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TGF-β1-induced LPP Expression Dependant on Rho Kinase during Differentiation and Migration of Bone Marrow-derived Smooth Muscle Progenitor Cells*

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Summary: Lipoma preferred partner (LPP) has been identified as a protein which is highly selective for smooth muscle progenitor cells (SMPCs) and regulates differentiation and migration of SMPCs, but mechanisms of LPP expression are not elucidated clearly. The aim of the present study was to discuss the mechanisms by which LPP expression is regulated in the differentiation and migration of SMPCs induced by TGF-β1. It was found that TGF-β1 could significantly increase the expression of LPP, smooth muscle α-actin, smooth muscle myosin heavy chain (SM-MHC), and smoothelin in SMPCs. Moreover, inactivation of Rho kinase (ROK) with ROK inhibitors significantly inhibited LPP mRNA expression in TGF-β1-treated SMPCs and mouse aortic smooth muscle cells (MAoSMCs). At the same time, LPP silencing with short interfering RNA significantly decreased SMPCs migration. In conclusion, LPP appears to be a ROK-dependant SMPCs differentiation marker that plays a role in regulating SMPCs migration.
Key words: lipoma preferred partner; smooth muscle progenitor cells; differentiation; migration; Rho kinase

Although differentiation of smooth muscle cells (SMCs) is an essential event for vascular development, little is known regarding the specific factors that promote specific differentiation of pluripotential mesenchymal cells into the SMCs lineage or maturation of early SMCs into a mature contractile form[1]. Transforming growth factor (TGF)-β1, a potent multifunctional cytokine, coordinately up-regulates a variety of SMCs differentiation marker genes including smooth muscle α-actin (αSMA), smooth muscle myosin heavy chain (SM-MHC), and h1-calponin in cultured SMCs derived from mature blood vessels[2, 3], and is thought to play a key role in modulating vascular development, although its specific effects on SMCs and their precursors during development remain unclear. Smooth muscle progenitor cells (SMPCs) can be isolated from bone marrow and they are able to self-renew and differentiate into SMCs in response to TGF-β1[4-7]. Moreover, SMPCs have a potential to home to damaged vessels and differentiate into smooth muscle-like cells (SMLCs), thereby contributing to the pathogenesis of vascular diseases[8-11].

Recently, the Lin-11, Isl-1 and Mec-3 (LIM) domain protein lipoma preferred partner (LPP) was identified as a novel SMCs marker[12-14] and in a followed-up study[15] LPP was shown to regulate SMCs migration. LPP[16] belongs to a subclass of LIM related proteins which includes zyxin, Trip6, Ajuba, LIMD1, WTIP, and Cal[17]. Like other members of this gene family, LPP possesses multiple protein-protein interaction domains including C terminally located LIM domains, proline-rich motifs, and ENA/VASP-homology regions in structure. It suggests that LPP and its closest homologue, Trip6[18], can enhance cell migration *in vitro*. Meanwhile, LPP expression is highly selective in the SMCs of adult animals[19-22]. Although LPP appears to be highly selective in regulating differentiation and migration of SMCs, as yet no studies have examined the mechanisms by which the SMCs marker gene expression and migration of SMPCs were regulated. This study demonstrated that LPP appears to be a Rho-kinase (ROK)-dependent SMPCs differentiation marker that plays a positive role in regulating SMPCs migration.

1 MATERIALS AND METHODS

1.1 Cell Culture

Mononuclear cells obtained by the Ficoll-Paque protocol from murine (C57BL6 mouse) bone marrow (2×10^5 cells/mL) were cultured in Dexter-type condition. Bone marrow-derived adhesion cells appeared within a week (early-phase adhesion cells) and gradually grew into bone marrow mesenchymal stem cells (BMSCs) during the later 3 to 7 weeks. A 510-base fragment of the mouse sm22 promoter that works specifically in SMPCs was obtained by polymerase chain reaction (PCR) from the plasmid psm22α-441 (including sm22α promoter from –441 to +441 bp, kindly provided by Dr. Gary K. Owens, University of Virginia, USA). The sequences of the for-

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ward and reverse primers were 5'-GCCGCGAATTCCA-AGTCAAGTTCCCACC-3' and 5'-GCGCCGGATCCA-TAGCTGGAAGGAGAGTAG-3', respectively. The fragment was integrated into a promoterless green fluorescent protein (GFP) vector (kindly provided by Dr. Zuoyan ZHU, Institute of Hydrobiology, Chinese Academy of Science, China). By the lipofection method (Invitrogen, USA), the plasmid was transfected to the early-phase adhesion cells on the 5th day after seeding of the mononuclear cells[7, 8]. Using Fluorescence Activated Cell Sorter (FACS) Vantage, GFP-positive BMSCs were successfully obtained.

BMSCs were plated at a density of $5\times10^3/cm^2$ (day 0) and cultured in hygromycin-free Dulbecco's modification of Eagle's medium (DMEM, Sigma, USA) supplemented with 7.5% fetal bovine serum (FBS, HyClone, USA), 200 μg/mL L-glutamine, 100 U/mL penicillin/streptomycin. Then the cells were treated with TGF-β1 (10 ng/mL, Sigma, USA) on the day 1. The medium (the same as the day 1) was changed on the day 3, and the cells were collected on the day 5. Some cells were treated with a ROK inhibitor H1152 (0.5 μmol/L, Calbiochem, USA) 1 h before, during and after differentiation. The medium was replaced by basal medium 24 h after the treatment, and then the cells were collected after culture for 6 days for mRNA extraction, or fixed for fluorescence imaging with their corresponding time controls. Mouse aortic media-derived SMCs (MAoSMCs) were cultured in DMEM containing 10% fetal cattle serum (FCS) and served as controls. The confluent cells cultured in gelatin-coated wells (Biocoat, Bencton Dickinson, USA) were treated with the ROK inhibitor H1152 (0.5 μmol/L) for 24 h with low serum concentration (0.5%). After 12 h, the cells were harvested for mRNA extraction, or fixed for fluorescence imaging.

1.2 RNA Interference and Cell Transfection

The small interfering RNA (siRNA) constructs against LPP were synthesized by *in vitro* transcription according to the manufacturer's instructions (Ribobio, China) using the following primers: for Si-LPP-A (5'-AACAAGGTCACCCAAATACCTCCTGTCTC-3' and 5'-AAAGGTATTTGGGTGACCTTGCCTGTCTC-3'); for Si-LPP-B (5'-AAATGACTCTGACCCTACCT-ACCTGTCTC-3' and 5'-AATAGGTAGGGTCAGAG-TCATCCTGTCTC-3'). Antisense reverse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) oligonucleotides were used as controls (Ribobio, China). SMPCs were split and seeded into 6-cm dishes. After 24 h, cells with about 70% confluence were transfected with 1 μL of 20 μmol/L LPP-siRNA or control oligonucleotides. Transient transfections were performed by Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, USA).

1.3 Cell Proliferation Assay

Both SMPCs and MAoSMCs stimulated by TGF-β1 (10 ng/mL, Sigma, USA) were seeded at a density of 5×10^4 cells per well on a 24-well plate coated with type Ⅰ collagen and incubated overnight with DMEM supplemented with 5% FCS. Similar initial seeding density was confirmed 12 h after plating by using cell-titer MTS assay (Cell-Titer AQ, No. G5421, Promega, USA). This generated a baseline seeding absorbance (*A*) for both cell types. All cells had growth arrest for 24 h in serum-free DMEM, and then released from growth arrest with addition of DMEM supplemented with 5% FCS, and the cell number in each well was determined by cell-titer assay at 2nd, 4th, 6th, and 8th day after serum stimulation. Proliferation of SMPCs and MAoSMCs was expressed as ratio of the *A* generated at each time point to the initial seeding *A* for each cell type.

1.4 Isolation of RNA and Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from cultured cells using TRIzol (Invitrogen Corp., USA) and the RNA quality was assessed on an Agilent Technologies bioanalyzer system. Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed using Promega's Access RT-PCR kit (Promega, USA). The sequences of the forward and reverse primers were as follows: (+) 5'-TACA-AGCAGCCTGGAGGAGAG-3' and (–) 5'-GCACTTT-GAAAGCCCCTTCATC-3' for mouse LPP; (+) 5'-CGC-TGTCAGGAACCCTGAGA-3' and (–) 5'-CGAAGC-CGGCCTTACAGA-3' for mouse αSMA; (+) 5'-TGG-ACACCATGTCAGGGAAA-3' and (–) 5'-ATGGACA-CAAGTGCTAAGCAGTCT-3' for mouse SM MHC; (+) 5'-GCTGGCATCCGCCGAGTG-3' and (–) 5'-GCACC-TTACCAGGGTCCAATGT-3' for mouse smoothelin; (+) 5'-GGCTCATGACCACAGTCCAT-3' and (–) 5'-GCCTGCTTCACCACCTTCT-3' for mouse GAPDH. Expression levels were normalized to an internal control gene GAPDH.

1.5 Immunostaining

Cultured cells were fixed with 3.0% formaldehyde in phosphate buffered saline (PBS) for 10 min at room temperature, rinsed with PBS and permeabilized with 0.5% Triton X100 in PBS for 5 min, and then immunolabelling was carried out by standard methods. Anti-KDR mouse antibody (1:500; Biolegend, USA), anti-LPP goat polyclonal antibody (1:500; Santa Cruz, USA), and anti-α-SMA mouse antibody (1:500; Sigma, USA) were used as primary antibodies. The secondary antibodies used were as follows: FITC-conjugated goat anti-mouse IgG (1:500; Jackson ImmunoResearch Laboratories, USA) or a rhodamine Red-X anti-mouse (1:500, Boster, China). Captured images were imported into NIH Image base, and an intensity threshold level was set to discern the positive cells from background labeling, and then the positive cell percentages were calculated.

1.6 Western Blot Analysis

The cells were assayed 48 h after transfection for Western blotting. Western blotting was carried out using Supersignal West Dura Extended Duration Substrate (Pierce, USA) and the following primary antibodies, mouse monoclonal αSMA (1:1000, Sigma, USA), goat polyclonal anti-LPP antibody (1:1500, Santa Cruz, USA), and β-actin (1:400, Boster, China) were used for normalization. All blots were detected with appropriate secondary antibodies. Proteins were quantified by densitometric analysis.

1.7 Cell Migration Assay

The migration assay was performed using Transwell culture inserts with polyethylene terephthalate membranes (8-μm pores, Corning, USA). Twenty-four h after transfection with LPP-siRNA or control oligonucleotides, the TGF-β1-induced SMPCs were harvested with trypsin-ethylene diamine tetraacetic acid (EDTA), resus-

pended in serum-free medium, counted, and distributed at a density of 5×10^4 cells/well in the inserts or seeded in separate wells. Each determination was performed in triplicate. The cells were allowed to settle for 1 h before the addition of epidermal growth factor (EGF) (10 nmol/L) in the lower chamber and then allowed to migrate to the underside of the insert's membrane for 4 h at 37°C in 5% CO_2. At the end of the experiment, the cells were fixed in methanol. The cells on the upper surface of the membrane were mechanically removed with a cotton swab. The cells that migrated to the lower surface of the membrane were counted from three different fields with a magnification of 20×.

1.8 Statistical Analysis

All data were expressed as $\bar{x}\pm s_x$. For analysis of differences between two groups, Student's *t* test was performed. For multiple groups, ANOVA was carried out followed by Student-Newman-Keuls test. The level of statistical significance was set at $P<0.05$.

2 RESULTS

2.1 Morphological and Proliferation Kinetic Characteristics of SMPCs during TGF-β1-Induced Differentiation

The SMPCs stimulated by TGF-β1 were large spindle-shaped cells from clone-like SMPCs that assumed a characteristic "hill and valley-like" (fig. 1A and B) and "vessel-like" growth morphology (fig. 1C and D). The SMPCs were undifferentiated progenitor cells that expressed progenitor cell maker KDR+ but did not express smooth muscle cell differentiated marker αSMA before the TGF-β1 treatment. At the same time the MAoSMCs, as a kind of differentiated SMCs, expressed αSMA but did not express progenitor cell marker KDR+ (fig. 1E). Moreover, SMPCs had a significantly increased rate of proliferation as compared with that of MAoSMCs when induced by TGF-β1 ($P<0.05$, fig. 1F).

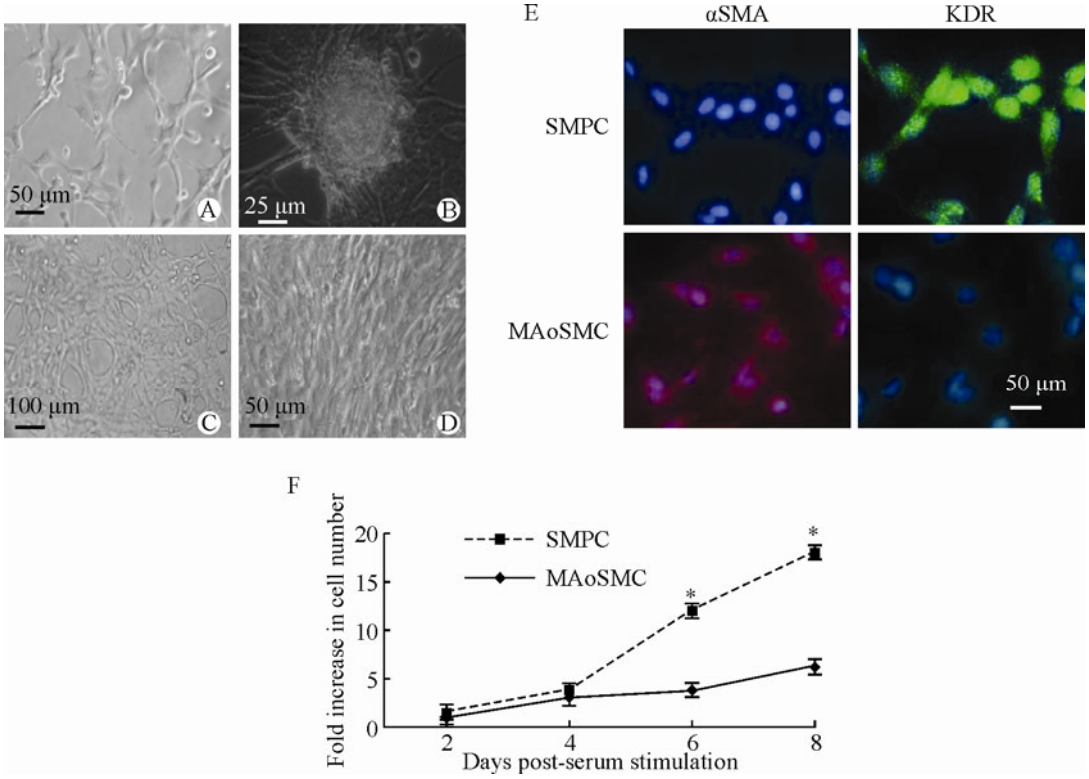

Fig. 1 Morphological and proliferation kinetic characteristics of SMPCs

A–D: Morphological characteristics of SMPCs during TGF-β1-induced differentiation; E: Immunocytochemial analysis of α-SMA and KDR expression in the MAoMSCs and SMPCs; F: Proliferation of SMPCs and MAoSMCs after release from growth arrest with 10 ng/mL TGF-β1 (n=3 for all experiments; $^*P<0.05$ *vs*. MAoSMC at the same time period after growth arrest)

2.2 Increased LPP Expression during TGF-β1-induced SMPCs Differentiation

SMPCs were shown to activate expression of SMC differentiation marker genes when treated with TGF-β1, which induced an intense increase of LPP and αSMA expression (fig. 2A), as well as mRNA expression of LPP and SM marker genes ($P<0.05$, fig. 2B and C). These results suggested that expression of the LPP gene and other SM marker genes could be up-regulated by TGF-β1.

2.3 ROK-dependent LPP Expression

As expression of a number of SMC genes is dependent on RhoA/ROK-mediated serum response factor (SRF) activation, we examined whether TGF-β1 induced LPP expression in a ROK dependent manner. SMPCs and MAoSMCs were treated with ROK inhibitor H1152. The LPP expression in SMPCs or MAoSMCs was down-regulated by approximately 50% by H1152 (1 μmol/L) either before ($P<0.05$, fig. 3A and B) or after TGF-β1 ($P<0.05$, fig. 3C and D) treatment. Similarly, H1152 treatment inhibited protein expression of LPP in SMPCs stimulated by TGF-β1 ($P<0.05$, fig. 3E and F).

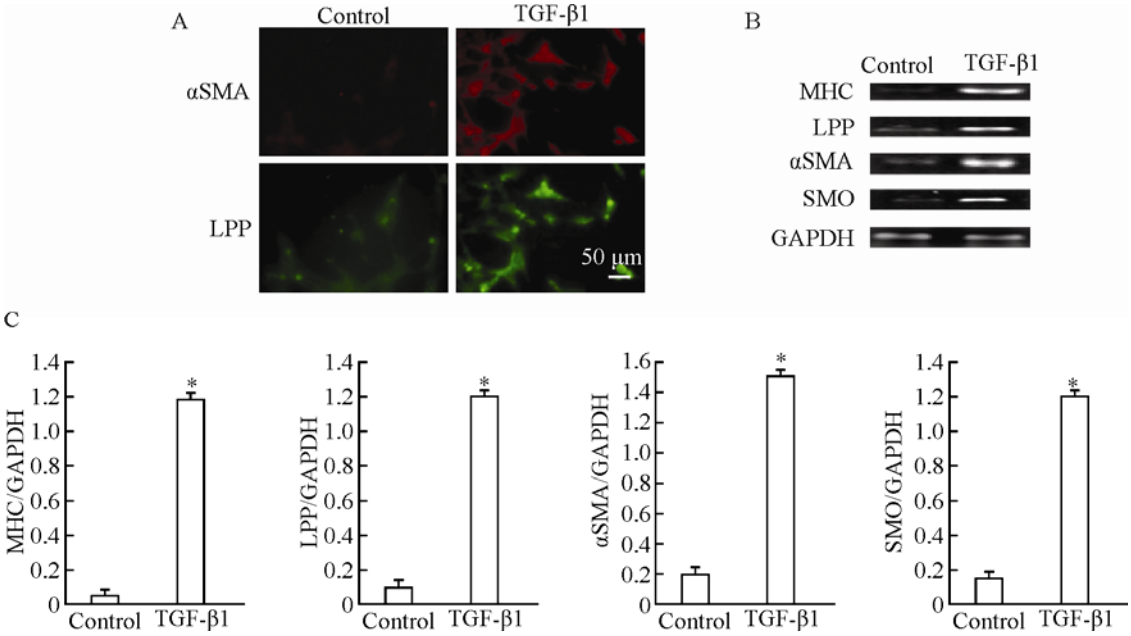

Fig. 2 TGF-β1-induced LPP, αSMA, MHC, and SMO expression in SMPCs

The cells were left untreated (control) or treated with 10 ng/mL TGF-β1 as described in Materials and Methods. A: Immunofluorescence of SMPCs stained with anti-LPP and anti-αSMA antibodies; B: RT-PCR analysis of MHC, LPP, αSMA and SMO expression in the SMPCs; C: Bar graphs of MHC, LPP, αSMA and SMO expression in the SMPCs by RT-PCR analysis (n=3 for all experiments, $^{*}P$<0.05 *vs*. the untreated cells)

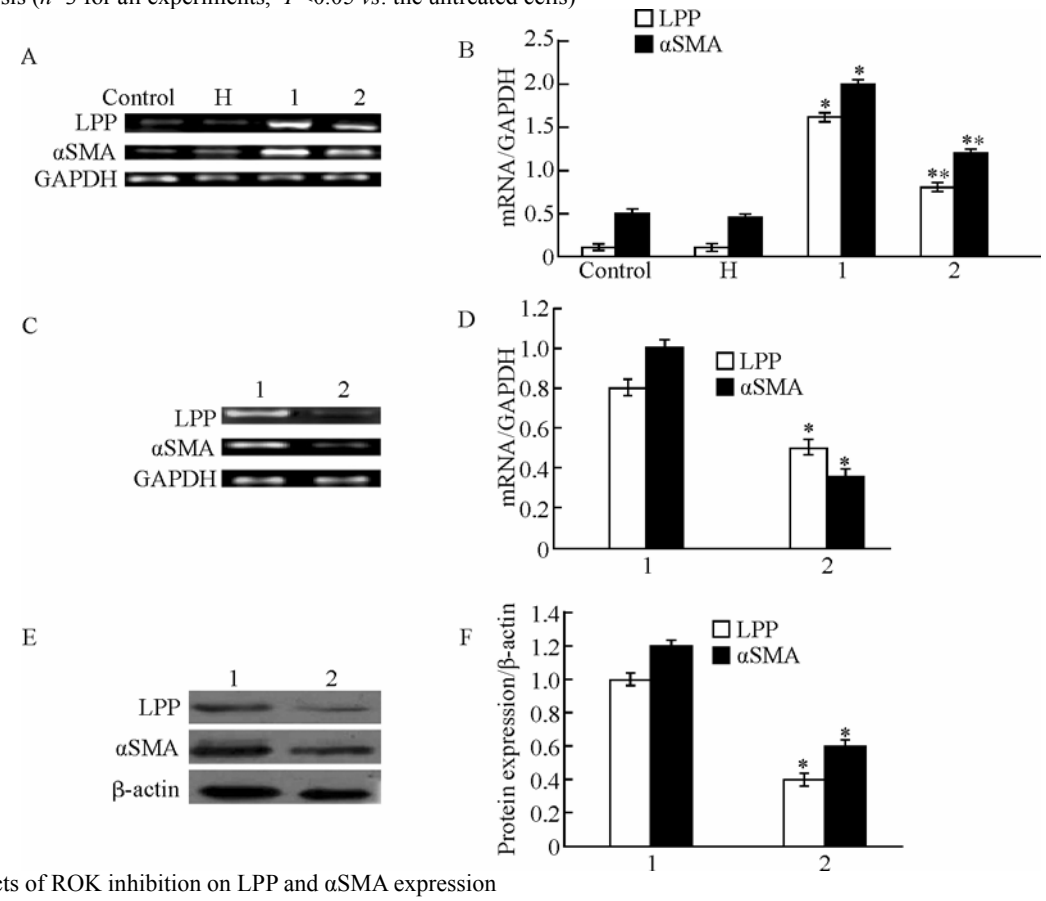

Fig. 3 Effects of ROK inhibition on LPP and αSMA expression

A, B: SMPCs were incubated with the ROK inhibitor H1152 (H) (0.5 μmol/L) 1 h before TGF-β1 addition and left in the medium during differentiation. Levels of LPP and αSMA mRNA were quantitated by RT-PCR and normalized to GAPDH. The untreated cells served as control (n=3 for all experiments, $^{*}P$<0.05 *vs*. control, $^{**}P$<0.05 *vs*. TGF-β1 treated group); C, D: Alternatively, MAoSMCs were incubated with H1152 for 24 h following TGF-β1 treatment. The cells were treated for 24 h with H1152 before measurement of LPP and αSMA expression by RT-PCR (n=3 for all experiments, $^{*}P$<0.05 *vs*. TGF-β1 treated group); E, F: Protein extraction and Western blotting for LPP and αSMA in the SMPCs incubated with H1152 for 24 h following the TGF-β1 treatment. LPP and αSMA protein contents are expressed as percentage of β-actin signal. 1: TGF-β1 treated group; 2: TGF-β1+H1152 treated group (n=3 for all experiment, $^{*}P$<0.05 *vs*. TGF-β1 treated group).

2.4 Modulation of TGF-β1-induced-SMPCs Migration by LPP Expression

SMPCs were treated with 10 ng/mL TGF-β1 as described in Materials and Methods. Forty-eight h after the transfection, the cells were seeded and settled in the upper well for 1 h before addition of EGF (10 nmol/L) to the lower chamber. After 4 h, the cells or the lower surface of the membranes were fixed, stained with crystal violet, and counted. The number of SMPCs that migrated in the lower chamber in response to EGF, used as chemoattractant, was counted. Figure 4A–D shows representative fields of the membrane after cell fixation. The percentage of cells stimulated with EGF that migrated to the lower surface of the membrane was significantly higher (fig. 4E, $P<0.05$) than that of the cells without stimulation (vehicle). In addition, in the presence of EGF, the total number of migrated cells transfected with LPP-siRNA was lower than that of the cells transfected with GAPDH-siRNA (fig. 4E, $P<0.05$). Moreover, Si-LPP treatment inhibited protein expression of LPP in the SMPCs with TGF-β1 treatment ($P<0.05$, fig. 4F and G).

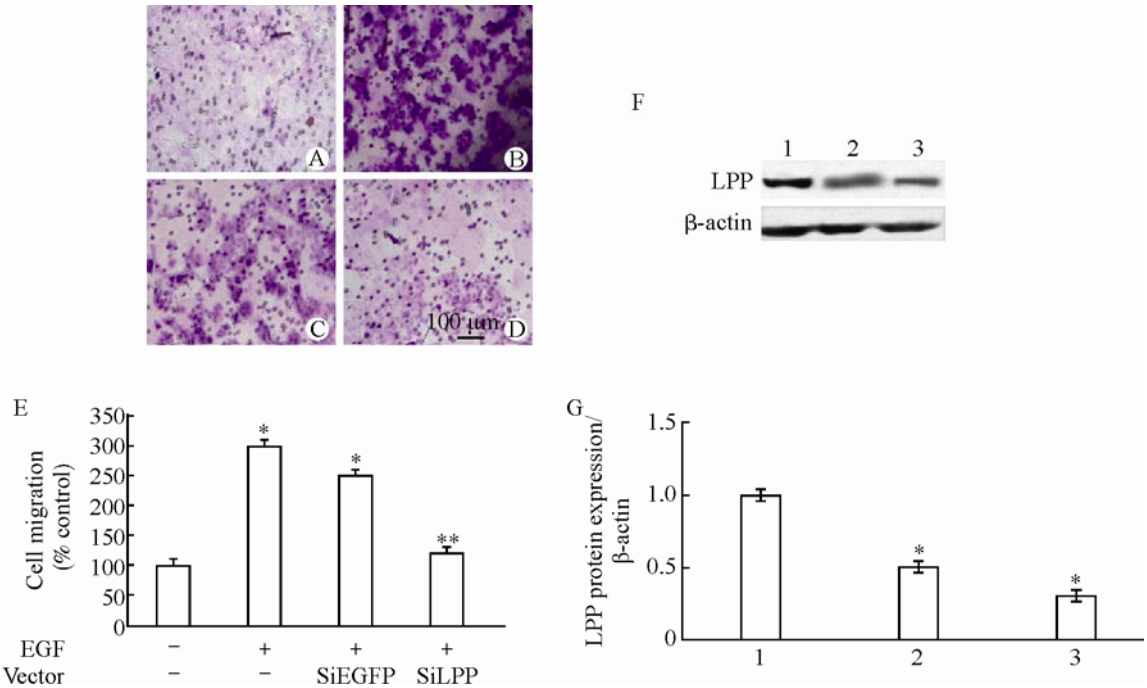

Fig. 4 Effects of LPP silencing on TGF-β1-treated SMPCs migration

A–D: Transwell assays of SMPCs migration. SMPCs were treated with 10 ng/mL TGF-β1 as described in Materials and Methods. Forty-eight h after the transfection, the cells were seeded and settled in the upper well for 1 h before addition of EGF (10 nmol/L) to the lower chamber. After 4 h, the cells or the lower surface of the membranes were fixed, stained with crystal violet, and counted (A: Untreated group; B: EGF treated group; C: SiEGFP transfected+EGF treated group; D: SiLPP transfected +EGF treated group); E: Bar graph of SMPCs migration (n=3, $^{*}P<0.05$ *vs*. untreated group, $^{**}P<0.05$ *vs*. SiEGFP+EGF treated group); F, G: LPP protein contents were quantitated by Western blotting and normalized to β-actin. 1: SiEGFP transfected group; 2: SiLPP-A transfected group; 3: SiLPP-B transfected group (n=3, $^{*}P<0.05$ *vs*. transfected SiEGFP group).

3 DISCUSSION

Although little is known about the mechanisms underlying the differentiation of SMCs from progenitor or stem cells (including pluripotential mesenchymal cells), TGF-βs are recognized as multifunctional proteins that play important roles in many aspects of embryonic development and adult homeostasis. These factors have various functions in different cells, and a completely different effect is shown to be dependent on the concentration of TGF-βs, the environment, or cellular stage. TGF-β1 has been shown to induce some smooth muscle markers from multipotent stem cells, such as neural crest stem cells[22, 23], embryonic cells[1], and mesenchymal stem cells[24]. TGF-βs exert their effects through heteromeric receptor complexes consisting of type Ⅰ and type Ⅱ serine/threonine kinase receptors. Intracellular signaling downstream of these receptor complexes is mediated by the Smad family. Several intracellular signaling pathways have also been shown to be activated by TGF-β1, including phosphatidylinositol 3-kinase (PI3K)/Akt, RhoA, and the mitogen-activated protein kinase superfamily[25, 26]. Inhibition or activation of the RhoA pathway may impact on the transcription of SMC marker genes in multiple ways including through the regulation of cytoskeletal dynamics, as well as through alterations in the expression and/or activity of myocardin or the myocardin related factors A and B (MKL1/1, MRTF-A, -B). This study demonstrated that the ROK inhibitor significantly depressed the expression of LPP, and SMCs marker genes in SMPCs differentiation induced by TGF-β1 (fig. 3). These results provide strong evidence that TGF-β1 is capable of inducing the SMC phenotype of mesenchymal progenitor cells through the RhoA/ROK signaling pathway.

Recent findings demonstrate that LPP is a novel marker of differentiated SMCs and expressed in the differentiated SMCs the same as a large panel of SMCs differention markers, αSMA, SM-MHC, SM22, and SM calponin[27, 28].

Interestingly, our study indicates that all promoters of these SMCs are regulated by the RhoA/ROK pathway as previously described[19, 29].

A special feature of LPP is the polyproline rich regions FPPPPP and FLPPPPPP at the amino-terminal half of the molecule[16]. The second proline sequence is also found in other actin regulatory proteins and is implicated in actin-based motility. Transwell migration assays showed that down-regulation of LPP induced by siRNA significantly reduced cell migration, which was further enhanced in the presence of EGF. The ability of the ROK inhibitor significantly depressed LPP mRNA expression in the differentiation of SMPCs induced by TGF-β1.

In conclusion, LPP is a differentiation marker of SMPCs and regulated through ROK-dependent pathway, also involved in regulating many other differentiation marker genes of SMCs. Of major interest, results of the present study also demonstrated that LPP plays a key role in regulating the migration of SMPCs induced by TGF-β1.

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