SUPPLEMENTAL MATERIAL

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

Short Title: Ren. PKCδ accelerates endothelial regeneration

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Subject codes: Vascular Biology, Peripheral Vascular Disease, Endothelium, Restenosis

Detailed Methods

Reagents

Dulbecco's Modified Eagle's Medium (DMEM) and cell culture reagents were purchased from Gibco Life Technologies (Carlsbad, CA). Endothelial Basal Medium (EBM-2) and EGM-2 SingleQuot Supplement & Growth Factors were purchased from Lonza (Allendale, NJ). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (St Louis, MO). Cell Proliferation ELISA, BrdU (colorimetric) kit was purchased from Roche (Mannheim, Germany). CellTiter-Glo® Reagent was purchased from Promega (Madison, WI). Rat Cytokine and Chemokine PCR array was purchased from SABiosciences (Frederick, MD). Dulbecco's phosphate-buffered saline (PBS) were purchased from Gibco (Grand Island, NY). Other chemicals and reagents, if not specified, were purchased from Sigma-Aldrich.

Adenoviral vector construction, amplification, and titration

Adenoviral vectors were constructed using AdEas XL Adenoviral Vector System (Agilent Technologies, Santa Clara, CA) according to manufacturer's protocols. Briefly, CMV promoter in the pShuttle-CMV vector was replaced with a chimeric smooth muscle (SM)-specific enhancer/promoter composed of the rabbit SM-myosin heavy chain (SM-MHC) enhancer (-1332~-1225) and the SM22α promoter (-445~+65) [1]. The new vector was named pShuttle-enSM22α. PKCδ-EGFP and EGFP coding sequences were amplified from pEGFP-PKCδ (a kind gift from Dr. Mary Reyland at University of Colorado Denver) and inserted into the multiple cloning sites (MCSs) of pShuttle-enSM22α to generate pShuttle-enSM22α-PKCδ-EGFP and pShuttle-enSM22α-EGFP, respectively. The Shuttle vectors were transferred into the adenovirus genome by means of homologous recombination in BJ5183 E. coli to produce recombinant adenoviral plasmids. Then, the recombinant adenoviral plasmids were digested with PacI and transfected into the AD-293 adenovirus packaging cell line to pack recombinant adenoviral particles. Recombinant adenoviral particles were further amplified in AD-293 cells and purified by CsCl gradient centrifuging and titrated as described previously [2]. The specific of enSM22α-driven adenovirus was tested in endothelial cells (ECs), smooth muscle cells (SMCs), and adventitial fibroblasts (Adv). Specifically, ECs, SMCs, and Adv were infected with either AdCMV-lacZ or AdenSM22α-lacZ at the m.o.i. of $5x10^4$ in DMEM containing 2% FBS overnight. 48h after infection followed by 24h serum starvation, cells were fixed and stained for β-galactosidase activity (Genlantis, San Diego, CA).

Lentiviral vector construction, amplification, and titration

Lentiviral vectors that express shRNA against *Prkcd* (pZIP-CMV-ZsGreen-IRES-sh PKCδ) or non-targeting shRNA (pZIP-CMV-ZsGreen-IRES-NT) were purchased from TransOMIC (Huntsville, AL). To attenuate injured-induced downregulation of SM22α activity, a GC-rich region (5' -GGCCGCCC- 3') within SM22α promoter was deleted and the truncated promoter was named SM22αgc. The CMV promoter in pZIP vectors was replaced with enSM22αgc and generated pZIP-enSM22αgc-ZsGreen-IRES-shPKCδ and ZIP-enSM22αgc-ZsGreen-IRES-NT. To overexpress *Cxcl1* or *Cxcl7* and knockdown *Prkcd* simultaneously, cDNA of ZsGreen in the pZIP-enSM22αgc-ZsGreen-IRES-shPKCδ was replaced with *Cxcl1* or *Cxcl7* cDNA and generated pZIP-enSM22αgc-CXCL1-IRES-shPKCδ and pZIP-enSM22αgc-CXCL7-IRES-shPKCδ, respectively. To generate lentiviral particles, lentiviral vector (pZIP-enSM22αgc-ZsGreen-IRES-shPKCδ or ZIP-enSM22αgc-ZsGreen-IRES-NT), lentiviral packaging plasmid (psPAX2) and the VSV-G-expressing envelope vector (pMD2.G) were co-transfected into HEK-293T cells. After transfection, cell culture supernatant containing lentiviral particles were collected, pooled, and concentrated using Lenti-X Concentrator (Clontech Laboratories, Fitchburg, WI). The titer of concentrated lentiviral particles was determined using qPCR Lentivirus Titration Kit (ABM Inc., Richmond, BC). The specific of enSM22αgc-driven Lentivirus was tested in ECs, SMCs, and Adv. Specifically, ECs, SMCs, and Adv were infected with either Lenti-CMV-ZsGreen-IRES-shPKCδ or Lenti-enSM22αgc-ZsGreen-IRES-shPKCδ lentiviral particles at the m.o.i. of 500 in DMEM containing 2% FBS overnight. 48h after infection followed by 24h serum starvation, cells were imaged for ZsGreen

expression. To test knockdown efficacy, cells there harvested in RIPA buffer and the whole-cell lysates were subjected to immunoblot analysis.

SMC-specific *Prkcd* knockout mice and mouse femoral artery wire injury model

All experimental protocols were approved by the Institute Animal Care and Use Committee at University of Wisconsin-Madison (#M002285) and conformed to the Guide for the Care and Use of Laboratory Animals published by the NIH Publication No. 85-23, 1996 revision. The inducible SMC-specific and *Prkcd* knockout mice were generated by breeding *Prkcd*$^{fl/fl}$ mice with SMMHC-CreER^{T2} mice. The congenic *Prkcd*$^{fl/fl}$ mice on a predominantly C57BL/6 background were generated by Bezy *et al.* (a kind gift from Dr. C. Ronald Kahn at Harvard Medical School) [3]. SMMHC-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. The presence of the floxed *Prkcd* gene and DNA sequences encoding SMMHC-CreER^{T2} recombinase were confirmed with the following primer pairs: floxed *Prkcd* forward, 5'- CTG CTG GGT AAC TTA ACA ACA AGA CC-3' and reverse 5'- CTG CTA AAT AAC ATG ATG TTC GGT CC -3'; SMMHC-CreER^{T2} forward, 5'-TGA CCC CAT CTC TTC ACT CC-3' and reverse, 5'-AGT CCC TCA CAT CCT CAG GTT-3'. Heterozygotes of SMC-specific *Prkcd* knockout mice were further bred to generate homozygous SMC-specific *Prkcd* knockout mice and male mice were employed in the study. The rationale of using male animals include: 1) Clinical studies suggest that restenosis after vascular intervention is more prevalent in males than females [4]. 2) Estradiol is reported to accelerate reendothelialization in mouse carotid artery [5]. 3) The promoter of Myh11 has high SMC lineage specificity [6] and the tamoxifen inducible system avoids leakiness of Cre expression during development. This Cre allele is inserted into the Y chromosome, which excluded its application in female mice. 8-12 weeks male *Prkcd*$^{fl/fl}$; SMMHC-CreER^{T2}; *Prkcd*$^{wt/wt}$ or SMMHC-CreER^{T2}; *Prkcd*$^{fl/fl}$ mice were treated with 75 mg/kg tamoxifen (Sigma-Aldrich) through intraperitoneal injection for 5 consecutive days as previously described [7]. All studies were performed 3 weeks post tamoxifen treatment to allow *Prkcd* excision and degradation. Mouse femoral artery wire injury was performed blindly as described previously [8]. Briefly, the left femoral artery was exposed by blunt dissection while mice were under anesthesia. The distal and proximal ends of the femoral artery were looped with 9-0 silk suture for temporary control of blood flow. An arteriotomy was made on the femoral artery muscular branch, and a 0.015-inch diameter guide wire (Cook Medical, Bloomington, IN) was inserted at 2 mm in depth and remained in the artery for 2 minutes to denude and dilate the artery. After removal of the wire, the muscular branch was ligated, and blood flow in the femoral artery was restored. The skin incision was closed with a 6-0 nylon suture. Mice were allowed to recover and femoral arteries were harvested 4 weeks after the injury and fixed in 4% formalin overnight. Sections were stained with hematoxylin and eosin. For Evans blue staining, anesthetized mice were perfused with 100 µl of 5% Evans blue, then washed with phosphate-buffered saline (PBS). Injured femoral arteries were harvested, cut longitudinally and photographed using a digital camera. Endothelial denuded areas were defined as the areas spanning from the bifurcation 2 mm towards the proximal using the ImageJ software (National Institutes of Health, Bethesda, MD). Reendothelialization was determined by the percentage of Evans blue negative area over total denuded areas.

Rat angioplasty model and Evans blue staining

After induction of anesthesia with 2.5% isoflurane, arterial denudation was performed in male Sprague-Dawley rats (8~12 weeks) obtained from Charles River Laboratories through carotid balloon angioplasty as described before [9, 10]. As stated in the above section, to avoid the gender differences in prevalence of restenosis and exclude effect of estradiol level differences on reendothelialization, male rats were employed in the study. Common carotid arteries were injured from the bifurcation 1.5 cm towards the proximal. 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 and incubating for 30 min after balloon-injury as described previously [10]. A sham group

underwent surgery without balloon angioplasty/viral infection. Arteries were harvested on days 3, 7, and 14 by perfusion fixation with 4% paraformaldehyde at physiologic pressure (90 mmHg) for immunohistochemistry analysis. For Evans blue staining, anesthetized rats were perfused with 1ml of 5% Evans blue, then washed with PBS. Injured common carotid arteries were harvested, cut longitudinally and photographed using a digital camera. Endothelial denuded areas were defined as those areas spanning from the bifurcation 1.5 cm towards the proximal using the ImageJ software (National Institutes of Health, Bethesda, MD). Reendothelialization was determined by the percent of Evans blue negative area over total denuded areas.

Cell culture and conditioned medium preparation

Primary SMCs were isolated from rat carotid arteries according to a method described previously [11]. SMCs were maintained at 37°C with 5% CO_2 in DMEM modified to contain 4mM L-Glutamine, 1g/L D-Glucose, and 110mg/L Sodium Pyruvate (Life Technologies) supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin. For experiments using conditioned medium, rat SMCs (6×10^5 cells/10-cm dish) were infected with adenovirus at the m.o.i. of $5x10^4$ in DMEM containing 2% FBS for overnight, followed by starvation in DMEM containing 0.5% FBS for 24 hours. The cells were then incubated with PMA (1nM) or solvent (DMSO) for additional 6h or 48h. Medium conditioned by SMCs was collected and used for EC function assays as described below. Cells were harvest for RNA isolation. Mouse aortic ECs were isolated from male C57BL/6J mice, as described previously [12]. ECs were grown on 1% gelatin-coated dishes at 30°C with 5% CO_2 in DMEM containing 20% FBS, 2 mM L-glutamine, 2 mM sodium pyruvate, 20 mM HEPES, 1% non-essential amino acids, 100 μg/ml streptomycin, 100 U/ml penicillin, freshly added heparin at 55 U/ml (Sigma), endothelial growth supplement 100 μg/ml (Sigma), and murine recombinant interferon-γ (R&D, Minneapolis, MN) at 44 units/ml [12].

RNA Isolation, cytokine and chemokine qPCR array

Total RNA was extracted from cultured cells using Trizol reagent (Life Technologies) according to manufacturer's protocols. Two micrograms of RNA were used for the first-strand cDNA synthesis (Applied Biosystems, Carlsbad, CA). A no-RT (reverse transcriptase) control was included in the same PCR mixtures without reverse transcriptase to confirm the absence of DNA contamination in RNA samples. cDNA was subjected to Cytokine and Chemokine qPCR array (Qiagen, Valencia, CA) analysis according to manufacturer's protocol. qPCR primers for *Cxcl1* and *Cxcl7*, and *Gapdh* were purchased from Qiagen. Triplicate 20-μl reactions were carried out in 96-well optical reaction plates using SYBR® Green PCR Master Mix (Applied Biosystems) with gene-specific primers, and the qPCR was run in the 7500 Fast Real-Time PCR System (Applied Biosystems). Relative differences in each PCR sample were corrected using *Gapdh* mRNAas an endogenous control and normalized to the level of control by using the $2^{-\Delta\Delta Ct}$ method.

Endothelial wound closure assay

The endothelial wound healing assay was performed as described previously [13] with certain modifications. Briefly, 5×10^4 mouse aortic ECs were seeded on a 24-well plate in growth medium until became a confluent monolayer. After starvation in DMEM containing 0.5% FBS for 24 hours, a 200-μl pipette tip was used to create a straight cell-free wound. Wells were washed twice with PBS to remove cell debris. Then, ECs were then cultured in SMCs conditioned medium for 16h. For immunodepletion studies, SMCs conditioned medium was incubated with 10 μg/ml neutralization antibody against MCP-1 (BioLegend, San Diego, CA. Cat. No, 505901), CXCL1 (R&D, Minneapolis, MN. Cat. No. AF-515-NA), CXCL7 (R&D, Minneapolis, MN. Cat. No. AF1116), or normal IgG (BioLegend, San Diego, CA. Cat. No 400901 or R&D, Minneapolis, MN. Cat. No. AB-108-C) for 1h prior to addition to the well. To block CXCR2-mediated signal, ECs were pretreated with an anti-CXCR2 antibody (R&D, Minneapolis, MN. Cat. No. MAB2164) (10 μg/ml) or normal IgG (R&D, Minneapolis, MN. Cat. No. MAB006) for 1h before switching to conditioned medium. After incubation, ECs were labeling with CMFDA fluorescence

dye for visualization. Reendothelialization was determined by the percentage of the recovered area over the total injured area using ImageJ software (National Institutes of Health, Bethesda, MD).

Proliferation assay

The proliferation of ECs was determined using Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche, Mannheim, Germany). Briefly, ECs were seeded in 1% gelatin-coated 96-well plates at a density of 2×10^4 cells per well for 24h, followed by starvation in DMEM containing 0.5% FBS for 24h. Medium was switched to SMC conditioned medium. BrdU was then added to ECs, and the cells were reincubated for an additional 24h. BrdU incorporation was determined according to manufacturer's protocols.

CellTiter-Glo® Luminescent Cell Viability Assay

ECs were seeded in 1% gelatin-coated 96-well plates at a density of 2×10^4 cells per well for 24h, followed by starvation in DMEM containing 0.5% FBS for 24 hours. The medium was then replaced with SMCs conditioned medium. After culturing for another 24h, CellTiter-Glo® Reagent was added. Luminescence was recorded 10 minutes after reagent addition using a FlexStation 3 Microplate Reader (Molecular Devices, CA).

Transwell migration assay

ECs were placed in the upper chamber (1×10^4 cells/well) of Costar 24-well transwell plates with 8 μm pore filters (Corning, Inc., Corning, NY). SMC conditioned medium (70%) was added to the lower chambers to induce cell migration. After 16 hours incubation at 37°C, cells migrated to the bottom of filters were imaged and counted.

Immunohistochemistry

Tissue sections were permeabilized with 0.1% Triton X-100 in Tris-buffered saline (TBS) or ice-cold acetone for 10 minutes at room temperature. Non-specific sites were blocked using 5% bovine serum albumin (BSA), 10% normal donkey serum in TBS for 2 hours at room temperature. Indicated primary antibody diluted in TBS with 5% BSA were then applied to arterial sections and incubated overnight at 4°C. Primary antibodies included anti-PKCδ (Santa Cruz Biotechnology, Dallas, TX. Cat. No. sc-213), anti-vWF (DAKO, Cat. No. A0082), anti-VE-cadherin (Santa Cruz Biotechnology, Dallas, TX. Cat. No. sc-28644), anti-CD31 (R&D, Minneapolis, MN. Cat. No. AF3628), anti-SMA (Abcam, Cambridge, MA. Cat. No. ab5694), anti-CXCL1 (Novus Biologicals, Centennial, CO. Cat. No. NBP1-51188), anti-CXCL7 (Bioss Antibodies, Woburn, MA. Cat. No. bs-2550R), anti- ZsGreen (Takara Bio USA, Mountain View, CA. Cat. No. 632474), anti-GFP (Abcam, Cambridge, MA. Cat. No. ab5665). Normal rabbit IgG isotype control (Thermo Fisher Scientific, Waltham, MA. Cat. No. 31235) or goat IgG isotype control (Thermo Fisher Scientific, Waltham, MA. Cat. No. 31245) was used as negative control to distinguish background signaling. Antibody specificity was confirmed through either using knockout cells, exogenous overexpression, or vendor provided information. The next day, arterial sections were rinsed with TBS plus 0.025% Triton X-100, followed by incubating with fluorophore-conjugated secondary antibodies diluted in TBS with 1% BSA for 1 hour at room temperature. After counterstaining with DAPI, stained tissue sections were visualized with a Nikon Eclipse Ti inverted microscope system, and digital images were acquired using a Nikon DS-Ri1 digital camera.

Morphometric analysis

Paraffin-embedded arteries were cut into 5 μm sections for analysis. Morphometric analysis was carried out on elastin-stained arteries, and 3-5 sections were randomly selected for quantification. The areas encompassed by the lumen surface (luminal area), internal elastic lamina-lumen surface (intimal area), and external elastic lamina (EEL)-internal elastic lamina (medial area) were measured. For the evaluation of the degree of intimal hyperplasia, the ratio of intimal area to medial area (I/M ratio) was calculated and compared using digital imaging software, as described previously[10].

Immunoblotting

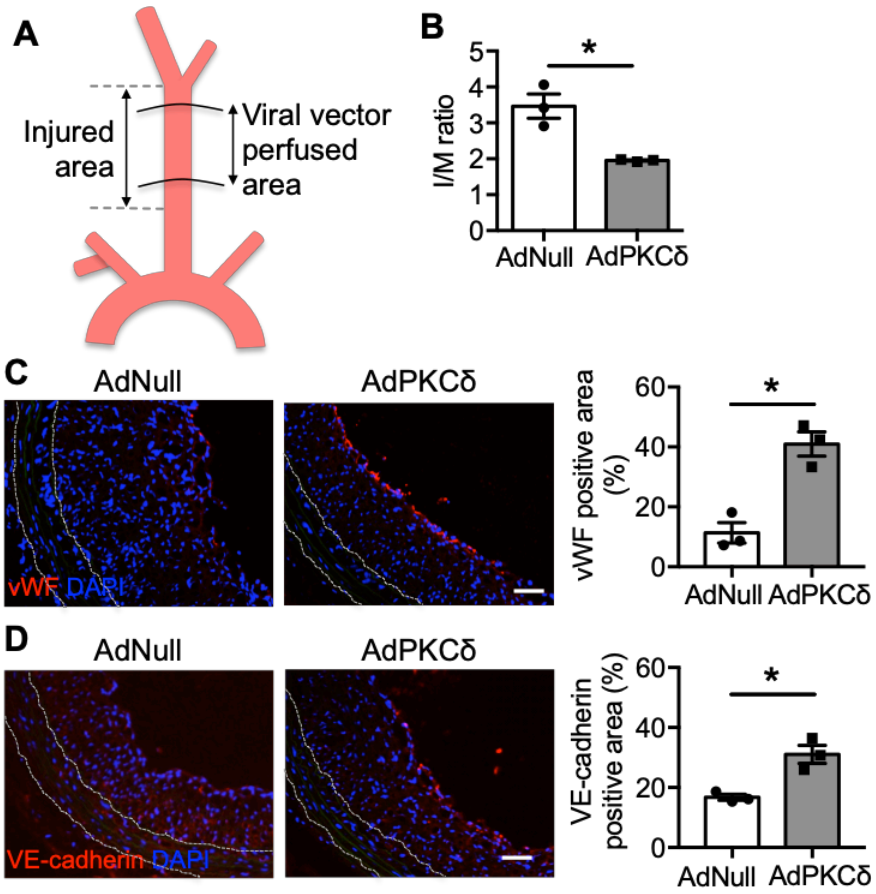
Cells were lysed in RIPA buffer (Sigma) and total protein was extracted. Equal amounts of protein extract were separated by SDS–PAGE and transferred to PVDF membranes. The membranes were then incubated with primary antibody overnight at 4°C. Primary antibodies included Phospho-Stat3 (Ser727) (Cell Signaling Technology, Danvers, MA. Cat. No. 9134), Stat3 (Cell Signaling Technology, Danvers, MA. Cat. No. 9139), Cleaved caspase-3 (Cell Signaling Technology, Danvers, MA. Cat. No. 9661), and β-actin (Sigma, St. Louis, MO. Cat. No. A5441). The next day, membranes were washed and incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit or anti-mouse immunoglobulin G (Bio-Rad, Hercules, CA. Cat. No. 1706515 and 1721011). Labeled proteins were visualized with an enhanced chemiluminescence system (Thermo Fisher Scientific, Waltham, MA.).

Statistical analysis

Results are presented as mean ± SEM. Data were assessed for normality using Shapiro-Wilk normality test. Data not exhibiting a normal distribution were log2-transformed and retested for normality. Two-tailed Student's 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 two conditions. One-way Analysis of Variance (ANOVA) with Tukey post hoc test for normally distributed data and Kruskal–Wallis nonparametric test for skewed data after transformation were used to compare ≥ three 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.

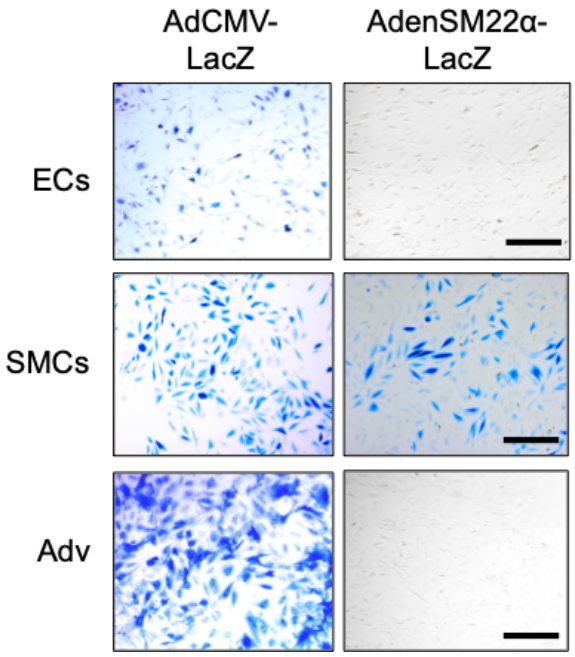
Online Figures and Figure Legends

Online Figure I



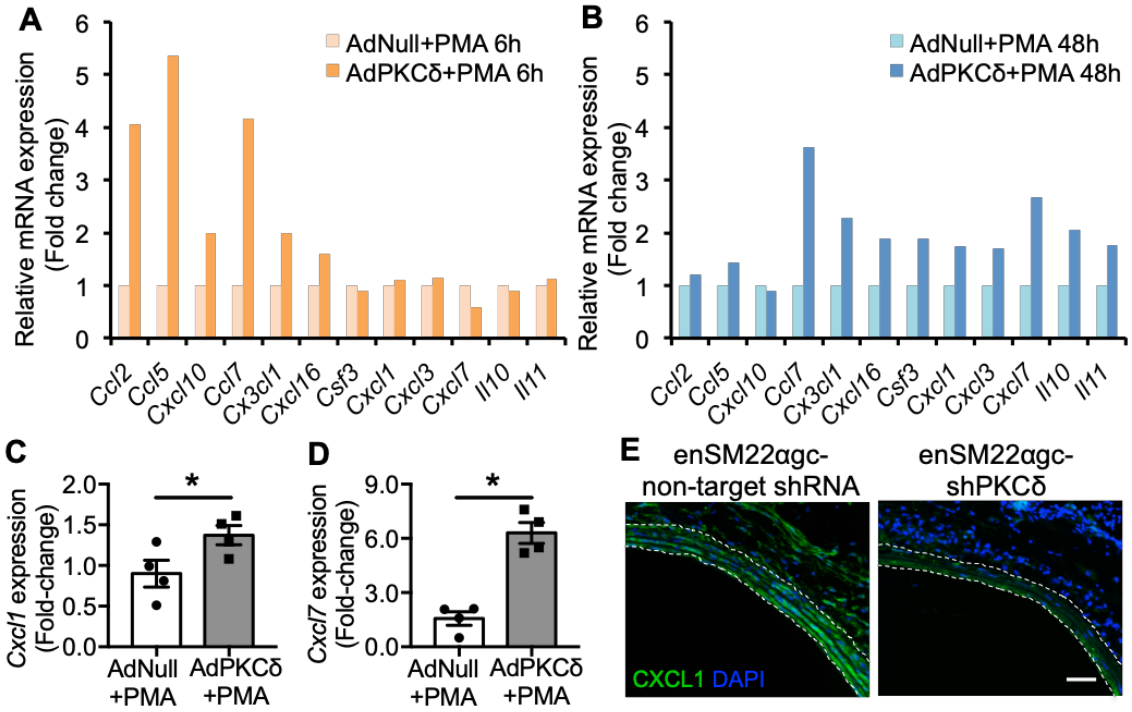
Online Figure I. Overexpression of PKCδ accelerates re-endothelialization
(A) Schematics of rat carotid injury model. Rat carotid arteries were injured with a balloon catheter. Gene transfer was achieved by intraluminal perfusion with adenoviral or lentiviral vectors within injured area.
(B) Carotid arteries were harvested 14 days after injury from AdNull- or AdPKCδ-treated rats, and the intima to media area (I/M) ratio was measured. Results are expressed as mean±SEM. n=3, * p <0.05, Two-tailed Student's *t*-test.
(C-D) Representative images and quantifications of rat carotid cross-sections harvested 14 days after angioplasty. AdNull and AdPKCδ: rats underwent carotid balloon injury followed by luminal perfusion with respective viruses. Sections were immunostained for vWF (C) or VE-cadherin (D). 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, Two-tailed Student's *t*-test.

Online Figure II



Online Figure II. SMC specificity of AdenSM22α-lacZ *in vitro*.
ECs, SMCs, and adventitial fibroblasts (Adv) were infected with either AdCMV-lacZ or AdenSM22α-lacZ. 72h after infection, cells were fixed and stained for β-galactosidase activity. AdenSM22α-lacZ virus produced lacZ in SMCs but not in ECs or Adv. In contrast, AdCMV-lacZ produced lacZ expression in all cell types tested. Scale bar = 50 μm.

Online Figure III



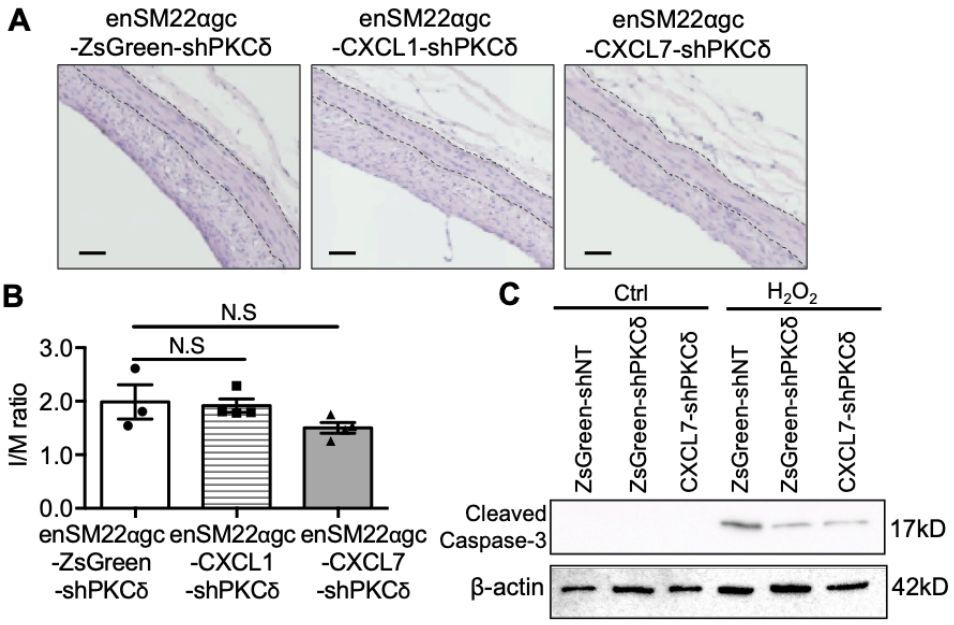
Online Figure III. PKCδ regulates chemokine expression in SMCs

(A-B) mRNA expression levels of cytokines and chemokines in SMCs infected with AdNul or AdPKCδ followed by incubation with PMA (1nM) for 6h (A) or 48h (B). Results were expressed as fold induction of mRNA expression levels compared with that of AdNull-infected SMCs. n=1.

(C-D) SMCs were infected with AdNull or AdPKCδ followed by incubation with PMA for 48h, *Cxcl1* and *Cxcl7* mRNA expression was analyzed using qPCR. Results are expressed as mean±SEM. n=4, **p*<0.05, Two-tailed Student's *t*-test.

(E) Representative images of CXCL1 stained rat carotid cross-sections harvested from injured Lenti-enSM22αgc-non-targeting shRNA-treated or injured Lenti-enSM22αgc-shPKCδ-treated rats (7 days post-angioplasty). Nuclei were indicated by positive stains to 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.

Online Figure IV



Online Figure IV. Restoration of CXCR2 ligands in the absence of PKCδ has minimal effect on apoptosis and I/M ratio.

(A&B) Representative images (A) and quantifications (B) of rat carotid arteries harvested 21 days after angioplasty from Lenti-enSM22αgc-ZsGreen-shPKCδ-, Lenti-enSM22αgc-CXCL1-shPKCδ-, or Lenti-enSM22αgc-CXCL7-shPKCδ-treated rats. Sections were immunostained for Haemotoxylin and Eosin (H&E). Scale bar = 50 μm. Results are expressed as mean±SEM. n= 3-4, Kruskal–Wallis nonparametric test.

(C) SMCs were transduced with Lenti-ZsGreen-shNT, Lenti-ZsGreen-shPKCδ-, or Lenti-CXCL7-shPKCδ followed by H_2O (Ctrl) or H_2O_2 treatment. Cells were harvested and whole-cell lysates were subjected to immunoblot analysis. n=3.

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