantly reduced the neointimal formation (Online Figure IB). Comparing with AdNull (empty vector), intraluminal delivery of AdPKCδ (adenoviral vector expressing *Prkcd* gene) increased the intimal area positive of vWF and VE-cadherin from 7.92±0.71% to 40.97±4.03% and 16.73±1.01% to 31.05±3.04%, respectively (Online Figure IC and ID). Since viral vectors delivered intraluminal predominately infect the medial cells of injured vessels,[23,32] we postulated that AdPKCδ affects endothelial repair indirectly, likely through a mechanism mediated by SMCs. To test this hypothesis more rigorously, we constructed a new adenoviral vector that expresses PKCδ under the chimeric enSM22α promoter (Figure 4A). Similar to what we showed with the lentivirus, enSM22α-containing adenoviruses drove EGFP (enhanced green fluorescent protein) expression in SMCs but not in ECs or adventitial cells (Figure 4B and Online Figure II). The en face Evens blue assay showed that AdenSM22α-PKCδ accelerated the endothelium restoration from 25.38±7.52% to 59.60±5.01% 14 days postinjury (Figure 4C).

SMCs Influence Endothelial Functions Through a PKCδ-Mediated Paracrine Mechanism

Regeneration of the endothelium after denudation involves migration and proliferation of ECs residing at the border zones adjacent to injured area.[14,16] Using an in vitro endothelial

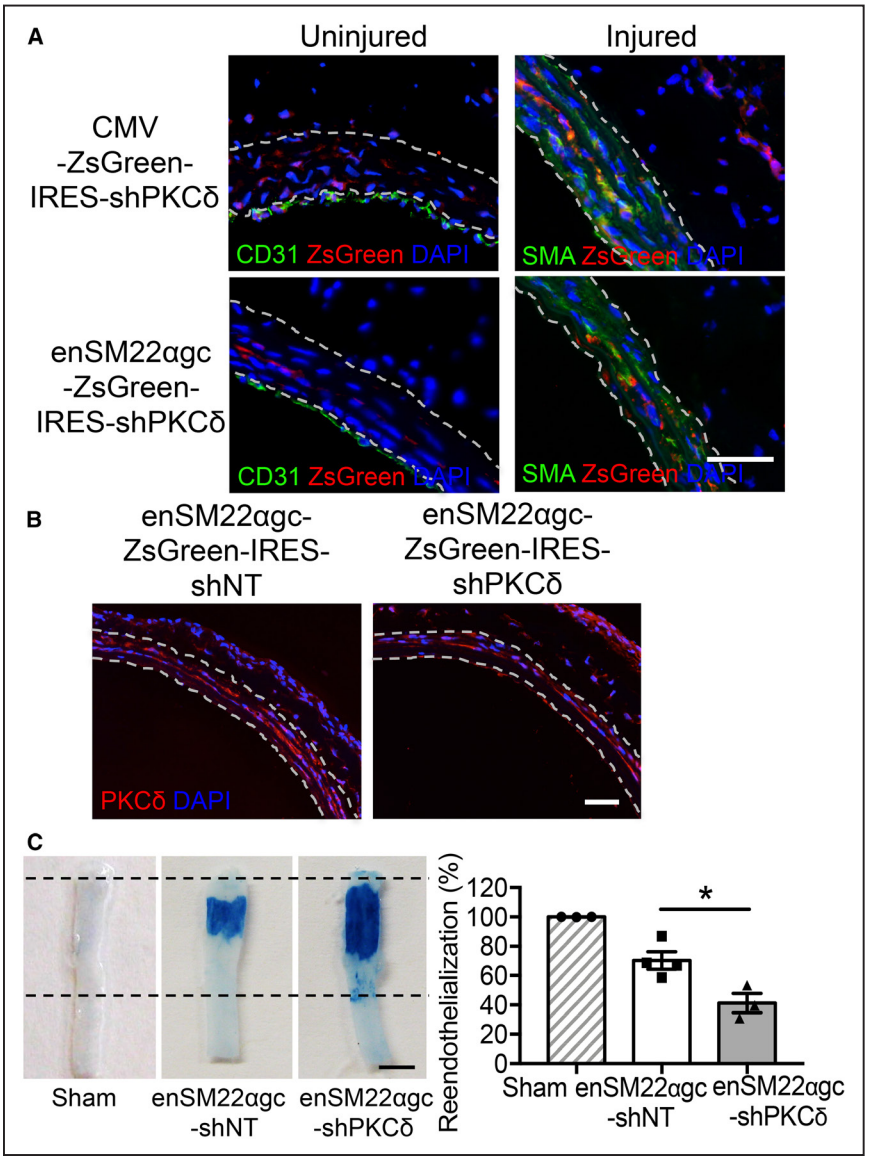

Figure 3. Smooth muscle cell (SMC)-specific knockdown of PKCδ (protein kinase C-δ) delays reendothelialization. A, Representative images of rat carotid cross-sections harvested 2 days after intraluminal perfusion with indicated lentiviral vectors. Sections were coimmunostained for CD31 (green) and ZsGreen (red) or SMA (green) and ZsGreen (red). Nuclei were stained with DAPI (blue). The locations of the internal and external elastic lamina defining boundaries of the media are shown as white dashed lines. Scale bar=50 μm. **B**, Representative images of rat carotid cross-sections harvested 3 days after balloon-injury followed by intraluminal perfusion with indicated lentiviral vectors. Sections were immunostained for PKCδ (red). Nuclei were stained with DAPI (blue). The locations of the internal and external elastic lamina defining boundaries of the media are shown as white dashed lines. Scale bar=50 μm. **C**, Representative Evans blue dye-stained carotid arteries harvested 21 days after angioplasty from sham, injured and Lenti-enSM22αgc-nontargeting shRNA treated, or injured and Lenti-enSM22αgc-shPKCδ-treated rats. Boundaries of the injured areas are indicated by dashed lines. Reendothelialization was quantitatively expressed by the percentage of Evans blue negative area over the total injured area using ImageJ software. Scale bar=3 mm. Results are expressed as mean±SEM. n=3–4, **P*<0.05, 1-way ANOVA. CMV indicates cytomegalovirus; enSM22αgc, a chimeric promoter that contains an SM-MHC (smooth muscle myosin heavy chain) enhancer and a modified SM22α (smooth muscle protein 22-α) promoter whose G/C-rich cis-element is deleted; IRES, internal ribosome entry site; shNT, nontargeting shRNA; and shPKCδ, shRNA against PKCδ.

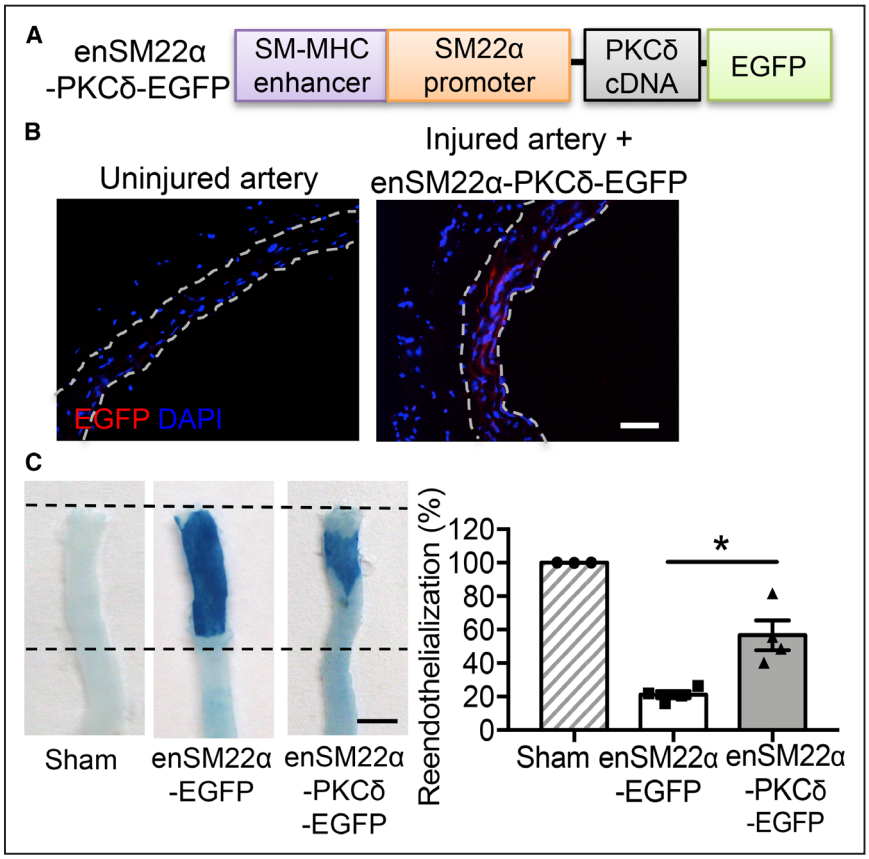

Figure 4. Smooth muscle cell (SMC)-specific overexpression of PKCδ (protein kinase C-δ) accelerates reendothelialization. A, Schematic design of SMC-specific PKCδ expression vector. Expression of PKCδ cDNA and EGFP (enhanced green fluorescent protein) cDNA is driven by a chimeric promoter (enSM22α) that contains an SM-MHC (smooth muscle myosin heavy chain) enhancer and the SM22α (smooth muscle protein 22-α) promoter. **B**, Representative images of uninjured carotid arteries or AdenSM22α-PKCδ-EGFP infected arteries. Sections were immunostained for EGFP. Nuclei were stained with DAPI (blue). The locations of the internal and external elastic lamina defining boundaries of the media are shown as white dashed lines. Scale bar=50 μm. **C**, Representative Evans blue-stained carotid arteries harvested 14 days after angioplasty from sham, injured AdenSM22α-EGFP-treated, or injured AdenSM22α-PKCδ-treated rats. Boundaries of the injured areas are indicated by the dashed lines. Reendothelialization was determined by the percentage of Evans blue negative area over the total injured area using ImageJ software. Scale bar=3 mm. Results are expressed as mean±SEM. n=3–4, *P<0.05, 1-way ANOVA.

wound closure model, we tested whether paracrine signals from SMCs influence EC proliferation or migration. To mimic the pathological environment, we titrated the dosages of PKCδ-expressing adenovirus (AdPKCδ) to upregulate PKCδ protein to a level comparable to the injury-associated elevation of PKCδ and incubated infected SMCs with TNFα (tumor necrosis factor-α; 1 ng/mL), a critical IH-associated physiological stimulus.[45–48] Medium conditioned by AdPKCδ-infected SMCs significantly promoted endothelial wound closure compared with medium conditioned by AdNull (empty vector)-infected SMCs (Figure 5A). Similar outcomes were produced with a low concentration of PKC activator phorbol-12-myristate-13-acetate (1 nmol/L) (Figure 5B), which alone has minimal effect on chemokine expression in SMCs.[22] To further characterize the paracrine function of PKCδ, we used the transwell assay and showed that medium conditioned by AdPKCδ-infected SMCs significantly induced migration of ECs (Figure 5C). EC proliferation, as well as viability, appeared to be similar regardless whether they were cultured in the medium conditioned by AdNull- or AdPKCδ-infected SMCs (Figure 5D and 5E).

CXCR2 Ligands Mediate Crosstalk Between SMCs and ECs

To search for a paracrine factor(s) that may mediate the crosstalk between SMCs and ECs, we conducted polymerase chain reaction arrays to identify PKCδ-dependent expression of cytokines and chemokines. SMCs were infected with AdNull, or AdPKCδ followed by incubation with phorbol-12-myristate-13-acetate (1 nmol/L). Compared with AdNull control, activation of PKCδ for 6 hours increased expression of *Ccl2*, *Ccl7*, *Cxcl16*, and *Cx3cl1* (Online Figure IIIA) in AdPKCδ-infected SMCs, which is consistent with our prior study.[22] However, the 48 hours activation resulted in a different expression profile. Since reendothelialization trails SMC apoptosis and other injury responses, we focused on factors that were uniquely upregulated at 48 hours in AdPKCδ-infected SMCs, particularly the 2 CXCR2 ligands CXCL1 (chemokine [C-X-C motif] ligand 1) and CXCL7 (chemokine [C-X-C motif] ligand 7); Online Figure IIIB). Consistent with the array results, PKCδ significantly upregulated *Cxcl7* and to a lesser extent *Cxcl1*. (Online Figure IIIC and IIID). In injured arteries, CXCL7 was readily detectable, and its levels were decreased by shPKCδ and increased by AdPKCδ, respectively (Figure 6A and 6B). Similar observations were made with CXCL1 (Online Figure IIIE).

To determine the role of CXCR2 ligands in the SMC-EC crosstalk, we added neutralizing antibodies against CXCL1 or CXCL7 to the EC wound healing assay. Neutralizing CXCL7 significantly attenuated the stimulatory effect of medium conditioned by AdPKCδ-infected SMCs on EC wound closure (Figure 6C and 6D). Also, preincubating ECs with an anti-CXCR2 antibody diminished the ability of EC to respond to AdPKCδ-infected SMCs conditioned medium (Figure 6C and 6D). Neutralizing CXCL1 produced a moderate reduction in EC wound closure, but this trend did not reach statistical significance (Figure 6C and 6D). Taken together, these results support that SMCs recruit ECs predominantly through a PKCδ-dependent release of CXCL7.

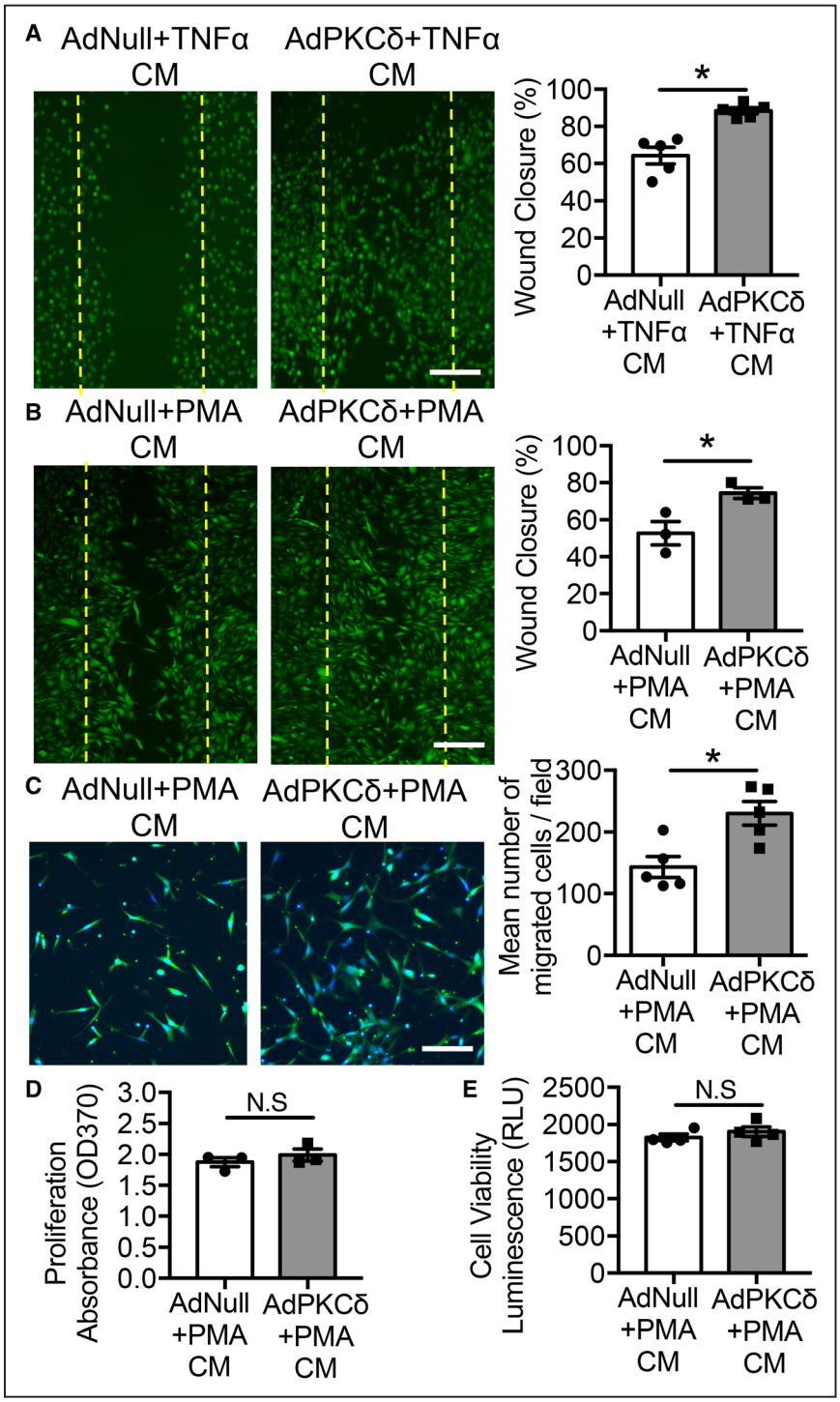

Figure 5. Smooth muscle cells (SMCs) regulate endothelial cell migration through a PKCδ (protein kinase C-δ)-mediated paracrine mechanism. **A** and **B**, Wound closure of endothelial monolayers grown in medium condition by AdNull- or AdPKCδ (adenoviral vector expressing *Prkcd* gene) -infected and TNFα (tumor necrosis factor-α; 1 ng/mL) or phorbol-12-myristate-13-acetate (PMA,1 nmol/L) treated SMCs. Endothelial cells (ECs) were labeled with 5-chloromethylfluorescein diacetate (CMFDA) green fluorescence dye for visualization. Dashed lines mark the wound edges generated by scratching. Wound closure was determined by the percentage of the recovered area over the total injured area using ImageJ software. Scale bar=50 μm. **C**, EC migration toward medium condition by AdNull-, or AdPKCδ-infected and PMA- (1 nmol/L) treated SMCs. ECs were labeled with CMFDA green fluorescence dye and counterstained with DAPI for visualization. Representative images of migrated cells are shown. Data are expressed as the mean number of migrated cells/fields±SEM. Scale bar=50 μm. **D** and **E**, Proliferation and viability of endothelial cells grew in medium conditioned by AdNull- and AdPKCδ-infected and PMA (1 nmol/L) treated SMCs were measured by BrdU incorporation and CellTiter-Glo luminescent cell viability assay, respectively. Results are expressed as mean±SEM. n=3–5, *P<0.05, 2-tailed Student $t$test.

PKCδ Regulates CXCL7 Expression in SMCs Through STAT3

Next, we sought to determine the mechanism underlying up-regulation of CXCL7 by PKCδ. In cultured rat carotid SMCs, TNFα increased *Cxcl7* mRNA levels in a PKCδ-dependent manner; the TNFα effect was diminished by shPKCδ but enhanced by AdPKCδ (Figure 7A and 7B). Since STAT1 (signal transducer and activator of transcription-1) and STAT3 play a critical role in regulating TNFα-mediated signaling,[49,50] we tested whether small interfering RNA (siRNA)–mediated knocking down of STAT1 and STAT3 affects *Cxcl7* expression in SMCs. Knockdown of STAT3 completely eliminated the effect of TNFα on *Cxcl7* mRNA expression (Figure 7C), whereas knockdown of STAT1 had minimal effects (Figure 7D). Furthermore, knockdown of STAT3 abolished the effect of AdPKCδ on *Cxcl7* expression (Figure 7E), suggesting PKCδ regulates *Cxcl7* through STAT3. The relationship between PKCδ and STAT3 was further demonstrated by examining STAT3 phosphorylation at Ser727, which regulates its transcriptional activation.[51,52] Treatment of SMCs with TNFα did not change the total level of STAT3, but rapidly increased STAT3 phosphorylation at

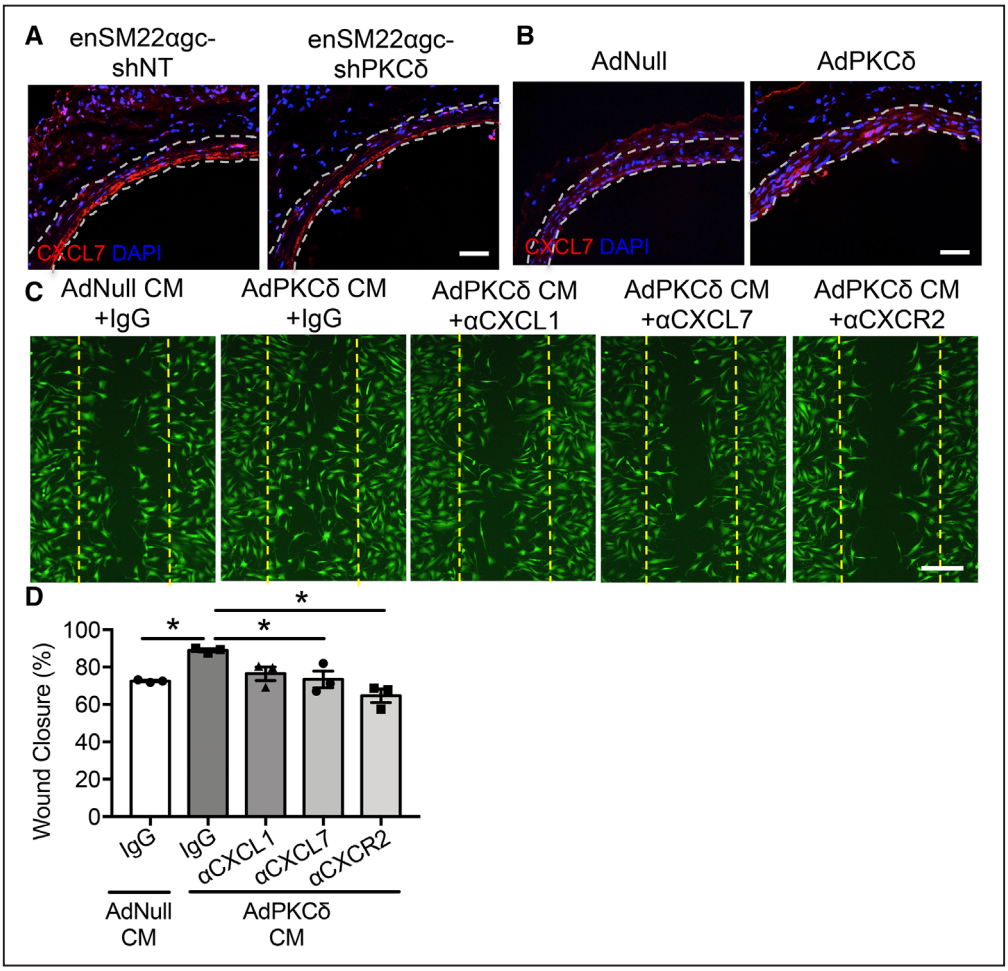

Figure 6. CXCR2 (chemokine [C-X-C Motif] receptor 2) ligands function downstream from PKCδ (protein kinase C-δ) in the recruitment of endothelial cells (ECs). A, Representative images of CXCL7 (chemokine [C-X-C motif] ligand 7) stained rat carotid cross-sections harvested from injured Lenti-enSM22αgc-nontargeting shRNA treated or injured Lenti-enSM22αgc-shPKCδ-treated rats (7 d post angioplasty). Nuclei were indicated by positive stains with DAPI (blue). The locations of the internal and external elastic lamina defining boundaries of the media are shown as white dashed lines. Scale bar=50 μm. **B**, Representative images of CXCL7 stained rat carotid cross-sections harvested 3 days after angioplasty from injured AdNull-treated or injured adenoviral vector expressing *Prkcd* gene (AdPKCδ) infected rats. Nuclei were indicated by positive stains with DAPI (blue). The locations of the internal and external elastic lamina defining boundaries of the media are shown as white dashed lines. Scale bar=50 μm. **C**, Wound closure of mouse ECs cultured in medium conditioned by AdNull- or AdPKCδ-infected and phorbol-12-myristate-13-acetate (PMA;1 nmol/L) treated SMCs in the presence of IgG, neutralizing antibody anti-CXCL1, anti-CXCL7, or anti-CXCR2. ECs were labeled with 5-chloromethylfluorescein diacetate (CMFDA) green fluorescence dye for visualization. Dashed lines indicate the cell-free gap right after the scratch. Scale bar=50 μm (**D**) Quantification of endothelial wound closure, determined by the percentage of the recovered area over the total injured area using ImageJ software. Results are expressed as mean±SEM. n=3, **P*<0.05, 1-way ANOVA.

Ser727 (Figure 7F and 7G). Ser727 phosphorylation was attenuated by knockdown of PKCδ but enhanced by overexpression of PKCδ (Figure 7F and 7G). Collectively, these results indicate the PKCδ regulates *Cxcl7* expression via STAT3 in SMCs, likely through modulating its phosphorylation at Ser727.

Restoring CXCL7 Expression in SMCs Rescues Reendothelialization

We reasoned that if SMCs facilitate endothelial repair through CXCL7, inserting the *Cxcl7* cDNA to the *Prkcd* shRNA lentiviral vector would overcome the adverse effect of *Prkcd* knockdown on endothelial regeneration (Figure 8A). Using the Evans blue dye exclusion assay, we demonstrated that the *Cxcl7* cDNA rescued reendothelialization (Evans blue negative area: *Prkcd* shRNA; 58.97±5.90% versus *Prkcd* shRNA+*Cxcl7* cDNA 90.45±5.03%, Figure 8B). In contrast, insertion of the *Cxcl1* cDNA produced a moderate effect on reendothelialization, which did not reach statistical significance (Figure 8B). Although restoration of CXCL7 in shPKCδ-treated arteries rescued endothelialization, it did not fully counter the adverse effect of PKCδ on intima/media ratio (Online Figure IVA and IVB). We speculate that CXCL7 is not responsible for all functions of PKCδ in injury response. Indeed, knocking down PKCδ caused apoptosis resistance in SMCs. However, the apoptosis resistance to H_2O_2 was similar between of the shPKCδ vectors with or without the *Cxcl7* cDNA (Online Figure IVC). Taken together, these results coupled with our previous findings suggest that PKCδ plays multiple roles in vascular injury response—acceleration of reendothelialization likely in part through upregulating CXCL7 and inhibition of intimal thickening through promoting SMC apoptosis[25]

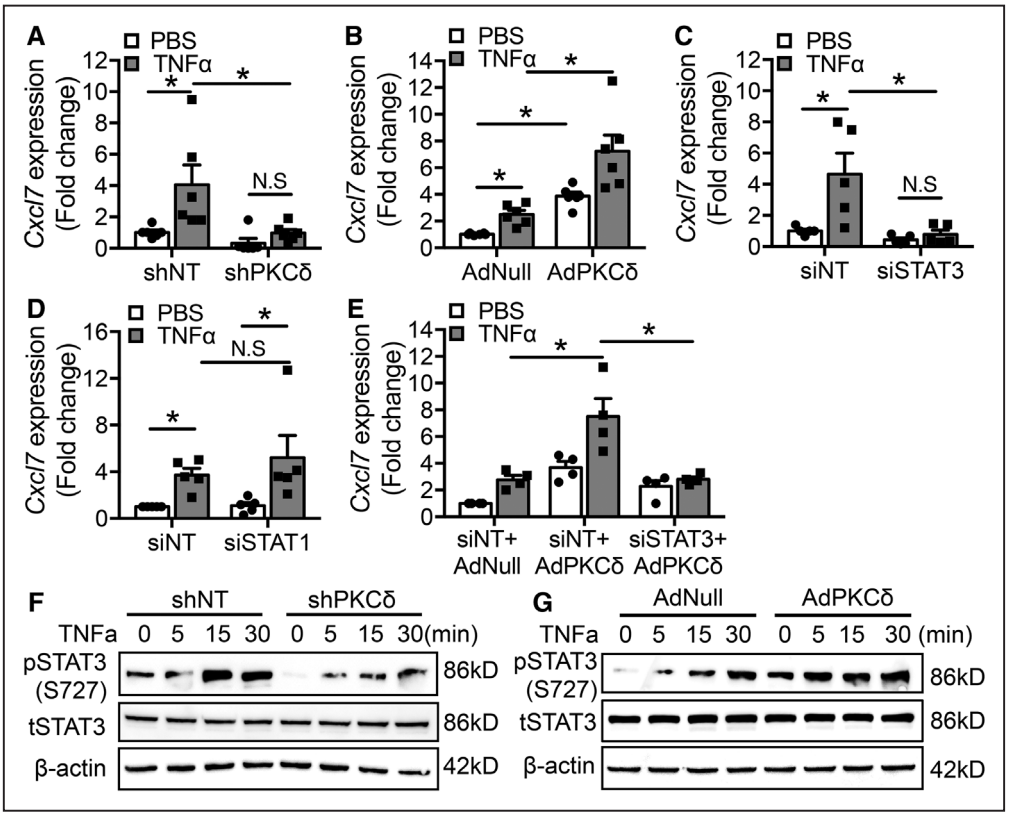

Figure 7. PKCδ (protein kinase C-δ) regulates Cxcl7 expression through STAT3 (signal transducer and activator of transcription 3). **A** and **B**, SMCs were infected with Lenti-nontargeting shRNA (shNT) or Lenti-PKCδ shRNA (shPKCδ) (**A**), AdNull or AdPKCδ (adenoviral vector expressing *Prkcd* gene) (**B**) followed by incubation with PBS or TNFα for 12 h. Levels of *Cxcl7* mRNA were analyzed using quantitative polymerase chain reaction (qPCR). Results are expressed as mean±SEM. n=6, **P*<0.05, 1-way ANOVA. **C** and **D**, SMCs were transduced with nontargeting siRNA (siNT), STAT3 siRNA (siSTAT3) (**C**), or STAT1 siRNA (siSTAT1) followed by incubation with PBS or TNFα for 12 h. Levels of *Cxcl7* mRNA expression were analyzed using qPCR. Results are expressed as mean±SEM. n=5, **P*<0.05, 1-way ANOVA. **E**, SMCs were transduced with indicated siRNA and adenoviral vectors followed by incubation with PBS or TNFα (tumor necrosis factor-α) for 12 h, *Cxcl7* mRNA expression was analyzed using qPCR. Results are expressed as mean±SEM. n=4, **P*<0.05, 1-way ANOVA. **F** and **G**, TNFα induced STAT3 S727 phosphorylation in SMCs infected with lentivirus shNT or shPKCδ (**F**) and in SMCs infected with adenovirus AdNull or AdPKCδ (**G**). Cells were harvested at the indicated time and whole-cell lysates were subjected to immunoblot analysis. n=3.

Discussion

The rate of restoring damaged endothelium inversely correlates with neointima formation in atherosclerosis and restenosis.[8–10] Our current study provides new mechanistic insights by demonstrating injured vascular SMCs play an active role in the reendothelialization process. Using a combination of gene therapy and conditional knockout approach, we demonstrated in rodent arterial injury models that SMCs facilitate endothelial regeneration in a PKCδ–dependent manner, likely in part through the release of CXCL7 which recruits ECs (Figure 8C). This novel SMC-to-EC communication, along with the established EC-to-SMC and SMC-fibroblast crosstalks, underscores the concept that different vascular cell types work in a coordinated fashion when responding to injury.

Our data further emphasize the complex roles of PKCδ in vascular injury response. Prior studies from our group and others established PKCδ as a central stress mediator in SMCs whose expression is markedly upregulated in human restenotic lesions.[25] Global deletion of *Prkcd* gene causes apoptosis resistance in the injured vessel wall thus increases the size of neointimal lesion.[25,26] The exacerbated IH in *Prkcd*$^{-/-}$ mice is accompanied by delayed reendothelialization as shown by Bai et al[29] in a mouse wire injury model. They attributed this phenotype to a defective migratory property of *Prkcd*$^{-/-}$ ECs.[29] Although our findings are consistent with Bai's study regarding the impeded endothelial repair, we provide a novel explanation that is different but not necessarily mutually exclusive from Bai's. Through SMC-specific knockdown and knockout of *Prkcd* gene in rats and mouse arteries, respectively, we explicitly illustrated inhibition of PKCδ in medial SMCs alone is sufficient to delay reendothelialization. However, because of technical difficulties associated with intraluminal delivery, we did not test whether restoring *Prkcd* expression rescues the endothelial phenotype of SMC-specific *Prkcd* knockout mice. In addition, no available assays allowing us to monitor EC migration in injured arteries. However, ectopic expression of PKCδ in medial SMCs of injured rat carotid arteries support the role of PKCδ in SMC-EC communication. This notion is further illustrated by in vitro studies in which medium conditioned by AdPKCδ-infected SMCs promoted migration of ECs.

The plasticity of vascular SMCs has been demonstrated in atherosclerosis, restenosis, hypertension as well as abdominal aortic aneurysm.[6,7,53] In response to numerous stimuli, vascular SMCs switch from a quiescent contractile state to more migratory, proliferative, synthetic, endocytic, phagocytic, or even osteoblastic phenotypes. The current work coupled with our previous studies highlights SMCs as an important source of chemokines.[22] Using conditioned medium as well as gene

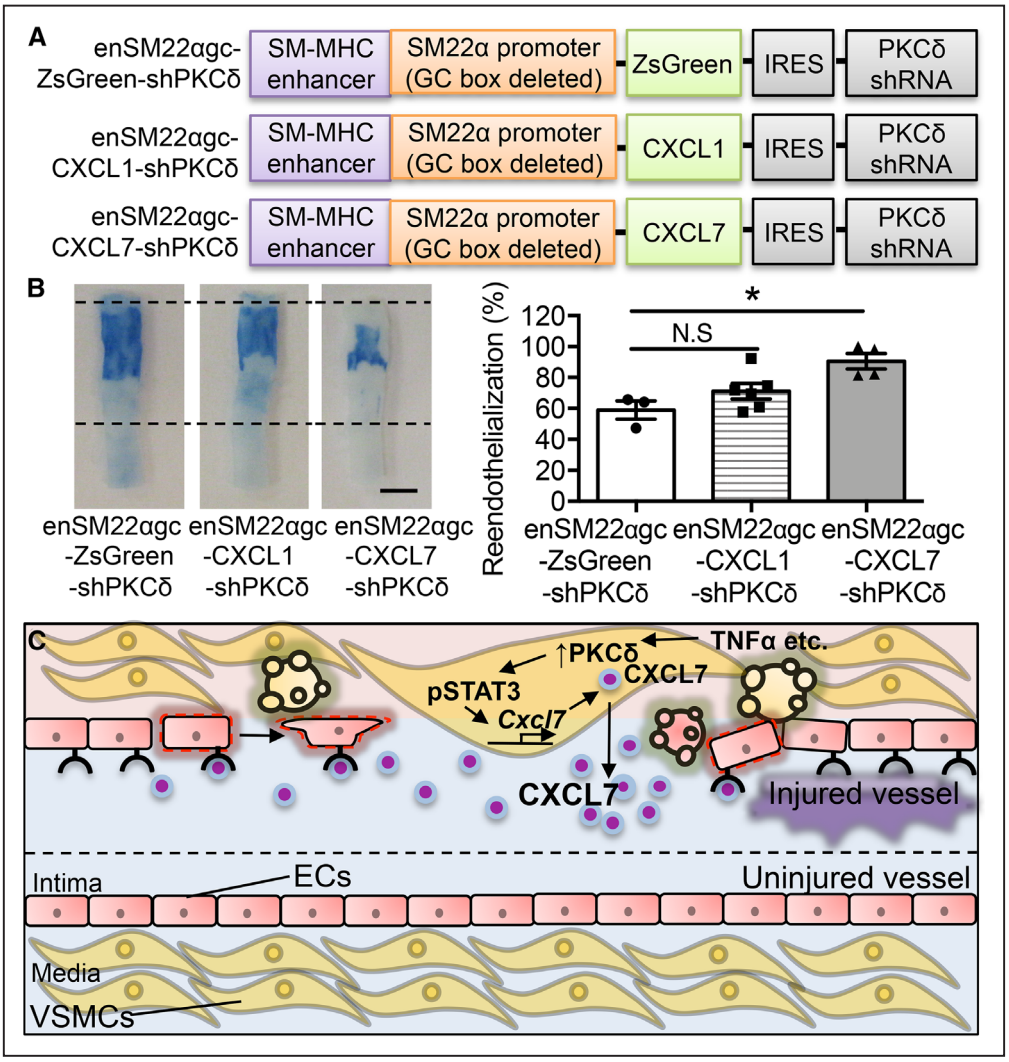

Figure 8. Restoration of CXCL7 (chemokine [C-X-C motif] ligand 7) rescues reendothelialization. **A**, Schematics of SMC-specific expression vectors expressing both PKCδ (protein kinase C-δ) shRNA and CXCR2 (chemokine [C-X-C Motif] receptor 2) ligand. **B**, Representative Evans blue dye-stained carotid arteries harvested 21 days after angioplasty from Lenti-enSM22αgc-ZsGreen-shPKCδ-, Lenti-enSM22αgc-CXCL1-shPKCδ-, and Lenti-enSM22αgc-CXCL7-shPKCδ-treated rats. Boundaries of the injured areas are indicated by the dashed lines. Reendothelialization was determined by the percentage of Evans blue negative area over the total injured area using ImageJ software. Scale bar=3 mm. Results are expressed as mean±SEM. n=3–6, *P<0.05, 1-way ANOVA. **C**, Proposed mechanisms through which SMCs facilitate endothelial regeneration. Vascular injury, which leads to endothelial denudation, increases PKCδ (↑ PKCδ). Activation of PKCδ causes SMC apoptosis and stimulates the production of paracrine factors including STAT3 (signal transducer and activator of transcription 3) activation-mediated CXCL7, which in turn triggers migration of neighboring ECs to the denuded region. EC indicates endothelial cells; enSM22αgc, a chimeric promoter that contains an SM-MHC (smooth muscle myosin heavy chain) enhancer and a modified SM22α (smooth muscle protein 22-α) promoter whose G/C-rich cis-element is deleted; IRES, internal ribosome entry site; shNT, nontargeting shRNA; shPKCδ, shRNA against PKCδ; and VSMC, vascular smooth muscle cells.

transfer approaches, we demonstrated both in vitro and in vivo that SMCs employ a PKCδ-dependent mechanism to produce chemokines. The observation that restoration of *Cxcl7* expression in *Prkcd* knockdown arteries rescued reendothelialization suggests the central role of this particular CXCR2 ligand. As such, the current study provides the first evidence that PKCδ upregulates CXCR2 ligands. CXCR2 plays an essential role in mediating migration of microvascular ECs in response to IL-8.[54,55] Liehn et al[56] showed that antibody blockade of CXCL1 inhibits endothelial recovery and enhances plaque formation in *ApoE*-deficient mice. Here, immunodepletion or genetic knockdown of CXCL7, but not CXCL1, markedly diminished EC wound closure, suggesting CXCL7 plays a predominant role in the PKCδ-mediated SMC-EC crosstalk. It is widely accepted that the regeneration of the endothelial lining is largely attributed to the migration and proliferation of neighboring cells. However, our data do not exclude the possibility that SMCs may influence endothelial repair through paracrine effects on circulating progenitors or progenitor-like cells.

Despite the important role of CXCL7 in vascular diseases,[57] the mechanism underlying CXCL7 regulation after arterial injury is rather poorly understood. Our results suggest the SMCs produce CXCL7 in a PKCδ-dependent manner in injured arteries. Vascular injury, caused by overstretching of an artery during vascular interventions, produces apoptotic bodies, necrotic cell debris, and increased expression of cytokines.[20] Among the injury-induced cytokines, TNFα has been implicated in vascular injury in patients who underwent

percutaneous coronary intervention as well as in preclinical models of restenosis.[45–47] Therefore, we use TNFα to activate PKCδ in vitro, although PKCδ is likely to be activated by multiple factors associated with injured vessels. We demonstrated that PKCδ regulates *Cxcl7* mRNA expression in cultured rat carotid SMCs. Interestingly, the regulation of *Cxcl7* by PKCδ appears to depend on STAT3, but not STAT1 although both have been implicated in PKCδ-mediated signaling.[58,59] Phosphorylation of STAT3 is linked to its transcriptional activation and function.[51,52] After TNFα stimulation, we observed that STAT3 was phosphorylated at Ser727, which is required for maximal activation by diverse stimuli.[60,61] Deficiency in PKCδ significantly attenuated Ser727 phosphorylation whereas overexpression of PKCδ enhanced TNFα-induced STAT3 Ser727 phosphorylation. Taken together, our results suggest PKCδ regulates CXCL7 through STAT3 in SMCs, likely by increasing STAT3 phosphorylation at Ser727.

In summary, the current study reveals a novel function of medial SMCs in vascular injury repair. Specifically, high expression of PKCδ in SMCs is required for rapid regeneration of denuded endothelium, likely in part through CXCR2 ligands-mediated EC migration.

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Disclosures

None.

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