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Rho-associated protein kinase isoforms stimulate proliferation of vascular smooth muscle cells through ERK and induction of cyclin D1 and PCNA

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ABSTRACT

Abnormal proliferation and migration of vascular smooth muscle cells (VSMC) plays an important role in vascular diseases. The Rho-associated protein kinase (ROCK) signaling pathway is now well recognized for its role in VSMC migration and proliferation. Recently, a number of studies revealed that different isoforms of ROCK have distinct functions in VSMCs. We have reported that ROCK1, rather than ROCK2, induces platelet-derived growth factor (PDGF)-BB-stimulated migration of VSMCs. In the current study, we aimed to investigate the roles of ROCK1/2 in PDGF-induced rat aorta VSMC proliferation by manipulating ROCK gene expression. The results revealed that knock-down of both ROCK1 and ROCK2 by siRNA technology decreased PDGF-BB-generated VSMC proliferation by inhibiting the expression of proliferating cell nuclear antigen (PCNA) and cyclin D1. In addition, up-regulation of ROCK1 expression through transfection, further increased the proliferation of VSMCs induced by PDGF-BB. The ERK inhibitor U0126 reduced the proliferation and expression of PCNA and cyclinD1, and ROCK1 and ROCK2 siRNA decreased the level of ERK in the nucleus. These results demonstrated that ROCK1 and ROCK2 could promote VSMC proliferation through ERK nuclear translocation, regulating the expression of PCNA and cyclin D1 protein.

1. Introduction

The incidence of cardiovascular diseases has increased remarkably over the past decade, and atherosclerosis and intimal hyperplasia are major causes of morbidity and mortality [1]. There is a large number of reports on the mechanisms of atherosclerosis and it is becoming clear that vascular smooth muscle cells (VSMCs) play an important role in the development of atherosclerotic plaques [2]. However, detailed knowledge on the regulation of VSMC proliferation and the signal transduction pathways implicated in this process, is still lacking.

Rho-associated protein kinases (ROCKs) and mitogen activated protein kinases (MAPKs) are serine-threonine protein kinases with multiple downstream effects. They have been described to be important in PDGF-induced cellular responses [3–6]. These molecules transduce various extracellular stimuli through a cascade of protein phosphorylations, leading to activation of transcription factors. Extracellular signal-regulated kinase (ERK) and p38 MAPK (p38) are known to play a major role in stress-induced cellular responses, including growth, proliferation, differentiation, migration and apoptosis of cells [7,8]. It has been reported that ROCK is involved in the migration and proliferation of glioblastoma multiforme (GBM) cells via linkage to the ERK signaling pathway [9].

Two isoforms of ROCK protein, ROCK1 (also referred to as ROKβ or p160ROCK) and ROCK2 (also referred to as ROKα or Rho-kinase), have been characterized. ROCK1 and ROCK2 do not only exhibit a 65% sequence identity, but also have a very similar tissue distribution pattern [10,11]. Recently, a number of studies have revealed the ROCK isoform-specific functions in vascular diseases. ROCK1 mediates leukocyte recruitment and neointima formation after vascular injury [12]. In addition, ROCK1 and ROCK2 regulate the phosphorylation of myosin light-chain phosphatase (MLCP) and myosin light-chain in VSMC through different mechanisms [13]. Inhibition of ROCK1 and ROCK2 activates vascular endothelial growth factor (VEGF)-driven angiogenesis *in vitro* and *in vivo*[14]. We have recently reported that activated ROCK1 can promote VSMC migration through phosphorylation and nuclear translocation of ERK protein [15]. In this study, the specific role of both ROCK isoforms in PDGF-induced rat aorta VSMC proliferation was investigated.

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2. Materials and methods

2.1. Cell culture

The A7r5 vascular smooth muscle cell line, originally derived from embryonic rat aorta [16], was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in a CO_2 incubator (in a humidified atmosphere of 5% CO_2 and 95% air). Cells were grown to 80–90% confluence. Quiescence was achieved by serum starvation overnight.

2.2. Cell proliferation assessment

To evaluate cell viability, 5×10^3 cells were plated in each well of a flat-bottom 96-well culture plate, incubated in 100 μL DMEM medium for 24 H and treated according to the described experimental conditions. After treatment, MTT was added to each well to a final concentration of 0.5 mg/mL and incubated for 4 H at 37 °C in a humidified incubator containing 5% CO_2. To dissolve the cells, we used 100 μL of DMSO and measured the absorbance of the resulting solutions at 570 nm using a microplate reader (Multiskan Ascent, Thermo Scientific, Barrington, IL, USA). Results were obtained as percentage cell viability (optical density [OD] of the experiment samples/OD of the control).

2.3. Transfection of siRNAs

Sequences of ROCK1 and ROCK2 siRNA were pre-designed and synthesized by Takara Bio Incorporated (Japan). ROCK1 siRNA sequences were: 5′-GAAGCGAAUGACUUACUUATT-3′ (sense) and 5′-UAAGUAAGUCAUUCGCUUCTT-3′ (antisense). ROCK2 siRNA sequences were: 5′-GAUCAGUGGAAUUGGGAUATT-3′ (sense) and 5′-UAUCCCAAUUCCACUGAUCTT-3′ (antisense). The negative control siRNA was purchased from Takara Biotechnology. A7r5 cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.4. Construction of ROCK1 expression vectors and selection of stable tranfectants

The 4.1-kb cDNA fragment containing the coding region of ROCK1 was amplified from A7r5 cells by reverse transcription-polymerase chain reaction (RT-PCR) using the ROCK1-specific forward primer 5′-GGTACCATGTCGACTGGGGACAGTTT-3′ and the reverse primer 5′-CCCGGGTTAACTAGTTTTTCCAGATGTATT-3′. The PCR product (*Kpn I* and *Sma I* restriction) was cloned into the pMD19-T simple cloning vector (Takara Biomedical, Japan) subsequently subcloned into a pEGFP-C1 (BD Biosciences Clontech, USA) expression vector that was digested by *Kpn I* and *Sma I* restriction enzymes (Takara Biomedical, Japan), and then purified by agarose gel electrophoresis. The ROCK1-GFP fusion gene was produced, and the orientation of the inserted ROCK1 gene was confirmed by sequencing. The resulting recombinant plasmid was named pEGFP-C1/ROCK1 plasmid, which was stably transfected into A7r5 cells.

A7r5 cells were co-transfected with 4 μg pEGFP-C1/ROCK1 expression plasmid and Lipofectamine 2000 (10 μL/well) according to manufacturer's instructions. The empty vector of pEGFP-C1 was transfected as a control. Forty-eight H after transfection, the selection medium containing 600 μg/mL G418 was added to the transfected cells until the resistant colonies were formed. These colonies were further subcloned using cloning cylinders, and individual colonies were tested for ROCK1 expression by immunoblotting using an anti-ROCK1 antibody. After two months of screening and culture, several colonies expressing ROCK1-GFP fusion protein were selected.

2.5. Western blotting

A7r5 cells were pretreated with ROCK inhibitor Y-27632 (Alexis Biochemicals, Lausen, Switzerland) and the MAPK/ERK kinase 1 (MEK1) inhibitor U0126 (Cell Signaling Technology, Danvers, MA, USA) for 1 H at the indicated concentrations, respectively. After terminating the reactions with the addition of trichloroacetic acid, total cellular proteins of each of the experimental groups were extracted with sodium dodecyl sulfate (SDS) sample buffer. The sample proteins (20 μg/lane) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Membranes were washed with Tris-buffered saline (TBS) and blocked with 5% (w/v) non-fat milk in TBS-Tween 20 (1%, v/v, TBST) for 1 H at room temperature. After three washes of 5 min each with TBST, membranes were incubated with a rabbit PCNA polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse cyclin D1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit actin polyclonal antibody (Cell Signaling Technology), ERK (Bioworld Technology, St. Louis Park, MN, USA) and a phospho-ERK antibody (Bioworld Technology, St. Paul, MN, USA) in TBST with 5% (w/v) bovine serum albumin (BSA) overnight at 4 °C. After three washes with TBST, the blots were incubated with horseradish peroxidase (HRP)-linked anti-rabbit immunoglobulin (Ig)G antibody (Jackson ImmunoResearch, USA) for 1 H at room temperature. Bands were visualized by enhanced chemiluminescence (ECL) detection after Western blotting (detection reagents were from Amersham Bioscience, Piscataway, NJ, USA). Quantitative analysis was performed using a Scion Image System (Frederick, MD, USA). Band intensities were analyzed using Image Gauge® Ver. 4.0 software (Fuji Film, Tokyo, Japan).

2.6. Preparation of nuclear extracts

Nuclear protein was extracted as previously described [17]. Briefly, after stimulation with PDGF-BB or vehicle, cells were washed in ice-cold phosphate-buffered saline/phosphatase inhibitor buffer (PBS/PIB). Cells were suspended in 1 mL ice-cold hypotonic buffer (HB; 20 mM HEPES, pH 7.5, 5 mM NaF, 10 mM Na_2MoO_4 and 0.1 mM ethylenediamine tetra-acetic acid [EDTA]) for incubation on ice for 15 min. 50 μL of 10% Nonidet P-40 (final concentration, 0.5%) was added to the cells. The homogenate was centrifuged at 300*g* for 30 s at 4 °C. The nuclear pellets were resuspended in 50 mL complete lysis buffer, and the tube was rocked gently on ice for 30 min. After centrifugation at 14,000*g* for 5 min at 4 °C, the supernatants (nuclear cell extracts) were stored at −80 °C until use.

2.7. Flow cytometry

A7r5 cells were harvested and fixed in 70% ice-cold ethanol for 10 min and incubated with RNase A (100 μg/ml) and propidium iodide (50 μg/ml) for 30 min, and 1×10^4 cells from each sample were measured with a BD FACSCalibur system (BD Biosciences, Bedford, MA). Data were analyzed using ModFit (Verity Software House, Topsham, ME).

2.8. Statistical analyses

Results are expressed as means ± standard deviations. Statistical analysis for significance was performed using the Student's *t*-test. $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. ROCK1 and ROCK2 are involved in PDGF-BB-induced proliferation of VSMC

We have shown that PDGF-BB induces the expression of ROCK isoforms, and that ROCK1, rather than ROCK2, is involved in PDGF-BB-stimulated migration of VSMCs [15]. To confirm the role of ROCK1/2 in PDGF-BB-stimulated proliferation of VSMC, we applied loss- and gain-of-function experiments using siRNA and transfection of expression constructs. siRNA technology was employed to specifically reduce the levels of target gene expression in A7r5 cells (Fig. 1A). In the transfectants, neither ROCK1 nor ROCK2 expression could be induced regardless of PDGF-BB stimulation (Fig. 1B). A previous study has shown that ROCK isoforms lose their specificity when overexpressed [18], and therefore only overexpression of ROCK1 was used. Assessment of proliferation by MTT assays showed that the knock-down of ROCK1 and ROCK2 by siRNA technology suppressed the proliferation of VSMCs (Fig. 1C). The need for ROCK activity in this assay was confirmed by similar results after exposure to the ROCK inhibitor Y27632. Overexpression of ROCK1 increased the proliferation of VSMCs as compared to cells transfected with the empty vector (Fig. 1D).

3.2. ROCK1 and ROCK2 knock-down suppressed the expression of cyclin D1 and PCNA in PDGF-BB-treated VSMCs

Cyclin D1 is a key regulator in the progression from G1 to S phase in the mammalian cell cycle. Furthermore, PCNA is a well-defined regulator of DNA replication and cell cycle control [19-21]. To investigate whether these regulators play a role in ROCK1/2 signaling, siRNAs against the ROCKs and the ROCK-inhibitor Y27632 were used. Expression levels of cyclin D1 and PCNA were assessed. Knock-down of both ROCKs, as well as treatment with Y27632, suppressed the expression of cyclin D1 and PCNA in A7r5 cells (Fig. 2A). In addition, the effect of ROCK isoform expression on the cell cycle distribution was examined by flow cytometry. Cells were transfected with ROCK1 and ROCK2 siRNA, and an increase in the number of cells in the G1 phase and a reduction of cells in the S phase of the cell cycle was observed (Fig. 2B). Taken together, these findings suggest a prominent role for ROCK1/2 during cell cycle.

3.3. ROCK1/2 are involved in the proliferation of VSMC via nuclear translocation of ERK

The relevance of ERK MAPK signaling pathways in PDGF-BB-induced VSMC proliferation was investigated. We found that the

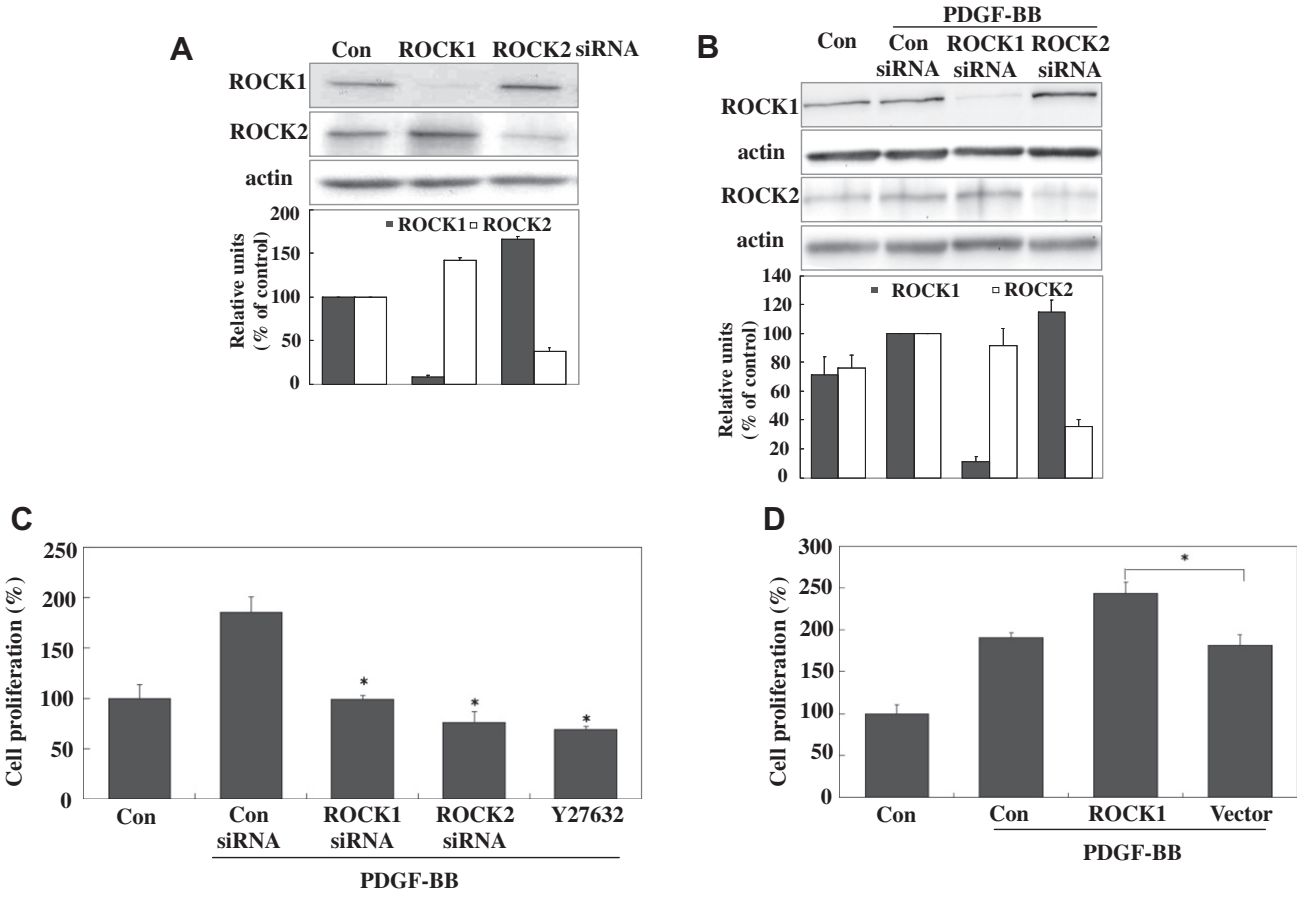

Fig. 1. Effects of ROCK isoform knock-down and overexpression on PDGF-BB-induced proliferation of VSMC. (A, B) Validation of ROCK isoform siRNAs in A7r5 cells without (A) or with (B) PDGF-BB stimulation. A7r5 cells were transfected with ROCK isoform silencing, and the expressions of ROCK isoforms were analyzed by Western blotting and quantified by densitometric analyses. Antibodies to actin were served as protein loading controls (major point 1). (C) Proliferation of A7r5 cells transfected with ROCK isoform specific siRNAs or pre-treated with Y27632. (D) Proliferation of A7r5 cells transfected with ROCK isoform expression constructs ("vector" indicates the empty vector control). In both A and B, cells were treated with 10 ng/mL PDGF-BB for 24 H, when indicated. Proliferation was determined using the MTT assay. All results are presented as means ± SEM ($n = 3$), $^{*}p < 0.05$.

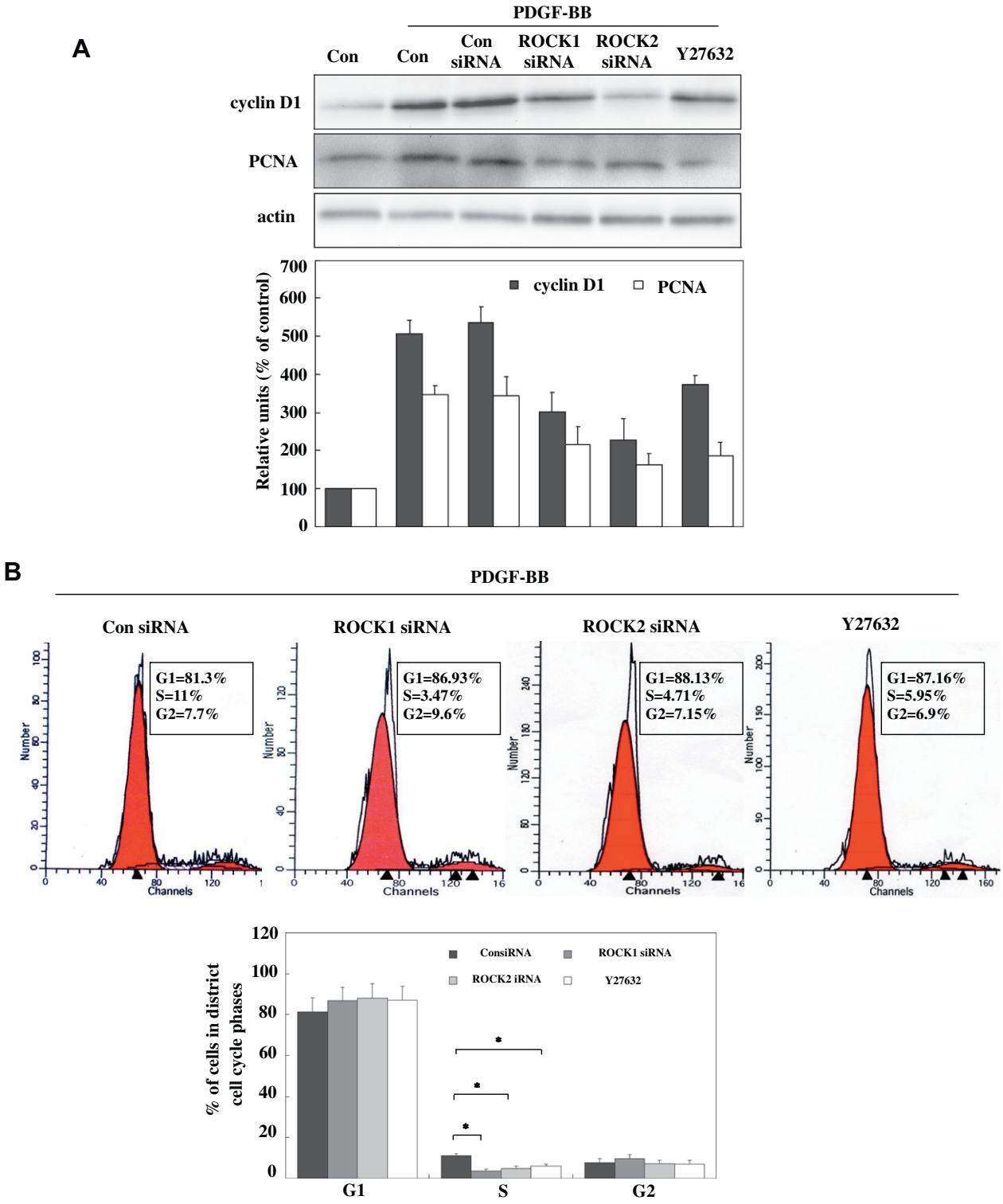

Fig. 2. Effect of ROCK isoform knock-down on cell cycle. (A) Expression of proliferation-associated proteins cyclin D1 and PCNA in PDGF-BB-exposed VSMCs. Cell lysates were analyzed by Western blotting using antibodies against cyclin D1 or PCNA. A7r5 cells were cultured in serum-free DMEM for 24 H to arrest cell cycle progression at G_0/G_1 phase. The cells were subsequently transfected with ROCK isoform specific siRNAs or were pre-treated with Y27632 before exposure to 10 ng/mL PDGF-BB for 24 H. (B) Cell cycle progression was assessed by flow cytometric analysis of DNA content. Results are expressed as means ± SEM ($n = 3$), $^*p < 0.05$.

MAPK/ERK kinase 1 (MEK1) inhibitor U0126 significantly suppressed the PDGF-BB induced proliferation of A7r5 cells (Fig. 3A). Meanwhile, U0126 decreased the expression levels of cyclin D1 and PCNA (Fig. 3B). Cell cycle analysis of A7r5 cells pretreated with U0126 showed an increase in the number of cells in the G1 phase and a reduction in DNA synthesis activity (S phase, Fig. 3C). To ascertain the role of ROCK1/2 in VSMC proliferation, the effects of ROCK1 and ROCK2 on p-ERK were examined by siRNA knockdown. ERK needs to translocate to the nucleus to exert its biological effects. Moreover, the participation of ROCK in serotonin-mediated mitogenesis of VSMCs occurs via cytoplasmic-to-nuclear translocation of ERK [22,23]. This issue was also investigated in

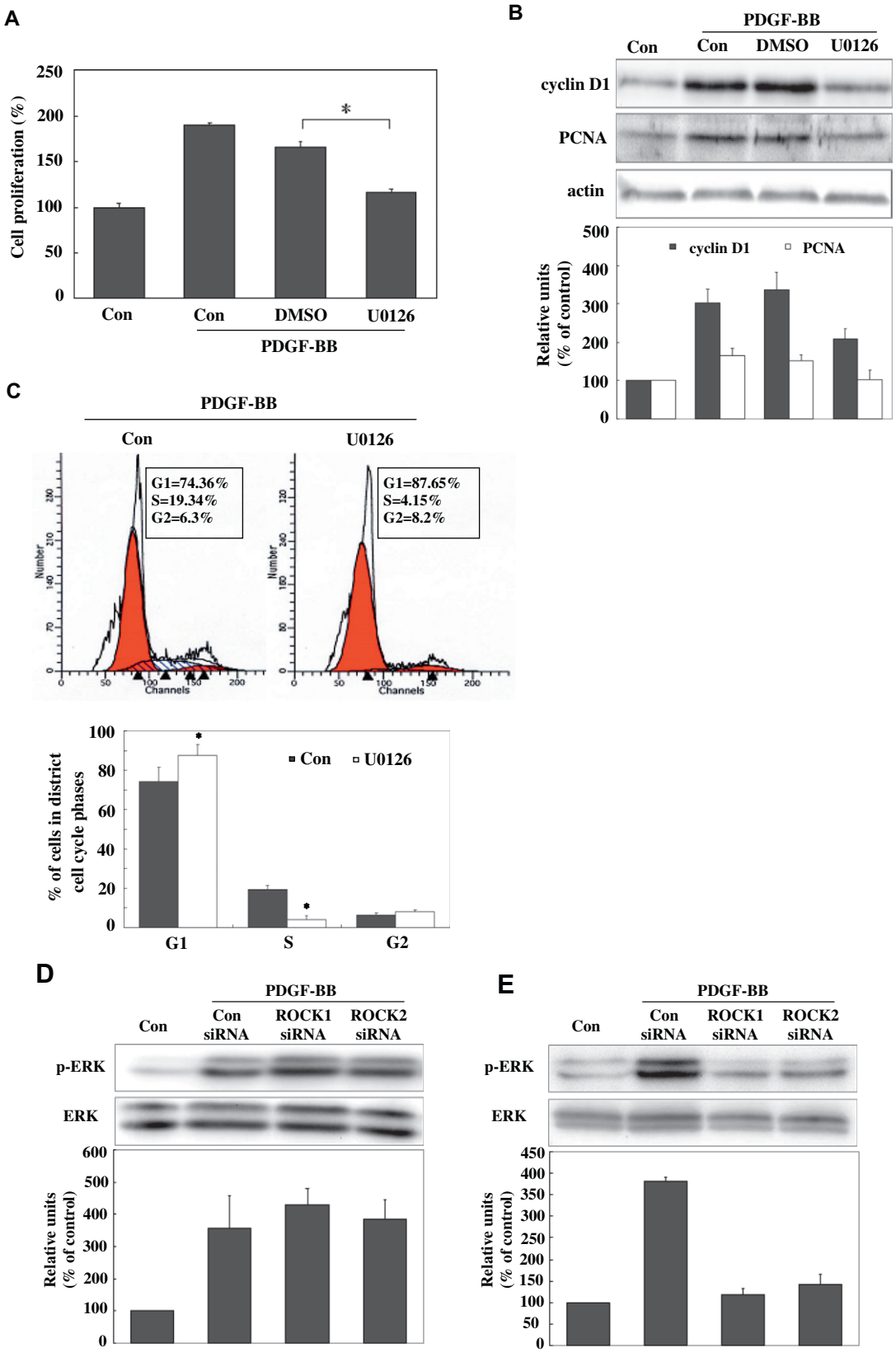

Fig. 3. Effect of the MAPK/ERK kinase 1 (MEK1) inhibitor U0126 on PDGF-BB-induced proliferation of VSMC, and effects of ROCK-isoform silencing on ERK phosphorylation. (A) Expression of proliferation-associated proteins cyclin D1 and PCNA in VSMCs in response to PDGF-BB, with or without pretreatment of U0126. The degree of proliferation was determined using the MTT assay. (B) Cell lysates were analyzed by Western blotting using antibodies against cyclin D1, PCNA, or actin. All results are means ± SEM ($n = 3$), $^*p < 0.05$. (C) Cell cycle progression was assessed by flow cytometric analysis of DNA content. Results are expressed as means ± SEM ($n = 3$), $^*p < 0.05$. A7r5 cells were transfected with ROCK isoform specific siRNAs, and the expression of phosphorylated ERK was analyzed by Western blotting after 5 H of PDGF-BB stimulation in total cell lysates (D) or in nuclear lysates. (E) Equal loading of protein was confirmed with antibodies against ERK. Results are expressed as means ± SEM ($n = 3$).

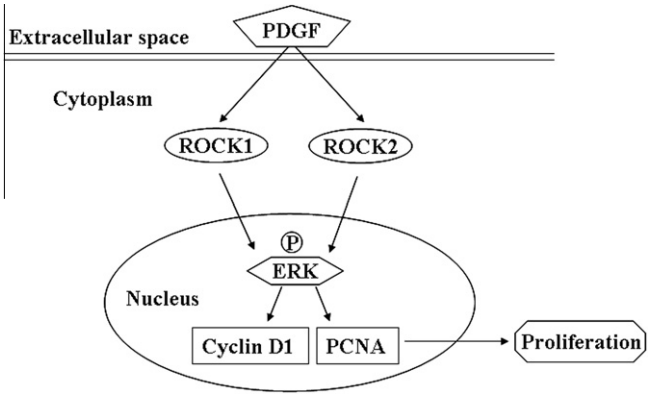

Fig. 4. A proposed model for the involvement of ROCK1 and ROCK2 in the signaling pathway of PDGF-BB-regulated cell proliferation.

this study by investigating the intracellular distribution of ERK MAPKs after ROCK1/2 knock-down in VSMCs. Repression of ROCK1 and ROCK2 did not affect the cytoplasmic level of p-ERK during PDGF-BB-induced proliferation (Fig. 3D). However, in the nucleus, ROCK1 and ROCK2 siRNA treatment blocked the level of p-ERK during PDGF-BB-induced proliferation (Fig. 3E). This may explain the potential and possible mechanisms of how ROCK1 and ROCK2 can affect VSMC proliferation: (1) the depletion of ROCK1/2 decreased the expression of ERK phosphorylation in the nucleus; (2) the depletion of ROCK1/2 decreased the expression of cyclin D1 and PCNA; and (3) an inhibitor of ERK was able to down-regulate the expression of cyclin D1 and PCNA. These findings suggest that ROCK1 and ROCK2 play a critical role in the proliferation of PDGF-BB-treated VSMCs. Furthermore, this effect is regulated through ERK nuclear translocation, resulting in the enhanced expression of PCNA and cyclin D1 (Fig. 4).

Our study shows that ROCK1 and ROCK2 have distinct roles in VSMC function. ROCK1 has a predominant role in VSMC migration, while ROCK1 and ROCK2 play a similar role in PDGF-BB-induced proliferation of VSMC, through different mechanisms. The current results may have a potential impact on therapeutic applications in the management of human cardiovascular diseases.

Acknowledgments

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