Function of Ahnak protein in aortic smooth muscle cell migration through Rac activation

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Aims
Ahnak protein acts as a scaffold protein networking phospholipase C-γ and protein kinase C-α, which subsequently stimulate an extracellular signal-regulated kinase (Erk) pathway. In mouse aortic smooth muscle cells (ASMCs), the activation of the signalling cascade ultimately promotes the cell migration through an unknown mechanism. We aimed to dissect the Ahnak-mediated cell signalling network involved in the migration of ASMCs.

Methods and results
Migration of ASMCs from wild-type mice was significantly increased by platelet-derived growth factor (PDGF) stimulation in transwell chamber and wound-healing assays, whereas migration of ASMCs from Ahnak knockout mice was reduced. Consistently, stimulation of wild-type ASMCs with PDGF resulted in Rac activation-mediated lamellipodial protrusion in migrating cells. In contrast, Ahnak knockout ASMCs displayed lower activation of Rac in response to PDGF and slow lamellipodial protrusion rate and cell migration. Ahnak signalling complex was analysed by immunoprecipitation with antibody to p21-activated protein kinase (PAK). Ahnak protein was shown to function as the signalling scaffold interacting with the multiple protein complex of Erk, PAK, and p21-activated kinase-interacting exchange factor β. The proposed role of Ahnak in cell migration was examined using a restenosis model in which the carotid arteries of mice were subjected to post-ligation injury. We show neointimal formation and SMC migration after ligation injury in Ahnak knockout mice were significantly retarded compared with wild-type mice.

Conclusion
Ahnak protein plays an important scaffolding function connecting Erk and Rac activation in PDGF-dependent migration of ASMC.

Keywords
Ahnak • Smooth muscle cells • Migration • Rac • Restenosis

1. Introduction
Ahnak is a large (700 kDa) protein that was initially identified in human neuroblastomas and skin epithelial cells.1,2 It has been implicated in diverse essential cellular processes such as regulation of calcium signalling,2,3 plasma membrane support,4,5 regulated exocytosis,6 and T cell differentiation.7 Ahnak protein can be divided into three regions based on the protein structure: the amino-terminal consisting of 251 amino acids, the large central region of ≈4300 amino acids made up of 36 repeat units, and the carboxyl-terminal domain of 1002 amino acids. Several lines of recent evidence indicate that the C-terminal region plays an important role in the formation of cytoskeletal structure, calcium homeostasis, and muscle regeneration.8–10 In epithelial cells, Ahnak is distributed mainly on the cell membrane, suggesting a role in cell–cell adhesion. In non-epithelial cells, Ahnak is expressed in the nucleus and cytoplasm indicating diverse and cell-type-specific functions. Although several putative interacting proteins have been identified, the exact biological function of Ahnak is unknown.

Cell migration is an important process in a number of physiological responses including leucocyte trafficking and remodelling of the vasculature.11 Vascular smooth muscle cell (SMC) proliferation and migration are major underlying events in the development and progression of various forms of cardiovascular diseases, including lesion progression of atherosclerosis, post-angioplasty restenosis, transplant arteriopathy, and pulmonary hypertension.12 Several growth factors, such as platelet-derived growth factor-BB (PDGF-BB) and insulin-like growth factor-I, play crucial roles in vascular SMC migration.13 A variety of
intracellular signalling molecules have been implicated in cell migration, including members of Erk cascades, lipid kinases, phospholipases, Ser/Thr and Tyr kinases, and scaffold proteins. The Erk pathway is involved in the rapid responses to PDGF in several cell types, and the activation of this pathway is critical to the progression of vascular SMC proliferation and migration in response to vascular injuries or diseases.15,16

The Erk pathway can regulate the activity of the small GTPases Rac and RhoA and thus contribute to tumour cell motility and invasion.17 The GTPases Rac1, RhoA, and Cdc42 act together to control cytoskeleton dynamics in migrating cells. Cdc42 regulates filopodia formation, Rac regulates the formation of focal contacts and lamellipodia formation, and RhoA induces the formation of stress fibres and focal adhesions.18,19 The RhoA family of GTPases has been implicated for roles in almost every aspect of vascular biology, regulating behaviours of endothelial cells, SMCs, leucocytes, and platelets and influencing such diverse processes as hypertension, inflammation and wound healing, and neointimal formation, and thrombosis.20

Aortic smooth muscle cells (ASMCs) contain high levels of Ahnak protein compared with other cell lines and tissues.21 Since the Erk cascade regulates cell migration, we hypothesized that Ahnak protein is involved in SMC migration. In this report, we used aortic SMCs prepared from Ahnak knockout mice and investigated the role of Ahnak in SMC migration. We show that Erk and Rac activation in response to PDGF was impaired in Ahnak knockout aortic SMCs, resulting in slow lamellipodial protrusion rate and cell migration. The study provides experimental evidences for the physiological function of Ahnak protein in SMCs which play key roles in vascular remodelling.

2. Methods
For a detailed description, see also the expanded materials and methods section in the Supplementary material online.

2.1 Animals
Animal study protocols conformed to the Institutional Animal Care and Use Committee (IACUC) of Ewha Womans University and Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011). Ahnak knockout mice were generated and genotyped as described previously.22

2.2 Preparation of murine aortic SMCs
Murine aortic SMCs were isolated from the thoracic aorta of 8-week-old male wild-type and Ahnak knockout mice. Each mouse was sacrificed by carbon dioxide (CO2) inhalation. The midline of the abdomen was incised and the thorax opened to expose the heart and lungs, and then slowly perfused with 1 mL of Heparin (Sigma; 1000 U/mL) into the left ventricle of the heart. The adventitia and connective tissue that surround the aorta were carefully removed with fine forceps. The aorta was cut into small pieces and digested with the aforementioned collagenase type I (Worthington; 1.013 mg/mL, dissolved in serum-deprived Dulbecco’s modified Eagle’s medium (DMEM)). After incubation for 30 min at 37°C, the aorta was cut into small pieces and digested with the aforementioned collagenase type I solution. After incubation for 90 min at 37°C, the aorta was centrifuged at 2000 rpm (Costar Corning) coated with 10 mg/mL fibronectin. The cells were plated at a density of 8 × 10^5 cells/well in six-well plates. After the cells had reached 90% confluence, the cells were deprived of serum for 16 h and then the cells were incubated with mitomycin C (10 μg/mL, dissolved in culture medium), a potent inhibitor of cell proliferation, for 2 h. After this incubation, cells were wounded with 200 μL pipette tips and the starting point was marked with a marker pen at the bottom of the plate. The medium was replaced with or without serum-deprived medium-containing PDGF (50 ng/mL) and the cells were incubated for 0, 6, 12, and 24 h. Photos Images were captured using an Axiovert 40C inverted microscope (Carl Zeiss) equipped with a Powershot A640 digital camera (Canon).

2.3 Cell cultures
Murine aortic SMCs and COS7 cells were cultured at 37°C in an atmosphere of 5% CO2 in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic-antimycotic solution (GIBCO). NIH3T3 cells were cultured in DMEM supplemented with 10% calf serum. Experiments were performed using aortic SMCs between passages 4 and 8.

2.4 Migration assay
SMCs (8 × 10^5 cells) were seeded into upper chamber of a Transwell filter with 8 μm pores (Costar Corning) coated with 10 μg/mL fibronectin. The cells were deprived of serum for 16 h, and stimulating medium-containing PDGF (50 ng/mL) was added to the lower chamber. Transwell chambers were incubated at 37°C for 16 h. After incubation, cells on the underside of the filter were stained with haematoxylin and eosin. Non-migrating cells on the upper side of the filter were removed with cotton swabs.

2.5 Wound-healing assay
SMCs were plated at a density of 8 × 10^5 cells/well in six-well plates. After the cells had reached 90% confluence, the cells were deprived of serum for 16 h and then the cells were incubated with mitomycin C (10 μg/mL, dissolved in culture medium), a potent inhibitor of cell proliferation, for 2 h. After this incubation, cells were wounded with 200 μL pipette tips and the starting point was marked with a marker pen at the bottom of the plate. The medium was replaced with or without serum-deprived medium-containing PDGF (50 ng/mL) and the cells were incubated for 0, 6, 12, and 24 h. Photos Images were captured using an Axiovert 40C inverted microscope (Carl Zeiss) equipped with a Powershot A640 digital camera (Canon).

2.6 Preparation of recombinant PAK GST fusion protein and Rac activity assay
Rac activity was assessed by affinity isolation of GTP-bound Rac using binding domains of p21-activated protein kinase (PAK). Glutathione-S-transferase (GST) fusion construct was generated by inserting CDNA fragments of PAK-RBD into pGEX-4T1 vector plasmid. The resulting plasmid was introduced into Escherichia coli, and the cells were cultured at 30°C for 3 h. Expression of the fusion proteins was induced with 1 mM isopropyl-b-D-thiogalactopyranoside, and the cells were subsequently collected by centrifugation at 1900 × g for 15 min and lysed by sonication in 10-fold (weight/volume) PBS containing 1% Triton X-100 and protease inhibitors (1 μg/mL aprotinin, 1 μg/mL leupeptin, and 0.5 mM AEBSF). After centrifugation for 30 min at 20000 × g, the resulting supernatant was incubated with Glutathione-Sepharose 4B (Amersham Pharmacia Biotech.) overnight at 4°C. The mixture was centrifuged for 1 min at 250 × g, and the isolated pellet was washed with PBS containing 1% Triton X-100. For the Rac activity assay, the bead-conjugated GST–PAK–RBD was incubated for 1 h at 4°C in lysis buffer with lysates of wild-type and Ahnak knockout SMCs. The beads were then separated by centrifugation, washed three times and subjected to immunoblot analysis with monoclonal antibodies to Rac1 (Upstate Biotechnology).

2.7 Fluorescence resonance energy transfer analysis
SMCs were plated at a density of 2 × 10^4 cells/well in 12-well plates on poly-l-lysine-coated coverslips. The cells were transfected with 1 μg of pRaichu-Rac using Lipofectamine (Invitrogen) according to the manufacturer’s protocol and maintained in the complete medium for 24 h. After serum starvation for 16 h, cells were stimulated with or without 50 ng/mL PDGF for 10 min, and fixed for fluorescence resonance energy transfer (FRET) analysis.23,24 FRET images were obtained using a LSM510 Meta confocal microscope (Carl Zeiss). FRET analysis by the acceptor bleaching method and calculation of FRET efficiency were performed as described previously.25,26
2.8 Time-lapse live cell imaging
SMCs infected with adenovirus containing green fluorescent protein (GFP) were plated at a density of $2 \times 10^4$ cells/well on poly-L-lysine-coated coverslips positioned in wells of 12-well plates, cultured in the serum-containing medium for 24 h, and then deprived of serum with DMEM containing 1% FBS for 16 h, and then stimulated with 50 ng/mL PDGF. Differential interference contrast and fluorescence images were recorded in intervals of 1 min. Fluorescence signals were visualized by a LSM510 Meta confocal microscope (Carl Zeiss).

2.9 Carotid artery ligation
Five male 8-week-old wild-type and four Ahnak knockout mice weighing 20–25 g were used in all experiments. A transluminal wire injury to the left common carotid artery was performed as described. The animals were anesthetized (isoflurane inhalation) and the left common carotid artery was dissected and ligated near the carotid bifurcation. Two weeks after the injury, the common carotid arteries were excised after transcardiac perfusion-fixation with heparinized saline containing 3.7% formaldehyde and paraffin embedded. Five serial tissue sections (100 μm interval and 3 μm thickness) were obtained from the middle area of common carotid arteries, and stained with haematoxylin and eosin. Cross-sectional areas of medial and neointimal layers were measured and analysed using a computerized imaging system.

2.10 Statistics
Statistical analysis was performed with a two-tailed unpaired t-test. A P-value <0.05 were accepted as statistically significant. Data are expressed as means ± standard deviations (SD) of values from three to five independent experiments. All western blots in figures were representative of three independent experiments.

3. Results
3.1 Ahnak enhances PDGF-induced migration of SMCs
Previously, we reported that Ahnak can act as a key molecule in PDGF-mediated cell signal by networking PLCγ, PKC, Raf, and Erk

![Figure 1](image-url)
into a signalling cascade.\textsuperscript{22,28} It is well known that PDGF serves as a growth factor in cell proliferation as well as a potent chemoattractant for cell motility leading to SMC migration from the media layer to the intima layer.\textsuperscript{12} Moreover, the Erk signalling pathway is activated in response to PDGF in SMCs and mediate cell migration and consequent-ly vascular remodelling.\textsuperscript{15,16} Although the Ahnak protein has been identified in neuronal and skin fibroblast cells, we noticed that Ahnak was also highly expressed in vascular SMCs (Supplementary material online, Figure S1).

To examine the function of Ahnak in vascular remodelling, we isolated primary SMCs from the aorta of wild-type or Ahnak knockout mice. The identity of SMCs was confirmed using antibody to SMA (data not shown). Because Ahank-dependent PKC-Raf-Erk activation is essential for cell proliferation, we first examined whether Ahnak promoted cell proliferation of aortic SMCs. Stimulation of Ahnak knockout aortic SMCs with PDGF resulted in significantly decreased cell proliferation compared with that of aortic SMCs from wild-type mice (Supplementary material online, Figure S2). This was in the absence of significant cell death in either of the cell populations based on examination of nuclear integrity (data not shown).

It has been well established that cell migration is critical event in vascular remodelling. To investigate whether Ahnak-mediated PDGF-induced migration in SMCs, SMC populations from the wild-type and Ahnak knockout mice were used in the transwell migration assay. Migration of wild-type aortic SMCs in response to PDGF was increased, whereas aortic SMCs from Ahnak knockout mice failed to show such response (Figure 1A). Previously, we demonstrated that four central repeated units (4CRUs) of Ahnak protein play an essential role in the Raf-MEK-Erk pathway in response to PDGF. Thus, we performed an add-back experiment of four CRUs of Ahnak into Ahnak knockout SMCs. Add-back expression of 4CRUs of Ahnak protein resulted in restored PDGF-dependent migration (Figure 1A and B), demonstrating that the Ahnak is critical for SMC migration in response to PDGF. To confirm the effect of Ahnak on the SMC migration, we also performed a wound-healing assay. Aortic SMCs from wild-type and Ahnak knockout mice were grown to the same density. To exclude the effect of Ahnak-dependent proliferation on a wound-healing assay, we pre-treated the cells with mitomycin for 2 h before ‘wounding’ the cell monolayer with a pipette tip. Wild-type aortic SMCs (number of migrated cells for 24 h in response to PDGF stimulation: 79.7 ± 8.4) exhibited cell reorientation responses along the wounded edge margin and migrated into the wounded area in response to PDGF, whereas aortic SMCs from Ahnak knockout mice (number of migrated cells for 24 h in response to PDGF stimulation: 38.7 ± 6.5) showed only limited cell reorientation responses along the wounded edge margin and did not efficiently repopulate the

Figure 2 Ahnak mediates the activation of the Erk pathway in SMC by PDGF. (A) Effect of Ahnak on Erk phosphorylation. SMCs were incubated without or with PDGF for indicated time and then subjected to western blot analysis with anti-phospho-Erk1/2 and actin (left panel). Add-back expression of adenovirus containing GFP-4CRUs was confirmed with polyclonal antibody to Ahnak (right panel). (B) NIH3T3 cells were stimulated without or with 100 ng/mL of PDGF for 10 min. GST and GST-4CRUs of Ahnak fusion proteins were incubated with NIH3T3 cell lysates. The interaction between Ahnak and Erk was determined by western blot using anti-Erk (upper panel). TCL means total cell lysates. (C and D) SMCs were stimulated without or with PDGF for the indicated time. Cell lysates were then subjected to western blot analysis with antibodies to PDGFR, actin (C) phospho-Akt, and Akt (D).
wound space (Figure 1C and D). These results indicate that the Ahnak protein is essential for aortic SMC migration and wound healing.

3.2 Ahnak interacts and activates the Erk

Cell migration is known to be mediated by PLCγ, PI3K, Rac, and Erk in response to growth factors. In addition, we have previously reported that Ahnak mediates the activation of the c-raf/MEK/Erk pathway in MEF cells. Therefore, we investigated the effect of Ahnak on the Erk phosphorylation in aortic SMCs in response to PDGF. Stimulation of Ahnak knockout aortic SMCs with PDGF showed a lower Erk phosphorylation than wild-type aortic SMCs (Figure 2A). Moreover, add-back expression of 4CRUs of Ahnak into aortic SMCs from Ahnak knockout mice resulted in a visible recovery of Erk phosphorylation in response to PDGF (Figure 2A). Furthermore, we found that 4CRUs of Ahnak is associated with Erk (Figure 2B), but not with Ras, Raf, and MEK in NIH3T3 cells (data not shown). Although PDGF-dependent Erk phosphorylation showed a different pattern in the wild-type and Ahnak knockout SMC, there was no difference in the PDGF receptor expression level in wild-type and Ahnak knockout SMCs (Figure 2C).

Next, we examined whether Ahnak activated the PI3K pathway in both aortic SMC populations. Ahnak protein had no effect on phosphorylation of Akt (Figure 2D), supporting the notion that Ahnak protein specifically modulates Erk activity in aortic SMCs, and not the PI3K pathway.

3.3 Ahnak stimulates Rac activity

The RhoA family of small G proteins including RhoA, Rac, and CDC42 plays a critical role in cell migration. Especially, Rac1 stimulates pathways implicated in lamellipodial protrusions for forward motions. To evaluate the effect of Ahnak protein on the RhoA family GTPase activity in aortic SMCs, we analysed active GTP-bound forms of Rac using a glutathione-S-transferase-fused Rac-binding domain (GST–PAK–RBD) of PAK. The results indicated that the PDGF significantly increased the active form of Rac (GTP-Rac) in wild-type aortic SMCs. However, Ahnak knockout aortic SMCs failed to activate Rac proteins in response to PDGF (Figure 3A).

To confirm that Ahnak was involved in Rac activation, we performed the acceptor photobleaching FRET analysis with Raichu-Rac. A reporter protein pair Rac and protein RBD of PAK fused to the cyan fluorescent protein and yellow fluorescent protein (CFP/YFP), respectively, was prepared for this purpose. Upon stimulation, active Rac protein binds RBD of PAK, mediating an intra-molecular association that positions CFP in close proximity to YFP. This causes FRET, which quenches CFP and facilitates the emanation of the YFP fluorescence. The CFP/YFP ratio allows a convenient determination
of FRET efficiency. PDGF stimulation led to a significantly higher FRET efficiency in wild-type aortic SMCs compared with Ahnak knockout aortic SMCs (Figure 3B). The result suggested that Ahnak protein mediates Rac activation in response to PDGF. Moreover, immunohistochemical analysis with laser-based confocal microscopy confirmed that Rac was translocated to the plasma membrane at the leading edge in wild-type aortic SMCs after PDGF treatment, but such translocation was not seen in Ahnak knockout aortic SMCs (Figure 3C).

Interestingly, Ahnak was diffusely stained in the cytoplasm of the control wild-type aortic SMCs, whereas the stimulation of wild-type aortic SMCs with PDGF resulted in increased localization of Ahnak to the plasma membrane (Supplementary material online, Figure S3).

### 3.4 Ahnak is co-localized with ternary complex of Erk–PAK–β-PIX

Previous studies showed that Erk binds and phosphorylates PAK in SMC and the activation of Erk regulates the integrity of the complex between PAK, β-PIX, and GIT1.31,32 To link Erk and Rac in the activation of SMC migration, we examined Ahnak-Erk downstream signalling cascade. Specifically, we transfected PAK, β-PIX, and 4CRUs of Ahnak into COS7 cells and complex formation was analysed with immunoprecipitation with antibody to PAK. It was readily shown that 4CRUs of Ahnak form a multiple protein complex with Erk, PAK, and β-PIX (Figure 4A). β-PIX protein is known to serve as the upstream guanine nucleotide exchange factor for Rac providing a regulatory input for various cellular activities including cell migration. Interestingly, immunohistochemical analyses with laser-based confocal microscopy showed that β-PIX and 4CRUs of Ahnak were translocated to leading edge of plasma membrane in response to PDGF (arrow head, Figure 4B). The physical association and co-translocation of Ahnak with β-PIX to leading edge were disrupted by pre-treatment of an Erk inhibitor, PD98059 (Figure 4B). These results suggest that the activation of Ahnak-Erk complex plays an important role in the formation of the signalling complex with PAK and β-PIX ultimately leading to cell migration.
3.5 Ahnak-dependent lamellipodia formation

To investigate whether Ahnak-mediated cell migration reflected increased plasma membrane dynamics as a result of changes in the formation of lamellipodia and membrane ruffling, a kymograph analysis for PDGF-dependent membrane protrusion of aortic SMCs expressing GFP protein was carried out. Kymograph analysis of membrane dynamics in both aortic SMCs showed that wild-type aortic SMCs had large lamellipodial protrusions upon PDGF stimulation, whereas Ahnak knockout aortic SMCs produced thin lamellipodia (Figure 5A). Protrusion activity was analysed as a number of spatial protrusions formed over time and was expressed as a box and whisker plot. Kymographic analysis was performed with 47 protrusions from eight wild-type aortic SMCs and 32 protrusions from eight Ahnak knockout aortic SMCs. The frequency of membrane protrusions was significantly higher in wild-type aortic SMCs than in Ahnak knockout aortic SMCs (Figure 5B and Supplementary material online, Figure S4). The results indicated that Ahnak regulated the formation of protrusion and cell migration through Rac activation.

3.6 Effect of Ahnak on neointimal formation after mouse carotid artery ligation injury

Migration of aortic SMCs is induced by arterial injuries. Such cellular activities can result in the lesion progression of restenosis and atherosclerosis. Substantial evidences suggest that Erk activation is involved in intimal hyperplasia induced by balloon injury. To determine the effect of Ahnak on intimal hyperplasia, we performed an in vivo experiment examining the neointimal accumulation of smooth muscle cells during restenosis. Large intimal hyperplasia lesions developed in the wild-type arteries after carotid artery ligation injury, whereas the hyperplasia was significantly inhibited in Ahnak knockout arteries (Figure 6A and B). The carotid arteries of uninjured wild-type and Ahnak knockout mice showed no evidence of neointimal thickening (Supplementary material online, Figure S5). Flk-1 expressed predominantly on endothelial cells and SMA for SMCs in the mouse carotid artery was used for immunohistochemical analysis. Undetectable Flk-1 signal (green colour) indicated the denudation of endothelial cells by injury. Importantly, SMCs (red colour) migrated from the media layer into the intima layer in wild-type arteries, but not in Ahnak knockout arteries (Figure 6C). These results support the hypothesis that Ahnak protein is involved in the formation of intimal hyperplasia after mouse carotid artery ligation injury.

4. Discussion

Atherosclerosis is a major cause of mortality in Western countries. The disease is considered to be an ultimate consequence of lipid accumulation and chronic inflammation resulting from concerted dys-function of endothelial cells, oxidation of lipoproteins, infiltration of macrophages, and formation of foam cells. During atherosclerotic progression, SMCs are activated and migrate towards the lesion in response to inflammatory molecules including various cytokines and most notably PDGF. First identified from platelets, PDGF is also expressed in vascular cells including endothelial cells and SMCs. Several lines of evidence indicate an important role for PDGF in an inducing proliferation of aortic SMCs and subsequent migration into the neointima layer after artery injury and during atherogenesis. Importantly, migration of aortic SMCs is regarded as the essential step leading to neointimal hyperplasia. It is also known that the activation of the Erk pathway contributes to intimal SMC proliferation and intimal thickening subsequent to balloon injury. Consistently, scavenging of PDGF or the blockade of its receptors leads to reduction in neointimal hyperplasia induced by artery injury in animal
PDGF-mediated cell signalling cascades trigger cytoskeletal remodelling required for migration through the well-known pathways of PI3K, calcium mobilization, and Erk.

Ahnak protein is an exceptionally large protein and features repeated sequence domains unique to this protein in the central region. The CRUs of Ahnak form a β-strand and a thin polyionic rod structure that provides the site for interaction with S100B, a calcium- and zinc-binding protein and PLCγ isozyme. Molecular functions of Ahnak protein have been examined in epithelia, muscles and heart, and Ahnak was shown to play the role of a scaffold protein relaying multiple signals. Of significant relevance to the current study, Ahnak protein is believed to be involved in pseudopod protrusion in tumour cell migration. Although the Ahnak protein is highly expressed in aortic SMCs, the detailed biological function of Ahnak in vasculature remains to be elucidated. Previously, we demonstrated that Ahnak stimulates disruption of the PKC–PP2A complex and that the release of PKC from the complex leads to the activation of c-Raf/MEK/Erk cascade in mouse embryonic fibroblasts. Given that Ahnak is essential for pseudopod protrusion in cell migration, we hypothesized that Ahnak regulates SMC migration via activation of Erk. We first showed that Erk phosphorylation in response to PDGF was enhanced in wild-type aortic SMCs compared with Ahnak knockout aortic SMCs (Figure 2A). Migration of wild-type aortic SMCs was significantly increased by PDGF stimulation, whereas the migration of ASMCs from Ahnak knockout mice was reduced. Importantly, add-back expression of 4CRUs into Ahnak knockout aortic SMCs resulted in a recovery of migration in response to PDGF (Figure 1A). These results clearly demonstrate the critical function of Ahnak protein in aortic SMC migration in response to PDGF and further confirm the role of Erk pathway activation.

Actin cytoskeleton plays an important role in defining cell shape and morphology and in orchestrating many of the dynamic aspects of cell migration. Contractile SMCs have highly organized long actin stress fibres which disintegrate in the mobile edges of non-contractile SMCs in response to environmental stimuli including growth factors. It is known that PDGF regulates the expression of non-contractile SMC-specific genes as well as the formation of stress fibres. The RhoA GTPase family plays a key role in the regulation of the actin cytoskeleton during cell migration, with Rac inducing membrane protrusion at the front of the cell. Our results show that Ahnak protein is involved in PDGF-induced actin reorganization through the activation of small G-proteins including Rac (Figure 3).

It has been well established that the function of scaffolding proteins is required for efficient cell signalling as they simultaneously interact with other proteins in signal transduction pathways.
with several proteins providing a signal relay networks in cells. Our results presented in this study show that Ahnak protein, with its highly repeated units in the central region, coordinates a relay network from PDGF to Erk cascade. Moreover, Ahnak protein interacts with Erk, PAK, and β-PIX indicating that it serves as a scaffolding module for a large signalling complex consisting of Erk, PAK, and β-PIX (Figures 4 and 6D). The results also indicate that Ahnak protein promotes interaction of Erk–PAK–β-PIX protein complex with Rac. Ahnak is thus shown to be involved in SMC migration via Erk and Rac activation mechanisms. Consistently in vivo, Ahnak contributes to neointima formation via mediating increased SMC migration in response to mechanical arterial injury. These novel findings raise the possibility that Ahnak will be useful as a new therapeutic target for treatment of vascular diseases, such as restenosis and atherosclerosis.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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