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Surfactant protein A, a novel regulator for smooth muscle phenotypic modulation and vascular remodeling

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Abstract

Objective: The objective of this study is to determine the role of surfactant protein A (SPA) in vascular smooth muscle cell (SMC) phenotypic modulation and vascular remodeling.

Approach and Results: Platelet-derived growth factor-BB (PDGFBB) and serum induced SPA expression while downregulating SMC marker gene expression in SMCs. SPA deficiency increased the contractile protein expression. Mechanistically, SPA deficiency enhanced the expression of myocardin and TGF-β, the key regulators for contractile SMC phenotype. In vivo, SPA was induced in medial and neointimal SMCs following mechanical injury in both rat and mouse carotid arteries. SPA Knockout in mice dramatically attenuated the wire injury-induced intimal hyperplasia while restoring SMC contractile protein expression in medial SMCs. These data indicate that SPA plays an important role in SMC phenotype modulation and vascular remodeling in vivo.

Conclusion: SPA is a novel protein factor modulating SMC phenotype. Blocking the abnormal elevation of SPA may be a potential strategy to inhibit the development of proliferative vascular diseases.

Graphical Abstract

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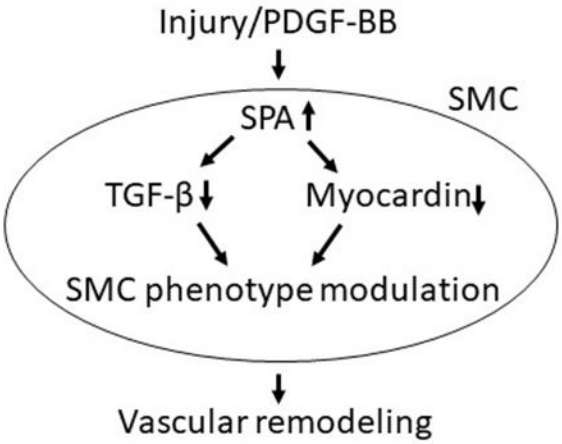
Disclosures: None



Keywords

Smooth muscle; Phenotypic modulation; Surfactant protein A; Vascular remodeling; Vascular smooth muscle

Introduction

Vascular remodeling is a pathological process occurring during the development of a number of vascular diseases such as restenosis after angioplasty, atherosclerosis, and aneurysm, etc. Smooth muscle cell (SMC) phenotypic modulation from a contractile to a synthetic phenotype accompanied by SMC migration and proliferation play key roles in vascular remodeling following the injury of blood vessel walls [1–3]. However, the protein factors causing SMC phenotypic modulation and the underlying regulatory mechanisms are not completely understood.

Surfactant protein A (SPA) is a large multimeric protein mainly expressed in the airway and alveoli in the lung under physiological conditions. SPA has diverse roles in the control of the pulmonary immune cell proliferation and lung cancer cell migration[4, 5]. However, expression of SPA has been reported in several extra-pulmonary tissues such as kidney, small intestine, colon, human thymus and prostate, etc [6]. We previously reported that SPA is expressed at a low level in epithelial cells of healthy kidney. However, unilateral ureter obstruction injury significantly induces its expression [7]. These results promoted us to hypothesize that SPA may be induced in other cells when the tissues are injured. Since SMCs express a slightly higher level of SPA than kidney in the normal condition (Biogps.org), we tested if SPA is regulated during SMC phenotypic modulation. We found that SPA was induced in platelet-derived growth factor-BB (PDGF-BB)-treated SMCs. More importantly, SPA appears to play an essential role in SMC phenotypic modulation and vascular remodeling.

Methods:

The authors declare that all supporting data are available within the article (and its online supplementary files).

Animals:

Male retired breeder of Sprague-Dawley rats weighing 450 to 500 g were purchased from Envigo. SPA deficient (SPA−/−) mice were purchased from The Jackson Laboratory (Sftpa1^{tm1Kor}, stock number: 004964). Portions of exons 3 and 4 (base pairs 795 through 1049) of the *sftpa1* gene were deleted by the insertion of a PGK-neo cassette [8]. All animals were housed under conventional conditions in the animal care facilities and received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals. All animal surgery procedures were approved by the Institutional Animal Care and Use Committee of The University of Georgia. Sex is a critical biological variable in cardiovascular diseases [9]. Since this is the first report investigating SPA function in vascular diseases, only male animals were used in this study. However, we will investigate if there is a sex-specific role of SPA in vascular remodeling by including both male and female animals in our future studies.

Cell Culture:

SMCs were collected by enzyme digestion method from rat or mouse aorta as described previously [2, 10, 11]. Briefly, the aortas were isolated and digested with 1 mg/ml collagenase for 30 mins. The adventitia and endothelium were then removed and the remaining media tissues were either used for protein extraction or cultured overnight in Dulbecco's modification of Eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS, Hyclone) and 5% L-glutamine. Then, the tissues were cut into small pieces and incubated with DMEM containing 0.25 mg/ml trypsin, 1 mg/ml collagenase and 0.5 mg/ml elastase for 30 mins. The digestion was stopped by adding 10 ml of DMEM with 10% FBS. The cells were collected and washed with culture medium for 2 times. The cells were then maintained in DMEM containing 10% FBS and 5% L-glutamine at 37°C in a humidified atmosphere of 5% CO_2 in air. Culture medium was changed every 48 hr. The primary cultured SMCs were confirmed by the expression of smooth muscle α-actin (ACTA2) and smooth muscle myosin heavy chain (MYH11). SMCs <6 passages with 70% of confluence were used for subsequent experiments. The SMCs were not clonal populations in this study. The cells were starved for 24 prior to PDGF-BB or 20% FBS treatment.

Western Blot Analysis:

Western blot was performed as described previously [12]. The proteins were extracted from freshly isolated SMCs or cultured primary SMCs with RIPA lysis buffer (50 mmol/L Tris-HCI, pH 7.4, 1% Triton X-100, 0.25% wt/vol sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EGTA, 0.1% SDS) containing protease inhibitors and phosphatase inhibitors (Thermo Scientific). The protein concentration was determined using BCA Protein Assay Reagent (Thermo Scientific). The proteins were separated with 9% SDS-PAGE and transferred to PVDF membrane (Bio-Rad). The membranes were blocked with 5% non-fat

milk for 1 hour at room temperature and then incubated with a primary antibody at 4°C overnight, followed by incubation with HRP-conjugated secondary antibodies (Sigma-Aldrich) for 1 hour. Protein bands were detected using enhanced chemiluminescence reagents (Millipore). Primary antibodies against SPA (Santa Cruz, Cat# SC13977, 1 μg/ml), smooth muscle α-actin (ACTA2, Sigma-Aldrich, Cat# A2547, 1μg/ml), myocardin (Myocd, Abcam, Cat# Ab22073, 1 μg/m), or smooth muscle myosin heavy chain (MYH11, Biomedical Technologies Inc., Cat # BT-562, 2 μg/ml) were used for immunoblotting.

Quantitative PCR (qPCR):

qPCR was performed as described previously[12]. Briefly, 1 ml of Trizol reagent (Invitrogen) was used to lyse artery tissues or SMCs. The lysates were then transferred into RNase-free microcentrifuge tubes and incubated at room temperature (RT) for 5 min. Then 0.2 ml chloroform was added to each tube and shaken vigorously for 15 seconds. Following incubation at RT for 2 min and centrifugation at 14,000×g at 4°C for 15 min, the upper aqueous phase was transferred to a new tube containing 0.5 ml isopropyl alcohol and incubated at RT for 10 min. RNA was then precipitated by centrifugation (14,000×g) at 4°C for 15 min. The supernatant was removed, and the RNA pellet was washed with 1 ml of 70% ethanol. RNA was dissolved in 20 μl RNase-free water. 1 μg of total RNA were reversely transcribed to cDNA using iScript cDNA Synthesis kit (Bio-Rad). qPCR was performed on a Stratagene Mx3005 qPCR thermocycler using SYBR Green master mix (Agilent Technologies, La Jolla, CA). The sequences of primers used in this study were as follows: 5’ TCC TGG AGA CTT CCA CTA CCT 3’ (forward) and 5’ CAG GCA GCC CTT ATC ATT CC 3’ (reverse) for SPA; 5’ CGC CAT CTA TGA GAA AAC C 3’ (forward) and 5’ GAT ACG CCA GGA ATT GT 3’ (reverse) for TGF-β1; 5’ GAT GGG CTC TCT CCA GAT CAG 3’ (forward) and 5’ GGC TGC ATC ATTC TTG TCA CTT 3’ (reverse) for Myocardin; 5’ GCT AAT CCA CCC CCG GAG TA 3’ (forward) and 5’ TCG CTG AGC TGC CCT TTC T 3’ (reverse) for MYH11; 5’ AAT GGC TCT GGG CTC TGT AAG 3’ (forward) and 5’ CAC GAT GGA TGG GAA AAC AGC 3’ (reverse) for ACTA2, and 5’ CAG ACG CCA CTG TCG CTT T 3’ (forward) and 5’ TGT CTT TGG AAC TTT GTC TG 3’ (reverse) for cyclophilin.

Rat Carotid Artery Injury Model:

The rat carotid artery balloon-injury model was generated as described previously [1, 2, 11]. Briefly, rats were anesthetized by isoflurane inhalation. A 2F Fogarty arterial embolectomy balloon catheter (Baxter Edwards Healthcare, Irvine, CA, USA) was inserted into the left external carotid artery and advanced 4 cm toward the thoracic aorta into the common carotid artery. The balloon was inflated with 20 μl saline and then withdrawn back to the carotid bifurcation with constant rotation during denudation of the endothelium. This procedure was repeated for two additional times to ensure complete endothelial denudation. 1, 3, 7, and 14 days following the surgery, the balloon-injured artery segments were perfused with saline and removed. The vessel segments were then fixed with 4% paraformaldehyde and embedded in paraffin followed by morphometric and histological analyses.

Mouse Carotid Artery Injury Model:

Mechanically induced endothelial denudation in mouse carotid artery was performed as described previously [13]. Briefly, an epon resin probe used for the arterial injury was made through forming epon beads on a 3-0 nylon suture. The beads on the suture were slightly larger than the diameter of the carotid artery. Eight-week-old WT or SPA−/− mice were anesthetized by continuous isoflurane inhalation. The mice were immobilized, and the fur covering the neck was removed with lotion hair remover. The entire left carotid artery, including its distal bifurcation, was exposed under a dissection microscope. The external branch of carotid artery was looped proximally and ligated distally with 6-0 silk sutures (Fine Science Tools, U.S.A). A transverse arteriotomy was made between the 6-0 silk sutures, and the resin probe was inserted, advanced and withdrawn in the common carotid artery for five times. The procedure was completed within 60 min. 14 days following the surgery, injured artery segments were perfused with saline and removed. The vessel segments were then fixed with 4% paraformaldehyde and embedded in paraffin followed by morphometric and histological analyses.

Histomorphometric analyses and immunohistochemistry staining (IHC):

The paraffin sections with 5 μm thickness were made by a serial cutting of the control and injured artery segments. 10 sections that were evenly distributed in the vessel segments for each group were collected for analyses. These sections were stained using modified hematoxylin and eosin (H&E) or Elastica van Gieson reagents by following the standard procedures. Images were captured with a Nikon microscope (Nikon America Inc). The circumference of the lumen, internal elastic lamina, and external elastic lamina of the injured arteries were measured by Image-Pro Plus Software. The neointima areas of injured SPA−/− arteries were normalized to the injured WT arteries, which were set as 1. For IHC staining, the sections were rehydrated and boiled in a citrate buffer (pH 6.0, Abcam, ab93678) with a microwave oven for 10 min to retrieve antigens. The sections were then incubated with 0.3% hydrogen peroxide, permeabilized with 0.3% Triton X-100 in PBS for 30 min, and blocked with 5% goat serum for 1 h, and incubated with primary SPA antibody at 4°C overnight. HRP-conjugated secondary antibody (Sigma-Aldrich) was incubated at RT for 1 h. 3,3’-Diaminobenzidine (DAB) was used to detect the SPA staining, and hematoxylin was used to counterstain the sections. Staining images were capture using a Nikon microscope, and Image J software was used to measure the intensity of IHC positive staining. Mean value of the staining intensity for each group was acquired from 10 artery sections. To quantify the protein level, the mean value of IHC positive signal of each group less the background signal was calibrated to the mean value of the staining intensity in uninjured vessels, in which the background signal was also subtracted. The protein level relative to the control group was shown as a fold increase of the signal intensity that was assessed by the following formula: [(Mean value of IHC staining intensity of injured vessels – background signal) / (Mean value of IHC staining intensity of uninjured vessels – background signal)].

Expression and purification of SP-A protein:

Human SPA cDNA clone was obtained from DNASU Plasmid Respository (Cat# HsCD00951577). The SPA cDNA in pDONR221 (Gateway donor) was recombined into the

mammalian gateway destination vector pDEST™17 (ThermoFisher, Cat# 11803012) using LR clonase II enzyme mix (ThermoFisher, Cat# 11791020). This vector (pDEST-SPA) was then transformed into BL21(DE3) competent cells (New England Biolabs, Cat # C2527I), and SPA protein expression was induced with 1 mM isopropyl β-d-thiogalactopyranoside (ThermoFisher, Cat # 15529019) for 24 h. SPA proteins were extracted using xTractor Buffer (Takara, Japan, Cat # 63562) and purified using Capturem™ His-Tagged purification miniprep kit (Takara, Japan, Cat # 635710). Purified SP-A protein was concentrated using Microcon-10kDa centrifugal filter unit (Millipore, Cat# MRCPRT010).

Statistical analysis:

There is a limitation for the sample size in some of the in vitro experiments. Most in vitro cell culture experiments were performed with 3-9 independent experiments (at least 10^6 cells/each group in each experiment), so n=3-9 was used for statistical analyses. All data were expressed as mean ± SEM. SPSS16 version was used for analysis. Two-tailed, unpaired Student t tests were used to compare 2-group data. One-way ANOVA followed by Fisher's least significant difference (LSD) tests were used to compare multiple group data. The normality and variance were not tested to determine whether the applied parametric tests were appropriate. $P<0.05$ were considered significant.

Results

SPA mediated SMC phenotypic modulation by regulating myocardin and TGF-β1 expression

In order to test if SPA is involved in SMC phenotypic modulation, we isolated SMCs from wild type mouse aorta and treated the cells with vehicle or PDGF-BB (20 ng/ml) for 0, 2, 8 and 24 hours. PDGF-BB time-dependently induced SPA expression along with the downregulation of SMC marker proteins ACTA2 and MYH11 (Figure 1A–1B). SPA was also induced when SMCs were treated with serum (20% fetal bovine serum or FBS), which also downregulated MYH11 and ACTA2 (Online Figure I, A–D). To test if SPA plays a role in SMC marker protein expression, we collected SMCs from wild type or SPA−/− mouse aorta and extracted the proteins. Western blot analyses showed that SPA deficiency increased SMC marker ACTA2 and MYH11 protein expression (Figure 1C–1D), suggesting that SPA is a negative regulator for the contractile SMC phenotype and important for maintaining the proper level of SMC contractile proteins.

Since myocardin and TGF-β1 are well-known protein factors regulating contractile SMC phenotype, we detected if SPA affects their expression. As shown in Figure 1E–1F, SPA deficiency increased the expression of both myocardin and TGF-β1 in medial SMCs. SPA knockout also caused higher levels of TGF-β1 and myocardin mRNA expression in mouse aorta media as compared to the wild type control (Online Figure II). Since SPA is a secretive protein, we treated the SPA−/− SMCs with recombinant SPA protein, which diminished the SPA deficiency-increased expression of TGF-β1, myocardin, as well as SMC markers MYH11 and ACTA2 (Online Figure III). Consistently, knockout of SPA increased Smad3 phosphorylation, which was blocked by TGF-β1 neutralizing antibody (Online Figure IV). TGF-β1 antibody also inhibited the increased MYH11 expression in SPA−/− cells (Online

Figure IV). These results suggest that SPA inhibits SMC marker protein expression through inhibiting myocardin and TGF-β1 expression/signaling during SMC phenotypic modulation.

SPA deficiency attenuated injury-induced neointimal formation/vascular remodeling.

In order to test if SPA is involved in SMC phenotypic modulation in vivo, we generated artery injury models with mechanically induced endothelial denudation in rat and mouse left carotid arteries. Balloon injury caused progressive neointima formation in rat carotid artery (Online Figure V). Importantly, injury induced SPA expression initially in medial SMCs and subsequently in neointimal SMCs (Online Figure V), suggesting that SPA may play a role in injury-induced vascular remodeling. SPA-positive staining was also presented in adventitia cells and extracellular matrix of the injured arteries because SPA is a secreted protein. In mouse carotid artery, wire injury also caused significant neointimal formation (Figure 2A) and induced SPA expression in both medial and neointimal SMCs (Figure 2A). Of importance, knockout of SPA significantly attenuated the injury-induced neointimal formation, as shown by both the H&E and elastin staining (Figure 2B). SPA deficiency reduced both the neointima area and the intima/media ratio (Figure 2C), demonstrating that SPA played a critical role in injury-induced vascular remodeling. To determine if SPA is involved in SMC phenotypic modulation in vivo, we detected the expression of the most specific SMC marker protein MYH11. As shown in Figure 2B–2C, MYH11 was strongly expressed in medial SMCs in the wild type carotid artery, but injury diminished its expression. However, SPA deficiency restored the MYH11 expression in the medial SMCs (Figure 2B–2C). These data indicated that SPA promoted the injury-induced vascular remodeling by modulating SMC phenotype.

Discussion

Although SPA is mainly expressed in the lung to maintain minimal surface tension and regulate respiratory immunity and inflammation [6], it is also presented in other tissues including blood vessel wall [14]. In this study, we have identified SPA as an important novel regulator for SMC phenotypic modulation. SPA is expressed at a low level in normal contractile SMCs. However, the expression is significantly increased when SMCs undergo phenotypic modulation. Deletion of SPA markedly increased the expression of SMC contractile proteins, suggesting that SPA is essential for maintaining SMC phenotype homeostasis. Consistent with the in vitro observations, a low level of SPA is expressed in normal healthy arterial wall. However, mechanical injury significantly elevated the SPA expression in both medial and neointimal SMCs. Of importance, SPA deficiency attenuated the injury-induced neointima formation. More importantly, SPA deficiency preserved the expression of SMC marker protein MYH11 that was downregulated due to the injury, suggesting that SPA plays an essential role in SMC phenotypic modulation in injury-induced vascular remodeling.

A number of protein factors have been shown to regulate SMC phenotypes. TGF-β signaling plays an important role in SMC differentiation and phenotype determination although the effects of TGF-β on proliferation remain controversial [15–19]. TGF-β activates SMC marker gene expression through Smad2 or Smad3 signaling [15, 17, 19]. Targeted disruption of TGF-β/

Smad3 signaling leads to enhanced neointimal hyperplasia and increased SMC proliferation [18]. However, overexpression of Smad3 via adenoviral delivery enhanced intimal hyperplasia and increased SMC proliferation [16]. The discrepancy could be due to the different responses of SMCs to the Smad3 levels and/or potential distinct roles of Smad3 at the various stages of vascular remodeling. It is likely that TGF-β/Smad3 is required to maintain contractile SMC phenotype while excessive Smad3 signaling may promote synthetic SMCs to proliferate. Our results show that SPA deficiency moderately but significantly increases TGF-β level along with increased Smad3 phosphorylation. TGF-β neutralizing antibody, however, blocks the SPA deficiency-caused Smad3 activation and the increased SMC marker gene expression, indicating that TGF-β signaling is essential in promoting the contractile phenotype of SPA−/− SMCs. Interestingly, SPA is found to bind TGF-β and modulates the induction of regulatory T cells via TGF-β in immune cells[20, 21]. However, in SMCs, SPA appears to inversely regulate TGF-β signaling in order to maintain the contractile phenotype. These results suggest that SPA is likely to have both extracellular and intracellular functions, and its functions may be different in different cell types.

In addition to TGF-β, myocardin is an essential regulator for both SMC differentiation and phenotypic modulation. Myocardin and TGF-β/Smad3 interacts and regulates each other's function in SMC marker gene expression [17, 22, 23]. Myocardin haploinsufficiency augments neointima formation due to increased SMC migration and proliferation while ectopic expression of myocardin in a murine carotid artery injury model decreases neointimal formation [24]. We found that SPA knockout increases the expression of both myocardin and TGF-β, indicating that SPA regulates SMC phenotype by maintaining proper levels of myocardin and TGF-β in the artery wall, which may be important for SMC and artery homeostasis. Moreover, SPA could also modulate the interaction of myocardin with Smad3 to regulate SMC marker expression, which could be investigated in the future.

One limitation of this study is the use of global SPA knockout mice, which does not address the cell-specific role of SPA in vascular remodeling. In addition to medial and neointimal SMCs, adventitia cells including macrophages could also express SPA following the injury. The cell-specific roles of SPA in vascular remodeling may be investigated using tissue-specific SPA knockout mice in the future. In addition, SPA is a secreted protein, and supplementation of SPA lessens the effect of SPA deficiency. Therefore, both extracellular and intracellular SPA appear to be important for SMC phenotype regulation and vascular remodeling, which are important subjects for future inquiry.

Taken together, our studies demonstrate for the first time that SPA is a novel regulator for SMC phenotype, and inhibition of SPA may be a potential therapeutic strategy for hindering the development of proliferative vascular diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations:

SPA	surfactant protein A
SMC	vascular smooth muscle cell
TGF-β	transforming growth factor β
ACTA2	smooth muscle α-actin
MYH11	smooth muscle myosin heavy chain

Reference

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Highlights:

1. SPA is induced in vascular smooth muscle cells following vascular injury.
2. SPA mediates SMC phenotypic modulation.
3. SPA deficiency promotes myocardin and TGFβ1 expression in SMCs.
4. SPA deficiency attenuates injury-induced neointima formation.

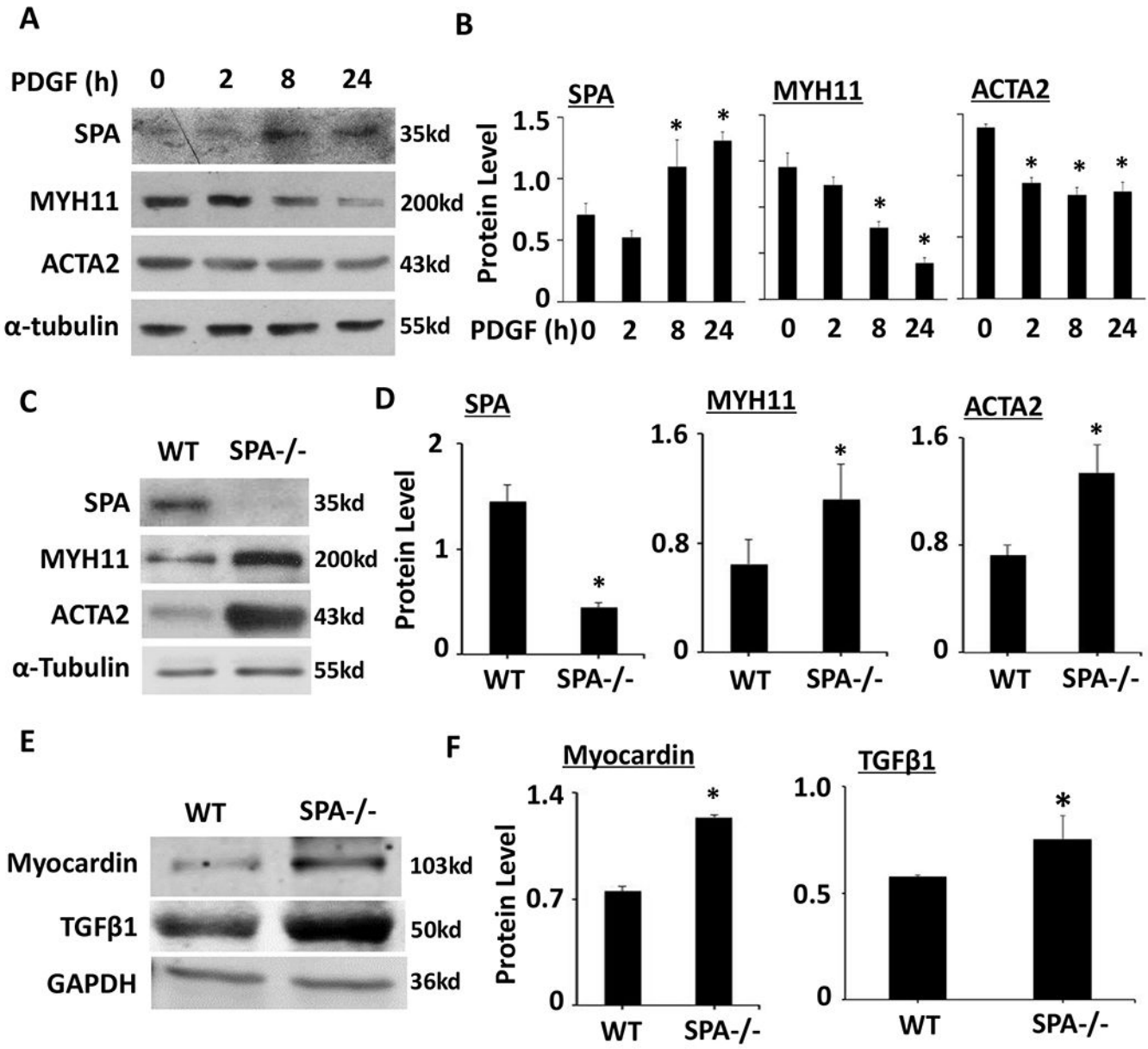

Figure 1: SPA mediated SMC phenotypic modulation through regulating Myocardin and TGFβ1 expression.

A, SMCs were isolated from mouse aorta, serum-starved for 24 h, and then treated with PDGF-BB (20 ng/ml) for the times indicated. SPA was increased along with the downregulation of SMC markers during PDGF-BB-induced SMC phenotypic modulation. **B,** Quantification of the protein expression shown in A by normalizing to α-tubulin. *P<0.05 vs control group (0 h), n=3. **C,** SMC marker expression in artery SMCs freshly isolated from aortas of wild (WT) or SPA knockout (SPA−/−) mice, and Western blots were performed to detect the expression of proteins indicated. **D,** Quantification of the protein expression shown in C by normalizing to α-tubulin. *P<0.05 vs WT group, n=5. **E,** Myocardin and TGFβ1 expression in arterial SMCs freshly isolated from WT and SPA

knockout aortas. **F,** Quantification of the protein expression shown in E by normalizing to GAPDH. *P<0.05 vs WT group, n=5.

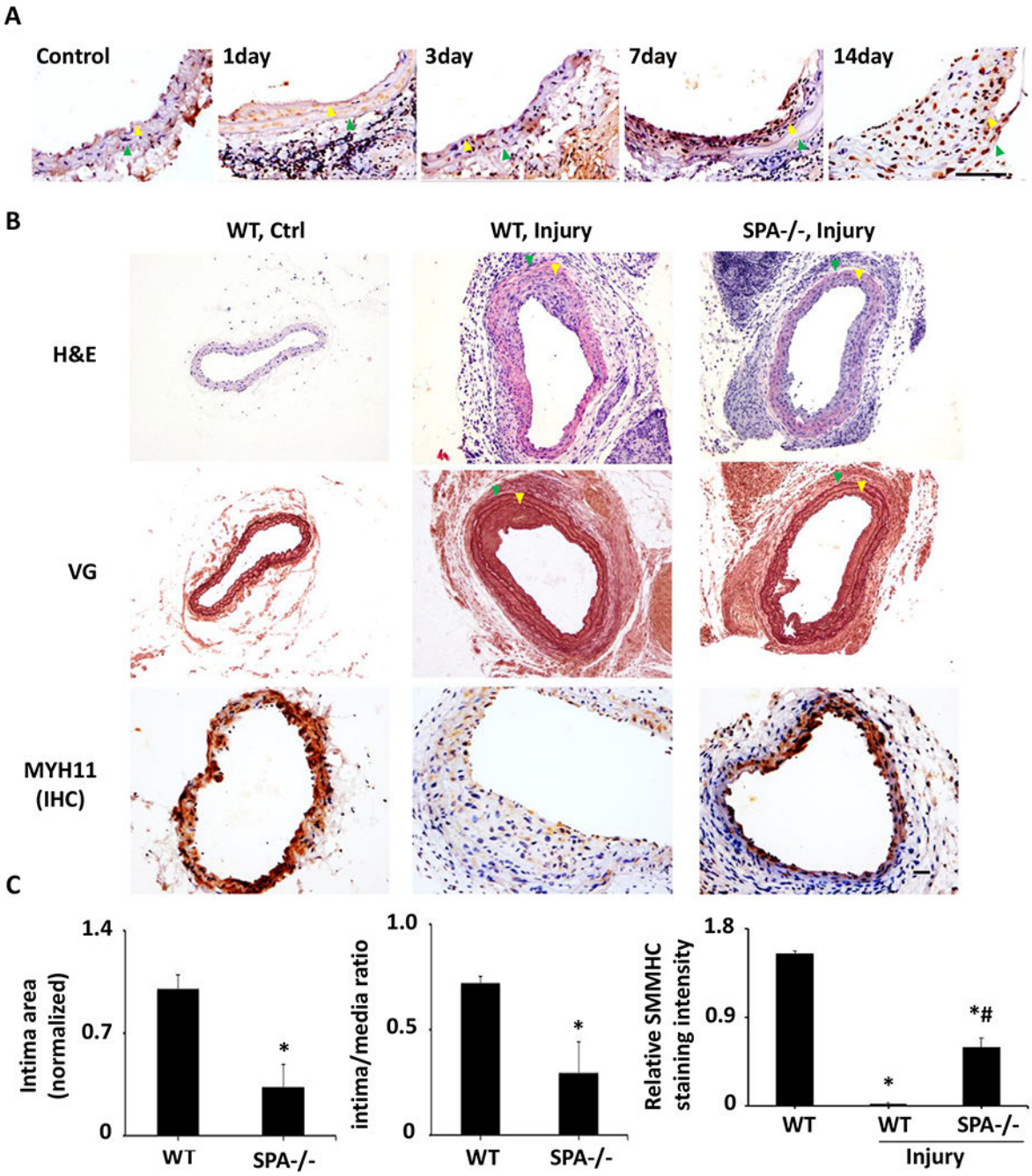

Figure 2: SPA was essential for injury-induced vascular remodeling.

Mouse left carotid arteries were injured through mechanical endothelial denudation. The arteries were collected at 1, 3, 7, 14 days following the surgery. **A,** SPA was induced in the mouse carotid arteries following the injury. **B,** Carotid artery sections of wild (WT) and SPA knockout (SPA−/−) mice with 14 days of injury were stained with H&E (upper panel), Elastica van Gieson (middle panel), or MYH11 antibody via immunohistochemistry (IHC) staining (lower panel). Yellow and green Arrows indicate the internal and external elastic lamina, respectively. Scale bar: 50μm for both A and B. **C,** Quantification of intima area,

intima/media ratio, or MYH11 staining intensity as indicated. *P<0.05 vs WT group; #P<0.05 vs injured WT group (n=8).