Lysophosphatidic acid signaling regulates the KLF9-PPARγ axis in human induced pluripotent stem cell-derived neurons

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Running title: LPA-KLF9-PPARγ axis in neuron

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ABSTRACT

Lysophosphatidic acid (LPA) is a lipid signaling molecule that plays several significant roles in the nervous system during development and injury. In this study, we differentiated human induced pluripotent stem cells (iPSCs) into neurons as an *in vitro* model to examine the specific effects of LPA. We demonstrated that LPA activates peroxisome proliferator-activated receptor gamma (PPARγ), a ligand-activated nuclear receptor, as well as its cognate receptor LPA_1 on human iPSC-derived neurons to enhance proliferation and neurite outgrowth. Furthermore, we found that the gene expression of Kruppel-like factor 9 (KLF9), a member of the large KLF transcription factor family, was induced by LPA treatment. Knockdown of KLF9 decreased proliferation and neurite outgrowth in vehicle- and LPA-treated IPSC-derived neurons compared to cells expressing KLF9. In conclusion, LPA plays dual roles as a ligand mediator through the activation of cell surface G-coupled protein receptors and as an intracellular second messenger through the activation of PPARγ. We discuss the contribution of the LPA_1-PPARγ-KLF9 axis to neurite outgrowth and proliferation in human iPSC-derived neurons.

Keywords: induced pluripotent stem cells; Kruppel-like factor 9; lysophosphatidic acid; peroxisome proliferator-activated receptor gamma.

Abbreviations: iPSC, induced pluripotent stem cell; KLF9, Kruppel-like factor 9; LPA, lysophosphatidic acid; PPARγ, peroxisome proliferator-activated receptor gamma.

INTRODUCTION

An important advantage of human induced pluripotent stem cell (iPSC)-derived neuronal cultures is that they offer a physiologically relevant system for the study of common drug targets such as G-protein-coupled receptors (GPCRs) and associated cell-type specific signaling pathways in the brain. In particular, lysophosphatidic acid (LPA) signaling has been shown to affect the formation of the central nervous system (1) (2) (3). LPA is present in the embryonic brain, neural tube, spinal cord, and cerebrospinal fluid at nanomolar to micromolar concentrations (4) (5) (6). The gene expression of LPA_1 receptor is enriched in the ventricular zone during embryonic cortical development (4,7). In the adult brain, LPA receptors are differentially expressed in various neural cell types (5); for example, the LPA_1 receptor has effects on cerebral cortical neuron growth, growth cone and process retraction, survival, migration, adhesion, and proliferation. LPA-mediated proliferation through the LPA_1 receptor is attenuated by treatment with a LPA receptor antagonist (8) (9) or a peroxisome proliferator-activated receptor gamma (PPARγ) antagonist (10). Indeed, different neuronal cell types express several LPA receptors, and their interactions with PPARγ suggests the potential for diverse and complex effects of LPA signaling in neurons. Similar to LPA, PPARγ has been previously implicated in neuronal differentiation from stem cells (11) (12); PPARγ activation induces the expression of a differentiation factor that plays a role in central nervous system development (13) (12). Here, we examined whether and how LPA regulates the proliferation and differentiation of human iPSC-derived neurons. First, we evaluated whether these cells express mRNA for LPA receptors. Then, we use pharmacological tools to determine the functional roles of LPA receptors and intracellular PPARγ in proliferation and neurite outgrowth. Finally, we explore the role of KLF9 in LPA-mediated effects; KLF9 is expressed in various tissues including the brain (14) and plays a key role in neuronal maturation in the central nervous system (15). Our results inform the importance of the LPA-KLF9-PPARγ axis in human iPSC-derived neurons.

EXPERIMENTAL PROCEDURES

Cells, chemicals, and antibodies

To obtain mature human matured neurons in a culture system, ReproNeuro, a neuron progenitor derived from human iPSCs, was purchased from ReproCELL (Yokohama, Japan) and maintained in ReproNeuro maturation medium for 14 days according to the manufacturer's instructions. All assays were performed on cells between passages 3 and 12 and were repeated at least 3 times in duplicate or triplicate. LPA (18:1) was purchased from Cayman Chemical (Ann Arbor, MI); LPA was dissolved in phosphate-buffered saline containing 0.1% lipid-free bovine serum albumin (Sigma-Aldrich, MO) to generate a 10-mM stock solution. GW9662 and Ki16425 were purchased from Sigma-Aldrich (St. Louis, MO). We used antibodies against PPARγ (rabbit monoclonal, sc-7196) and KLF9 (rabbit monoclonal, GTX129316), horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit secondary antibodies (Cell Signaling Technologies, Danvers, MA), and an antibody against β-actin (mouse monoclonal, sc-47778).

Quantitative real-time PCR

Total RNA was isolated from cells by using a NucleoSpin® RNA II kit (TAKARA, Otsu, Japan). Then, 0.5 μg total RNA was used for cDNA synthesis with a ReverTra Ace qPCR RT Kit (Toyobo) as per the manufacturer's instructions. mRNA levels were quantified using an ECO Real-Time PCR system (Illumina, Inc., San Diego, CA). All PCRs were performed in 10 μL volumes in 48-well PCR plates (Illumina) with SYBR Green Real-time PCR Master Mix-Plus (Toyobo) and the following primer pairs: PPARγ_1, 5′-GTGGCCGCAGATTTGAAAGAAG-3′ (F) and 5′-TGTCAACCATGGTCATTTCG-3′ (R); PPARγ_2, 5′-CAAACCCCTATTCCATGCTGTT-3′ (F) and 5′-AATGGCATCTCTGGTCAACC-3′ (R); LPA1, 5′-GCTGCCATCTCTACTTCCATC-3′ (F) and 5′-AAGCGGCGGTTGACATAGATT-3′ (R); LPA2, 5′-TGTCGAGCCTGCTTGTCTTC-3′ (F) and 5′-TGAGCGTGGTCTCTCGGTAG-3′ (R);

LPA3, 5′-GCTGCCGATTTCTTCGCTG-3′ (F) and 5′-AGCAGTCAAGCTACTGTCCAG-3′ (R); LPA4, 5′-TCCTTACCAACATCTATGGGAGC-3′ (F) and 5′-ACGTTTGGAGAAGCCTTCAAAG-3′ (R); LPA5, 5′-ATCTTCCTGCTGTGCTTCGT-3′ (F) and 5′-TGGTCTACAGCTTGGTGCTG-3′ (R); LPA6, 5′-TTGTATGGGTGCATGTTCAGC-3′ (F) and 5′-GCCAATTCCGTGTTGTGAAGT-3′ (R); KLF1, 5′-CCCCTCCTTCCTGAGTTGTT-3′ (F) and 5′-GTGGGAGCTCTTGGTGTAGC-3′ (R); KLF2, 5′- CTACACCAAGAGTTCGCATCTG-3′ (F) and 5′-CCGTGTGCTTTCGGTAGTG-3′ (R); KLF3, 5′-GCACGGAATACAGATGGAGCC-3′ (F) and 5′-TGTGAGGACGGGAACTTCAGA-3′ (R); KLF4, 5′-CCCACATGAAGCGACTTCCC-3′ (F) and 5′-CAGGTCCAGGAGATCGTTGAA-3′ (R); KLF5, 5′-CCTGGTCCAGACAAGATGTGA-3′ (F) and 5′-GAACTGGTCTACGACTGAGGC-3′ (R); KLF6, 5′-GGCAACAGACCTGCCTAGAG-3′ (F) and 5′-CTCCCGAGCCAGAATGATTTT-3′ (R); KLF7, 5′-AGACATGCCTTGAATTGGAACG-3′ (F) and 5′-GGGGTCTAAGCGACGGAAG-3′ (R); KLF8, 5′-CCCAAGTGGAACCAGTTGACC-3′ (F) and 5′-GACGTGGACACCACAAGGG-3′ (R); KLF9, 5′-AGAGTGCATACAGGTGAACGG-3′ (F) and 5′-AGTGTGGGTCCGGTAGTGG-3′ (R); KLF10, 5′-ACTGCCAAACCTCACATTGC-3′ (F) and 5′-ACGAATCACACTTGTTGCCTG-3′ (R); KLF11, 5′-GCATGACAGCGAAAGGTCTAC-3′ (F) and 5′-GGGGTCTTATCCGCAACAGG-3′ (R); KLF12, 5′-CGGCAGTCAGAGTCAAAACAG-3′ (F) and 5′-CGGCTTCCATATCGGGATAGT-3′ (R); KLF14, 5′-TTACAAGTCGTCGCACCTCAA-3′ (F) and 5′-TCTGGATGATAGGTTGGGTGG-3′ (R); KLF15, 5′-TTCTCGTCGCCAAAATGCC-3′ (F) and 5′-CCTGGGACAATAGGAAGTCCAA-3′ (R); KLF16, 5′-CAAGTCCTCGCACCTAAAGTC-3′ (F) and 5′-AGCGGGCGAACTTCTTGTC-3′ (R); KLF17, 5′-GCTGCCCAGGATAACGAGAAC-3′ (F) and 5′-ATCTCTGCGCTGTGAGGAAAG-3′ (R); and GAPDH, 5′-GTCGCTGTTGAAGTCAGAGG-3′ (F) and 5′-GAAACTGTGGCGTGATGG-3′ (R). The cycling conditions were 95°C for 10 min (polymerase activation) followed by 40 cycles of

95°C for 15 s, 55°C for 15 s, and 72°C for 30 s. After amplification, the samples were slowly heated from 55°C to 95°C and fluorescence was measured continuously to obtain a melting curve. Relative mRNA levels were quantified by using the formula $2^{-\Delta\Delta Cq}$, where ΔCq is the difference between the threshold cycle of a target cDNA and an endogenous reference cDNA.

Cell proliferation

Cell proliferation was determined using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Human iPSCs (1×10^4 cells/ml) were grown in 96-well plates for 48 h. Then, 10 μl of Cell Counting Kit-8 reagent was added to the medium and incubated for 1 h under a 5% CO_2 atmosphere. The orange formazan dye was measured by determining absorbance at 450 nm using a microplate reader (Awareness Technology Inc., USA). Cell viability was expressed as a percentage of control (untreated) cell values.

Reporter gene assays

pSV40-β-galactosidase and pcDNA3.1 plasmids were purchased from Promega (Madison, WI, USA) and Invitrogen Corp. (Carlsbad, CA, USA), respectively. The pcDNA3.1-PPARγ and pGL3b-PPRE (ACO)-Fluc plasmids were constructed as described previously (16). pcDNA3.1-FLAG-PPARγ was purchased from Addgene (Cambridge, MA, USA). PPARγ activation was determined in cells transfected with 125 ng of pGL3-PPRE-acyl-CoA oxidase luciferase, 62.5 ng of pcDNA3.1-PPARγ, and 12.5 ng of pSV-β-galactosidase (Promega), constructed as previously reported. Briefly, cells were seeded on a 96-well plate at a density of 1×10^4 cells/well. Twenty-four hours after transfection, the media was changed to Opti-MEM (Invitrogen) containing test compound dissolved in DMSO (up to 0.1%) and cultured for an additional 20 h. Luciferase activity was measured with a ONE-Glo Luciferase Assay System (Promega) using a LuMate microplate luminometer (Awareness Technology).

Western blotting

Proteins were separated on 5–20% SDS-PAGE gels (e-PAGEL; ATTO, Tokyo, Japan) and electrotransferred to Immobilon-P membranes (Millipore). The membranes were blocked in Block Ace (DS Pharma Biomedical Co. Ltd., Osaka, Japan) for 1 h and then incubated with a primary antibody in Tris-buffered saline-Tween 20 with 5% Block Ace for 12 h at 4°C. Bands were visualized with EzWestLumi plus (ATTO).

Small interfering RNAs

We suppressed KLF9 expression in human iPSC-derived neurons by transfecting the cells with small interfering RNAs (siRNAs) targeting KLF9 (SASI_Hs01_00084009, Sigma-Aldrich, Tokyo, Japan); Lipofectamine RNAiMAX (Invitrogen) was used for transfections. Cells were plated in 24-well plates (Iwaki, Tokyo, Japan) at a density of 5×10^4 cells/well in DMEM containing 10% FBS and then transfected with 100 pmol/mL of mRNA-specific siRNAs or scrambled siRNAs (control). Reductions in the gene expression of KLF9 were confirmed by real-time PCR and western blotting.

Determination of total neurite length

Human iPSC-derived neurons were plated on film-bottom dishes (FD10300, Matsunami Glass, Ltd., Japan). To assess the influence of KLF9 knockdown on neuritogenesis, neurons were transfected with KLF9 siRNA for 24 h, exposed to 10 μM LPA for 24 h, and total neurite outgrowth was measured. Coverslips were washed and mounted on microscope slides and analyzed by microscopy on a Shimadzu-rika BA210EINT microscope with a digital camera. Digital images of individual neurons were captured, and total neurite lengths were quantified using Motic Images Plus (Motica China Group CO., Ltd.). Total neurite length was calculated by adding all of the traced neurite lengths measured on individual neurons. At least 5 neurons randomly selected from 2 different cultures were evaluated for each treatment group.

Statistical analysis

Student's *t* tests were used for statistical comparisons and $p < 0.05$ was considered to be statistically significant.

RESULTS and DISCUSSION

LPA exerts its effects through interaction with 1 of 6 cognate GPCRs: LPA_1, LPA_2, LPA_3, LPA_4, LPA_5, and LPA_6. The LPA_1 receptor is ubiquitously expressed in the central nervous system and conducts several essential functions (17) (18). We first evaluated the gene expression of the LPA_{1-6} receptors in human iPSC-derived neurons. As shown in Fig. 1, only LPA_1 and LPA_2 mRNA were detected; LPA_1 mRNA levels were at least 5-fold higher than LPA_2 levels. Other LPA receptors (LPA_{3-6}) were not detected. This finding was consistent with previous reports that downstream activation of LPA_1 elicits cellular responses related to cell proliferation, survival, and migration in the nervous system (3).

A previous report identified PPARγ as an intracellular receptor for LPA (19). In line with this observation, treatment of human iPSC-derived neurons with 10 μM LPA induced $PPAR\gamma_1$ but not $PPAR\gamma_2$ mRNA expression by up to 3-fold compared to vehicle treatment alone (Fig. 2A). Next, we evaluated the effect of a specific LPA_1 antagonist Ki16425 on LPA-mediated $PPAR\gamma_1$ mRNA expression. Ki16425 significantly reduced LPA-induced $PPAR\gamma_1$ mRNA expression, whereas GW9662, a specific PPARγ antagonist, had no effect (Fig. 2B). We then directly investigated the interaction of LPA with the ligand-binding domain of PPARγ; 10 μM LPA elicited activation of a PPRE-ACox-Luc reporter gene expressed in human iPSC-derived neurons. LPA-induced activation of the reporter gene was abolished by treatment with GW9662, while Ki16425 had no effect (Fig. 3A). It has been previously reported that LPA promotes a variety of responses that include signals for cell proliferation, migration, and survival via interaction with LPA receptors expressed on the cell surface (19). Recently, it was reported that LPA also induces proliferation by acting as an intracellular agonist of PPARγ in human cultured cells (10). Consistent with this concept, we found that LPA-mediated proliferation was attenuated by either GW9662 or Ki16425

treatment in human iPSC-derived neurons (Fig. 3B). These results indicated that the effects of LPA on human iPSC-derived neuronal proliferation were mediated through both LPA_1-dependent and PPARγ-dependent pathways.

Central nervous system neurons lose their ability to regenerate early in development (20), yet the underlying mechanisms of this characteristic are still unclear. To inform this research question indirectly, we next screened LPA-regulated genes in human iPSC-derived neurons and found that LPA treatment profoundly induced the gene expression of KLF9 (Fig. 4A). KLF9 has been associated with several functions such as improved survival and neurite outgrowth in some neuronal subtypes (14). Furthermore, studies in embryonic cortical neurons revealed that KLF9 knockdown decreased neurite branching (21). Accordingly, we investigated the relationship between KLF9 and neurite outgrowth in human iPSC-derived neurons and found that KLF9 knockdown significantly decreased neurite outgrowth (Fig.4B, C). Moreover, KLF9 knockdown had negative effects on proliferation in both vehicle-treated and LPA-treated cells. These results suggested that KLF9 expression was required for proliferation and neurite outgrowth in human iPSC-derived neurons.

The present study is, to the best of our knowledge, the first to identify functional expression of LPA receptors in human iPSC-derived neurons. Although little is known about the pharmacological effects of LPA receptor stimulation on human iPSC-derived neurons, the detection of LPA_1 receptor functional expression in this study supports the idea that LPA_1 receptors are important modulators of neuronal proliferation. Furthermore, we found that the effects of LPA on neurite outgrowth and proliferation were also mediated through the PPARγ pathway. Taken together, these results suggest that the LPA-KLF9-PPARγ axis is a significant contributor to neurite outgrowth and proliferation in human iPSC-derived neurons.

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collection, analysis and interpretation of data; in writing of the report; or in the decision to submit the article for publication.

AUTHOR CONTRIBUTIONS

T.T. conceived and designed the project. T.T., S. Y., Y.M., and H.H. acquired the data; T.T. and H.H. analyzed and interpreted the data; and T.T. wrote the article. All authors have read and approved the final version of the article.

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FIGURE LEGENDS

Figure 1.

(A) Real-time PCR measurement of $LPA_{1\text{-}6}$ receptor mRNA in induced pluripotent stem cell (iPSC)-derived neurons. To obtain mature human neurons in a culture system, we maintained ReproNeuro human iPSC-derived neuronal progenitors in ReproNeuro maturation medium for 14 days according to the manufacturer's instructions. Total RNA was extracted from the cells and the expression of each LPA receptor was determined using quantitative real-time PCR. The relative levels of $LPA_{1\text{-}6}$ receptors normalized to GAPDH are expressed as the mean ± standard error of the mean (n = 3, ** $p < 0.01$).

Figure 2.

(A) Real-time PCR measurement of the expression of $PPAR\gamma_1$ and $PPAR\gamma_2$mRNA in induced pluripotent stem cell (iPSC)-derived neurons. Cells were incubated with 10 μM LPA (18:1) for 12 h. Relative mRNA levels of $PPAR\gamma_1$ and $PPAR\gamma_2$ normalized to GAPDH, are expressed as the mean ± standard error of the mean (n = 3, ** $p < 0.01$). (B) Real-time PCR measurement of the expression of $PPAR\gamma_1$ after treatment with Ki16425 or GW9662 normalized to GAPDH and expressed as the mean ± standard error of the mean (n = 3, ** $p < 0.01$).

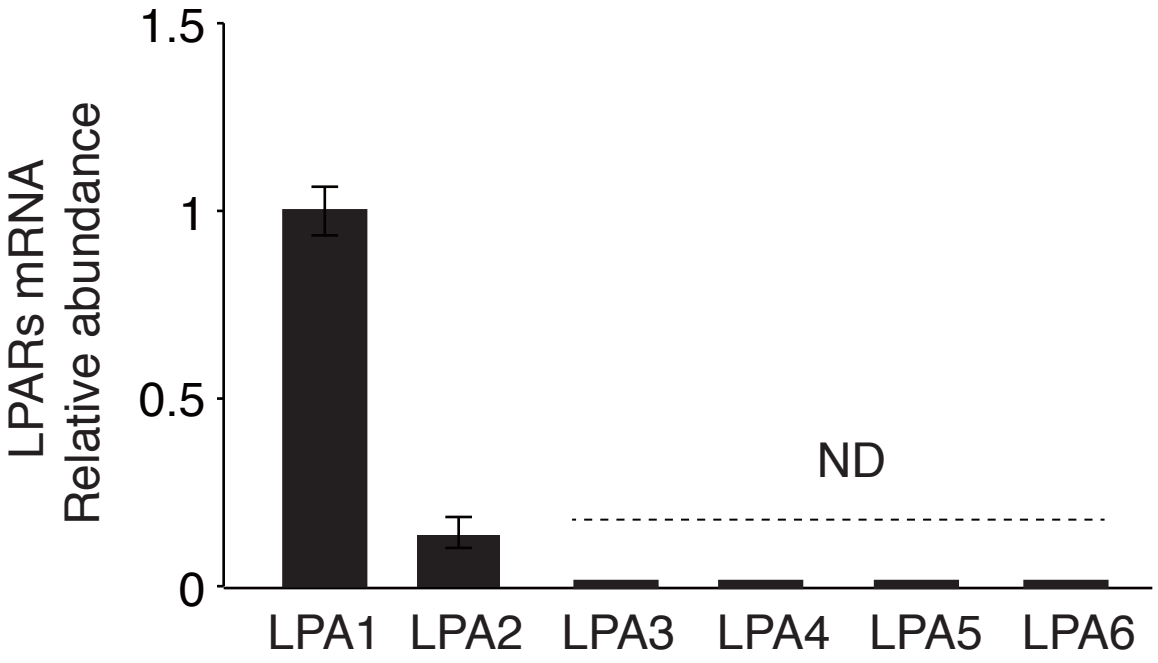
Figure 3.

(A) Induced pluripotent stem cell (iPSC)-derived neurons were transfected with the PPRE-luc and CMV-β-galactosidase plasmids for 72 h and then treated with LPA in the presence or absence of Ki16425 or GW9662 for 24 h. Luciferase activity was measured in cell lysates and normalized to β-galactosidase activity. Data represent the mean ± standard error of the mean (n = 4, ** $p < 0.01$). (B) Effects of LPA on the proliferation of iPSC-derived neuronal cells were tested in 0.1% BSA (fatty acid-free) medium. Cell proliferation was measured using the Cell Counting Kit-8 after a 24-h incubation period in medium alone (A) or in medium containing 10 μM LPA with or without Ki16425 or GW9662.

Figure 4.

Effects of KLF9 on LPA-induced neurite outgrowth. (A) Real-time PCR was used to measure KLFmRNA in induced pluripotent stem cell (iPSC)-derived neurons. (B) Representative images (scale bar = 10 μm) and (C) expression and quantification of the effects of KLF9 on LPA-enhanced neurite outgrowth at 24 h after plating. Neurons were treated with 10 μM LPA in the presence or absence of KLF9 siRNA and outgrowth was quantified for each exposure condition. (D) Effect of KLF9 siRNA on cell proliferation. At 24 h post-transfection, cells were re-plated in 96-well plates (5×10^3 cells/ well) and incubated for 24 h in the presence or absence of 10 μM LPA. Cell proliferation was determined using the Cell Counting Kit-8 (Dojindo).

Figure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0.5
0
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LPA2
LPA3
LPA4
LPA5
LPA6

Fig.1

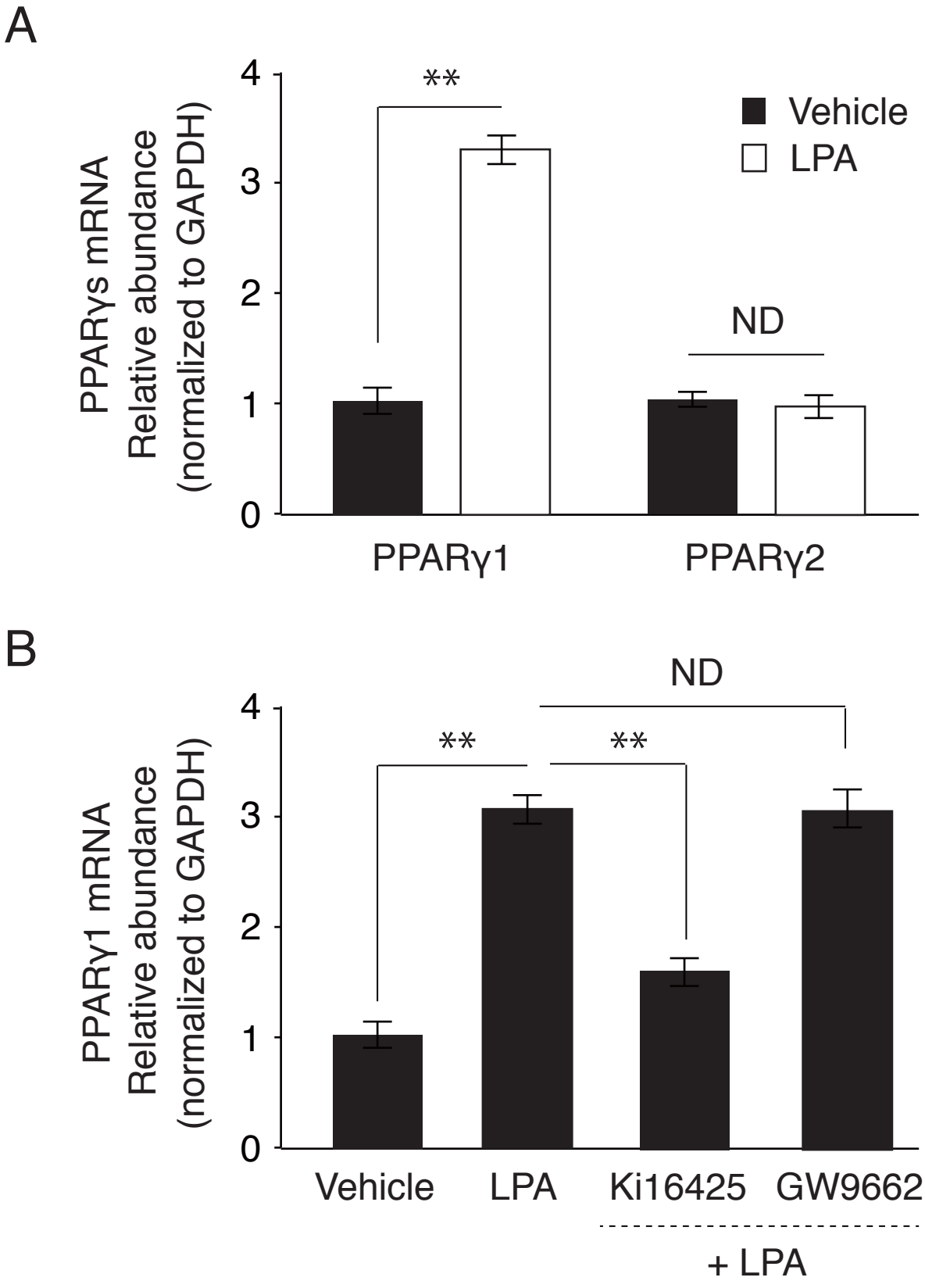
A
PPARγs mRNA
Relative abundance
(normalized to GAPDH)
4
3
2
1
0
**
ND
Vehicle
LPA
PPARγ1
PPARγ2
B
PPARγ1 mRNA
Relative abundance
(normalized to GAPDH)
4
3
2
1
0
ND
**
**
Vehicle
LPA
Ki16425
GW9662
+ LPA

Fig.2

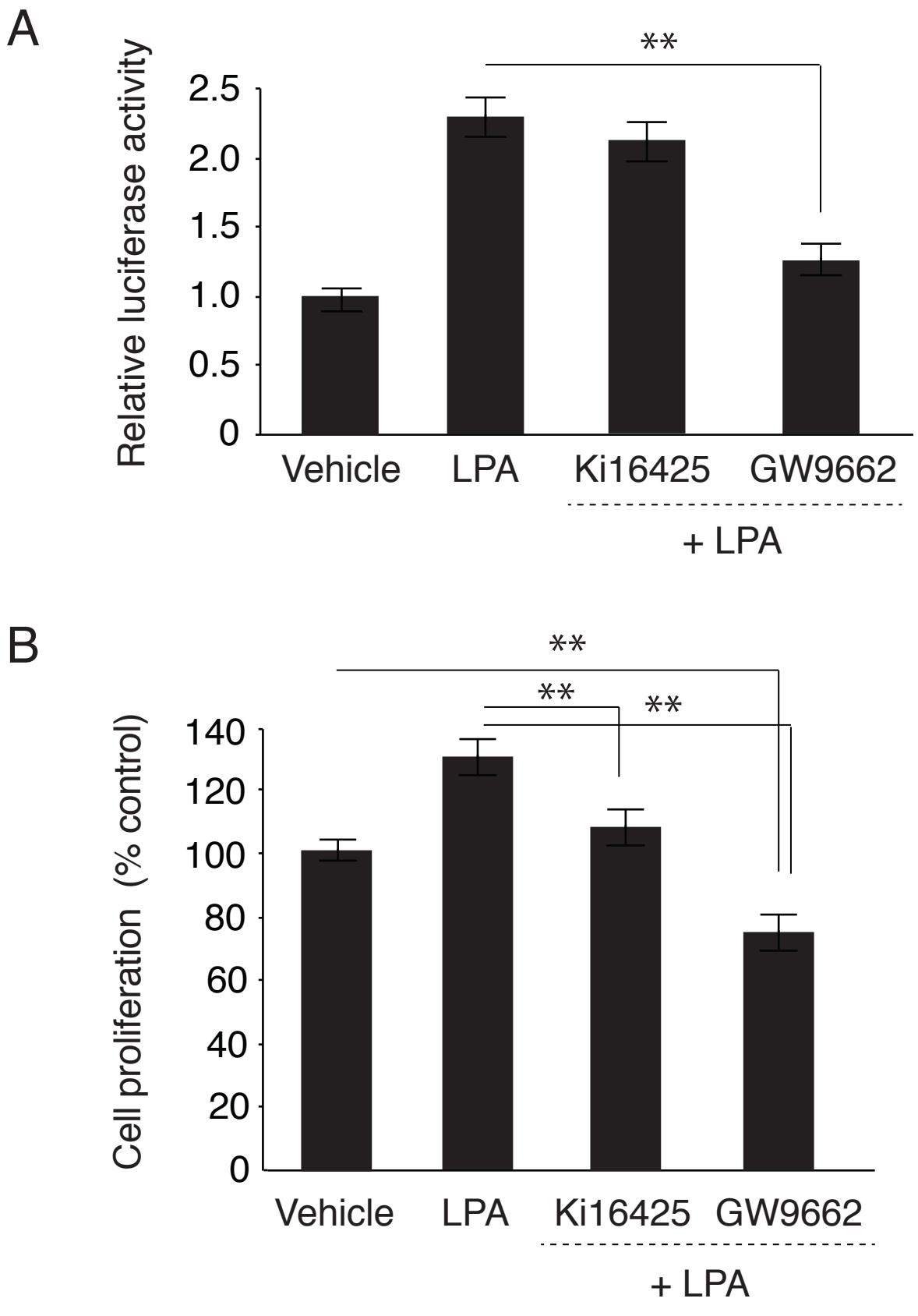
A
Relative luciferase activity
2.5
2.0
1.5
1.0
0.5
0
**
Vehicle
LPA
Ki16425
GW9662
+ LPA
B
Cell proliferation (% control)
140
120
100
80
60
40
20
0
**
**
**
Vehicle
LPA
Ki16425
GW9662
+ LPA

Fig.3

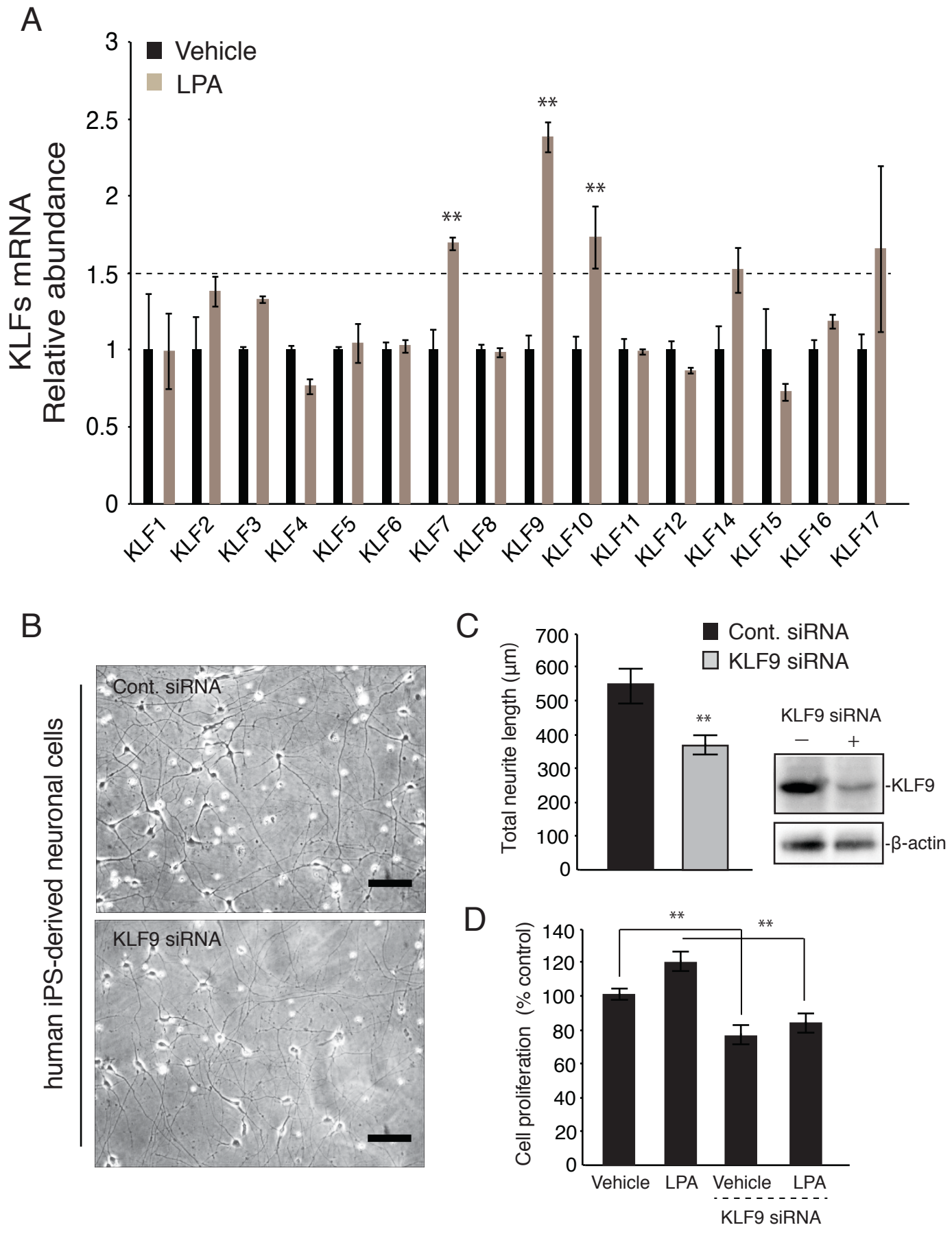
A
Vehicle
LPA
KLFs mRNA
Relative abundance
3
2.5
2
1.5
1
0.5
0
**
**
**
KLF1
KLF2
KLF3
KLF4
KLF5
KLF6
KLF7
KLF8
KLF9
KLF10
KLF11
KLF12
KLF14
KLF15
KLF16
KLF17
B
human iPS-derived neuronal cells
Cont. siRNA
KLF9 siRNA
C
Cont. siRNA
KLF9 siRNA
Total neurite length (μm)
700
600
500
400
300
200
100
0
**
KLF9 siRNA
–
+
-KLF9
-β-actin
D
Cell proliferation (% control)
140
120
100
80
60
40
20
0
**
**
Vehicle
LPA
Vehicle
LPA
KLF9 siRNA

Fig.4

***Conflict of Interest**

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