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LPA receptor1 antagonists as anticancer agents suppress human lung tumours

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ABSTRACT

Lysophosphatidic acid (LPA), as a bioactive lipid, plays a variety of physiological and pathological roles via activating six types of G-protein-coupled LPA receptors (LPA1–6). Our preliminary study found that LPA1 is highly expressed in lung cancer tissues compared with paracancerous tissues, but the role of LPA1 in lung carcinoma is unclear. This study aimed to elucidate the association between LPA1 and lung tumour behaviour at the cellular and animal model levels. We found that LPA promoted the migration, proliferation and colony formation of a lung cancer cell line (A549). LPA1 and LPA3 are preferentially expressed in A549 cells, and both Ki16425 (LPA1 and LPA3 antagonist) and ono7300243 (LPA1 antagonist) completely blocked the LPA-induced actions. These results were further verified by experiments of the LPA1/3 overexpression and LPA1 knockdown A549 cells. Furthermore, LPA1 overexpression and knockdown A549 cells were used to assess the *in vivo* tumour-bearing animal model and the mechanism underlying LPA-induced actions. In the animal model, A549 cell-derived tumour volume was significantly increased by LPA1 overexpression and significantly decreased by LPA1 knockdown respectively, suggesting that LPA1 is a regulator of *in vivo* tumour formation. Our results also indicated that the LPA1/Gi/MAP kinase/NF-κB pathway is involved in LPA-induced oncogenic actions in A549 cells. Thus, targeting LPA1 may be a novel strategy for treating lung carcinoma.

1. Introduction

Lysophosphatidic acid (LPA) is a bioactive lipid composed of a phosphate group, a glycerol backbone, and a single acyl chain that varies in length and saturation. LPA activates six class A G-protein-coupled receptors to provoke various cellular reactions. The six LPA receptors are subdivided into the endothelial differentiation gene (EDG) family (LPA1-LPA3) and the phylogenetically distant non-EDG family (LPA4-LPA6)(Choi et al., 2010). LPA is a serum-derived growth factor that is involved in several cellular functions, such as proliferation, migration, cytokine/chemokine secretion, and neurite retraction; LPA and LPA receptors have been implicated in various human diseases, including cancer, cardiovascular system diseases, and fibrosis (Zhao and Natarajan, 2013).

Xu initially reported LPA as a potential marker for epithelial ovarian cancer due to its high level of expression in these patients compared to patients with benign diseases and/or healthy controls (Xu et al., 1998). Another research group also found that the LPA level was much higher in the pleural effusions of patients with lung cancer than in benign pleural effusions (Bai et al., 2014). Therefore, an interesting conclusion is that the LPA level is significantly higher in malignant pleural effusions than in benign pleural effusions.

According to cancer statistics, lung cancer has the highest incidence rate among all cancers and is the leading cause of cancer-related death worldwide (Siegel et al., 2017; Torre et al., 2016). Regarding the roles of LPA and LPARs in lung cancer, BrP-LPA (a panantagonist for four LPA receptors and an inhibitor of the lysophospholipase D activity of autotaxin) was shown to inhibit A549 cell migration and invasion *in vitro* (Xu and Prestwich, 2010). Furthermore, conditional medium overexpressing ATX had higher LPA levels than medium not overexpressing ATX and stimulated cell migration (Zhao et al., 2011). In the tumour microenvironment, LPA-induced βig-h3 expression is mediated by the LPA1- Rho kinase pathway in human adipose tissue-derived mesenchymal stem cells, and βig-h3 was shown to elicit A549 cell

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adhesion and proliferation (Shin et al., 2012). Importantly, LPA was able to increase intracellular calcium levels, extracellular signal-regulated kinase 1/2 phosphorylation, and A549 cell contraction (Carmona-Rosas et al., 2017). Furthermore, genetic deletion of Lpar1 in mice significantly attenuated urethane-induced lung carcinogenesis (Magkrioti et al., 2018). Thus far, the roles of LPA and LPARs in the tumour biology of lung cancer remain obscure. Therefore, in this study, we explored the molecular mechanisms by which LPARs enhance the oncogenicity of lung cancer.

2. Materials and methods

2.1. Ethics statement

This investigation was conducted in accordance with ethical standards, the Declaration of Helsinki and national and international guidelines. All experiments were performed in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Inner Mongolia University (approval number 2019006).

2.2. Cell line and cell culture conditions

The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC; Gaithersburg, MD, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing high glucose and HEPES supplemented with heat-inactivated foetal bovine serum (FBS) (10% v/v), penicillin (100 U/ml), and streptomycin (100 μg/ml). The starvation medium had the same composition as the complete medium but did not contain FBS. A549 cell lines in which LPA1 was overexpressed or knocked down were constructed using a lentiviral vector. 1-Oleoyl-sn-glycero-3-phosphate (LPA, C18:1), fatty acid-free BSA, pertussis toxin (PTX), and epidermal growth factor (EGF) were obtained from Sigma-Aldrich. Ki16425, PD98059, SP600125, BAY11–7082, H89, ono7300243, staurosporine (STS) and SB203580 were purchased from Selleck Chemicals. These reagents were dissolved in phosphate-buffered saline (PBS) to make stock solutions, which were further diluted to the appropriate concentration with culture medium before each experiment. Importantly, LPA was diluted in basic medium containing 0.1% BSA.

2.3. Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis

To measure the expression levels of LPA1, LPA2, LPA3, LPA4, LPA5 and LPA6, cDNA was synthesized from individual cells using a Transcriptor First Strand cDNA Synthesis Kit (TaKaRa Bio Inc., Shiga, Japan). For quantitative real-time PCR analysis, SYBR Premix Ex Taq (Tli RNaseH Plus) (TaKaRa Bio Inc., Shiga, Japan) and a Smart Cycler II System (TaKaRa) were used. The PCR conditions were as follows: 1 cycle for 3 min at 95 °C, followed by 45 cycles for 10 s at 95 °C, 20 s at 60 °C, and 20 s at 72 °C. The expression levels of the target genes were normalized to those of human GAPDH. The human sequence-specific primers are presented in Table 1.

Table 1
Sequences of primers used for qRT-PCR.

Name	Forward primer sequence	Reverse primer sequence
LPA1	TCCTTCGTCAGGGCCTCATT	ACCGTAATGTGCCTCTCGATT
LPA2	GCTTCCACCAGCCCATCTAC	TGAGGAAGAGGTAGGCCACG
LPA3	GCAACCTGACCAAAAAGAGGG	GCAATTCCAGCCCAGTGTG
LPA4	TCCTTACCAACATCTATGGGAGC	ACGTTTGGAGAAGCCTTCAAAG
LPA5	ACTCGGTGGTGAGCGTGTA	GTGCAGTGCGTAGTAGGAGA
LPA6	CACCCGCCGTTTTTGTTCAG	ATGTTTTCCATGTGGCTTCTGG
GAPDH	CCAGGTGGTCTCCTCTGACTTC	GTGGTCGTTGAGGGCAATG

2.4. Cell viability assay

Cell viability was tested using a TransDetect TM Cell Counting Kit (CCK, TransGen Biotech). Cells were seeded at 4000 cells/well in 96-well plates containing complete medium. After the cells adhered to the well, the culture medium was replaced with starvation medium overnight. The cells were stimulated for 24 h with different reagents, including LPA (1 μM), EGF (10 ng/ml), Ki16425 (1 μM), ono7300243 (1 μM), PD98059 (5 μM), SB203580 (1 μM), SP600125 (5 μM), BAY11-7082 (1 μM) and PTX (100 ng/ml). At the indicated time points, 10 μl of CCK reagent was added to each well, and the cells were incubated at 37 °C for 3 h. Finally, the absorbance of each well was measured at a wavelength of 450 nm using a Cytation3 cell imaging multimode reader (Bio-Tek Instruments Inc). Independent experiments were repeated in triplicate.

2.5. Cell migration assay

For the cell migration assay, 1×10^5 cells were plated on a cell culture insert with an 8 mm pore size in 200 μl of starvation medium (upper chamber). The filters were then placed in 24-well plates (lower chamber) containing 500 μl of starvation medium with various stimulants, including LPA (0.1, 1, 5, and 10 μM), EGF (10 ng/ml), SB203580 (1 μM), PD98059 (5 μM), SP600125 (10 μM), BAY11-7082 (1 μM), H89 (10 μM) and STS (0.1 μM), and incubated for 6 h. The cells were pretreated with 100 ng/ml PTX for 24 h, and 1 μM ono7300243 or 1 μM Ki16425 for 30 min before the assay. After crystal violet staining, the number of cells that had moved to the lower side of the filter was counted under a microscope.

2.6. Colony formation assay

Cells were seeded in 6-well culture plates at various concentrations (10, 25 100, 200, and 300 cells/well) in 2 ml of complete medium with LPA (1 μM). The plates were incubated for 14 days, and the medium was replaced every third day. The colonies were stained with Giemsa solution for 20 min, and the colony numbers were counted under a Zeiss microscope.

2.7. Animal studies

BALB/c nude mice were obtained from Charles River Laboratories (Beijing, China). Tumour xenograft experiments were performed by overexpressing and knocking down LPA1 in stable A549 cells. Two types of cells were inoculated into 5 mice. The control and experimental group cells were suspended at a density of 2×10^7 cells/ml in PBS and inoculated subcutaneously into the right and left flank regions of each male BALB/c nude mouse at 4 weeks of age. The tumour size was assessed by external measurements of the length (L) and width (W) using a Vernier calliper. The tumour volume (TV; expressed in mm^3) was calculated using the following equation: $TV = (L \times W^2)/2$. Then, the animals were killed after 30 days, and the xenograft tumours were subjected to qRT-PCR and immunohistochemistry analyses.

2.8. Immunohistochemistry

All tumour tissues were fixed with 4% paraformaldehyde (pH 7.4) and embedded in paraffin. Four-micron-thick sections were cut from paraffin blocks and mounted on charged slides. The slides were deparaffinized in methylcyclohexane and hydrated with an ethanol series after baking for 30 min; antigen retrieval was then performed using a microwave, and the slides were blocked with 3% H_2O_2. The slides were incubated with goat serum for 10 min and with an LPA1 primary antibody (Santa Cruz, 1:400 dilution) overnight at 4 °C. On the second day, the slides were incubated with biotinylated polyclonal rabbit anti-rat immunoglobulin (Dako Cytomation) for 10–15 min. After washing

with PBS, the slides were stained with DAB and haematoxylin and then examined by a Zeiss microscope.

2.9. Tissue microarrays

Tissue arrays containing multiple human lung cancer tissues were obtained from Shanghai Outdo Biotech. Immunohistochemical staining microarrays were scored by multiplying the intensity and extent of staining for each tissue sample as previously described by (Azim et al., 2015).

2.10. Western blot analysis

Cells lysates were loaded onto a 10% Tris-acetate gel (Invitrogen) and subjected to electrophoresis under reducing conditions. After electrophoresis, the proteins were transferred onto Immobilon transfer membranes (Merck Millipore), which were incubated with anti-p-ERK1/2 (CST), anti-ERK1/2 (CST), anti-p-JNK (Santa Cruz Biotech.), anti-JNK (Santa Cruz Biotech.), anti-p38 (CST), anti-p-p38 (CST) or anti-α-tubulin (Sigma-Aldrich) antibodies. The bound antibodies were detected by a horseradish peroxide (HRP)-conjugated donkey anti-rabbit secondary antibody and an HRP-conjugated anti-mouse secondary antibody (Jackson, ImmunoResearch Laboratories, Inc.; 1:10000 dilution). The bands were visualized by reacting with an enhanced chemiluminescence (ECL) substrate (Bio Rad).

2.11. Statistical analysis

The data are presented as the mean ± S.E.M from three independent experiments, and all statistical analyses were performed using GraphPad Prism 7.0 software. The means were compared by *t*-test or one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. LPA promoted the migration, proliferation and colony formation of A549 lung cancer cells

We examined the effects of LPA on the migration, proliferation and colony formation activity of A549 lung cancer cells. A549 cells were treated with different gradient concentrations of LPA (0.01, 0.1, 1, and 10 μM), and our findings showed that LPA significantly promoted A549 cell migration at the 1 and 10 μM concentrations (Fig. 1A). LPA-induced A549 cell proliferation was detected by a cell viability assay. The proliferation rates of the group treated with LPA (1 μM) were markedly increased compared to those of the control group (Fig. 1B). EGF was a positive control and observably promoted A549 cell migration and proliferation. A549 cells seeded at different concentrations (10, 25, 50, 100, 200, and 300 cells) in 6-well culture plates were treated with or without LPA, and LPA obviously improved the colony formation ability of A549 cells (Fig. 1C).

3.2. LPA induced A549 cell migration, proliferation and colony formation through LPA1 coupled with the Gi protein

LPA exerts its cellular functions by acting on its six receptors (LPA1-6), and we thus detected LPAR expression profiles in human lung cancer A549 cells. LPA1/3 were shown to be expressed at higher levels than the other LPA reporters in A549 cells (Fig. 2A), indicating that LPA1/3 play an important role in the LPA-induced migration, proliferation and colony formation of A549 cells. To illustrate this point, we treated A549 cells with Ki16425 (a specific antagonist of LPA1 and LPA3) and ono7300243 (a specific antagonist of LPA1). As shown in Fig. 2B and C, both the migration and proliferation rates of A549 cells treated with Ki16425 and ono7300243 were decreased significantly compared to those of the control group. Then, we constructed LPA1-and LPA3-overexpressing stable transgenic cells. Interestingly, LPA induced A549 cell migration and proliferation through LPA1 but not through LPA3 (Fig. 2D–E). Cell proliferation and migration were inhibited in LPA1-knockdown A549 cells (Fig. 2F–G). Moreover, compared with control conditions, LPA1 overexpression enhanced colony formation (Fig. 2H). To further examine G protein involvement in the LPA-induced proliferation and migration of A549 cells, we treated cells with different G protein inhibitors. Our data indicate that PTX, a specific inhibitor of the Gi protein, abolished LPA-induced proliferation and migration (Fig. 2I–J).

3.3. LPA induced in vivo tumourigenesis via LPA1

To verify the pathological relevance of our *in vitro* findings, we extended our investigation to a nude mouse model of human lung cancer cells. Primary tumours of the axillae of nude mice were produced by injecting control group cells, LPA1-overexpressing cells and LPA1-knockdown A549 cells. After four weeks, the nude mice were killed, and the tumours were dissected, weighed and subjected to immunohistochemistry analysis. During this period, TVs were measured weekly, and the TVs were significantly higher in the LPA1-overexpressing group than in the control group (Fig. 3A–B). The tumours were excised and processed for qRT-PCR and immunohistochemical staining, revealing that LPA1 and Ki-67 (a tumour proliferation marker) were expressed at higher levels in the LPA1-overexpression group than in the control group (Fig. 3C and G). In contrast, compared with control conditions, LPA1 knockdown significantly reduced the TVs (Fig. 3D–E).

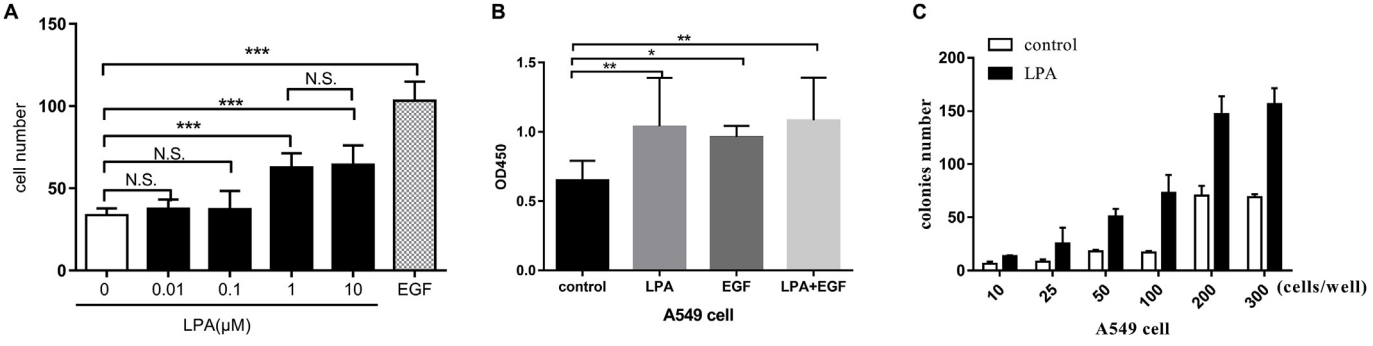

Fig. 1. LPA induced the migration, proliferation and colony formation of A549 cells
(A) Transwell assays were used to assess cell migration. The cells were stimulated with LPA at various concentrations (0.01, 0.1, 1, and 10 μM) for 6 h. EGF (10 ng/ml) was used as a positive control. (B) Cell proliferation was assessed using a CCK assay according to the manufacturer's instructions after the cells were treated with LPA (1 μM) and EGF (10 ng/ml) for 24 h. (C) Colony formation assay: different concentrations of cells (10, 25 100, 200, and 300 cells) seeded in 6-well plates were stimulated by LPA (1 μM). The values are the means ± S.D. and represent three independent experiments (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

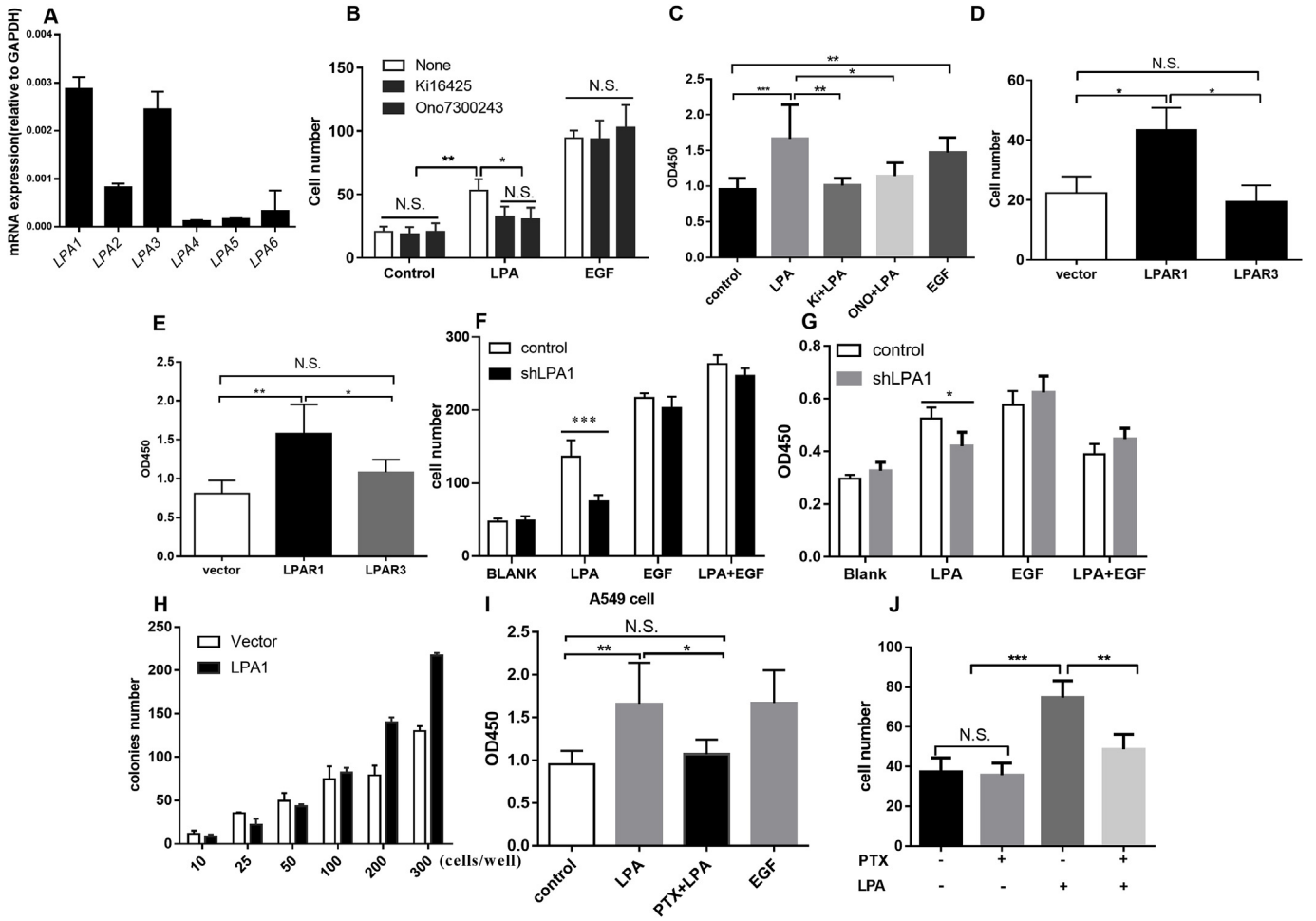

Fig. 2. LPA induced A549 cell migration, proliferation and colony formation through LPA1 coupled with the Gi protein
(A) LPAR expression was measured by quantitative real-time qRT-PCR. (B) A549 cells were treated with or without 1 μM LPA, 1 μM Ki16425 and 1 μM ono7300243 for 6 h, and migration was then measured using Transwell assays. (C) A549 cells in 96-well plates were treated with or without 1 μM LPA, 1 μM Ki16425, 1 μM ono7300243, and 10 ng/ml EGF for 24 h before the cells were grown under starvation conditions. Then, the absorbance of the 96-well plates was measured by a microplate reader at 450 nm. (D) LPA1 and LPA3 transgenic cells were stimulated with 1 μM LPA to detect their migration activity. (E) LPA1 and LPA3 transgenic cells were incubated with 1 μM LPA for 24 h, and cell proliferation was then detected with a CCK assay. (F) Cell motility was measured by Transwell assays in LPA1-knockdown A549 cells. (G) LPA1-knockdown A549 cells were treated with 1 μM LPA for 24 h, and cell proliferation was monitored by CCK assay. (H) LPA1-overexpressing and vector A549 cells were seeded at different concentrations (10, 25, 50, 100, 200, and 300 cells) in 6-well plates. (I) A549 cell proliferation were determined by the CCK assay after PTX pre-treated for 24 h. (J) Transwell assay was performed after.A549 cells treated with PTX for 6 h, as determined by the values are the means ± S.D. and represent three independent experiments (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

LPA1 expression was reduced in tumours treated with shLPA1 compared with control group tumours (Fig. 3F). Because laboratory studies may not recapitulate clinical lung malignancies, we extended our study to detect LPA1 expression in patient specimens by immunohistochemistry analysis. The LPA1 expression rate was also increased in cancer specimens compared with normal specimens, and the difference was statistically significant (Fig. 3H–I).

3.4. Signalling pathways of LPA-induced proliferation and migration in A549 cells

To explore the signalling pathways of LPA via LPA1-induced proliferation and migration in A549 cells, we examined LPA-induced MAP kinase responses in A549 cells. As shown in Fig. 4A, treatment with LPA resulted in the activation of ERK1/2, JNK and p38 MAP kinase expression in A549 cells, as measured by increases in the phosphorylated forms of these enzymes. In contrast, the levels of p38 and ERK1/2 phosphorylation were much higher in the LPA1-overexpressing group than in the control group (Fig. 4A–C). To determine whether the ERK1/2 and p38 MAP kinases mediated the LPA-induced changes in A549 cell proliferation and migration, we used specific inhibitors of these enzymes. Treatment with the p38 MAP kinase inhibitor SB203580 markedly suppressed LPA-induced migration in the LPA1-overexpressing group. In addition, the presence of PD98059, an ERK1/2 MAPK inhibitor, significantly decreased the LPA-induced proliferation of the LPA1-overexpressing group compared to the control group. However, the inhibition of JNK using SP600125 was hardly effective against LPA-induced proliferation and migration (Fig. 4D and E).

H89 effectively inhibits protein kinase A (PKA) at 10 μM, and staurosporine (STS) effectively inhibits protein kinase C (PKC) at 0.1 μM A549 cells were treated with H89 and STS for 20 min before stimulated by LPA. The PKC inhibitor STS significantly inhibited LPA-induced migration, but the PKA inhibitor H89 had no remarkable effect (Fig. 4E). Nuclear factor kappa B (NF-κB) is a transcription factor that controls many of genes transcription related to cancer. In our results, BAY11-7082, an NF-κB inhibitor, significantly inhibited LPA-induced cell migration and proliferation in the LPA1-overexpressing group compared with the control group (Fig. 4D and E).

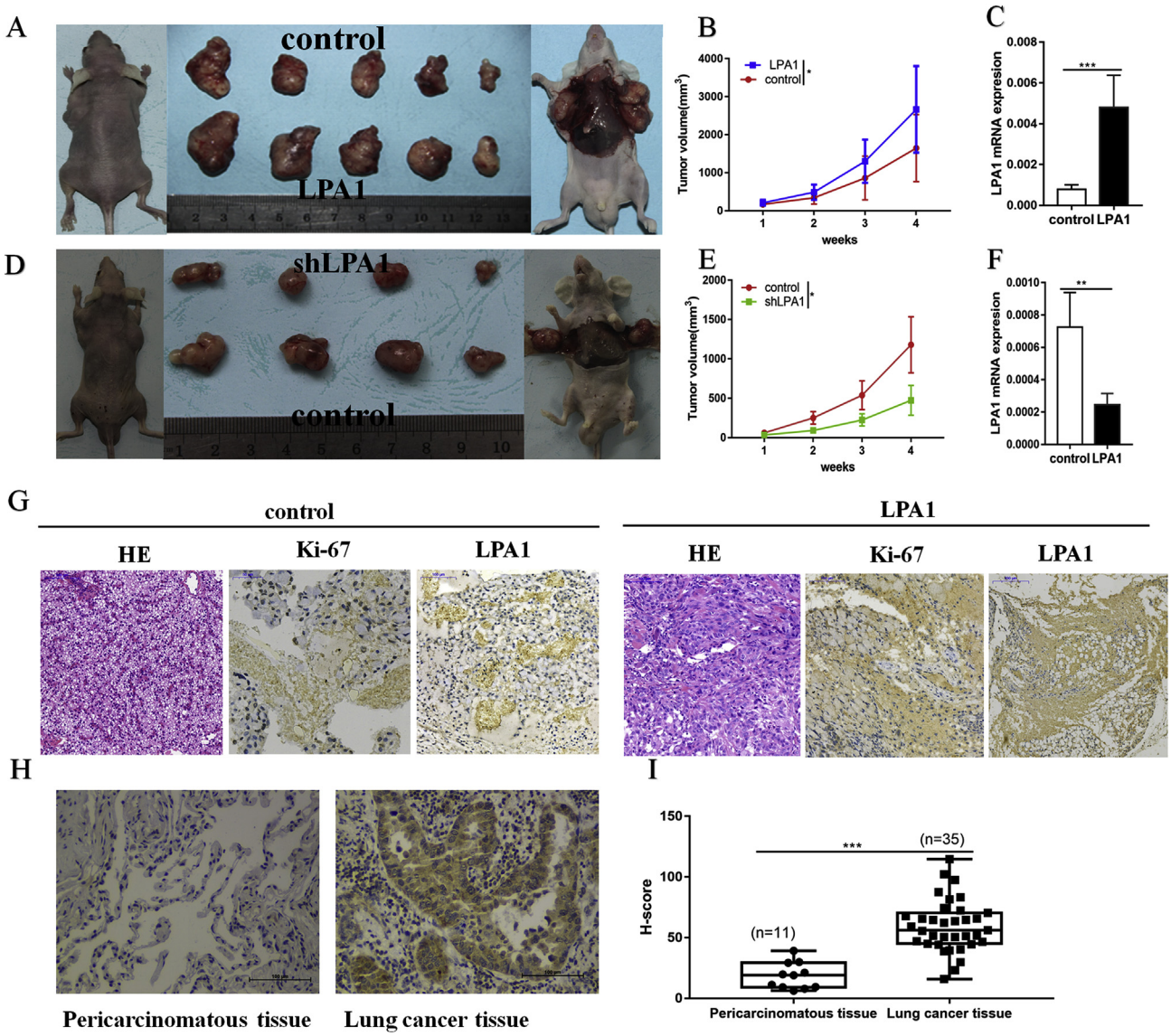

Fig. 3. LPA induced tumourigenesis *in vivo* via LPA1
(A) Representative images of tumours formed in BALB/c nude mice that were injected with either A549 cells alone or LPA1-overexpressing transgenic cells. The effects of tumour growth with or without LPA1 overexpression in BALB/c nude mice. (B) TV was monitored and measured once a week. (C) After 4 weeks, the tumours were excised and subjected to qRT-PCR analysis. (D) Representative macroscopic findings of lung tumours. A549 vector cells and A549 shLPA1 cells were injected subcutaneously into the right and left flanks of mice. (E) Tumour size was monitored and measured once a week. (F) On day 30, the tumours were excised and subjected to qRT-PCR analysis. (G) Histological morphology analysis of tumour tissue sections from A549 tumour-bearing mice stained with haematoxylin and eosin. Tumour tissue sections were subjected to immunohistochemistry detection using antibodies for Ki-67 and LPA1. (H) Immunohistochemistry staining results for LPA1 in lung cancer and pericarcinomatous tissues from patients (40 ×). (I) Expression of LPA1 as determined by immunohistochemistry using the H-scores of tissues from patients with lung cancer and pericarcinomatous tissues.

4. Discussion

Currently, lung cancer is the leading cause of cancer-related death worldwide (Siegel et al., 2017; Torre et al., 2016). Lung cancer is divided into non-small cell lung cancer and small cell lung cancer. Non-small cell lung cancer accounts for approximately 80–85% of cases and is divided into adenocarcinoma, squamous cell carcinoma and large cell carcinoma. The A549 cell line is derived from human squamous cell lung carcinoma and is frequently used as model for type II pulmonary epithelial cells (Croce et al., 1999; Lieber et al., 1976). The level of LPA is reportedly much higher in pleural effusions from patients with lung cancer than in benign pleural effusions from patients (Bai et al., 2014). Therefore, in this work, we explored a variety of actions of LPA in A549 cells. In a review paper published in Cell, Hanahan summarized that the hallmarks of cancer comprise eight biological capabilities that are acquired during the multistep development of human tumours (Hanahan and Weinberg, 2011). Among the cancer hallmarks are sustained proliferative signalling and increased tumour metastasis and invasion abilities. Therefore, we first examined A549 cell proliferation and migration abilities. Interestingly, our findings showed that LPA promoted the proliferation, migration and colony formation of A549 cells (Fig. 1).

We further identified the LPA receptor subtypes involved in LPA induced actions in A549. The expression profile of LPA receptors showed that LPA1 and LPA3 were markedly expressed in A549 cells, indicating that LPA1 or LPA3 was involved in LPA-induced actions in A549 cells. We previously reported that LPA stimulated the proliferation and migration of human umbilical cord mesenchymal stem cells and human artery smooth muscle cells through LPA1 (Bao et al., 2018; Li et al., 2017). Moreover, the hypoxic tumour microenvironment

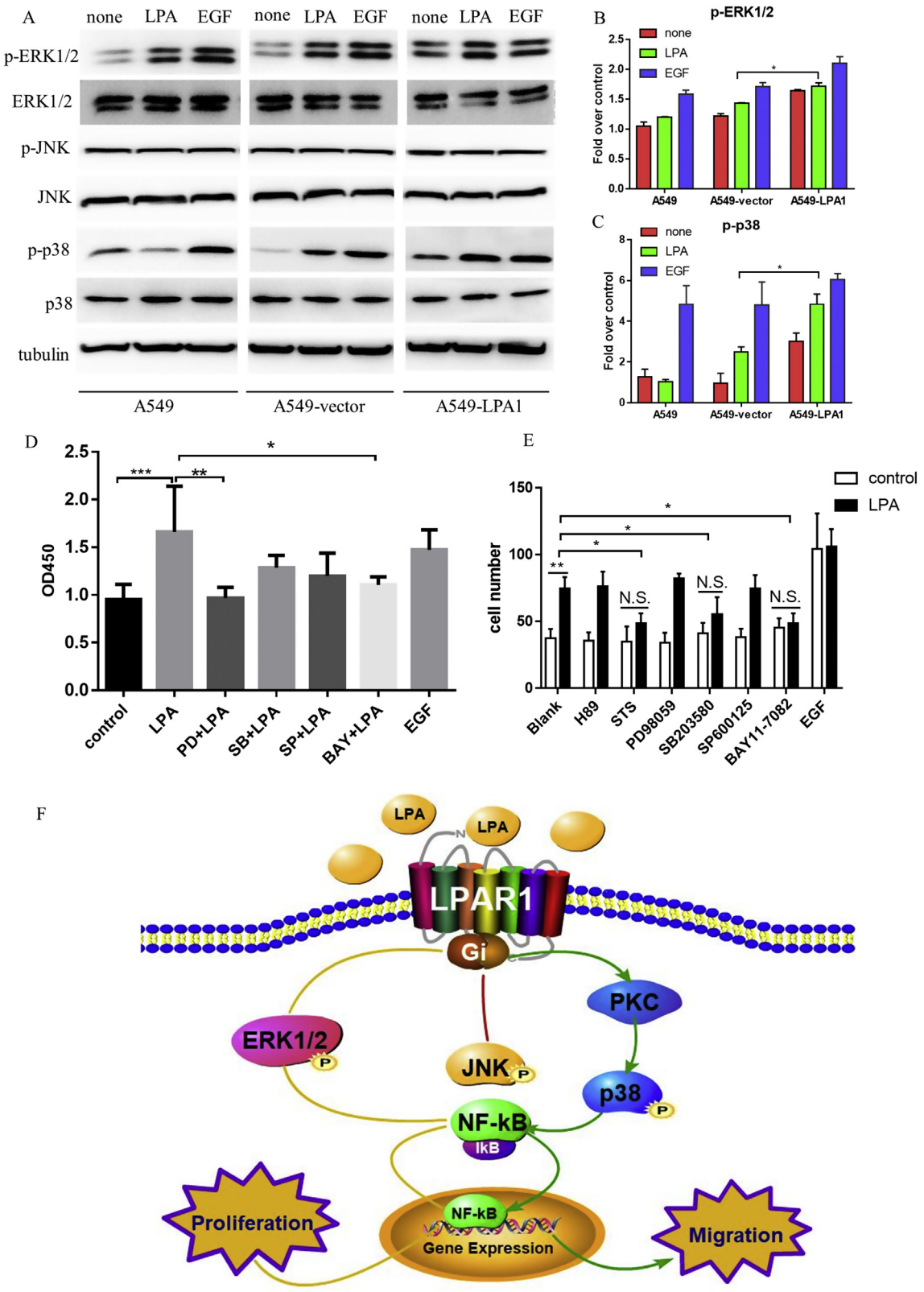

Fig. 4. Signalling pathways involved in A549 cell proliferation and migration induced by LPA
(A) Significant changes in MAP kinase phosphorylation were detected by western blotting. A549 cells with or without LPA1 overexpression were stimulated with 1 μM LPA and 10 ng/ml EGF for 15 min. (B) Analysis of ERK1/2 phosphorylation. (C) Analysis of p38 phosphorylation. (D) Proliferation was measured with CCK assay. A549-LPA1 cells in 96-well plates were treated with or without 1 μM LPA, 5 μM PD98059, 1 μM SB203580, 5 μM SP600125, 10 ng/ml EGF and 1 μM BAY11-7082 for 24 h. (E) A549 cells were stimulated with 1 μM LPA, 10 μM H89, 0.1 μM STS, 5 μM PD98059, 1 μM SB203580, 5 μM SP600125, 10 ng/ml EGF and 1 μM BAY11-7082 for 6 h and subjected to Transwell assay. (F) LPA effects via the LPA1 signalling pathway.

promoted invadopodia formation and metastasis through LPA1 (Harper et al., 2018). LPA enhanced the cell invasion activity through LPA1 in pancreatic cancer cells (Fukushima et al., 2018a). Therefore, we speculate that LPA1 has a high correlation with lung cancer A549 cells. We tried to further elucidate the roles of LPA1 in lung cancer at the cell, tissue and animal levels in combination with clinical and pathological data. Our data demonstrated that LPA1 was involved in LPA-induced proliferation, migration and colony formation, which were detected by LPAR inhibitors, LPA1/3 transgenic cells and LPA1-knockdown A549 cells (Fig. 2B–H). We also verified this conclusion with an *in vivo* experiment. LPA1 overexpression promoted tumour growth in tumour-bearing nude mouse experiments, while tumour growth was inhibited in LPA1-knockdown cells. High LPA1 expression was observed in lung cancer patients by immunohistochemical experiments. These results indicated that LPA1 plays an important role in the occurrence and development of lung tumours.

LPAR genes are expressed in a clear cell type-selective manner; tumour cells predominantly express LPA1-3, while macrophages and T cells also express LPA5 and LPA6 at high levels, indicating cell type-specific LPA signalling pathways (Reinartz et al., 2019). Bioinformatics tools were used to analyse LPA1, which was identified as a hub gene associated with the progression of gastric cancer (Gong et al., 2019). HT1080 cells treated with chemotherapy drugs had improved LPA2 expression, which promoted tumour cell invasion (Takahashi et al., 2018). LPA3 was identified as a tumourigenic gene, which showed that LPA3 expression was correlated with a lower overall survival rate (Ihara et al., 2017). Lysophosphatidic acid signalling via LPA4 and LPA6 negatively regulated the cell mobility activities of colon cancer cells (Takahashi et al., 2017). In addition, cell mobility activity was regulated through LPA5 by phorbol ester and anticancer drug treatments in melanoma A375 cells (Fukushima et al., 2018b). LPA1 usually couples with different G proteins, including Gi, G12/13 and Gq, depending on the cell type and the environment. To identify which G protein is involved in LPA-induced A549 cell proliferation and migration, several G protein inhibitors were used in the cell proliferation and migration assays. Excitingly, LPA enhanced A549 cell proliferation and migration in a PTX-dependent manner, which illustrated that LPA1 belongs to the Gi protein-sensitive receptors (Fig. 2I and J). Gi protein activation reduces intracellular cAMP levels in mammalian cells and results in decreased PKA activation. The PKC signalling pathway likely responds to LPA1 coupled with the Gi protein (Carmona-Rosas et al., 2017). Based on current research, we again demonstrated that the activated Gi protein regulates the PKC signalling pathway but not the PKA signalling pathway after treatment with two inhibitors, H89 and STS (Fig. 4D and E).

In numerous cell types, PKC activation enhances the transcription of specific genes. To date, PKC is known to activate very powerful MAPK cascades, which are some of the largest protein kinases in cells. The phosphorylated forms of p38, ERK1/2 and JNK were detected by western blotting in A549 cells, and the results revealed that LPA-induced migration and proliferation occurred via LPA1, indicating that the three MAP kinases were involved in this phenomenon (Fig. 4A). The ERK1/2 module plays a central role in controlling cell proliferation. ERK1/2 activity is rapidly stimulated by mitogenic agents, and in normal cells, sustained activation of these kinases is required for efficient G1-to S-phase progression (Meloche and Pouyssegur, 2007). Our findings showed that ERK1/2 was phosphorylated in a time-dependent manner and that PD98059 inhibited LPA-induced cell proliferation, indicating that ERK1/2 was involved in the cell proliferation process. Moreover, p38 mediated LPA-induced cell migration through its phosphorylation. However, JNK was highly phosphorylated and was not involved in cell proliferation or migration. Similar to p38 MAPKs, JNK is strongly activated in response to various cellular stresses; in addition, JNK is activated by growth factors, some GPCR ligands, and serum (Bogoyevitch et al., 2010). Furthermore, most stimuli that activate p38 MAPKs also stimulate JNK isoforms, and many MAPKKKs in the p38 module are shared by the JNK module (Cargnello and Roux, 2011). Unfortunately, we do not clearly understand the reasons for JNK phosphorylation.

NF-κB refers to a group of transcription factors (RelA, RelB, c-Rel, NF-κB1/p50 and NF-κB2/p52) that form homo- and heterodimers to upregulate or suppress the expression of many genes; consequently, these transcription factors play a role in many steps of cancer initiation and progression (Hoesel and Schmid, 2013; Sokolova and Naumann, 2017). During these latter processes, NF-κB cooperates with multiple other signalling molecules and pathways. BAY11-7082, an inhibitor of NF-κB, blocked LPA-induced migration and proliferation, indicating that NF-κB plays a role in LPA-induced actions in A549 cells.

One hallmark of cancer is its ability to replicate itself indefinitely. We found an LPA-mediated signalling pathway (LPA/LPA1/ERK1/2/NF-κB) in the lung cancer cell proliferation process. Metastasis is responsible for more than 90% of cancer-associated mortality in clinical oncology (Mehlen and Puisieux, 2006). In our study, we discovered that the LPA/LPA1/Gi/PKC/p38/NF-κB signalling pathway was involved in lung cancer cell migration (Fig. 4F). According to the cancer database, LPA1 is highly expressed in lung cancer compared with other cancers, and lung cancer patients with high LPA1 expression have a low survival rate (Koster, 2019). Therefore, combined with our previous studies and the cancer database, we propose LPA1 antagonists to be potential therapeutic and preventative drugs for the treatment of lung cancer.

Declaration of competing interest

We have no conflicts of interest to declare.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2019.172886.

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