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Lysophosphatidic acid receptor-5 negatively regulates cellular responses in mouse fibroblast 3T3 cells

Yan Dong [a,1], Miku Hirane [a,1], Mutsumi Araki [a], Nobuyuki Fukushima [b], Toshifumi Tsujiuchi [a,*]

[a] Division of Cancer Biology and Bioinformatics, Department of Life Science, Faculty of Science and Engineering, Kinki University, 3-4-1, Kowakae, Higashiosaka, Osaka 577-8502, Japan
[b] Division of Molecular Neurobiology, Department of Life Science, Faculty of Science and Engineering, Kinki University, 3-4-1, Kowakae, Higashiosaka, Osaka 577-8502, Japan

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ABSTRACT

Lysophosphatidic acid (LPA) signaling via G protein-coupled LPA receptors (LPA_1–LPA_6) mediates a variety of biological functions, including cell migration. Recently, we have reported that LPA_1 inhibited the cell motile activities of mouse fibroblast 3T3 cells. In the present study, to evaluate a role of LPA_5 in cellular responses, *Lpar5* knockdown (3T3-L5) cells were generated from 3T3 cells. In cell proliferation assays, LPA markedly stimulated the cell proliferation activities of 3T3-L5 cells, compared with control cells. In cell motility assays with Cell Culture Inserts, the cell motile activities of 3T3-L5 cells were significantly higher than those of control cells. The activity levels of matrix metalloproteinases (MMPs) were measured by gelatin zymography. 3T3-L5 cells stimulated the activation of Mmp-2, correlating with the expression levels of *Mmp-2* gene. Moreover, to assess the co-effects of LPA_1 and LPA_5 on cell motile activities, *Lpar5* knockdown (3T3a1-L5) cells were also established from *Lpar1* over-expressing (3T3a1) cells. 3T3a1-L5 cells increased the cell motile activities of 3T3a1 cells, while the cell motile activities of 3T3a1 cells were significantly lower than those of control cells. These results suggest that LPA_5 may act as a negative regulator of cellular responses in mouse fibroblast 3T3 cells, similar to the case for LPA_1.

1. Introduction

Lysophosphatidic acid (LPA) is a simple bioactive lipid consisting of a glycerol, a fatty acid and a phosphate. LPA interacts with six G protein-coupled transmembrane LPA receptors (LPA receptor-1 (LPA_1)–LPA_6) [1,2]. LPA signaling via LPA receptors mediates a variety of cellular functions, including cell proliferation, motility, differentiation, morphogenesis and protection from apoptosis [1–4]. The effects of each LPA receptor are not functionally uniform. For example, LPA_1 and LPA_2 enhance cell proliferation activities, phospholipase C activation, intracellular calcium mobilization, and adenylyl cyclase inhibition. In neuronal cells, almost all LPA receptor subtypes mediate LPA-induced neurite retraction and growth cone collapse, but not LPA_3 [5,6]. In contrast, LPA_3 stimulated axon branching via the activations of Gq subunit and Rnd2 [7].

It is known that cell motility is one of essential physiological functions of cells, such as embryogenesis, morphogenesis, regeneration, inflammation and wound healing [8]. LPA signaling via LPA receptors contributes to the regulation of the cell motile activities in normal cells as well as cancer cells [3,4]. The effects of the individual LPA receptors are varied, depending on the cell types. In our recent studies, LPA_1 suppressed the cell motile activities of mouse fibroblast 3T3 cells [9]. Moreover, LPA_1 inhibited and LPA_3 enhanced the cell motile activities stimulated by hydrogen peroxide in 3T3 cells [10]. In neuroblastoma cells, LPA_2 and LPA_3 enhanced the cell motile and invasive activities, but not LPA_1 [11]. LPA_3 stimulated the cell motility, invasion and tumorigenicity in liver tumor cells [12]. In contrast, the cell motile activities of lung cancer cells were significantly suppressed by LPA_3 [13].

In the present study, to assess a role of LPA_5 in cellular functions in 3T3 cells, *Lpar5* knockdown cells were generated from 3T3 cells. In addition, we investigated the interaction of LPA_5 and other LPA receptors on the cell motile activities of 3T3 cells. We here demonstrate that LPA signaling via LPA_5 inhibits the cellular responses in 3T3 cells.

Abbreviations: LPA, lysophosphatidic acid; LPA_5, LPA receptor-5; RT, reverse transcription; PCR, polymerase chain reaction.

* Corresponding author. Fax: +81 6 6721 2721.
E-mail address: ttujiuch@life.kindai.ac.jp (T. Tsujiuchi).

[1] These authors contributed equally to this work.

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2. Materials and methods

2.1. Cell culture and establishment of Lpar5 knockdown cells

All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Wako Pure Chemical Industries Ltd., Osaka, Japan) containing 10% fetal bovine serum (FBS) in a 5% CO_2 atmosphere at 37 °C. *Lpar5* knockdown (3T3-L5) cells were generated by transfection of a short hairpin RNA plasmid for the *Lpar5* (Origene, Rockville, MD) [10]. 3T3-GFP (vector) cells were used as control cells.

2.2. Cell proliferation assay

Cell were seeded at 3000 cells/well in 96-well plates and cultured in 100 μl of DMEM containing 10% FBS. To measure cell proliferation rates for 3 days, solution from a Cell Counting Kit-8 (CCK-8) (Dojin Chemistry, Kumamoto, Japan) was added to each well at 0, 1, 2, or 3 days and the cells were further incubated for 1 h. The absorbance of the culture medium at 450 nm was determined [11,14]. To evaluate the effects of LPA treatment on cell growth, cells were cultured with 100 μl of DMEM containing 5% charcoal stripped FBS (Sigma, St. Louis, MO, USA). Cells were 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 plates. These assays were performed in triplicate [11,14].

2.3. Cell motility assays

For motility assays, a Cell Culture Insert with 8 μm pore size (BD Falcon, Franklin Lakes, NJ) was used. The cells were seeded on the filters at 1×10^5 cells in 200 μl of serum-free DMEM (upper chamber). The filters were placed in 24-well plates (lower chamber) containing 800 μl of DMEM supplemented with 5% charcoal stripped FBS with or without LPA (10 μM) (Avanti Polar Lipids, Inc., AL), and incubated for 20 h. The cells remaining on the upper side of the filters were removed with cotton swabs. After Giemsa staining, the numbers of cells that had migrated to the lower side of the filters were counted [9–11].

Before cell motility assays, cells were pretreated with hydrogen peroxide at 0.1 and 1 μM concentrations for 48 h which were added every 24 h [9]. In addition, some cells were pretreated with 10 μM dioctylglycerol pyrophosphate (DGPP) (Avanti Polar Lipid) for 30 min. After the pretreatment, cells were seeded into the filter at 1×10^5 cells and incubated for 20 h [9,10].

2.4. Quantitative real-time reverse transcription (RT)-polymerase chain reaction (PCR) analysis

After RNA extraction, cDNA was synthesized with Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics Co. Ltd., Mannheim, Germany). To measure the expression levels of the target genes, quantitative real-time RT-PCR analyses using SYBR *Premix Ex Taq* (Tli RNaseH Plus) (TaKaRa Bio Inc., Shiga, Japan) and a Smart Cycler II System (TaKaRa) were performed according to the manufacturer's protocol. The expression levels of each target gene were normalized to those of rat *Gapdh* [9,10].

2.5. Gelatin zymography

The activities of matrix metalloproteinase (Mmp)-2 and Mmp-9 were measured by gelatin zymography. Briefly, supernatants from individual cells cultured in serum-free DMEM were loaded on a 10% SDS–PAGE containing 0.1% gelatin. The gels were washed twice with washing buffer (50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 2.5% Triton X-100) for 30 min, and then incubated in 50 mM Tris–Cl (pH 7.5), 150 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3 for 16 h at 37 °C. After incubation, the gels were stained with 0.25% Coomassie Brilliant Blue R250 [15].

3. Results

3.1. Cell proliferation activities of Lpar5 knockdown cells generated from 3T3 cells

3T3-L5 cells were generated from 3T3 cells, which expressed the *Lpar5* gene (Fig. 1A). The expression patterns of the *Lpar5* gene in 3T3-L5 and 3T3-GFP cells were confirmed by RT-PCR analysis (Fig. 1B). The cell proliferation rates between 3T3-L5 and 3T3-GFP cells showed the same levels in DMEM containing 10% FBS (Fig. 1C). To examine the effects of LPA treatment on cell proliferation, cells were cultured for 3 days in DMEM containing 5% charcoal stripped FBS with or without LPA (1 or 10 μM). LPA stimulated the cell growth at a concentration of 10 μM in both cells, but not 1 μM. The cell growth rates in 3T3-L5 cells treated with LPA (10 μM) were significantly higher than those in 3T3-GFP cells (Fig. 1D).

3.2. Effects of LPA_5 on cell motile activities of 3T3 cells

In cell motility assays, the cell motile activities of 3T3-L5 cells were markedly higher than those of 3T3-GFP cells, while LPA enhanced the cell motile activities of both cells (Fig. 2A). Moreover, to assess an involvement of LPA_5 in the cell motile activities of 3T3 cells stimulated by hydrogen peroxide, cells were pretreated with hydrogen peroxide for 48 h [9]. The cell motile activities of 3T3-GFP and 3T3-L5 cells treated with hydrogen peroxide (0.1 and 1 μM) were significantly higher than those of untreated cells. 3T3-L5 cells treated with hydrogen peroxide indicated markedly high cell motile activities, in comparison with 3T3-GFP cells (Fig. 2B).

3.3. The expression and activation levels of MMPs in Lpar5 knockdown cells

The expression levels of the *Mmp-9* in 3T3-L5 cells were significantly higher than 3T3-GFP cells, but not the *Mmp-2* gene (Fig. 2C). In gelatin zymography, 3T3-L5 cells indicated the enhanced activity of the Mmp-9, compared with 3T3-GFP cells. No significant change of the Mmp-2 activities was detected in both cells (Fig. 2D). LPA treatment did not affect the expression levels and activities of the Mmp-2 and Mmp-9 in both cells (data not shown).

3.4. Co-effects of LPA_5 and other LPA receptors on cell motile activities of 3T3 cells

We next investigated the co-effects of LPA_5 and other LPA receptors on the cell motile activities of 3T3 cells. Before the cell motility assays, cells were pretreated with DGPP which is an antagonist of LPA_1/LPA_3 [16], and incubated in DMEM supplemented with 5% charcoal stripped FBS with LPA (10 μM). The cell motile activities of 3T3-L5 cells treated with DGPP were significantly lower than those of DGPP-untreated 3T3-L5 cells, similar to the case for 3T3-GFP cells (Fig. 3A).

Furthermore, to assess the co-effects of LPA_1 and LPA_5 on the cell motile activities of 3T3 cells, *Lpar5* knockdown 3T3a1-L5 and 3T3AB-L5 cells were generated from *Lpar1* over-expressing (3T3-a1) and 3T3-AB (vector) cells, respectively. As control (vector) cells, 3T3a1-G and 3T3AB-G cells were also established from 3T3-a1 and 3T3-AB cells, respectively [9,11,14]. In cell motility

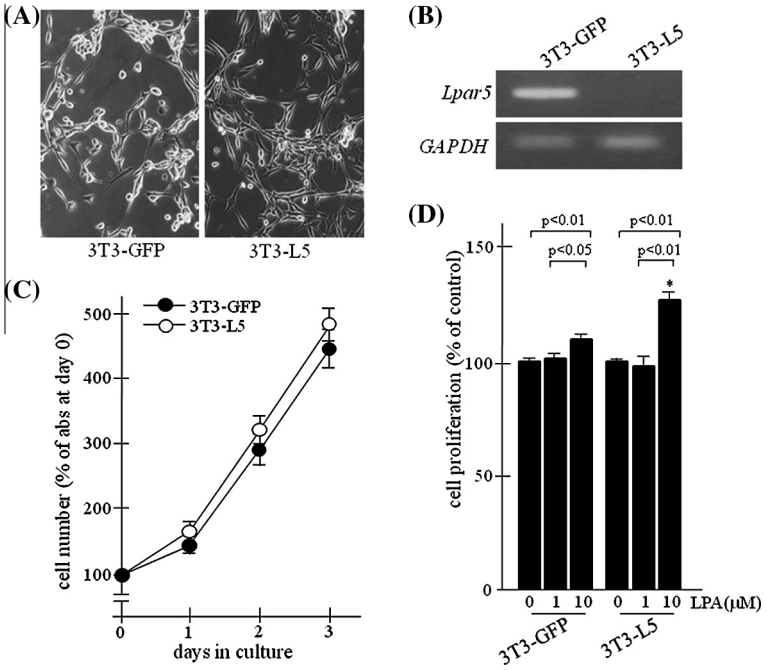

Fig. 1. *Lpar5* knockdown cells generated from fibroblast 3T3 cells. (A) Morphology of 3T3-GFP (control) and *Lpar5*-expressing 3T3-L5 cells in DMEM containing 10% FBS. (B) Expression patterns of the *Lpar5* gene by semi-quantitative RT-PCR analysis. (C) Cell proliferation rates. Cells were cultured in DMEM containing 10% FBS and cell proliferation was measured using a CCK-8. Data are expressed as the percentage of cell number on day 0. (D) Effects of LPA on cell proliferation activities of 3T3-GFP and 3T3-L5 cells. Cells were cultured with or without LPA (1 or 10 μM), and cell proliferation rates were measured using a CCK-8. Columns indicate the mean of four studies; bars indicate SD. $^{*}p < 0.01$ vs. LPA (10 μM)-treated 3T3-GFP cells.

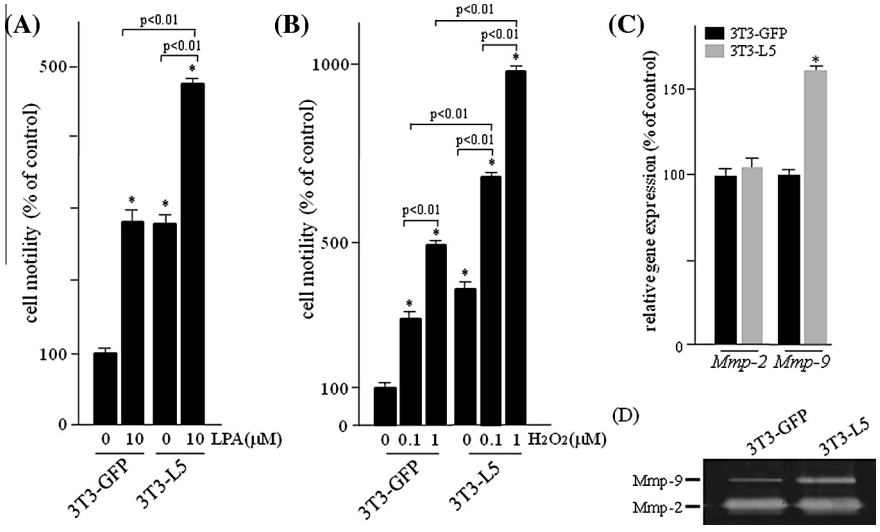

Fig. 2. Effects of *Lpar5* knockdown on motile activities and MMP activations in 3T3 cells. (A) The cell motile activities of *Lpar5* knockdown cells. Cells were incubated for 20 h with or without LPA (10 μM). Columns indicate the mean of three studies. Bars indicate SD. $^{*}p < 0.01$ vs. LPA-untreated 3T3-GFP cells. (B) The cell motile activities stimulated by hydrogen peroxide. After the treatment of hydrogen peroxide (0.1 or 1 μM) for 48 h, cells were seeded and incubated for 20 h in DMEM containing 10% FBS. Columns indicate the mean of three studies. Bars indicate SD. $^{*}p < 0.01$ vs. untreated 3T3-GFP cells. (C) Expression levels of *Mmp-2* and *Mmp-9* genes by quantitative real-time RT-PCR analysis. Columns indicate the mean of three studies; bars indicate SD. $^{*}p < 0.01$ vs. 3T3-GFP (control) cells. (D) Representative results of gelatin zymography for Mmp-2 and Mmp-9 activities in supernatants from cultured cells for 48 h.

assays, the cell motile activities of 3T3a1-G and 3T3AB-L5 cells were significantly lower and higher than those of 3T3AB-G cells, respectively. 3T3a1-L5 cells showed the low cell motile activities, compared with 3T3AB-G cells. Interestingly, the elevated cell motile activities of 3T3AB-L5 cells were markedly inhibited in 3T3a1-L5 cells. LPA treatment suppressed the cell motile activities of 3T3a1-G and 3T3a1-L5 cells, while those of 3T3AB-G and 3T3AB-L5 cells were stimulated (Fig. 3B).

3.5. Effects of LPA_5 on cell proliferation and motile activities of endothelial F-2 and lung cancer A549 cells

To confirm whether LPA_5 can inhibits cell proliferation and motile activities of other cell types, *Lpa5* knockdown F2-L5 and A549-L5 cells were generated from mouse endothelial F-2 and human lung cancer A549 cells, respectively. As control (vector) cells, F2-GFP and A549-GFP cells were also used. The cell proliferation

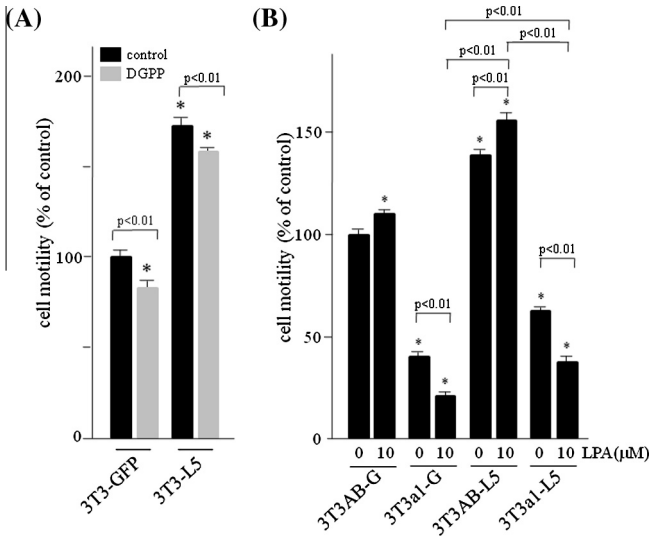

Fig. 3. Interaction of LPA_5 and other LPA receptors on cell motile activities of 3T3 cells. (A) Effects of DGPP on cell motile activities of *Lpar5* knockdown cells. Cells were pretreated with DGPP (10 μM) for 30 min. After the pretreatment, cells were seeded into the filter and incubated for 20 h. (B) Co-effects of LPA_5 and LPA_1 on cell motile activities of 3T3 cells. *Lpar5* knockdown 3T3a1-L5 cells were generated from *Lpar1* over-expressing 3T3-a1 cells. Cells were incubated for 20 h with or without LPA (10 μM). Columns indicate the mean of three studies. Bars indicate SD. $^*p < 0.01$ vs. LPA-untreated 3T3AB-G cells.

rates of F2-L5 and A549-L5 cells treated with LPA (10 μM) were significantly higher than those of control cells (Fig. 4A). In cell motility assays, F2-L5 and A549-L5 cells indicated the high cell motile activities, compared with control cells. LPA treatment enhanced the cell motile activities of all cells (Fig. 4B).

4. Discussion

The present study indicated that Lpar5 knockdown enhanced the cell proliferation and motile activities and increased the expression and activation levels of the Mmp-9 in 3T3 cells. Moreover, *Lpar5* knockdown significantly elevated the cell motile activities of *Lpar1* over-expressing 3T3 cells, while LPA_1 inhibited the cell motile activities of 3T3 cells. These results suggest that LPA signaling via LPA_5 may act as a negative regulator of the cellular responses in 3T3 cells, similar to the case for LPA_1. In addition, we confirmed that LPA_5 suppressed the cell proliferation and motile activities of other cell lines, endothelial F-2 and lung cancer A549 cells.

The expression patterns of LPA_5 are varied in normal tissues. LPA_5 is expressed at low levels in embryonic brain, moderate levels in skin, spleen, stomach, thymus, lung and liver, and high levels in small intestine [17]. LPA_5 is coupled with Gq and G12/13 proteins and mediates a variety of biological functions, such as the increase of cAMP, platelet activation and neuropathic pain [17,18]. Recently, we have demonstrated that the high expression levels of the *Lpar5* gene were detected in lung and liver tumors induced by nitroso-compounds, compared with normal lung and liver tissues. Moreover, LPA stimulated the cell growth and motile activities of highly *Lpar5* expressing lung tumor (RLCNR) and hepatoma (RH7777) cells. These findings suggested that LPA_5 is involved in the acquisition of growth advantage of rat lung and liver tumor cells [19]. Taken together with the present results, it seems that the different biological effects of LPA_5 may be dependent on the cell types. In fact, exogenously expressed LPA_3 inhibited the cell motile activities of RLCNR cells, while LPA_3 enhanced those of 3T3 cells [9,13]. In addition, RH7777 cells expressed the *Lpar5* gene but did not express other LPA receptor genes [19,20].

Recently, we have demonstrated that LPA_1 suppresses and LPA_3 enhances the cell motile activities of 3T3 cells. To assess a role of LPA_1 in the cell motile activities, *Lpar1* over-expressing cells were generated from 3T3 cells which expressed endogenous LPA_1 [14]. The cell motile activities of *Lpar1* over-expressing cells were significantly lower than those of 3T3 cells. In contrast, mutated LPA_1 which lacked the carboxyl terminal region markedly elevated the cell motile activities of 3T3 cells [9,11]. Moreover, the cell motile activities of 3T3 cells were significantly elevated by the treatment of hydrogen peroxide, correlating with the expression levels of the *Lpar3* gene. The cell motile activities stimulated by hydrogen peroxide were inhibited in *Lpar1* over-expressing cells [9].

To investigate the interaction of LPA_5 and other LPA receptors, we used the antagonist of LPA_1/LPA_3, DGPP [16]. The treatment of DGPP inhibited the cell motile activities of both cells, while the cell motile activities of *Lpar5* knockdown cells were significantly higher than those of control cells. Since LPA_1 inhibited and LPA_3 enhanced the cell motile activities of 3T3 cells, the cell motile activities decreased by DGPP may be mainly due to the inhibition of LPA_3. Furthermore, to evaluate the co-effects of LPA_1 and LPA_5 on the cell motile activities of 3T3 cells, we generated *Lpar5* knockdown cells from *Lpar1* over-expressing cells. The cell motile activities elevated

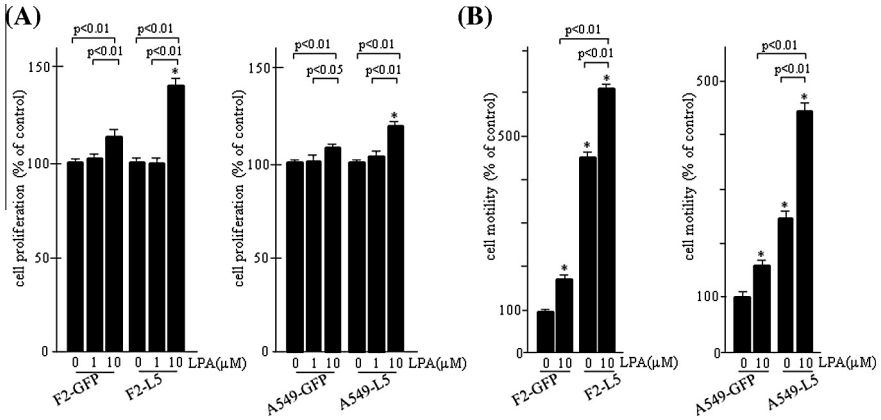

Fig. 4. Cell proliferation and motile activities of *Lpar5* knockdown cells generated from F-2 and A549 cells. (A) Effects of LPA on cell proliferation activities of *Lpar5* knockdown cells. Cells were cultured with or without LPA (1 or 10 μM), and cell proliferation rates were measured using a CCK-8. Columns indicate the mean of four studies; bars indicate SD. $^*p < 0.01$ vs. LPA (10 μM)-treated control cells. (B) Cell motile activities of *Lpar5* knockdown cells. Cells were incubated for 20 h with or without LPA (10 μM). Columns indicate the mean of three studies. Bars indicate SD. $^*p < 0.01$ vs. LPA-untreated control cells.

by *Lpar5* knockdown were markedly inhibited by LPA_1. Therefore, it is suggested that the cell motile activities of 3T3 cells may be strongly suppressed by LPA signaling via both LPA_1 and LPA_5.

Recently, it has been reported that LPA signaling is involved in the expressions and activations of MMPs in cancer cells. It is considered that MMP-2 and MMP-9 essentially promote the invasion and metastasis of cancer cells through extracellular matrix degradation [21,22]. LPA_1 contributes to the stimulation of invasive activity and MMP-9 expression in human liver cancer cells which expressed LPA_3 at low levels [23]. In contrast, LPA_1 decreased the activations of Mmp-2 and Mmp-9 in hamster pancreatic cancer cells [24]. In addition, mutated LPA_1 markedly enhanced the induction of Mmp-2 expression and activation in rat neuroblastoma cells, but not LPA_1 [15].

In conclusion, LPA signaling via LPA_5 negatively regulates the cellular responses of 3T3 cells. Moreover, LPA_5 inhibited the cell proliferation and motile activities of F-2 and A549 cells. It has been considered that each LPA receptor has diverse cellular functions, depending on the cell types involved [3,4]. Therefore, we are currently investigating the effects of LPA_5 on the cell motile and invasive activities of other cancer cells.

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