Slingshot Isoform-Specific Regulation of Cofilin-Mediated Vascular Smooth Muscle Cell Migration and Neointima Formation

Rebecca A. Torres, Douglas A. Drake, Viktoriya Solodushko, Rashmi Jadhav, Erika Smith, Petra Rocic, David S. Weber

Objective—We hypothesized that cofilin activation by members of the slingshot (SSH) phosphatase family is a key mechanism regulating vascular smooth muscle cell (VSMC) migration and neointima formation following vascular injury.

Methods and Results—Scratch wound and modified Boyden chamber assays were used to assess VSMC migration following downregulation of the expression of cofilin and each SSH phosphatase isoform (SSH1, SSH2, and SSH3) by small interfering RNA (siRNA), respectively. Cofilin siRNA greatly attenuated the ability of VSMC migration into the “wound,” and platelet-derived growth factor (PDGF)-induced migration was virtually eliminated versus a 3.5-fold increase in nontreated VSMCs, establishing a critical role for cofilin in VSMC migration. Cofilin activation (dephosphorylation) was increased in PDGF-stimulated VSMCs. Thus, we assessed the role of the SSH family of phosphatases on cofilin activation and VSMC migration. Treatment with either SSH1 or SSH2 siRNA attenuated cofilin activation, whereas SSH3 siRNA had no effect. Only SSH1 siRNA significantly reduced wound healing and PDGF-induced VSMC migration. Both SSH1 expression (4.7-fold) and cofilin expression (3.9-fold) were increased in balloon injured versus noninjured carotid arteries, and expression was prevalent in the neointima.

Conclusion—These studies demonstrate that the regulation of VSMC migration by cofilin is SSH1 dependent and that this mechanism potentially contributes to neointima formation following vascular injury in vivo. (Arterioscler Thromb Vasc Biol. 2011;31:2424-2431.)

Key Words: PDGF ■ VSMC migration ■ neointima ■ slingshot phosphatase ■ vascular injury

Vascular smooth muscle cell (VSMC) migration is a critical aspect of pathological remodeling in response to vascular injury (restenosis and neointima formation).1,2 Given the importance of cell chemotaxis in this processes, understanding the mechanisms that regulate VSMC migration are of high clinical relevance.

A key component of cell migration is the dynamic regulation of the actin cytoskeleton at the leading edge of the cell. This process is regulated by a family of actin-binding proteins, including actin-related protein 2/3 (Arp 2/3), which assembles new actin filaments (F-actin), and the actin depolymerizing factor cofilin, which is responsible for depolymerization (severing) of existing F-actin to provide substrate for Arp 2/3.3–4 Thus, cofilin is likely to play a critical role in directional cell migration.5 In further support of the important role for cofilin in migration are findings that cells deficient in cofilin exhibit impaired motility, whereas those overexpressing cofilin are highly motile.6,7 It is, however, important to note that these facts may not translate to the unique physiology of smooth muscle, where, unlike in any other cell type, the actin cytoskeleton is anchored into dense bodies, structures characteristic of smooth muscle cells and, importantly, intimately related to VSMC function in intact vessels, including VSMC positioning with respect to blood flow, force transduction, and maintenance of vessel diameter under varying blood pressures. In addition, this knowledge was accumulated in cell types that are far less phenotypically plastic than VSMCs and, unlike VSMCs, do not acquire a migratory phenotype in either physiological or pathological situations in vivo.

Activity of cofilin is regulated by phosphorylation, so that the dephosphorylated form is the active form. Stimulation by platelet-derived growth factor-BB (PDGF-BB), one of the most potent promigratory growth factors, has recently been reported to increase cofilin dephosphorylation, but the signaling pathways involved in the regulation of cofilin activity have not been fully identified. LIM kinase (LIMK) has been shown to inactivate cofilin by phosphorylation at Ser3, resulting in abolition of actin depolymerization in other cell types.4,5 Of several phosphatases that have been reported to...
activate cofilin by dephosphorylation, the slingshot (SSH) family of phosphatases (SSH1, SSH2, and SSH3) has emerged as the primary mediator in restoring cofilin’s actin depolymerizing activity and inducing cell motility.9–11 Most studies have focused on the role of SSH1,6,8,12,13 but additional studies indicate a crucial role of the other SSH isoforms in mediating cell motility.12,14 suggesting that the regulation of cofilin during chemotaxis is highly complex. To date, there has been a single study that has examined the role of SSH1 in mediating VSMC migration.15 No studies have compared the influence of the other SSH isoforms (SSH2 and SSH3) during VSMC chemotaxis. Also, a role for cofilin in VSMC migration has not been definitively established.

As SSH-mediated cofilin activation potentially regulates VSMC migration, one physiologically relevant process in which it could be seminal is during the neointimal formation in response to vascular injury. Following artery damage, such as that associated with percutaneous transluminal coronary angioplasty, the injured vascular wall is repaired by cells derived from adjacent normal tissue. The initial injury to the artery results in destruction of the endothelium and extensive medial VSMC death. After this, both medial smooth muscle migration and proliferation result in neointima growth. Although the SSH isoforms mediate cofilin activation and cell chemotaxis in vitro, to date there are no studies that have examined their contribution in vivo. In our current study, we examined changes in SSH and cofilin expression following balloon injury as a novel mechanism contributing to neointimal formation, presumably via mediating VSMC migration. Thus, in these studies, we tested 2 hypotheses: (1) that activation of cofilin by SSH phosphatase-mediated dephosphorylation is a key mediator of both nondirectional VSMC migration and PDGF-induced directional VSMC migration in vitro, and (2) that SSH1 and cofilin expression are increased during neointima formation following vascular injury in vivo. Our findings demonstrate that (1) downregulation of cofilin expression by small interfering RNA (siRNA) attenuates VSMC migration, (2) siRNA targeting specific SSH isoforms results in differential effects on the regulation of VSMC migration and cofilin dephosphorylation, and (3) SSH1 and cofilin are upregulated in the neointima following vascular injury in vivo. Our study is the first to compare the roles of the SSH isoforms in cofilin activation and chemotaxis in VSMCs and demonstrate increased SSH1 and cofilin expression following vascular injury in vivo.

Methods

Cell Culture
Male 8-week-old Sprague-Dawley rats were anesthetized with pentobarbital sodium (100 mg/kg IP). Thoracic aortas were removed and VSMCs isolated by enzymatic digestion as described previously.16

siRNA Transfection Procedures
VSMCs in the log growth phase (~70%–80% confluent) were trypsinized, collected by centrifugation (1000g, 10 minutes), resuspended in Opti-MEM medium, counted and plated at a density of 5 × 10^4 cells/well in 6-well plates. Following 24 hours in culture, Opti-MEM medium was replaced with fresh medium containing DharmaFECT 1 transfection reagent and either 100 nmol of nontargeting siRNA or siRNA specifically targeting cofilin, SSH1, SSH2, or SSH3 (Dharmacon SMARTpool). Nontargeting siRNA pools, which have no gene targets in rat cells, serve as a control to discriminate sequence-specific silencing from nonspecific effects. Transfection efficiency was confirmed by harvesting VSMCs 48 to 72 hours posttransfection for determination of protein expression by immunoblotting or mRNA expression using real-time polymerase chain reaction. All siRNA sequences used are included in Supplemental Table IV, available online at http://atvb.ahajournals.org.

Transwell Migration Assay
VSMC migration was assayed 48 to 72 hours after siRNA transfection using collagen-coated (5 ng/mL) transwell dishes (Greiner Bio-One) containing 8-μm pores as described previously.16

Scratch Wound Assay
VSMCs were cultured to 80% to 90% confluence. Artificial wounds were created by scratching the monolayer with a 10-μL pipette tip, and medium was replaced with Dulbecco’s modified Eagle’s medium containing 10% calf serum. Cells were incubated at 37°C in a 5% CO2 incubator for 12 hours. VSMCs were fixed in 5% formaldehyde and stained with Coomassie Blue, and images were captured using phase-contrast microscopy.17 Open area was calculated using analysis algorithm-based T-scratch software.18

Immunoblotting
Quiescent VSMCs at 80% to 90% confluence were stimulated with PDGF (20 ng/mL) at 37°C in serum-free Dulbecco’s modified Eagle’s medium for specified durations. Following treatment, cells were washed with ice-cold PBS, and lysed with 500 μL of ice-cold lysis buffer, containing 1% Triton X-100, as previously described.19 For carotid artery samples, equal protein loading was verified by immunoblotting for β-actin. The data were normalized as the ratio of protein expression in the injured left carotid to protein expression the noninjured right carotid to obtain the left carotid/right carotid ratio within each animal. Antibody sources were as follows: phosphocofilin (Ser3) and cofilin were from Cell Signaling Technology, α-actin and β-actin were from Sigma, GAPDH and SSH1 were from ABR Affinity BioReagents, and SSH3 was from Abcam.

Rat Carotid Injury
Sprague-Dawley rats were anesthetized with ketamine/xylazine (75/5 mg/kg IP), and the left external carotid artery was isolated by blunt dissection. A Fogarty 2F arterial embolectomy catheter (Edwards Life Sciences) was inserted into the common carotid artery in the direction of the aortic arch via the external carotid, and the balloon was inflated for 1 minute 3 times. The external carotid branch was ligated following catheter removal.20,21 The right carotid artery served as a noninjured contralateral control in each animal. Carotid tissue was harvested from rats 4, 7, or 14 days following injury for immunoblotting or immunohistochemistry. All animal procedures were conducted with approval of the institutional animal care and use committee of the University of South Alabama and in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1996).

Immunohistochemistry
For histological analysis, carotids were placed in OCT and cut in 7-μm frozen sections before staining with hematoxylin and eosin. Additional sections were fixed in methanol before blocking in 3% goat serum/tris-buffered saline, rinsed with tris-buffered saline, and placed in primary antibody recognizing SSH1 or cofilin. Sections were then incubated with Alexa Fluor 568–conjugated secondary antibody (Invitrogen), mounted on microscope slides using Vectashield, and visualized by fluorescent microscopy.

Statistical Analysis
Summary data for all experiments are presented as mean±SEM. Statistical significance was determined by either t test or 1-way ANOVA followed by a Newman-Keuls multiple-comparison post
Results

PDGF Activates Cofilin in VSMCs

Initial studies were completed to determine whether cofilin activation and expression were regulated by PDGF in VSMCs. Quiescent VSMCs were treated with PDGF for increasing times and cofilin activity was assessed using a phospho-specific antibody recognizing cofilin phosphorylated at Ser3. PDGF stimulation of VSMCs resulted in time-dependent dephosphorylation of cofilin, without changes in cofilin expression (Supplemental Figure I).

Treatment With Cofilin siRNA Attenuates VSMC Migration

Immunoblot analysis for total cofilin expression indicated that transfection of VSMCs with siRNA targeting cofilin were effective at decreasing protein expression (Figure 1A). No change in cofilin expression was observed as a result of DharmaFECT treatment alone or when VSMCs were transfected with the control nontargeting siRNA. Real-time polymerase chain reaction also confirmed decreased cofilin expression vs nontransfected control VSMCs (P<0.05). Cntrl indicates control.

Figure 1. Cofilin small interfering RNA (siRNA) treatment inhibits vascular smooth muscle cell (VSMC) migration. A, Treatment with cofilin siRNA suppresses endogenous protein expression. VSMCs were transfected with either control nontargeting siRNA (siCntrl) or cofilin siRNA (siCofilin) as indicated. After 48 hours, cell lysates were analyzed by immunoblotting with anticofilin antibody (top) or α-actin antibody (bottom), which served as a loading control. Summary data are shown in bar graph (n=3). *Significant decrease in cofilin expression vs nontransfected control VSMCs (P<0.05). Cntrl indicates control. B, Phase-contrast images illustrate that cofilin siRNA expression vs nontransfected nonstimulated control VSMCs (P<0.01); †significant decrease in migration vs nontransfected control siRNA–treated cells. C, Migration in response to platelet-derived growth factor (PDGF) (20 ng/mL, 4 hours) was measured using a modified Boyden chamber transwell migration assay (n=5). *Significant increase in migration vs nontransfected nonstimulated control VSMCs (P<0.001).

Multiple SSH Isoforms Attenuate PDGF-Induced Cofilin Dephosphorylation

Cofilin has recently been identified as a target of the SSH1 phosphatase in VSMCs, but to date there has been no assessment of the role of other SSH isoforms on cofilin activity and ultimately VSMC migration. The effectiveness of VSMC transfection with siRNA targeting SSH1, SSH2, or SSH3, was confirmed by real-time polymerase chain reaction and Western blotting (Supplemental Figure II). Each of the SSH siRNAs were specific to the isoform that they targeted and did not change mRNA levels of other nontargeted SSH isoforms, cofilin, or GAPDH (data not shown). We also confirmed that cofilin protein expression was unaffected by any of the siRNA targeting the SSH isoforms (Figure 2). To determine whether PDGF indeed regulates cofilin dephosphorylation via the SSH phosphatases, VSMCs were transfected with siRNA targeting SSH1, SSH2, or SSH3 before stimulation with PDGF. As expected, PDGF induced a significant dephosphorylation of cofilin at Ser3. Decreasing the expression of either SSH1 or SSH2 was effective in attenuating PDGF mediated cofilin activation (Figure 2A and 2B and Supplemental Figure III). In contrast, siRNA targeting SSH3 had no effect on PDGF-induced dephosphorylation (Figure 2C). Thus, although each of the SSH isoforms is expressed in VSMCs, these findings suggest that their ability to regulate cofilin in response to PDGF activation is varied.

VSMC Migration Is Attenuated Following Treatment With SSH1 But Not SSH2 or SSH3 siRNA

Based on the differential regulation of the SSH isoforms in mediating cofilin dephosphorylation, we wanted to determine the role of each isoform as a mechanism mediating VSMC migration. VSMCs were transfected with siRNA targeting the SSH isoforms, and migration studies were conducted. In the
absence of nonspecific effects of the transfection procedure, siRNA targeting SSH1 significantly attenuated wound healing (Figure 3A and Supplemental Table V), as well as PDGF-induced directional VSMC migration quantified in the Transwell assay (Figure 3B). Somewhat surprisingly, siRNA targeting SSH2 had virtually no effect on wound healing (Figure 3A) or PDGF-induced transwell migration (Figure 3C). Similar to decreasing SSH2 expression, siRNA targeting SSH3 had no effect on wound healing (Figure 3A) or transwell migration (Figure 3D). As the results with siRNA targeting SSH1 were similar to those of transfection with siRNA targeting cofilin, this further supports the notion that both cofilin and SSH1 are mediators of VSMC migration under varying conditions. However, our findings also suggest

Figure 2. Slingshot (SSH)-1 and SSH2 small interfering RNA (siRNA) treatments prevent dephosphorylation of cofilin in platelet-derived growth factor (PDGF)-stimulated vascular smooth muscle cells (VSMCs). Following 48 to 72 hours of transfection with control siRNA (nontargeting), SSH1 siRNA (n=6) (A), SSH2 siRNA (n=5) (B), or SSH3 siRNA (n=5) (C) cells were stimulated with PDGF for 15 minutes (20 ng/mL). Immunoblot analysis was performed with anti-phospho-specific (Ser3) cofilin antibody (top). Total cofilin was used as a loading control (bottom). Summary data are shown in bar graphs. Cntrl indicates control. *Significant decrease in phosphorylation vs nontransfected nonstimulated VSMC (P<0.05); †Significant increase in phosphorylation vs nontransfected cells stimulated with PDGF (P<0.01).

Figure 3. Transfection with slingshot (SSH)-1 small interfering RNA (siSSH1) reduces vascular smooth muscle cell (VSMC) migration; however, treatment with SSH2 or SSH3 siRNA has no effect. Both a scratch wound assay and a modified Boyden chamber Transwell migration assay were used to determine VSMC migration following 48 to 72 hours of transfection with a control nontargeting siRNA (siCntrl), SSH1, SSH2, or SSH3 siRNA. A, Phase-contrast images were used to demonstrate VSMC movement in response to a scratch wound 12 hours postinjury. B, VSMCs were transfected with either control siRNA or SSH1 siRNA for 72 hours. Platelet-derived growth factor (PDGF)-induced migration (20 ng/mL, 4 hours) was measured using a modified Boyden chamber transwell migration assay (n=4). Cntrl indicates control. C, Same as B, except VSMCs were transfected with control siRNA or SSH1 siRNA for 48 hours (n=5). D, Same as B, except VSMCs were transfected with control siRNA or SSH3 siRNA for 48 hours (n=5). *Significant increase in migration vs nontransfected nonstimulated control VSMCs (P<0.05); †Significant decrease in migration vs nontransfected cells stimulated with PDGF (P<0.05).
a potentially complex regulation of cofilin during migration in that although both the SSH1 and SSH2 isoforms regulate cofilin activity, clearly their function deviates when their role in mediating cell chemotaxis is examined.

**Cofilin and SSH1 Expression Are Increased Following Balloon Injury**

Although cofilin and each of the 3 SSH isoforms is expressed basally in rat carotid artery (data not shown), in vitro studies implicate SSH1 regulation of cofilin as a key mediator of VSMC migration. Thus, a major goal of these studies was to assess whether SSH1 and cofilin are physiologically relevant regulators of neointima formation following balloon injury. Immunohistochemistry demonstrated neointima formation at both 7 and 14 days following balloon injury (Figure 4A, a–h). At these time points, both cofilin and SSH1 were expressed in the neointima of the artery (Figure 4A, i–p), which is consistent with their potential function in vascular repair following injury. To quantify changes in expression, 14 days postprocedure, both injured and uninjured contralateral carotid arteries were harvested. Balloon injury resulted in approximately a 4-fold increase in cofilin expression (Figure 4B and 4C) and a 5-fold increase in SSH1 expression (Figure 4D and 4E) compared with noninjured control arteries at the time point tested. Immunoblotting detected no change in the expression of either SSH2 or SSH3 at any time point following the balloon injury (data not shown).

**Discussion**

In response to vascular injury, VSMC migration is in part, critical for neointima formation. Several signaling pathways in migrating VSMCs are clearly defined; however, the mechanisms regulating changes in the actin cytoskeleton during VSMC migration are poorly understood. These studies establish cofilin and its activation by SSH1 as a key regulatory mechanism of VSMC migration, presumably through the regulation of the actin cytoskeleton of migrating VSMCs. Downregulation of cofilin expression by siRNA attenuated VSMC migration during in vitro wound healing and completely blocked directional PDGF-induced migration. Furthermore, we demonstrate that although all the SSH isoforms are present in VSMCs, only SSH1 mediates VSMC migration, via control of cofilin. Finally, we demonstrate for the first time that both cofilin and SSH1 expression are found within the neointima of carotid arteries following vascular injury at both 7 and 14 days postinjury, suggesting that both cofilin and SSH1 may be key mediators of neointima formation in vivo.

The active/dephosphorylated form of cofilin has been reported to localize to structures that require high turnover of actin filaments, such as the lamellipodia, filopodia, and invadopodia. In the lamellipodia of migrating fibroblasts and in neuronal growth cones, cofilin may be responsible for the polarization of lamellipodia. In essence, active cofilin establishes the directional regulation of migration. Our findings in this study demonstrate that the highly potent promigratory stimulus PDGF facilitates cofilin dephosphorylation, suggesting that PDGF-mediated VSMC migration is dependent on cofilin activation, and this may be the potential mechanism by which PDGF regulates the actin cytoskeleton. This is strongly supported by our current results that demonstrate that ~80% downregulation in cofilin expression results in a large decrease in the ability of VSMCs to migrate into the “wounded” area in vitro and in complete blockade of directional migration in response to PDGF (Figure 1), suggesting that cofilin is a critical regulator of both types of VSMC migration.

Signaling mechanisms that regulate cofilin activity are complex and have not been fully characterized in VSMCs. In other cell types, potential regulators include changes in pH, the binding of polyphosphoinositides, and phosphorylation, which is by large, the most potent regulator. Both LIMK and testicular protein kinase can inactivate cofilin via phosphorylation at Ser3, whereas several protein phosphatases, including the SSH family, chronophin, PP1, PP2A, and PP2B activate cofilin by dephosphorylation. Of these, the SSH family and chronophin have been shown to be specific to cofilin, but the chronophin phosphatase appears to play a more significant role in processes such as cell division, where no directional cellular protrusion or movement is required. In contrast, SSH1 activity is specifically associated with directional formation of both lamellipodia and actin cytoskeleton-mediated chemotaxis.

Much of the function of SSH phosphatases has been examined in neuronal or immortalized cell lines and is not fully understood. Downstream targets of SSH1 include cofilin and LIMK, both of which have been reported to be mediators of cell migration. Our observation that SSH1 siRNA significantly attenuated cofilin dephosphorylation in response to PDGF (Figure 2A) is in agreement with the findings of San Martin. This previous study, however, falls short of definitively linking SSH1-mediated cofilin dephosphorylation to cofilin-dependent VSMC migration. In combination with our current data demonstrating the crucial role of cofilin in mediating PDGF-induced VSMC migration, as well as in vitro wound healing (Figure 1), these results conclusively link SSH1-mediated regulation of cofilin activity with VSMC migration. It remains to be determined whether this effect of SSH1 on cofilin is mediated exclusively via direct dephosphorylation or whether SSH1 also targets LIMK in VSMCs.

In human keratinocytes, expression of phosphatase-dead versions of the SSH isoforms singularly (SSH1, SSH2, or SSH3) increased cofilin phosphorylation while decreasing lamellipodia formation and directional motility. To date, no study has examined SSH2 or SSH3 regulation of cofilin phosphorylation in VSMCs or the potential contribution of these SSH isoforms in mediating VSMC migration, and thus a complete assessment of SSH isoform participation in VSMC migration is vital to fully understand cofilin regulation of VSMC chemotaxis. Similar to the downregulation of SSH1, decreasing SSH2 expression via siRNA almost completely blocked cofilin dephosphorylation (Figure 2). This somewhat unexpected finding suggested some redundancy between the isoforms in VSMCs. Basically, both isoforms are capable of inducing cofilin dephosphorylation, but in the absence of 1 isoform, the expression of the remaining isoform is sufficient to almost entirely dephosphorylate and activate cofilin. Our observation that decreasing SSH1 expression
Figure 4. Both cofilin and slingshot (SSH) 1 expression are increased after carotid injury. A, Immunohistological analysis in sequential carotid artery cross-sections of neointimal formation stained with hematoxylin/eosin (H&E) (magnification: a–d, ×10; e–h, ×40), cofilin expression (i–l, ×40), and SSH1 expression (m–p, ×40) at days 0, 4, 7, and 14 after balloon injury. Scale bars=50 μm. B, Representative immunoblots for cofilin expression in carotid arteries from sham operated and balloon-injured rats 14 days postsurgery. LC indicates left carotid; RC, right carotid. C, Summary data reported as the ratio of cofilin expression in the LC to that in the RC within each animal in rats following either a sham surgery or balloon injury (n=6). D, Representative immunoblots for SSH1 expression in sham-operated and injured carotid arteries 14 days postsurgery. E, Summary data reported as the ratio of SSH1 expression in the LC to that in the RC within each animal in rats following either a sham surgery or balloon injury (n=6). *Significant increase in expression following carotid injury relative to noninjured control arteries (P<0.01).
virtually eliminates PDGF-induced VSMC migration, as well as severely impairing in vitro wound healing (Figure 3), is consistent with the previously reported role of SSH1 in directional actin cytoskeleton-mediated chemotaxis.\textsuperscript{5,15,25} Interestingly, downregulation of neither SSH2 nor SSH3 had any effect on wound healing or PDGF-induced VSMC migration. These data suggest an isoform specific effect by the SSH phosphatase family on VSMC chemotaxis. Thus, although either SSH1 or SSH2 is capable of almost complete dephosphorylation of cofilin, the distinct subcellular localization of these isoforms may determine their differential effects on migration.

The lack of participation by SSH2 in cell migration in our studies is in contrast to recent findings in which knockdown of SSH2 by siRNA was more efficacious than SSH1 siRNA at attenuating tumor cell infiltration into a mesothelial cell monolayer.\textsuperscript{14} One plausible explanation for this discrepancy in findings could be cell-specific functions of the SSH family of phosphatases during dynamic cellular processes that require changes in the actin cytoskeleton. Overexpression of either wild-type or phosphatase inactive forms of mouse SSH isoforms in HeLa cells revealed interesting findings in regard to the cellular location of these proteins; both mouse SSH1 and mouse SSH2 colocalized with F-actin fibers, but only SSH2 demonstrated a propensity to localize to focal adhesions at the terminal end of stress fibers, further confirmed by vinculin colocalization.\textsuperscript{26} Taken together, these findings suggest differences in the specific intracellular localization of versus SSH2 or differential effects of SSH1 versus SSH2 on other signaling pathways involved in migration. Furthermore, cofilin has been identified as a mediator of invadopodia formation, which is a specialized cell protrusion rich in actin filaments and characteristic of cells with a high propensity for invasive behavior such as tumor cells. Given the differences observed between SSH1 and SSH2 in both cellular localization and cell invasion, it would be interesting to determine whether SSH2 is more critical to maintaining cofilin functionality in distinct subcellular locations.

Accumulated data suggest that LIMK is a crucial mediator necessary for cell migration, albeit that the mechanism by which it regulates cell movement is not yet clear.\textsuperscript{6,7,27,28} To date, SSH1 is the only phosphatase that has been shown to dephosphorylate LIMK.\textsuperscript{8} One plausible explanation for these seemingly disparate findings between SSH1 and SSH2 may be that although SSH2 can dephosphorylate cofilin, it may not dephosphorylate LIMK, thus being unable to create a positive feedback loop fully allowing for cell migration.

SSH3 siRNA treatment had minimal effect on either cofilin dephosphorylation or VSMC migration (Figures 2 and 3). SSH3, the shortest isoform of the SSH family, lacks expression of the region, which (1) allows for F-actin interaction potentially reducing its propensity to localize in cellular regions of increased actin density, such as the leading edge during migration, and (2) may explain why it is thought to have the weakest phosphatase activity of the isoforms for cofilin.\textsuperscript{11,26,29}

As our in vitro data suggest that the SSH1 isoform is crucial in mediating both cofilin activity and VSMC migration, to determine the physiological relevance of these findings, we turned to in vivo studies. Following balloon injury, mimicking percutaneous transluminal coronary angioplasty, VSMCs contribute to neointimal formation via both proliferation and migration. We examined both cofilin and SSH1 expression at 3 different time points throughout the process of healing. Our findings showed an increased expression of both cofilin and SSH1 following injury (Figure 4). It is thought that proliferation occurs early during the repair process, whereas migration is likely playing a key role in neointima formation during the later stages.\textsuperscript{30,31} The localization of cofilin and SSH1 in cells of the neointima (associated with a high propensity for migration) further supports our theory that SSH1 and cofilin may play a critical role during the in vivo repair process by mediating VSMC migration. One limitation of the current study is that it does not fully establish a cause-effect relationship between cofilin, SSH1, and neointima formation following vascular injury. Additional studies in which these proteins are either inhibited or overexpressed in animal models are required to definitively establish this mechanistic link in vivo.

In summary, our study is the first to definitively test the contribution of each of the SSH isoforms in the control of cofilin activity and migration in VSMCs. Our findings demonstrate a critical role for cofilin and the control of its phosphorylation state by SSH1 in 2 distinct settings, during in vitro wound healing, involving nondirectional VSMC migration and during PDGF-induced directional VSMC migration. Our study also demonstrate differential function for SSH family of phosphatases in VSMC chemotaxis. Finally, we identify SSH1 and cofilin as a potential mediators of neointimal formation. Thus, our study provides an important step in furthering the understanding of the mechanisms which underlie VSMC migration both in vitro and in vivo.

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Disclosures
None.

References


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Supplement Material

Methods

RNA isolation and Real Time PCR: Total RNA was purified from cultured VSMCs using the RNeasy Mini Kit (Qiagen) according to the manufacturer guidelines and quantified by UV spectroscopy at $A_{260}$ (nm). Reverse transcription was carried out using SuperScript III First-Stranded Synthesis System (Invitrogen), and resulting cDNA template was used for Real-time PCR amplification. Primers to amplify SSH1 were: (forward) 5'-AGT TGT TGA GAG TGT GTA CC -3' and (reverse) 5'- ACT TCT AAT CCT TCT GCT TCC -3' and for GAPDH were: (forward) 5'- GGT GCC AAC CCC AAA CGT AT -3' and (reverse) 5'- CTT TCA CAG CCT CCT TGA TAG CA -3' (IDT, Coralville, IA). Primers to amplify cofilin, SSH2, and SSH3 were designed by and purchased from Qiagen (QuantiTect Primer Assays). For real-time PCR analysis, duplicate amplifications for each sample were completed using the following thermal cycling conditions: initial denaturation at 95 °C for 3 minutes, 40 cycles of 95 °C for 10 seconds and 52 °C for 45 seconds (iCycler system, Bio-Rad Laboratories, Inc.). To determine the decrease in mRNA levels, results were normalized to GAPDH expression and analyzed using the $\Delta\Delta$Ct method.
Figure Legends

Supplemental Figure I. PDGF activates cofilin in a dose and time-dependent manner in VSMCs.  
A. VSMCs were stimulated with PDGF (20ng/mL) for the indicated times (0-30 minutes) (n=4).  
B. VSMCs were stimulated with PDGF (20ng/mL) for the indicated times (0-6 hours) confirming that PDGF-induced cofilin dephosphorylation was maintained for the time frame of transwell migration studies (n=4).  
Immunoblot analysis was performed with an anti-phospho-specific cofilin (Ser3) antibody (top). Total cofilin was used as a loading control (bottom). Summary data is show in bar graphs.  * indicates a significant dephosphorylation vs. non-stimulated control VSMCs (p<0.01).

Supplemental Figure II. Treatment with SSH siRNA down regulates endogenous SSH expression. 
VSMCs were transfected with either control siRNA (non-targeting) or SSH1 (72 hours) (n=4), SSH2 (48 hours) (n=5), or SSH3 (48 hours) (n=5). Following transfection, both protein and total RNA were isolated.  Real-time PCR confirmed siRNA efficacy of each respective siRNA (A-C).  * indicates a significant decrease in fold expression vs. non-transfected control VSMCs (p<0.05). Immunoblot analysis confirmed decreased protein expression of SSH1 (D, n=4) with α-actin serving as a loading control and SSH3 (E, n=5) with GAPDH as a loading control.

Supplemental Figure III. The inhibition of cofilin dephosphorylation following PDGF-stimulation in SSH1 and SSH2 siRNA transfected VSMCs. 
Following 48-72 hours of transfection with control siRNA (non-targeting), SSH1 siRNA (upper) or SSH2 siRNA (lower), cells were stimulated with PDGF for 6 hours (20 ng/mL). Representative immunoblots of anti-phospho-specific (Ser3) cofilin antibody (top) and total cofilin (bottom) are shown (all lanes are from the same gel).
A

PDGF       0        2        5       10     15     30     min
p-cofilin
cofilin

% of control phosphorylation

B

PDGF       0       0.5     1      2       4      6     hours
p-cofilin
cofilin

% of control phosphorylation

Supplemental Figure I
D

SSH1

α-actin

Supplemental Figure II

E

SSH3

GAPDH

Supplemental Figure II
Supplemental Figure III

cofilin

$\begin{array}{cccc}
p\text{-cofilin} & - & - & + & + \\
\text{cofilin} & - & - & + & + \\
\text{Ctrl} & \text{SSH1} & \text{Ctrl} & \text{SSH1} & \text{PDGF (6 hrs)} \\
\text{siRNA} & \text{PDGF (6 hrs)} & \text{siRNA} & \\
\end{array}$
### Supplemental Table IV. siRNA sequences

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-targeting control</td>
<td>N/A</td>
</tr>
<tr>
<td>(catalog # D-001210-01)</td>
<td></td>
</tr>
<tr>
<td>Cofilin</td>
<td>Sequence 1: 5'-CAG AAG AAG UGA AGA AAC G-3’</td>
</tr>
<tr>
<td>(catalog # M-092986)</td>
<td>Sequence 2: 5'-GAG GAC CUG GUA UUC AUU U-3’</td>
</tr>
<tr>
<td></td>
<td>Sequence 3: 5'-GAA ACU AGG UGG CAG CGC C-3’</td>
</tr>
<tr>
<td></td>
<td>Sequence 4: 5'-GCA CGA AUU ACA AGC UAA C-3’</td>
</tr>
<tr>
<td>SSH1</td>
<td>Sequence 1: 5'-GAU AAG AAC GCA GCC AAU AUU-3’</td>
</tr>
<tr>
<td>(catalog # M-081325)</td>
<td>Sequence 2: 5'-GCA CAA GAC GGG ACG AAU UUU-3’</td>
</tr>
<tr>
<td></td>
<td>Sequence 3: 5'-GAA UGA GGC CUA UCA GUU CUU-3’</td>
</tr>
<tr>
<td></td>
<td>Sequence 4: 5'-CAA UUG CUC UUC AUG AAA UUU-3’</td>
</tr>
<tr>
<td>SSH2</td>
<td>Sequence 1: 5'-UGA CAG UGU UGG CGU GUU A-3’</td>
</tr>
<tr>
<td>(catalog # M-085487)</td>
<td>Sequence 2: 5'-CCA UGC AUC UAA AGC CUU A-3’</td>
</tr>
<tr>
<td></td>
<td>Sequence 3: 5'-AAA GAC UAC UUA CCG GAU A-3’</td>
</tr>
<tr>
<td></td>
<td>Sequence 4: 5'-CGA ACA GAA AGG CUU AUU A-3’</td>
</tr>
<tr>
<td>SSH3</td>
<td>Sequence 1: 5'-GGA GAC ACA UCG AUU CAU U-3’</td>
</tr>
<tr>
<td>(catalog # M-085292)</td>
<td>Sequence 2: 5'-AAA CAG AGC ACC CAU GGA A-3’</td>
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<tr>
<td></td>
<td>Sequence 3: 5'-GAA CGA UUC ACC UAU CAC A-3’</td>
</tr>
<tr>
<td></td>
<td>Sequence 4: 5'-AGU GUU AAC GUC UAA AGA GA-3’</td>
</tr>
</tbody>
</table>

### Supplemental Table V. Summary data of remaining open areas measured 12 hours following scratch wounding in VSMCs previously treated with siRNAs.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of remaining open area</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-treated</td>
<td>20.37 ± 4.6</td>
<td>5</td>
</tr>
<tr>
<td>Control siRNA</td>
<td>25.4 ± 4.4</td>
<td>5</td>
</tr>
<tr>
<td>Cofilin siRNA</td>
<td>48.56 ± 10.3</td>
<td>5</td>
</tr>
<tr>
<td>SSH1 siRNA</td>
<td>58.83 ± 18.2</td>
<td>3</td>
</tr>
<tr>
<td>SSH2 siRNA</td>
<td>26.71 ± 6.4</td>
<td>5</td>
</tr>
<tr>
<td>SSH3 siRNA</td>
<td>35.75 ± 9.4</td>
<td>3</td>
</tr>
</tbody>
</table>