

CFTR Suppresses Neointimal Formation Through Attenuating Proliferation and Migration of Aortic Smooth Muscle Cells

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Abstract: Cystic fibrosis transmembrane conductance regulator (CFTR) plays important roles in arterial functions and the fate of cells. To further understand its function in vascular remodeling, we examined whether CFTR directly regulates platelet-derived growth factor-BB (PDGF-BB)-stimulated vascular smooth muscle cells (VSMCs) proliferation and migration, as well as the balloon injury-induced neointimal formation. The CFTR adenoviral gene delivery was used to evaluate the effects of CFTR on neointimal formation in a rat model of carotid artery balloon injury. The roles of CFTR in PDGF-BB-stimulated VSMC proliferation and migration

were detected by mitochondrial tetrazolium assay, wound healing assay, transwell chamber method, western blot, and qPCR. We found that CFTR expression was declined in injured rat carotid arteries, while adenoviral overexpression of CFTR in vivo attenuated neointimal formation in carotid arteries. CFTR overexpression inhibited PDGF-BB-induced VSMC proliferation and migration, whereas CFTR silencing caused the opposite results. Mechanistically, CFTR suppressed the phosphorylation of PDGF receptor β , serum and glucocorticoid-inducible kinase 1, JNK, p38 and ERK induced by PDGF-BB, and the increased mRNA expression of matrix metalloproteinase-9 and MMP2 induced by PDGF-BB. In conclusion, our results indicated that CFTR may attenuate neointimal formation by suppressing PDGF-BB-induced activation of serum and glucocorticoid-inducible kinase 1 and the JNK/p38/ERK signaling pathway.

Key Words: CFTR, VSMCs proliferation, neointimal formation, PDGF-BB, SGK1

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INTRODUCTION

Vascular surgical procedures, such as angioplasty, vascular bypass surgery, and solid organ transplantation, have adverse impacts on vasculature and often result in an increased intra-arterial thickness and decreased lumen diameter, which ultimately lead to neointimal formation.¹ The proliferation and migration of vascular smooth muscle cells (VSMCs) induced by growth factors and inflammatory cytokines play predominant roles in the hyperplastic response in the vessel wall and neointimal formation. Platelet-derived growth factor-BB (PDGF-BB) is a classical growth factor, produced in response to primary vessel damage and secondary inflammation, which acts as a potent stimulator of injury-induced VSMC proliferation and migration.^{2,3} The protein levels of proliferating cell nuclear antigen (PCNA), Ki67, and matrix metalloproteinase-9 (MMP9), commonly used as indicators of injury-induced VSMC proliferation and migration, are markedly elevated by treatment with PDGF-BB.^{4,5} PDGF receptor β (PDGFR β)-mediated mitogen-activated protein kinase (MAPK) signaling activation is an important mechanism for injury-induced VSMC proliferation and migration.^{6,7} In addition, serum and glucocorticoid-inducible kinase 1 (SGK1), a growth factor-responsive kinase, has recently been implicated as a novel regulator of injury-induced VSMC proliferation.⁸ SGK1 has been reported as

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the most positively co-expressed gene with PDGFR β in lung metastasis cells.⁹ However, the precise mechanisms by which PDGF-BB-stimulated VSMC migration, proliferation, and neointimal formation are not fully understood.

Cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-dependent chloride channel, is highly expressed in VSMCs and has been implicated in basal functions of vascular arteries, such as aortic tension and vasoconstriction.¹⁰ Moreover, studies from our laboratory and others have revealed critical roles of CFTR in the fate of cells. We have demonstrated that CFTR is involved in H₂O₂-induced apoptosis in basilar arterial smooth muscle cells.¹¹ Li et al¹² found that overexpression of CFTR reduces proinflammatory cytokine levels in the aorta and prevents atherogenesis in atherosclerotic apolipoprotein E-deficient mice. Another *in vitro* study suggested that miR-1246 promotes VSMC proliferation, invasion, and differentiation to synthetic phenotypes by regulating CFTR¹³. However, it is not clear whether CFTR contributes to injury-induced VSMC proliferation, migration, and neointimal formation.

In this study, the roles of CFTR in the development of neointimal formation were evaluated using the classic rat carotid artery balloon injury model by adenovirus-mediated intravascular CFTR overexpression. *In vivo* and *in vitro* results showed that CFTR deficiency exacerbates carotid artery neointimal formation and PDGF-BB-induced VSMC proliferation and migration, whereas overexpression of CFTR results in opposite effects. Mechanistically, CFTR regulates VSMC proliferation through activation of SGK1 and MAPK signaling pathways. These findings indicated that CFTR may be a novel therapeutic target for vascular proliferative diseases.

MATERIALS AND METHODS

Animals

Adult male Sprague–Dawley rats were supplied by Sun Yat-sen University Animal Care and housed in the SFP Experimental Animals Center at Sun Yat-sen University, Guangzhou, China. All animal experimental procedures were performed in accordance with protocols approved by Sun Yat-sen University Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals of the National Institute of Health in China.

Rat Carotid Artery Balloon Injury Model

The rat carotid artery balloon injury model was established according to previously described methods, with some modifications.¹⁴ Briefly, male Sprague–Dawley rats (250–300 g) were anesthetized by intraperitoneal injection of pentobarbital (60 mg/kg). The left carotid sinus was exposed, and the common carotid artery was injured by a catheter of 1.5 mm in diameter from an external carotid arteriotomy incision. The balloon was inflated and withdrawn 3 times with rotation. Then, the catheter was removed, and the injured vessel segments were subjected to various treatments. After treatment, the incision was closed, and the carotid artery was fully recovered. Animals were then returned to the animal care facility and provided standard rat chow. At 14 days after surgery, rats were euthanized, and the carotid arteries were harvested for specific experiments. For

morphometric histological analyses, the artery tissues were perfused with Krebs solution, fixed with 4% paraformaldehyde, embedded in OCT, and frozen sectioned. We selected arteries within 1 mm from the center of the balloon catheter for sectioning, and 5 slides per sample were randomly chosen for subsequent staining and quantification. The sections were then stained, and the morphometric parameters were measured using Image-pro Plus (National Institutes of Health, Bethesda, MD).

Adenovirus-Mediated Gene Transfer of CFTR

For *in situ* CFTR gene delivery in the rat balloon injury model, after the catheter was removed, 1×10^9 pfu of adenovirus was injected into the arteriotomy incision. Then, the injured artery was clipped with a temporary vascular clip proximally and incubated with CFTR-overexpressed adenovirus (Ad-CFTR) for 30 minutes. The Ad-CFTR was kindly provided by Professor John Engelhardt (University of Iowa, Department of Anatomy and Cell Biology). The adenovirus expressing a small hairpin RNA sequence targeting the rat CFTR was successfully constructed as described previously.¹¹ The VSMCs were infected with the adenovirus for 48 h, followed by stimulation with PDGF-BB.

VSMC Isolation and Culture

Primary VSMCs were isolated from rat aortas as we described previously.¹⁵ Briefly, the isolated VSMCs were collected and grown in high-glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gibco, Waltham, MA), 100 U/mL penicillin, and 100 μ g/mL streptomycin. The VSMCs were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Passages 4–9 of the cultured VSMCs were used for the experiments.

Cell Proliferation Assay

Cell viability was assessed using the mitochondrial tetrazolium assay (MTT) kit (Roche, Basel, Switzerland). VSMCs were starved by serum-free DMEM for 24 hours and infected with adenovirus at an MOI of 100 for 48 hours, followed by stimulation with PDGF-BB (20 ng/mL) for 24 hours. MTT solution (10 μ L/well) was then added to examine the cell viability. The optical density was read at 570 nm using a BioTek Elx-800 plate reader (BioTek, Winooski). Cells alone were used as the control group, and the cell viability in the control group was taken as 100%.

Migration Assay

VSMC migration was investigated as described previously.¹⁶ Briefly, for the wound healing assay, confluent VSMCs in 6-well plates were starved in serum-free DMEM for 24 hours. A scratch lesion was created on confluent VSMC cultures with a pipette tip, and 3 randomly selected fields at the lesion border were examined using an inverted microscope (Zeiss, Oberkochen, Germany).

For the transwell assay, VSMCs were harvested after the adenovirus transfection and resuspended in the starvation medium. Then, the cells were loaded in the upper chamber of an 8.0- μ m hanging cell culture component (Millipore, Billerica, MA), and the lower chamber was contained in the medium with PDGF-BB (20 ng/mL). After incubation for 24

hours, the membrane was fixed and stained using crystal violet. Cells migrating through the membrane were counted in 3 randomly chosen regions of each well under an inverted microscope (Olympus IX-70, Tokyo, Japan).

Western Blot Analysis

Cellular proteins were harvested, and immunoblotting was performed as described previously.¹⁶ Briefly, samples were resolved by 8% SDS-PAGE and transferred to PVDF membranes. The bound proteins were determined by immunoblotting with the indicated antibodies. The immunodetection was processed using the following primary antibodies: CFTR (168 kDa, Cat. No. NB300-511, 1:1000; NOVUS Biologicals), Ki67 (358 kDa, Cat. No. ab 16667, 1:1000; Abcam), PCNA (36 kDa, Cat. No. 2586, 1:1000; Cell Signaling Technology), PDGF receptor β (190 kDa, Cat. No. 3162, 1:1000; Cell Signaling Technology), p-PDGF receptor β (190 kDa, Cat. No. 3166, 1:1000; Cell Signaling Technology), p38 (38 kDa, Cat. No. 9212, 1:1000; Cell Signaling Technology), p-p38 (38 kDa, Cat. No. 9211, 1:1000; Cell Signaling Technology), SGK1 (55 kDa, Cat. No. S5188, 1:1000; Merck), p-SGK1 (48 kDa, Cat. No. SAB4503834, 1:1000; Merck), JNK (56/42 kDa, Cat. No. sc-7345, 1:500; Santa Cruz Biotechnology), p-JNK (56/42 kDa, Cat. No. sc-6254, 1:500; Santa Cruz Biotechnology), ERK (44/42 kDa, Cat. No. 9102, 1:1000; Cell Signaling Technology), and p-ERK (44/42 kDa, Cat. No. 4370, 1:1000; Cell Signaling Technology). The primary antibodies were detected using secondary antibodies (anti-mouse or anti-rabbit horseradish peroxidase conjugate). The membranes were detected using a ChemiDoc XRS system (Bio-rad, Hercules) and quantified with ImageJ software.

Real-Time PCR

A quantitative real-time PCR experiment was conducted as described previously.¹⁶ Briefly, total RNA was isolated using TRIzol reagent. The RNA concentration was determined using a spectrophotometer (NanoDrop 2000, Thermo, Wilmington, DE). In total, 1 μ g of total RNA was reverse-transcribed and used for the SYBR Green-based real-time PCR, as described previously. The samples were run in triplicate with RNA preparations from 5 independent experiments. The QuantiTect primers were as follows: MMP2 forward: GATCCGTGGTGAGATCTTCTTC, reverse: AGAACACAGCCTTCTCTTCCTG; MMP9 forward: TGGCCTTTAGTGTCTCGCTGT, reverse: CACACAGCTGGCAGAGATT; and α -tubulin forward: TCATCACTATTGGCAACGAGC, reverse: AACAGTCCGCTAGAAGCAC. Real-time PCR reactions were performed using the iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA) in a volume of 20 μ L, containing 10 μ L of FastStart Universal SYBR Green Master (Roche, Indianapolis, IN) and 0.4 μ L (10 μ mol) of each primer. The reaction profile consisted of 40 cycles (qPCR conditions: 95°C for 15 seconds and 60°C for 45 seconds) after an initial 10-min incubation period at 95°C. A dissociation stage was performed at the end of the reaction, consisting of 50 cycles of 10 seconds with a temperature increase of 0.5°C/cycle to demonstrate the specificity of the amplification. The fold change in the mRNA expression level of each gene was determined by the $2^{-\Delta\Delta C_t}$ method using α -tubulin mRNA as an internal control.

Immunohistochemistry

The immunohistochemical staining experiment was conducted as described previously.¹⁶ Briefly, mice were perfused through a cardiac puncture with 4% PFA, and then, carotid arteries were collected and sequentially cut into 8- μ m thick sections. The sections were heated in a tissue-drying oven for 45 minutes at 60°C, blocked with endogenous peroxidases for 15 minutes at 95°C, cooled down to room temperature, and washed 3 \times 5 minutes with PBS. The sections were incubated for 30 minutes at 37°C with 5% BSA. The sections were covered with the primary antibody, which was diluted in the blocking buffer (1:100) overnight at 4°C. Then, the sections were washed 3 \times 5 minutes with PBS. The sections were covered with the secondary antibody for 30 minutes at 37°C and washed 3 \times 5 minutes with PBS. The sections were stained with DAB for 30 seconds and counterstained with hematoxylin. The integral optical density (IOD) of CFTR (IOD sum/area) in each section was scanned using a confocal system (Olympus FV500-IX81) and analyzed using Image-Pro Plus 6.0 software. The average IOD of at least 3 section values was obtained as the mean IOD of a sample.

Statistical Analysis

All data are expressed as means \pm SEM. Student's *t* test was used to compare 2 groups. A one-way analysis of variance, followed by a Bonferroni multiple comparison post hoc test with a 95% CI was used for comparisons among 3 or more groups. Statistical analyses were performed using Prism 6 (GraphPad Software, San Diego, CA). *P* < 0.05 was considered statistically significant.

RESULTS

CFTR Expression is Decreased in Proliferating VSMCs in Rat Balloon Injury Models

We have previously shown that the CFTR gene is widely expressed in VSMCs.^{11,17} To determine the role of CFTR in pathological vascular remodeling, we examined its protein expression in balloon-injured rat carotid arteries. The CFTR protein levels in the VSMC media of carotid arteries decreased progressively (Fig. 1A). Immunohistochemical staining showed that significant intimal hyperplasia developed at 14 days after balloon injury, accompanied by decreased expression of CFTR in vascular tissue (Fig. 1B). These data suggest that CFTR expression is altered in proliferative VSMCs. CFTR might be involved in balloon injury-induced vascular intimal hyperplasia and VSMC proliferation.

In Vivo CFTR Overexpression Suppressed Injury-Induced Neointimal Formation and VSMC Proliferation

To explore the role of CFTR in balloon injury-induced VSMC proliferation, we used Ad-CFTR to infect injured rat carotid arteries in vivo. As shown in Figure 1C and **Supplemental Digital Content 1** (see **Figure S1C**, <http://links.lww.com/JCVP/A796>), we initially confirmed that the protein expression of CFTR was enhanced in carotid arteries from Ad-CFTR-transferred mice in vivo using western blot and

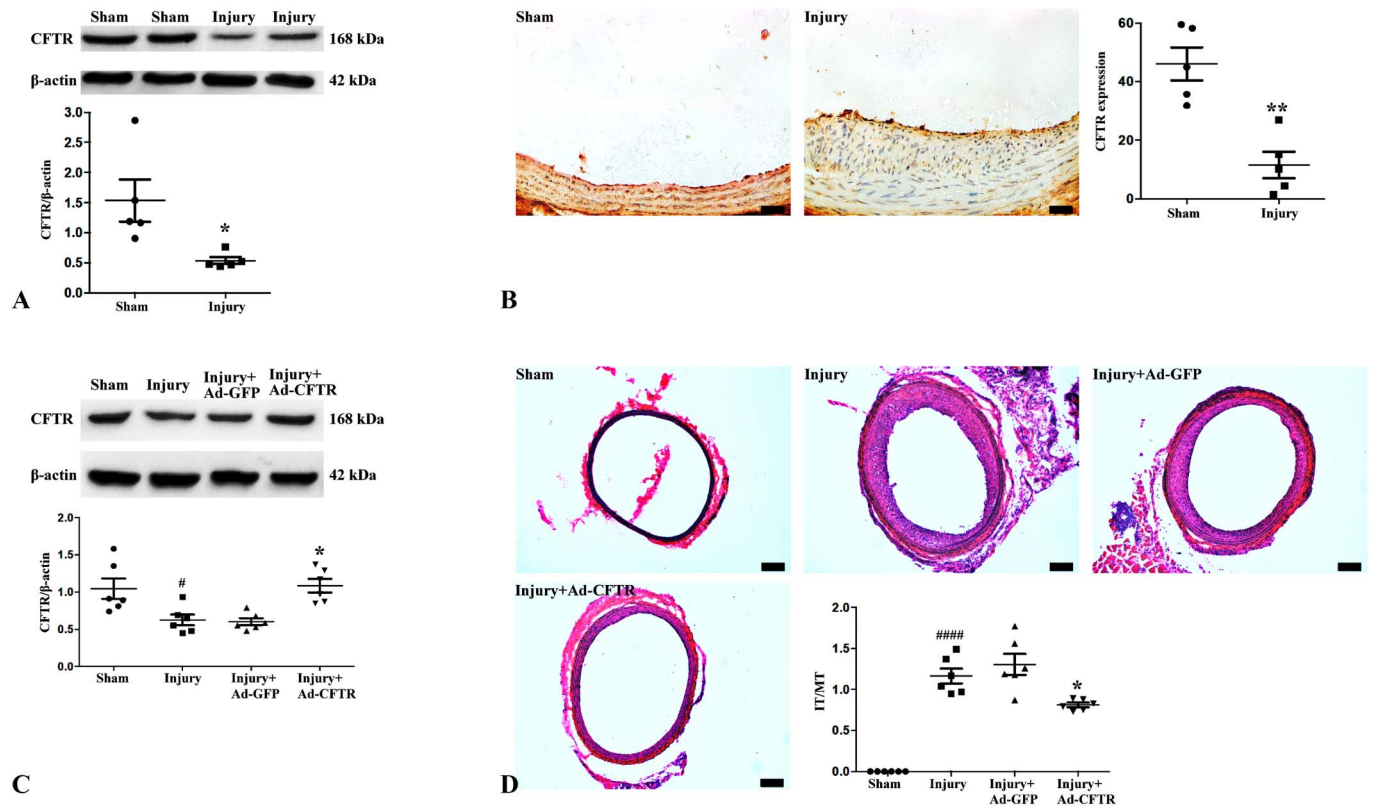


FIGURE 1. CFTR expression decreased as neointimal formation progressed in balloon-injured rat carotid arteries. **A,** Representative western blot images and plot graphs show the protein expression levels of CFTR in VSMC media of carotid arteries isolated from sham-operated (Sham) and balloon-injured (Injury) rats at 14 days after surgery. $n = 5$ per group, $*P < 0.05$ versus Sham. **B,** Representative images show immunohistochemical staining for CFTR (yellow brown) in sections from sham-operated (Sham) or balloon-injured carotid arteries at 14 days after surgery (scale bar = 20 μm). Plot graphs show relative CFTR expression. $n = 5$ per group, $**P < 0.01$ versus Sham. **(C, D),** Rat carotid arteries were injured with a balloon and locally infected with adenovirus expressing green fluorescent protein (GFP) alone (Ad-GFP) or Ad-CFTR (1×10^9 PFU) for 30 minutes. **C,** Injured arteries were isolated for staining after 14 days. Western blot analysis showed the adenovirus transfection were effective in VSMC media of carotid arteries. **D,** Representative H&E-stained carotid arterial sections and the intima/media ratio of rat injured arteries (scale bar = 100 μm). $n = 6$ per group, $\#P < 0.05$, $####P < 0.0001$ versus Sham, $*P < 0.05$ versus Injury.

immunohistochemistry assay. The expression of CFTR in the Ad-CFTR group was 150% of that in the corresponding control group. H&E staining showed that the overexpression of CFTR remarkably attenuated balloon injury–induced neointimal formation (Fig. 1D). The average intima/media ratio with CFTR overexpression decreased to 38.97% that of the sham group. The western blot analysis showed that CFTR overexpression markedly suppressed VSMC proliferation in injured carotid arteries as evidenced by PCNA protein expression (Fig. 2A).

Pathological proliferation in VSMCs is the major process in neointimal formation. We thus investigated the biological function of CFTR in VSMCs using the adenovirus-mediated gene transfer method. The transfection efficiency of adenovirus-mediated CFTR cDNA overexpression in carotid arteries and VSMCs is shown in **Supplemental Digital Content 1** (see **Figure S1A, B**, <http://links.lww.com/JCVP/A796>). The MTT assay demonstrated that Ad-CFTR significantly suppressed PDGF-BB–triggered VSMC proliferation compared with the untreated control. Inversely, CFTR shRNA silencing adenovirus (Ad-shCFTR) promoted PDGF-BB–triggered VSMC proliferation (Fig. 2B). On average, Ad-CFTR reduced PDGF-BB–triggered VSMC proliferation by 18.81%, while Ad-shCFTR increased PDGF-BB–

triggered VSMC proliferation by 21.83%, compared with the corresponding control. PDGF-BB–triggered VSMC proliferation was also examined for the protein expression of the proliferation marker PCNA and Ki67. Western blot analysis showed that Ad-shCFTR further increased PCNA expression induced by PDGF-BB, whereas Ad-CFTR did the opposite (Figs. 2C, D). Overexpression of CFTR effectively suppressed the Ki67 expression in PDGF-BB–stimulated VSMCs, whereas knockdown of CFTR promoted PDGF-BB–triggered Ki67 enhancement (Figs. 2E, F). These results suggest that the upregulation of CFTR may be enough to suppress PDGF-BB–triggered VSMC proliferation, and the downregulation of CFTR promoted it.

CFTR Suppressed PDGF-BB–Triggered VSMC Proliferation by DownRegulation of SGK1 Activity and the PDGF Signaling Pathway

We next determined how CFTR regulates the signaling pathway downstream of the PDGF-BB stimulation of VSMCs. PDGF-BB binds to PDGFR β and enhances PDGFR β phosphorylation to activate downstream signaling molecules. Western

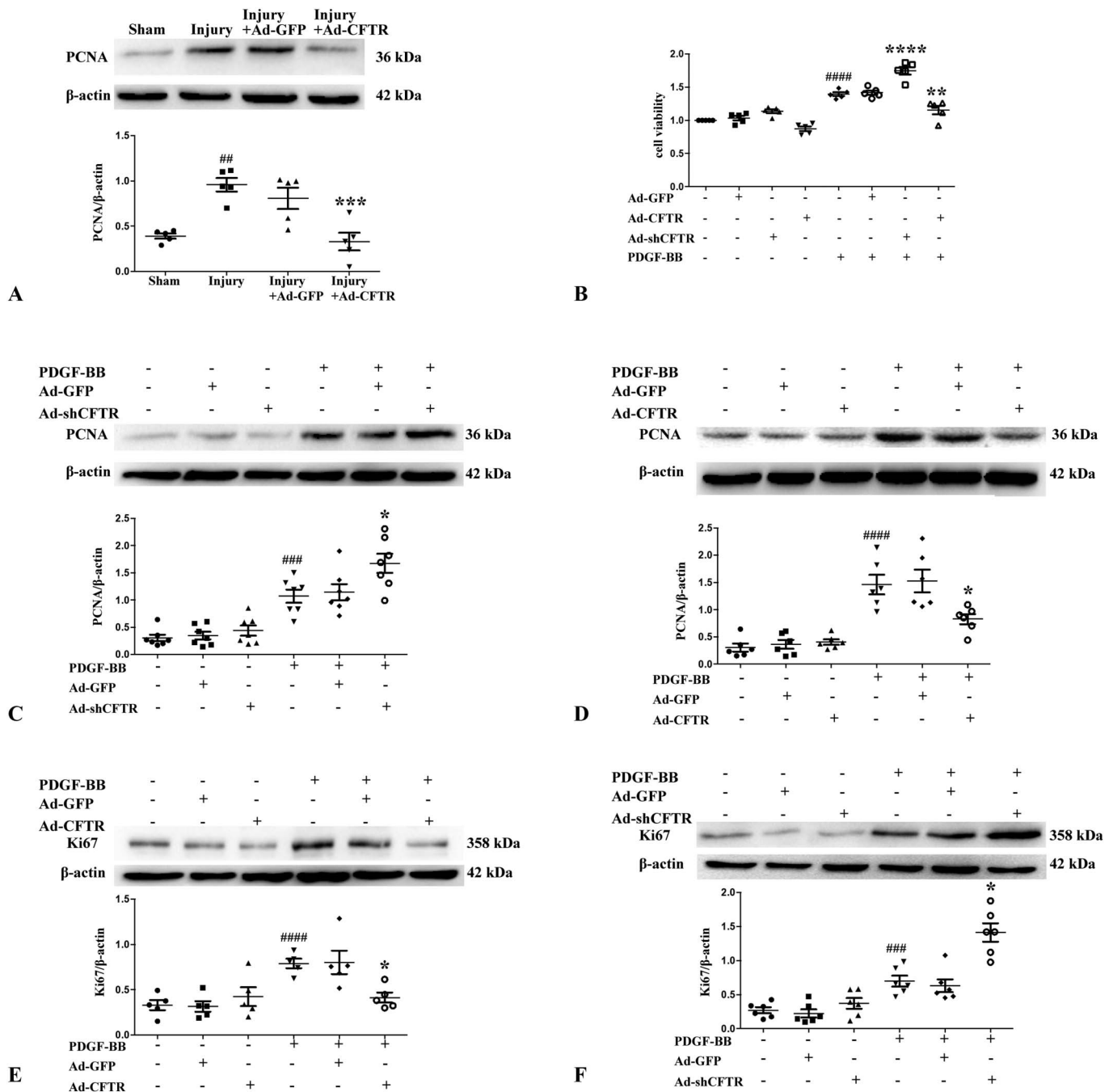


FIGURE 2. CFTR suppresses balloon injury-induced and PDGF-BB-stimulated VSMC proliferation. **A**, Protein expression of PCNA in injured arteries transfected with Ad-CFTR or Ad-GFP alone. $n = 5$, $^{\#}P < 0.01$ versus Sham, $^{***}P < 0.001$ versus Injury. **B**, VSMCs were treated with Ad-CFTR, Ad-GFP, or Ad-shCFTR and then exposed to PDGF-BB (20 ng/mL) for 24 hours. Cell viability was quantified by the MTT assay. $n = 6$, $^{####}P < 0.0001$ versus control cells. $^{**}P < 0.01$, $^{****}P < 0.0001$ versus PDGF-BB-stimulated cells. **(C, D)**, Protein expression of PCNA in VSMCs infected with Ad-GFP, Ad-shCFTR, or Ad-CFTR, respectively. $n = 6$, $^{###}P < 0.001$, $^{####}P < 0.0001$ versus control cells. $^{*}P < 0.05$ versus PDGF-BB-stimulated cells. **(E, F)**, Expression of Ki67 in PDGF-BB-stimulated VSMCs infected Ad-GFP, Ad-shCFTR, or Ad-CFTR, respectively. $n = 5$, $^{*}P < 0.05$ versus PDGF-BB-stimulated cells. $n = 5$, $^{###}P < 0.001$, $^{####}P < 0.0001$ versus control cells, $^{*}P < 0.05$ versus PDGF-BB-stimulated cells.

blotting showed that the PDGFRβ phosphorylation in VSMCs increased dramatically in response to PDGF-BB stimulation. Ad-CFTR abolished PDGF-BB-triggered PDGFRβ phosphorylation, whereas Ad-shCFTR further increased PDGFRβ

phosphorylation induced by PDGF-BB (Figs. 3A, B). However, CFTR gene transfection did not influence the total protein expression of PDGFRβ. Immunohistochemical staining of p-PDGFRβ showed that the phosphorylated PDGFRβ

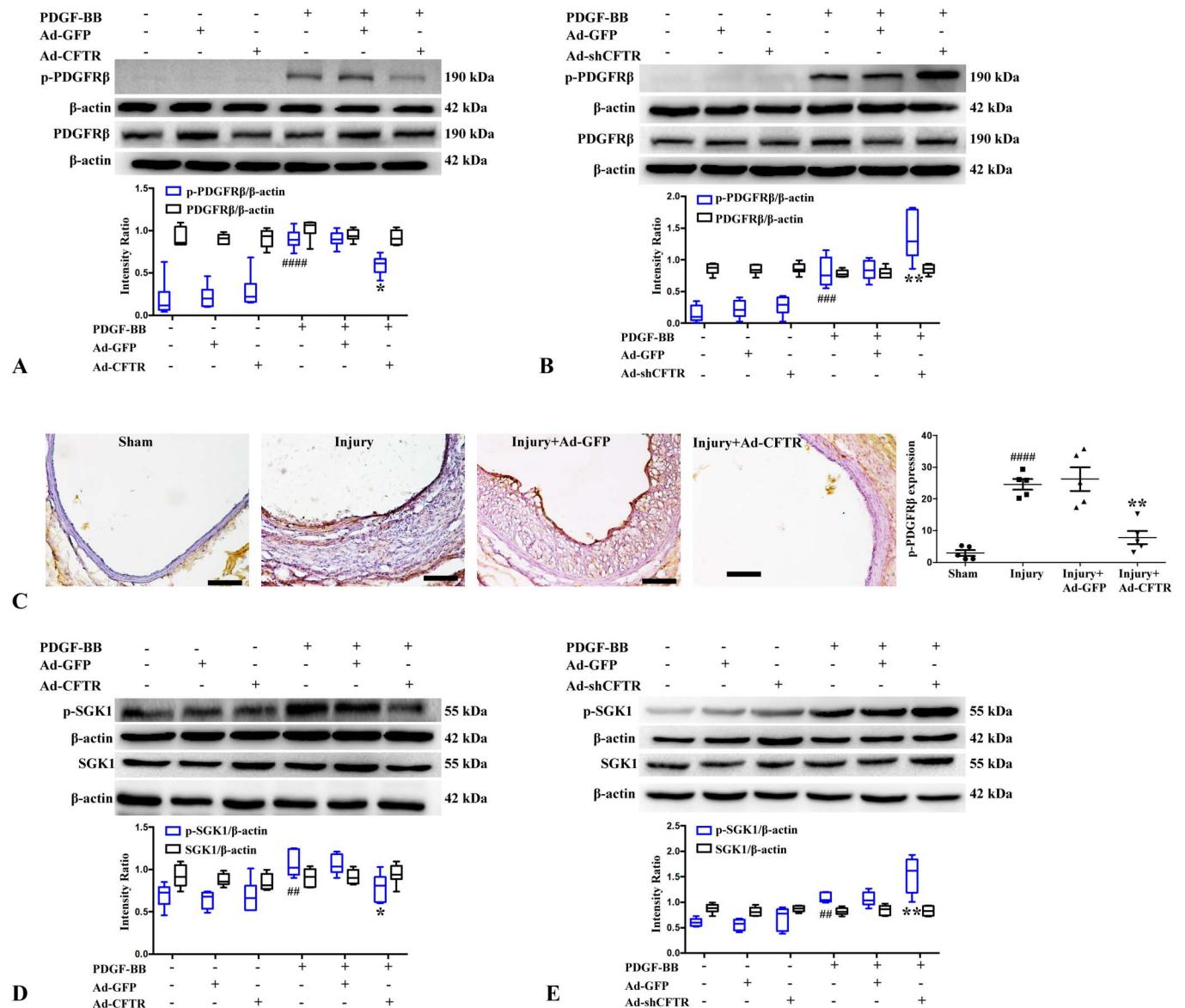


FIGURE 3. CFTR inhibits the activation of PDGFR β and SGK-1. (A, B), Ad-shCFTR- or Ad-CFTR-infected VSMCs were treated with or without 20 ng/mL PDGF-BB for 2 minutes, and then, PDGFR β protein and p-PDGFR β expression were analyzed by western blotting. $n = 6$, $###p < 0.001$, $####p < 0.0001$ versus control cells, $*p < 0.05$, $**p < 0.01$ versus PDGF-BB-stimulated cells. C, Representative images show immunohistochemical staining for p-PDGFR β in sections from sham-operated, balloon-injured carotid, Ad-CFTR-treated, or Ad-GFP-treated injured arteries at 14 days after surgery (scale bar = 50 μ m). Plot graphs show relative p-PDGFR β expression. $n = 5$ per group, $####p < 0.0001$ versus Sham, $**p < 0.01$ versus Injury. (D, E), Protein expression of SGK1 and p-SGK1. $n = 6$, $##p < 0.01$ versus control cells. $*p < 0.05$, $**p < 0.01$ versus PDGF-BB-stimulated cells.

expression increased dramatically in injured carotid arteries, and overexpression of CFTR attenuated the p-PDGFR β protein level in VSMC media (Fig. 3C).

As SGK1 is the main inflammatory regulator in VSMCs,⁸ we hypothesized that SGK1 may be a downstream effector of CFTR in PDGF-BB-triggered VSMC proliferation. Remarkably, PDGF-BB stimulation increased SGK1 phosphorylated to 155% of the corresponding control. Ad-CFTR significantly abrogated PDGF-BB-induced upregulation of p-SGK1, whereas Ad-shCFTR further enhanced it (Figs. 3D, E).

CFTR Regulated PDGF-BB-Stimulated VSMCs through Regulation of the MAPK Signaling Pathway

Because MAPK signaling contributes to the regulation of VSMC inflammation and proliferation,¹⁸ we further investigated whether CFTR regulates the activation of p38, ERK, and JNK activation in PDGF-BB-induced VSMCs. Western blotting showed that neither Ad-CFTR nor Ad-shCFTR affected the total protein expression of p38, ERK, and JNK. However, the phosphorylation of p38, ERK, and JNK was increased in response to

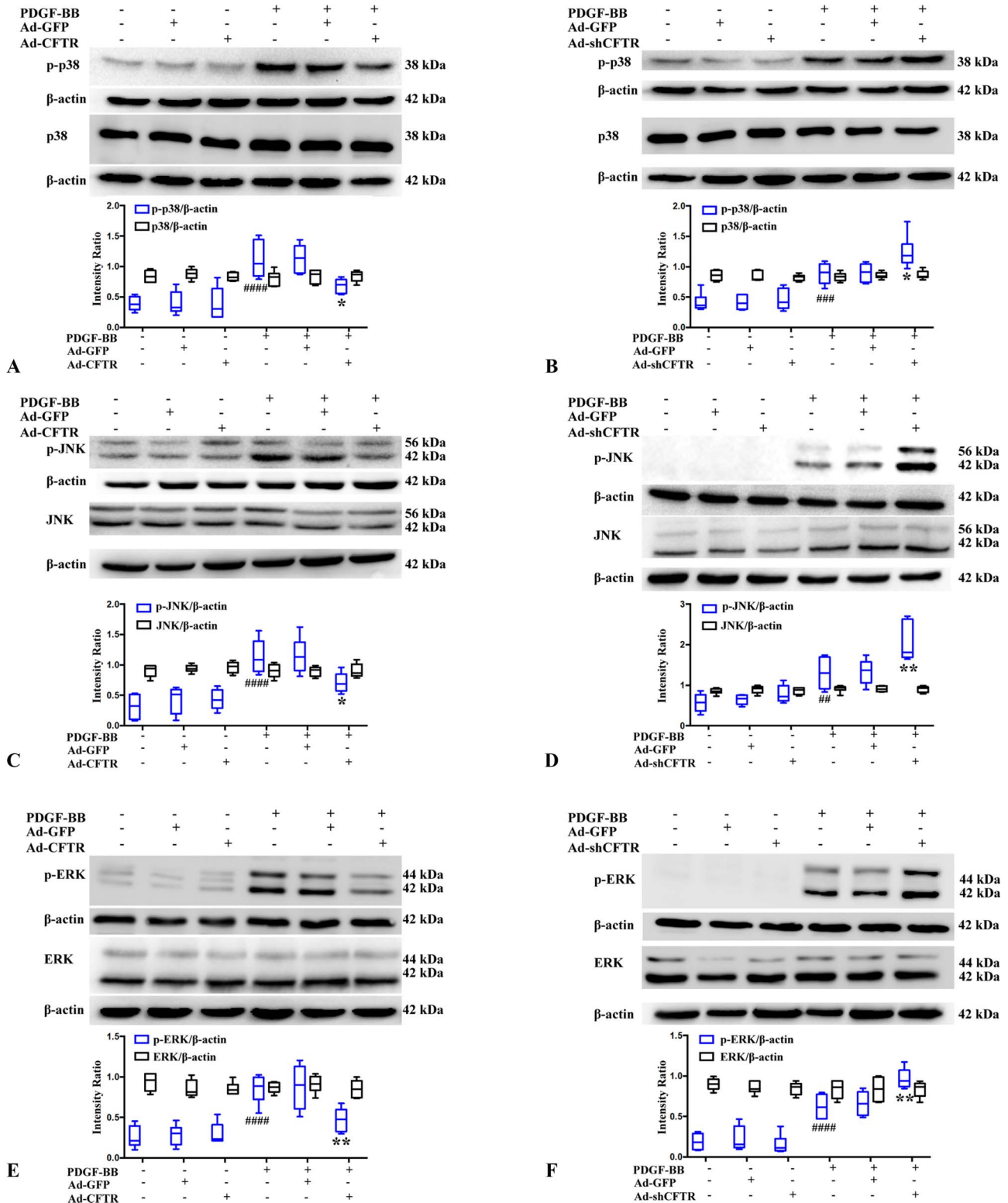


FIGURE 4. CFTR inhibits the activation of p38, JNK, and ERK. (A, B), Ad-shCFTR- or Ad-CFTR-infected VSMCs were treated with or without 20 ng/mL PDGF-BB for 2 minutes, and p38 expression was analyzed by western blotting. $n = 6$, $###P < 0.001$, $####P < 0.0001$ versus control cells, $*P < 0.05$ versus PDGF-BB-stimulated cells. (C, D), Protein expression of JNK and p-JNK in VSMCs treated with 20 ng/mL PDGF-BB for 15 minutes $n = 6$, $##P < 0.01$, $####P < 0.0001$ versus control cells, $*P < 0.05$, $**P < 0.01$ versus PDGF-BB-stimulated cells. (E, F) Protein expression of ERK and p-ERK in VSMCs treated with 20 ng/mL PDGF-BB for 1 minute $n = 6$, $####P < 0.0001$ versus control cells, $**P < 0.01$ versus PDGF-BB-stimulated cells.

PDGF-BB stimulation in a time-dependent manner. Ad-CFTR significantly suppressed the increased phosphorylation of p38, ERK, and JNK induced by PDGF-BB, whereas Ad-shCFTR further enhanced the levels of p38, ERK, and JNK phosphorylation (Figs. 4A–F).

Effects of in Vitro CFTR Gene Transfer on VSMC Migration and MMP2/9 Expression

We also established PDGF-BB-induced VSMC migration models in vitro. VSMC migration was measured by wound healing and transwell assays. Ad-CFTR-overexpressing

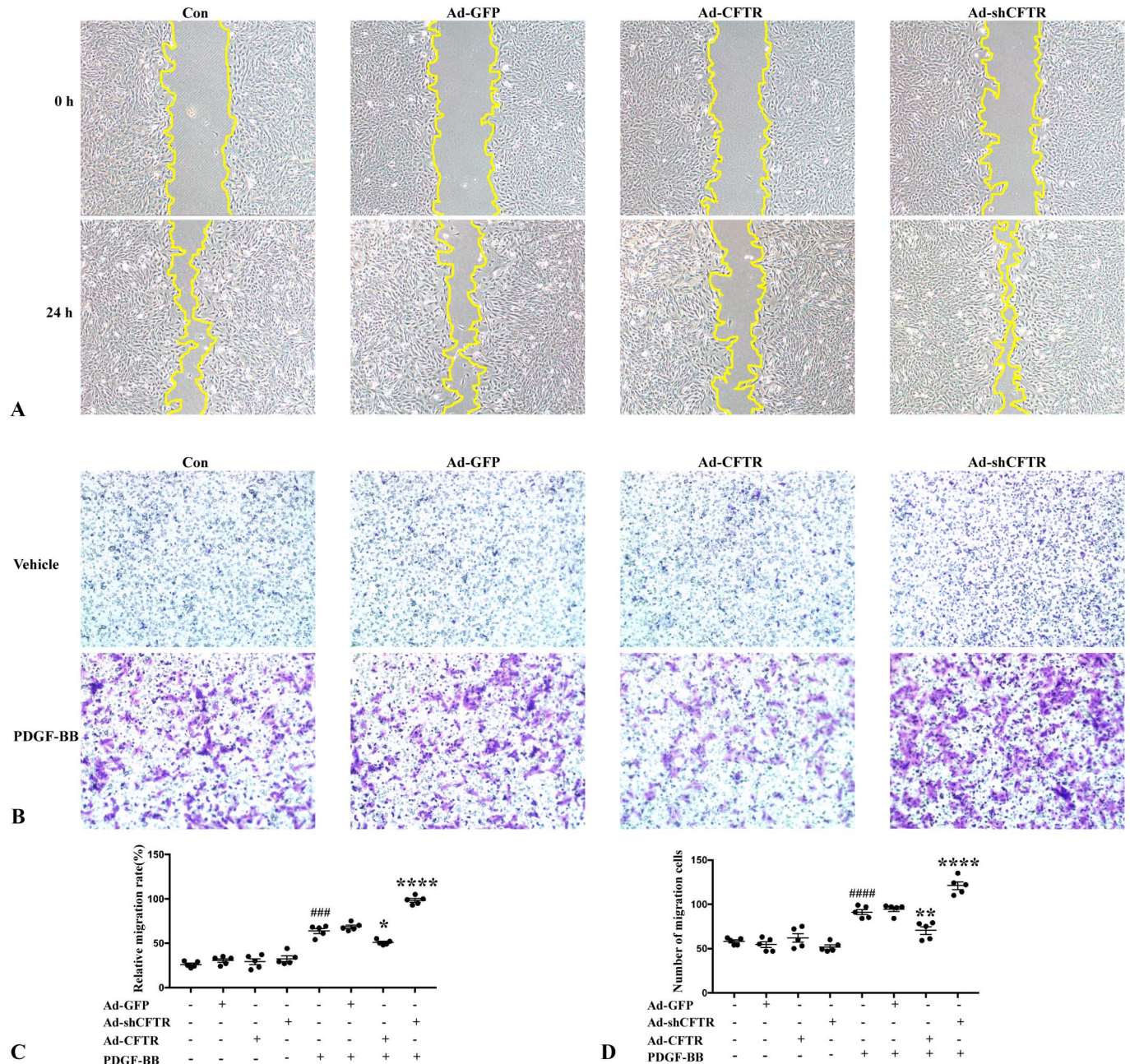


FIGURE 5. CFTR suppresses PDGF-BB-induced VSMC migration. VSMC migration was evaluated by a scratch assay (A and C) and transwell assay (B and D). (A and C) Representative images of the scratch assay and the quantification results for the wound closure assay $n = 5$, $###P < 0.001$ versus control cells, $*P < 0.05$, $****P < 0.0001$ versus PDGF-BB-stimulated cells. (B and D). Ad-shCFTR- or Ad-CFTR-infected VSMCs were transferred to transwell filters, and the migration ability of VSMCs in response to 20 ng/mL PDGF-BB was determined. Representative transwell filters are shown (100 \times) from 5 independent experiments. $n = 5$, $####P < 0.0001$ versus control cells, $*P < 0.05$, $****P < 0.0001$ versus PDGF-BB-stimulated cells.

VSMCs showed a 25.14% reduction in wound healing compared with that in the corresponding control cells at 24 h after scratch. By contrast, Ad-shCFTR-knockdown increased VSMC migration by 52.41% compared with that in the corresponding control at 24 h after scratch. The effect of CFTR on PDGF-BB-induced VSMC migration was further confirmed by the transwell assay. Ad-CFTR strongly inhibited VSMC migration by 15.49% compared with that in the control, whereas Ad-shCFTR increased migration by 35.79% compared with the corresponding control (Figs. 5A–D).

Finally, we examined the effect of CFTR on the expression and activity of MMP2 and MMP9, which have been reported to promote VSMC migration.¹⁹ The stimulation of VSMCs with PDGF-BB for 24 hours increased the mRNA expression of *MMP2* and *MMP9*. Ad-shCFTR further increased *MMP2* and *MMP9* expression induced by PDGF-BB. Conversely, Ad-CFTR suppressed PDGF-BB-induced *MMP2* and *MMP9* expression (Figs. 6A, B).

DISCUSSION

In this study, we obtained multiple lines of evidences for the roles of CFTR in the regulation of VSMC proliferation. First, CFTR expression was downregulated in balloon-injured rat arteries. Second, the overexpression of CFTR inhibited VSMC proliferation and limited balloon injury-induced neointimal formation. Finally, CFTR stabilized PDGFRβ, downregulated SGK1 phosphorylation, and attenuated VSMC proliferation through MAPK signaling.

VSMC dedifferentiation contributes to neointimal formation, which results in various vascular diseases.^{20,21} An important response to vascular injury is a dramatic increase in VSMC proliferation in media, which occurs 1–3 days after injury, followed by the migration of VSMCs from media to intima, where the cells undergo extensive proliferation by day 14 after injury.²² CFTR is widely expressed in the cardiovascular system, including in VSMCs, endothelial cells, myocardial cells,

and platelets.^{23,24} Accumulating studies have indicated that CFTR is involved in the cardiopathological process, including prolonging the cardiac action potential and myocardial lesions.²⁵ Robert et al¹⁰ reported that the degree of constriction of the aorta ring was greater in CFTR knockout mice than that in wild-type mice. Recently, the role of CFTR on cell fate has been revealed by researchers.¹³ In this study, we demonstrated CFTR attenuated neointimal formation and downregulated the protein expression of PCNA in VSMCs from a rat model of carotid artery balloon injury. This in vivo data indicating that CFTR could suppress the injury-induced proliferation of VSMCs, but the role of CFTR in arterial restenosis in clinical practice needs to be studied further.

PDGF-BB, which was upregulated in the progression of neointimal formation, directly contributes to vascular response to injury.²⁶ Pathways activated by PDGF-BB promote VSMC proliferation.²⁷ PDGF-BB-induced PDGFRβ activation is a key process involved in the dysregulation of VSMC proliferation and migration.²⁸ The expression of PDGFRβ is upregulated in injured arteries and in the early stage of atherosclerosis, whereas inhibition of PDGFRβ signaling attenuates neointimal formation and atherosclerotic lesion progression.²⁹ Regarding the intracellular signaling of PDGFRβ-related VSMC proliferation and migration, it has been reported that extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) are involved in PDGF-BB-induced rat aortic VSMC migration.³⁰ JNK, p38, and ERK, the MAPK family members, participated in a series of physiological and pathological processes associated with PDGF-BB, including cell proliferation and migration.^{31,32} Our results showed that CFTR abolishes PDGFRβ activation, thereby inhibiting downstream MAPK signaling. The induction of MMP2 and MMP9 by p38 and JNK activation is one of the key steps in neointimal formation.³³ Furthermore, we found that CFTR strongly inhibited the increased mRNA levels of *MMP2* and *MMP9* in response to PDGF-BB stimulation.

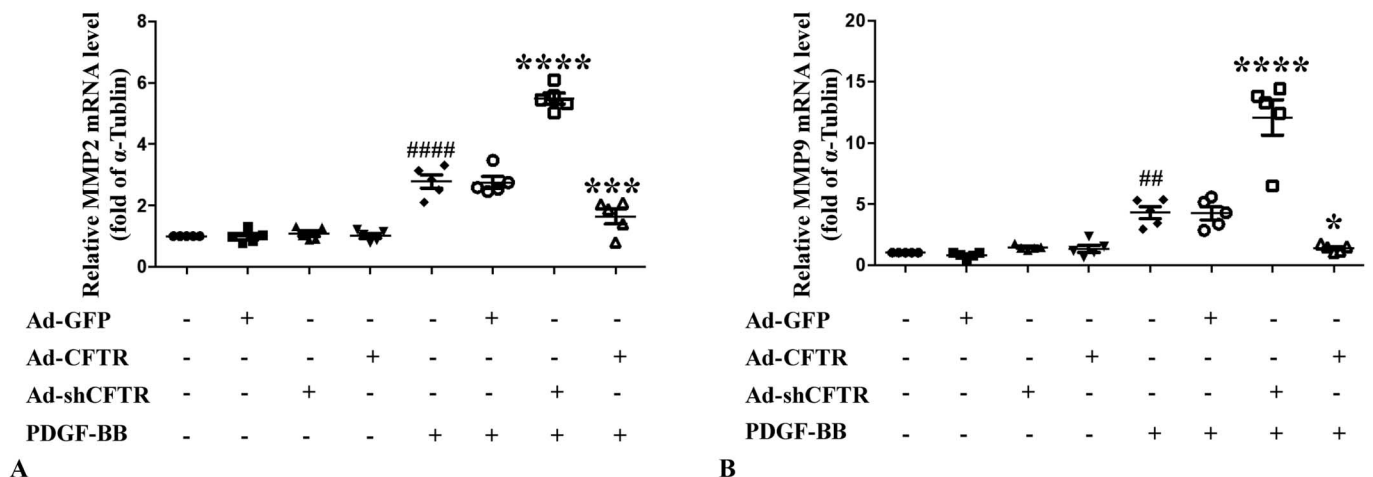


FIGURE 6. CFTR downregulated MMP2/9 expression in VSMCs. Ad-shCFTR- or Ad-CFTR-infected VSMCs were transferred to VSMCs and stimulated with 20 ng/mL PDGF-BB for 24 hours. Quantitative real-time RT-PCR analysis of mRNA levels of *MMP2* (A) and *MMP9* (B). n = 5, ###P < 0.01, ####P < 0.0001 versus control cells, *P < 0.05, ***P < 0.001, ****P < 0.0001 versus PDGF-BB-stimulated cells.

Taken together, our data have shown that CFTR suppressed the VSMC proliferation and migration by inhibiting of the MAPK signaling pathway and its downstream effectors.

SGK1, a member of the protein kinase A, G, and C (AGC) family, has well-documented roles in the regulation of various ion channels, including CFTR, which indicated that SGK1 may serve as a potential therapeutic target based on the pathophysiological functions of these ion channels.³⁴ SGK1 can stimulate CFTR-mediated chloride currents by increasing CFTR protein levels in the plasma membrane. Recently, CFTR has been demonstrated to be critical for epithelial inflammation through an SGK1-dependent signaling pathway.³⁵ SGK1 is also important for vascular remodeling.³⁶ Zhong et al⁸ reported that SGK1 expression is significantly increased in PDGF-BB-stimulated VSMCs, and the activation of SGK1 activity promotes VSMC proliferation. They also reported that SGK1 and PDGF signaling act in concert to promote migration efficiency in metastasis-initiating cells.⁸ Here, we found that CFTR attenuated the activation of SGK1 and PDGFR β stimulated by PDGF-BB, which established a link between CFTR and the PDGF-BB/SGK1 signaling pathway in vascular proliferation, further supporting the proposal that the inhibition of SGK1 activity may be a therapeutic approach for neointimal formation.

Although the role of CFTR in regulating VSMC proliferation, migration, and balloon injury-induced neointimal formation was revealed in this study by both in vivo and in vitro assays, several limitations still existed in this study. Mechanistically, it is not clear how CFTR cross talk with SGK1 or PDGFR β , which may need additional experimental work in our further research. What is more, the antiproliferative effect of CFTR would be strengthened confirmed through studies using mice with specific knockout or knockin of CFTR in VSMCs and blocking of the proposed signaling mechanisms (PDGFR β , SGK1, p38, JNK, or ERK), followed by rescue experiments.

Collectively, our results showed that CFTR regulates neointimal formation by targeting VSMC proliferation and migration. Mechanistically, CFTR inhibited VSMC migration and proliferation by suppressing SGK1 and MAPK activation through PDGFR β signaling pathway. We suggested that CFTR may be a potential target for preventing arterial restenosis.

CONCLUSIONS

In conclusion, our results indicated that CFTR functions as a reparative regulator of neointimal formation and inhibits the migration and proliferation of VSMCs through the PDGFR β signaling pathway.

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