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Cyclic Strain Modulates Migration and Proliferation of Vascular Smooth Muscle Cells via Rho-GDIα, Rac1, and p38 Pathway

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ABSTRACT

Cyclic strain is an important inducer of proliferation and migration of vascular smooth muscle cells (VSMCs) which are involved in vascular remodeling during hypertension. However, its mechanism remains to be elucidated. VSMCs of rat aorta were exposed to cyclic strains in vitro with defined parameters, the static, 5%-strain (physiological) and 15%-strain (pathological), at 1.25 Hz for 24 h respectively. Then the possible signaling molecules participated in strain-induced VSMC migration and proliferation were investigated. The results showed that 15%-strain significantly increased VSMC migration and proliferation in comparison with 5%-strain. Expression of Rho GDP dissociation inhibitor alpha (Rho-GDIα) was repressed by 15%-strain, but expressions of phospho-Rac1 and phospho-p38 were increased. Expressions of phospho-Akt and phospho-ERK1/2 were similar between the static, 5%-strain and 15%-strain groups. Rho-GDIα "knock-down" by target siRNA transfection increased migration and proliferation of VSMCs, and up-regulated phosphorylation of Rac1 and p38 in all groups. Rac1 "knock-down" repressed migration and proliferation of VSMCs, down-regulated phosphorylation of p38, but had no effect on Rho-GDIα expression. When siRNAs of Rho-GDIα and Rac1 were co-transfected to VSMCs, the expressions of Rho-GDIα and phospho-Rac1 were both decreased, and the effects of Rho-GDIα "knock-down" were blocked. Rho-GDIα "knock-down" promoted while Rac1 "knock-down" postponed the assembly of stress fibers and focal adhesions in static. The results demonstrate that the pathological cyclic strain might induce migration and proliferation of VSMCs via repressing expression of Rho-GDIα, which subsequently verified phosphorylations of Rac1 and p38. J. Cell. Biochem. 109: 906–914, 2010.

KEY WORDS: CYCLIC STRAIN; VASCULAR SMOOTH MUSCLE CELLS; MIGRATION; PROLIFERATION; Rho-GDIα

Arterial wall is exposed to three main hemodynamic forces in vivo: shear stress, caused by the dragging frictional force of blood flow; transmural pressure, caused by the hydrostatic forces of blood within the blood vessel; and cyclic strain, defined as the repetitive deformation of the cells as the arterial wall rhythmically distends and relaxes with the cardiac cycle [Haga et al., 2007]. Vascular smooth muscle cells (VSMCs) in the media of arterial wall are primarily subjected to the cyclic strain. There is growing evidence that cyclic strain can profoundly modulate morphology [Halka et al., 2008; Qu et al., 2008] and functions, for example, migration, proliferation, and apoptosis of VSMCs [Williams, 1998]. It had been proved that physiological cyclic strain is an atheroprotective factor which prevents pathological changes in vascular wall [Williams, 1998; Morrow et al., 2005]. While under pathological conditions, such as hypertension, chronically elevated cyclic strain stimulates VSMC proliferation and migration, and subsequently causes vascular remodeling [Williams, 1998; Bobik, 2005; Morrow et al., 2005].

Studies had shown that cyclic strain can evoke various intracellular signaling pathways, such as Rho family GTPases, mitogen-activated protein kinases (MAPKs) and PI3K/Akt [Mayr et al., 2002; Li and Xu, 2007], which initiated differentiation,

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migration, and proliferation of VSMCs. The molecular mechanism of pathological cyclic strain induced VSMC migration and proliferation, however, remains to be further elucidated.

Rho GDP dissociation inhibitor alpha (Rho-GDIα), defined as the negative regulator of several Rho family GTPases, is ubiquitously expressed in all cell types [Dovas and Couchman, 2005]. Our previous work had revealed that decreased expression of Rho-GDIα promoted migration and apoptosis of VSMCs, which might participated in low-shear-stress induced vascular remodeling [Qi et al., 2008]. Recent study demonstrated that different frequencies of cyclic strain changed the expression of Rho-GDIα [Qu et al., 2008]. Hence, we hypothesized that Rho-GDIα might be a crucial regulator on mechanotransduction in VSMCs, and its different expression might contribute to pathological cyclic strain induced VSMC migration and proliferation via modulating the activation of Rho family GTPases.

In the present study, expression of Rho-GDIα was examined in cultured VSMCs subjected to different magnitudes of cyclic strain in vitro, 5%-strain (normal or physiological strain) [Asanuma et al., 2003] and 15%-strain (elevated or pathological strain) [Morrow et al., 2005] respectively. Furthermore, the expressions of Rho-GDIα and Rac1 were silenced by target siRNA transfection to demonstrate the roles of Rho-GDIα and Rac1 on cyclic strain induced VSMC migration and proliferation, and then the possible signaling molecules involved in were elucidated. Our findings demonstrated that the decreased expression of Rho-GDIα participated in pathologically elevated cyclic strain-induced migration and proliferation of VSMCs, which involved the activations of Rac1 and p38.

MATERIALS AND METHODS

VSMCs ISOLATION AND CULTURE

The animal care and experimental protocols conforms to the Animal Management Rules of China (Documentation 55, 2001, Ministry of Health, China), and the investigation is approved by the Animal Research Committee of Shanghai Jiao Tong University.

Primary cultures of VSMCs were isolated from the thoracic aorta of male Sprague–Dawley rats, 220–260 g, by the explant method [Stavri et al., 1995]. The media of aorta was isolated surgically and minced into small pieces, which were plated onto 25-cm^2 culture flasks for culture in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin, and incubated at 37°C in a humidified incubator of 95% air and 5% CO_2. VSMCs were characterized by immunohistochemical staining for smooth muscle-specific α-actin monoclonal antibody (Sigma). VSMCs monolayers were passaged every 3–4 days after trypsinization and passages 6–9 were used for all experiments.

CYCLIC STRAIN APPLICATION

For the application of cyclic strain, VSMCs were plated on type I collagen-coated flexible silicone bottom plates (Flexcell International, USA) at initial density of 3×10^5 cells per well (9.32 cm^2). After seeding for 24 h, the cells were incubated with 1% FBS/DMEM for 24 h prior to the experiments to arrest growth and synchronize the cells. VSMCs were then subjected to cyclic strain which produced by computer-controlled vacuum (FX-4000T Strain Unit, Flexcell International) as previously described [Qu et al., 2008]. The following mechanical parameters were applied: strain magnitudes of 5% and 15% at a constant frequency of 1.25 Hz, and a constant duration of 24 h. VSMCs cultured under the same conditions but no mechanical strain applied was regarded as the static control.

RNA INTERFERENCE AND INHIBITOR TREATMENT

The double strands of small interfering RNAs (siRNA) for Rho-GDIα were: 5′-AGCA CUCU GUGA ACUA CAAdT dT-3′, and 5′-UUGU AGUU CACA GAGU GCUC dG-3′, and those for Rac1 were: 5′-CAAA CAGA CGUG UUCU UAAT T-3′, and 5′-UUAA GAAC ACGU CUGU UUGC G-3′. For RNA interference studies, VSMCs were seeded at a density of 3.0×10^5 cells per well in Flexcell plate and grown in 10% FBS/DMEM. After seeding for 24 h, the cells were transfected with siRNA and Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instruction. Briefly, 100 nmol siRNA and 5 μl Lipofectamine™ 2000 were diluted in opti-MEM (Invitrogen), serum- and antibiotic-free, to a final volume of 800 μl. After mixing for 20 min at room temperature, the siRNA/Lipofectamine™ 2000 mixture was added dropwise onto the cells and incubated for 6 h at 37°C in a humidified CO_2 incubator. Following incubation, the mixture was replaced with 1% FBS/DMEM for 24 h prior to cyclic strain. Nonsilencing siRNA that does not recognize any known homology to rat genes was used as a mock control.

WESTERN BLOTTING

The protein concentrations were determined by the Bradford method (Bio-Rad). Then proteins, 30 μg per lane, were subjected to electrophoretic separation with 12% SDS–PAGE and transferred to nitrocellulose membranes (Hybond, Amersham). Western blots were performed by using antibodies directed against Rho-GDIα (1:500, Santa Cruz Technologies), phospho-Rac1 (1:500, Cell Signaling Technologies), Rac1/2/3 (1:500, Cell Signaling Technologies), phospho-Akt (1:500, Cell Signaling Technologies), total-Akt (1:500, Cell Signaling Technologies), phospho-p38 (1:500, Cell Signaling Technologies), total-p38 (1:500, Santa Cruz Technologies), phospho-ERK1/2 (1:500, Cell Signaling Technologies), total-ERK1/2 (1:500, Cell Signaling Technologies), and GAPDH (1:500, Santa Cruz Technologies), respectively. After incubation with alkaline phosphatase conjugated secondary antibodies (Jackson Immunoresearch), the signals were detected by nitroblue tetrazolium–bromochloroindolyl phosphate (Bio Basic, Inc.).

CELL MIGRATION ASSAY

VSMC migration assay was performed with the Transwell (Costar) system, which allows cells to migrate through a polycarbonate membrane with a pore size of 8 μm in 6-well plates as described previously [Qi et al., 2008]. Briefly, VSMCs were plated at a concentration of 1.0×10^6 cells/ml on the upper chamber, and the lower chamber was filled with 800 μl 50% FBS/DMEM to serve as the chemoattractant. After 6 h of incubation, the number of migrated cells was counted under a microscope (magnification 200×, Olympus). Six randomly chosen fields were evaluated per transwell membrane.

CELL PROLIFERATION ASSAY

Cell proliferation was analyzed by using a colorimetric bromodeoxyuridine (BrdU) kit (Roche Diagnostics). Six hours prior to the end of cyclic strain application, BrdU labeling reagent was added into the culture medium (1:1,000). After cyclic strain application, the cells were seeded at a density of 1.0×10^4 per well in 96-well plates and labeled according to the manufacturer's instructions. Briefly, the cells were fixed with FixDenat solution for 30 min at room temperature, and then incubated with anti-BrdU peroxidase working solution (freshly diluted 1:100) for 90 min. Following three rinses with washing buffer, 100 μl/well substrate solution was added onto the cells and incubated for 20 min at room temperature. Thereafter, 25 μl H_2SO_4, 1 mol/L, were added to each well, and the 96-well plates were shake at 300 rounds per minute. The absorbance at 450 nm was measured in an ELISA plate reader (Bio-Rad 680).

IMMUNOFLUORESCENCE

VSMCs transfected with Rho-GDIα siRNA or/and Rac1 siRNA for 24 h were seeded at a density of 2.0×10^4 cells per well in 24-well plates with a cover glass in the well. After incubated for 1 h, 3 h and 5 h with 10% FBS/DMEM at 37°C in a humidified 5% CO_2, VSMCs attached to the cover glass were fixed in 4% paraformaldehyde for 20 min, permeabilized for 5 min with 0.4% Triton X-100 in PBS, and blocked in PBS containing 1% BSA for 30 min. Filamentous F-actin was visualized by using rhodamine phalloidin (Molecular Probes). Focal adhesions (FAS) were stained with an anti-paxillin antibody (Neomarker) and a FITC-conjugated secondary antibody (Jackson Immunoresearch). The cell staining was observed under a fluorescence microscope (Olympus IX71). Nonsilencing siRNA that does not recognize any known homology to rat genes was used as a mock control.

STATISTICAL ANALYSIS

Each experiment was performed at least in triplicates, and all values are expressed as mean ± SD. The one-way ANOVA was used to compare the results between the two groups followed by Fisher *t*-test for multiple comparisons. A value of $P < 0.05$ was considered significant.

RESULTS

CYCLIC STRAIN REGULATED MIGRATION AND PROLIFERATION OF VSMCs IN A NONLINEAR MAGNITUDE-DEPENDENT MANNER

The migration of VSMCs treated with 15%-strain was significantly higher than that of the static and 5%-strain ($P < 0.05$, Fig. 1A). With 5%-strain, the migration of VSMCs was a little decreased as compared to the static, but the difference was not statistically significant ($P > 0.05$, Fig. 1A). VSMC proliferation was evaluated by measuring BrdU incorporation in newly synthesized DNA. As shown in Figure 1B, the proliferation of VSMCs exposed to 15%-strain was higher than that of the static and 5%-strain ($P < 0.05$). While in 5%-strain group, the VSMC proliferation was repressed compared with the static ($P < 0.05$, Fig. 1B).

These data indicate that cyclic strain modulates the migration and proliferation of VSMCs in a nonlinear magnitude-dependent manner in vitro. The elevated cyclic strain increases migration and proliferation of VSMCs in comparison with the normal cyclic strain. Our studies in vitro suggest that physiological cyclic strain may be a protective factor for the vascular homeostasis.

EXPRESSIONS OF Rho-GDIα, Phospho-Rac1, Phospho-Akt, Phospho-p38, AND Phospho-ERK1/2 IN VSMCs TREATED WITH DIFFERENT CYCLIC STRAINS

After VSMCs were treated with different magnitudes of cyclic strain for 24 h, the protein expressions of Rho-GDIα were analyzed by Western blotting. The activations of Rac1, Akt, p38, and ERK1/2 were assessed by their specific phospho-antibodies individually, which recognize the phosphorylated Ser71 residue on Rac1, Thr308 residue on Akt, Thr180 and Tyr182 residues on p38, Thr202 and Tyr204 residues on ERK1/2. These phosphorylated residues are the critical sites required for the activation of these factors respectively.

As shown in Figure 1C, the expression of Rho-GDIα was higher in VSMCs treated with 5%-strain than that of 15%-strain and the static ($P < 0.05$). The variation of phospho-Rac1 regulated by different cyclic strains was quite contrary to that of Rho-GDIα (Fig. 1C), which consists with the widely reported function of Rho-GDIα as the negative regulator of Rac1 [Dovas and Couchman, 2005]. Phosphorylation of p38 in VSMCs was repressed by 5%-strain treatment as compared with 15%-strain ($P < 0.05$, Fig. 1C). In comparison with the static, 5%-strain slightly decreased phosphorylation of p38, but the difference had no statistical significance ($P > 0.05$, Fig. 1C). The expressions of phospho-Akt and phospho-ERK1/2 in VSMCs had no significant difference between the static, 5%-strain and 15%-strain ($P > 0.05$, Fig. 1C).

The expressions of Rho-GDIα, phospho-Rac1, and phospho-p38 in VSMCs also changed with nonlinear magnitude-dependent manners under 24-h treatment of different cyclic strains. Therefore, it provided evidence that these factors might participate in the intracellular signal transduction of cyclic strain modulated VSMC migration and proliferation, but phospho-Akt and phospho-ERK1/2 might not.

THE EFFECTS OF Rho-GDIα ON CYCLIC STRAIN-INDUCED VSMC MIGRATION AND PROLIFERATION

To assess the possible roles of differently expressed Rho-GDIα in the cyclic strain-induced VSMC migration and proliferation, the target siRNA transfection was used to "knock down" the expression of Rho-GDIα in VSMCs under all conditions, the static, 5%-strain and 15%-strain application.

As compared with the mock control, the expressions of Rho-GDIα were remarkably suppressed by target siRNA transfection in all groups ($P < 0.05$, Figs. 2A, 3A, and 4A), and the expressions of phospho-Rac1 and phospho-p38 were significantly increased ($P < 0.05$, Figs. 2A, 3A, and 4A). The decreased expression of Rho-GDIα was associated with significant enhancement of VSMC migration and proliferation ($P < 0.05$, Figs. 2B,C, 3B,C, and 4B,C). Even in VSMCs subjected to 5%-strain, the increased expression of Rho-GDIα and the decreased migration and proliferation of VSMCs induced by 5%-strain, compared with 15%-strain ($P < 0.05$, Fig. 1), were significantly reversed by Rho-GDIα siRNA transfection ($P < 0.05$, Fig. 3).

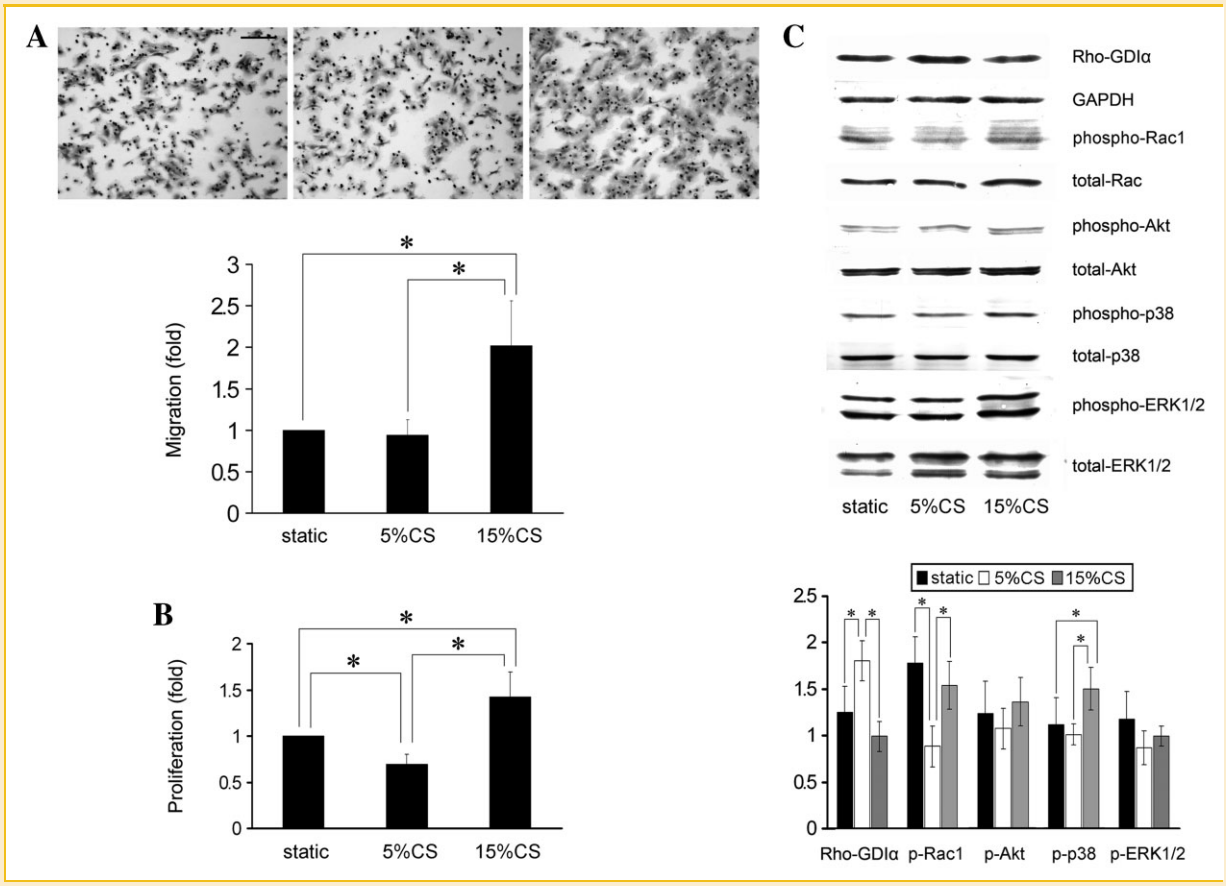

Fig. 1. The effect of different magnitudes of cyclic strain (CS) on migration and proliferation of VSMCs and expressions of Rho-GDIα, phospho-Rac1, phospho-Akt, phospho-p38, and phospho-ERK1/2. A: VSMC migration was significantly increased by 15%-CS which was determined by transwell assays. Representative microscopic images illustrate VSMC migration. Bar = 100 μm. Histogram shows fold change in VSMC migration relative to the static control. Six images were random acquired in each independent experiment. B: VSMC proliferation was significantly increased by 15%-CS, but decreased by 5%-CS, which was determined by a colorimetric BrdU kit. Histogram shows fold change in VSMC proliferation relative to the static. C: Expression of Rho-GDIα was up-regulated by 5%-CS, but expressions of phospho-Rac1 and phospho-p38 were down-regulated. Expressions of phospho-Akt and phospho-ERK1/2 were similar between the static, 5%-CS and 15%-CS. The top panel represents the western blotting results from a typical study; the bottom histogram shows densitometric quantification. Shown values are the mean ± SD for each condition from four independent experiments; $^{*}P < 0.05$.

These results demonstrated that the expression of Rho-GDIα was a crucial regulator of mechano-induced migration and proliferation of VSMCs. Physiological cyclic strain represses VSMC migration and proliferation might via up-regulating expression of Rho-GDIα, while pathological cyclic strain acts the contrary.

THE EFFECTS OF Rac1 ON CYCLIC STRAIN-INDUCED VSMC MIGRATION AND PROLIFERATION

Specific Rac1 siRNA transfection significantly suppressed the expressions of both total-Rac (Rac1/2/3) and phospho-Rac1 in all groups, the static, 5%-strain and 15%-strain ($P < 0.05$, Figs. 2A, 3A, and 4A). Compared with the mock control which substitutes the specific Rac1 siRNA with nonsilencing siRNA, the decreased expression of phospho-Rac1 repressed the activation, that is, phosphorylation, of p38 ($P < 0.05$, Figs. 2A, 3A, and 4A), but had no significant effect on the expression of Rho-GDIα ($P > 0.05$, Figs. 2A, 3A, and 4A). The expression of phospho-Rac1 and VSMC migration and proliferation, which up-regulated by 15%-strain, compared with 5%-strain ($P < 0.05$, Fig. 1), was significantly reduced after specific Rac1 siRNA transfection ($P < 0.05$, Fig. 4).

Co-transfection of siRNAs specific to Rho-GDIα and Rac1 decreased the expressions of Rho-GDIα, total-Rac and phospho-Rac1 in all groups ($P < 0.05$, Figs. 2A, 3A, and 4A). It revealed that the effects of Rho-GDIα siRNA transfection on phosphorylation of p38, migration and proliferation of VSMCs could be reversed by Rac1 co-transfection ($P < 0.05$, Figs. 2, 3, and 4).

The present data demonstrate that Rho-GDIα is a predominant molecular sensor of cyclic strain, and the effect of Rho-GDIα on VSMC migration and proliferation is phospho-Rac1 dependent and might via p38 MAPK pathway.

THE EFFECTS OF Rho-GDIα AND Rac1 ON STRESS FIBER FORMATION AND FOCAL ADHESIONS ASSEMBLY IN VSMCs

Several studies revealed that Rac1 is a crucial regulator in cytoskeleton arrangement and FAS signaling transduction [Turner, 2000; van Nieuw Amerongen and van Hinsbergh, 2001; Onoue et al., 2008]. F-actin, one kind of cytoskeleton, associated with paxillin, a

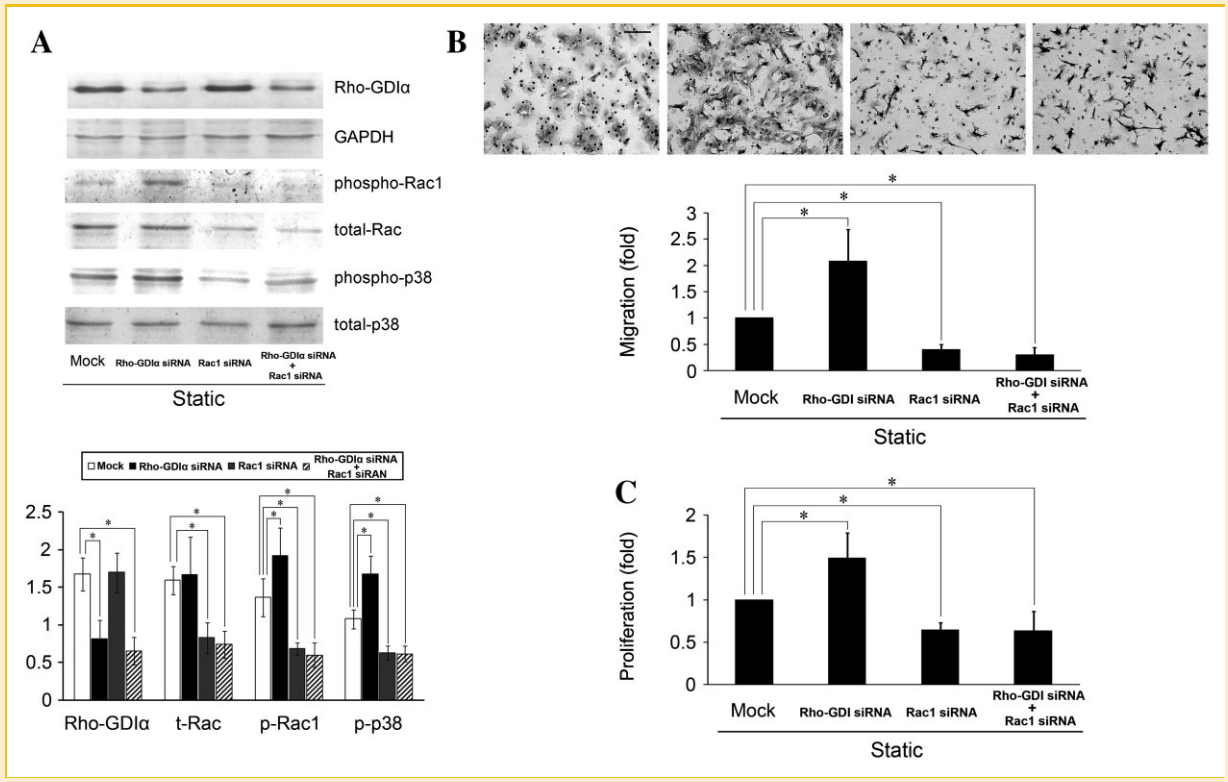

Fig. 2. The role of Rho-GDIα, Rac1 and p38 on VSMC migration and proliferation under static condition. A: Rho-GDIα siRNA transfection suppressed the expression of Rho-GDIα, but increased expressions of phospho-Rac1 and phospho-p38. Rac1 siRNA transfection suppressed expressions of total-Rac, phospho-Rac1, and phospho-p38, but had no statistical effect on expression of Rho-GDIα. Rho-GDIα siRNA and Rac1 siRNA co-transfection blocked the effect of Rho-GDIα siRNA transfection which showed a similar effect as Rac1 siRNA transfection. The top panel represents the Western blotting results from a typical study; the bottom histogram shows densitometric quantification. B: VSMC migration was increased by Rho-GDIα siRNA transfection but decreased by Rac1 siRNA transfection. The effect of Rho-GDIα was blocked by Rac1 siRNA co-transfection. Representative microscopic images illustrate VSMC migration. Bar = 100 μm. Histogram shows fold change in VSMC migration relative to the mock control. Six images were random acquired in each independent experiment. C: VSMC proliferation was enhanced by Rho-GDIα siRNA transfection, but suppressed by Rac1 siRNA transfection. The increase of VSMC proliferation by Rho-GDIα siRNA transfection could be reversed by Rac1 siRNA co-transfection. Histogram shows fold change in VSMC proliferation relative to the mock. Shown values are the mean ± SD for each condition from four independent experiments; $^{*}P < 0.05$.

crucial component of FAS, are important for various cell behaviors, including migration and proliferation [Woodring et al., 2003; Revenu et al., 2004]. Therefore, the possible roles of Rho-GDIα and Rac1 on the F-actin rearrangement and paxillin redistribution were examined.

At 1 h, the filaceous F-actin in VSMC cytoplasm of all groups, the mock control, Rho-GDIα siRNA transfection, Rac1 siRNA transfection and Rho-GDIα siRNA and Rac1 siRNA co-transfection, was indistinct (Fig. 5Aa–Da). In the mock control VSMCs, at 5 h after plating the aggregated F-actin filaments were visible but still not prominent (Fig. 5Ac). However, in the Rho-GDIα siRNA transfected VSMCs, the filaceous F-actin staining at cytoplasm was observable at 3 h (Fig. 5Bb), and marked stress fibers were present at 5 h (Fig. 5Bc). Rac1 siRNA transfection postponed the rearrangement of F-actin (Fig. 5Ca,Cb,Cc). Until 5 h after plating the cytoplasmic F-actin filaments were still not distinct (Fig. 5Cc). Co-transfection of both Rho-GDIα and Rac1 siRNAs showed a similar change on F-actin rearrangement with Rac1 siRNA transfection (Fig. 5Da,Db,Dc).

Paxillin, typically localizes in the cell member primarily at FAS, was undetectable in all groups at 1 h after plating (Fig. 4Ad,Bd,Cd,Dd). Compared with the mock control, Rho-GDIα siRNA transfection significantly increased the redistribution of paxillin, which distributed as dotted patterns at the periphery of VSMCs at 3 h (Fig. 5Be) and significantly increased size and typical FAS appearance at 5 h (Fig. 5Bf). The effect of Rho-GDIα siRNA transfection was thoroughly reversed by Rac1 siRNA co-transfection (Fig. 5Dd,De,Df).

These results indicate that the reduced expression of Rho-GDIα promote actin-containing microfilaments formation and FAS assembly in the cultured VSMCs, which is in a Rac1-dependent manner.

DISCUSSION

Increasing studies had demonstrated that persistent elevation of cyclic mechanical strain during hypertension is strongly associated with vascular remolding, which is characterized as VSMC migration from tunica media into subendothelial layer and abnormal proliferation [Williams, 1998; Bobik, 2005; Haga et al., 2007; Li and Xu, 2007; Yan et al., 2009]. In the present study, the physiological cyclic strain (5%) [Chapman et al., 2000; Asanuma

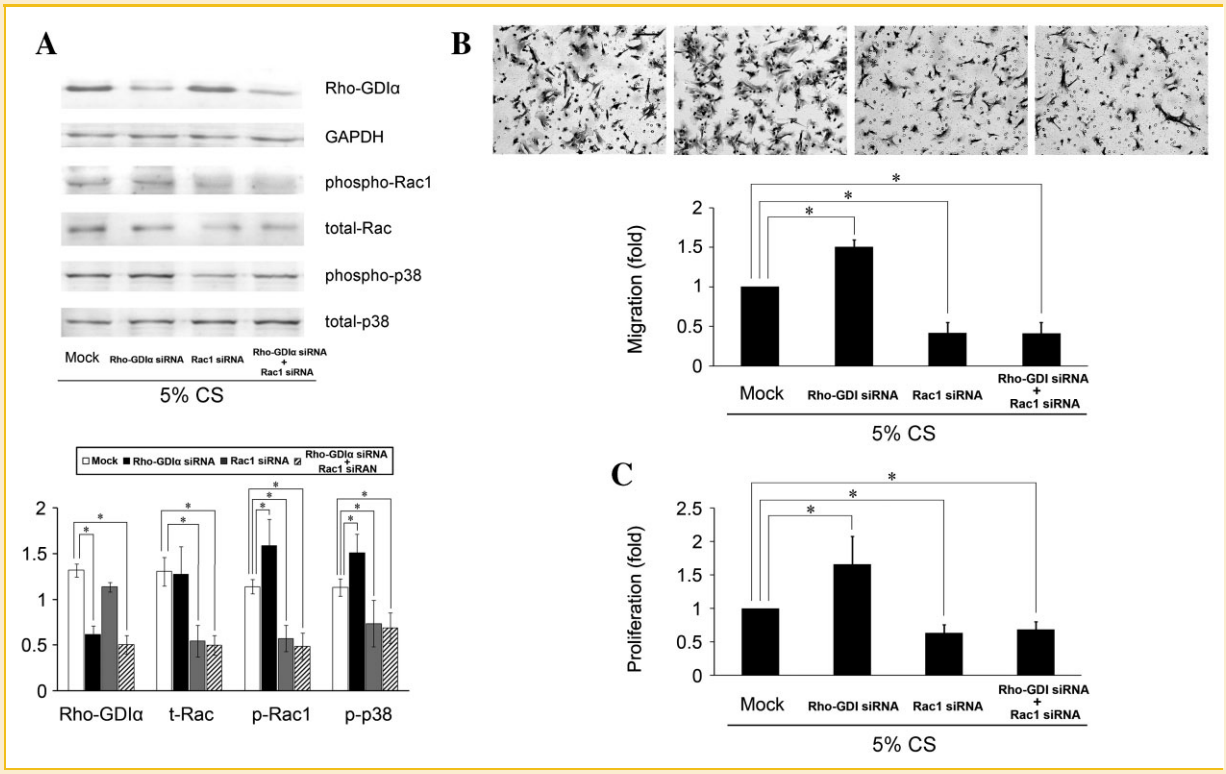

Fig. 3. The role of Rho-GDIα, Rac1 and p38 on VSMC migration and proliferation under 5%-cyclic-strain (CS) application. A: Rho-GDIα siRNA transfection increased the phosphorylation of Rac1 and p38 which were repressed by 5%-CS application. Rac1 siRNA transfection decreased expressions of total-Rac, phospho-Rac1, and phospho-p38. The effect of Rho-GDIα siRNA transfection was reversed by Rac1 siRNA co-transfection. The top panel represents the western blotting results from a typical study; the bottom histogram shows densitometric quantification. B: The repressed migration of VSMCs caused by 5%-CS was increased by Rho-GDIα siRNA transfection, which was blocked by Rac1 siRNA co-transfection. Representative microscopic images illustrate VSMC migration. Bar = 100 μm. Histogram shows fold change in VSMC migration relative to the mock control. Six images were random acquired in each independent experiment. C: VSMC proliferation down-regulated by 5%-CS was up-regulated by Rho-GDIα siRNA transfection, which was blocked by Rac1 siRNA co-transfection. Histogram shows fold change in VSMC proliferation relative to the mock. Shown values are the mean ± SD for each condition from four independent experiments; $^{*}P < 0.05$.

et al., 2003] and pathological elevated cyclic strain (15%) [Morrow et al., 2005] were applied to cultured VSMCs respectively. The pathological elevated cyclic strain was found to enhance migration and proliferation of VSMCs while the physiological cyclic strain significantly repressed VSMC proliferation. Our results in vitro suggest that the physiological cyclic strain may be a protective factor for the vascular homeostasis.

How do VSMCs convert different cyclic strains to biochemical signals? Our previous proteomic analysis on low-shear-stress induced vascular remodeling had demonstrated that Rho-GDIα can respond to the shear stress and modulate VSMC migration and apoptosis [Qi et al., 2008]. Hence, it was hypothesized that Rho-GDIα might participate in the mechanism by which VSMCs sense and transduce the extracellular cyclic strain stimuli.

Rho-GDIα is a member of Rho GDP dissociation inhibitors (Rho-GDIs), which have been proved to negatively regulate the activities of small G proteins of Rho family by shutting off their GDP (inactive)/GTP (active) cycling and cytosol (inactive)/membrane (active) translocation [Hoffman et al., 2000; Heasman and Ridley, 2008]. Our findings demonstrated that the pathological cyclic strain down-regulated the expression of Rho-GDIα in comparison with the physiological cyclic strain. Using target siRNA transfection, the expression of Rho-GDIα was decreased which was accompanied with the enhancement of VSMC migration and proliferation. When the up-regulated expression of Rho-GDIα induced by the physiological cyclic strain was "knocked-down," the protective effect of physiological cyclic strain, that is, repressed migration and proliferation of VSMCs in vitro, was markedly reduced. These results suggest that Rho-GDIα plays a significant role in mechanotransduction and regulation of VSMC functions in response to cyclic strain, and higher expression of Rho-GDIα may be beneficial to vascular wall.

Rac1 is one of small G proteins of Rho family whose activation could be negatively regulated by Rho-GDIα [Dovas and Couchman, 2005]. In this regard, we investigated the activations of Rac1 and the reported downstream signaling molecules, such as Akt, p38, and ERK1/2 [Mayr et al., 2002; Haga et al., 2007; Takashima et al., 2008], under different magnitudes of cyclic strain and target siRNA transfection. Contrary to the expression of Rho-GDIα, expressions of phospho-Rac1 and phospho-p38 were enhanced by the pathological cyclic strain compared with the physiological strain. Therefore, we hypothesized that different cyclic strains might modulate the expression of Rho-GDIα and then subsequently regulate the phosphorylation of Rac1 and p38. Supporting of this

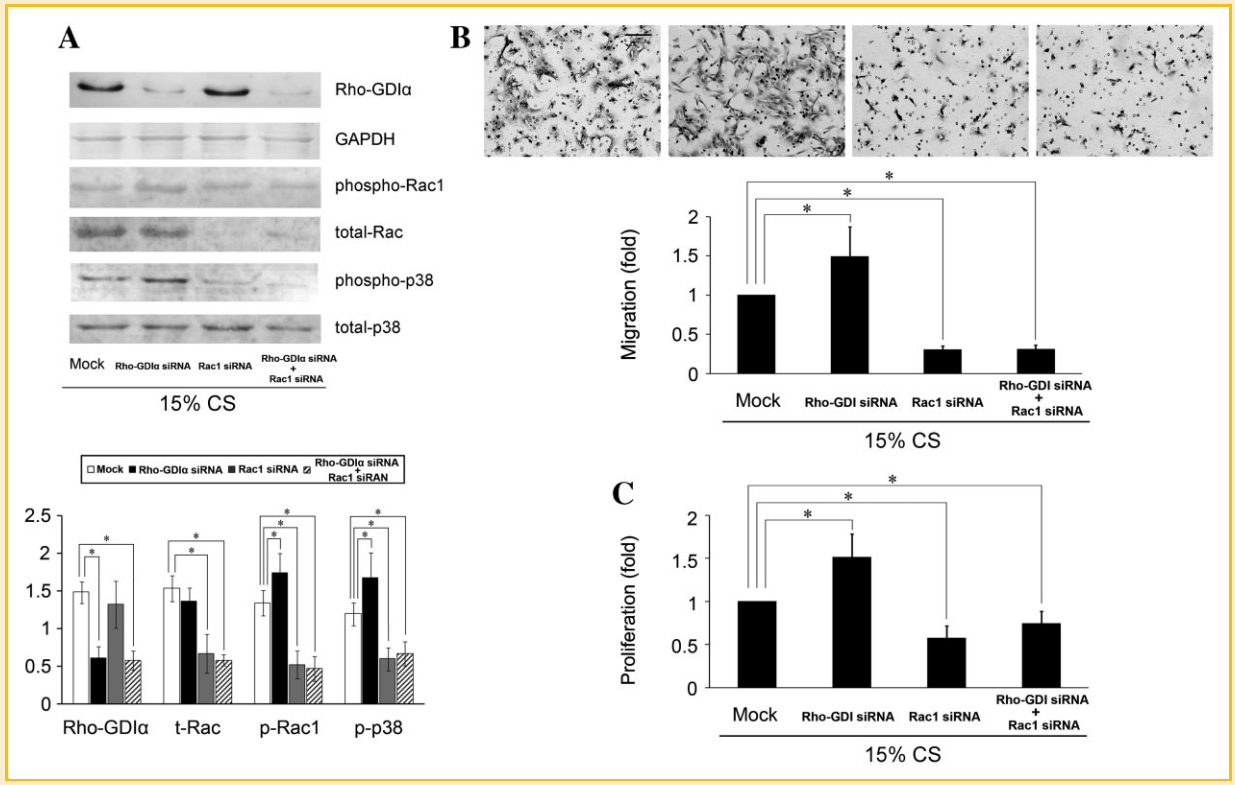

Fig. 4. The role of Rho-GDIα, Rac1, and p38 on migration and proliferation of VSMCs under 15%-cyclic-strain (CS) application. A: Rho-GDIα siRNA transfection suppressed the expression of Rho-GDIα, but increased expressions of phospho-Rac1 and phospho-p38. Rac1 siRNA transfection repressed the up-regulated expression of phospho-Rac1 and phospho-p38 induced by 15%-CS, but had no remarkable effect on the expression of Rho-GDIα. The top panel represents the Western blotting results from a typical study; the bottom histogram shows densitometric quantification. B: Rho-GDIα siRNA transfection increased migration of VSMCs. The enhanced VSMC migration induced by 15%-CS or Rho-GDIα siRNA transfection was diminished by Rac1 siRNA transfection. Representative microscopic images illustrate VSMC migration. Bar = 100 μm. Histogram shows fold change in VSMC migration relative to the mock. Six images were random acquired in each independent experiment. C: Rho-GDIα siRNA transfection increased proliferation of VSMCs. VSMC proliferations up-regulated by 15%-CS or Rho-GDIα siRNA transfection were reversed by Rac1 siRNA transfection. Histogram shows fold change in VSMC proliferation relative to the mock. Shown values are the mean ± SD for each condition from four independent experiments; $^{*}P < 0.05$.

hypothesis is the experiments on target siRNA transfection. Rho-GDIα "knock-down" by target siRNA transfection increased phosphorylation of Rac1 and p38. Rac1 "knock-down" reversed the effects of Rho-GDIα "knock-down" on phospho-p38 expression and VSMC migration and proliferation. The up-regulated migration and proliferation of VSMCs and phosphorylation of p38 induced by 15%-strain were repressed by Rac1 "knock-down."

Our results demonstrated that there was no significant difference in expressions of phospho-Akt and phospho-ERK1/2 among the static, 5%- and 15%-strains, which did not consistent with works of Sedding et al. [2003] and Li et al. [1999]. The differences may be attributable to the differences in experimental variables, such as duration and frequency of cyclic strain application, duration of cell synchronize step, type of cells, passage of cells, and coating of the flexible silicone bottom. For example, the duration and frequency of cyclic strain applied in our work was 24 h and 1.25 Hz respectively, 15 min or even less and 0.5 Hz in Sedding's, 6 h or even less and 1 Hz in Li's.

VSMC migration and proliferation are both the anchorage-dependent processes, during which cytoskeletal reorganization and FAS formation are important events [Revenu et al., 2004; Gros et al., 2006]. F-actin cytoskeleton is a fundamental component of all eukaryotic cells which provides force and stability of the cell [Woodring et al., 2003; Revenu et al., 2004]. The initial events of cell migration, such as exploration, adhesion and polarization, require regulated assembly and disassembly of F-actin and FAS [Woodring et al., 2003]. Paxillin is a multi-domain protein that localizes at FAS of the cells which serves as an attachment point of actin stress fiber to the membrane [Turner, 2000; Revenu et al., 2004]. Several studies suggested that Rho family proteins play an important role in cytoskeleton rearrangement and FAS organization [van Nieuw Amerongen and van Hinsbergh, 2001; Ohtsu et al., 2005; Onoue et al., 2008], but the effect of Rho-GDIα on these processes is still unknown. Immunofluorescence of F-actin and paxillin were examined in VSMCs treated with Rho-GDIα siRNA after 1, 3, and 5 h attachment. The results indicated that the decreased expression of Rho-GDIα promoted stress fiber formation and FAS assembly in VSMCs in a Rac1-dependent manner. With special attention on the roles of Rho-GDIα, there is a need for further studies on the effects of cytoskeleton rearrangement and FAS organization in VSMC migration and proliferation induced by cyclic strain.

In summary, our results demonstrate that Rho-GDIα is a predominant signaling molecule in VSMC migration and proliferation modulated by different magnitudes of cyclic strain. The

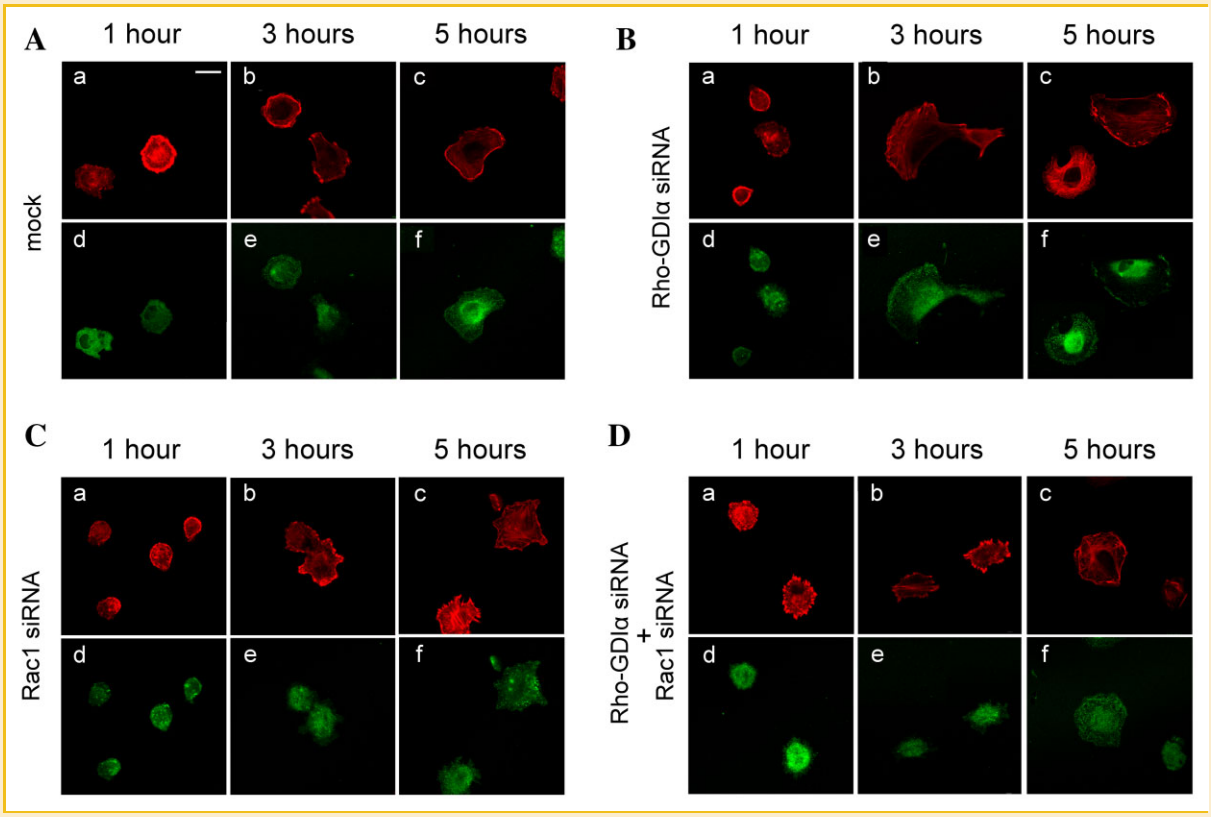

Fig. 5. The role of Rho-GDIα and Rac1 on stress fiber formation and FAS assembly in VSMCs. A: VSMCs transfected with nonsilencing siRNA were seeded in wells for 1 h (a,d), 3 h (b,e), and 5 h (c,f), and stained with F-actin (red, a–c) and paxillin (green, d–f). B: Rho-GDIα siRNA transfection promoted the rearrangement of F-actin (red, a–c) and the redistribution of paxillin (green, d–f). C: Target Rac1 siRNA transfection postponed the formation of F-actin (red, a–c) and the assembly of paxillin (green, d–f). D: Rho-GDIα siRNA and Rac1 siRNA co-transfection blocked the effect of Rho-GDIα siRNA transfection, which showed a postponed formation of F-actin (red, a–c) and the assembly of paxillin (green, d–f) as Rac1 siRNA transfection (C). Photographs were taken with a fluorescence microscope. Bar = 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

physiological cyclic strain up-regulate the expression of Rho-GDIα, while the pathological elevated cyclic strain down-regulate, which subsequently verify phosphorylation of Rac1 and p-38, and modulate the reorganization of cytoskeleton and FAS. Given the importance of Rho-GDIα in mediating vascular remodeling induced by the pathological strain, Rho-GDIα may be a potential target for the treatment of hypertensive vascular diseases.

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