Epac1 Deficiency Attenuated Vascular Smooth Muscle Cell Migration and Neointimal Formation

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Objective—Vascular smooth muscle cell (SMC) migration causes neointima, which is related to vascular remodeling after mechanical injury and atherosclerosis development. We previously reported that an exchange protein activated by cAMP (Epac) 1 was upregulated in mouse arterial neointima and promoted SMC migration. In this study, we examined the molecular mechanisms of Epac1-induced SMC migration and the effect of Epac1 deficiency on vascular remodeling in vivo.

Approach and Results—Platelet-derived growth factor-BB promoted a 2-fold increase in SMC migration in a primary culture of aortic SMCs obtained from Epac1+/+ mice (Epac1+/+-ASMCs), whereas there was only a 1.2-fold increase in Epac1−/−-ASMCs. The degree of platelet-derived growth factor-BB–induced increase in intracellular Ca2+ was smaller in Fura2-labeled Epac1+/+-ASMCs than in Epac1+/+ASMCs. In Epac1+/+-ASMCs, an Epac-selective cAMP analog or platelet-derived growth factor-BB increased lamellipodia accompanied by cofilin dephosphorylation, which is induced by Ca2+ signaling, whereas these effects were rarely observed in Epac1−/−-ASMCs. Furthermore, 4 weeks after femoral artery injury, prominent neointima were formed in Epac1+/+ mice, whereas neointima formation was significantly attenuated in Epac1−/− mice in which dephosphorylation of cofilin was inhibited. The chimeric mice generated by bone marrow cell transplantation from Epac1+/+ into Epac1−/− mice and vice versa demonstrated that the genetic background of vascular tissues, including SMCs rather than of bone marrow–derived cells affected Epac1-mediated neointima formation.

Conclusions—These data suggest that Epac1 deficiency attenuates neointima formation through, at least in part, inhibition of SMC migration, in which a decrease in Ca2+ influx and a suppression of cofilin-mediated lamellipodia formation occur.

Key Words: arterial injury ■ intima-media thickness ■ migration ■ signal transduction ■ vascular smooth muscle

Intimal thickening occurs after injury to the luminal surface of arteries during percutaneous transmural angioplasty and coronary artery stenting, as well as during coronary artery bypass vein grafting.1 Excessive intimal thickening leads to the failure of these interventions. Intimal thickening is also associated with early- and advanced-stage atherosclerotic lesions.1 Despite the relative success of drug-eluting stents and pharmacological regimens to lower cholesterol levels, therapeutic strategies to obstructive arterial remodeling have not been fully established.2

Arterial smooth muscle cell (SMC) migration to the intima is a fundamental process that leads to intimal thickening in diseased vessels and plays an important role in the pathogenesis of vascular stenosis and occlusion.3,4 Platelet-derived growth factor (PDGF)-BB is released at the vascular injury sites and atherosclerotic lesions.2 Studies using a blockade of PDGF-B or PDGF receptor (PDGFR)-β, or genetic deletion of PDGFR-β in rodents or nonhuman primates have demonstrated that PDGFR-β signaling contributes to ≤80% of SMC migration and accumulation after acute vascular injury.2,3 Consistent with this evidence, exogenously administered PDGF-BB into the rat carotid artery after endothelial denudation increased neointimal lesions.2,4 In addition, genetic deletion of PDGFR-β decreased SMC accumulation in atherosclerotic lesions.2,5 This accumulating evidence suggests that PDGF-BB and PDGF-β signaling play a prominent role in vascular SMC migration into the neointima after vascular injury and during the development of atherosclerosis.2

It has been suggested that PDGF-BB–mediated cell migration is attributed to the activation of intracellular signal transduction pathways, including phosphatidylinositol 3-kinase, mitogen-activated protein kinase cascade, and
extracellular signal–regulated kinase.\textsuperscript{6,7} In addition to these signaling pathways, it has been recognized that PDGF-BB increases intracellular cAMP via a G protein–coupled receptor transactivation mechanism.\textsuperscript{8,9} However, the role of PDGF-BB–induced cAMP in SMC migration remains unknown.

A major second messenger cAMP activates protein kinase A (PKA) and an exchange protein activated by cAMP (Epac).\textsuperscript{10} Epac has 2 isoforms, Epac1 and Epac2, both of which are activated in living cells by physiologically relevant concentrations of cAMP.\textsuperscript{10} Epac1 was found to be expressed in most tissues, including the blood vessels, whereas Epac2 is localized in the adrenal gland and the brain.\textsuperscript{10} In our previous study, we demonstrated that Epac1 expression was increased in the area of intimal thickening after mouse femoral arteries were injured, and Epac1 activation promoted vascular SMC migration.\textsuperscript{11}

On the basis of these studies, we hypothesized that Epac1 was involved in PDGF-BB–induced SMC migration and neointimal thickening. In this study, we examined the molecular mechanisms of Epac1-induced migration using a primary culture of SMCs from Epac1-deficient (Epac1\textsuperscript{−/−}) mice.\textsuperscript{12} We also investigated whether Epac1 deficiency inhibited neointimal formation after vascular injury in vivo.

**Materials and Methods**
Materials and Methods are available in the online-only Data Supplement.

**Results**

**Epac1 Deficiency Attenuated Vascular SMC Migration**
First, we examined PDGF-BB–mediated cAMP production in aortic SMCs from Epac1\textsuperscript{+/+} (Epac1\textsuperscript{+/+}-ASMCs) and Epac1\textsuperscript{−/−} mice (Epac1\textsuperscript{−/−}-ASMCs). In accordance with the previous reports on vascular SMCs,\textsuperscript{8,9} stimulation of PDGF-BB led to a 1.30±0.07-fold increase in intracellular cAMP in Epac1\textsuperscript{+/+}-ASMCs (\textit{P}<0.05 versus control of Epac1\textsuperscript{+/+}-ASMCs, \textit{n}=6). A similar effect of PDGF-BB on cAMP production was also detected in Epac1\textsuperscript{−/−}-ASMCs (1.30±0.02-fold; \textit{P}<0.01 versus control of Epac1\textsuperscript{−/−}-ASMCs; \textit{n}=6). Subsequent PKA activation induced by PDGF-BB similarly occurred in both Epac1\textsuperscript{+/+}-ASMCs (2.90±0.22-fold; \textit{P}<0.001 versus control; \textit{n}=6) and Epac1\textsuperscript{−/−}-ASMCs (2.98±0.13-fold; \textit{P}<0.001 versus control; \textit{n}=6).

Because there was no significant difference in the effects of PDGF-BB on cAMP production between Epac1\textsuperscript{+/+}- and Epac1\textsuperscript{−/−}-ASMCs (\textit{P}=0.50; \textit{n}=6), we examined the migratory effect of PDGF-BB on Epac1\textsuperscript{+/+}-ASMCs and Epac1\textsuperscript{−/−}-ASMCs. In the basal condition, cell migration determined by total path length of ASMCs was similar in both Epac1\textsuperscript{+/+}-ASMCs and Epac1\textsuperscript{−/−}-ASMCs (Figure 1A and 1B; Movies I–IV in the online-only Data Supplement). In Epac1\textsuperscript{+/+}-ASMCs, PDGF-BB significantly promoted migration, whereas PDGF-BB–induced migration was significantly attenuated in Epac1\textsuperscript{−/−}-ASMCs (Figure 1A and 1B; Movies I–IV in the online-only Data Supplement). Furthermore, PDGF-BB promoted the invasion of Epac1\textsuperscript{−/−}-ASMCs across Matrigel-coated filters, whereas this effect was significantly attenuated in Epac1\textsuperscript{−/−}-ASMCs across Matrigel-coated filters, whereas this effect was significantly attenuated in Epac1\textsuperscript{−/−}-ASMCs (Figure I in the online-only Data Supplement). The expression level of PDGFR-\(\beta\), which primarily contributes to PDGF-BB–induced vascular SMC migration,\textsuperscript{2} did not differ between Epac1\textsuperscript{+/+} and Epac1\textsuperscript{−/−} ASMCs (0.72±0.12 and 0.53±0.04 arbitrary unit; \textit{P}=0.20; \textit{n}=4). Therefore, these data suggest that Epac1 is involved in PDGF-BB–induced vascular SMC migration.

**Epac1 Deficiency Decreased PDGF-BB–Induced Intracellular Ca\textsuperscript{2+} Elevation**
It is well recognized that PDGF-mediated intracellular Ca\textsuperscript{2+} elevation regulates cell migration.\textsuperscript{13,14} Because activation
of Epac1 increases intracellular Ca²⁺ concentration by activating Ras-related protein (Rap)-phospholipase C (PLC)ε signaling. We investigated whether Epac1 was involved in the PDGF-BB–mediated increase in intracellular Ca²⁺ concentration. Intracellular Ca²⁺ concentrations acquired by monitoring Fura-2-AM were significantly increased in PDGF-BB–stimulated Epac1⁺⁻⁻ASMCs (Figure 2A and B). In Epac1⁺⁻⁻ASMCs, however, PDGF-BB–mediated intracellular Ca²⁺ elevation was significantly attenuated (Figure 2A and 2B) Ionomycin, which was used as a positive control, significantly increased intracellular Ca²⁺ in both Epac1⁺⁻⁻ and Epac1⁺⁻⁺ASMCs. These data suggest that Epac1 is involved in PDGF-BB–induced intracellular Ca²⁺ elevation in vascular SMCs.

Epac Stimulation Increased Lamellipodia Formation

Migrating cells are polarized with membrane protrusions at the leading edge. Protrusion with lamellipodial growth forms new adhesions and plays crucial roles in cell migration. It is well recognized that the elevation of intracellular Ca²⁺ concentration dephosphorylates cofilin to form lamellipodia by accelerating actin filament turnover. To assess the role of Epac in the establishment of lamellipodia formation, lamellipodia accompanied by dephosphorylated cofilin in ASMCs was evaluated by immunocytochemistry. In untreated Epac1⁺⁻⁻ASMCs, cofilin phosphorylation was maintained at the cell membrane, and lamellipodia were not formed in >80% of cells (Figure 3A and 3B). In contrast, stimulation of Epac using the Epac-selective cAMP analog 8-pCPT-2-O-Me-cAMP significantly increased the number of lamellipodia accompanied by dephosphorylated cofilin in Epac1⁺⁻⁻ASMCs (Figure 3A and 3B). This effect was not observed in Epac1⁺⁻⁻ASMCs (Figure 3A and 3B), suggesting that the effect of 8-pCPT-2-O-Me-cAMP on lamellipodia formation is mediated by Epac1. In accordance with these immunocytochemistry data, Western blotting analysis revealed that 8-pCPT-2-O-Me-cAMP significantly decreased phosphorylated cofilin protein expression in Epac1⁺⁻⁺ASMCs but not in Epac1⁺⁻⁻ASMCs (Figure 3C and 3D). Motile cells typically contain fewer, thinner, and more dynamic stress fibers. Stimulation of Epac decreased F-actin stress fibers in Epac1⁺⁻⁻ASMCs (Figure 3A). In contrast, in Epac1⁺⁻⁻ASMCs, stress fibers seemed to be increased in the basal condition, and 8-pCPT-2-O-Me-cAMP did not affect stress fiber formation (Figure 3A), which is consistent with the previous reports showing that Epac inhibits RhoA via Rap1, resulting in decreased stress fiber formation.

Figure 3. Epac activation increased lamellipodia formation in aortic smooth muscle cells (ASMCs). A, Representative images of lamellipodia formation in ASMCs. F-actin (red) and phosphorylated cofilin (green; p-cofilin) were visualized by immunofluorescent staining. Top and Middle, p-cofilin and F-actin expressions. Bottom, Merged images of p-cofilin and F-actin expressions. B, Quantification of the number of lamellipodia that exhibited lamellipodia formation in ASMCs. F-actin (red) and phosphorylated cofilin (green; p-cofilin) were visualized by immunofluorescent staining. Arrows indicate lamellipodia formation accompanied by dephosphorylated cofilin. The insets are the images of lamellipodia magnified 2 additional times. Scale bars, 30 μm. B, Quantification of the number of ASMCs that exhibited lamellipodia accompanied by dephosphorylated cofilin. Lamellipodia-positive cell rates are presented as the percent of total cell number; n=235 (control Epac1⁺⁻⁻-ASMCs), 136 (O8Me-cAMP Epac1⁺⁻⁻-ASMCs), 681 (control Epac1⁺⁻⁻-ASMCs), and 907 (O8Me-cAMP Epac1⁺⁻⁻-ASMCs). The data were obtained from >4 independent experiments. C, Representative images of Western blotting showing p-cofilin protein expression. D, Quantification of C; n=5 to 6. Epac1⁺⁻⁻ ASMCs and Epac1⁺⁻⁻ASMCs were incubated with 3 μmol/L of O8Me-cAMP for 20 minutes. ***P<0.001. NS indicates not significant.

Epac1-Rap1α Is Involved in PDGF-BB–Induced Lamellipodia Formation

On the basis of the above findings, Epac1 seems to play a role in migration through regulation of lamellipodia formation. On binding of cAMP, Epac stimulates the exchange GDP for GTP on Rap1, followed by a PLCε-mediated increase in intracellular Ca²⁺. In our previous reports, we demonstrated that Epac1 activates Rap1 in vascular SMCs. We then examined the involvement of Epac1 and the subtype-specific effect of Rap1 in lamellipodia formation using Epac1⁺⁻⁻ and Epac1⁺⁻⁺ASMCs.

Rap1-targeted siRNAs significantly decreased expression levels of Rap1a in Epac1⁺⁻⁻ASMCs (0.12±0.01-fold
versus control siRNA; P<0.001; n=4) and Epac1−/−-ASMCs (0.15±0.01-fold versus control siRNA; P<0.001; n=4) but not of Rap1b in Epac1+/+/-ASMCs (0.99±0.06-fold versus control siRNA, n=4) and Epac1−/−-ASMCs (1.10±0.07-fold versus control siRNA, n=4). Under transfection of control siRNAs, PDGF-BB increased the number of lamellipodia accompanied by dephosphorylated cofilin in Epac1+/+-ASMCs but not in Epac1+/+-ASMCs (Figure 4A and 4B). In accordance with this result, Western blotting using whole-cell lysates showed that PDGF-BB decreased phosphorylation of cofilin expression in Epac1+/+-ASMCs transfected with control siRNAs but not in Epac1+/+-ASMCs (Figure 4C and 4D). We confirmed similar findings of immunocytochemistry and Western blotting in untransfected Epac1+/+- and Epac1−/−-ASMCs (Figure II in the online-only Data Supplement). These data suggest that Epac1 was involved in the PDGF-mediated dephosphorylation of cofilin and the subsequent lamellipodia formation.

**Neointimal Thickening Was Attenuated in Epac1−/− Mice**

Our in vitro data suggested that a deficiency of Epac1 attenuated PDGF-BB–induced migration in vascular SMCs. We therefore examined the effect of Epac1 deficiency on the neointimal thickening in vivo. There was no morphological difference between the femoral arteries of Epac1+/+, Epac1 heterozygous deficiency (Epac1+/-), or Epac1−/− mice under basal conditions (Figure 5A, top). After 4 weeks, transmural mechanical injury was induced in femoral arteries, prominent neointimal thickening formation was observed in Epac1+/+ mice, whereas neointimal thickening formation was less pronounced in Epac1−/− than in Epac1+/+ mice (Figure 5A, bottom). The quantitative data also demonstrated that intima cross-sectional area was smaller in Epac1−/− than in Epac1+/+ mice (Figure 5B). Because media thickness was similar in Epac1+/+ and Epac1−/− mice, the ratio of the area of neointimal thickening to that of the smooth muscle layer was smaller in Epac1−/− than in Epac1+/+ mice, and the internal lumen was significantly greater in Epac1−/− than in Epac1+/+ mice (Figure 5B). Although there was no statistical difference in wire injury–induced intimal thickening between Epac1+/+ and Epac1−/− mice, the intimal thickening of Epac1−/− mice seemed moderately suppressed (Figure 5B).

Neointima formation was significantly developed at 2 weeks after injury, and it reached a maximum at 4 weeks after injury in Epac1+/+ mice (Figure 5C). However, for Epac1−/− mice, only slight neointima formation was observed at 2 weeks after injury, and this formation was not as developed as in Epac1+/+ mice at even 4 weeks after injury (Figure 5C). Furthermore, immunofluorescence staining using an antiphosphorylated cofilin antibody revealed that levels of p-cofilin expression were smaller in the area of intimal thickening in Epac1+/+ than in Epac1−/− mice. Similar changes in p-cofilin expression were observed in the medial layer of Epac1+/+ and Epac1−/− mice, although the degree of changes was moderate compared with that of the intimal thickening area (Figure 5C), which is in accordance with the in vitro data.

Neointimal thickening is attributed to multiple cellular mechanisms in SMCs, such as proliferation and phenotypic modulation.21–23 Because the previous reports suggest that Epac1 inhibits SMC proliferation in vitro,21,22 we examined Rap1a-targeted siRNAs significantly attenuated PDGF-BB–induced lamellipodia formation in Epac1+/+-ASMCs (Figure 4A and 4B). In Epac1−/−-ASMCs, Rap1a-targeted siRNA did not affect lamellipodia formation (Figure 4A and 4B). We observed similar findings using another set of Rap1a-targeted siRNA (Figure III in the online-only Data Supplement). In addition, Rap1a-targeted siRNAs attenuated PDGF-BB–mediated downregulation of phosphorylation of cofilin in whole-cell lysates of Epac1+/+ ASMCs, but the silencing of Rap1a did not affect cofilin phosphorylation in Epac1−/−-ASMCs (Figure 4C and 4D). These data suggest that Epac1−/− is involved in PDGF-BB–induced cofilin dephosphorylation and lamellipodia formation.
SMC proliferation in vitro and in vivo, and found that Epac1 deficiency promotes fetal bovine serum-induced SMC proliferation in vitro (Figure IVA in the online-only Data Supplement). These in vitro data are consistent with the previous reports. However, immunohistochemistry using an anti-Ki67 antibody did not detect an increase in proliferative cells in injured Epac1−/− arteries compared with injured Epac1+/− arteries (Figure IVB in the online-only Data Supplement). Phenotypic modulation of SMCs, especially the upregulation of myosin heavy chain isoform SMemb, is well recognized in injured arteries. The expression level of SMemb mRNA did not differ between Epac1+/− and Epac1−/− ASMCs (Figure VA in the online-only Data Supplement). SMemb protein expression was also similar in both injured Epac1+/− and Epac1−/−-arteries (Figure VB in the online-only Data Supplement), suggesting that Epac1 did not directly modulate the SMC dedifferentiation marker.

Involvement of Vascular Tissue Rather Than Bone Marrow–Derived Cells in Epac1-Mediated Neointimal Thickening

In addition to the role of SMC migration, it has been demonstrated that bone marrow–derived cells (BMCs) and perivascular fibroblasts contribute to intimal thickening. We, therefore, performed total BMC transplantation after irradiation from Epac1+/+ into Epac1−/− mice and vice versa (Epac1−/− recipient and Epac1+/+ recipient, respectively). Successful reconstitution of transplanted bone marrow was demonstrated by a shift in the Ly-antigen expression on peripheral blood CD11b-positive cells 4 weeks after transplantation using FACS (fluorescence activated cell sorting) analysis. Ly5.1 was expressed on Epac1+/+ CD11b-positive cells, whereas Ly5.2 was expressed on Epac1−/− CD11b-positive cells (Figure 6A). According to each donor BMCs, we found exclusive Ly5.1 expression (98.8±0.3%) in mice with Epac1+/+ recipient CD11b-positive cells and exclusive Ly5.2 expression (98.8±0.5%) in mice with Epac1−/− recipient CD11b-positive cells in peripheral blood samples (Figure 6B). As a sham control, we transplanted BMCs from Epac1+/+ into Epac1+/+ mice (Epac+/+ sham) and from Epac1−/− into Epac1−/− mice (Epac−/− sham). After reconstitution, we induced vascular injury of the femoral arteries in the transplanted mice and analyzed vascular remodeling 4 weeks after arterial injury. Morphometric analysis revealed that Epac1−/− recipient (bone marrow: Epac+/+) and Epac1+/+ sham exhibited significant reduction of intimal thickening compared with Epac+/+ sham. However, reduction of intimal thickening in Epac1−/− recipient (bone marrow: Epac−/−) compared with Epac+/+ sham did not reach a statistical difference (Figures 6C and 6D). These data suggest that the extent of intimal thickening was significantly associated with the genetic background of the vascular tissues, such as SMCs and perivascular fibroblasts, rather than with that of BMCs.

In addition to the migratory roles of SMCs, perivascular fibroblast migration is implicated in contributing to intimal thickening. To examine the role of Epac1 in perivascular fibroblasts, we performed a migration assay using a primary culture of periaortic fibroblasts of Epac+/+ and Epac−/− mice. In the basal condition, cell migration determined by total path length of ASMC was similar in both Epac1+/+ and Epac1−/− fibroblasts (Figure VI in the online-only Data Supplement). In Epac1+/− fibroblasts, PDGF-BB significantly promoted migration, whereas PDGF-BB–induced migration was significantly attenuated in Epac1−/− fibroblasts (Figure VI in the online-only Data Supplement).

Together with in vitro data, these data suggest that Epac1 in SMCs plays an important role in intimal thickening, and perivascular fibroblasts may contribute to intimal thickening as well.

Discussion

We demonstrated, using Epac1-deficient vascular SMCs, that PDGF-BB–induced intracellular Ca2+ elevation, lamellipodia formation accompanied by dephosphorylated cofilin, and migration were significantly attenuated. Conversely, this study showed in Epac1+/−-ASMCs that stimulation of Epac promoted lamellipodia formation accompanied by dephosphorylated cofilin and
that PDGF-BB–induced lamellipodia formation was inhibited by the silencing of Rap1a. These data suggested that Epac1 promoted cofilin dephosphorylation, and thus promoted lamellipodia formation and subsequent vascular SMC migration. In accordance with these in vitro data, the in vivo study revealed that Epac1 deficiency inhibited vascular injury–induced intimal thickening in which cofilin dephosphorylation was inhibited.

PDGF-BB is a potent stimulator that promotes migration in various cell types and plays a prominent role in neointimal thickening.2,14 It has also been reported that 2 single-nucleotide polymorphisms located in the PDGF-B gene were associated with cardiac allograft vasculopathy, which is a condition of atherosclerotic-like changes in the coronary arteries.26 During the development of intimal thickening, multiple signaling pathways of PDGF-induced migration such as phosphatidylinositol 3-kinase, mitogen-activated protein kinase, and extracellular signal-regulated kinase signaling have been proposed.2 Since the late 1990s, it has been recognized that PDGF-BB activates cytosolic phospholipase A2 and produces prostaglandin E2, resulting in G protein–coupled receptor activation and intracellular cAMP elevation.8,9 Although some reports suggested that PKA, a conventional downstream effector of cAMP, is activated by PDGF-BB stimulation in human arterial SMCs,5 not much attention has been paid to the physiological or pathological roles of PDGF-BB–mediated cAMP downstream signaling pathways. To the best of our knowledge, this is the first study showing the involvement of Epac in PDGF-BB signaling pathways. Accumulating evidence demonstrated the migratory role of Epac in various types of cells including vascular SMCs, tumor cells, fibroblasts, and leukocytes,11,27–31 although some reports showed conflicting results in epithelial cells and prostate carcinoma cells.32,33 Rap1, an immediate effector of Epac, has been shown to contribute to Epac-mediated cell migration.11,27,31,34 In addition, Rap1 has been demonstrated to promote cell migration via regulation of integrin and actin polymerization.35–37 However, downstream molecular mechanisms of Epac-mediated cell migration are beginning to be relatively defined. Baljinnyam et al29 reported Epac1-Ca2+-mediated migration in melanoma cells. Using various inhibitors, the authors demonstrated that Epac1 increased intracellular Ca2+ concentration through PLCε inositol 1,4,5-triphosphate receptor signaling, which promoted actin assembly at the leading edge through Ca2+-binding protein S100A4-induced activation of MHCIIA (non-muscle myosin heavy chain II A).29 In this study, we found that Epac1 activation promoted cofilin dephosphorylation, resulting in lamellipodia formation at the leading edge of SMCs, and thus migration. Cofilin is a protein belonging to the actin depolarizing factor family of proteins, and it mediates lamellipodial extension and polarized cell migration by stimulating actin-filament disassembly at the leading edge of migrating cells.38–40 Phosphorylated cofilin is dephosphorylated by Ca2+-dependent serine–threonine phosphatase calcineurin to be converted to active form.41 Calcineurin-mediated cofilin activation may be a mechanism of Epac1-induced cell migration, although this study did not show direct evidence. Previous reports suggesting that cofilin is activated under PDGF stimulation in vascular SMCs42,43 also support our findings.

The elevation of intracellular Ca2+ is important in cell migration.44 It is well recognized that PLCγ contributes to...
BB−mediated PLC activity is attributed to PLCε using PLCε−deficient fibroblasts revealed that 50% of PDGF-BB−mediated PLC activity is attributed to PLCε. The authors demonstrated that the majority of the PLCε−deficient cells that migrated were biased toward the PDGF gradient, whereas PLCε-deficient cells did not exhibit directional migration. However, the mechanisms of PLCε activation under PDGF stimulation have not been demonstrated. Our data suggested that Epac1 was responsible for 50% of the PDGF-BB−induced intracellular Ca2+ elevation. Epac1 activates PLCε via Rap1 or Rap2 and enhances intracellular Ca2+ release. Although this study did not show direct evidence of the involvement of PLCε, Epac1 may contribute to PDGF-PLCε−induced intracellular Ca2+ elevation. Recent studies suggest that PLCε cooperates with nuclear factor-kB to induce proinflammatory-related mediators, such as CXCL2 and cyclooxygenase 2, which are upregulated at the vascular injury site. In addition to the potential role of PLCε in Epac1−mediated migration, further studies on the roles of PLCε as a downstream signaling effector of PDGF-BB-Epac are required.

As mentioned above, Rap1 contributes to Epac1−mediated cell migration in various cell types. However, a subtype-specific differential effect of Rap1 on cell migration has been reported. A study using Rap1α−deficient mice demonstrated that Rap1α reduced the ability for directed migration of monocytes toward chemokines. Severson et al also demonstrated that Rap1α, but not Rap1b, promoted epithelial cell migration. However, Infante et al indicated that Rap1b, but not Rap1α, inhibited migration in leukemia cells. Although a subtype-specific effect of Rap1 on vascular SMC migration has not been reported, in accordance with these previous reports on other cell types, our data showed that Rap1α was involved in PDGF-BB−induced SMC lamellipodia formation. Another line of study demonstrated that the CDC25 homology domain of PLCε, which exhibits guanine nucleotide exchange factor activity toward Rap1, is critical for the prolonged activation of PLCε under PDGF stimulation. The authors also demonstrated that Rap1α, rather than Rap2α or Rap2b, is likely to be responsible for PDGF−mediated prolonged activation of PLCε signaling. Because Rap1α binds the Ras/Rap1−associating domain of PLCε and induces subsequent activation of PLCε, Rap1α and PLCε seem to synergistically activate each other, thus prolonging the activation of PLCε. These data support our concept that Epac1−Rap1α contributes to PDGF-mediated migration.

Epac acts independently or sometimes together with PKA. Although both PKA and Epac can be activated on cAMP production, the reported roles of PKA and Epac in vascular SMC migration and neointimal thickening opposed one another. The activation of cAMP-PKA signaling has been reported to inhibit vascular SMC migration. However, Epac activation stimulated vascular SMC migration. In accordance with these reports, this study demonstrated that Epac1 deficiency significantly attenuated PDGF-BB−induced migration. Under stimulation of PDGF-BB, cAMP seems to, at least in part, substantially stimulate Epac and promote migration. The opposing roles of PKA and Epac in neointima formation were also demonstrated in in vivo studies. Local administration of cAMP and phosphodiesterase inhibitor drugs to rats markedly inhibited neointima formation after balloon injury in vivo, which was completely reversed by the inhibition of PKA. In contrast, Epac activation promoted intimal thickening in an organ culture of rat arteries. In addition, this study using Epac1−/− mice demonstrated that Epac deficiency inhibited cofilin dephosphorylation in SMCs and neointimal thickening after intramural vascular injury, which mimicked clinical restenosis after percutaneous coronary intervention. Our previous data suggested that the expression of regulatory and catalytic subunits of PKA was downregulated after intramural vascular injury in mice in a time-dependent manner. In contrast, the expression of Epac1, but not of Epac2, was increased after arterial injury. A change in expression of PKA and Epac after vascular injury may lead to a preferential stimulation of Epac rather than of the PKA pathway.

The chimeric mice generated using bone marrow cell transplantation demonstrated that the genetic background of vascular tissues rather than of BMCs affected Epac1−mediated vascular remodeling. Although our data demonstrated the contribution of Epac1 of SMCs in intimal thickening, perivascular fibroblast migration has been indicated to be associated with neointimal thickening. Our data showed that Epac1 deficiency attenuated PDGF-BB−induced migration in perivascular fibroblasts as well as in SMCs, suggesting the potential role of Epac1 in perivascular fibroblasts in intimal thickening in vivo. Further studies are required for understanding the cell type−specific relative contribution in Epac1−induced intimal thickening.

In conclusion, our in vitro and in vivo study data suggested that Epac plays a role in vascular SMC migration via cofilin dephosphorylation under PDGF-BB stimulation and intimal thickening after vascular injury. In addition to PDGF-BB, various G protein−coupled receptor ligands, such as catecholamine, are released at diseased vessels, and inhibition of Epac1 might offer a new pharmacological therapeutic strategy for intimal thickening.

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Disclosures
None.

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Vascular smooth muscle cell migration causes neointima, which is strongly related to the vascular remodeling after mechanical injury and the development of atherosclerosis. To date, therapeutic strategies to obstructive arterial remodeling have not been fully established. Using a primary culture of vascular smooth muscle cells of exchange protein activated by cAMP 1 (Epac1)-deficient mice, we demonstrated that Epac1 contributed to platelet-derived growth factor-BB–induced intracellular Ca2+ elevation, lamellipodia formation accompanied by dephosphorylated cofilin, and smooth muscle cell migration. Our in vivo study further revealed that vascular injury–induced intimal thickening was significantly inhibited in Epac1−/− mice, in which cofilin dephosphorylation was also inhibited. In addition to platelet-derived growth factor-BB, various G protein–coupled receptor ligands, such as catecholamine, are released at diseased vessels. Therefore, the inhibition of Epac1 might offer a new pharmacological therapeutic strategy for intimal thickening.
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SUPPLEMENTAL MATERIAL

Detailed Methods

Reagents
Anti-phosphorylated cofilin antibody (sc-12912-R), anti-Ki67 antibody (sc-7846), and anti-cofilin antibody (sc-8441) were purchased from Santa Cruz Biotechnology (San Diego, CA, USA). Anti-myosin heavy chain isoform SMemb antibody (ab684) was purchased from abcam (Cambridge, UK). Rhodamine-conjugated anti-F-actin antibody (R415), Alexa Fluor 488 anti-Rabbit IgG (A-11008), and Hoechst 33342 (H3570) were purchased from Life Technologies (Carlsbad, CA, USA). Recombinant human PDGF-BB (100-14B) was purchased from PeproTech (Rocky Hill, NJ, USA). 8-pCPT-2-O-Me-cAMP (O8Me-cAMP) (C8988) and poly-L-lysine (P9155) were purchased from Sigma (St. Louis, MO, USA). Ionomycin (407950) was purchased from Calbiochem (San Diego, CA, USA). Buffered formalin (10%) (066-03847) was purchased from Wako (Osaka, Japan). Elastase type II-A (E0127), trypsin inhibitor type I-S (17075-029), bovine serum albumin V (A4919), penicillin-streptomycin solution (P4333), and Dulbecco’s modified Eagle’s medium (DMEM) (D5030) were purchased from Sigma-Aldrich. Collagenase II (4174 X8B10273A) was purchased from Worthington Biochemical (Lakewood, NJ, USA). Collagenase-dispase (10269638001) was purchased from Roche Diagnostics (Tokyo, Japan). Fetal bovine serum (FBS) (F9423) was purchased from Equitech-Bio (Kerrville, TX, USA).

Animals
All animals were cared for in compliance with the guiding principles of the American Physiological Society. The experiments were approved by the Ethical Committee of Animal Experiments at Yokohama City University School of Medicine. Generation of Epac1-deficient mice has been described previously. All mice were littermates from heterozygote crosses. Experiments for comparing results between WT and Epac1 KO were performed on 3–8-month-old homozygous Epac1 KO mice and WT littermates.

Isolation and culture of mouse vascular smooth muscle cells and periaortic fibroblasts
ASMCs and periaortic fibroblasts were isolated from the thoracic aorta of mice using an explant method. Briefly, the mice were euthanized with 100 mg/kg of pentobarbital (DY11441) purchased from Kyoritsu Seiyaku (Tokyo, Japan), the aortas were excised, and the fat and surrounding connective tissue were removed. To remove smooth muscle layers, the aortas were digested by a collagenase enzyme mixture (1.5 mg/ml collagenase-dispase, 0.5 mg/ml elastase
type II-A, 1 mg/ml trypsin inhibitor type I-S, and 1 mg/ml collagenase II) at 37°C for 9 min. The smooth muscle layers were separated from adventitia. The smooth muscle layers and the adventitia were then cut into small sections and plated onto poly-L-lysine-coated dishes. These cells were grown in Dulbecco’s modified essential medium supplemented with 20% fetal bovine serum (FBS) and penicillin-streptomycin and in air supplemented with 5% CO₂ at 37°C. Confluent cells within nine passages were used in the experiments.

**Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)**

Isolation of total RNA, generation of cDNA, and RT-PCR analysis were carried out as described previously². The primers were designed based on mouse nucleotide sequences of mouse PDGF receptor β (PDGFRβ) (NM_008809.2) (5’-TTGCCTTACGACTCCACCT -3’ and 5’-CAGCATCTTGACAGCCACTT-3’), RAS-related protein (Rap) 1a (NM_00116356.1) (5’-CATCATGCCTGAGTACAAGCTA-3’ and 5’-TTGCTGTAAATTGCTCGGTTC-3’), Rap1b (NM_024457.2) (5’-GCTTGAAATCTTGGACACTGC-3’ and 5’-GGTTCTGACCTTGACCGCCACTGC-3’), and myosin heavy chain isoform SMemb (NM_001256012) (5’-AACAGTCTTCAGGAGCAGCAG-3’ and 5’-GGCGGTTCTTGGTCTTCTCTC-3’). Each primer set was designed between multiple exons, and PCR products were confirmed by sequencing. The abundance of each gene was determined relative to the 18S transcript.

**Measurement of intracellular cAMP concentration**

After ASMCs were cultured on 24-well plates with 20%FBS/DMEM, ASMCs were serum-starved for 48 h. ASMCs were then incubated with 0.2 mmol/L of 3-Isobutyl-1-methylxanthine (IBMX, 100 µM) for 20 min, followed by incubation with or without PDGF-BB (10 ng/mL) for 30 min. The reaction was terminated by aspirating the supernatant. Following the instructions of the manufacturer, ASMCs were lysed with 120 µl of 0.25% solution of dodecyltrimethylammonium bromide, and 100 µl of the lysate was used for the measurement of cAMP using an enzyme-linked immunosorbent assay (ELISA) (RPN225, GE Healthcare Life Sciences, Piscataway, NJ, USA). Following the instructions of the manufacturer, the data was obtained from ELISA.

**Cell migration**

ASMCs or periaortic fibroblasts on four-well glass slides were maintained in DMEM containing 20% FBS, and serum-starved for 48 h. ASMCs were placed in the temperature control incubator (5% CO₂ at 37°C), and then treated with or without 10 ng/mL of PDGF-BB for 8 h. Cell migration was evaluated through measurement of the path length of ASMCs via time-lapse microscopy.
(TE2000 Eclipse, Nikon, Tokyo, Japan). To track migration paths, images were recorded at 20-min intervals, and the nucleus of each cell was manually traced for each frame.

**Measurement of intracellular Ca\(^{2+}\) Concentration**
Measurement of intracellular Ca\(^{2+}\) Concentration was performed as previously described\(^3\) with some modifications. ASMCs were loaded with fura-2/AM (F015, Dojindo, Kumamoto, Japan) in a Tyrode solution (137 mM NaCl, 2.7 mM KCl, 1.4 mM CaCl\(_2\), 5.6 mM glucose, 0.5 mM MgCl\(_2\), 0.3 mM NaH\(_2\)PO\(_4\), 12 mM NaHCO\(_3\), pH 7.4). The ASMCs on a 96-well microplate (235304, Nunc, Roskilde, Denmark) were incubated with 5 µM of fura-2/AM for 40 min at 37°C. After the dye loading, the loading buffer was removed, and the cells were washed twice with loading buffer. Intracellular Ca\(^{2+}\) concentration was detected using the ARVOTMMX fluorescence microplate reader (PerkinElmer Life Sciences, Boston, MA, USA). Fura-2/AM was excited at 340 and 390 nm with fluorescence emission detected using a 510-nm band pass filter. Intracellular Ca\(^{2+}\) concentration was evaluated using the observed fluorescence ratio 340/390 nm.

**SMC proliferation assay**
ASMC proliferation was measured by a BrdU colorimetric cell proliferation ELISA kit (1-647-229, Roche Diagnostics, Basel, Switzerland). ASMCs were plated into a 96-well culture plate in DMEM containing 20% FBS at an initial density of 4 × 10\(^3\) cells/well and cultured for 48 h. After 24-h serum starvation, the medium was replaced with DMEM containing 10% FBS or serum-free DMEM, followed by 24-h incubation with BrdU (10 µM). Incorporation of BrdU was quantified according to the manufacturer’s instructions.

**PKA activity assay**
Quantification of PKA activity was performed as previously described\(^4\). Briefly, after ASMCs were cultured on six-well plates with 20% FBS/DMEM, ASMCs were serum-starved for 48 h. ASMCs were then incubated with 100 ng/mL PDGF-BB for 15 min. Cells were then scraped into lysis buffer (20 mM Mops, 50 mM β-glycerol phosphate, 50 mM NaF, 1 mM NaVO\(_4\), 5 mM EGTA, 2 mM EDTA, 1% Nonidet P-40, 1 mM DTT, and protease inhibitor mixture from Sigma), incubated for 10 min, and then sonicated three times at 20-s intervals. Homogenates were centrifuged at 15,000 × g for 15 min. PKA activity was measured in the resulting supernatant using the PKA activity assay kit (Enzo life sciences, Farmingdale, NY, USA), following the manufacturer’s instructions.

**Immunohistochemistry**
Immunohistochemical analysis was performed as previously described. Paraffin-embedded sections containing the aorta subjected to organ culture were stained. Slides were incubated with primary antibody overnight at 4°C. The slides were washed three times with PBS for 5 min and sequentially incubated with secondary antibodies for 30 min. After three washes with PBS, the slides were incubated with streptavidin peroxidase at room temperature for 30 min followed by incubation with DAB chromogen substrate solution (Nichirei, Tokyo, Japan) to detect Ki-67 or SMemb proteins.

**Immunocytochemistry and evaluation of lamellipodia formation**

Immunocytochemical analysis was performed as previously described. ASMCs cultured on 12-mm glass coverslips were serum starved for 48 h and then stimulated for 30 min in media alone (control), or PDGF-BB (10 ng/mL). Cells were then fixed in 10% buffered formalin for 10 min, washed twice with PBS, and permeabilized in 0.3% Triton X-100 and PBS for 10 min. ASMCs were washed twice with Tween 20 (0.1%)/PBS and incubated with 1% BSA/Tween 20 (0.1%)/PBS for 20 min, and then incubated with anti-phosphorylated cofilin and rhodamine-conjugated anti-F-actin antibodies for 24 h at 4°C. After three washes with Tween 20/PBS, cells were incubated with a secondary antibody, Alexa Fluor 488 anti-rabbit IgG, for 1 h. After six washes with Tween 20/PBS, DNA was stained with Hoechst 33342 solution. After two washes with PBS, coverslips were mounted for microscopic imaging. All incubation and wash steps were conducted at room temperature. Lamellipodia formation was evaluated morphologically using immunocytochemistry. ASMCs showing lamellipodial formation accompanied by dephosphorylated cofilin was counted as positive. Lamellipodia-positive cell rate was calculated as the ratio of the positive cell number to the total cell number. Morphometric analyses were performed using Nikon TE2000-E (Tokyo, Japan).

**Immunoblot Analysis**

Proteins from whole cells were analyzed by immunoblotting as described previously. ASMCs were prepared in a cell lysis buffer (150mM Na$_2$CO$_3$ [pH 11.0], 1 mM EDTA) and homogenized by sonication. Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane by electroblotting. Membranes were blocked in 1% Tween/3% BSA/PBS and incubated with primary antibody overnight at 4°C. Specific binding was visualized by the Super Signal Western Lightning ECL Pro (NEL122001EAPerkinElmer, Waltham, MA, USA).

**RNA Interference**
Rap1-targeted siRNAs (siGENOME SMARTpools; M-057058–01) and scrambled siRNA pool were purchased from Dharmacon (Lafayette, CO, USA). Epac1+/− and Epac1−/−-ASMCs were transfected with siRNAs (2 pmol/well of six-well plate) using the RNAiMAX transfection reagent (Invitrogen) as previously described4. Forty-eight hours after transfection, the cells were subjected to each assay for Figure 4. For Supplemental Figure III, Rap1a-targeted siRNAs (M-057058-01) and negative siRNA (SI00237097, SI00222565) were purchased from QIAGEN (Hilden, Germany). ASMCs were transfected with siRNA (500 pmol/well of six-well plate) using the RNAiMAX transfection reagent (Invitrogen). Forty-eight hours after transfection, the cells were subjected to each assay.

**Femoral artery injury model**

Surgery was carried out using a dissecting microscope SMZ-800 (Nikon, Tokyo, Japan). Transluminal mechanical injury of the femoral artery was induced by the insertion of a large wire (0.38 mm in diameter, C-SF-15-15, Cook, Bloomington, IN, USA) as previously described5,7. Mice were euthanized by an intraperitoneal administration with 100 mg/kg of pentobarbital at four weeks after injury. After systemic perfusion with 0.9% NaCl solution, arteries were harvested two weeks after injury. Femoral arteries were fixed in 4% paraformaldehyde overnight at 4°C, and embedded in paraffin for elastica van Gieson staining. The neointima area, the area of the medial layer, the ratio of the neointima area to the medial layer, the thickness of the medial layer, the length of the external elastic laminae, and the area of the internal lumen were measured on digitized images using image-analysis software (Image-Pro Plus version 4.5, Media Cybernetics, San Diego, CA, USA).

**Bone marrow-derived cell (BMC) transplantation**

BMCs were harvested from femora and tibia. After γ-irradiation of 9.5 Gy of 10–13-week-old male recipient mice, 1 x 10⁶ BMCs from Epac1+/− mice were suspended in 0.4 mL RPMI1640 (Life Technologies, Carlsbad, CA) / 10%FBS and injected intravenously by tail vein puncture into Epac1−/− mice and vice versa, as well as from Epac1−/− into Epac1+/− mice and from Epac1+/− into Epac1−/− mice as sham controls. Four weeks after transplantation, peripheral blood samples were collected from the tail vein, and red blood cells were lysed with ACK buffer (NH₄Cl: 0.15 M; KHCO₃: 0.01 M; EDTA·2Na: 0.1 mM). White blood cells were washed with PBS containing 0.5% bovine serum albumin and 2 mM EDTA and subjected to flow cytometry. CD11b-positive cells were gated and analyzed CD45.1 (Ly5.1) which was expressed only by Epac1+/− mice, and CD45.2 (Ly5.2) which was expressed only by Epac1−/− mice. Blood cell populations were counted according to their FSC and SSC properties. Four-color immunofluorescence staining was
performed using anti-CD11b antibodies conjugated with BV421, anti-CD45.1 antibodies conjugated with APC-Cy7, anti-CD45.2 antibodies conjugated with FITC, and anti-CD115 antibodies conjugated with APC. All antibodies were purchased from BioLegend (San Diego, CA). Four weeks after BMT, wire-induced femoral artery injury was performed as described above. Four weeks after BMT, the femoral arteries were fixed in 4% paraformaldehyde and analyzed.

**Invasion assay**

Invasion assay was performed using 24-well Transwell culture inserts with polycarbonate membranes containing 8 µm pores (3422 Corning, NY, USA). The membrane was coated with 20 µL of the 0.76 mg/mL Matrigel solution (356230, Corning) and polymerized at 37°C for 30 min. ASMCs in serum-free DMEM were plated at a density of 8 × 10^4 cells/100 µL per each insert. The lower chambers were filled with 600 µL serum-free DMEM for 1 h as a preincubation. ASMCs were then stimulated with or without 10 ng/mL PDGF-BB, which was administered into the lower chambers for 14 h at 37°C at 5% CO₂. At the end of the experiment, cells were fixed in 10% buffered formalin (Wako Pure Chemical Industries, Osaka, Japan). After cells on the upper surface of the membrane had been mechanically removed with a cotton swab, invaded cells on the lower surface of the membrane were stained with Cyto Quick (Muto Pure Chemicals, Tokyo, Japan) and then manually counted from three different fields (0.5 mm²/field) in a microscope using Image J software. We performed at least three independent experiments.

**Statistical analysis**

Data are expressed as means ± SEM. Statistical analysis was performed using the unpaired Student’s t-test in the comparison of two groups, such as the data in Supplemental Figures IIIA-B and VA. A one-way ANOVA followed by Bonferroni’s multiple comparison test was applied to Figure 5B and 6C. A two-way ANOVA followed by Bonferroni’s multiple comparison test was used for Figures 1B, 2B, 3B, 3D, 4B, and 4D and Supplemental Figures IIB, IID, IIDD, IVA, and VIB. A value of p<0.05 was considered significant.
Supplemental Figure I

Supplemental Figure I. The effect of Epac1 deficiency on cell invasion
A. Quantification of ASMCs that were invaded across Matrigel. n=6. *p<0.05; ***p<0.001; NS, not significant.
Supplemental Figure II

A. Representative images of lamellipodia formation in Epac1+/+ and Epac1−/− ASMC. p-cofilin (green; upper panels) and F-actin (red; middle panels) were visualized by immunofluorescent staining. ASMCs were incubated with or without 10 ng/mL of PDGF-BB for 30 min. Merged images of p-cofilin and F-actin staining are shown in the lower panels. Arrows indicate lamellipodia formation accompanied by dephosphorylated cofilin. The insets are the images of lamellipodia magnified two additional times. Scale bars: 30 μm.

B. Quantification of the number of ASMCs that exhibited lamellipodia accompanied by dephosphorylated cofilin. Lamellipodia-positive cell rates are presented as the percent of total cell number. The data were obtained from more than five independent experiments. n=353 (control Epac1+/+ASMCs), 227 (PDGF-BB Epac1+/+ASMCs), 195 (control Epac1−/−ASMCs), and 487 (PDGF-BB Epac1−/−ASMCs).

C. Representative images of western blotting showing protein expression of p-cofilin. ASMCs were incubated with or without 10 ng/mL of PDGF-BB for 30 min.

D. Quantification of C. n=4-6. **p<0.01; ***p<0.001; NS, not significant.
Supplemental Figure III

A and B. The mRNA expression determined by quantitative RT-PCR of Rap1a and Rap1b, respectively, in Epac1+/−-ASMCs transfected Rap1a-targeted siRNAs. Data were normalized to 18s RNA level. n=4-8.

C. Representative images of PDGF-BB-induced (10 ng/mL, 30 min) lamellipodia formation in Epac1+/−-ASMCs transfected with negative siRNA or Rap1a-targeted siRNAs. F-actin (red) and p-cofilin (green) were visualized by immunofluorescent staining. Marged images of p-cofilin and F-actin staining are shown in the lower panels. Arrows indicate lamellipodia formation accompanied by dephosphorylated cofilin. The insets are the images of edge of cell or lamellipodia magnified two additional times. Scale bars: 30 μm.

D. Quantification of the number of Epac1+/−-ASMCs that exhibited PDGF-BB-induced lamellipodia accompanied by dephosphorylated cofilin. Lamellipodia-positive cell rates are presented as the percent of total cell number. The data were obtained from more than four independent experiments.

n=244 (control Epac1+/−-ASMCs), 153 (PDGF-BB Epac1+/−-ASMCs), 218 (control Epac1−/−-ASMCs), and 468 (PDGF-BB Epac1−/−-ASMCs). **p< 0.01; ***p<0.001; NS, not significant.
Supplemental Figure IV

A. Proliferation induced by FBS were measured by BrdU incorporation using ELISA in Epac1\textsuperscript{+/+}-ASMCs and Epac1\textsuperscript{-/-}-ASMCs. \textit{n}=10. **, \textit{p}<0.01; ***, \textit{p}<0.001; NS, not significant.

B. Representative image of injured femoral arteries stained with an anti-Ki67 antibody. Dotted lines indicate internal elastic laminae. Scale bars: 50 \textmu m.

Supplemental Figure IV. The effect of Epac1-deficiency on proliferation in ASMCs
Supplemental Figure V. The effect of Epac1-deficiency on cell differentiation in ASMCs

A. The mRNA expression of SMemb in Epac1+/+ ASMCs and Epac1−/− ASMCs determined by quantitative RT-PCR. Data were normalized against 18sRNA levels. n=9-10. NS, not significant.

B. Representative images of injured femoral arteries stained with an anti-SMemb antibody. Dotted lines indicate internal elastic lamina. Scale bars: 50 µm.
Supplemental Figure VI. The effect of Epac1 deficiency on PDGF-BB-induced fibroblast migration

A. Analyses of fibroblast migration under stimulation with or without PDGF-BB (10 ng/ml) for 8 h. Trajectories of fibroblast migration from origin (center of the graphs) to the end point are shown.

B. Quantification of total path length of fibroblasts. n=32 (control Epac1+/+), 29 (PDGF-BB Epac1+/+), 40 (control Epac1−/−), and 29 (PDGF-BB Epac1−/−). ***, p<0.001; NS, not significant.
Supplemental References


Legends for the Video files

Time-lapse movies monitored ASMC migration for 8 h.

**Supplemental movie I:** WT-ASMC without stimulation.

**Supplemental movie II:** Epac1KO-ASMC without stimulation.

**Supplemental movie III:** WT-ASMC migration under stimulation with 10 ng/mL of PDGF-BB.

**Supplemental movie IV:** Epac1KO-ASMC migration under stimulation with 10 ng/mL of PDGF-BB.
Supplemental Figure I. The effect of Epac1 deficiency on cell invasion

A. Quantification of ASMCs that were invaded across Matrigel-coated filters. n=6. *p<0.05; ***p<0.001; NS, not significant.
Supplemental Figure II. The effects of Epac1 deficiency on lamellipodia formation

A. Representative images of lamellipodia formation in Epac1+/+- and Epac1-/--ASMC. p-cofilin (green; upper panels) and F-actin (red; middle panels) were visualized by immunofluorescent staining. ASMCs were incubated with or without 10 ng/mL of PDGF-BB for 30 min. Merged images of p-cofilin and F-actin staining are shown in the lower panels. Arrows indicate lamellipodia formation accompanied by dephosphorylated cofilin. The insets are the images of lamellipodia magnified two additional times. Scale bars: 30 µm.

B. Quantification of the number of ASMCs that exhibited lamellipodia accompanied by dephosphorylated cofilin. Lamellipodia-positive cell rates are presented as the percent of total cell number. The data were obtained from more than five independent experiments. n=353 (control Epac1+/+-ASMCs), 227 (PDGF-BB Epac1+/+-ASMCs), 195 (control Epac1-/--ASMCs), and 487 (PDGF-BB Epac1-/--ASMCs).

C. Representative images of western blotting showing protein expression of p-cofilin. ASMCs were incubated with or without 10 ng/mL of PDGF-BB for 30 min.

D. Quantification of C. n=4-6. **p<0.01; ***p<0.001; NS, not significant.
Supplemental Figure III. Involvement of Rap1 isoforms in lamellipodia formation in ASMCs

A and B. The mRNA expression determined by quantitative RT-PCR of Rap1a and Rap1b, respectively, in Epac1+/+-ASMCs transfected Rap1a-targeted siRNAs. Data were normalized to 18s RNA level. n=4-8.

C. Representative images of PDGF-BB-induced (10 ng/mL, 30 min) lamellipodia formation in Epac1+/+-ASMCs transfected with negative siRNA or Rap1a-targeted siRNAs. F-actin (red) and p-cofilin (green) were visualized by immunofluorescent staining. Merged images of p-cofilin and F-actin staining are shown in the lower panels. Arrows indicate lamellipodia formation accompanied by dephosphorylated cofilin. The insets are the images of edge of cell or lamellipodia magnified two additional times. Scale bars: 30 μm.

D. Quantification of the number of Epac1+/+-ASMCs that exhibited PDGF-BB-induced lamellipodia accompanied by dephosphorylated cofilin. Lamellipodia-positive cell rates are presented as the percent of total cell number. The data were obtained from more than four independent experiments. n=244 (control negative siRNA), 153 (PDGF-BB negative siRNA Epac1+/+-ASMCs), 218 (control Rap1-targeted siRNA), and 468 (PDGF-BB Rap1-targeted siRNA).

**p<0.01; ***p<0.001; NS, not significant.
Supplemental Figure IV. The effect of Epac1-deficiency on proliferation in ASMCs

A. Proliferation induced by fetal bovine serum (FBS) were measured by BrdU incorporation using ELISA in Epac1+/+-ASMCs and Epac1-/--ASMCs. n=10. **p<0.01; ***p<0.001; NS, not significant.

B. Representative image of injured femoral arteries stained with an anti-Ki67 antibody. Dotted lines indicate internal elastic laminae. Scale bars: 50 μm.
Supplemental Figure V. The effect of Epac1 deficiency on SMC differentiation

A. The mRNA expression of SMemb in Epac1+/+ ASMCs and Epac1−/− ASMCs determined by quantitative RT-PCR. Data were normalized against 18sRNA levels. n=9-10. NS, not significant.

B. Representative images of injured femoral arteries stained with an anti-SMemb antibody. Dotted lines indicate internal elastic laminae. Scale bars: 100 µm.
Supplemental Figure VI. The effect of Epac1 deficiency on PDGF-BB-induced perivascular fibroblast migration

A. Analyses of fibroblast migration under stimulation with or without PDGF-BB (10 ng/mL) for 8 h. Trajectories of fibroblast migration from origin (center of the graphs) to the end point are shown.

B. Quantification of total path length of fibroblasts. n=32 (control Epac1+/+), 29 (PDGF-BB Epac1+/+), 40 (control Epac1−/−), and 29 (PDGF-BB Epac1−/−). ***p<0.001; NS, not significant.
Legends for the Video files

Time-lapse movies monitored ASMC migration for 8 h.

**Supplemental movie I:** Epac1\(^{+/−}\)-ASMC without stimulation.

**Supplemental movie II:** Epac1\(^{−/−}\)-ASMC without stimulation.

**Supplemental movie III:** Epac1\(^{+/−}\)-ASMC migration under stimulation with 10 ng/mL of PDGF-BB.

**Supplemental movie IV:** Epac1\(^{−/−}\)-ASMC migration under stimulation with 10 ng/mL of PDGF-BB.