Dedicator of Cytokinesis 2, A Novel Regulator for Smooth Muscle Phenotypic Modulation and Vascular Remodeling

Xia Guo, Ning Shi, Xiao-Bing Cui, Jia-Ning Wang, Yoshinori Fukui, Shi-You Chen

Rationale: Vascular smooth muscle cell (SMC) phenotypic modulation and vascular remodeling contribute to the development of several vascular disorders such as restenosis after angioplasty, transplant vasculopathy, and atherosclerosis. The mechanisms underlying these processes, however, remain largely unknown.

Objective: The objective of this study is to determine the role of dedicator of cytokinesis 2 (DOCK2) in SMC phenotypic modulation and vascular remodeling.

Methods and Results: Platelet-derived growth factor-BB induced DOCK2 expression while modulating SMC phenotype. DOCK2 deficiency diminishes platelet-derived growth factor-BB or serum-induced downregulation of SMC markers. Conversely, DOCK2 overexpression inhibits SMC marker expression in primary cultured SMC. Mechanistically, DOCK2 inhibits myocardin expression, blocks serum response factor nuclear location, attenuates myocardin-binding to serum response factor, and thus attenuates myocardin-induced smooth muscle marker promoter activity. Moreover, DOCK2 and Kruppel-like factor 4 cooperatively inhibit myocardin–serum response factor interaction. In a rat carotid artery balloon-injury model, DOCK2 is induced in media layer SMC initially and neointima SMC subsequently after vascular injury. Knockdown of DOCK2 dramatically inhibits the neointima formation by 60%. Most importantly, knockout of DOCK2 in mice markedly blocks ligation-induced intimal hyperplasia while restoring SMC contractile protein expression.

Conclusions: Our studies identified DOCK2 as a novel regulator for SMC phenotypic modulation and vascular lesion formation after vascular injury. Therefore, targeting DOCK2 may be a potential therapeutic strategy for the prevention of vascular remodeling in proliferative vascular diseases. (*Circ Res. 2015;116:e71-e80. DOI: 10.1161/CIRCRESAHA.116.305863*)

Key Words: cell proliferation ■ dedicator of cytokinesis 2 ■ vascular remodeling.

Vascular remodeling contributes to the development of several vascular disorders including restenosis after angioplasty, vein graft stenosis, transplant vasculopathy, and atherosclerosis, etc. Although neointimal formation may be controlled by different mechanisms, medial smooth muscle cell (SMC) phenotypic modulation from a contractile to a synthetic phenotype triggered by damaging to blood vessel walls followed by SMC migration and proliferation plays a major role in injury-induced vascular remodeling. Elucidating mechanisms underlying SMC phenotypic modulation, therefore, is critical for understanding the pathogenesis of above-mentioned vascular proliferative disorders and for developing effective therapeutics to block the narrowing of vessel lumen because of vascular remodeling.

Dedicator of cytokinesis 2 (DOCK2) is an atypical guanine nucleotide exchange factor for the Rho-small guanine triphosphatase. Under physiological conditions, DOCK2 is mainly expressed in hematopoietic cells and is involved in lymphocyte activation and migration via regulating actin cytoskeleton through Rac activation. Deletion of DOCK2 enables long-term cardiac allograft survival via suppressing graft tissue infiltration of alloreactive T cells. DOCK2 also controls various immunologic functions including helper T-cell differentiation, neutrophil chemotaxis, and type I interferon induction. It is unknown, however, whether DOCK2 is involved in regulating vascular function.

In this study, we found that platelet-derived growth factor-BB (PDGF-BB) induced DOCK2 expression in SMC while modulating SMC phenotype. Knockout of DOCK2 (DOCK2−/−) enhanced SMC marker gene expression in primary cultured SMCs isolated from DOCK2−/− mice. Ectopic expression of DOCK2 inhibited the marker protein expression. DOCK2 seemed to modulate SMC phenotype by suppressing myocardin/serum response factor (SRF)–mediated...
transcription of SMC marker genes. In vivo study using rat carotid artery balloon-injury model showed that DOCK2 is essential for injury-induced neointima formation because knockdown of DOCK2 dramatically inhibited the neointima formation by 60%. Most importantly, knockout of DOCK2 markedly blocked ligation-induced intimal hyperplasia and SMC phenotypic modulation in mouse arteries. Our study demonstrates that DOCK2 is a novel regulator of SMC phenotypic modulation and an essential factor contributing to vascular remodeling.

**Methods**

**Animals**

Male Sprague–Dawley rats weighing 450 to 500 g were purchased from Harlan. DOCK2−/− mice were previously described.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. Animal surgical procedures were approved by the Institutional Animal Care and Use Committee of The University of Georgia.

**Cell Culture**

SMCs were cultured by enzyme digestion method from rat or mouse thoracic aorta as described previously.13,14 The primary cultured SMCs were confirmed by the expression of smooth muscle α-actin (α-SMA) and SM22α. SMCs <6 passages with 70% of confluence were used in the experiments.

**Construction of Adenovirus**

Adenoviral vectors expressing scramble or DOCK2 short hairpin RNA (shRNA) (shDOCK2) were constructed, and the viruses were purified as described previously.15 The top and bottom strand sequences for scramble and shDOCK2 were included in Online Data Supplement.

**Western Blot Analysis**

Western blot was performed as described previously.15 Antibodies against DOCK2 (Millipore), α-SMA (Sigma), calponin (Abcam), SM22α (Abcam), SRF (Santa Cruz Biotechnologies), myocardin (Myocd, Abcam), smooth muscle myosin heavy chain (SMMHC, Biomedical Technologies Inc.), Kruppel-like factor 4 (KLF4, Abcam), Flag (Sigma), GAPDH (Sigma), and α-tubulin (Sigma) were used for immunoblotting.

**Real-Time Quantitative Polymerase Chain Reaction**

Quantitative polymerase chain reaction was performed as described previously.16 The primer sequences for the involved genes are listed in the Online Table I.

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>α-SMA</td>
<td>smooth muscle α-actin</td>
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<tr>
<td>DOCK2</td>
<td>dedicator of cytokinesis 2</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>KLF4</td>
<td>Kruppel-like factor 4</td>
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<tr>
<td>PDGF-BB</td>
<td>platelet-derived growth factor</td>
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<td>shRNA</td>
<td>short hairpin RNA</td>
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<td>SMC</td>
<td>smooth muscle cell</td>
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<td>SMMHC</td>
<td>smooth muscle myosin heavy chain</td>
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<td>SRF</td>
<td>serum response factor</td>
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**Figure 1. Dedicator of cytokinesis 2 (DOCK2 [DK2])–mediated smooth muscle cell (SMC) phenotypic modulation.**

A, Temp-dependent induction of DK2 and SMC markers by platelet-derived growth factor-BB (PDGF-BB; 10 ng/mL). B, Quantitative analysis of protein expression shown in A by normalizing to α-tubulin. *P<0.05 vs vehicle-treated group (0 hour). n=3. C, Knockout of DOCK2 blocked PDGF-BB–induced downregulation of SMC marker proteins. SMCs isolated from wild type (WT) and DOCK2 knockout (DK2−/−) mice were treated with vehicle (−) or PDGF-BB (+) for 36 hours. DK2, smooth muscle α-actin (α-SMA), and calponin protein expression were detected by Western blot. D, Protein levels shown in C were quantified by normalizing to GAPDH. Fold changes are shown. *P<0.05 vs vehicle-treated WT group (−); #P<0.05 vs vehicle-treated DK2−/− group, n=3. E, Serum (fetal bovine serum [FBS])-induced SMC phenotypic modulation in WT and DK2−/− SMCs. Cells were treated with vehicle (−) or 10% FBS (+) for 48 hours. SM22α, calponin, and DK2 protein expression was detected by Western blot. F, Protein levels shown in E were quantified by normalizing to GAPDH. *P<0.05 vs vehicle-treated WT group (−); #P<0.05 vs vehicle-treated DK2−/− group; ##P<0.05 vs vehicle-treated DK2−/− group, n=3. G, DOCK2−/− attenuated FBS-induced SMC phenotypic modulation. The percentage downregulation of SMC markers by FBS was calculated by the following formula: [(untreated value–FBS-treated)/untreated value]×100. *P<0.05 vs WT group for each corresponding marker, n=3. SMMHC indicates smooth muscle myosin heavy chain.
Transfection and Luciferase Assay
Transfection of α-SMA promoter construct with myocardin and DOCK2 plasmids into SMCs was described previously.17 Luciferase assay was performed as described previously.16 Experiments were repeated for 3×. Results shown were from a representative experiment with SDs.

Immunofluorescent Staining
SMCs cultured form wild-type (WT) and DK2−/− mice or Rat were grown on glass coverslips. Immunofluorescent staining was performed using SRF or DOCK2 antibody as described previously.18 Stained cells were imaged using a Nikon fluorescent microscope.

Rat Carotid Artery Injury Model and Adenoviral Gene Transfer
Rat carotid artery balloon-injury model and the method that introduces adenovirus into the injured artery have been previously described.14 The balloon-injured and adenovirus-dwelled segments were collected at 3, 7, and 14 days later. Subsequent morphometric analyses were performed in a double-blinded manner.

Mouse Carotid Artery Ligation-Injury Model
The mouse carotid artery ligation model was described previously.19 Mouse left common carotid arteries were exposed and ligated. The ligation-injured segments were collected at 4 weeks later. Contralateral noninjured carotid arteries were used as controls. Subsequent morphometric analyses were performed in a double-blinded manner.

Histomorphometric Analysis and Immunohistochemistry Staining
Vessel segments were removed for analysis. The dissected arteries were stained with modified hematoxylin and eosin or Elastica van Gieson staining and captured using a Nikon microscope. The circumference of lumen, internal elastic lamina, and external elastic lamina were measured by Image-pro Plus Software. Immunohistochemistry staining was performed using DOCK2, α-SMA, or SMMHC antibody as described previously.20

Statistical Analysis
All data were evaluated with a 2-tailed, unpaired Student t test, or compared by 1-way ANOVA followed by Fisher t test and are expressed as mean±SD. A value of P<0.05 was considered statistically significant.

Results
DOCK2 Was Essential for SMC Phenotypic Modulation
PDGF-BB is a potent stimulator of SMC phenotypic modulation.21 Although DOCK2 is expressed in a low level in normal SMC, its expression was significantly induced in SMCs when the cells were treated with PDGF-BB. Dose-dependent study showed that 5 ng/mL of PDGF-BB significantly elevated DOCK2 expression, whereas 10 ng/mL of PDGF-BB induced a high level of DOCK2 expression (Online Figure IA and IB). Therefore, we used 10 ng/mL of PDGF-BB for all subsequent experiments. Because SMC phenotypic modulation is characterized by the reduction of SMC contractile proteins, we detected whether DOCK2 induction by PDGF-BB correlates with the attenuation of SMC marker proteins. As shown in Figure 1A and 1B, PDGF-BB induced DOCK2 expression in

Figure 2. Dedicator of cytokinesis 2 (DOCK2 [DK2]−)-modulated smooth muscle cell (SMC) phenotype through downregulating myocardin. A, Knockout of DOCK2 (DK2−−) increased myocardin (Myocd) but not serum response factor (SRF) mRNA expression. SMCs isolated from wild type (WT) or DK2−− mice were treated with vehicle (−) or platelet-derived growth factor-BB (PDGF-BB; +) for 24 hours. Myocd and SRF mRNA expression was detected by quantitative polymerase chain reaction (qPCR). *P<0.05 vs vehicle-treated (−) WT group, #P>0.05 vs vehicle-treated DK2−− group. n=3. B, Ectopic expression of DK2 inhibited Myocd but not SRF mRNA expression. Myocd and SRF mRNA expression was detected by qPCR. *P<0.05 compared with control plasmid group (Ctrl), n=3. C, Ectopic expression of DK2 blocked Myocd and SMC marker protein expression. The protein expression of DK2, myocardin, and smooth muscle myosin heavy chain (SMMHC) was detected by Western blot. D, Quantification of protein levels shown in C by normalized to α-tubulin. *P<0.05 compared with Ctrl group, n=3. E, Knockdown of DK2 by short hairpin RNA (shRNA) enhanced Myocd and SMMHC protein expression in SMC. Primary cultured SMCs were transfected with control (shCtrl) or DOCK2 shRNA (shDK2) followed by PDGF-BB-treatment. The protein expression of DK2, Myocd, and SMMHC was detected by Western blot. F, Quantification of protein levels shown in E by normalized to α-tubulin. *P<0.05 compared with shCtrl group, n=3.
DOCK2 Inhibited Myocard While Modulating SMC Phenotype

SRF and its coactivator Myocd are well-known factors regulating SMC contractile protein expression. PDGF-BB has been shown to induce SMC phenotypic modulation by inhibiting Myocard and SRF expression or blocking Myocard–SRF interaction. To determine the mechanisms underlying DOCK2 function in SMC phenotypic modulation, we first tested whether DOCK2 regulates Myocard or SRF expression. We found that PDGF-BB inhibited Myocard and SRF expression (Figure 2A). Knockout of DOCK2, however, dramatically increased Myocard but not SRF expression. PDGF-BB treatment failed to reduce Myocard expression in DOCK2−/− SMCs, suggesting that DOCK2 can block Myocard expression (Figure 2A). SRF expression was inhibited by PDGF-BB in DOCK2−/− SMCs similarly as in WT SMCs (Figure 2A), suggesting that DOCK2 is not involved in SRF expression. Conversely, overexpression of DOCK2 blocked Myocard, but not SRF expression (Figure 2B) while blocking α-SMA and calponin expression (Online Figure II). Importantly, DOCK2 overexpression attenuated Myocard expression while inhibiting SMMHIC expression (Figure 2C and 2D), suggesting that DOCK2 modulating SMC phenotype, at least partially, through blocking Myocard expression. Indeed, knockdown of DOCK2 enhanced Myocard expression while increased SMMHIC expression (Figure 2E and 2F). These data demonstrated that downregulation of Myocard is one of the
DOCK2 Mediated PDGF-BB Function in Blocking SRF Nuclear Location and Attenuated Myocd-Induced SMC Marker Promoter Activity

Although DOCK2 did not affect SRF expression, SRF plays an important role in SMC marker gene expression and SMC phenotype. Because SRF has to be translocated into nuclei to serve as a transcription factor, we sought to determine whether DOCK2 is involved in SRF nuclear location. PDGF-BB inhibited SRF nuclear location (Figure 3A and 3B), consistent with a previous report. However, DOCK2 blunt the effect of PDGF-BB and restored SRF nuclear location (Figure 3A and 3B), suggesting that DOCK2 mediated PDGF-BB function in inhibiting SRF nuclear location.

Because Myocd is a coactivator of SRF in regulating SMC marker gene transcription, we sought to determine whether DOCK2 alters Myocd/SRF-activated SMC marker gene transcription. As shown in Figure 3C, Myocd strongly induced α-SMA promoter activity (Figure 3C). These data demonstrate that DOCK2 regulates SMC phenotype through inhibiting Myocd-activated transcription of SMC contractile genes. Previous studies have shown that DOCK2 function in lymphocyte migration is mainly mediated by Rac1. We thus tested whether DOCK2 function in SMC promoter activity is also associated with Rac1. As shown in Figure 3D, a specific inhibitor CPYPP (4-[3′-(2′-chlorophenyl)-2′-propen-1′-ylidene]-1-phenyl-3,5-pyrazolidinedione) that blocks DOCK2 activity in activating Rac1 (Online Figure III) did not affect DOCK2 function in inhibiting Myocd-induced α-SMA promoter activity, suggesting that DOCK2 modulates SMC phenotype independent of its role in activating Rac1.

The role of DOCK2 in SMC marker gene transcription motivated us to hypothesize that DOCK2 may be a nuclear factor in SMCs. Indeed, immunostaining of DOCK2 showed that PDGF-BB–induced DOCK2 were located in the nuclei of SMCs in addition to the cytoplasm membrane and the cytoplasm (Figure 3E), consistent with its function in inhibiting Myocd/ SRF-activated α-SMA promoter activity and SRF nuclear location.

DOCK2 Inhibited Myocd–SRF Interaction

Because PDGF-BB also inhibits Myocd binding to SRF, which blocks SMC marker transcription, we explored whether DOCK2 modulates SMC phenotype by influencing SRF–SRF interaction. Thus, Flag-tagged Myocd plasmids were transfected into SMCs, and the interaction of Myocd with endogenous SRF was detected. As shown in Figure 4A and 4B, Myocd indeed interacted with SRF in SMCs, and PDGF-BB inhibited their interaction. However, when DOCK2 was knocked down by shRNA, the reduced Myocd–SRF interaction by PDGF-BB was significantly increased. These data suggest that DOCK2 mediates PDGF-BB–induced reduction of mycardin binding to SRF. To confirm DOCK2 function in blocking myocardin–SRF binding, we overexpressed DOCK2 in SMCs and found that DOCK2 indeed significantly blocked the interaction between Myocd and SRF (Figure 4C and 4D). To determine how DOCK2 inhibits Myocd–SRF interaction, we tested whether DOCK2 interacts with myocardin or SRF. As shown in Figure 4E and 4F, DOCK2 interacted with Myocd, but not SRF. PDGF-BB treatment significantly enhanced DOCK2–Myocd interaction. These data suggest that DOCK2 may inhibit Myocd–SRF interaction by directly interacting with Myocd, which likely diminished the availability of Myocd for SRF.

DOCK2 and KLF4 Cooperatively Blocked Myocd–SRF Interaction

KLF4 is found to be a critical factor in modulating PDGF-BB–induced SMC phenotypic modulation. Because both KLF4 and DOCK2 modulate SMC phenotype by blocking Myocd–SRF interaction, we sought to determine whether KLF4 and DOCK2 work together to regulate Myocd–SRF interaction. As shown in Figure 5A and 5B, individual expression of either DOCK2 or KLF4 significantly attenuated Myocd–SRF binding. Coexpression of DOCK2 and KLF4 further and almost completely blocked the Myocd–SRF interaction (Figure 5A and 5B), suggesting that DOCK2 and KLF4 cooperatively blocked Myocd–SRF interaction.
and KLF4 cooperatively regulate Myocd–SRF interaction in SMC phenotypic modulation. In addition, knockdown of DOCK2 enhanced Myocd–SRF interaction. But this effect was blocked by the overexpression of KLF4 (Figure 5D and 5E), probably because of a deprivation of SRF by a strong interaction between KLF4 and SRF, as shown in Figure 5A and previous findings. KLF4 seemed to compete with Myocd for binding to SRF because the KLF4–SRF binding was significantly increased, whereas Myocd–SRF binding was diminished (Figure 5D–5F). Interestingly, the KLF4–SRF interaction was regulated by DOCK2 because overexpression of DOCK2 attenuated KLF4 binding to SRF (Figure 5A and 5C), whereas knockdown of DOCK2 increased KLF4–SRF binding (Figure 5D and 5F). This effect of DOCK2 was likely attributed to the role of DOCK2 in SRF nuclear location (Figure 3A and 3B). Collectively, these data indicate that DOCK2 can block both the Myocd–SRF interaction and the KLF4–SRF interaction in phenotype-modulated SMCs.

**DOCK2 Expression Was Activated During Injury-Induced SMC Phenotypic Modulation and Was Essential for Injury-Induced Vascular Remodeling**

Mechanical injury to artery causes phenotypic modulation of medial layer SMC followed by neointimal formation. DOCK2 is clearly important for PDGF-BB–induced SMC phenotypic modulation in vitro. To determine whether DOCK2 is involved in SMC phenotypic modulation and vascular remodeling in vivo, we used balloon catheter to mechanically injure rat carotid artery. As shown in Figure 6A, balloon injury induced a progressive neointima formation, consistent with previous reports. DOCK2 expression is barely detectable in SMCs of the normal artery, but was highly induced in the medial layer SMCs 3 days after the injury (Figure 6B), a time when SMC phenotypic modulation occurs without evident neointima formation. Seven or 14 days after the injury, DOCK2 was expressed in the neointimal SMCs (Figure 6B). Notably, strong DOCK2 expression was mainly observed in the neointimal SMCs near or on the luminal surface of arteries with 14 days of injury (Figures 6B and 7A), consistent with its role in modulating SMC phenotype because luminal surface SMCs exhibit de-differentiated state, whereas SMCs in other neointima area start to redifferentiate at this stage. Time course quantitative analysis of DOCK2 expression revealed that DOCK2 protein was highly induced as early as 3 days after the injury (Figure 6C and 6D), indicating that DOCK2 may be involved in vascular lesion formation.

To test whether DOCK2 plays a role in balloon injury–induced vascular remodeling, we used adenovirus to deliver DOCK2 shRNA to the injured arteries. As shown in Figure 7A, DOCK2 shRNA effectively blocked DOCK2 expression in neointima SMCs. Saline or control shRNA did not affect the injury-induced neointima formation. However, knockdown of DOCK2 dramatically blocked
Knockout of DOCK2 in Mice Blocked Ligation Injury–Induced Intimal Hyperplasia and SMC Phenotypic Modulation

Adenovirus-mediated shRNA delivery approach may not be able to fully reveal DOCK2 function in vascular remodeling because adenoviral vector can only modify DOCK2 expression in cells with a direct contact with the virus. Therefore, we used DOCK2−/− mice and carotid artery ligation-injury model to further confirm the role of DOCK2 in vascular remodeling. Mouse carotid artery ligation injury elicits a remodeling response similar to rat carotid artery balloon injury although the small size of the vessel and thrombus often limit the sample size for molecular analysis. In addition, the degree of neointima formation in areas with the same distance from the ligation among mice with artery ligation is a critical role of DOCK2 in injury-induced vascular remodeling.

Knockdown of dedicator of cytokinesis 2 (DOCK2) blocked neointima formation. A, DK2 expression was efficiently blocked by adenovirus delivery of DK2 short hairpin RNA (shRNA). Immediately after balloon injury, the injured rat carotid arteries were incubated with sterile saline solution, adenovirus expressing scramble (Ad-shCtrl), or DK2 shRNA (Ad-shDK2) as indicated. Fourteen days later, artery sections were stained with DK2 antibody. DK2 expression was visualized by DAB (3,3′-diaminobenzidine) staining. B, DK2 knockdown blocked injury-induced neointima formation. Artery sections were stained with Elastica van Gieson solution. Yellow arrows indicate internal elastic lamina. C and D, Quantification of intima area and intima/media ratio. *P<0.05 compared with saline- or Ad-shCtrl–treated arteries (n=6).

To determine whether DOCK2−/− affects injury-induced SMC phenotypic modulation in vivo, we detected α-SMA and SMMHC expression in WT and DOCK2−/− mouse carotid arteries with or without ligation injury. As shown in Figure 8E–8H, in control arteries without ligation, DOCK2−/− mice as compared with the WT mice (0.009±0.001 versus 0.048±0.001 mm²; P<0.01; n=6; Figure 8C). The intima/media area ratios (Figure 8D) showed similar results as in Figure 8C. These data further demonstrate a critical role of DOCK2 in injury-induced vascular remodeling.

Discussion

In the present study, we have identified DOCK2 as a novel regulator in SMC phenotypic modulation and vascular remodeling. DOCK2 is scarcely detectable in normal SMCs. However, it is induced during SMC phenotypic modulation induced by PDGF-BB or serum in vitro and vascular...
injury in vivo. DOCK2 deficiency increased the expression of SMC marker proteins downregulated by PDGF-BB or serum in vitro and by vascular injury in vivo, demonstrating that DOCK2 promotes SMC phenotypic modulation, leading to vascular remodeling/neointima formation. Interestingly, DOCK2 knockout in normal SMCs also causes a dramatic increase of contractile proteins, suggesting that a low level of DOCK2 in quiescent SMCs may be necessary for maintaining the proper level of contractile proteins while excessive DOCK2 expression results in SMC phenotype alteration, similar to the treatment with PDGF-BB or serum. Importantly, vascular injury induces DOCK2 expression initially in medial SMCs and subsequently neointima SMCs. The initial expression of DOCK2 in medial SMCs is likely attributed to the SMC phenotypic modulation in injured arteries because at this time (1–3 days after injury), SMCs are responding to injury-triggered serum factors such as PDGF-BB or serum. DOCK2 may inhibit Myocd expression and SRF nuclear location requiring further investigation, DOCK2 is likely to block Myocd–SRF interaction through diminishing the availability of Myocd for SRF binding because DOCK2 inhibits Myocd expression as well as binding to Myocd. The combined effects of DOCK2 on Myocd/SRF function via inhibiting Myocd expression, SRF nuclear location, and Myocd–SRF interaction. DOCK2 may inhibit Myocd expression by regulating Myocd transcription similar to SMC marker genes because DOCK2 is a nuclear factor. DOCK2 blocks SRF nuclear translocation probably via an indirect mechanism because DOCK2 does not directly interact with SRF. These mechanisms may include SRF nuclear transporting or SRF exporting machinery. Although the mechanisms by which DOCK2 inhibits Myocd expression and SRF nuclear location require further investigation, DOCK2 is likely to block Myocd–SRF interaction through diminishing the availability of Myocd for SRF binding because DOCK2 inhibits Myocd expression as well as directly binding to Myocd. The combined effects of DOCK2 on Myocd expression, SRF nuclear location, and Myocd–SRF interaction make DOCK2 a powerful regulator mediating SMC phenotypic modulation.

DOCK2 and KLF4 seem to cooperatively mediate SMC phenotypic modulation because coexpression of DOCK2 and KLF4 has a greater effect in reducing Myocd–SRF interaction comparing with each of the individual effect. KLF4 is known to inhibit SMC marker expression and modulate SMC phenotype also by inhibiting Myocd expression and Myocd–SRF interaction. However, the mechanisms underlying DOCK2 and KLF4 are different. DOCK2 interacts with Myocd, but not SRF to attenuate Myocd–SRF interaction, whereas KLF4 interacts with SRF, but not Myocd to block Myocd–SRF binding. DOCK2 also inhibits KLF4 binding to SRF (Figure 5), which is likely because of the role of DOCK2 in blocking SRF nuclear translocation (Figure 3A and 3B). Because PDGF-BB induces the expression of both DOCK2 and KLF4, the cooperative action of DOCK2 and KLF4 may...

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Disclosures
None.
Novelty and Significance

What Is Known?

- Smooth muscle cell (SMC) phenotypic modulation is an essential process initiating vascular remodeling/neointima formation in proliferative vascular diseases.
- Myocardin–serum response factor (SRF) interaction is critical for maintaining the contractile SMC phenotype.
- Kruppel-like factor 4 (KLF4) blocks myocardin–SRF interaction by binding to SRF, resulting in SMC phenotypic modulation.

What New Information Does This Article Contribute?

- Dedicator of cytokinesis 2 (DOCK2) is a novel protein factor regulating SMC phenotypic modulation and vascular remodeling. Deletion of DOCK2 blocks injury-induced vascular remodeling while restoring SMC contractile protein expression.
- DOCK2 modulates SMC phenotype by inhibiting myocardin expression, blocking SRF nuclear localization, attenuating myocardin binding to SRF, and consequently diminishing smooth muscle marker promoter activity.
- DOCK2 and KLF4 cooperatively modulate SMC phenotype. Unlike KLF4 that binds to SRF, DOCK2 attenuates myocardin–SRF interaction by binding to myocardin.

Prior studies have shown that SMC phenotypic modulation plays an important role in injury-induced vascular remodeling, but the mechanisms underlying this process remain incompletely elucidated. Here, we used a DOCK2 knockout mouse model as well as molecular and cellular analyses to identify a novel mechanism underlying SMC phenotypic modulation. Our studies demonstrate for the first time that DOCK2 is a novel regulator for SMC phenotype modulation and injury-induced vascular remodeling. DOCK2 modulates SMC phenotype by decreasing myocardin expression, blocking SRF nuclear localization, and attenuating myocardin binding to SRF, thus inhibiting myocardin-induced SMC marker promoter activity. Importantly, our studies indicate that mechanisms underlying DOCK2 and KLF4 functions are different. DOCK2 interacts with myocardin, but not SRF, to attenuate myocardin–SRF interaction, whereas KLF4 interacts with SRF, but not myocardin, to block myocardin–SRF binding. In a rat carotid artery balloon-injury model, DOCK2 is induced in medial SMC initially and neointimal SMC subsequently after vascular injury. Knockdown of DOCK2 inhibits the neointima formation by 60%. Most importantly, knockout of DOCK2 in mice markedly blocks ligation-induced intimal hyperplasia while restoring SMC contractile protein expression, demonstrating a critical role of DOCK2 in SMC phenotypic modulation in vivo.
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Dedicator of cytokinesis 2, a novel regulator for smooth muscle phenotypic modulation and vascular remodeling

Xia Guo\textsuperscript{1}, Ning Shi\textsuperscript{1}, Xiao-Bing Cui\textsuperscript{1}, Jia-Ning Wang\textsuperscript{2}, Yoshinori Fukui\textsuperscript{3}, Shi-You Chen\textsuperscript{1,2}

\textsuperscript{1} Department of Physiology & Pharmacology, University of Georgia, Athens, GA;
\textsuperscript{2} Institute of Clinical Medicine & Department of Cardiology, Renmin Hospital, Hubei University of Medicine, Shiyan, Hubei 442000, China;
\textsuperscript{3} Department of Immunobiology and Neuroscience, Kyushu University, Fukuoka, Japan

Detailed Methods:

Animals
Male Sprague-Dawley rats weighing 450-500 g were purchased from Harlan. DOCK2\textsuperscript{-/-} mice were previously described\textsuperscript{1}. 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. Animal surgical procedures were approved by the Institutional Animal Care and Use Committee of The University of Georgia.

Cell culture
SMCs were cultured by enzyme digestion method from rat or mice thoracic aorta as described previously\textsuperscript{2,4}. Briefly, animal thoracic aortas were removed and digested with collagenase for 30 mins. The adventitia was then removed and the artery was cultured overnight in culture medium. The artery was cut into small pieces and digested with collagenase and elastase for 30 mins. The digestion was stopped by 10 ml culture medium and the collected cells were washed with culture medium for 2 times. The cells were maintained in Dulbecco's modification of Eagle's medium (Invitrogen) containing 10\% fetal bovine serum (FBS, Hyclone) and 5\% L-glutamine at 37°C in a humidified atmosphere of 5\% CO\textsubscript{2} in air. Medium was changed every 48 hr. The primary cultured SMCs were confirmed by the expression of smooth muscle α-actin (α-SMA) and SM22α.

Construction of Adenovirus
Adenoviral vectors expressing scramble (control) or DOCK2 short hairpin RNA (shRNA) (shDOCK2) were constructed, and the viruses were purified as described previously\textsuperscript{5}. ShDOCK2 sequences were as follows: 5' - CGC GTC GGA CCT AAT TGCA TGG CAG ATC CCC TTC CTT CAA GAG AGG ATG CAT GCA ATT AGG TCC TTT TTT CCA AA-3' (top strand) and 5' - AGC TTT TGG AAA AAA GGA CCT AAT TGC ATG GCA GAT CCC CTT CCT CTC TTG AAG GAA GGG GAT CTG CCA TGC AAT TAG GTG CGA-3' (bottom strand). Control shRNA (shCtrl) sequences were as follows: 5' - CGC GTC GAT CGA TGA TTC GCC CGG CGT CTT CAT AAT TCA AGA GAT TAT GAA GAC GCC GGG CGA ATC ATC GAT CTT TTT TCC AAA-3' (top strand) and 5' - AGC TTT TGG AAA AAA GAT CGA TGA TTC GCC CGG CGT CTT CAT AAT CTC TTG AAT TAT GAA GAC GCC GGG CGA ATC ATC GAT CGA-3’ (bottom strand). Both strands were annealed and ligated...
Guo et al. DOCK2 in vascular remodeling

into pRNAT-H1.1/Adeno (Genscript corporation) digested with Mlu I and Hind III. Recombinant adenoviral vector was produced by homologous recombination in AD-1 competent. The resultant recombinant vector pAd-shDOCK2 digested with Pac I was transfected into AD-293 cells to package viral particles expressing shDOCK2 (Ad-shDOCK2). The adenovirus was purified using gradient density ultracentrifugation of cesium chloride and dialyzed in dialysis buffer.

Western Blot Analysis
Western blot was performed as described previously 6. Cultured SMCs were washed twice with PBS followed by protein extraction using RIPA buffer (50 mmol/L Tris-HCl, 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. SMCs from Rat or mice thoracic aorta were homogenized in RIPA buffer containing protease inhibitors. The protein concentration was measured using BCA Protein Assay Reagent (Thermo Scientific). Lysates were denatured by boiling with SDS and 2-mercaptoethanol solution. Cell lysates or proteins collected from arteries were resolved on a 10% SDS-PAGE and were transferred to PVDF membrane (Bio-Rad). Membranes were blocked with 5% nonfat dry milk for regular antibodies, and then incubated for 1 to 2 hours with primary antibodies in blocking buffer followed by incubation with HRP-conjugated secondary antibody for 1 hour (Sigma). Detection was performed with enhanced chemiluminescence (Millipore). Antibodies against DOCK2 (Millipore), α-SMA (Sigma), calponin (Abcam), SM22α (Abcam), SRF (Santa Cruz Biotechnologies), Myocardin (Myocd, Abcam), smooth muscle myosin heavy chain (SMMHC, Biomedical Technologies Inc.), Kruppel-like factor 4 (KLF4, Abcam), Flag (Sigma), GAPDH (Sigma), and α-Tubulin (Sigma) were used for immunoblotting.

Real-time Quantitative PCR (qPCR)
Quantitative PCR was performed as described previously 7. Total RNA from cells was extracted using Trizol Reagent (Invitrogen) according to the manufacturer’s protocol. Briefly, add 1 ml of Trizol per well to collect samples to RNase free microcentrifuge tubes and incubate 2 min at room temperature (RT). Next add 0.2 ml chloroform to each tube and shake by hand for 30 seconds. Incubate the samples for 5 min at RT and centrifuge for 15 min at 12,000×g at 4°C. Transfer the upper aqueous phase about 0.5 ml to a new tube and add 0.5 ml isopropyl alcohol to the aqueous phase to mix well. Incubate samples at RT for 10 min and spin for 15 min at 12,000 x g at 4°C. Take off the supernatant and wash pellet with 1 ml 70% ethanol. Air dry the pellet for 10min and dissolve the RNA in 30 μl of RNase free water to do later experiment. cDNA was synthesized from 1μg of total RNA by 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 the forward and reverse primers used in this study were listed in Supplementary Table S1.

Transfection and Luciferase Assay
α-SMA promoter construct, myocardin expression plasmid, DOCK2 plasmid, and/or control or DOCK2 shRNA ((Dharmacon) was transfected individually or in combination into SMCs as described previously 8. Cells were then starved in serum-free medium for 12 h followed by treatment with vehicle, 10 ng/ml of PDGF-BB or 10 % FBS for times needed for each experiment. Luciferase assay was performed using Progema Luciferase kit as described previously 7. Experiments were repeated for three times, and results from a representative experiment were shown with standard deviations.

Rat Carotid Artery Injury Model and Adenoviral Gene Transfer
Rat carotid artery balloon injury was performed as described previously 9, 10. Briefly, rats were anesthetized by an
intra-peritoneal injection of Ketamine (80 mg/kg) and Xylazine (5 mg/kg). A 2F Fogarty arterial embolectomy balloon catheter (Baxter Edwards Healthcare) was introduced through the left external carotid artery and advanced 4 cm toward the thoracic aorta. The method that introduces adenovirus into rat balloon-injured carotid artery has been previously described.5,11 The injured artery was washed with saline, and incubated with 100 µl saline or adenovirus (5x10⁹ pfu) for 20 minutes. 3, 7, and 14 days later, the balloon-injured segment of the artery from the proximal edge of the omohyoid muscle to the carotid bifurcation was perfused with saline and excised. The balloon-injured and adenovirus-dwelled segments were fixed with 4% paraformaldehyde (PFA) and embedded in paraffin. Subsequent morphometric analyses were performed in a double-blinded manner.

**Mouse Carotid Artery Ligation-injury Model**
The mouse carotid artery ligation model was described previously.12,13 Mice were anesthetized with ketamine hydrochloride (80 mg/kg IP) and xylazine (5 mg/kg IP). The left common carotid artery was exposed and ligated by a 6-0 propylene suture about 2 mm proximal from the carotid bifurcation. Four weeks later, the mice were sacrificed and the ligation-injured segments were removed and fixed with 4% PBS-buffered PFA and embedded in paraffin. Subsequent morphometric analyses were performed in a double-blinded manner.

**Histomorphometric Analysis and Immunohistochemistry (IHC) Staining**
Vessel segments were cut by serial sectioning and 10 sections that were evenly distributed in the vessel segment were collected for analysis. The dissected arteries were stained with modified hematoxylin and eosin (HE) or Elastica van Gieson staining and captured using a Nikon microscope. The circumference of lumen, internal elastic lamina, and external elastic lamina were measured by Image-pro Plus Software. For immunohistochemistry, sections were rehydrated, blocked with 10% goat serum and permeabilized with 0.01% Triton X-100 in PBS, and incubated with DOCK2, α-SMA, or SMMHC primary antibody at 4°C overnight followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody. The sections were counterstained with hematoxylin.

**Immunofluorescent Staining**
SMCs were grown on glass coverslips in 24-well cell culture plates at a density of 10³ cells/cm² and incubated overnight in normal cell growth conditions. For immunostaining, cells were washed with phosphate-buffered saline (PBS) twice and fixed with 4% PFA in PBS, or 1% PFA for 1 min and permeabilized with 0.01% Triton X-100 in PBS. Tissue sections were treated the same as in IHC staining above. The cells/sections were then blocked with 5% goat serum for 30 min and incubated overnight with SRF or DOCK2 primary antibodies. Cells were then rinsed with PBS for three times and incubated with FITC-conjugated secondary antibodies (1:100). Cell nuclei were stained with DAPI (Molecular Probe). Stained cells were imaged using a Nikon microscope.

**Rac activation assay**
Rat primary vascular smooth muscle cells were cultured in 10-cm dishes and transfected with pcDNA, Myocad in and/or DOCK2 plasmids. Cells were then treated with 25 µM of CPYPP 4 h prior to harvest. Rac activity was determined by using Rac1 and Rac2 activation assay kits (STA-401-1-T and STA-401-2-T, Cell Biolabs Inc) as previously described. Briefly, cell lysates were cleared by centrifugation. Lysates were diluted to equal concentrations, and RAC pull-down assays were performed by using PAK PBD agarose beads according to manufacturer’s instructions followed by Western blot analyses.

**Statistical analysis**
All data were evaluated with a 2-tailed, unpaired Student t test or compared by one-way ANOVA followed by Fisher
t test and are expressed as mean±SD. A value of P<0.05 was considered statistically significant.

Reference:


Online Table I: Primer sequences for qPCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
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<td>DOCK2</td>
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<td>5’GATGCTGTTGAGCAGTCCCA3’</td>
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<tr>
<td>α-SMA</td>
<td>5’TTCATGCTCCCTGCCCATGTA3’</td>
<td>5’GAAGGAATAGCCACGCTAG3’</td>
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<td>Calponin</td>
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<td>Myocardin</td>
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<tr>
<td>SRF</td>
<td>5’CCTAGTCCCCATGCACTGAT3’</td>
<td>5’TGCTGTGAGGAACACCTGC3’</td>
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Online Figure I

Online Figure I. PDGF-BB induced DOCK2 expression along with SMC phenotypic modulation. A, PDGF-BB induced DOCK2 protein expression in a dose-dependent manner. SMC were treated with different dosages of PDGF-BB for 48 hr. The protein expression of DOCK2 (DK2) and SMC marker α-actin (α-SMA) and calponin was detected by western blot. B, Quantitative analysis of protein expression shown in A by normalizing to α-Tubulin. *, P<0.05 vs vehicle-treated group (0 ng).
Online Figure II

**Online Figure II.** DOCK2 inhibited VSMC marker expression. SMCs transfected with pcDNA (Ctrl) or DOCK2 (DK2) cDNA were starved for 24 hr. DK2 and SMC marker mRNA expression was detected by qPCR. *, $P<0.05$ compared with Ctrl groups, n=3.

Online Figure III

**Online Figure III.** CPYPP blocked DOCK2-mediated Rac activation in VSMCs. A, CPYPP completely inhibited DOCK2 (DK2)-mediated Rac activation. pcDNA, myocardin (Myocd), and/or DOCK2 (DK2) plasmids were transfected into primary rat VSMCs and treated with or without CPYPP (25 µM) as indicated for 4 h. Rac1 and Rac2 activation assay was performed. GTP bound and total Rac 1 and Rac 2 were detected by western blot. B and C, quantification of active Rac by normalizing to total Rac shown in A. *, $P<0.05$ compared with Myocd-transfected groups. #, $P<0.05$ compared with DK2-transfected groups without CPYPP (n=3).