

Targeting AGGF1 (angiogenic factor with G patch and FHA domains 1) for Blocking Neointimal Formation After Vascular Injury

Yufeng Yao, PhD;* Zhenkun Hu, BS;* Jian Ye, PhD;* Changqing Hu, BS;* Qixue Song, BS; Xingwen Da, BS; Yubin Yu, BS; Hui Li, BS; Chengqi Xu, PhD; Qiuyun Chen, PhD; Qing Kenneth Wang, PhD, MBA

Background—Despite recent improvements in angioplasty and placement of drug-eluting stents in treatment of atherosclerosis, restenosis and in-stent thrombosis impede treatment efficacy and cause numerous deaths. Research efforts are needed to identify new molecular targets for blocking restenosis. We aim to establish angiogenic factor AGGF1 (angiogenic factor with G patch and FHA domains 1) as a novel target for blocking neointimal formation and restenosis after vascular injury.

Methods and Results—AGGF1 shows strong expression in carotid arteries; however, its expression is markedly decreased in arteries after vascular injury. AGGF1^{+/-} mice show increased neointimal formation accompanied with increased proliferation of vascular smooth muscle cells (VSMCs) in carotid arteries after vascular injury. Importantly, AGGF1 protein therapy blocks neointimal formation after vascular injury by inhibiting the proliferation and promoting phenotypic switching of VSMCs to the contractile phenotype in mice in vivo. In vitro, AGGF1 significantly inhibits VSMCs proliferation and decreases the cell numbers at the S phase. AGGF1 also blocks platelet-derived growth factor-BB–induced proliferation, migration of VSMCs, increases expression of cyclin D, and decreases expression of p21 and p27. AGGF1 inhibits phenotypic switching of VSMCs to the synthetic phenotype by countering the inhibitory effect of platelet-derived growth factor-BB on SRF expression and the formation of the myocardin/SRF/CArG-box complex involved in activation of VSMCs markers. Finally, we show that AGGF1 inhibits platelet-derived growth factor-BB–induced phosphorylation of MEK1/2, ERK1/2, and Elk phosphorylation involved in the phenotypic switching of VSMCs, and that overexpression of Elk abolishes the effect of AGGF1.

Conclusions—AGGF1 protein therapy is effective in blocking neointimal formation after vascular injury by regulating a novel AGGF1-MEK1/2-ERK1/2-Elk-myocardin-SRF/p27 signaling pathway. (*J Am Heart Assoc.* 2017;6:e005889. DOI: 10.1161/JAHA.117.005889.)

Key Words: restenosis • vascular disease • vascular smooth muscle

Coronary artery disease (CAD) is the leading cause of death and disability in the United States and other developed countries.^{1,2} The most common treatment strategy for CAD is the coronary revascularization procedure called percutaneous transluminal coronary angioplasty (PTCA), in which the atherosclerotic blockage in a coronary artery is opened by a balloon to restore blood flow to the heart. PTCA is performed on

nearly half a million people a year in the United States.³ However, PTCA is associated with vascular injury, which consequently results in restenosis (reocclusion of the artery) in 32% to 55% of patients.^{4,5} Incidence of restenosis has been reduced to 17% to 41% with use of bare-metal stents and further reduced to 10% with the use of drug-eluting stents.⁴⁻¹⁷ However, drug-eluting stents presented with an unexpected problem of an increased rate of in-

From the Key Laboratory of Molecular Biophysics of the Ministry of Education, Cardio-X Center, College of Life Science and Technology and Center for Human Genome Research, Huazhong University of Science and Technology, Wuhan, China (Y. Yao, Z.H., J.Y., C.H., Q.S., X.D., Y. Yu, H.L., C.X., Q.K.W.); Department of Molecular Cardiology, Center for Cardiovascular Genetics, Lerner Research Institute, Cleveland Clinic, Cleveland, OH (Q.C., Q.K.W.); Department of Molecular Medicine, Cleveland Clinic Lerner College of Medicine, Cleveland, OH (Q.C., Q.K.W.); Department of Genetics and Genome Sciences, Case Western Reserve University, Cleveland, OH (Q.K.W.).

*Dr Yao, Dr Zhenkun Hu, Dr Ye, and Dr Changqing Hu contributed equally to this work.

Correspondence to: Qiuyun Chen, PhD, Department of Molecular Cardiology, Center for Cardiovascular Genetics, Lerner Research Institute, Cleveland Clinic, Cleveland, OH. E-mail: chenq3@ccf.org and Qing Kenneth Wang, PhD, MBA, Center for Human Genome Research and College of Life Science and Technology, Huazhong University of Science and Technology, 1037 Luoyu Rd, Wuhan, China. E-mail: qkwang@mail.hust.edu.cn, wangq2@ccf.org

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Clinical Perspective

What Is New?

- Despite important improvements in percutaneous transluminal coronary angioplasty (PTCA) and implantation of drug-eluting stents to treat coronary artery disease (CAD) and myocardial infarction (MI), restenosis continues to occur in numerous patients, and in-stent thrombosis poses a serious threat to patients and causes death.
- We report a novel angiogenic factor angiogenic factor with G patch and FHA domains 1-based protein therapy to block restenosis associated with treatments of atherosclerosis, coronary artery disease, and myocardial infarction.
- We have identified 1 novel molecular mechanism and signaling pathway for restenosis after vascular injury, which is composed of AGGF1-MEK1/2-ERK1/2-EIk-VSMC phenotypic switching-VSMC proliferation and migration.

What Are the Clinical Implications?

- The angiogenic factor with G patch and FHA domains 1-targeted therapies may become a potentially effective treatment for blocking restenosis associated with the implantation of stents or revascularization therapies of atherosclerosis, coronary artery disease, and myocardial infarction, such as percutaneous transluminal coronary angioplasty.
- The angiogenic factor with G patch and FHA domains 1 protein may be used in drug-eluting stents in blocking restenosis after vascular injury associated with treatments of atherosclerosis, coronary artery disease, and myocardial infarction.

stent thrombosis (0.5–3.1%),^{4–7,18,19} which causes a significantly high 6.3% rate of MI and death.^{18,20–22} This led to a 20% reduction in the use of drug-eluting stents.²³ Thus, despite important improvements in PTCA and placement of drug-eluting stents to treat CAD and MI, restenosis continues to occur in about 10% of patients and in-stent thrombosis poses a serious threat to patients. To resolve these issues, it is critical to identify new molecular targets for blocking restenosis and for use in drug-eluting stents.^{19,23}

Restenosis is caused mainly by neointimal formation, which results from the proliferation and migration of vascular smooth muscle cells (VSMCs).^{19,21,24–26} Therefore, genes and proteins involved in VSMCs functions become candidates for developing strategies for blocking restenosis.

Both the human AGGF1 (angiogenic factor with G patch and FHA domains 1) gene and mouse AGGF1 gene were molecularly cloned by our group.²⁷ The AGGF1 gene encodes an AnGiogenic Factor with G Patch and FHA Domains protein of 714 amino acids.^{27–32} In subsequent studies, we have found that the AGGF1 protein can promote angiogenesis as potently as VEGFA (vascular endothelial growth factor A)²⁷; however, it functions independently from VEGFA signaling.³³ During embryogenesis, AGGF1 is

required for differentiation of multipotent hemangioblasts, which eventually differentiate into vascular cells and blood cells in zebrafish.³⁴ AGGF1 is required for specific differentiation of veins and development of intersegmental vessels during zebrafish embryogenesis,³⁵ which was confirmed by Kashiwada et al.³⁶ Our recent studies using AGGF1^{+/-} knockout (KO) mice showed that AGGF1 is essential for early embryogenesis and vascular development, and blocks vascular permeability.³⁷ We have found that AGGF1 is an important signaling factor that can induce autophagy by activating JNK,³³ regulate angiogenesis and vascular development by activating PI3K, AKT, GSK3 β , and S6K and inhibiting ERK1/2 (also reported by Hu et al.³⁸), and maintain vascular integrity by inhibiting VE-cadherin phosphorylation.^{35,37} We also showed that AGGF1 protein therapy can robustly treat CAD and MI by inducing autophagy and therapeutic angiogenesis.³³ AGGF1 is a much better agent than VEGFA for treating CAD and MI because AGGF1 blocks vascular permeability,³⁷ whereas VEGFA promotes vascular permeability, leading to formation of leaky vessels that cause no-reflow.³⁹

Our previous work focused solely on endothelial cells.^{27,30,33–35,40} However, we have reported that AGGF1 is also highly expressed in VSMCs;²⁷ thus, AGGF1 may play an important role in VSMCs functions. To explore the role of AGGF1 in VSMCs functions, we studied neointimal formation, a process caused mostly by VSMCs proliferation and migration^{19,21,24–26} using AGGF1^{+/-} knockout mice, AGGF1 protein therapy, and cellular signaling. We have found that AGGF1 regulates neointimal formation after vascular injury by regulating the phenotypic switching of VSMCs via the MEK-ERK-EIk signaling pathway.

Methods

PDGF-BB, Antibodies, and Plasmids

Recombinant platelet-derived growth factor-BB (PDGF-BB) was from R&D. The 6xHis-tagged AGGF1 protein was purified as described by us previously.^{27,33,37}

Antibodies against AGGF1, SM22, α -SMA, MYH11, serum response factor (SRF), myocardin, and Enhanced Green Fluorescent Protein (EGFP) were from Proteintech. Antibodies against phosphorylated ERK1/2 and total ERK1/2 were from Affinity Biosciences.

SRF cDNA was subcloned into pGFP-N1 (pGFP-SRF). Myocardin cDNA was subcloned into pcDNA3.1 (pcDNA3.1-myocardin). CARG box core promoter sequences were cloned into pGL3-luc promoter vector (Promega), resulting in luciferase reporters α -SMA-luc, SM22-luc, and MYH11-luc. All plasmid constructs were verified by Sanger DNA sequencing. The polymerase chain reaction (PCR) primers used for making the luciferase reporters are: Primers for α -SMA CARG core promoter: Forward 5'-atgcctcagggatctctgccattaccctagctc-3' and Reverse 5'-

atgcaagcttctcccactgtcttccaacaag-3'; Primers for SM22 CARG core promoter: Forward 5'-atgctctgagccccgtagactgctccaactt-3' and Reverse 5'-atgcaagcttccgccgtggtgatgtcagc-3'; Primers for MYH11 CARG core promoter: Forward 5'-atgcctcgagccggtggc tgcgtgaagg-3' and Reverse 5'-atgcaagctt tcccagcgccgcatacc-3'.

Cell Culture and Transfection

VSMCs in this study are referred to as the mouse VSMC line MOVAS-1, which is an immortalized mouse aorta VSMC line purchased from ATCC (American Type Culture Collection). Primary VSMCs were isolated from mouse aortas as described previously.⁴¹ Cells were maintained in the Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco Life Technologies, Gaithersburg, MD). Cells were cultured at 37°C with 5% CO₂ in a humidified cell incubator.

Small interfering RNA against SRF was designed by use of the Block-It TM RNAi Designer and chemically modified by the manufacturer (RioboBio). Transfection of VSMCs with the small interfering RNA (100 nmol) was performed using Lipofectamine 2000 (Invitrogen).

The transfection of VSMCs using plasmid DNA (2 µg) was also performed using Lipofectamine 2000 (Invitrogen).

Wire-Induced Vascular Injury of the Carotid Artery

Male C57BL/6 mice were used for all studies (n=6–8/group). The AGGF1^{+/-} knockout (KO) mice with a gene-trap allele were created by us and described previously.^{33,37} Animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Cleveland Clinic and the Ethics Committee on Animal Research of College of Life Science and Technology at Huazhong University of Science and Technology and performed in accordance with institutional guidelines.

The carotid artery restenosis model was created by guidewire injury with male mice at the age of 10 to 12 weeks (20–25 g). Male C57BL/6 mice or AGGF1^{+/-} mice under the C57BL/6 background (n=6–8/group) were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). The left common carotid artery was injured by inserting and removing a 0.014-in guidewire 3 times.

For AGGF1 protein therapy, the mice were injected intravenously with AGGF1 (0.25 mg/kg body weight in PBS) or the same amount of PBS (n=6–8/group) 1 day after the surgery, twice a week for 4 weeks.

Real-Time PCR Analysis

Quantitative real-time PCR analysis was performed using the FastStart Universal SYBR Green Master (Roch) and a

7900 HT Fast Real-Time PCR System (ABI) as described previously.^{42–44}

Western Blot Analysis

Western blot analysis was carried out as described previously.^{27,30,33,37,45,46} In brief, protein extracts from mouse carotid artery samples or cultured VSMCs were resolved by SDS-PAGE. The membranes were incubated with a primary antibody, followed by an appropriate secondary antibody. Images from Western blot analysis were captured and quantified using 1-D Analysis Software and Quantity One (Bio-Rad).

Cell Proliferation Assays

MOVAS-1 VSMCs or primary VSMCs isolated from mouse aortas were seeded onto 96-well plates, cultured for 36 hours, and assayed for proliferation using a CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instruction and as described.^{47–49} VSMC proliferation was measured by reading the absorbance at 450 nm using a microplate reader.

Immunostaining

Consecutive frozen sections of carotid arteries were immunostained with antibodies against α -SMA, SM22, and MYH11, which was followed by incubation with a biotinylated secondary antibody as described previously.^{33,37,45,50} Sections were also counterstained with hematoxylin and eosin as described.^{33,37,45,50} The intensity of the immunostaining signal was determined by measurement of the integrated optical density with light microscopy using a computer-based Image-Pro System as described previously.^{33,37,45,50}

Luciferase Reporter Assays

Relative luciferase activities were measured 24 hours after transfection and PDGF-BB treatment of MOVAS-1 VSMCs or primary VSMCs isolated from mouse aortas using the Dual-Glo luciferase assay kit (Promega) as described.^{30,47–49,51–53}

Co-Immunoprecipitation

VSMCs were transfected with pCDNA3.1-myocardin (10 µg) and pGFP-SRF (10 µg) and lysed. The lysates were incubated with anti-myocardin before immunoprecipitation with protein A/G agarose beads (Vigorous). Precipitated proteins were resolved by 10% SDS-PAGE gel electrophoresis and then immunoblotted with anti-SRF.

Chromatin Immunoprecipitation

VSMCs were transfected with pGFP-SRF (10 μ g) using Lipofectamine 2000 (Invitrogen). After 24 hours, cells were serum starved for 24 hours and then stimulated with PDGF-BB (25 μ g/L) with or without AGGF1 (100 μ g/L) for 2 hours. Cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature. The cross-linked chromatin was sonicated, and immunoprecipitated with 10 μ L of anti-SRF antibody or normal IgG, together with single-strand salmon sperm DNA saturated with protein A agarose beads (Vigorous). The precipitated chromatin DNA bound to SRF was then purified.

Data Analysis

Data are presented as mean \pm SEM. Two-group comparisons were analyzed by a Student *t* test or nonparametric Wilcoxon rank test when the sample size was small and/or the distribution was not normal. For comparisons of more than 2 groups, 1-way ANOVA or the generalized linear regression approach was employed for data with normal distribution and the Kruskal–Wallis test for non-normal distribution data or small samples. $P < 0.05$ was considered statistically significant.

Results

AGGF1 Expression Is Markedly Decreased in Carotid Arteries With Neointimal Formation After Vascular Injury

Our previous work focused solely on endothelial cells.^{27,30,33–35,40} In this study, we explored the potential function of AGGF1 in VSMCs. Immunostaining with an AGGF1-antibody showed that AGGF1 expression colocalized with expression of VSMCs marker α -SMA in carotid arteries (Figure 1A). These data are consistent with our previous report that AGGF1 is also highly expressed in VSMCs.²⁷

To explore the role of AGGF1 in VSMCs functions, we studied neointimal formation, a process caused mostly by VSMCs proliferation and migration.^{19,21,24–26} Immunostaining showed that AGGF1 expression was dramatically reduced in carotid arteries with neointimal formation after vascular injury (Figure 1B and 1C).

Heterozygous AGGF1 Knockout Exacerbates Neointimal Formation After Vascular Injury

We investigated the role of AGGF1 in neointimal formation in vivo using AGGF1^{+/-} KO (AGGF1^{-/-} is embryonically lethal) and wild-type (WT) mice (AGGF1^{+/+} littermates) at the age of 16 to 20 weeks (Figure 2). After wire-induced vascular injury in the carotid arteries, AGGF1^{+/-} mice showed a

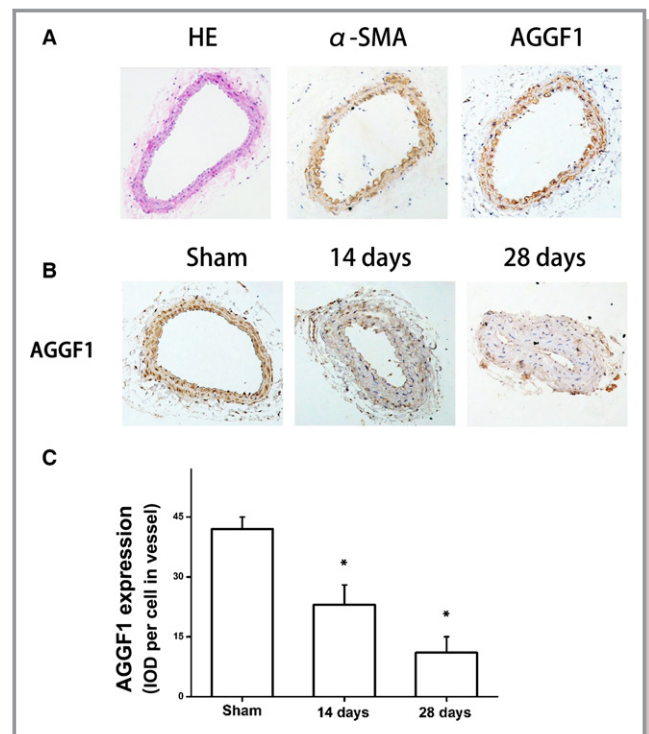


Figure 1. AGGF1 expression is markedly reduced with neointimal formation after vascular injury. A, Immunostaining showing strong AGGF1 protein expression in aortic vascular smooth muscle cells. B and C, Immunohistochemistry showing dramatic reduction of AGGF1 protein expression in wire-injured carotid arteries compared with sham-operated vessels 14 and 28 days after surgery. The AGGF1 expression level was quantified by measuring the integrated optical density (IOD) per cell in vessel from the images from (A and B). * $P < 0.05$ ($n = 6$ /group).

significantly higher degree of neointimal formation (ie, increased intima-to-media ratios) as compared with WT mice, particularly at day 28 after vascular injury (Figure 2A and 2B).

AGGF1 Protein Therapy Blocks Neointimal Formation After Vascular Injury

Most interestingly, intravenous injection of AGGF1 protein (0.25 mg/kg body weight) twice a week dramatically decreased neointimal formation in both WT and AGGF1^{+/-} KO mice after vascular injury as compared with control PBS treatment (Figure 2A and 2B). These results indicate that AGGF1 plays an important role in neointimal formation after vascular injury.

AGGF1 Regulates Proliferation, But Not Apoptosis, of VSMCs in Carotid Arteries After Vascular Injury

We determined the effects of arterial injury on cell proliferation in vivo by staining the neointimal area using

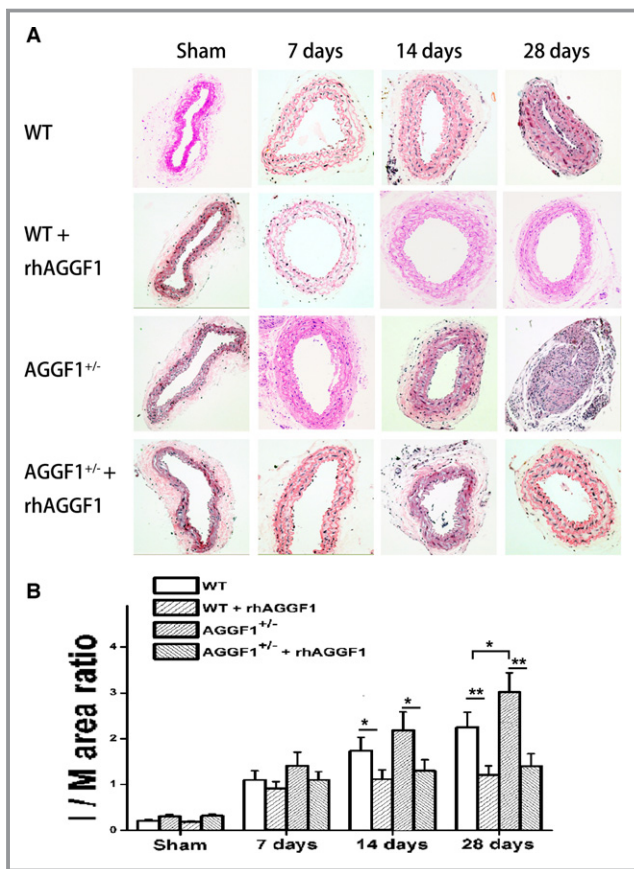


Figure 2. AGGF1 haploinsufficiency (heterozygous AGGF1^{+/-} knockout or KO) increases neointimal formation after vascular injury in mice and AGGF1 protein therapy blocks neointimal formation. A, Hematoxylin and eosin staining showing the intimal formation among different groups of mice after vascular injury. B, The intima/media (I/M) ratios of carotid arteries are graphed for different groups of mice. Heterozygous AGGF1^{+/-} mice show a significantly higher degree of neointimal formation (higher I/M ratios) at day 28 after vascular injury as compared with WT mice. Tail-vein injection of AGGF1 protein significantly decreases neointimal formation in both WT and heterozygous AGGF1^{+/-} mice after vascular injury. * $P < 0.05$, ** $P < 0.01$ ($n = 6$ /group).

proliferating cell nuclear antigen. After wire injury of the carotid artery, proliferating cell nuclear antigen staining significantly increased in the neointimal area in AGGF1^{+/-} KO mice as compared with WT mice (Figure 3A and 3C). Interestingly, AGGF1 protein treatment dramatically reduced proliferating cell nuclear antigen staining (cell proliferation) (Figure 3A and 3C). These data demonstrate the critical role of AGGF1 in neointimal formation via regulating cell proliferation.

We also examined the effects of AGGF1 on apoptosis in carotid arteries after vascular injury. Terminal deoxynucleotidyl transferase dUTP nick-end labeling staining showed that AGGF1 KO or protein treatment did not have any effect on apoptosis (Figure 3B and 3C).

AGGF1 Protein Inhibits PDGF-BB-Induced Proliferation and Migration of VSMCs

We treated MOVAS1 VSMCs using recombinant AGGF1 protein, and assessed their proliferation. The AGGF1 protein treatment significantly inhibited the proliferation of VSMCs (Figure 4A) and decreased the cell numbers at the S phase during mitosis (Figure 4B). Moreover, we also found that the AGGF1 protein inhibited PDGF-BB-induced proliferation and migration of VSMCs (Figure 4C and 4E). PDGF is one of the most important factors involved in neointimal formation after vascular injury via its potent stimulating effects on VSMCs proliferation and migration,²³ and therefore is frequently used as a cellular model for investigating signaling events involved in neointimal formation (Figure 4). As shown in Figure 4C, PDGF-BB stimulated VSMCs proliferation, but AGGF1 protein treatment significantly inhibited PDGF-BB-induced VSMCs proliferation. PDGF significantly increased the number of cells at the S-phase during cell division (Figure 4D), which is usually correlated with increased cell proliferation. However, the effect was blocked by AGGF1 protein treatment (Figure 4D). Similarly, cell migration assays using the scratch-wound healing assay showed that PDGF-BB stimulated VSMCs migration, but the effect was blocked by AGGF1 protein treatment (Figure 4E).

AGGF1 Protein Inhibits PDGF-BB-Induced Increases of Cyclin D and Reduction of Expression of p21 and p27 in VSMCs

To identify the molecular mechanism by which AGGF1 protein inhibits cell division and proliferation, we used Western blot analysis to examine the expression levels of key cell cycle regulatory proteins cyclin D1, p21Cip1, and p27Kip1 in VSMCs stimulated with PDGF-BB. PDGF significantly increased the expression level of cyclin D and reduced the expression levels of p21Cip1 and p27Kip1 (Figure 4F). These effects were reversed by AGGF1 protein (Figure 4F). These results suggest that AGGF1 inhibits VSMCs proliferation by reducing expression of cyclin D and inhibiting expression of p21 and p27.

AGGF1 Regulates VSMCs Phenotypic Switching In Vivo After Vascular Injury

VSMCs proliferation and migration are regulated by their phenotypic switching between a contractile/differentiated phenotype and a synthetic/dedifferentiated phenotype, a critical step in the regulating of vascular function and disease.^{24,54–57} The contractile phenotype of VSMCs is characterized by a low rate of cell proliferation and migration and high expression of contractile proteins such as α -SMA,

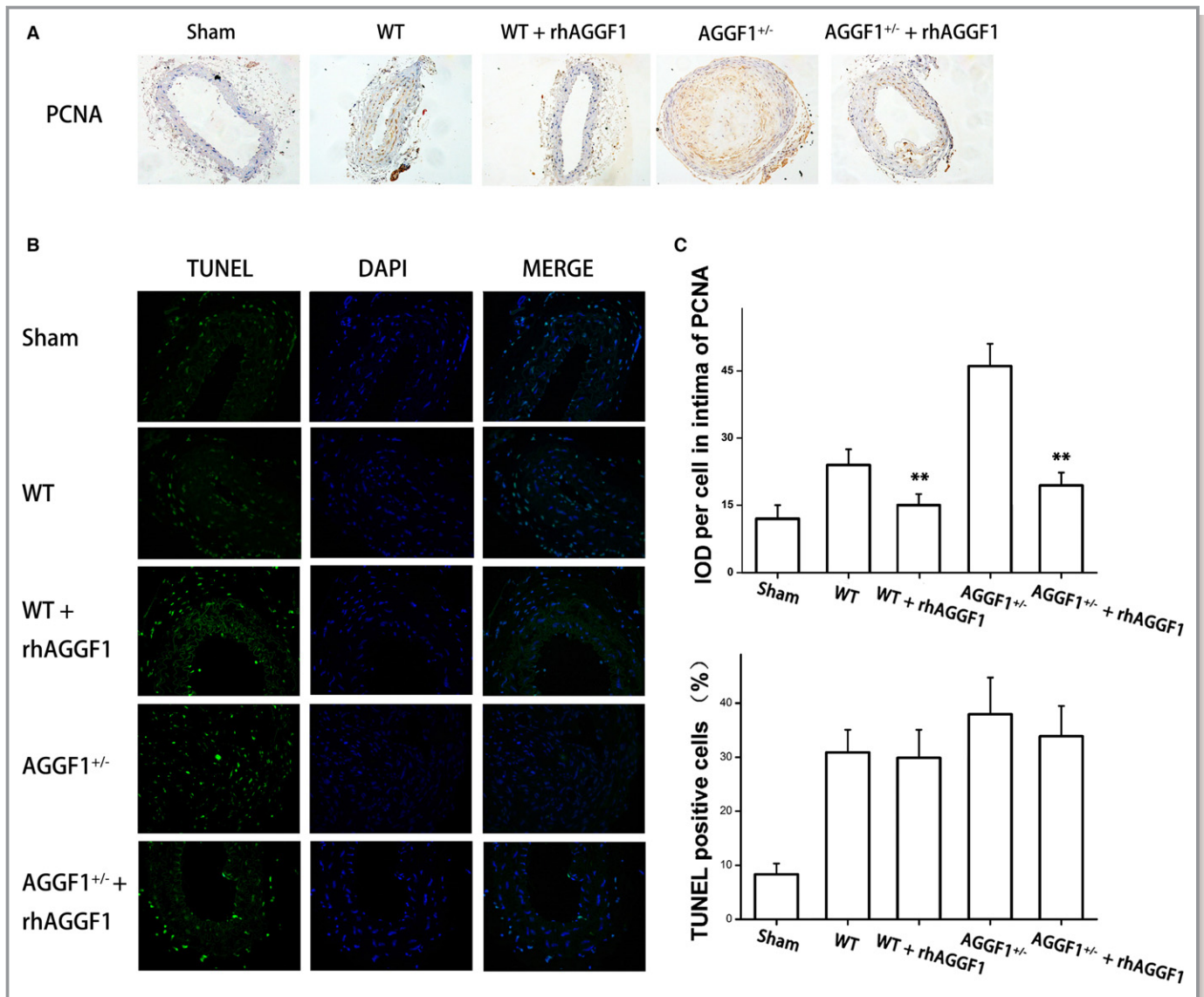


Figure 3. AGGF1 regulates cell proliferation in injured carotid arteries. A, Immunostaining with an antibody against proliferating cell nuclear antigen (PCNA) in carotid arteries at day 28 after vascular injury. B, Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining for apoptosis in carotid arteries at day 28 after vascular injury. DAPI indicates 4',6-diamidino-2-phenylindole. C, Summary data for PCNA and TUNEL signal intensities. ** $P < 0.01$ ($n = 8$ /group). IOD indicates integrated optical density.

SM22 (transgelin), and smooth muscle myosin heavy chain 11 (MYH11).^{24,54–57} The synthetic phenotype is characterized by a high rate of cell proliferation and migration and decreased expression of contractile proteins.^{24,54–57} Disruption of the balance of the VSMCs phenotypes (for example, a transition from a contractile phenotype to a synthetic phenotype) will favor VSMCs proliferation and migration, leading to development of neointimal formation and restenosis after vascular injury.^{24,55–58} Immunostaining showed that 28 days after the surgery, vascular injury significantly reduced the expression levels of α -SMA, SM22, and MYH11 in the WT group compared with the sham group, and further reduced α -SMA, SM22, and MYH11 expression in the AGGF1^{+/-} KO mice (Figure 5A and 5B).

Intravenous injection of the AGGF1 protein significantly increased the expression levels of α -SMA, SM22, and MYH11 after vascular injury and blocked neointimal formation and restenosis in both WT and AGGF1^{+/-} KO mice (Figure 5A and 5B). These data suggest that AGGF1 regulates VSMCs phenotypic switching after vascular injury.

However, the AGGF1 protein treatment did not affect the expression levels of α -SMA, SM22, and MYH11 in carotid arteries from normal control mice (Figure 5C), probably because of high endogenous expression levels of α -SMA, SM22, and MYH11. Together, our data suggest that AGGF1 regulates VSMCs phenotypic switching only under a pathological condition, for example, after vascular injury, but not under a normal condition.

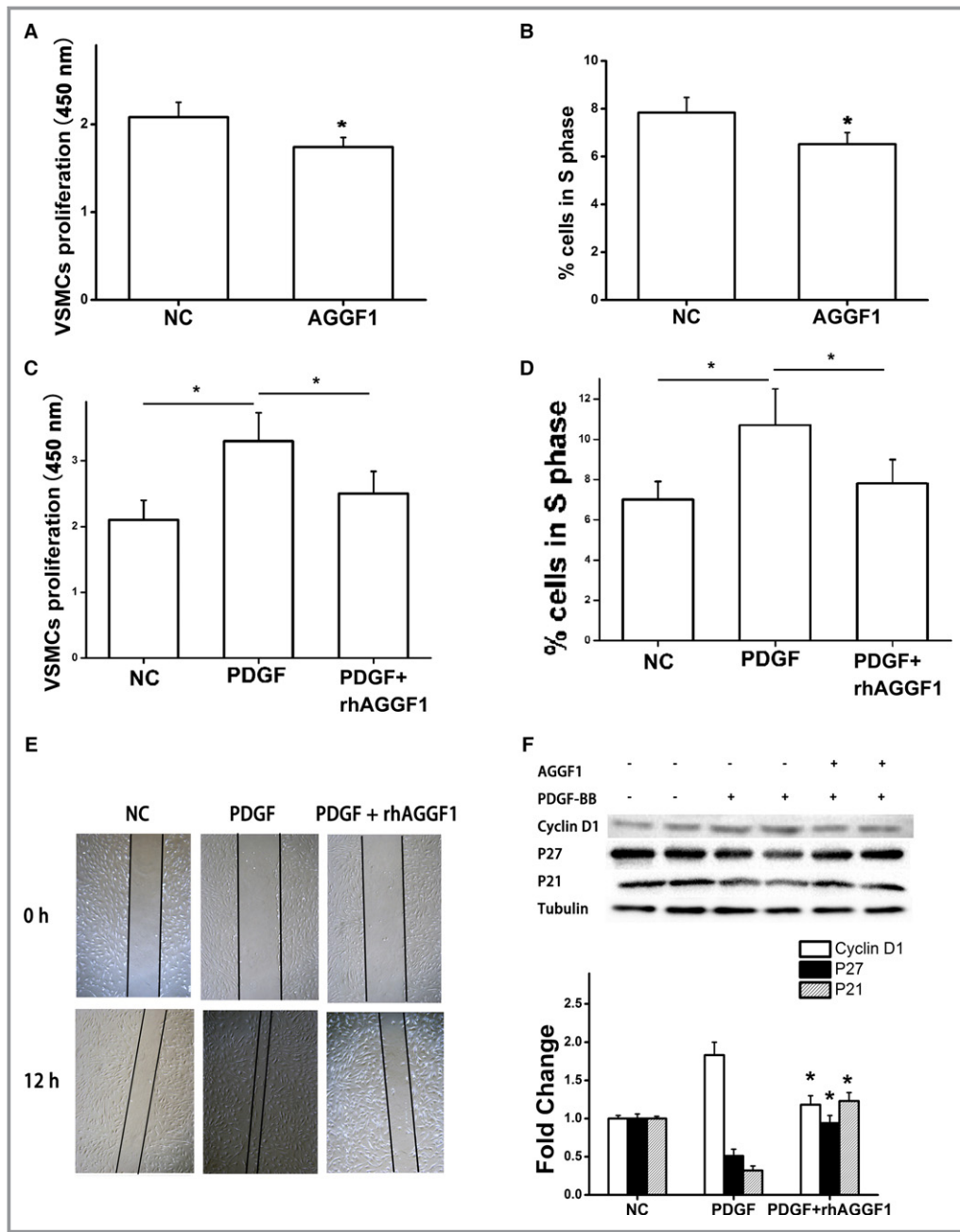


Figure 4. AGGF1 protein inhibits the proliferation and migration of vascular smooth muscle cells (VSMCs) induced by the platelet-derived growth factor subunit B homodimer (PDGF-BB). A, AGGF1 significantly inhibits the proliferation of VSMCs. B, AGGF1 significantly decreases the number of S-phase cells. C, AGGF1 blocks the PDGF-BB-induced proliferation of VSMCs. D, AGGF1 decreases the number of S-phase cells. E, AGGF1 decreases the migration of VSMCs (scratch-wound healing assay). F, AGGF1 downregulates cyclin D and upregulates p21 and p27. NC indicates negative control (PBS). * $P < 0.05$ ($n = 3$ /group).

AGGF1 Regulates VSMCs Phenotypic Switching In Vitro

The critical role of AGGF1 in phenotypic switching of VSMCs was further demonstrated by the finding that VSMCs treated with PDGF-BB, one of the most important factors involved in

neointimal formation after vascular injury, showed a reduced expression level of α -SMA, SM22, or MYH11 on both the mRNA and protein levels, but the effects were reversed by AGGF1 protein treatment (Figure 6A through 6C). Note that PDGF-BB did not affect the expression level of AGGF1 in VSMCs (Figure 6A).

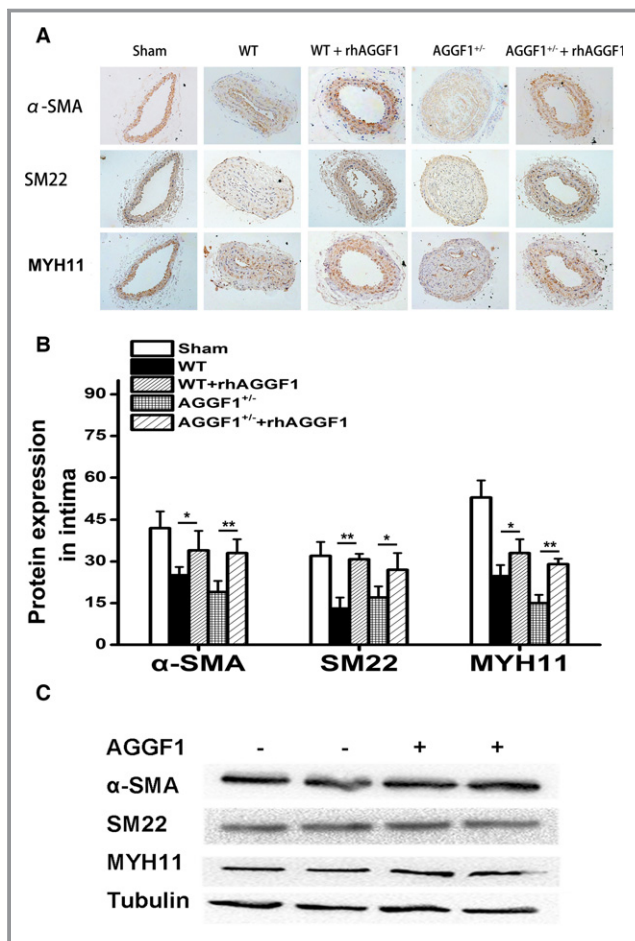


Figure 5. AGGF1 regulates the phenotypic switching of vascular smooth muscle cells in injured carotid arteries in mice. A, Immunostaining analysis showing that the expression of contractile markers α -SMA (α smooth muscle actin), SM22 (smooth muscle protein 22- α or transgelin), and MYH11 (myosin heavy polypeptide 11, smooth muscle) is downregulated after vascular injury, but the effect was blocked by intravenous injection of AGGF1 protein. B, Summary data for (A). C, Western blot analysis showing that the expression levels of α -SMA, SM22, and MYH11 are not affected in carotid arteries from normal mice with intravenous injection of recombinant AGGF1 protein vs control PBS. * P <0.05, ** P <0.01 (n =8/group).

To further examine the role of AGGF1 in VSMCs phenotypic switching, we treated MOVAS-1 VSMCs with the recombinant AGGF1 protein or control PBS and carried out Western blot analysis. Our data showed that the AGGF1 protein significantly increased the expression levels of α -SMA, SM22, and MYH11 (P <0.05) (Figure 6D and 6F). Similar results were obtained from primary VSMCs isolated from mouse aortas (at passage 5) (Figure 6E and 6F). The MOVAS-1 VSMCs used in our studies are likely to be synthetic VSMCs with relatively fewer expression levels of α -SMA, SM22, and MYH11 than primary VSMCs isolated from mouse aortas (Figure 6F).

AGGF1 Regulates VSMCs Phenotypic Switching by Countering the Effects of PDGF-BB on SRF

The SRF is a key transcription factor for phenotypic switching of VSMCs by binding to the promoter/regulatory regions of VSMCs-specific contractile genes, specifically to the cis-acting DNA sequence CarG box (CC[A/T]6GG), to induce their expression.^{55–58} Western blots and real-time PCR analyses showed that although AGGF1 protein treatment can reverse downregulation of contractile genes/proteins α -SMA, SM22, and MYH11 by PDGF-BB in VSMCs, but the effects were completely lost in cells with knockdown of SRF expression by small interfering RNA (+siSRF) (Figure 6G and 6H).

Luciferases assays with promoter-luciferase reporters (α -SMAp-luc, SM22p-luc, or MYH11p-luc) containing the CarG box showed that overexpression of SRF stimulated the transcriptional activation of the α -SMA promoter, SM22 promoter, and MYH11 promoter (Figure 7A). The AGGF1 protein treatment significantly increased the luciferase activities from the α -SMA, SM22, and MYH11 promoters (Figure 7A; compare SRF and SRF+rhAGGF1). On the other hand, the PDGF-BB treatment significantly reduced the luciferase activities from the VSMCs contractile marker gene promoters (Figure 7B). Treatment with AGGF1 protein reversed the effects of PDGF-BB (Figure 7B). Chromatin immunoprecipitation-qPCR revealed that AGGF1 reversed the effects of PDGF-BB on reduced SRF binding to α -SMA, SM22, and MYH11 promoter regions (Figure 7C). Co-immunoprecipitation assays showed that AGGF1 increased the interaction between myocardin and SRF (Figure 7D). Also, PDGF-BB reduced the interaction between myocardin and SRF, but the effect was reversed by AGGF1 (Figure 7E). Together, our data suggest that AGGF1 regulates phenotypic switching of VSMCs to the synthetic phenotype (downregulation of contractile genes/proteins) by countering the effects of PDGF-BB on SRF.

AGGF1 Regulates VSMCs Phenotypic Switching by Regulating the ERK-Elk Pathway

Elk is a transcriptional factor belonging to the ternary complex factors of the E26 transformation-specific-domain family.^{59,60} Phosphorylated Elk displaces myocardin from the SRF–CarG complex, thereby inhibiting expression of contractile markers and regulating VSMCs phenotypic switching.^{59,60} PDGF-BB induced increased phosphorylation of Elk, MEK1/2, and ERK1/2, but AGGF1 protein treatment blocked the effects (Figure 8A).

Overexpression of Elk blocked expression of α -SMA, SM22, and MYH11 (Figure 8B). Treatment with AGGF1 protein failed to rescue the effect of Elk (Figure 8B), indicating that AGGF1 acts upstream of Elk.

Luciferase assays showed that SRF increased transcriptional activation of α -SMA, SM22, and MYH11 promoters;

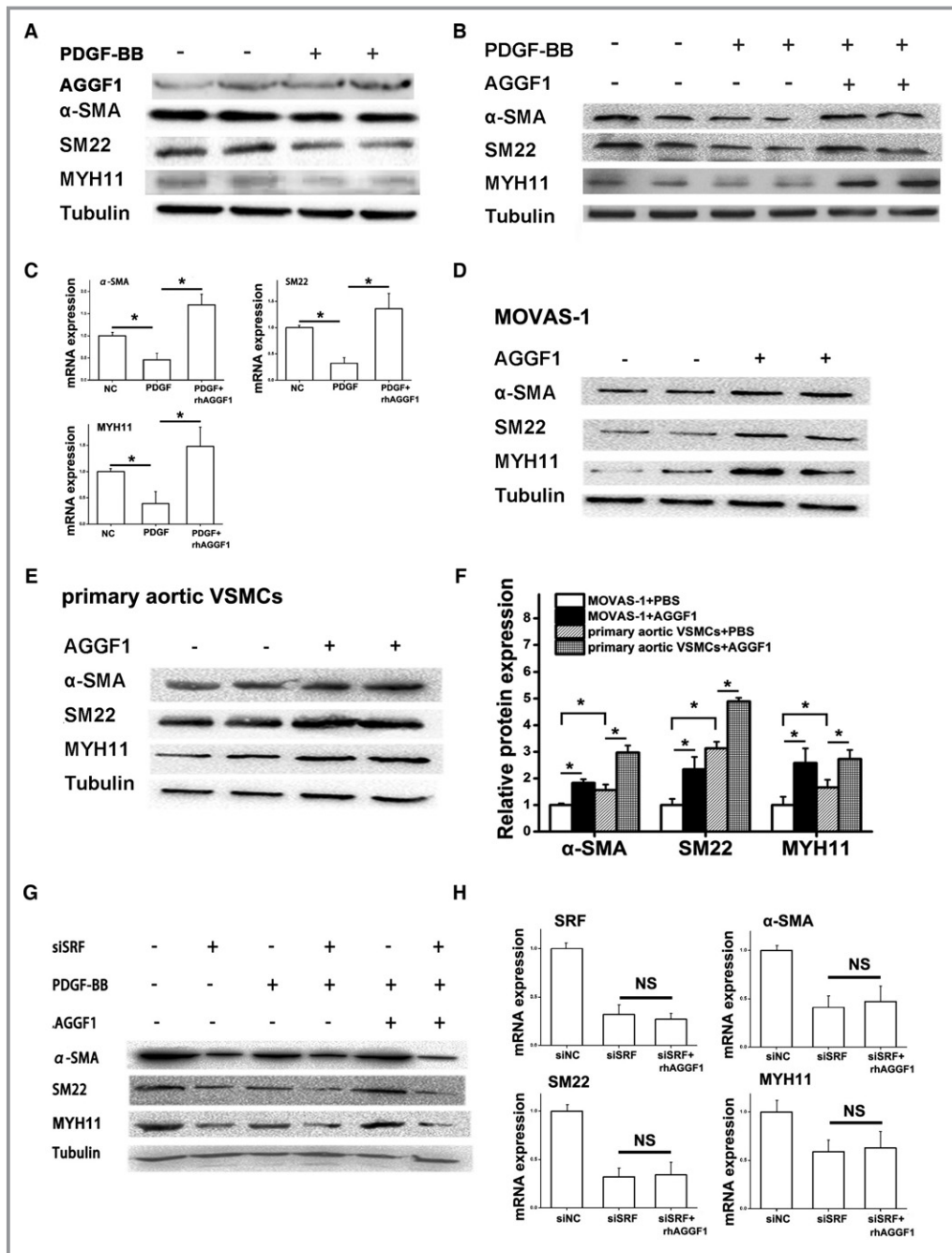


Figure 6. AGGF1 regulates expression of phenotypic switching markers of vascular smooth muscle cells (VSMCs). A, The platelet-derived growth factor subunit B homodimer (PDGF-BB) decreases the expression levels of α -SMA (α smooth muscle actin), SM22 (smooth muscle protein 22- α or transgelin), and MYH11 (myosin heavy polypeptide 11, smooth muscle) at the protein level. PDGF-BB does not affect the expression level of AGGF1. B, AGGF1 blocks PDGF-induced downregulation of contractile markers at the protein level. C, AGGF1 blocks PDGF-induced downregulation of contractile markers at the mRNA level. NC indicates negative control. D, AGGF1 increases the expression levels of α -SMA, SM22, and MYH11 in mouse VSMC line MOVAS-1 VSMCs. E, AGGF1 increases the expression levels of α -SMA, SM22, and MYH11 in primary VSMCs isolated from mouse aortas. F, The expression levels of α -SMA, SM22, and MYH11 in MOVAS-1 VSMCs are significantly less than in primary mouse aortic VSMCs. G, Knockdown of SRF encoding the serum response factor by siRNA (siSRF) abolishes the effect of AGGF1 on PDGF at the protein level. H, Knockdown of SRF by siRNA (siSRF) abolishes the effect of AGGF1 on PDGF at the mRNA level. * P <0.05 (n=3/group). NS indicates not significant.

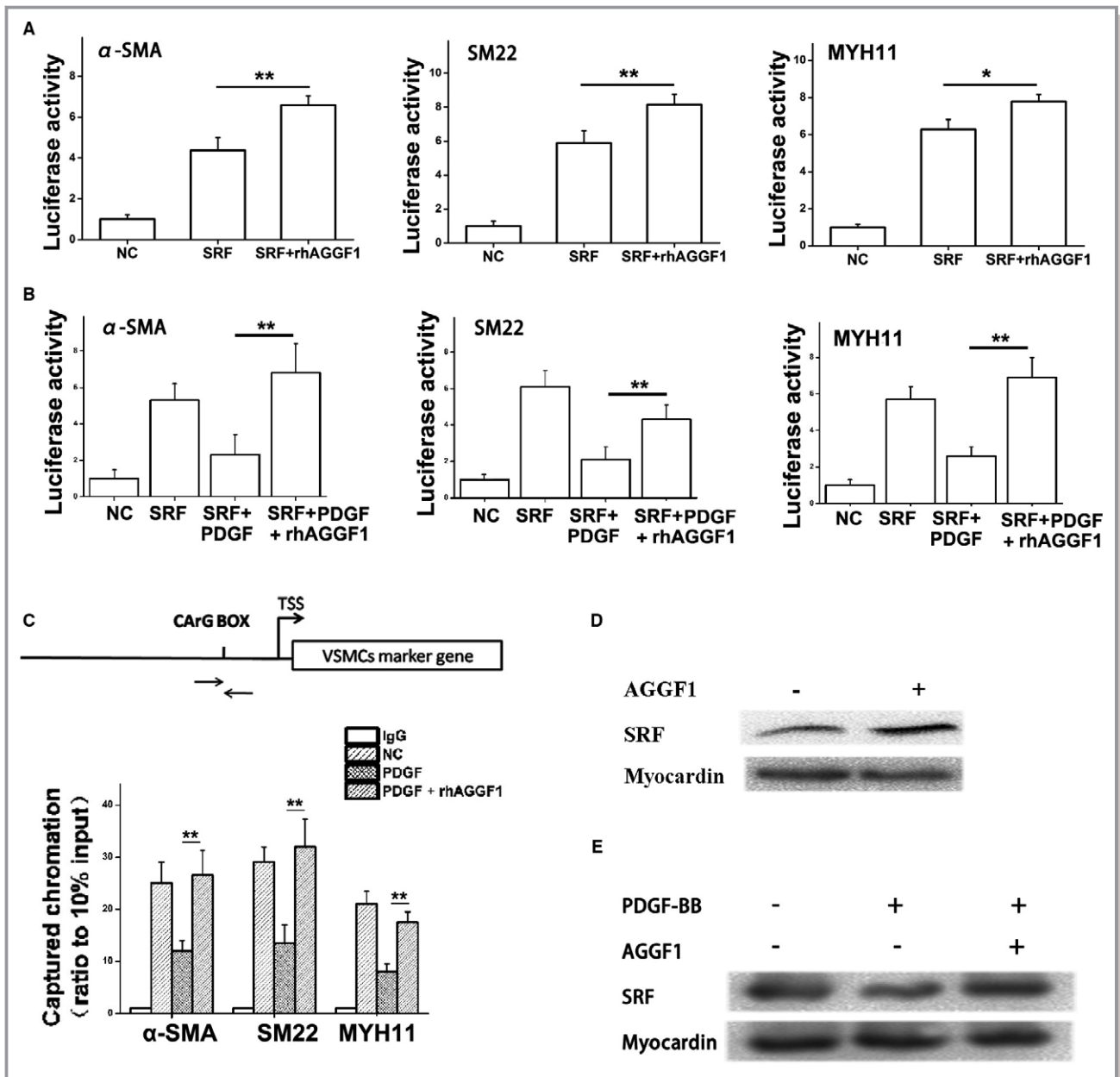


Figure 7. AGGF1 regulates transcriptional activation of vascular smooth muscle cells (VSMCs) phenotypic switching markers. A, Luciferase assays showing that AGGF1 increases transcriptional activation of VSMCs contractile marker genes encoding α -SMA (α smooth muscle actin), SM22 (smooth muscle protein 22- α or transgelin), and MYH11 (myosin heavy polypeptide 11, smooth muscle) in the presence of SRF (serum response factor) (SRF vs SRF+rhAGGF1). NC indicates negative control. B, Luciferase assays showing that the platelet-derived growth factor subunit B homodimer (PDGF-BB) represses SRF-induced transcriptional activation of VSMCs contractile marker genes encoding α -SMA, SM22, and MYH11, but the effects are abolished by AGGF1 protein. C, Chromatin immunoprecipitation assays to detect protein–DNA interaction between SRF and the CARg elements at the promoter/regulatory regions of VSMCs contractile marker genes. PDGF reduces the SRF binding to CARg elements, but the effects are abolished by AGGF1 protein. D, Co-immunoprecipitation assays showing that the AGGF1 protein increases the interaction between SRF and myocardin in MOVAS-1 VSMCs with overexpression of both myocardin and SRF. An anti-myocardin antibody was used for immunoprecipitation, and an anti-SRF antibody was used for immunoblotting. E, Co-immunoprecipitation assays showing that PDGF reduced the interaction between SRF and myocardin, but the effect was reversed by AGGF1. * P <0.05 and ** P <0.01 (n =3/group).

however, the effects were blocked by overexpression of Elk (Figure 8C). Treatment with AGGF1 protein failed to rescue the effect of Elk (Figure 8C), further indicating that AGGF1 acts upstream of Elk.

Discussion

In this study, we show that angiogenic factor AGGF1 is a novel target for developing strategies to block neointimal formation,

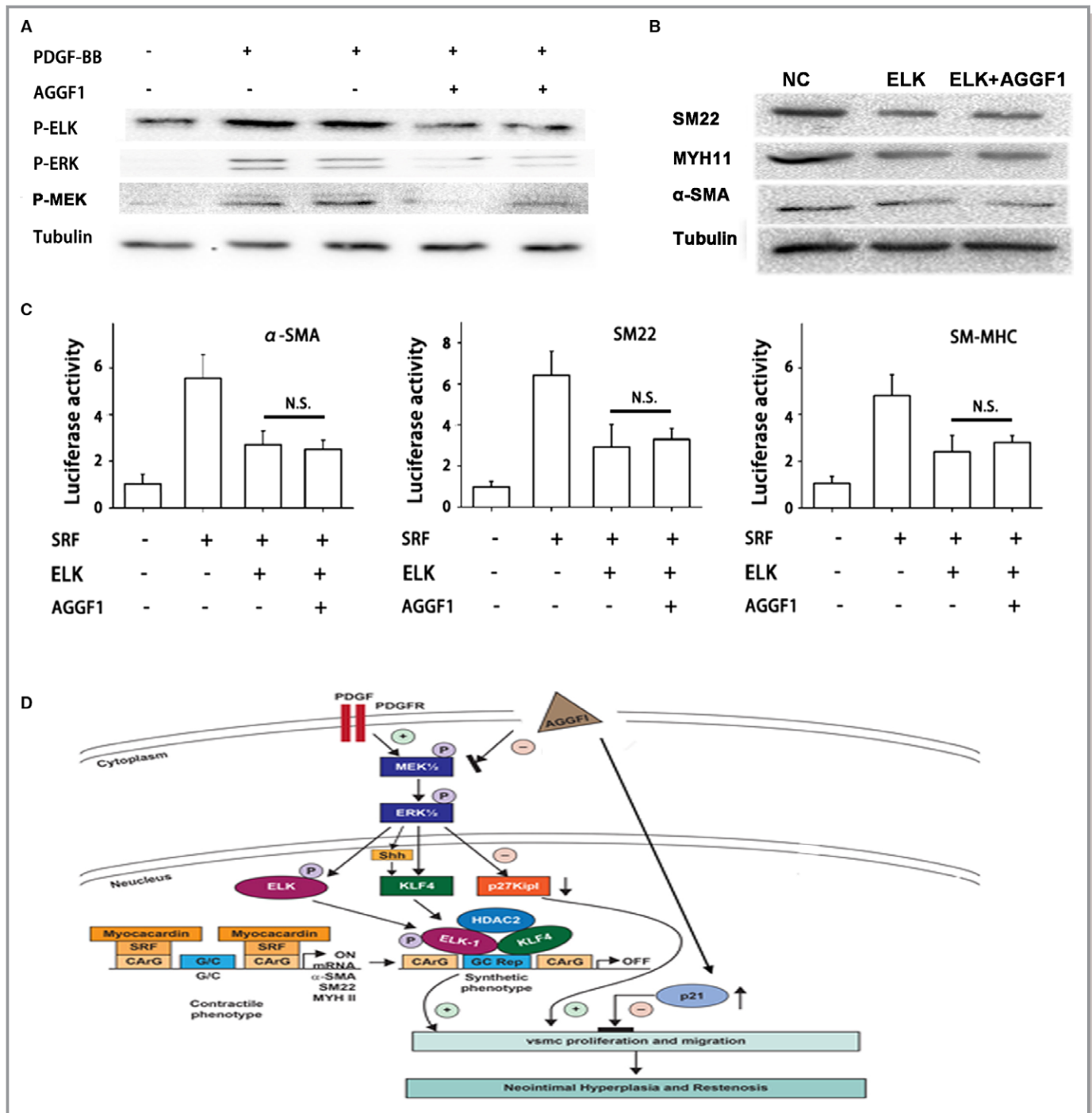


Figure 8. AGGF1 regulates the phenotypic switching of vascular smooth muscle cells (VSMCs) by regulating the MEK-ERK-Elk pathway. A, Western blot analysis showing that AGGF1 protein treatment blocks the platelet-derived growth factor subunit B homodimer (PDGF-BB)–induced increases of phosphorylation of Elk, MEK1/2, and ERK1/2. B, Overexpression of Elk blocks expression of α-SMA (α smooth muscle actin), SM22 (smooth muscle protein 22-α or transgelin), and MYH11 (myosin heavy polypeptide 11, smooth muscle) while treatment with AGGF1 protein failed to rescue the effect of Elk. NC indicates negative control. C, Luciferase assays showing that treatment with AGGF1 protein failed to rescue the effect of Elk, which inhibits the transcriptional activation of contractile marker genes. N.S. indicates not significant. D, The molecular signaling pathway for neointimal formation after vascular injury.

which causes restenosis after vascular injury associated with treatments of atherosclerosis, CAD, and MI using PTCA and placement of stents. First, we demonstrated that AGGF1 plays

an important role in neointimal formation after vascular injury in mice. The expression level of AGGF1 was dramatically reduced in carotid arteries with neointimal formation after a

wire-induced vascular injury (Figure 1). Then, we showed that heterozygous AGGF1^{+/-} KO mice displayed an increased level of neointimal formation after vascular injury, which was associated with increased proliferation of VSMCs (Figures 2 and 3). Most interestingly, AGGF1 protein therapy by intravenous injection blocked VSMCs proliferation and neointimal formation after wire-induced vascular injury in mice. Therefore, we conclude that AGGF1 protein therapy or alternative delivery strategies of AGGF1 may become an effective method to treat restenosis after revascularization therapies of atherosclerosis, CAD, and MI. Alternatively, AGGF1 protein may be used in drug-eluting stents in blocking restenosis after vascular injury.

Our study has identified a novel signaling pathway and a fundamental mechanism for the pathobiology of neointimal formation after vascular injury, consisting of AGGF1, MEK, ERK, and Elk (Figure 8D). Revascularization procedures such as PTCA and placement of stents cause vascular injury, which exposes VSMCs to growth factors PDGF and thrombin, the 2 potent stimulators of VSMCs proliferation and migration and neointimal formation.²³ PDGF induces phosphorylation of MEK and ERK, which then induces phosphorylation of Elk. Phosphorylated Elk displaces myocardin from the SRF-CarG complex, which inhibits expression of contractile markers α -SMC, SM22, and MYH11, leading to VSMCs phenotypic switching to the synthetic phenotype with a great potential of proliferation and migration (Figure 8).^{59,60} We show that AGGF1 blocks all effects of PDGF (Figures 4 through 8). It is likely that the AGGF1-MEK-ERK signaling pathway may not be the only molecular mechanism by which AGGF1 regulates VSMCs functions and neointimal formation. Identification of a non-ERK pathway may be particularly interesting. We have reported that in endothelial cells, AGGF1 can activate the PI3K/AKT signaling pathway important for angiogenesis,³⁷ regulate phosphorylation of VE-cadherin important for maintenance of vascular integrity,³⁷ and activate JNK, which is important for autophagy and therapeutic angiogenesis for CAD and MI.³³ Based on our extensive analyses of reported data in the literature and our preliminary data, we concluded that the AGGF1-mediated ERK signaling pathway is the most relevant and logical signaling pathway for AGGF1 in VSMCs. Therefore, our research plans focus on the AGGF1-ERK signaling, which has been well documented in the literature.^{37,38} It should be interesting to explore other AGGF1-mediated signaling pathways, such as AKT, JNK, and other pathways, in VSMCs functions and neointimal formation in the future.

We have shown that AGGF1 can block PDGF-induced downregulation of p27Kip1 (Figure 4D), which may be caused by AGGF1-mediated inhibition of ERK1/2. It is possible that p27Kip1 may also be a downstream effector of AGGF1-ERK signaling. Inhibition of ERK1/2 by specific inhibitors or

overexpression of dominant negative ERK inhibits PDGF-induced downregulation of p27Kip1.^{61,62} p27Kip1 is a cyclin-dependent kinase inhibitor that regulates cell cycle progression and cell proliferation.⁶³ Downregulation of p27Kip1 was shown to promote PDGF-induced VSMCs proliferation.⁶⁴ We have also shown that AGGF1 blocked PDGF-induced upregulation of cyclin D and downregulation of p21. The molecular mechanism for the regulation of p21 and p27Kip1 by AGGF1 remains to be identified in the future.

During the preparation of this article, Zhou et al⁶⁵ reported that overexpression of AGGF1 using adenoviruses inhibited neointimal formation induced by carotid artery ligation. However, there are several major differences between our study and that by Zhou et al. First, we presented the data on neointimal formation from AGGF1^{+/-} KO mice, but the Zhou et al study did not have such data. Second, different signaling pathways were identified by the 2 groups. Zhou et al argued that AGGF1 interacted with myocardin, but we did not find such interaction even though we performed such studies. Our comprehensive analysis showed that AGGF1 inhibits PDGF-BB-induced activation of MEK1/2, ERK1/2, and Elk, which displaces myocardin from the SRF-CarG complex, and inhibits expression of contractile markers α -SMC, SM22, and MYH11, leading to VSMCs phenotypic switching to the synthetic phenotype with a great potential of proliferation and migration (Figure 8). Moreover, AGGF1 has been previously reported to inhibit activation of ERK1/2 by Hu et al³⁸ and Zhang et al.³⁷ Third, Zhou et al found that PDGF-BB reduced expression of AGGF1, but our study showed that treatment of VSMCs with PDGF-BB did not affect the expression level of AGGF1 (Figure 6A). Fourth, we used the wire-induced vascular injury model, whereas Zhou et al used a carotid artery ligation model.

The data in this study and in other reported publications^{27,30,33,37} suggest that there is a receptor for AGGF1. The receptor for AGGF1 has not been reported yet and we are actively pursuing it. Moreover, we have reported recently that the AGGF1 signaling pathway is independent from the VEGFA signaling pathway and knockdown of VEGFR2 expression did not have any effect on AGGF1 signaling.³⁷ It is interesting to note that the intravenous administration of the recombinant AGGF1 protein into mice under a normal physiological condition did not affect the expression levels of VSMC contractile markers α -SMA, SM22, and MYH11 in carotid arteries (Figure 5C). The VSMCs in mice under a normal condition are mostly in the contractile state with high endogenous expression levels of α -SMA, SM22, and MYH11. Therefore, they may not be responsive to the AGGF1 stimulation to further increase the levels of the contractile phenotypic markers. However, VSMCs in mice under a pathological condition such as those after vascular injury are more likely to be in the synthetic state so that they are

responsive to the AGGF1 stimulation to increase the expression levels of the contractile markers α -SMA, SM22, and MYH11 (Figures 5 through 7). Alternatively, it is also possible that both MOVAS cells and primary aortic VSMCs used in our study (Figure 6) are stem cell–derived progeny as purported by Yuan et al.⁶⁶ Hence, it may not only be the process of phenotypic switching that is targeted by AGGF1 but also modulation of the appearance of stem cell–derived progeny. Together, our data suggest that AGGF1 regulates VSMCs phenotypic switching under a pathological condition, but not under a normal condition. This interesting effect may be advantageous for AGGF1 protein therapy because it may not cause unwanted effects.

Conclusions

In summary, the present study identifies a critical role of AGGF1 on the proliferation, migration, and phenotypic switching of VSMCs and neointimal formation after vascular injury. Our study also identifies novel molecular mechanisms and signaling pathways for neointimal formation after vascular injury, which provides fundamental understanding of the pathobiology of this important process. Finally, our data establish AGGF1 as a novel target for developing strategies to block restenosis associated with treatments of atherosclerosis, CAD, and MI.

Author Contributions

Yao and Wang conceived and designed the study; Yao, Z. Hu, Ye, Chen, and Wang performed experiments; Yao, C. Hu, Chen, and Wang analyzed data; Song, Li, Xu, and Da contributed reagents/materials; Yao and Wang drafted the manuscript; Yao, Chen, and Wang critically revised the manuscript; and Wang supervised the study; all authors reviewed the manuscript.

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Disclosures

None.

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