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Platelet-derived growth factor regulates vascular smooth muscle phenotype *via* mammalian target of rapamycin complex 1

Jung Min Ha [a], Sung Ji Yun [a], Young Whan Kim [a], Seo Yeon Jin [a], Hye Sun Lee [a], Sang Heon Song [b], Hwa Kyoung Shin [c], Sun Sik Bae [a, *]

[a] Medical Research Institute, Department of Pharmacology, Pusan National University School of Medicine, Yangsan, Republic of Korea
[b] Department of Internal Medicine, Pusan National University Hospital, Busan, Republic of Korea
[c] Department of Anatomy, Pusan National University School of Korean Medicine, Yangsan, Republic of Korea

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ABSTRACT

Mammalian target of rapamycin complex (mTORC) regulates various cellular processes including proliferation, growth, migration and differentiation. In this study, we showed that mTORC1 regulates platelet-derived growth factor (PDGF)-induced phenotypic conversion of vascular smooth muscle cells (VSMCs). Stimulation of contractile VSMCs with PDGF significantly reduced the expression of contractile marker proteins in a time- and dose-dependent manner. In addition, angiotensin II (AngII)-induced contraction of VSMCs was completely blocked by the stimulation of VSMCs with PDGF. PDGF-dependent suppression of VSMC marker gene expression was significantly blocked by inhibition of phosphatidylinositol 3-kinase (PI3K), extracellular signal-regulated kinase (ERK), and mTOR whereas inhibition of p38 MAPK had no effect. In particular, inhibition of mTORC1 by rapamycin or by silencing of Raptor significantly blocked the PDGF-dependent phenotypic change of VSMCs whereas silencing of Rictor had no effect. In addition, loss of AngII-dependent contraction by PDGF was significantly retained by silencing of Raptor. Inhibition of mTORC1 by rapamycin or by silencing of Raptor significantly blocked PDGF-induced proliferation of VSMCs. Taken together, we suggest that mTORC1 plays an essential role in PDGF-dependent phenotypic changes of VSMCs.

1. Introduction

Vascular smooth muscle cells (VSMCs) play a pivotal role in asthma, hypertension and atherosclerosis [1]. Blood vessel tone, blood flow and blood pressure are regulated by highly specialized VSMCs. These cells exhibit low synthetic activity, and express unique contractile proteins and ion channels. Mature VSMCs exhibit the contractile phenotype by expression of marker proteins including smooth muscle actin (SMA), SM22α, calponin, myosin heavy chain (MHC) and myosin light chain kinase (MLCK). Unlike cardiac or skeletal muscle cells, VSMCs undergo phenotypic modulation in response to environmental cues [2,3]. Cellular events underlying VSMC-related physiological and pathological processes, such as wound healing, restenosis, hypertension, or atherosclerosis can promote phenotypic change from contractile, non-proliferative VSMCs to the migratory, proliferative synthetic phenotype [4,5]. Thus, understanding the mechanism that controls phenotypic conversion of VSMCs is important for elucidating pathological diseases.

Platelet-derived growth factor (PDGF) family consists of five proteins, PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD, which act through two receptors, PDGFRα and β. PDGF-BB is a critical mitogen for fibroblasts, mesangial cells and VSMCs, and plays crucial roles in pathophysiological conditions, such as development, neoplasia and atherosclerosis [6]. All PDGFs and PDGFRs are expressed in atherosclerotic lesions and VSMCs in vessel walls, respectively. Released PDGF plays a key role in the VSMC migration from media into the intima layer. In addition, infusion or transfection of PDGF induces SMC proliferation and intima thickening [7]. It has been reported that PDGF-BB or PDGF-DD inhibits the expression of contractile VSMC marker proteins, including SMA, SM22α, and MHC in cultured VSMCs [5,8,9]. In addition, zinc finger motif-1 (ZFM-1) attenuates PDGF-induced

* Corresponding author. Department of Pharmacology, Pusan National University School of Medicine, Bumeo-ri, Mulgeum-eup, Yangsan-si, Kyungnam 626-870, Republic of Korea.
E-mail address: sunsik@pusan.ac.kr (S.S. Bae).

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phenotypic change and growth in VSMCs [4]. However, the regulatory mechanism by PDGF in contractile VSMCs is poorly understood.

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase, and consists of two distinct complexes, mTORC1 and mTORC2. Regulatory-associated protein of mTOR (Raptor) and proline-rich Akt substrate (PRAS40) binding to mTOR forms mTORC1 [10,11]. mTORC1 regulates cell growth and proliferation in response to growth factors, oxygen and amino acids by controlling 4E-BP1 and S6K1. It has been reported that PRAS40, an inhibitor of mTORC1, is phosphorylated by Akt, a downstream effector of PI3K, and induces the dissociation of PRAS40 from Raptor. This event regulates cell growth and protein synthesis *via* phosphorylation of S6K1 and 4E-BP1 [12–14]. In contrast to mTORC1, mTORC2 contains rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated map kinase-interacting protien1 (mSin1), and protein observed with rictor1 and 2 (protor1/2). mTORC2 plays an important role in cell survival and cytoskeletal organization [15]. In addition, mTORC2 is insensitive to nutrients, but responds to growth factors that require PI3K signaling [11,16]. Indeed, mTORC2 directly phosphorylates Akt at Ser473 [17].

In the present study, the role of the mTORC1 signaling pathway in the PDGF-induced phenotypic change of VSMCs has been elucidated. We provide novel evidence that mTORC1 is important for modulating phenotypic conversion of VSMCs by PDGF.

2. Materials and Methods

2.1. Materials

All culture media were obtained from Hyclone Laboratories, Inc. (Logan, UT, USA). Anti-SMA and anti-calponin antibodies were purchased from Sigma–Aldrich (St. Louis, MO, USA). Anti-MHC antibody was purchased from Proteintech Group, Inc. (Chicago, IL, USA). Anti-MLCK antibody was obtained from Epitomics, Inc. (Burlingame, CA, USA). Antibodies against pan-Akt, phospho-Akt (Ser473), S6K1, phospho-S6K1, ERK, phospho-ERK (Thr202/Tyr204), mTOR, Rictor, and Raptor were purchased from Cell Signaling Technology (Boston, MA, USA). Anti-SM22α antibody was obtained from Abcam (Cambridge, UK). Anti-actin antibody was purchased from MP Biomedicals (Aurora, OH, USA). PDGF-AA, PDGF-BB, PDGF-DD, rapamycin and PF-4708671 were obtained from Sigma–Aldrich (St. Louis, MO, USA). SB203580 and LY294002 were purchased from Calbiochem (Darmstadt, Germany). U0126 was obtained from Cell Signaling Technology (Boston, MA, USA). 4′,6-diamidino-2-phenylindole (DAPI), Alexa Fluor 488-conjugated goat anti-mouse secondary antibody and Cy3-conjugated goat anti-rabbit secondary antibody were purchased from Molecular Probes, Inc. (Carlsbad, CA, USA). IRDye700-and IRDye800-conjugated rabbit/mouse secondary antibodies were obtained from Li-COR Bioscience (Lincoln, NE, USA). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise indicated.

2.2. Cell isolation and cell culture

To isolate VSMCs, Sprague–Dawley rats (3 weeks old) were euthanized *via* intraperitoneal injection of sodium pentobarbital (60 mg/kg) by a tissue explanting method. Thoracic aorta was isolated and surrounding fat and connective tissues were discarded. Vessels were longitudinally cut and the lumen side was scraped with a razor blade to remove the intima. Vessels were fragmented into 3–5 mm lengths and explanted lumen side down on collagen-coated culture dishes. After seven days of explanting, tissue fragments were discarded and sprouted VSMCs were collected (referred as P0). Synthetic VSMCs were cultured at low density (<20%). To induce phenotypic conversion of VSMCs, synthetic VSMCs (P0) were cultured at high density (~100%) and passages between P2 and P5 were defined as contractile VSMCs. The precise method is described in a previous report [18].

2.3. Cell proliferation assay

For measurement of VSMC proliferation, VSMCs (5×10^3) were plated on six-well plates and stimulated with PDGF (25 ng/ml) for 1, 3 or 5 days in 0.5% FBS medium. Cells were fixed with 4% paraformaldehyde, and the nuclei were stained with DAPI. Images of stained cells were captured with a fluorescence microscope at ×20 magnification.

2.4. Collagen gel contraction assay

For collagen gel contraction assay, confluent VSMCs were cultured by trypsin digestion and resuspended in serum-free DMEM. The cell suspension was mixed on ice with collagen gel solution (8 mg/ml of collagen type I in 2X PBS, pH 8.0) to give 5×10^5 cells/ml and 4 mg/ml of collagen gel solution. 12-well plates were prepared, and 100 μl of VSMC-collagen gel solution was slowly added into the wells. The plates were incubated at 37 °C to allow for polymerization. After 30 min, the gels were floated in serum-free DMEM. After 6 h, angiotensin II (AngII) was added to initiate contraction and images were captured using a digital camera. Collagen gel contraction was analyzed by the decrease in gel area using image J (National Institutes of Health, MD, USA). Relative gel area was obtained by dividing the area at each time point by the initial area of the gel.

2.5. Short hairpin RNA constructs

To silence Rictor or Raptor, oligonucleotides tagged with a 5′-end *AgeI* site and a 3′-end *EcoRI* site were designed for sh-Rictor (5′-CCG GTT CTG TGA ACT AGC ACT TCA GAC TCG AGT CTG AAG TGC TAG TTC ACA GAT TTT TG-3′) and sh-Raptor (5′-CCG GTG GAG AAT GAA GGA TCG GAT CTC GAG ATC CGA TCC TTC ATT CTC CTT TTT G -3′) and both sense and anti-sense oligonucleotides were synthesized (XENOTECH, Daejeon, Korea). Both complementary oligonucleotides were mixed and heated at 98 °C for 5 min and cooled to room temperature. Annealed nucleotides were subcloned into the *AgeI*/*EcoRI* site of a pLKO.1 lentiviral vector.

2.6. Lentiviral knockdown

For gene silencing, HEK293-FT packaging cells (Invitrogen) were grown to ~70% confluence in 100-mm cell culture dishes. Cells were triple transfected with 20 μg of pLKO.1 lentiviral vector containing sh-Rictor or sh-Raptor, 5 μg of Δ8.9, and 5 μg of pVSV-G using the calcium phosphate method. Medium was replaced with fresh medium 8 h post-transfection. Lentiviral supernatants were harvested 24 h and 48 h post-transfection and passed through 0.45-μm filters. Cell-free viral culture supernatants were used to infect contractile VSMCs in the presence of 8 μg/ml of polybrene (Sigma). Infected cells were isolated by selection with 10 μg/ml puromycin for 2 days.

2.7. Western blotting and immunocytochemistry

Cell lysates were loaded into 10% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, which were incubated with the indicated primary antibodies and IRDye-conjugated secondary antibodies (Li-COR Biosciences). Protein bands were visualized using Odyssey (Li-COR Biosciences). For

immunocytochemistry, contractile VSMCs were grown in 6-well plates on collagen-coated coverslips, and treated with PDGF (25 ng/ml) for 4 days. Cells were washed with ice-cold PBS and fixed with 4% paraformaldehyde. Cells were permeabilized with 0.2% Triton X-100 and incubated with the indicated antibodies for 2 h followed by Cy3-or Alexa Fluor 488-conjugated secondary antibodies for 30 min. Samples were mounted with anti-fading reagent (2% n-propylgalate in 80% glycerol/PBS solution) and images were obtained with a confocal microscope at ×60 magnification (FV1000-ZDC, Olympus, Japan).

2.8. Statistical analysis

Results are expressed as the mean ± SD of three independent experiments ($n = 3$ for each experiment). When comparing two groups, an unpaired Student's *t*-test was used to assess differences. *P*-values less than 0.05 were considered significant.

3. Results

3.1. PDGF-BB induces phenotypic conversion of VSMCs

Vascular smooth muscle cells (VSMCs) exhibit two distinct phenotypes [19]. Phenotypic conversion of VSMCs from contractile phenotype to synthetic phenotype is modulated by response to injury or extracellular stimuli [2]. Previously, it was reported that PDGF-BB and PDGF-DD regulate the expression of multiple VSMC differentiation marker proteins, including SMA, MHC, and SM22α, and modulate SMC differentiation [5,8,9]. To define the role of PDGF isoforms in the phenotypic change of VSMCs, we examined phenotypic conversion of VSMCs in response to treatment with PDGF-AA, PDGF-BB and PDGF-DD. The expression of SMA and SM22α was reduced by PDGF-BB or PDGF-DD stimulation, whereas PDGF-AA had no effect (Online Figure S1A). In addition, treatment of VSMCs with PDGF-BB or PDGF-DD for 15 min resulted in the activation of Akt, ERK, p38 MAPK and S6K1. However, PDGF-AA had no effect on expression of marker proteins, and activation of Akt, ERK, p38 MAPK, and S6K1 was relatively low (Online Figure S2B). Stimulation of VSMCs with PDGF-BB decreased the expression of marker proteins in a time- and dose-dependent manner (Fig. 1A and B). Immunofluorescence staining showed that treatment of contractile-type VSMCs with PDGF reduced the expression of SM22α (Fig. 1C). As shown in Fig. 1D, angiotensin II (AngII) significantly induced contraction of VSMCs, whereas treatment of VSMCs with PDGF abolished the effect of AngII, indicating that PDGF is an important regulator for phenotypic change of VSMCs.

3.2. mTORC plays an essential role in PDGF-induced phenotypic change of VSMCs

To delineate the regulatory mechanism underlying PDGF-induced phenotypic conversion of VSMCs, we explored the effect of various pharmacological inhibitors on the phenotypic change of VSMCs. Stimulation of VSMCs with PDGF resulted in the activation of S6K1, ERK, PI3K/Akt, and p38 MAPK within 15 min (Fig. 2A). As shown in Fig. 2B, PDGF-induced ERK activation was selectively blocked by pretreatment with U0126 (ERK inhibitor). Activation of Akt was blocked by both PI3K (LY294002) and mTOR (Torin1) inhibitors. However, selective inhibition of mTORC1 (rapamycin) did not affect PDGF-induced activation of Akt. Activation of S6K1 was blocked by the inhibition of PI3K, mTOR, and mTORC1. We also measured phenotypic conversion of VSMCs after stimulation with PDGF. As shown in Fig. 2C and D, pharmacological inhibition of ERK, PI3K, mTOR, or mTORC1 completely blocked PDGF-induced phenotypic conversion of VSMCs.

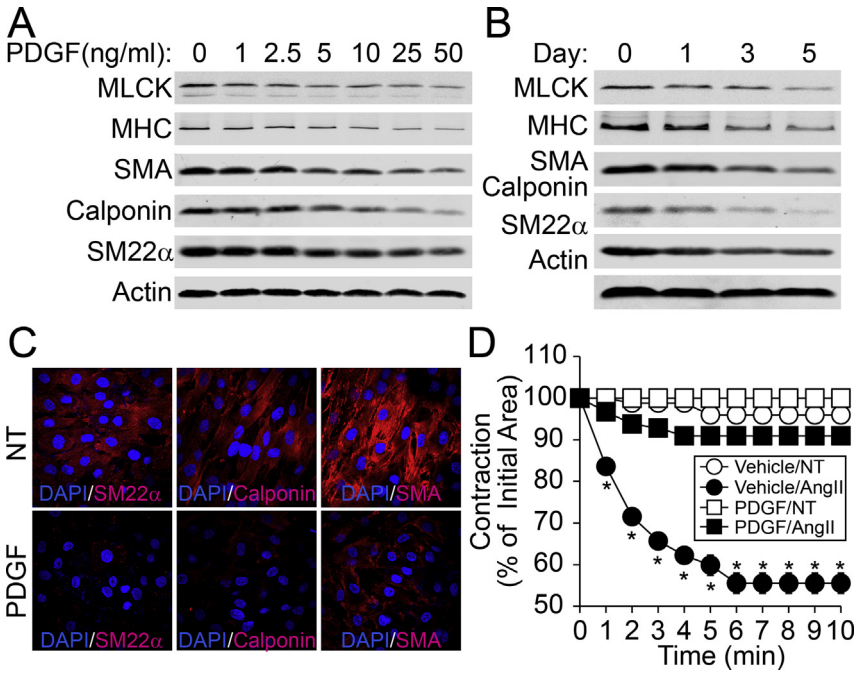

Fig. 1. PDGF-BB induces phenotypic conversion of VSMCs. (A, B) Contractile-type VSMCs were treated with PDGF (25 ng/ml) at the indicated doses and for the indicated time periods. Cells were harvested and expression of smooth muscle marker proteins was verified by western blot analysis. (C) Contractile-type VSMCs were stimulated in the presence or absence of PDGF for 4 days, and cells were stained by the indicated antibodies. Images were captured on confocal microscope at ×60 magnification. (D) Contractile-type VSMCs were stimulated with vehicle or PDGF for 4 days and embedded in collagen gel as described in "Materials and Methods". AngII (1 μM)-dependent contraction of collagen gel beads was recorded digitally and contraction was expressed as % of initial area. Data are means ± S.D. of three independent experiments ($n = 3$ for each experiment). Asterisks indicate statistical significance ($P < 0.05$).

3.3. mTORC1is essential for PDGF-induced phenotypic conversion of VSMCs

Since selective inhibitor of mTORC1 blocked PDGF-induced phenotypic conversion of VSMCs, we evaluated the role of Rictor and Raptor in the modulation of PDGF-dependent phenotypic change. As shown in Fig. 3A, lentiviral infection of Rictor and Raptor shRNA selectively disrupted the expression of Rictor and Raptor, respectively. Knockdown of Rictor selectively abrogated the activation of Akt whereas knockdown of Raptor selectively blocked the activation of S6K1 (Fig. 3B). Silencing of Raptor completely blocked PDGF-induced phenotypic conversion of VSMCs whereas silencing of Rictor had no effect (Fig. 3C and D). In addition, loss of AngII-induced contraction by PDGF stimulation was significantly blocked by silencing of Raptor (Fig. 3E). However, silencing of Rictor had no effect.

3.4. mTORC1 regulates PDGF-induced VSMC proliferation

Since the proliferation rates of synthetic- and contractile-type VSMCs are quite different, we next examined the proliferation of contractile VSMCs and synthetic VSMCs acquired by PDGF stimulation. As shown in Fig. 4A, the proliferation rate was highly elevated in synthetic VSMCs obtained from phenotypic switch by PDGF stimulation. In addition, proliferation of contractile VSMCs was highly elevated by PDGF stimulation and was abrogated by inhibition of ERK or mTORC1 (Fig. 4B). Furthermore, silencing of Raptor significantly blocked PDGF-induced proliferation of VSMCs whereas silencing of Rictor had no effect (Fig. 4C).

4. Discussion

In the present study, we demonstrated that mTORC1 mediates PDGF-induced phenotypic conversion of VSMCs by regulating expression of contractile marker proteins. In addition, we showed that PDGF-stimulated proliferation and promoter activity of contractile marker genes were controlled by the mTORC1 pathway. Notably, our data suggested that mTORC1, but not mTORC2, regulates the PDGF-induced phenotypic switch from contractile to synthetic VSMCs.

It has been reported that PDGF can modulate the phenotypic status of VSMCs [4,5,8]. In particularly, PDGF is required for development of neointima formation during atherosclerosis. In addition, PDGF expression is enhanced in atherosclerotic lesions and is involved in the intimal thickening [20]. Therefore, it is reasonable that PDGF can cause phenotypic switch of VSMCs from the non-proliferative contractile type to the proliferative synthetic type to facilitate neointima formation in atherosclerotic lesions. In correlation with this idea, our data showed that PDGF suppressed the expression of contractile marker proteins, such as SMA, SM22α,

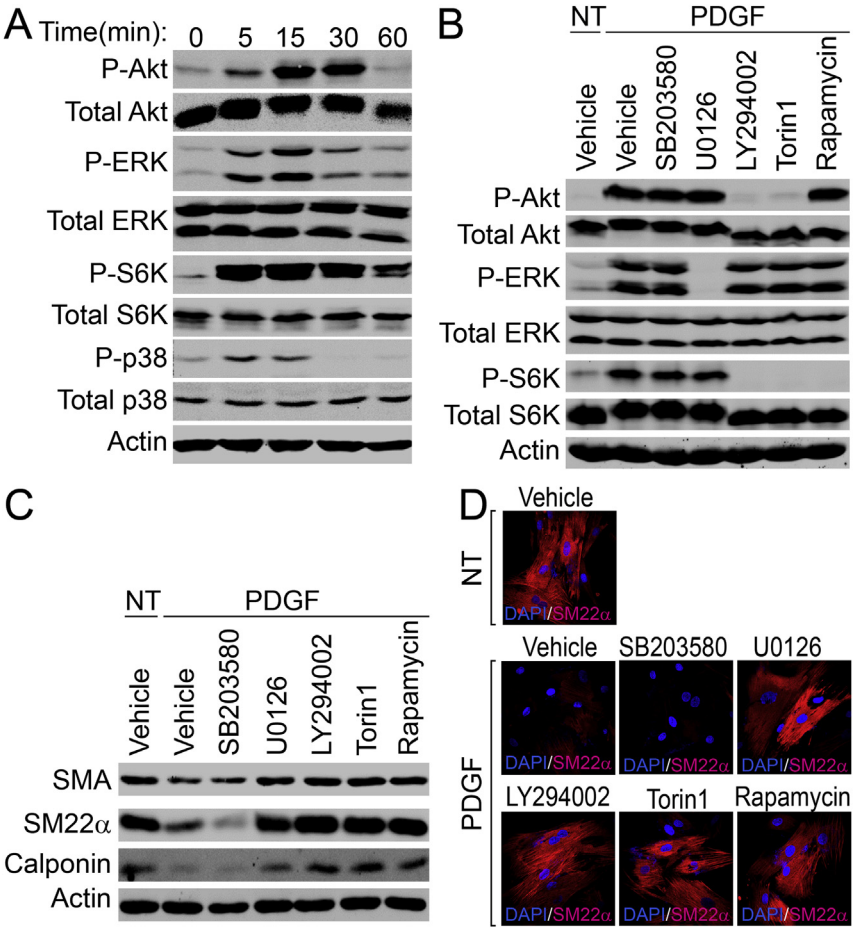

Fig. 2. mTOR is necessary for PDGF-dependent phenotypic conversion of VSMCs. (A) Contractile-type VSMCs were stimulated with PDGF at the indicated times, and activation of S6K1, ERK, Akt, and p38 MAPK was verified by western blot analysis. (B) Contractile-type VSMCs were pretreated with the indicated inhibitors (p38 MAPK inhibitor, SB203580, 10 μM; ERK inhibitor, U0126, 10 μM; PI3K inhibitor, LY294002, 50 μM; mTOR inhibitor, Torin1, 100 nM; mTORC1 inhibitor, Rapamycin, 100 nM) and PDGF-dependent activation of each signaling molecule was verified by western blot analysis. (C and D) Contractile-type VSMCs were stimulated with PDGF in the presence of the indicated inhibitors, and expression of marker proteins was measured by western blot analysis and immunocytochemistry.

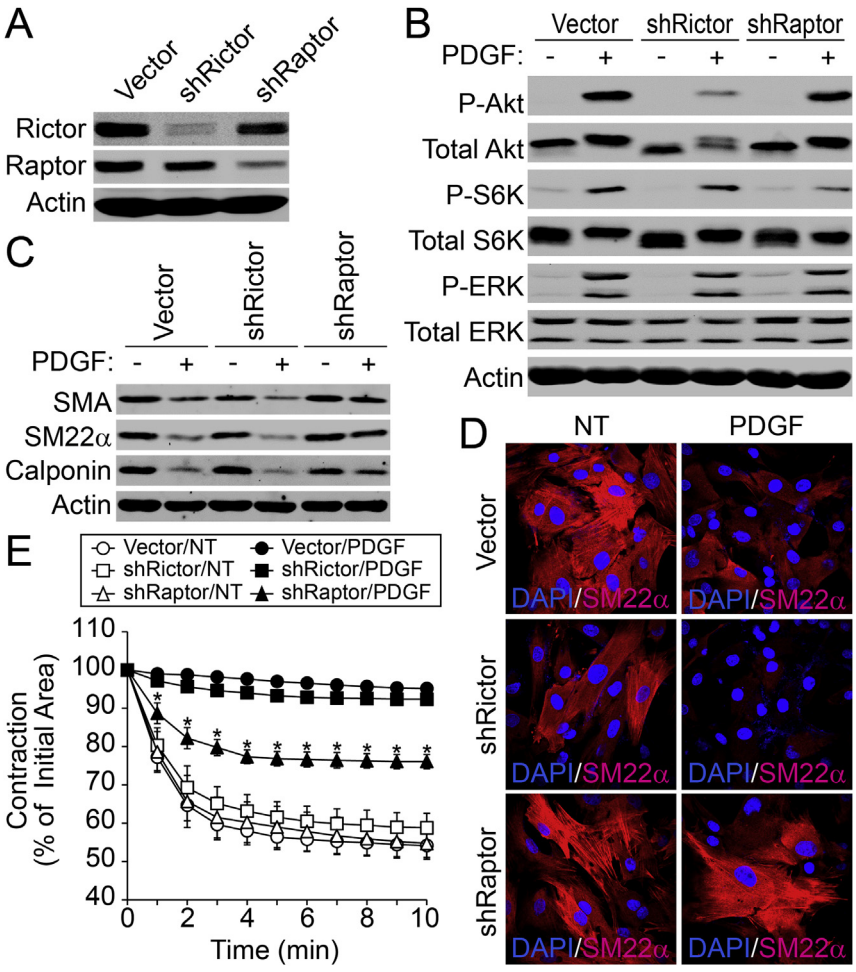

Fig. 3. mTORC1 is required for PDGF-induced phenotypic change of VSMCs. Either Rictor or Raptor was silenced in contractile VSMCs (A). Cells were stimulated with PDGF, and activation of S6K1, ERK, and Akt was examined by western blot analysis (B) or expression of the indicated smooth muscle marker proteins was verified by western blot analysis (C) and immunocytochemistry (D). (E) After silencing of either Rictor or Raptor in contractile VSMCs, cells were stimulated with PDGF for 4 days and then embedded in collagen gel. AngII-dependent contraction was quantified as described in "Materials and Methods". Data are means ± S.D. of three independent experiments ($n = 3$ for each experiment). Asterisks indicate statistical significance ($P < 0.05$).

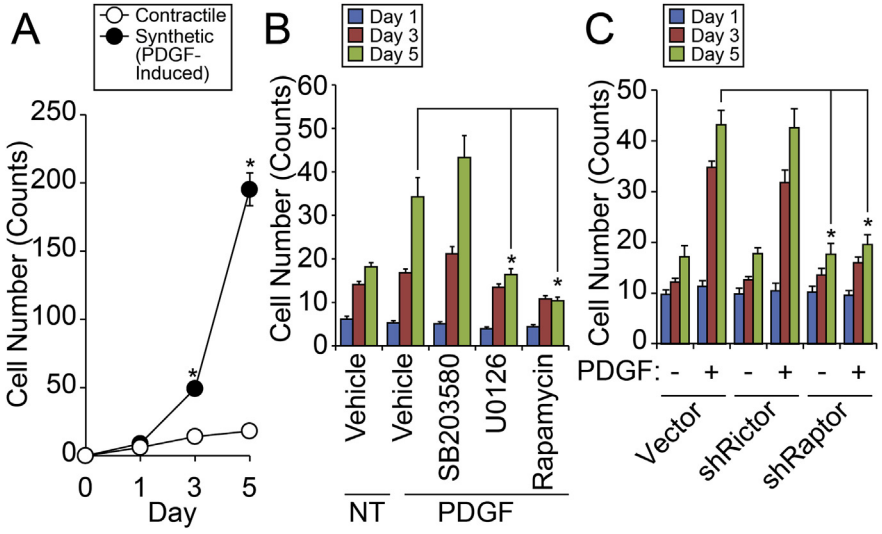

Fig. 4. mTORC1 regulates PDGF-dependent hyper-proliferation of VSMCs and promoter activity of smooth muscle marker genes. (A) Contractile VSMCs were stimulated with PDGF for 4 days, and proliferation was measured. (B) Contractile VSMCs were stimulated with PDGF in the presence or absence of the indicated inhibitors, and cell number was counted at the indicated time points. (C) Either Rictor or Raptor was silenced in contractile VSMCs and PDGF-dependent proliferation was measured at the indicated time points. Data are means ± S.D. of three independent experiments ($n = 3$ for each experiment). Asterisks indicate statistical significance ($P < 0.05$).

MLCK, MHC, and calponin (Fig. 1), as well as promoter activity of SMA or SM22α (Online Figure S2). In addition, phenotypic conversion of VSMCs by PDGF strongly enhanced their proliferation rate (Fig. 4A), suggesting pivotal role of PDGF in vascular hyperplasia.

It has been reported that the ERK signaling pathway regulates PDGF-dependent phenotypic conversion of VSMCs [21]. In addition to this finding, we also demonstrated that mTORC1 plays an essential role in PDGF-dependent phenotypic conversion of VSMCs. mTOR exists as a multi-protein subunit of two distinct complexes, mTORC1 and mTORC2. mTORC1, which is regulated by PI3K, and comprised of a rapamycin-sensitive complex, plays critical roles in cancer development, protein synthesis, cell proliferation and cell growth. mTORC2, which is also a downstream effector of PI3K and is a rapamycin-insensitive complex, mediates cancer cell migration and metastasis by selective activation of Akt1 [15,16]. Several lines of evidence support the involvement of mTORC1 rather than mTORC2 in PDGF-dependent phenotypic conversion of VSMCs. For example, our results showed that down-regulation of contractile marker genes, including SMA, SM22α and calponin, by PDGF was blocked by pharmacological inhibition of PI3K by LY294002 or of both mTORC1 and mTORC2 by Torin1 (Fig. 2C). Notably, inhibition of mTORC1 by rapamycin or disruption of mTORC1 by silencing Raptor blocked PDGF-dependent suppression of marker gene expression in VSMCs (Figs. 2C and 3C). However, disruption of mTORC2 by silencing Rictor had no effect (Fig. 3C). Blocking of the PDGF-dependent phenotypic switch by mTORC1 inhibition was further confirmed by measuring VSMC contractility. Loss of AngII-induced contraction in PDGF-stimulated VSMCs was significantly blocked by silencing of Raptor whereas silencing of Rictor had no effect (Fig. 3E). Therefore, it is likely that the mTORC1 pathway plays a crucial role in the regulation of the PDGF-induced phenotypic switch of VSMCs.

mTORC1 has various downstream substrates including S6K1, 4E-BP1, and Unc-51 like autophagy activating kinase (ULK1) [16]. Currently, the downstream substrate of mTORC1 that regulates phenotypic conversion of VSMC is still unclear. However, S6K1 might not be involved in this process since inhibition of S6K1 did not affect the PDGF-dependent phenotypic change of VSMCs (Online Figure S3). Recently, it has been reported that down-regulation of autophagy facilitates epithelial mesenchymal transition (EMT) [22]. mTORC1 down-regulates autophagy through phosphorylation of ULK1, and EMT is a similar physiological process to the phenotypic change of VSMCs. In this regard, we suggest that ULK1 is a possible target in the mTORC1-regulated phenotypic change of VSMCs.

In conclusion, PDGF stimulates phenotypic conversion of VSMCs through the activation of mTOR as well as ERK. mTORC1, but not mTORC2, plays an essential role in PDGF-dependent phenotypic conversion of VSMCs. PDGF-dependent activation of mTORC1 shifts the phenotype of VSMCs from contractile to synthetic, resulting in the hyper-proliferation of VSMCs, which is a typical characteristic in neointima formation of vascular walls. Therefore, mTORC1 may be a possible therapeutic target for atherosclerosis.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.097.

Transparency document

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