

ORIGINAL ARTICLE

Krüppel-like factor 4 mediates lysophosphatidic acid-stimulated migration and proliferation of PC3M prostate cancer cells

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Prostate cancer is the most frequently diagnosed malignancy and the second leading cause of cancer mortality among men in the United States. Accumulating evidence suggests that lysophosphatidic acid (LPA) serves as an autocrine/paracrine mediator to affect initiation, progression and metastasis of prostate cancer. In the current study, we demonstrate that LPA stimulates migration and proliferation of highly metastatic human prostate cancer, PC-3M-luc-C6 cells. LPA-induced migration of PC-3M-luc-C6 cells was abrogated by pretreatment of PC-3M-luc-C6 cells with the LPA receptor 1/3 inhibitor Ki16425 or small interfering RNA (siRNA)-mediated silencing of endogenous LPA receptor 1, implicating a key role of the LPA-LPA receptor 1 signaling axis in migration of PC-3M-luc-C6 cells. In addition, LPA treatment resulted in augmented expression levels of Krüppel-like factor 4 (KLF4), and siRNA or short-hairpin RNA (shRNA)-mediated silencing of KLF4 expression resulted in the abolishment of LPA-stimulated migration and proliferation of PC-3M-luc-C6 cells. shRNA-mediated silencing of KLF4 expression resulted in the inhibition of *in vivo* growth of PC-3M-luc-C6 cells in a xenograft transplantation animal model. Taken together, these results suggest a key role of LPA-induced KLF4 expression in cell migration and proliferation of prostate cancer cells *in vitro* and *in vivo*.

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INTRODUCTION

Prostate cancer continues to be the most common lethal malignancy diagnosed in men and the second leading cause of male cancer deaths.¹ Prostate cancer cells initially proliferate within a local area but later may metastasize, preferentially to bone, where the metastases may cause clinical problems, such as bone pain, suppressed mobility, replacement of hematopoietic tissue and compression of the spinal cord.² Cellular motility of highly metastatic prostate cancer cells is a critical step in progression and metastasis of prostate cancer. Nevertheless, the intracellular signaling pathways essential for migration of prostate cancer cells remain poorly understood.

Lysophosphatidic acid (LPA) is increasingly being recognized as an important multifunctional mediator that affects various cellular responses, including cell proliferation,

differentiation, adhesion and migration.³ LPA has been detected in many physiological and pathological biological fluids and tissues, including serum and malignant ascites from ovarian cancer patients,^{4,5} suggesting that LPA may serve as an autocrine/paracrine mediator in regulation of the functions of prostate cancer cells. LPA has been reported to promote the migration of prostate cancer cells, including primary prostate cancer from patients and various prostate cancer cell lines, such as LNCaP, DU145 and PC3 cells.^{6–8} In addition, expression of LPA receptors, such as LPA₁, LPA₂ and LPA₃, has been detected in prostate cancer cells.^{9,10} In particular, LPA₁ was found to have a key role in LPA-induced migration of prostate cancer cells, including LNCaP and PC3 cells. However, the downstream signaling mechanisms that mediate LPA₁-activated cell migration of prostate cancer cells are still elusive.

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The Krüppel-like factor (KLF) family proteins are transcription factors implicated in the regulation of a wide range of cellular processes, including proliferation, apoptosis, differentiation, inflammation, migration and tumor formation.¹¹ Krüppel-like factor 4 (KLF4), a member of the KLF family, is a zinc-finger transcription factor engaged in the regulation of differentiation and proliferation.¹² KLF4 has recently been reported as one of four factors (Oct4, Sox2, KLF4 and c-myc) having an ability to induce reprogramming of somatic cells into an embryonic stem cell-like state or pluripotent stem cells.^{13,14} Expression of KLF4 in primary breast ductal carcinoma and oral squamous carcinoma in association with an aggressive phenotype has been reported.¹¹ However, downregulation of KLF4 in several types of cancer, including prostate cancer, may contribute to cellular hyperproliferation and malignant transformation.^{15,16} Therefore, the role of KLF4 in tumorigenesis of prostate cancer is still unclear.

An increasing body of evidence suggests that KLF4 has a key role in chemotactic migration of several cell types, including mesenchymal stem cells, smooth muscle cells and cancer cells.^{17–19} Oxidized phospholipids have been reported to induce migration of vascular smooth muscle through a KLF4-dependent mechanism.¹⁷ We have previously demonstrated that platelet-activating factor or an oxidized phosphatidylcholine POVPC (1-palmitoyl-2-oxovaleroyl-*sn*-glycero-3-phosphorylcholine) stimulated migration of human bone marrow-derived mesenchymal stem cells by inducing expression levels of KLF4.^{18,19} On the contrary, overexpression of KLF4 resulted in suppressed migration of several cell types, including vascular smooth muscle cells, MDA-MB-231 breast cancer cells and RKO human colon cancer cells.^{20–22} Therefore, the role of KLF4 in the regulation of migration of prostate cancer cells is still unclear.

In this study, we provide evidence of migration and proliferation of prostate cancer cells stimulated by LPA through a KLF4-dependent mechanism *in vitro*, as well as a key role for LPA-stimulated expression of KLF4 in tumor growth of prostate cancer in a xenograft animal model.

MATERIALS AND METHODS

Materials

RPMI 1640 medium was purchased from HyClone (Thermo Fisher Scientific, Salt Lake City, UT, USA). Phosphate-buffered saline (PBS), trypsin and Lipofectamine reagent were purchased from Invitrogen (Carlsbad, CA, USA). LPA (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate) and Ki16425 were purchased from Sigma-Aldrich (St Louis, MO, USA). Platelet-derived growth factor-BB (PDGF-BB) was purchased from R&D Systems (Minneapolis, MN, USA). Anti-KLF4 rabbit antibody (ab26648) was purchased from Abcam (Cambridge, MA, USA). Antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, clone MAB374) was purchased from Millipore (Billerica, MA, USA). Anti-Ki67 rabbit antibody (NCL-Ki-67p) was purchased from Novocastra (Leica Microsystems, Buffalo Grove, IL, USA).

Cell culture

Luciferase-expressing PC-3M-luc-C6 cell line derived from PC-3M metastatic prostate cancer cells was purchased from PerkinElmer

(Waltham, MA, USA). PC-3M-luc-C6 cells were cultured in RPMI 1640 medium with 10% fetal bovine serum and penicillin/streptomycin, and maintained at 37 °C in 5% CO₂. Cells were serum-starved for 24 h before treatment with LPA.

Transfection with small interfering RNA (siRNA)

siRNA duplexes were synthesized, desalted and purified by Samchully Pharm Co Ltd (Siheung, Gyeonggi, Korea) as follows: LPA₁ 5'-GGACUUGGAAUCACUGUUUUU-3' (sense) and 5'-AAACAGU GAUCCAAGUCCUU-3' (antisense); LPA₂ 5'-CCGCGAGUCUGUC CACUAUUU-3' (sense) and 5'-AUAGUGGACAGACUCGCGGUU-3' (antisense); LPA₃ 5'-CAGCAGGUUACCUUUGUUUUU-3' (sense) and 5'-AACAAGGUAACUCCUGCUGUU-3' (antisense); KLF4 5'-GGTCTTGAGGAAGTGCTGA-3' (sense) and 5'-TGAGATGGGAA CTCTTTGTG-3' (antisense). The control siRNA (D-001206-13-05) was purchased from Dharmacon Inc. (Chicago, IL, USA).

Short-hairpin RNA (shRNA)-mediated silencing of gene expression

For generation of lentiviruses expressing shRNA, pLKO.1 constructs (2 µg) were co-transfected with pVSV-G (0.2 µg) and Δ8.9 (2 µg) using the calcium phosphate method in HEK293-FT cells. HEK293-FT cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and penicillin/streptomycin. Viral particles were harvested at 24 and 48 h, and infected into cells in the presence of 8 µg ml⁻¹ of polybrene. Puromycin (10 µg ml⁻¹) was used in selection of the lentivirus-infected cells. pLKO1-puro lentiviral vectors expressing LPA₁ shRNA (TRCN0000011368), KLF4 (TRCN000005313) or non-target control shRNA (SHC002) were purchased from Sigma-Aldrich. The functional sequence in the LPA₁ shRNA lentiviral vector is '5'-CCGGCCTTCTGAAGACTGTGGTCAT CTCGAGATGACCACAGTCTTCAGAAGGTTTTT-3'', targeting the LPA₁ gene sequence (5'-CCTTCTGAAGACTGTGGTCAT-3'); the sequence of sh-KLF4 is '5'-CCGGCCAGCCAGAAAGCACTACAAT CTCGAGATTGTAGTGCTTTCTGGCTGGTTTTT-3'', targeting the KLF4 gene sequence (5'-CCAGCCAGAAAGCACTACAAT-3').

Reverse transcription-polymerase chain reaction

Cells were treated as indicated, and total cellular RNA was extracted using the Trizol method (Invitrogen). For reverse transcription-polymerase chain reaction analysis, aliquots of 2 µg each of RNA were subjected to complementary DNA synthesis with 200 U of M-MLV reverse transcriptase (Invitrogen) and 0.5 µg of oligo (dT) 15 primer (Promega, Madison, WI, USA). The complementary DNA in 10 µl of the reaction mixture was amplified with 0.5 U of GoTaq DNA polymerase (Promega) and 10 pmol each of sense and antisense primers, as follows: LPA₁ 5'-GGACUUGGAAUCACUGUUUUU-3' (sense), 5'-AAACAGU GAUCCAAGUCCUU-3' (antisense), LPA₂ 5'-CCGCGAGUCUGUCCACUAUUU-3' (sense), 5'-AUAGUGGACA GACUCGCGGUU-3' (antisense); LPA₃ 5'-CAGCAGGAGUUACCUU GUUUU-3' (sense), 5'-AACAAGGUAACUCCUGCUGUU-3' (antisense); GAPDH 5'-TCCATGACAACCTTGGTATCG-3' (sense), 5'-TGTAGCCAAATTCGTTGTCA-3' (antisense). The thermal cycle profile was as follows: denaturing at 95.0 °C for 30 s, annealing at 52–58 °C for 45 s, depending on the primers used, and extension at 74.0 °C for 45 s. Each PCR reaction was carried out for 30 cycles, and PCR products were size-fractionated on 1.2% ethidium bromide/agarose gel and quantified under UV transillumination.

Cell migration and proliferation assays

Cell migration assays were performed using a 96-well chemotaxis chamber (ChemoTx, Neuro Probe, Gaithersburg, MD, USA). For the migration assay, the bottom side of the ChemoTx membrane was pre-coated overnight with 20 $\mu\text{g ml}^{-1}$ rat-tail type I collagen at 4 °C, and cell suspension in serum-free medium (5×10^3 cells per 100 μl) was added to the upper compartment of the chamber and separated from the lower chamber containing 100 μl of chemotactic agents by a polycarbonate filter (8- μm pore). To induce cell migration, serum-free medium containing LPA, PDGF-BB or vehicles was added in the lower compartment of the chemotaxis system. To assess the involvement of LPA receptors in LPA-stimulated migration, the LPA receptor inhibitor Ki16425 was added into the upper compartment of the chamber. After 12-h incubation at 37 °C in a 5% CO₂ atmosphere, the ChemoTx membrane was fixed with 4% paraformaldehyde, and non-migratory cells on the top side of the membrane were removed by gently wiping with a cotton swab. The membrane was stained with 4,6-diamidino-2-phenylindole (DAPI) and migrating cells were counted under a fluorescence microscope at $\times 100$ magnification.

To measure the effects of LPA on cell proliferation of PC-3M-luc-C6 cells, a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used: MTT is metabolized by NAD-dependent dehydrogenase to form a colored reaction product (formazan), and the amount of dye formed correlates directly with the number of cells. For determination of cell numbers, PC-3M-luc-C6 cells were seeded in a 24-well culture plate at a density of 2×10^4 cells per well, cultured for 48 h in normal growth medium, serum-starved for 24 h and treated with various reagents (or a vehicle control) for the indicated times. Cells were washed twice with PBS and incubated with 100 μl of MTT (0.5 mg ml^{-1}) for 2 h at 37 °C. Formazan granules generated by the cells were dissolved in 100 μl of dimethylsulfoxide, and the absorbance of the solution at 562 nm was determined using a PowerWave_x microplate spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT, USA) after dilution to a linear range.

Western blotting

Serum-starved PC-3M-luc-C6 cells were treated with appropriate conditions, washed with ice-cold PBS and then lysed in lysis buffer (20 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, 10 mM NaCl, 0.1 mM phenylmethyl sulfonyl fluoride, 1 mM Na₃VO₄, 30 mM sodium pyrophosphate, 25 mM β -glycerol phosphate, 1% Triton X-100, pH 7.4). Lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane and then stained with 0.1% Ponceau S solution (Sigma-Aldrich). After blocking with 5% nonfat milk, the membranes were immunoblotted with anti-KLF4 or anti-GAPDH antibodies for 2 h, and the bound antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence Western blotting system (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

Xenograft transplantation of tumor and *in vivo* monitoring

BALB/c-nu/nu mice were randomly divided into two groups (six mice in each group). Mice in the control groups received subcutaneous injection with sh-control-infected PC-3M-luc-C6 cells (1×10^6 cells per 200 μl PBS) and experimental groups received subcutaneous administration of sh-KLF4-infected PC-3M-luc-C6 cells (1×10^6 cells per 200 μl PBS). Tumor cells were subcutaneously injected into the right and left flanks of the mice. Imaging of mice was performed before treatment and weekly during 5 weeks using a IVIS Lumina bioluminescent imager (PerkinElmer) and data analysis was

performed using Xenogen living imaging software (V. 2.50). Mice were injected with D-Luciferin Firefly, potassium salt (Biosynth Inc., Itasca, IL, USA) 150 mg kg^{-1} I.P. shortly before imaging. All images were formatted with the same color-coded scale for visual assessment. The tumor volume was examined twice weekly: measurements of the length (mm) and width (mm) of the tumor mass were performed using electronic vernier calipers, and tumor volume (mm^3) was calculated as (length \times width \times width)/2. After 5 weeks, the mice were killed, and the weight of each xenograft tumor was determined.

Immunofluorescence staining

Immunostaining and confocal microscopy were used to determine the cellular expression patterns of proteins within xenograft tumor tissues. For immunostaining, specimens were incubated with anti-KLF4 or anti-Ki67 antibodies for 2 h, followed by incubation with Alexa Fluor 488-conjugated anti-rabbit secondary antibody for 1 h. The specimens were finally washed and mounted in Vectashield medium with DAPI for visualization of nuclei. Images of KLF4-positive cells, Ki67-positive cells and DAPI-positive nuclei were collected using a TCL-SP2 confocal microscope system (Leica Microsystems, Richmond Hill, Ontario, Canada).

Statistics

The results of multiple observations are presented as mean \pm s.d. Student's *t*-test was used for analysis of differences between the two groups. For multivariate data analysis, two-way analysis of variance was used for assessment of group differences, followed by *post hoc* comparisons tested using Scheffe's method.

RESULTS

LPA induces migration of PC-3M-luc-C6 cells through an LPA₁-dependent mechanism

To explore the role of LPA in migration of prostate cancer cells, we examined the effects of LPA on chemotactic migration of PC-3M-luc-C6 cells using the ChemoTx system from Neuroprobe. LPA augmented PC-3M-luc-C6 cell migration in a concentration-dependent manner with a maximal stimulation at 1 μM concentration (Figure 1a). In addition, LPA treatment resulted in stimulated migration of PC-3M-luc-C6 cells in a time-dependent manner (Figure 1b), suggesting that LPA positively regulates migration of prostate cancer cells.

To explore the role of LPA receptors in LPA-stimulated migration of PC-3M-luc-C6 cells, we examined the effects of Ki16425, an antagonist for LPA receptors 1 and 3, on the migration stimulated by LPA. As shown in Figure 2a, treatment with Ki16425 resulted in complete inhibition of LPA-stimulated migration of PC-3M-luc-C6 cells. To support the specific inhibition of LPA receptors by Ki16425, we examined the effects of Ki16425 on PDGF-BB-induced migration of PC-3M-luc-C6 cells. Treatment with Ki16425 had no significant impact on PDGF-BB-induced migration of PC-3M-luc-C6 cells, suggesting that LPA_{1/3} are responsible for LPA-induced migration of PC-3M-luc-C6 cells. Next, we examined the effects of siRNA-mediated depletion of LPA receptors on LPA-stimulated cell migration. Results of reverse transcription-polymerase chain reaction indicated expression of three LPA receptors (LPA₁, LPA₂ and LPA₃) in

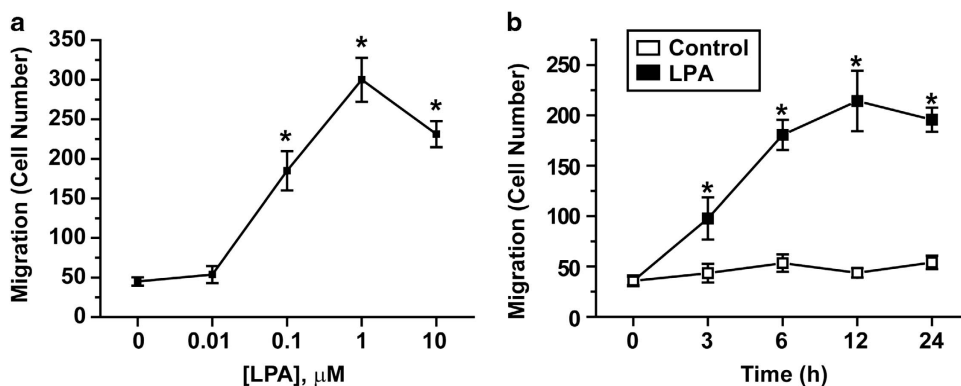


Figure 1 Effect of LPA on migration of PC-3M-luc-C6 cells. (a) Dose dependence of LPA-induced cell migration. PC-3M-luc-C6 cells were loaded into the upper compartments of a 96-well chemotaxis system and serum-free medium containing the indicated concentrations of LPA were placed in the lower chambers. The numbers of migratory PC-3M-luc-C6 cells were determined after 12 h. (b) Time dependence of LPA-induced cell migration. Serum-free medium containing $1\ \mu\text{M}$ LPA or vehicles (control) were added into the lower chambers and the number of PC-3M-luc-C6 cells migrated to the lower surface of filters was determined after the indicated time periods. Data indicate mean \pm s.d. ($n=4$). * $P<0.05$ versus control.

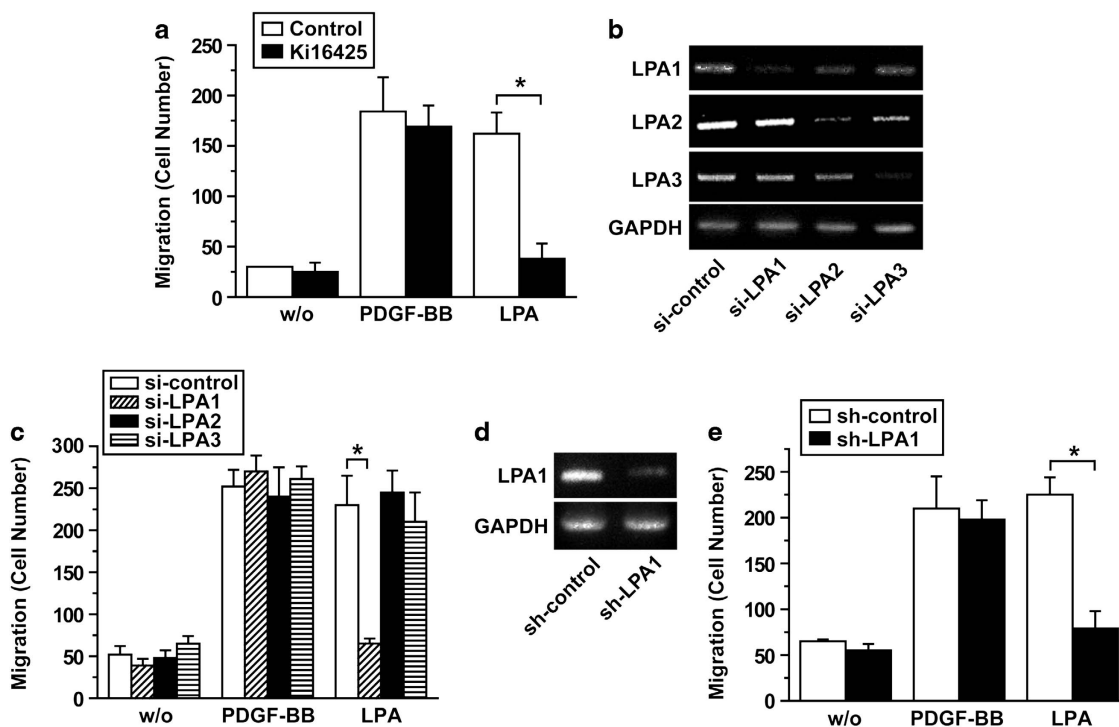


Figure 2 Role of LPA receptors in LPA-induced migration of PC-3M-luc-C6 cells. (a) Effects of Ki16425 on LPA-induced cell migration. PC-3M-luc-C6 cells were exposed to vehicles (w/o), $10\ \text{ng ml}^{-1}$ PDGF-BB or $1\ \mu\text{M}$ LPA in the absence or presence of $1\ \mu\text{M}$ Ki16425, followed by measurement of cell migration. (b) PC-3M-luc-C6 cells were transfected with control siRNA or siRNAs specific for LPA₁, LPA₂ or LPA₃, respectively. Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed for determination of mRNA levels of LPA₁, LPA₂, LPA₃ and GAPDH. (c) Effects of siRNA-mediated silencing of LPA receptors on cell migration. siRNA-transfected PC-3M-luc-C6 cells were treated with vehicles (w/o), $10\ \text{ng ml}^{-1}$ PDGF-BB or $1\ \mu\text{M}$ LPA for 12 h, followed by determination of the numbers of migrating cells. (d) PC-3M-luc-C6 cells were infected with lentiviruses expressing control shRNA or LPA₁-specific shRNA. RT-PCR was performed for determination of mRNA levels of LPA₁ and GAPDH. (e) Effects of LPA₁-specific shRNA on LPA-induced cell migration. shRNA-infected PC-3M-luc-C6 cells were treated with vehicles, $10\ \text{ng ml}^{-1}$ PDGF and $1\ \mu\text{M}$ LPA, followed by determination of the numbers of migrated cells. Data indicate mean \pm s.d. ($n=4$). * $P<0.05$.

PC-3M-luc-C6 cells (Figure 2b). The mRNA levels of LPA₁, LPA₂ or LPA₃ receptors in PC-3M-luc-C6 were specifically downregulated by transfection with specific siRNAs for LPA₁,

LPA₂ or LPA₃. Next, we examined the effects of silencing of endogenous expression of LPA₁, LPA₂ or LPA₃ receptors on LPA-stimulated migration. LPA-stimulated migration of

PC-3M-luc-C6 cells was markedly attenuated by depletion of the endogenous LPA₁ (Figure 2c), whereas depletion of the endogenous expression of LPA₂ and LPA₃ did not affect LPA-stimulated migration of PC-3M-luc-C6 cells. In addition, PDGF-BB-induced cell migration was not affected by silencing of LPA₁, LPA₂ or LPA₃ receptor, respectively. To confirm these results, we explored the effect of shRNA-mediated silencing of LPA₁ expression on LPA-stimulated cell migration. LPA₁ expression was downregulated by lentiviral infection of LPA₁-specific shRNA (Figure 2d). shRNA-mediated silencing of LPA₁ expression blocked the migration of PC-3M-luc-C6 cells stimulated by LPA but not PDGF-BB (Figure 2e). These results clearly indicate that LPA has a key role in LPA-stimulated migration through activation of LPA₁, but not LPA₂ or LPA₃.

KLF4 mediates LPA-stimulated migration and proliferation of PC-3M-luc-C6 cells

To explore the question of whether LPA treatment can induce expression of KLF4, PC-3M-luc-C6 cells were treated with LPA for 6 h and western blot analysis was then performed for determination of the expression levels of KLF4. As shown in Figure 3a, treatment with LPA resulted in a dose-dependent increase in the expression level of KLF4, with a maximal stimulation at 1 μM . In addition, treatment of PC-3M-luc-C6 cells with LPA showed a time-dependent increase in expression of KLF4 with a maximal stimulation at 6 h (Figure 3b).

We have previously reported on KLF4-mediated migration of human bone marrow-derived mesenchymal stem cells induced by oxidized low-density lipoprotein or POVPC.¹⁹ In order to explore the question of whether KLF4 is involved in LPA-induced migration of prostate cancer cells, we examined

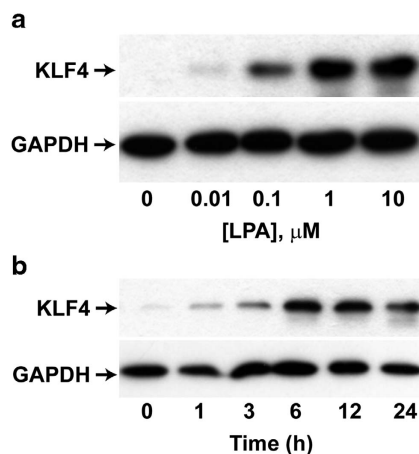


Figure 3 Effect of LPA on KLF4 expression in PC-3M-luc-C6 cells. (a) Dose dependence of LPA-induced KLF4 expression. Serum-starved PC-3M-luc-C6 cells were treated with the indicated concentration of LPA for 6 h. (b) Time dependence of LPA-induced KLF4 expression. Serum-starved PC-3M-luc-C6 cells were treated with 1 μM LPA for the indicated time periods. Western blotting was performed for determination of expression levels of KLF4 and GAPDH. Representative data from three independent experiments are shown.

the effects of siRNA-mediated silencing of KLF4 expression on LPA-stimulated migration of PC-3M-luc-C6 cells. Transfection of PC-3M-luc-C6 cells with KLF4-specific siRNA resulted in depleted expression of KLF4 (Figure 4a). siRNA-mediated knockdown of endogenous KLF4 in PC-3M-luc-C6 cells resulted in abrogation of LPA-induced migration of PC-3M-luc-C6 cells (Figure 4b). In order to provide support for these results, we further explored the effect of shRNA-mediated silencing of KLF4 on LPA-stimulated cell migration. As shown in Figures 4c and d, depletion of KLF4 expression with KLF4-specific shRNA showed inhibition of LPA-stimulated migration of PC-3M-luc-C6 cells. These results suggest a pivotal role of KLF4 in LPA-stimulated migration of PC-3M-luc-C6 cells.

Next, we explored the question of whether the LPA₁-KLF4 signaling pathway is implicated in LPA-stimulated cell proliferation of PC-3M-luc-C6 cells. PC-3M-luc-C6 cells were infected with lentiviruses expressing control shRNA, KLF4-specific shRNA or LPA₁-specific shRNA, followed by treatment with LPA for 3 days, and the numbers of PC-3M-luc-C6 cells were counted. LPA treatment resulted in augmented proliferation of PC-3M-luc-C6 cells, whereas shRNA-mediated silencing of KLF4 or LPA₁ in PC-3M-luc-C6 cells led to marked attenuation of LPA-induced cell proliferation (Figure 4e). Taken together, these results suggest that LPA-stimulated KLF4 expression may have critical roles in cell migration and proliferation of PC-3M-luc-C6 cells.

Knockdown of KLF4 expression inhibits *in vivo* tumor growth of PC-3M-luc-C6 cells

To explore the role of KLF4 in tumorigenesis, we examined the effect of silencing of KLF4 in PC-3M-luc-C6 cells on tumor growth *in vivo*. Using shRNA, we depleted KLF4 expression, followed by subcutaneous transplantation of PC-3M-luc-C6 cells into nude mice. As shown in Figures 5a and b, xenograft transplantation of control shRNA-infected PC-3M-luc-C6 cells, but not sh-KLF4-infected cells, resulted in formation of tumors. Control shRNA-infected PC-3M-luc-C6 cells, but not sh-KLF4-infected cells, exhibited a time-dependent increase in tumor volume (Figure 5c). Consistently, shRNA-mediated silencing of KLF4 resulted in reduced *in vivo* growth of PC-3M-luc-C6 xenograft tumors (Figure 5d), suggesting a critical role of KLF4 in tumor growth of PC-3M-luc-C6 cells *in vivo*. In addition, in order to determine whether expression of KLF4 was knocked down by infection with KLF4-specific shRNA *in vivo*, we performed immunofluorescence staining using Ki67 and KLF4 in tumor tissues. Injection of PC-3M-luc-C6 cells infected with lentivirus-expressing control shRNA resulted in detection of Ki67-positive and KLF4-positive immunoreactivity in the xenograft tumor. However, injection of PC-3M-luc-C6 cells infected with sh-KLF4 lentivirus resulted in abrogated expression of KLF4 in tumor tissues. In addition, the number of Ki67-positive cells was also decreased in xenograft tumor tissues transplanted with sh-KLF4-infected PC-3M-luc-C6 cells compared with the control xenograft tumor tissues (Figure 6). These results suggest a

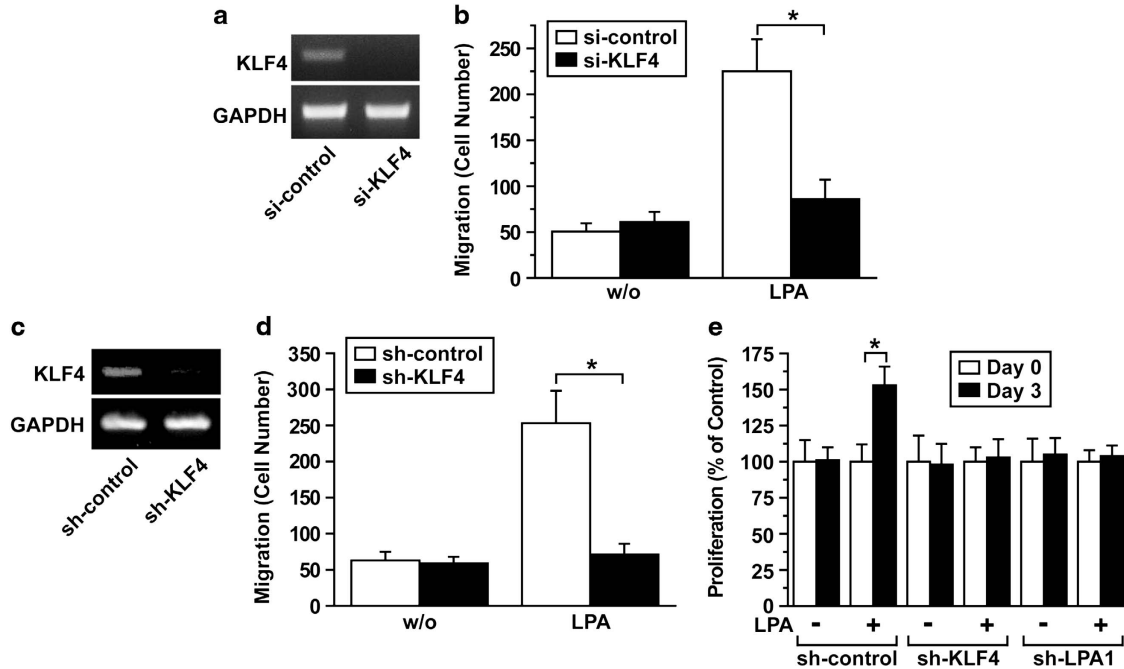


Figure 4 Role of KLF4 in LPA-induced migration and proliferation of PC-3M-luc-C6 cells. (a, c) siRNA or shRNA-mediated silencing of KLF4 expression. PC-3M-luc-C6 cells were transfected with siRNAs (a, control siRNA (si-control) or KLF4-specific siRNA (si-KLF4)) or infected with lentiviral shRNA (c, control shRNA (sh-control) or KLF4-specific shRNA (sh-KLF4)), followed by the determination of mRNA levels of KLF4 and GAPDH by RT-PCR. (b, d) Effects of KLF4 silencing on LPA-stimulated cell migration. siRNA-transfected (b) or shRNA-infected PC-3M-luc-C6 cells (d) were exposed to serum-free media containing vehicle or 1 μ M LPA and the number of cells that migrated to the lower surface of the filters was determined after 12 h. (e) PC-3M-luc-C6 cells were infected with sh-control, sh-LPA1 or sh-KLF4, followed by incubation with serum-free medium containing vehicles or 1 μ M LPA for 3 days, and the number of cells was quantified. Data indicate mean \pm s.d. ($n=4$). * $P<0.05$.

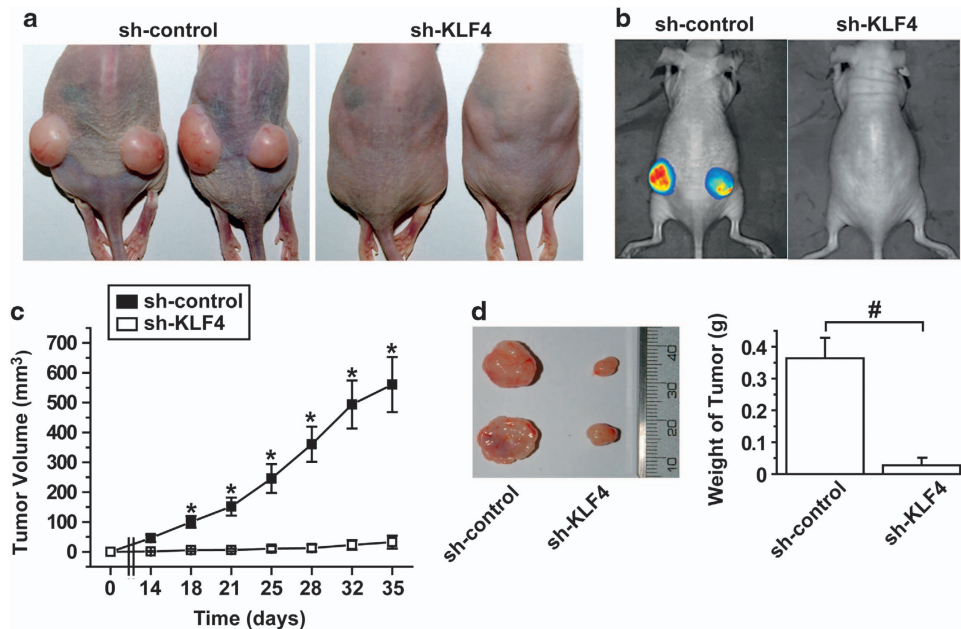


Figure 5 Role of KLF4 in growth of PC-3M-luc-C6 cells in a xenograft tumor model. (a) PC-3M-luc-C6 cells were infected with sh-control or sh-KLF4 lentiviruses and were then transplanted subcutaneously into nude mice. Mice were photographed 4 weeks after xenograft transplantation. Xenograft tumors were photographed after killing of mice. (b) Bioluminescence imaging of the xenograft tumors. (c) Volume of xenograft tumors was measured at the indicated time points. Data are expressed as mean \pm s.d. ($n=12$) * $P<0.05$ versus sh-control by two-way analysis of variance and Scheffe's *post hoc* test. (d) Weights of the tumor mass were quantified and expressed as mean \pm s.d. ($n=12$). # $P<0.05$ by Student's *t*-test.

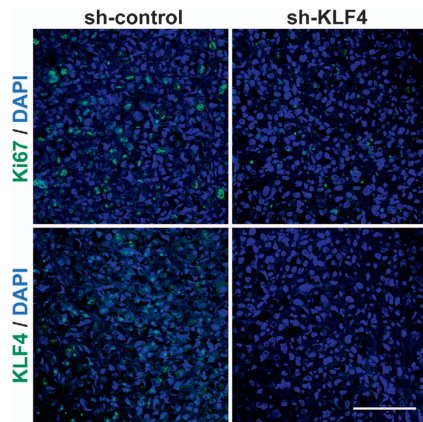


Figure 6 Role of KLF4 in proliferation of PC-3M-luc-C6 cells in the xenograft tumor. Immunostaining was performed for determination of expression of Ki67 and KLF4 in the xenograft tissues. Images of Ki67 and KLF4 (green color) are overlaid with images of nuclei (DAPI, blue color). Scale bar = 100 μ m.

pivotal role of KLF4 in the *in vivo* proliferation of human prostate tumor.

DISCUSSION

LPA has been implicated in invasion and metastasis of diverse cancer types.^{3,23} In the current study, we demonstrated that LPA stimulated migration of highly metastatic human prostate cancer, PC-3M-luc-C6 cells, through an LPA₁-dependent mechanism. Treatment with LPA_{1/3} inhibitor Ki16425 resulted in complete abrogation of LPA-induced migration of PC-3M-luc-C6 cells. In addition, siRNA- or shRNA-mediated depletion of LPA₁ showed specific abolishment of LPA-induced migration of PC-3M-luc-C6 cells. Prostate cancer cells have been shown to produce a high level of LPA and they respond to LPA via an autocrine/paracrine mechanism.⁷ In addition, LPA stimulated proliferation and invasion of prostate cancer cells,^{24,25} implying a critical role of LPA in development of prostate cancer. LPA exerts its cellular responses through its cognate G protein-coupled receptors.²⁶ LPA₁ has been implicated in LPA-induced cell migration in a variety of cell types.^{27–29} Together with previous reports demonstrating LPA-stimulated migration of prostate cancer cell lines through an LPA₁-dependent mechanism,⁸ results of the current study suggest that LPA₁, but not LPA₂ or LPA₃, is responsible for functional receptor-mediated LPA-stimulated migration of prostate cancer cells.

KLF4 has been implicated in differentiation during organogenesis of various tissues, including the skin, colon and eye.^{30,31} KLF4 also has a pivotal role in reprogramming of somatic cells for induction of pluripotent stem cells¹³ and maintenance of self-renewal and pluripotency of embryonic stem cells.³² Recent evidence has shown that KLF4 expression can be induced by several factors, including PDGF-BB, POVPC, platelet-activating factor and all-trans retinoic acid in vascular smooth muscle cells and human bone marrow-derived mesenchymal stem cells.^{18,19,22,33,34} POVPC has been reported to induce migration and phenotypic switching of

vascular smooth muscle cells through a KLF4-dependent mechanism.¹⁷ In the current study, we demonstrated that LPA treatment resulted in increased expression of KLF4, and siRNA- or shRNA-mediated silencing of KLF4 showed abrogation of LPA-stimulated cell migration. These results suggest a key role of KLF4 in LPA-stimulated migration of prostate cancer cells; however, further clarification of the signaling mechanisms involved in KLF4-mediated migration of PC-3M-luc-C6 cells is needed.

In this study, we demonstrated proliferation of PC-3M-luc-C6 cells by LPA through a KLF4-dependent mechanism. Silencing of KLF4 expression resulted in the decrease *in vivo* tumor growth of transplanted prostate cancer cells. High levels of KLF4 expression were reported in primary breast ductal carcinoma and oral squamous carcinoma.^{35,36} It has been reported that KLF4 was required for maintenance, cell migration and invasion of breast cancer stem cells. Knockdown of KLF4 in breast cancer cells (MCF-7 and MDA-MB-231) inhibited tumorigenesis in a xenograft tumor model.³⁷ In addition, KLF4 expression increases during progression of breast cancer and nuclear localization of KLF4 is associated with an aggressive phenotype.^{35,36} However, downregulated expression of KLF4 has been reported in various tumors, including colon, gastric, prostate and lung cancers, and downregulation of KLF4 expression was found to contribute to cellular hyperproliferation and malignant transformation.^{15,16} KLF4 expression was downregulated in hepatocellular carcinoma cells and KLF4 reverted epithelial mesenchymal transition by suppression of slug expression.³⁸ These controversial results regarding the function of KLF4 in tumorigenesis were partially resolved by results reported from a recent study, which demonstrated that p21Cip1 status might be a switch that determines the oncogene or tumor suppressor function of KLF4.³⁹ Therefore, these results support the current findings that KLF4 has a pivotal role in migration and proliferation of prostate cancer cells.

In conclusion, our study provides data demonstrating stimulation of cell motility of metastatic prostate cancer and tumorigenesis by LPA through a KLF4-dependent mechanism. However, further studies are needed in order to clarify the mechanisms of regulation of cell migration, proliferation and *in vivo* growth of prostate cancer cells by KLF4.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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