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# Diverse effects of $LPA_4$, $LPA_5$ and $LPA_6$ on the activation of tumor progression in pancreatic cancer cells

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ABSTRACT

Lysophosphatidic acid (LPA) is an extracellular biological lipid which interacts with G protein-coupled LPA receptors ($LPA_1$ to $LPA_6$). LPA signaling via LPA receptors mediates several cellular responses. In the present study, to assess the roles of $LPA_4$, $LPA_5$ and $LPA_6$ in cellular functions of pancreatic cancer cells, we generated LPA receptor knockdown cells from PANC-1 cells (PANC-sh4, PANC-sh5 and PANC-sh6 cells, respectively). In cell motility assay, PANC-sh4 and PANC-sh5 cells enhanced the cell motile activities, compared with control cells. In contrast, the cell motile activity of PANC-sh6 cells was suppressed. The invasive activities of PANC-sh4 and PANC-sh5 cells were markedly stimulated, while PANC-sh6 cells showed the low invasive activity. In colony assay, PANC-sh4 and PANC-sh5 cells formed the large sized colonies, but not PANC-sh6 cells. When endothelial cells were incubated with supernatants from PANC-sh4 and PANC-sh5 cells, the cell motility and tube formation of endothelial cells were significantly induced, but not PANC-sh6 cells. These results suggest that the diverse roles of $LPA_4$, $LPA_5$ and $LPA_6$ are involved in the activation of tumor progression in pancreatic cancer cells.



## 1. Introduction

Lysophosphatidic acid (LPA) is an extracellular physiological mediator and interacts with G protein-coupled transmembrane LPA receptors (LPA receptor-1 ($LPA_1$) to $LPA_6$) [1,2]. LPA signaling via LPA receptors induces a variety of cellular functions, such as cell proliferation, differentiation, morphogenesis, cell motility and protection from apoptosis [1,2]. In cancer cells, the individual LPA receptors exhibit the different responses, dependent on types of cells [3]. Recently, we reported that $LPA_3$ enhanced the cell motility, invasion and tumorigenicity in liver tumor cells [4]. In neuroblastoma cells, $LPA_2$ and $LPA_3$ stimulated the cell motile and invasive activities, while $LPA_1$ inhibited. Moreover, mutated $LPA_1$ increased the cell motile and invasive activities, matrix metalloproteinase (MMP) activation and angiogenesis in neuroblastoma cells [5,6]. In contrast, the cell motile activity was suppressed by $LPA_3$ in lung cancer cells [7].

Among LPA receptors, $LPA_4$, $LPA_5$ and $LPA_6$ are structurally distance from other LPA receptors [1,2]. $LPA_4$ and $LPA_5$ induced neurite retraction and stress fiber formation in neural cells. In addition, cell aggregation and adhesion were prompted by $LPA_4$ [1]. $LPA_6$ was involved in the pathogenesis of hypotrichosis simplex which is a group of hereditary isolated alopecias [8]. In some cancer cells, it has been reported that $LPA_4$ and $LPA_5$ negatively regulated the cell motile activities [9,10].

In the present study, to assess the roles of $LPA_4$, $LPA_5$ and $LPA_6$ in the activation of tumor progression in cancer cells, $LPA_4$, $LPA_5$ and $LPA_6$ knockdown cells were generated from human pancreatic PANC-1 cells. We demonstrate that the diverse functions of $LPA_4$, $LPA_5$ and $LPA_6$ regulate the activation of tumor progression, such as cell motility, invasion, tumorigenicity and angiogenesis in PANC-1 cells.

## 2. Materials and methods

### 2.1. Cell culture and establishment of LPA receptor knockdown cells

PANC-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Wako Pure Chemical Industries Ltd., Osaka, Japan)

*Abbreviations:* LPA, lysophosphatidic acid; $LPA_4$, LPA receptor-4; RT, reverse transcription; PCR, polymerase chain reaction.

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containing 10% FBS. To generate $LPA_4$, $LPA_5$ and $LPA_6$ knockdown (PANC-sh4, PANC-sh5 and PANC-sh6 cells, respectively) cells, a HuSH short hairpin RNA plasmid (29-mer) against target LPA receptor (Origene, Rockville, MD) was transfected into PANC-1 cells using X-tremeGENE HP Transfection Reagent (Roche Diagnostics GmbH). After transfection, cells were selected by the treatment of puromycin for at least 4 weeks and a stable clone was obtained. PANC-RFP cells were established as controls using a control (vector) plasmid without target sequence.

### 2.2. Cell proliferation assay

Cells were seeded at 3000 cells/well in 96-well plates and cultured in DMEM containing 10% FBS. The cell proliferation rates were measured at 0, 1, and 3 days using a Cell Counting Kit-8 (CCK-8) (Dojin Chemistry, Kumamoto, Japan). In addition, some cells were maintained in DMEM containing 5% charcoal stripped FBS (Sigma, St. Louis, MO, USA) and treated with LPA (Avanti Polar Lipids, Inc., AL, USA) at concentrations of 1 and 10 μM every 24 h. After 3 days, the CCK-8 was added to the individual plates. These assays were performed in triplicate [11].

### 2.3. Cell motility and invasion assays

Cells were seeded into a Cell Culture Insert (8 μm pore size) (BD Falcon, Franklin Lakes, NJ) at $5 \times 10^4$ cells in 200 μl serum-free DMEM (upper chamber) and placed in 24-well plates (lower chamber) containing 800 μl DMEM supplemented with 5% charcoal stripped FBS in the presence of LPA (10 μM). After 16 h incubation, the numbers of cells that had moved to the lower side of the filters were counted after Giemsa staining. For cell invasion assay, the filters were coated with Matrigel (12.5 μg/filter) (BD Falcon) and dried for at least 1 day. Cells were seeded into the filter at $1 \times 10^5$ cells and incubated for 20 h [4,5,7].

### 2.4. Gelatin zymography

To obtain supernatants from the individual cells, cells were cultured in serum-free DMEM with or without LPA at a concentration of 10 μM every 24 h for 2 days. The samples were loaded on a 10% SDS-PAGE containing 0.1% gelatin. After the electrophoresis, the gel was washed twice with washing buffer (50 mM Tris–HCl (pH 7.5), 100 mM NaCl and 2.5% Triton X-100) for 30 min, and then incubated in reaction buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 10 mM $CaCl_2$ and 0.02% $NaN_3$) at 37 °C for 16 h [6]. The gels were stained with 0.25% Coomassie Brilliant Blue R250 (Wako Pure Chemical). The bands were quantitated with image analysis software (NIH Image, Bethesda, MD).

### 2.5. Colony assay

For colony assay, cells were suspended in DMEM containing 0.4% low-melting-point agarose and 10% FBS, and plated on a bottom layer containing 0.8% agarose at $1 \times 10^4$ cells/3-cm diameter well. The cells were photographed on day 10 after plating and measured the size of ten colonies [4,5,11].

### 2.6. Cell motility and tube formation of endothelial cells

To assess the effects of the $LPA_4$, $LPA_5$ and $LPA_6$ on cellular functions of endothelial F-2 cells, LPA receptor knockdown cells were maintained in serum-free DMEM for 2 days and the supernatants were obtained. In cell motility assay, F-2 cells were seeded in the filter at $1 \times 10^5$ cells in 200 μl serum-free DMEM (upper chamber) and placed in 24-well plates (lower chamber) containing 800 μl of supernatants from each LPA receptor knockdown cells. For tube formation assay, Matrigel at 100 μl/well was plated in 96-well plates and incubated at 37 °C for 30 min. Cells were seeded onto Matrigel-coated plates at $3 \times 10^4$ cells in 100 μl supernatants from each LPA knockdown cells and cultured at 37 °C for 4 h. The total tube formation length was measured from three representative 100 x fields/well.

### 2.7. Quantitative real-time reverse transcription (RT) – polymerase chain reaction (PCR) analysis

Total RNA was extracted from each cells and cDNA was synthesized by a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics Co. Ltd., Mannheim, Germany). The expression levels of vascular endothelial growth factor (VEGF) genes were measured by quantitative real-time RT-PCR analysis using SYBR *Premix Ex Taq* (Tli RNaseH Plus) (TaKaRa Bio, Inc., Shiga, Japan) and a Smart Cycler II System (TaKaRa Bio, Inc.). The expression levels of target genes were normalized to those of *GAPDH*.

## 3. Results

### 3.1. Effects of LPA on cell proliferation and motile activities in LPA receptor knockdown cells

The expression patterns of LPA receptor knockdown cells are shown in Fig. 1(A). The growth rates of PANC-sh5 and PANC-sh6 cells were significantly higher than that of PANC-RFP cells, while PANC-sh4 cells indicated the low growth rate (Fig. 1(B)). To investigate the effects of LPA on cell proliferation, cells were maintained in DMEM supplemented with 5% charcoal stripped FBS which did not contain endogenous LPA. The cell proliferation activities of PANC-sh4 and PANC-sh6 were significantly inhibited by LPA as well as PANC-RFP cells. LPA enhanced the cell proliferation activity of PANC-sh5 cells (Fig. 1(C)). In cell motility assay, PANC-sh4 and PANC-sh5 cells indicated the high cell motile activities, in comparison with PANC-RFP cells. In contrast, the cell motile activity of PANC-sh6 cells was significantly lower than that of PANC-RFP cells. The cell motile activities of PANC-sh4 and PANC-sh5 cells were enhanced by LPA, while LPA suppressed the cell motile activity of PANC-sh6 cells (Fig. 1(D)).

### 3.2. Invasive activities in LPA receptor knockdown cells

For cell invasion assay, the filters were coated with Matrigel. The invasive activity of PANC-sh4 cells was markedly higher than that of PANC-RFP cells. Moreover, LPA stimulated the invasive activity of PANC-sh4 cells, but not PANC-RFP cells. PANC-sh6 cells indicated the low invasive activity. The cell invasive activity of PANC-sh6 cells were significantly suppressed by LPA. Although PANC-sh5 cells showed the low invasive activity in the absence of LPA, the treatment of LPA induced the high invasive activity of PANC-sh5 cells, compared with PANC-RFP cells (Fig. 2(A)).

### 3.3. Activities of MMP-2 and MMP-9 in LPA receptor knockdown cells

To obtain the supernatants for gelatin zymography, cells were cultured in serum-free DMEM with or without LPA for 2 days. Representative results of gelatin zymography are shown in Fig. 2(B). The activities of MMP-2 in PANC-sh5 and PANC-sh6 cells were significantly lower than that in PANC-RFP cells. LPA increased the activity of MMP-2 in PANC-sh4 cells, in comparison with PANC-RFP cells. On the other hand, PANC-sh4, PANC-sh5 and PANC-sh6 cells showed the elevated activities of MMP-9 by LPA (Fig. 2(C)).

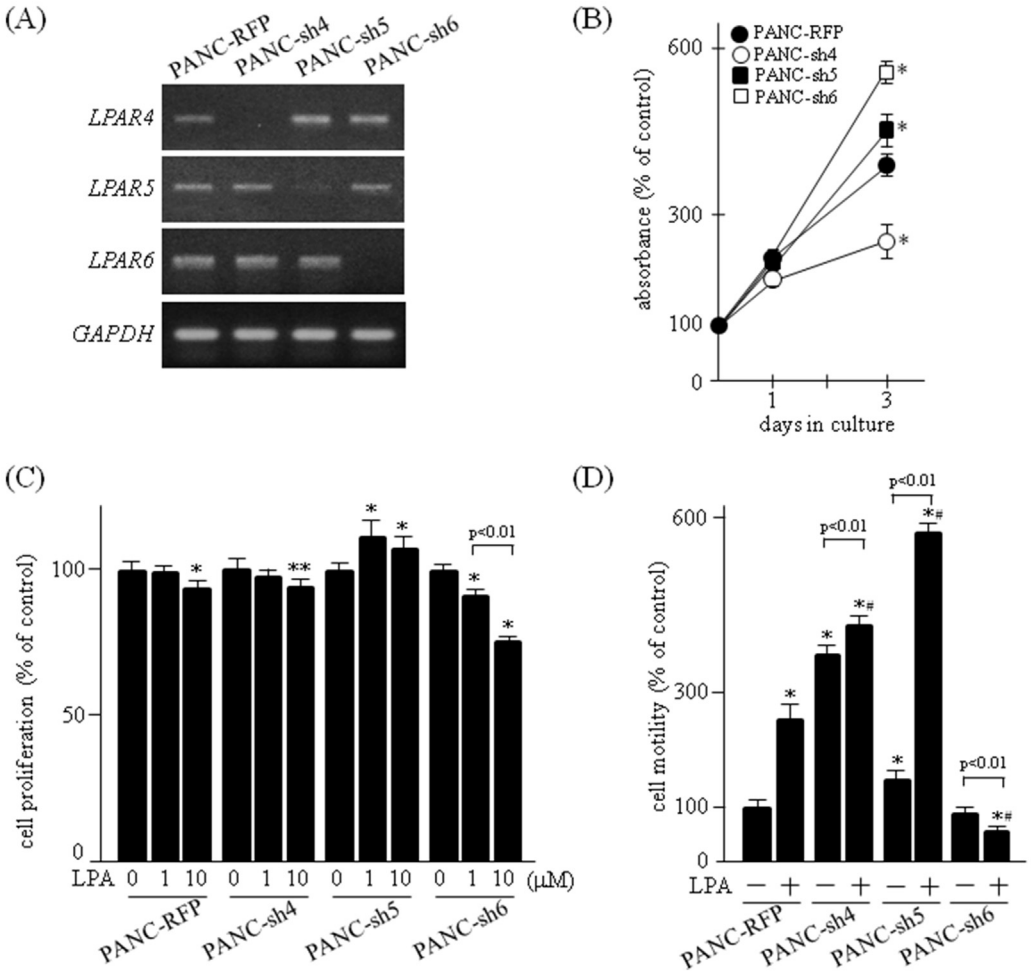


**Fig. 1.** Cell proliferation and motile activities of LPA receptor knockdown cells. (A) Expression patterns of LPA receptor knockdown cells by semi-quantitative RT-PCR analysis. (B) Cell proliferation rates of LPA receptor knockdown cells. Cells were cultured in DMEM containing 10% FBS. Cell proliferation rate was measured using the CCK-8. Data are expressed as the percentage of cell number on day 0. *; $p < 0.01$ vs. PANC-RFP cells. (C) The effects of LPA on cell growth in LPA knockdown cells. Cells were cultured with or without LPA (1 or 10 μM) and cell growth rates were measured using the CCK-8. Columns indicate the mean of three studies; bars indicate SD. *; $p < 0.01$ vs. LPA untreated cells. (D) Cell motility assays. Cells were incubated in DMEM supplemented with 5% charcoal stripped FBS with or without LPA (10 μM). Columns indicate the mean of three studies. Bars indicate SD. *; $p < 0.01$ vs. PANC-RFP cells treated without LPA. #; $p < 0.01$ vs. PANC-RFP cells treated with LPA.

### 3.4. Colony formations of LPA receptor knockdown cells in soft agar

To examine whether LPA receptor knockdown cells indicated colony formation, cells were seeded into condition mediums containing low-melting-point agarose. PANC-sh4 and PANC-sh5 cells formed markedly large sized colonies, in comparison with PANC-RFP cells. However, no colony formation was found in PANC-sh6 cells (Fig. 3(A) and (B)).

### 3.5. Effects of LPA receptor knockdown cells on cell motile activity and tube formation of F-2 cells

When F-2 cells were incubated with supernatants from PANC-sh4 and PANC-sh5 cells, the cell motile activity of F-2 cells was significantly stimulated, compared with PANC-RFP cells. In contrast, the cell motile activity of F-2 cells was inhibited by PANC-sh6 cells (Fig. 4(A)). Representative results of the tube formation assay are shown in Fig. 4(B). For tube formation assay, F-2 cells were seeded onto Matrigel-coated plates in supernatants from the individual LPA knockdown cells and incubated for 4 h. The tube formations of F-2 cells were facilitated by supernatants from PANC-sh4 and PANC-sh5 cells, but not PANC-sh6 cells (Fig. 4(C)). PANC-sh5 and PANC-sh6 cells showed the low expressions of the *VEGF*-A gene, but not PANC-sh4 cells. The expression levels of the *VEGF*-C gene in PANC-sh4, PANC-sh5 and PANC-sh6 cells were significantly lower than that in PANC-RFP cells (Fig. 4(D)).

## 4. Discussion

It has been reported that LPA signaling via LPA receptors is involved in the pathogenesis of cancer cells [3]. Frequent mutations of the *Lpar1* gene were detected in lung and liver tumors [12,13]. In addition, the aberrant expressions of LPA receptor genes due to DNA methylation occurred in colon cancer cells [14]. However, the biological functions of $LPA_4$, $LPA_5$ and $LPA_6$ are not fully understood in cancer cells. In this study, we investigated the roles of $LPA_4$, $LPA_5$ and $LPA_6$ in the cellular functions using LPA receptor knockdown cells generated from PANC-1 cells. The present results indicated that $LPA_4$ and $LPA_5$ knockdown stimulated the malignant properties, such as cell motility, invasion, tumorigenicity and angiogenesis in PANC-1 cells, while these properties were suppressed by $LPA_6$ knockdown. Therefore, it is suggested that the different roles of

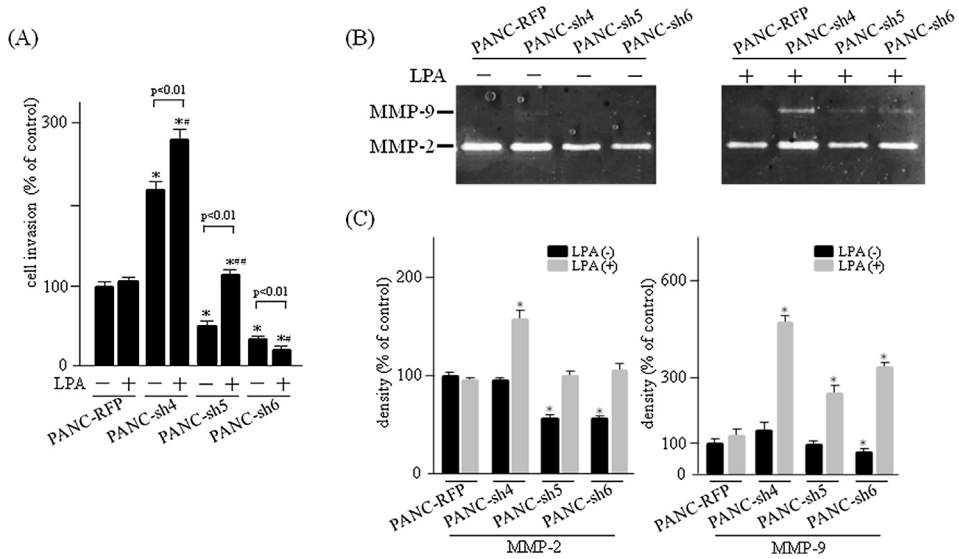


**Fig. 2.** The cell invasive activities of LPA receptor knockdown cells. (A) Cell invasion assays with the Matrigel-coated filters. Cells were incubated in DMEM supplemented with 5% charcoal stripped FBS with or without LPA (10 μM). Columns indicate the mean of three studies. Bars indicate SD. *; $p < 0.01$ vs. PANC-RFP cells treated without LPA. #; $p < 0.01$ vs. PANC-RFP cells treated with LPA. ##; $p < 0.01$ vs. PANC-RFP cells treated with LPA. (B) Gelatin zymography for MMP-2 and MMP-9 activities in supernatants from cultured cells in serum-free DMEM with LPA (10 μM) for 2 days. (C) Densitometric analysis of MMP-2 and MMP-9 activities. The bands were quantitated with NIH Image.

$LPA_4$, $LPA_5$ and $LPA_6$ are involved in the activation of tumor progression in PANC-1 cells.

In colon and neuroblastoma cells, the cell motile activities were inhibited by $LPA_4$ [9]. $LPA_5$ negatively regulated the cell motility of melanoma cells [10]. Recently, we have reported that $LPA_5$ reduced the cell motility and Mmp-9 activation in fibroblast 3T3 cells [15]. Moreover, the cell motile and invasive activities were inhibited by $LPA_5$ in sarcoma cells [16]. In contrast, $LPA_5$ increased the cell motile activities of *Lpar5*-expression lung and liver tumor cells [17]. In this study, the invasive activities of PANC-sh4 and PANC-sh5 cells were correlated with the levels of MMP-2 and MMP-9 activations, but not PANC-sh6 cells. One possibility is that the reduced invasive activity of PANC-6 cells may be due to the low cell motile activity.

To assess the tumorigenic activity of target gene, the colony assay using a soft agar is widely used [11]. In this study, $LPA_4$ and $LPA_5$ knockdown cells formed the large sized colonies, but not $LPA_6$ knockdown cells. In our previous reports, the colony formations were detected in *Lpar3*-expressing liver and neuroblastoma cells, correlating with the cell motile and invasive activities. On the other hand, no colony formation was observed in *Lpar2*-expressing neuroblastoma cells which stimulated the cell motile and invasive activities [5]. Therefore, it is suggested that the cell motile and

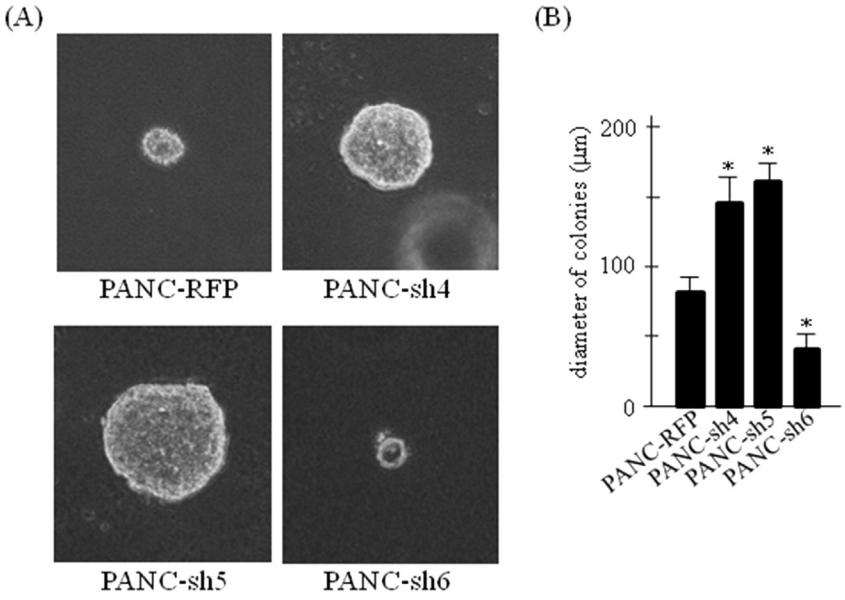


**Fig. 3.** The colony formation assay. (A) Phase contrast micrographs of LPA receptor knockdown cells in a clonogenic soft agar assay. Single cells were mounted in soft agar gel containing serum and cultured. The same cells were photographed on day 10 after plating. (B) Diameter of colonies in soft agar gel. Columns indicate the mean of ten colonies; bars indicate SD. *; $p < 0.01$ vs. PANC-RFP cells.

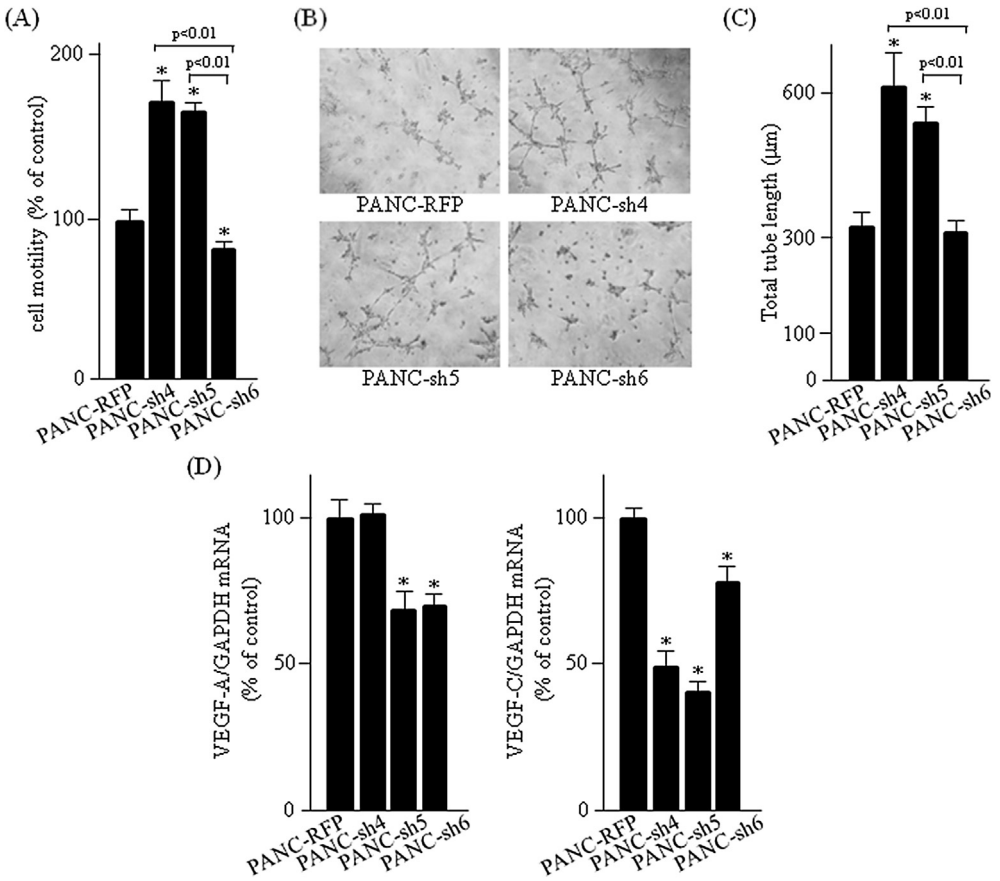


**Fig. 4.** Effects of LPA receptor knockdown cells on cell motile activity and tube formation of F-2 cells. (A) Effects of cell motile activities of F-2 cells by LPA receptor knockdown cells. F-2 cells were incubated in serum-free DMEM (upper chamber) with supernatants (lower chamber) from each LPA receptor knockdown cells. Columns indicate the mean of three experiments. Bars indicate SD. *;$P < 0.01$ vs. PANC-RFP cells. (B) Tube formation assays. Cells were seeded onto Matrigel-coated plates at $3 \times 10^4$ cells in 100 μl supernatants from each LPA knockdown cells and cultured at 37 °C for 4 h. (C) The total tube formation length was measured from three representative 100 x fields/well. (D) Expression levels of *VEGF-A* and *VEGF-C* gene mRNAs relative to *GAPDH* mRNA by quantitative real time RT-PCR analysis. Columns indicate the mean of three experiments; bars indicate SD. *; $P < 0.01$ vs. PANC-RFP cells.

invasive activities may be independent on the tumorigenic activity in cancer cells.

Solid tumors require oxygen and several nutrients from the surrounding blood vessels for cell growth. Angiogenesis is a process of constructing new vascular formation from the existing blood vessels and contributes to the activations of invasion and metastasis in cancer cells [18,19]. It has been reported that LPA receptors play important roles in the angiogenesis process of cancer cells as well as LPA *per se* [20,21]. LPA induced the expressions of VEGF mRNA and protein expressions in ovarian cancer cells [22]. Furthermore, LPA enhanced VEGF protein secretion through $LPA_1$ and $LPA_2$ in colon cancer cells [23]. When F-2 cells are cultured with supernatants from exogenous *Lpar1*-and *Lpar3*-expressing neuroblastoma cells, the cell motile activities of F-2 cells were significantly enhanced, correlating with the VEGF expression levels [24]. In the present study, the motile activity and tube formation of F-2 cells were stimulated by $LPA_4$ and $LPA_5$ knockdown. However, no relationship between these activities and VEGF expression levels in $LPA_4$ and $LPA_5$ knockdown cells was found. Identification of the factors which regulated the cellular functions of F-2 cells in $LPA_4$ and $LPA_5$ knockdown cells remains to be clarified.

In conclusion, we demonstrated that $LPA_4$ and $LPA_5$ negatively and $LPA_6$ positively regulated the malignant properties in PANC-1 cells. We are currently investigating the roles of $LPA_4$, $LPA_5$ and $LPA_6$ in the pathogenesis of other cancer cells.

## Conflict of interest

The authors declare that they have no conflict of interest.

## Acknowledgments

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## Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.169.

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