Regulation of Cell Signaling Pathways

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SM22α inhibits lamellipodium formation and migration via Ras-Arp2/3 signaling in synthetic VSMCs

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Submitted 4 February 2016; accepted in final form 7 September 2016

Lv P, Zhang F, Yin YJ, Wang YC, Gao M, Xie XL, Zhao LL, Dong LH, Lin YL, Shu YN, Zhang DD, Liu GX, Han M. SM22α inhibits lamellipodium formation and migration via Ras-Arp2/3 signaling in synthetic VSMCs. Am J Physiol Cell Physiol 311: C758–C767, 2016. First published September 14, 2016; doi:10.1152/ajpcell.00033.2016.—We previously demonstrated that smooth muscle (SM) 22α promotes the migration activity in contractile vascular smooth muscle cells (VSMCs). Based on the varied functions exhibited by SM22α in different VSMC phenotypes, we investigated the effect of SM22α on VSMC migration under pathological conditions. The results demonstrated that SM22α overexpression in synthetic VSMCs inhibited platelet-derived growth factor (PDGF)-BB-induced cell lamellipodium formation and migration, which was different from its action in contractile cells. The results indicated two distinct mechanisms underlying inhibition of lamellipodium formation by SM22α, increased actin dynamic stability and decreased Ras activity via interference with interactions between Ras and guanine nucleotide exchange factor. The former inhibited actin cytoskeleton rearrangement in the cell cortex, while the latter significantly disrupted actin nucleation activation of the Arp2/3 complex. Baicalin, a herb-derived flavonoid compound, inhibited VSMC migration via upregulation of SM22α expression in vitro and in vivo. These data suggest that SM22α regulates lamellipodium formation and cell migration in a phenotype-dependent manner in VSMCs, which may be a new therapeutic target for vascular lesion formation.

SM22α: vascular smooth muscle cells; phenotype; cortex actin dynamics; Arp2/3

THE MIGRATION of vascular smooth muscle cells (VSMCs) is a key early event in neointima formation after vascular injury (29). Migration induced by cytokines and growth factors is a dynamic and cyclic process, generally beginning with the extension of actin-rich protrusions called lamellipodia (25). The formation of these structures at the leading edge of the cells requires the protrusive forces that are generated by actin polymerization and increased actin branching (32). An important set of actin regulators initiates the formation of new F-actin via the nucleation process, including the actin-related protein (Arp) 2/3 complex, formins, and spire. The Arp2/3, a seven-subunit complex, was the first of these molecules to be identified and plays a crucial role in the formation of branched-actin-filament networks (3, 5, 17, 42).

The Ras-related small guanosine triphosphatase (GTPase) family is a known regulator that modifies actin filament organization (7, 15). Son of Sevenless 1 (SOS1) protein functions as a guanine nucleotide exchange factor (GEF) for the small GTPase Ras by stimulating the substitution of GDP for GTP (4, 24). Ras GTPase-activating proteins (RasGAPs) accelerate the intrinsically slow GTPase activity of Ras, thereby facilitating hydrolysis of GTP bound to Ras (2, 41). Thus SOS1 and RasGAP act as positive and negative regulators, respectively, of the Ras pathway.

Smooth muscle (SM) 22 (also known as transgelin), a differentiated VSMC marker, is a cytoskeleton-associated protein and is important for maintaining the differentiated phenotype of VSMCs (9, 14, 19). Disruption of SM22α is known to increase atherosclerotic lesions (13) and enhance arterial proinflammation (37, 39). Our previous study demonstrated that the overexpression of SM22α inhibits VSMC proliferation and neointima formation via blockade of the Ras-extracellular signal regulated kinase (ERK) 1/2 pathway (10). Furthermore, SM22α may have a link with ROS formation in VSMCs (27). Loss of SM22α reduces angiotensin II-induced contraction and hypertension (43). In contractile VSMCs, SM22α facilitates the activities of migration and contraction (19). Although there have been reports that SM22α is recruited to podosomes upon phorbol-12,13-dibutyrate stimulation in A7r5 cells (16), the exact role of SM22α in VSMC migration under pathological conditions is unknown.

Baicalin, as a flavonoid compound, obtained from the flowering plant Scutellaria baicalensis Georgi, has significant anti-inflammatory (45), antibacterial (33), antiviral (31), and free-radical scavenging properties (38). Based on our previous finding that baicalin inhibits VSMC proliferation and migration (10), we investigated if SM22α is involved in the inhibitory effect of baicalin on VSMC migration.

In the present study, we demonstrated that overexpression of SM22α inhibits lamellipodium formation and migration in proliferative/synthetic VSMCs in vitro and in vivo. SM22α increases actin dynamic stability and interferes with the Ras-Arp2/3 signaling pathway, leading to a weaker actin nucleation activation of Arp2/3 complex in the cell cortex and lamellipodium formation disorder. We also found that baicalin inhibits platelet-derived growth factor (PDGF)-BB-induced VSMC migration via upregulation of endogenous SM22α expression. Taken together, these
findings indicate a novel mechanism underlying the involvement of SM22α in vascular homeostasis and remodeling.

MATERIALS AND METHODS

Animals. The Sm22α−/− mouse line [B6.129S6-TgH1Sm22αcreYesc/J] carrying a Cre-recombinase gene inserted into the endogenous SM22α locus was purchased from The Jackson Laboratory (America). Eight- to 12-wk-old male Sm22α−/− mice and their littermate wild-type controls were anesthetized via isoflurane inhalation and the left common carotid artery was ligated with a 6-0 silk suture so that the common carotid artery blood flow was completely disrupted. Six animals were used per group.

Male Sprague-Dawley rats were obtained from the Experimental Animal Center of Hebei Medical University. Balloon denudation of the left common carotid artery was performed as previously described (27). Six animals were used per group. All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, and were approved by the Institutional Animal Care and Use Committee of Hebei Medical University.

Cell culture. VSMCs were isolated from the thoracic aorta of male Sprague-Dawley rats weighing 80–100 g, as previously described (20). VSMCs were grown in low-glucose Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells from passages 3 to 5 only were used in the experiments. The SMC identity of the cells was confirmed by staining with SMC-specific α-smooth muscle actin monoclonal antibody. The VSMCs of wild-type (Sm22α+/+) and Sm22α−/− mice (aged 8–12 wk) were isolated from the aorta with 1% collagenase and cultured in low-glucose DMEM supplemented with 20% FBS.

Cell migration assay. Directional cell migration of VSMCs was determined in a monolayer using an in vitro scratch wound as previously described (40). After achieving confluence, serum-starved VSMCs were subjected to injury using 200-μl sterilized pipette tips, washed, and stimulated with 20 ng/ml PDGF-BB (R&D) for the indicated time, then stained by gentian violet. This was followed by image capture with a microscope.

Adenovirus packaging and infection. Full-length cDNA of rat SM22α was cloned into plasmid pEGFP-C2. The pAd/CMV/V5-DEST Gateway Vector (Invitrogen) was used to pack green fluorescent protein (GFP)-tagged adenovirus (27). The VSMCs were infected with adenovirus (5 × 10⁹ pfu/ml) for 24 h, washed, and treated with 20 ng/ml PDGF-BB (R&D) for the indicated time, then stained by gentian violet. This was followed by image capture with a microscope.

MATERIALS AND METHODS

Immunochemistry (IHC) analysis. Sections were blocked with 0.3% hydrogen peroxide, followed by preincubation with 5% normal goat serum and then incubation with primary antibodies against MMP-2 (1:1,000, Abcam), MMP-9 (1:1,000, Abcam), ICAM-1 (1:500, Abcam), VCAM-1 (1:500, Abcam), osteopontin (OPN; 1:500, Epitomics), integrin β₃ (1:500, Abcam), or SM22α (1:1,000, Abcam) at 4°C overnight. Next, the sections were incubated with the biotinylated secondary antibody, followed by streptavidin-horseradish peroxidase and diaminobenzidine, and then counterstained with hematoxylin. Staining intensities were determined by measurement of the integrated optical density (IOD) by light microscopy using a computer-based Image-Pro Morphometric System.

Immunoprecipitation (IP) analysis. Lysate samples were preclreated with Protein A/G PLUS-Agarose (Santa Cruz) to reduce nonspecific binding. The supernatants were immunoprecipitated with indicated antibodies at 4°C overnight, followed by incubation with Protein A/G PLUS-Agarose beads for 2–4 h at 4°C. The agarose beads were then collected by centrifugation, washed with the lysis buffer, and resuspended in sample buffer. Bound proteins were resolved by SDS-PAGE followed by Western blot analysis as described above.

Ras activity assay. VSMCs were lysed and centrifuged, and an equivalent amount (at least 1 mg per experiment) of supernatant protein from each condition was assayed for active Ras (GTP-Ras) using Ras Activation Assay Kit (Millipore, 17–218) according to the manufacturer instructions.

Observation of the actin cytoskeleton. VSMCs cultured on coverslips were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 at room temperature for 30 min. Thereafter, the cells were stained with 1 μg/ml tetraethyl rhodamine isothiocyanate (TRITC)-phalloidin (Sigma-Aldrich) in a blocking solution [1% bovine serum albumin (BSA) and 0.1% Triton X-100 in phosphate-buffered saline (PBS)] for 120 min in the dark, at room temperature to determine F-actin localization. The cells were then observed and photographed under a laser scanning confocal microscope.

Immunofluorescence assay. VSMCs cultured on coverslips were fixed with 4% paraformaldehyde solution for 30 min at room temperature, washed with PBS, and treated with 10% normal goat serum blocking solution for 20 min in a humidified chamber at room temperature. Cells were incubated with anti-SM22α (1:1,000, Abcam) or anti-ARPC2 (1:500, Abcam) at 4°C overnight. Then, cells were washed 3 times with PBS and incubated with fluorescein-conjugated secondary antibodies (1:500, KPL) for 60 min at room temperature. The cells were then washed with PBS, mounted with DAPI (1:2,000, Sigma-Aldrich), and visualized using a laser scanning confocal microscope.

Small interfering RNA (siRNA) transfection. VSMCs were grown to 50% to 70% confluence and then transfected with specific duplex siRNA for SM22α (5'-GCU AGU GGA GUG GAU UGU ATT-3' and 5'-UAC AUR CCA CUC CAC UAG CTT-3') or nonspecific, scrambled siRNA (5'-GCU AGA GUA CGG GUG AAU UGC TT-3' and 5'-CGA AUU CAC CGC UAC UAC AGC TG-3') using Lipofectamine RNAiMAX reagent (Invitrogen). After transfection of siRNA for 6–12 h, VSMCs were treated with PDGF-BB as mentioned.

Statistical analysis. Data analysis was performed using SPSS version 16.0. Data are presented as means ± SE. Paired data were compared using Student’s t-test. Differences between groups were determined with one-way analysis of variance (ANOVA) with repeated measures. A probability value of <0.05 was considered significant.
RESULTS

Disruption of SM22α promotes the migration of synthetic VSMCs. Our previous study demonstrated that SM22α promotes the mobility of contractile VSMCs (19). Increased migration activity is a well-known characteristic of VSMC phenotype switching (15, 36), accompanied with decreased expression of SM22α, different from the contractile cells (9, 12, 18, 35). To determine the effects of SM22α on the migratory activity of synthetic/proliferative VSMCs, the cells were treated with PDGF-BB (20 ng/ml) for 0–24 h to induce

Fig. 1. Disruption of SM22α promotes the migration of synthetic VSMCs. A: Western blot analysis (left) and quantification (right) for the expression of SM22α, calponin, caldesmon, SM α-actin, or PCNA in VSMCs stimulated by PDGF-BB (20 ng/ml) for 0, 6, 12, or 24 h. β-Actin was used as an internal control. *P < 0.05, **P < 0.01, ***P < 0.001 vs. 0 h. B: serum-starved VSMCs were treated with PDGF-BB for indicated durations, and cell number was counted for cell proliferative activity. ***P < 0.001 vs. 0 h. C: serum-starved VSMCs were scratched and treated with PDGF-BB for indicated durations or remained untreated. Results are expressed as the relative width of the injury lines. Bars, 50 μm. *P < 0.05 vs. PDGF-BB(−) at 12 or 24 h. D: Western blot for SM22α expression after Ad-GFP or Ad-GFP-SM22α infection for 24 h. *P < 0.05 vs. Ad-GFP. E: VSMCs were cultured in serum-starved medium with Ad-GFP or Ad-GFP-SM22α for 24 h, and then scratched and stimulated by PDGF-BB for 12 h. Results are expressed as the relative width of the injury lines. Bars, 100 μm. *P < 0.05 vs. Ad-GFP. F: Western blot for SM22α expression in VSMCs from Sm22α−/− mice or their Sm22α+/− littermate. G: serum-starved VSMCs from mice were scratched and treated with PDGF-BB for 12 h. Results are expressed as the relative width of the injury lines. Bars, 100 μm. **P < 0.01 vs. Sm22α+/− littermate. H: serum-starved VSMCs from mice were treated with PDGF-BB for 24 h, and cell number was counted for cell proliferative activity. **P < 0.01 vs. Sm22α+/− littermate. Bar graphs show mean ± standard error of the mean (SE) values from 3 independent experiments (n = 3).
phenotypic switching. VSMCs exhibited decreased expression of differentiation markers (Fig. 1A), and increased directional proliferative (Fig. 1B) and migratory activity upon PDGF-BB stimulation (Fig. 1C). To identify the correlation between decreased expression of SM22α and increased migration of PDGF-BB-stimulated VSMCs (synthetic state), SM22α was overexpressed in VSMCs. We showed that overexpression of SM22α significantly inhibited PDGF-BB-evoked cell migration (Fig. 1, D and E). Conversely, VSMCs from Sm22α−/− mice displayed an enhanced migratory and proliferative response to PDGF-BB compared with wild-type cells (Fig. 1, F–H). These results suggested that the effect of SM22α on VSMC migration is opposite in contractile and synthetic VSMCs, and the disruption of SM22α promotes the migration of synthetic VSMCs.

Disruption of SM22α results in enhanced migratory activity of VSMCs during neointima formation. Migration is an early event in the development of restenosis. To determine the negative correlation between SM22α expression and VSMC migration during neointima formation, we performed carotid...
ligation of Sm22α−/− mice and their Sm22α+/+ littermates. At 1 wk postligation, the injured carotids from both Sm22α−/− and Sm22α+/+ mice swelled significantly more than their sham controls, and the carotid neointima of Sm22α−/− mice was thicker than that of Sm22α+/+ mice (Fig. 2A), consistent with previous studies (37, 39).

The upregulation of migration and adhesion-related molecule expression, including MMPs, VCAM-1, and ICAM-1, offers the molecular basis for VSMC migration (15). Our previous study had shown that the expression of these proteins in the arteries of Sm22α−/− mice was significantly elevated compared with wild-type mice at baseline (6). Using IHC, we found that the expression of ICAM-1, VCAM-1, MMP-2, MMP-9, OPN, and integrin β3 in the media and neointima of Sm22α−/− mice was approximately 2–3 times higher than that in their Sm22α+/+ littermates (Fig. 2, B and C), suggesting that the disruption of SM22α resulted in enhanced migration of VSMCs during vascular injury.

Overexpression of SM22α inhibits lamellipodium formation via promoting actin bundling in PDGF-BB-induced VSMCs. VSMCs form lamellipodia when they migrate and invade, wherein actin polymerization in the lamellipodia is thought to be the driving force for much of cell migration (15). It has been confirmed that SM22α localizes at actin structures such as stress fibers and lamellipodia in VSMCs via binding to F-actin (16, 19). In this study, we showed that there were widespread F-actin bundles in SM22α-overexpressed cells but not the Ad-GFP control (Fig. 3A). PDGF-BB induced a rapid rearrangement of actin cytoskeleton at the zone of lamellipodia located around the cell cortex, accompanied by formation of numerous lamellipodia at the leading edge in Ad-GFP-transduced cells, which was markedly attenuated by overexpression of SM22α (Fig. 3A). The normalized length of the lamellipodia around Ad-SM22α-infected cells was significantly reduced by 71 ± 8% (Fig. 3B). These results indicate that SM22α plays a key role in actin remodeling at the peripheral lamellipodia of migrating cells, via maintaining F-actin bundling in the cytosol and preventing the formation of remodeled actin structures at the cell periphery induced by PDGF-BB.

SM22α inhibits lamellipodium formation through sequestration of actin nucleation activity of Arp2/3. Although numerous polymerization nucleation factors play roles in lamellipodium formation, the crucial mode of filament assembly is via the Arp2/3 complex (17). Arp2/3 complex is active at the leading edge of motile cells and produces branches along the sides of existing filaments (3, 5, 17, 42). To elucidate the mechanism by
which SM22α reduces lamellipodium formation, we directly examined the effect of SM22α on Arp2/3 distribution. We showed that ARPC2 (a subunit of Arp2/3 complex) redistributed from the cytosol to the periphery of VSMCs upon PDGF-BB stimulation (Fig. 4A). SM22α overexpression impaired PDGF-BB-induced translocation of ARPC2 to the leading edge of VSMCs (Fig. 4A). In accordance with these data, as shown by membrane fractionation from whole cell lysates and Western blotting using specific antibodies, PDGF-BB-induced redistribution of ARPC2 in the membrane fraction was decreased by SM22α overexpression (Fig. 4B). These data support the notion that SM22α inhibits rearrangement of the

Fig. 5. SM22α prevents Arp2/3 activation by interfering with Ras signaling. A and B: fluorescence staining with Phalloidin (red; A) or ARPC2 (green; B) and nuclei (DAPI, blue). VSMCs were preincubated with FTase inhibitor (1 μM) for 24 h and were then either stimulated with PDGF-BB for 10 min or remained untreated. The representative images are from 3 independent experiments (n = 3). Bars, 25 μm. C: GTP-Ras was immunoprecipitated using agarose-bound RBD, then the amount of activated Ras (GTP-Ras) was determined by immunoblotting. *P < 0.05, ***P < 0.001 vs. 0 min. D: Ad-GFP- or Ad-SM22α-infected VSMCs were either stimulated by PDGF-BB for 2 min or remained untreated, followed by quantification of GTP-Ras. **P < 0.01 vs. Ad-GFP, #P < 0.05 vs. Ad-GFP plus PDGF-BB. E: Western blot for the expression of Ras, SOS1, RasGAP, or SM22α. F: IP for the interaction between SOS1 and Ras in cells treated as in D. *P < 0.05 vs. Ad-GFP, #P < 0.05 vs. Ad-GFP plus PDGF-BB. G: IP for the interaction between RasGAP and Ras in cells infected with adenovirus following treatment by PDGF-BB for 2 min. H: IP for the interaction between SOS1 and Ras in HeLa cells transfected with constructs following treatment by PDGF-BB for 2 min. Bar graphs show mean ± SE values from 3 independent experiments (n = 3).
actin cytoskeleton and lamellipodium formation via decrease of Arp2/3 actin-nucleating activity.

SM22α prevents Arp2/3 activation by interfering with Ras signaling. To address the molecular mechanism by which overexpression of SM22α inhibits the activation of Arp2/3, we focused on the intracellular signaling pathways. Our previous study demonstrated that SM22α overexpression markedly inhibits Ras signaling cascades (10). As small GTPase R-Ras regulates organization of actin and drives membrane protrusions (1), we hypothesize that Arp2/3 may act directly downstream of Ras to initiate PDGF-BB-dependent actin rearrangement. To test this hypothesis, farnesyltransferase (FTase) inhibitor was used to block Ras activation. We observed that pretreatment with FTase inhibitor decreased PDGF-BB-induced actin remodeling (Fig. 5A) and ARPC2 translocation (Fig. 5B), implying that Arp2/3 may be the main effector downstream of Ras in actin remodeling of VSMCs.

Next, we investigated the mechanism of SM22α acting as an upstream component to regulate Ras activity. Using the glutathione S-transferase (GST) pull-down assay, we showed that acute PDGF-BB stimulation increased Ras activation (GTP-Ras) in a time-dependent manner (Fig. 5C). SM22α overexpression resulted in a reduced GTP-Ras level (Fig. 5D), suggesting that SM22α may be responsible for the regulation of Ras activity. Combining the correlation between Ras and Arp2/3 described above, we speculated that SM22α suppressed Arp2/3 activity via blockade of Ras activation.

SOS1 or RasGAP serve as master regulators of the activation of Ras. We first tested the effect of SM22α on the expression of Ras, SOS1, and RasGAP, and found that SM22α overexpression did not alter the expression of Ras and SOS1; rather, it caused only a slight increase in the level of RasGAP in VSMCs (Fig. 5E). Further immunoprecipitation results showed that PDGF-BB stimulation increased the interaction between SOS1 and Ras by $97 \pm 18\%$, while this increased interaction was weakened by $35 \pm 4\%$ in Ad-GFP-SM22α-infected cells (Fig. 5F). However, no significant change in RasGAP was observed in Ras-immunoprecipitated complexes under the same conditions (Fig. 5G). To verify the involvement of SM22α in direct interaction of SOS1 and Ras, HeLa cells were transfected with GFP-SM22α constructs. The interaction of SOS1 and Ras induced by PDGF-BB was decreased in SM22α-overexpressed HeLa cells (Fig. 5H). These data suggest that SM22α inhibits Ras-SOS1 complex formation and subsequently blocks Ras activation.

**Antimigration role of baicalin is dependent on SM22α upregulation.** Our previous study demonstrated that baicalin, a herb-derived flavonoid compound, inhibits proliferation and migration induced by PDGF-BB in VSMCs (11). In the present study, we further verified this finding and showed that preincubation with baicalin (40 μM) inhibited PDGF-BB-induced cell migration (Fig. 6A) and lamellipodium formation in VSMCs (Fig. 6B). Baicalin also decreased PDGF-BB-induced, Ras-mediated ARPC2 translocation to the cell periphery (Fig. 6, C and D). Furthermore, the mechanism underlying baicalin blocking of Ras-Arp2/3 signaling was associated with a reduced interaction between SOS1 and Ras (Fig. 6E). We investigated whether SM22α mediated the inhibitory effect of baicalin on migration. Using the balloon injury model of rat carotid arteries, we found that administration with baicalin (70 mg·kg$^{-1}$·day$^{-1}$) significantly enhanced SM22α expression, which was accompanied with decreased expression of MMP-9, ICAM-1, and VCAM-1 in the neointima of carotid arteries (Fig. 6F). In vitro studies also showed that baicalin inhibited PDGF-BB-induced expression of MMP-2 and VCAM-1 in VSMCs and promoted the expression of differentiated markers, including SM22α, calponin, caldesmon, and SM α-actin (Fig. 6G). This implied that inhibition of cell migration by baicalin may be associated with upregulation of SM22α expression. To verify the involvement of SM22α in inhibition of cell migration by baicalin, SM22α-specific siRNA (si SM22α) was used to silence the expression of endogenous SM22α. SM22α knockdown significantly decreased the inhibitory effect of baicalin on expression of MMP-2, MMP-9, and VCAM-1 (Fig. 6H) as well as lamellipodium formation in VSMCs (Fig. 6I). Taken together, these data strongly suggest that baicalin, as a vascular protective agent, maintains the contractile phenotype of VSMC via upregulation of SM22α.

**DISCUSSION**

VSMC migration is an early event during vascular injury. To our knowledge, this study is the first to identify that SM22α, a differentiated VSMC marker, is a negative regulator of PDGF-BB-induced migration of VSMCs. SM22α overexpression inhibited migration of VSMCs induced by PDGF-BB, which differs from its role in contractile VSMCs. Sm22α$^{-/-}$ VSMCs displayed enhanced migratory activity in vitro and in vivo. Furthermore, we found that SM22α inhibits lamellipodium formation through sequestration of actin nucleation by Arp2/3.
SM22α suppressed Ras-mediated Arp2/3 activation by inhibiting Ras-SOS1 complex formation.

Migration of VSMCs is a critical step in the formation of atherosclerotic lesions (15, 36). The majority of intimal SMCs within atherosclerotic lesions are derived from resident medial SMCs that undergo phenotypic modulation and migration into the intima, where they proliferate (18, 34). PDGF-BB-treated VSMCs display increased activity of migration and proliferation, accompanied with decreased SM22α expression (8, 18, 21, 35), leading to the speculation of a negative correlation between VSMC migration and SM22α expression. The present study provided evidence that overexpression of SM22α inhibi-
its PDGF-BB-induced cell migration in synthetic VSMCs and that disruption of SM22α promotes migratory responses after artery ligation. This result is similar to the previously reported effects of SM22α on proliferation (10), oxidative stress (27), and proinflammation responses (6, 37, 39), suggesting that there is a causal relationship between decreased expression of SM22α and pathological changes during vascular disease. SM22α exerts a dual regulatory action on VSMC migration in a phenotype-dependent manner. SM22α facilitates cell mobility by inducing stress fiber formation in contractile VSMCs (19) and inhibits cell migration via suppression of lamellipodium formation in synthetic phenotype.

Although lamellipodium formation in different cell types involves focal contact remodeling, molecular motors, and contraction of the cell body (3, 15), rearrangement of the cytoskeleton is the most important step. It is known that dynamic instability of microtubules is necessary for adequate dissolution of focal contacts at the trailing edge of migrating cells (23). However, the effect of actin cytoskeleton stability on lamellipodium formation remains unclear. As actin-associated proteins, SM22α and Arp2/3 regulate cytoskeleton polymerization in different ways. SM22α directly binds to the actin cytoskeleton and induces actin bundling to facilitate the formation of cytoskeletal structure such as stress fibers, a stable cytoskeleton (19, 37). The Arp2/3 complex, on the other hand, binds to the side of an existing parent filament and nucleates the formation of new actin filaments, leading to the formation of branched filament networks that are required for efficient cell migration (30). Our data demonstrated that SM22α-mediated polymerization of the actin cytoskeleton inhibited lamellipodium formation in VSMCs. It seems plausible that the increased actin cytoskeleton stability by SM22α would be due, at least in part, to decrease F-actin nucleation at the cell cortex.

Caldesmon greatly retards Arp2/3-mediated actin nucleation and actin polymerization (44). SM22α has functions similar to those of caldesmon in VSMCs, such as regulation of cell contraction and organization of actin filaments (14, 19). In the present study, we found that Arp2/3 translocation to the leading edge induced by PDGF-BB was significantly impaired in Ad-GFP-SM22α-infected cells, meaning that actin nucleation activity of Arp2/3 was also inhibited by SM22α. There are two distinct mechanisms underlying SM22α regulation of lamellipodium formation, namely, stabilization of actin cytoskeleton and inhibition of cortex actin polymerization, which are essential for lamellipodium formation in VSMCs.

Previous studies demonstrated that activation of members of the Ras superfamily is essential for vascular disease (7, 10, 28). We therefore tested if SM22α interfered with Arp2/3 activity via blockade of Ras activation. Our results indicate that SM22α decreased the GTP-Ras level and the FTase inhibitor suppressed Arp2/3 translocation, suggesting that overexpression of SM22α inhibited lamellipodium formation via blockade of the Ras-Arp2/3 signaling pathway. Ras activity is regulated by balance between GEF and RasGAP (2, 4, 24, 41). To further demonstrate the mechanism by which SM22α regulates Ras activity, we examined the expression of SOS1 and RasGAP. We found that SM22α showed little effect on their expression. However, SM22α overexpression decreased the affinity of SOS1 and Ras, which is the rate-limiting step in Ras activation. SM22α regulation of Ras activity provides a potential treatment strategy to treat vascular pathologies.

Baicalin has been demonstrated to have antitumor (22, 26), antibacterial (33), and antiviral (31) properties. Our previous study has revealed the inhibitory effect of baicalin on PDGF-BB-induced proliferation and migration of VSMCs (10). Probably, both reduced cell proliferation and migration contribute to achieving the therapeutic goal of inhibiting vascular remodeling; therefore, baicalin may be considered as an effective cardiovascular drug as well. In the present study, we showed that baicalin inhibited PDGF-BB-induced expression of MMPs and VCAM-1, and promoted the expression of VSMC differentiated markers, including SM22α, calponin, caldesmon, and SM α-actin. This implies that baicalin protects VSMCs from proliferative disorders via inhibition of phenotype remodeling. This result was validated in vivo using an animal arterial balloon-injury model. Furthermore, baicalin inhibiting VSMC migration was also associated with blockade of Ras-Arp2/3 signaling pathway via upregulation of SM22α expression. Knockdown of SM22α reduced the effect of baicalin on the expression of MMPs and VCAM-1 as well as on lamellipodium formation. Taken together, SM22α may be a regulatory target for cell migration and vascular homeostasis.

In summary, SM22α plays a strong protective role in vascular injury. Therapeutic upregulation of SM22α expression by baicalin may represent a safe and effective approach to prevent vascular disease. Therefore, our findings provide new insights into multiple functions of SM22α in the regulation of VSMC migration during the development of vascular disorders.

GRANTS
This study was supported by the National Natural Science Foundation of China (Grant nos. 91439114, 31471092, and 32171222, awarded to M. Han; Grant no. 81300225 awarded to P. Lv; Grant no. 31401199 to F. Zhang), National Key Research and Development Program (Grant no. 2016YFF0202300, awarded to P. Lv), National Science Foundation of Hebei Province, China (Grant no. H2015206467, awarded to P. Lv), Outstanding Youth Foundations of the Department of Education of Hebei Province, China (Grant no. YQ2013018, awarded to P. Lv), and China Postdoctoral Science Foundation (Grant no. 2015M571277, awarded to P. Lv).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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