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Cancer Science

Lysophosphatidic acid stimulates the proliferation and motility of malignant pleural mesothelioma cells through LPA receptors, LPA_1 and LPA_2

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Short running title: LPA stimulates MPM via LPA receptors.

Key words: Lysophosphatidic acid; Lysophosphatidic acid receptors; malignant pleural mesothelioma; cell proliferation; cell motility

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Summary

Lysophosphatidic Acid (LPA) is one of the simplest natural phospholipids. This phospholipid is recognized as an extracellular potent lipid mediator with diverse effects on various cells. Although LPA is shown to stimulate proliferation and motility via LPA receptors, LPA_1 and LPA_2, in several cancer cell lines, the role of LPA and LPA receptors for malignant pleural mesothelioma (MPM) was unknown. MPM is an aggressive malignancy with a poor prognosis and the incidence is increasing and is expected to more increase for another 10 to 20 years in worldwide. Therefore development of novel effective therapies needs urgently. In this study, we investigated the effect of LPA on the proliferation and motility of MPM cells. We found that all twelve cell lines and four clinical samples of MPM expressed LPA_1, and some of them expressed LPA_2, LPA_3, LPA_4, and LPA_5. LPA stimulated the proliferation and motility of MPM cells in a dose-dependent manner. Moreover, LPA-induced proliferation was inhibited by Ki16425, an inhibitor of LPA_1, and small interfering RNA against LPA_1, but not LPA_2. Interestingly, LPA-induced motility was inhibited by small interfering RNA against LPA_2, but not LPA_1, unlike a number of previous reports. These results indicate that LPA is a critical factor on proliferation though LPA_1, and on motility

though LPA_2 in MPM cells. Therefore, LPA and LPA receptors, LPA_2 as well as LPA_1, represent potential therapeutic targets for patients with MPM.

Abbreviations

MPM, malignant pleural mesothelioma; SV40, the simian virus 40; LPA, Lysophosphatidic acid; GPCRs, GTP binding protein-coupled receptors; FBS, fetal bovine serum; RT-PCR, reverse transcription-PCR; BSA, bovine serum albumin; MAPK, mitogen activated protein kinase; SCID, severe combined immunodeficiency

Introduction

Malignant pleural mesothelioma (MPM) is an aggressive tumor arising from the mesothelial cells in the pleural cavity. It is frequently diagnosed at locally advanced stage and is refractory to conventional treatment regimens, such as chemotherapy and radiation therapy, and so its prognosis is extremely poor. Several etiological factors, including asbestos (1,2), iron (3), the simian virus 40 (SV40), radiation and thorotrast (4), have been reported to be involved in the development of MPM. Of these factors, exposure to asbestos is most closely related to the development of MPM. Prior to the recognition of its adverse health effects, large amounts of asbestos were used worldwide. As the latent period between the first exposure to asbestos and the occurrence of MPM tends to be from 30 to 40 years, it is expected that there will be rapid increases in the number of MPM patients in Europe and Australia from 2015 to 2020 (5) and in Japan from 2010 to 2040 (2). Thus, the incidence of MPM is expected to increase further for another 10 to 20 years worldwide, although the disease has already reached its peak incidence in the USA (5). The major reasons for the poor prognosis of MPM are 1) the highly proliferating and invasive characteristics of the disease and 2) its resistance to conventional

chemotherapy and radiotherapy. Therefore, novel effective therapeutic strategies based on the molecular mechanisms of proliferation and motility/invasion of MPM are required to improve the prognosis of this disease. While several growth factors, including platelet-derived growth factor A and B, epidermal growth factor, transforming growth factor β, and vascular endothelial growth factor, were reported to be involved in the progression of MPM (5), the molecular mechanisms of the proliferation and motility/invasion of MPM are not fully understood.

Lysophosphatidic acid (LPA) is one of the simplest natural phospholipids. It is present in serum at concentrations of 2–20 μM (6) and is derived from various enzymatic mechanisms, such as activated platelets, fibroblasts, adipocytes, and ovarian tumors (6–8). Recently, this phospholipid has attracted a great deal of attention as a potent lipid mediator responsible for multiple cellular processes (9), such as cell proliferation, platelet aggregation, smooth muscle contraction, and cytoskeletal reorganization. In fact, extracellular LPA has been shown to be associated with various diseases, including atherosclerosis (10) and cancer (11–13). LPA exerts its multiple biological functions *via* three types of G protein-coupled receptor (GPCR), LPA_1, LPA_2, and LPA_3. These receptors

share 50–54% amino acid homology and transmit their signals *via* three families of heterotrimeric G proteins, G_i, G_q, and $G_{12/13}$ (14). Furthermore, more recent studies have identified orphan GTP binding protein-coupled receptors (GPCRs), LPA_4 (15) and LPA_5 (16).

The mechanisms of action of LPA receptors differ among subtypes, and they are expressed at different levels in both normal and malignant tissues. In normal tissues, LPA_1 is highly expressed in the brain and heart, LPA_2 is highly expressed in the testis and leukocytes, LPA_3 is highly expressed in the kidney and prostate (17,18), and LPA_4 is expressed highly in the ovary (15). In malignant tumors, LPA_1 is dominant in brain tumors (19), LPA_2 is predominantly expressed in colon, stomach, thyroid, and breast cancers (20–22), and LPA_3 is expressed at relatively high levels in ovarian and prostate cancers (19,23). Another study showed that the expression pattern of LPA receptors is associated with cancer differentiation in gastric cancer cells (24). Moreover, LPA_1 has recently been reported to control tumor cell proliferation in prostate (25) and breast cancer cells (26) and LPA_2 was shown to mediate the growth of ovarian (27), thyroid (21), and colon cancer cells (28). Interestingly, LPA_1 also stimulates the motility of various types of cancer, such as pancreas, colon,

glioblastoma, and gastric cancer (19,24,28,29). Thus, LPA receptors play crucial roles at least in cancer proliferation and motility/invasion. However, the roles of LPA receptors and their downstream signaling pathways in MPM are unknown.

The present study was performed to explore the expression of LPA receptors in human MPM cell lines and further elucidate the roles of five LPA receptors in the malignant properties of MPM cells in terms of proliferation and motility.

Materials and Methods

Cell lines and culture conditions.

In this study, we used twelve human MPM cell lines, EHMES-1, EHMES-10, MSTO-211H, Y-MESO-8A, NCI-H28, NCI-H290, NCI-H513, NCI-H2052, NCI-H2373, NCI-H2452, ACC-MESO-1, and ACC-MESO-4. EHMES-1 and EHMES-10 (30,31) were kindly provided by Dr Hironobu Hamada (Ehime University, Matsuyama, Japan). NCI-H290 and NCI-H513 were kindly provided by Dr Adi F. Gazdar (University of Texas Southwestern Medical Center, Dallas, TX). Y-MESO-8A, ACC-MESO-1, and ACC-MESO-4 (32) were established in Aichi Cancer Center Research Institute. MSTO-211H, NCI-H28, NCI-H2052, NCI-H2373, and NCI-H2452 were purchased from American Type Culture Collection (Manassas, VA).

All human tumor cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (50 μg/ml); this was designated as RPMI 1640 in this study. Cells were cultured in a humidified CO_2 incubator at 37°C.

Clinical specimens.

All human MPM samples were obtained from the Department of Internal Medicine and Molecular Therapeutics, University of Tokushima Gradate School. Written informed consent was obtained from all subjects prior to tissue sampling.

Reagents.

1-Oleoyl-LPA (18:1) and Ki16425 were purchased from Sigma-Aldrich Inc. (St. Louis, MO).

Expression of LPA_1, LPA_2, LPA_3, LPA_4, and LPA_5.

LPA_1, LPA_2, LPA_3, LPA_4, and LPA_5 mRNA expression were determined by reverse transcription-PCR (RT-PCR). Total RNA samples were isolated using an RNeasy Mini kit and RNase-free DNase kits (Qiagen, Valencia, CA) in accordance with the manufacturer's recommendations. Total RNAs were reverse transcribed using an Omniscript RT kit (Qiagen). The primers for LPA_1, LPA_2, LPA_3, LPA_4, LPA_5, and β-actin were as follows:

LPA_1: 5'-TGGCTGCCATCTCTACTTCC-3' and

5'-AACCAATCCAGGAGTCCAGC-3', LPA_2:

5'-CATCATGCTTCCCGACAACG-3' and 5'-GGGCTTACCAAGGATACGCAG-3',

LPA_3: 5'-AGTGTCACTATGACAAGC-3' and 5'-GAGATGTTGCAGAGGC-3',

LPA_4: 5'-TGAAGGCTTCTCCAAACGTGTCTG-3' and

5'-GTTCAGAGTTGCAAGGCACAAGGT-3', LPA_5:

5'-CTGGATCTAAACCGCCACAG-3' and 5'-GCCTGGAAAAGGGGATGT-3',

and β-actin: 5'-AAGAGAGGCATCCTCACCCT-3' and

5'-TACATGGCTGGGGTGTTGAA-3'.

PCR was performed with Ex Taq Hot Start Version (Takara Bio, Shiga, Japan). The bands were visualized by ethidium bromide staining. Data shown are representative of three independent experiments.

Cell proliferation assay.

Cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye reduction method (33). Briefly, tumor cells seeded in 96-well plates (5×10^3 per well) were

incubated in RPMI 1640 for 24 h. The cells were starved for 24 h by replacing the medium with serum-free RPMI 1640 medium containing 0.1% fatty acid-free bovine serum albumin (BSA), with or without LPA and/or Ki16425, an inhibitor of LPA_1 (and LPA_3 at higher doses) (34). The cells were cultured for 48 h. Then, an aliquot of 50 μL of stock 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (2 mg/mL; Sigma, St. Louis, MO) was added to each plate and the cells were incubated for 2 h at 37°C. The media containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution were removed and the dark blue crystals were dissolved by adding 100 μL of DMSO. Absorbance was measured with an MTP-120 microplate reader (Corona Electric, Ibaraki, Japan) at test and reference wavelengths of 550 and 630 nm, respectively. Data shown are representative of three independent experiments.

Cell migration assay.

Cell migration was quantified in Falcon Cell Culture Inserts (pore size 8 μm; BD Biosciences, San Jose, CA). Cells suspended in serum-free RPMI 1640 containing 0.1% fatty acid-free BSA, with or without LPA and/or Ki16425, were

added to the upper chamber (1×10^5 cells in 100 μl/well). Serum-free RPMI 1640 containing 0.1% fatty acid-free BSA, containing human fibronectin (5 μg/ml; BD Biosciences) as a chemoattractant was placed in the lower chamber. When the cells treated with Ki16425, the cells were preincubated with 10 μM Ki16425 for 30 min. The cells were allowed to migrate for 3 h at 37°C. Non-migratory cells were removed from the top filter surface with a cotton swab. Migrated cells, attached to the bottom surface, were fixed with methanol, stained with Giemsa, mounted, and then counted. Data shown are representative of three independent experiments.

RNA interference.

Three different duplexed Stealth™ RNAi (Invitrogen, Carlsbad, CA) against LPA_1 and LPA_2, and Stealth RNAi Negative Control Kit (Invitrogen) were used for RNA interference assay. Briefly, aliquots of 1×10^5 211H, H28, or H2052 cells in 2 ml of antibiotic-free medium were plated on 6-well plates and incubated at 37°C for 24 h, and the cells were then transfected with siRNA (250 pmol) or scramble RNA (siSCR) using Lipofectamine 2000 (5 μl) in accordance with the

manufacturer's instructions. After 24-h incubation, the cells were washed twice with PBS, and incubated in antibiotic-containing medium. These cells were then used for proliferation or migration assay as described above. LPA_1 and LPA_2 knockdown were confirmed by RT-PCR analysis. The sense sequences of siRNA were as follows:

siLPA1#1: 5'- AACCAAUCCAGGAGUCCAGCAGAUG-3' and 5'-CAUCUGCUGGACUCCUGGAUUGGUU-3'; siLPA1#2: 5'-AUUACAGGGAUGGAAGUAGAGAUGG-3' and 5'-CCAUCUCUACUUCCAUCCCUGUAAU-3'; siLPA1#3: 5'-AUAGAUUGCCACCAUGACCAAUAGG-3' and 5'-CCUAUUGGUCAUGGUGGCAAUCUAU-3'; siLPA2#1: 5'-UACACAGCAGCAUUGACCAGUGAGU-3' and 5'-ACUCACUGGUCAAUGCUGCUGUGUA-3'; siLPA2#2: 5'-AUGUAUAGUGGACAGACUCGCGGGU-3' and 5'-ACCCGCGAGUCUGUCCACUAUACAU-3'; siLPA2#3: 5'-UGUGGAACAUGAGGAAGAGGUAGGC-3' and 5'-GCCUACCUCUUCCUCAUGUUCCACA-3'.

Western blotting.

Tumor cells were washed twice with PBS, harvested in cell lysis buffer (20 mM Tris, pH7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride), and flash-frozen on dry ice. After allowing the cells to thaw, the cell lysates were collected with a rubber scraper, sonicated, and centrifuged at 14000×*g* (4°C for 20 min). The total protein concentration was measured using a Pierce BCA Protein Assay Kit (Pierce, Rockland, IL). Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis, and the proteins were then transferred onto PVDF membranes (Atto, Tokyo, Japan). The membranes were blocked with Blocking One (Nacalai Tesque Inc, Kyoto, Japan) for 1 h at room temperature, and then the blots were incubated at 4°C overnight with anti-phospho-p44/42 mitogen-activated protein kinase (MAPK) (Thr202/Tyr204), anti-p44/42 MAPK (1:1000 dilution; Cell Signaling Technology, Beverly, MA), or anti-β-actin antibody (1:5000 dilution; Sigma), followed by incubation for 2 h at room temperature with secondary Ab (horseradish peroxidase-conjugated species-specific Ab). Immunoreactive bands were visualized using enhanced chemiluminescent substrate (Pierce).

RhoA activity assay.

We determined RhoA activation as the amount of RhoA-GTP binding using a G-LISA RhoA™ Activation Assay Biochem Kit™ (Cytoskeleton, Denver, CO), in accordance with the manufacturer's recommendations. Briefly, tumor cells (2×10^5 per well) seeded in 6-well plates were incubated in RPMI 1640 containing 10% FBS for 24 h. The cells were starved for 24 h by replacing the medium with serum-free RPMI 1640 medium containing 0.1% BSA. The dishes were then treated with LPA (10 μM) for 0, 1, 3, 6, 12, and 30 min. The tumor cells were washed twice with PBS and harvested in cell lysate with a cell scraper, sonicated, and centrifuged at 10000×*g* (4°C for 2 min). The total protein concentration was measured using Precision Red™ Advanced Protein Assay Reagent. Equal amounts of cell lysate protein were incubated in Rho-GTP affinity plates for 30 min at 4°C. To the plates was added anti-RhoA primary antibody, followed by secondary HRP-labeled antibody and HRP detection reagent, and incubated at room temperature for the time recommended by the manufacturer. Absorbance was measured with an MTP-120 microplate reader at wavelength of 490 nm. Data shown are representative of three independent

experiments with similar results.

Statistical analysis.

All data, expressed as means ± SE, were analyzed by one-way analysis of variance. The statistical significance of differences was assessed by the Fisher protected least-significant difference test. *P* values less than 0.05 were considered statistically significant. Statistical analyses were performed using StatView ver.5.0.

Results

Human MPM cell lines expressed LPA_1, LPA_2, LPA_3, LPA_4, and LPA_5 at various levels.

In the first set of experiments, we examined the mRNA expression of five LPA receptors (LPA_1, LPA_2, LPA_3, LPA_4, and LPA_5) in twelve human MPM cell lines by RT-PCR (Fig. 1A). All of the MPM cell lines examined expressed LPA_1 mRNA at various levels. Of these cell lines, 211H, H28, H290, H2052, and H2373 cells showed higher levels of expression as compared with the others. On the other hand, some of the MPM cell lines expressed LPA_2. Y-MESO-8A, H290, H2052, and ACC-MESO-4 cells showed high levels of LPA_2 expression as compared with the other cell lines. Similarly, some of the MPM cell lines expressed LPA_3, LPA_4, and LPA_5. In parallel experiments, we examined LPA_1, LPA_2, LPA_3, LPA_4, and LPA_5 mRNA expression in tumor specimens obtained from four patients with MPM (Fig. 1B). Consistent with the results in the cell lines examined, all samples expressed LPA_1 and some expressed LPA_2, LPA_3, LPA_4, and LPA_5.

LPA stimulated the proliferation of MPM cells via *LPA_1.*

We determined the effects of LPA on proliferation of MPM cells. Exogenous LPA at physiological concentrations (up to 20 μM) significantly stimulated the proliferation of 5 of the 11 MPM cell lines examined (data not shown). Of these cell lines, 211H and H28 cells responded well to LPA in a dose-dependent manner (Fig. 2A). Therefore, these two cell lines were used to investigate whether LPA receptors are involved in LPA-induced proliferation, when the effects of Ki16425, an inhibitor of LPA_1 (and LPA_3 at higher doses), on proliferation were examined. Ki16425 inhibited LPA-induced proliferation of both 211H and H28 cells in a dose-dependent manner (Fig. 2B). In a parallel experiment, we examined the effects of small interfering RNA (siRNA) against LPA_1 and LPA_2. Knockdown of LPA_1 resulted in significant inhibition of the LPA-induced proliferation of both 211H and H28 cells. These results were confirmed using serum-starved media with LPA, as well as media containing 10% FBS (data not shown). However, knockdown of LPA_2 had little impact on LPA-induced proliferation of 211H or H28 cells (Fig. 2C). These results indicated that LPA stimulated the proliferation of these two MPM cell lines *via* LPA_1 receptors.

LPA activates p44/42 MAPK through LPA_1.

To determine the mechanism by which LPA_1-mediated proliferation of MPM cells, we next examined the phosphorylation of MAPK, which is the downstream signal of GPCRs. MPM cells were stimulated with LPA (10 μM) for 0, 2, or 6 h, and the phosphorylation of MAPK was evaluated by Western blotting. While LPA did not affect MAPK protein expression, it induced the phosphorylation of MAPK (phospho-p44/42 MAPK) in both 211H and H28 cells. The level of phosphorylation became highest after 2 h and the effect was still substantial after 6 h (Fig. 3A). Therefore, we evaluated the phosphorylation of MAPK after 2-h incubation with LPA. In a parallel experiment, siRNA knockdown of LPA_1, but not of LPA_2, markedly reduced the phosphorylation of p44/42 MAPK in these two MPM cell lines (Fig. 3B), indicating that LPA activates the p44/42 MAPK pathway *via* LPA_1.

LPA stimulates cell motility* via *LPA_2.

We next examined the effect of LPA on the motility of MPM cells using a cell migration assay. LPA stimulated migration of 6 of 10 MPM cell lines (data not shown). Of these MPM cell lines, LPA induced a significant increase in

migration of H2052, H2373, and ACC-MESO-4 cells in a dose-dependent manner (Fig. 4A). However, it did not affect the proliferation of these MPM cells (data not shown). These observations exclude the possibility that the effect of cell motility was attributable to differences in proliferation. In turn, these cell lines were used to examine whether LPA receptors were involved in LPA-induced motility, when the effect of Ki16425, an inhibitor of LPA_1 (and LPA_3 at higher doses), on the motility was examined. Ki16425 had no effect on LPA-induced motility in these MPM cells (Fig. 4B). To clarify the involvement of LPA_1 and LPA_2, we further knocked down LPA_1 and LPA_2 expression, respectively, with siRNA using H2052 cells, because LPA induced the motility of H2052 cells most efficiently. Knockdown of LPA_2 markedly inhibited LPA-induced motility of H2052 cells, while knockdown of LPA_1 did not (Fig. 4C). These results strongly suggest that LPA induces motility *via* LPA_2 in H2052 cells.

LPA activates RhoA through LPA_2.

We next investigated the effects of LPA on the activation of RhoA, which is a downstream signal of GPCRs and one of the key molecules for cell motility.

H2052 cells were stimulated with LPA (10 μM) for 0, 1, 3, 6, 12, or 30 min, and activation of RhoA was evaluated by G-LISA RhoA^{TM} Activation Assay (Biochem Kit^{TM}). LPA induced activation of RhoA, the degree of which became strongest after 1 min, and the effect decreased within 30 min (Fig. 5A). Therefore, we evaluated RhoA after 1-min incubation with LPA. Knockdown of LPA_2 with siRNA, but not of LPA_1, significantly reduced RhoA activation in comparison with the control level (Fig. 5B). These results suggest that LPA stimulates cell motility through an LPA_2-mediated RhoA pathway.

Discussion

In the present study, we demonstrated that all of the twelve cell lines and four clinical samples of MPM examined expressed LPA_1, while other types of LPA receptors (LPA_2, LPA_3, LPA_4, and LPA_5) were expressed only in some of the cell lines and clinical samples, and LPA stimulated the proliferation and motility of several MPM cell lines in a dose-dependent manner. There was no apparent correlation between the response to LPA and the expression level of LPA receptors, although MPM cells with high level of LPA_1 tend to be stimulated proliferation by LPA (data not shown). The histological subtypes, such as epithelial, biphasic, and sarcomatoid, were not also particularly correlated with the response to LPA (data not shown). We also examined the role of LPA and its receptors using a non-cancerous mesothelial cell line, Met-5A. Met-5A cells expressed LPA_1 and LPA_2, and LPA induced the proliferation, but not migration (data not shown). These observations suggest that MPM is biologically heterogeneous and uses various factors and signaling pathways for cell proliferation and motility. Our results also clearly showed that particular populations of MPM cells essentially utilized the LPA/LPA receptor pathway for both cell proliferation and motility. As the biomarker for MPM, Lindholm et.al

reported that gene copy number losses are a major mechanism for carcinogenesis and copy number changes are associated with a recurrent pattern (35). Pass et al. reported to predict survival and progression of MPM patients, using a 27-gene expression profiles (36). However, to our knowledge, this is the first report of the involvement of LPA/LPA receptors in the malignant phenotype of MPM.

LPA is known to act on specific GPCRs to elicit a wide range of cellular responses, ranging from cell proliferation and motility of various types of solid tumor (37). MPM tends to advance locally with pleural effusion, rather than distant metastasis, and the extent of the disease is directly associated with the prognosis of MPM patients (5). It is important to understand the molecular mechanism of MPM pathogenesis in terms of proliferation and motility, because molecules responsible for MPM progression seem to be ideal targets for therapy of MPM.

Recently, the differential roles of five GPCRs as LPA receptors have attracted a great deal of attention. Of these receptors, LPA_1 and LPA_2 are thought to play important roles in the signaling pathways of cancer cells. Several studies indicated that LPA_1 controls cell proliferation in prostate cancer

(25) and breast cancer cells (26). Similarly, LPA_2 was found to mediate LPA-induced growth of ovarian (27), thyroid (21), and colon cancer cells (29). Thus, the role of LPA receptors in cell proliferation triggered by LPA appears to depend on the type of cancer cell line examined. In this study, we demonstrated that LPA_1, but not LPA_2, is a key receptor in LPA-stimulated proliferation of MPM cells using silencing of LPA receptors.

GPCRs, including LPA receptors, have multiple intracellular signaling mechanisms by small GTPase proteins, Ras, Rho, Rac, and their downstream cascades (37,38). The activation of Ras-MAPK responsible for cell proliferation can lead to the nuclear translocation of MAPK followed by gene transcription and cell cycle progression, and this signaling plays a pivotal role in cancer cell proliferation and differentiation. In the present study, we showed that LPA activated the p44/42 MAPK pathway and siRNA for LPA_1, but not LPA_2, caused marked inhibition of cell proliferation and phosphorylation of p44/42 MAPK. These results strongly suggest that MAPK is involved in the LPA_1-mediated proliferation of MPM cells induced by LPA.

MPM cells have high motility, facilitate free movement in the pleural cavity during respiration by extending lubricating glycoproteins, and show disordered

invasion into the thoracic cavity (5). LPA is already well known to stimulate cell motility in many cell types, such as fibroblasts, gliomas, and T-cell lymphomas (39). LPA-stimulated motility of cancer cells was recently reported to require signaling *via* LPA_1 in many types of solid tumor, including pancreas, colon, glioblastoma, and gastric cancer (19,24,28,29). However, this was not the case in our studies that indicated that the motility of MPM cells was augmented by LPA through LPA_2, but not LPA_1. This was confirmed in the present study using two different methods. First, treatment with Ki16425, an inhibitor of LPA_1, did not inhibit cell migration. Second, knockdown of LPA_2, but not LPA_1, effectively suppressed LPA-induced motility of MPM (H2052) cells. These results demonstrated that LPA-induced motility of MPM cells is mediated partly, if not completely, through its interaction with LPA_2.

RhoA is a small GTPase protein that mediates actin microfilament organization necessary for adhesion, motility, and changes in cell shape (40). Recent studies have implicated the pathway of RhoA activation, which acts downstream of LPA receptors, in tumor invasion (41,42). We demonstrated that LPA activated RhoA and that the effect was significantly, but not completely, diminished by knockdown of LPA_2, suggesting that LPA stimulated migration of H2052 cells

was mediated by LPA_2 through the RhoA activation pathway. Further studies are warranted to determine the involvement of other signaling pathways in LPA_2-mediated cell-motility of MPM cells.

To explore antitumor activity by inhibiting LPA receptors, we used an inhibitor of LPA_1, Ki16425, because it is only one commercially available LPA inhibitor at present. The severe combined immunodeficiency (SCID) mice bearing subcutaneously inoculated MSTO-211H cells were treated daily from day 19 to day 30 with s.c. injection of Ki16425 at 20 mg/kg per day. The tumor growth was measured every 3 days. Under these experimental conditions, treatment with Ki16425 did not significantly inhibit the tumor growth of MSTO-211 cells (data was not shown). However, we can not make conclusion by this experiment, because Ki16425 is an inhibitor of LPA_1, but not LPA_2. In addition, since limited amount of Ki16425 was available, only one dose of Ki16425 was used and treatment was given only 12 days started at late stage. Therefore, further experiments with higher doses of Ki16425 for longer duration or dual inhibitors of LPA_1 and LPA_2, if available, are warranted to elucidate real role of LPA receptors in mesothelioma cells *in vivo*.

In summary, we demonstrated that MPMs are heterogeneous in terms of the

response to LPA and the expression of LPA receptors. However, at least in particular populations of MPM, LPA stimulated proliferation *via* LPA_1 through MAPK and motility *via* LPA_2 through RhoA. These novel findings suggest that LPA_1 and LPA_2 may be one of therapeutic targets for controlling the proliferation and motility of MPM cells. As MPM develops into locally advanced disease, novel strategies targeting both LPA_1 and LPA_2 might be beneficial for suppressing the progression of MPM. Further *in vivo* experiments with dual inhibitors of LPA_1 and LPA_2, are warranted.

Acknowledgements

This work was supported by Grants-in-Aid 17016051 for Cancer Research from the Ministry of Education, Science, Sports and Culture of Japan (S.Sone). We thank Dr Hironobu Hamada for the EHMES-1 and EHMES-10 cell lines and Dr Adi F. Gazdar for the NCI-290 and NCI-H513 cell lines.

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Figure legends

Figure 1. Expression of LPA receptors, LPA_1, LPA_2, LPA_3, LPA_4, and LPA_5 in human MPM cell lines, and clinical specimens.

Reverse transcription-PCR was performed to determine expression of the LPA receptors, LPA_1, LPA_2, LPA_3, LPA_4, and LPA_5. Reverse transcription-PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. Data shown are representative of three independent experiments with similar results.

Figure 2. Effects of LPA on cell proliferation of human MPM cells, and inhibition by antagonist and knockdown of LPA_1.

Tumor cells (5×10^3 per well) plated in triplicate in 96-well plates were incubated overnight in RPMI 1640 containing 10% FBS. The cells were starved for 24 h by replacing the media with serum-free RPMI 1640 containing 0.1% fatty acid-free bovine serum albumin. Different doses of LPA and Ki16425, an inhibitor of LPA_1, were then added, and the cells were cultured for 48 h. Then, an aliquot of 50 μL of stock 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (2 mg/mL; Sigma, St. Louis, MO) was added to each plate and the cells were

incubated for 2 h at 37°C. LPA stimulated proliferation of 211H and H28 cells in a dose-dependent manner (A). LPA-induced proliferation of 211H and H28 cells was inhibited by Ki16425 in a dose-dependent manner (B). After transfection with siRNA or scramble RNA (siSCR) using Lipofectamine 2000 in accordance with the manufacturer's instructions, the cells were starved for 24 h by replacing the media with serum-free RPMI 1640 containing 0.1% fatty acid-free BSA. LPA (10 μM) was then added and cells were cultured for 48 h. The cells were then subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Knockdown of LPA_1 (siLPA1#1, siLPA1#2, and siLPA1#3) significantly inhibited LPA-induced proliferation of 211H and H28 cells, while knockdown of LPA_2 (siLPA2#1, siLPA2#2, and siLPA2#3) had little impact on LPA-induced proliferation of 211H or H28 cells (C). Data shown are representative of three independent experiments with similar results. * Significantly different from control (P<0.001). ** Significantly different from siSCR (P<0.005).

Figure 3. Effects of LPA on phospho-p44/42 MAPK, and inhibition by knockdown of LPA_1.

Tumor cells were incubated with LPA (10 μM) for 0, 2, and 6 h. Then, the cell

lysates were harvested and the protein level and phosphorylation of p44/42 MAPK were determined by immunoblotting analysis. LPA-induced phosphorylation in 211H and H28 cells occurred after 2 h, and the effect was still substantial after 6 h (A). Moreover, knockdown of LPA_1 with siRNA markedly reduced the activation of p44/42 MAPK. However, knockdown of LPA_2 had little impact (B). Data shown are representative of three independent experiments with similar results.

Figure 4. Effects of LPA on migration of human MPM cells, and inhibition by knockdown of LPA_2.

Cell migration was measured in Falcon Cell Culture Inserts (pore size 8 μm). Cells suspended in serum-free RPMI 1640 containing 0.1% fatty acid-free BSA with or without LPA and/or Ki16425 were added to the upper chamber (1×10^5 cells at 100 μl/well). Serum-free RPMI 1640 containing 0.1% fatty acid-free BSA and human fibronectin (5 μg/ml) as a chemoattractant was added to the lower chamber. When the cells treated with Ki16425, the cells were preincubated with 10 μM Ki16425 for 30 min. The cells were allowed to migrate for 3 h at 37°C. LPA stimulated migration of MPM cells, H2052, H2373, and ACC-MESO-4 in a

dose-dependent manner (A). Ki16425 had no effect on LPA-induced motility in these MPM cells (B). After transfection with siRNA or scramble RNA (siSCR), the cells were starved for 24 h by replacing the media with serum-free RPMI 1640 containing 0.1% fatty acid-free BSA. Knockdown of LPA_2 by siRNA significantly inhibited the LPA-induced motility of H2052 cells, while knockdown of LPA_1 did not (C). Data shown are representative of three independent experiments. * Significantly different from control (P<0.005). ** Significantly different from siSCR (P<0.005).

Figure 5. Effects of LPA on RhoA activity, and inhibition by knockdown of LPA_2.

To assess RhoA activation, we determined RhoA activation as the amount of RhoA-GTP binding using a G-LISA RhoA™ Activation Assay Biochem Kit™ (Cytoskeleton, Denver, CO) in accordance with the manufacturer's recommendations. H2052 cells were treated with LPA (10 μM) for 0, 1, 3, 6, 12, and 30 min, and the cell lysates were then prepared for the assay. LPA stimulation of RhoA activity in H2052 cell was highest after 1 min and the effect decreased within 30 min (A). Knockdown of LPA_2 with siRNA significantly

reduced the activation of RhoA, while knockdown of LPA_1 had little effect (B). Data shown are representative of three independent experiments. * Significantly different from control (P<0.05). ** Significantly different from siSCR (P<0.005).

Figure 1.

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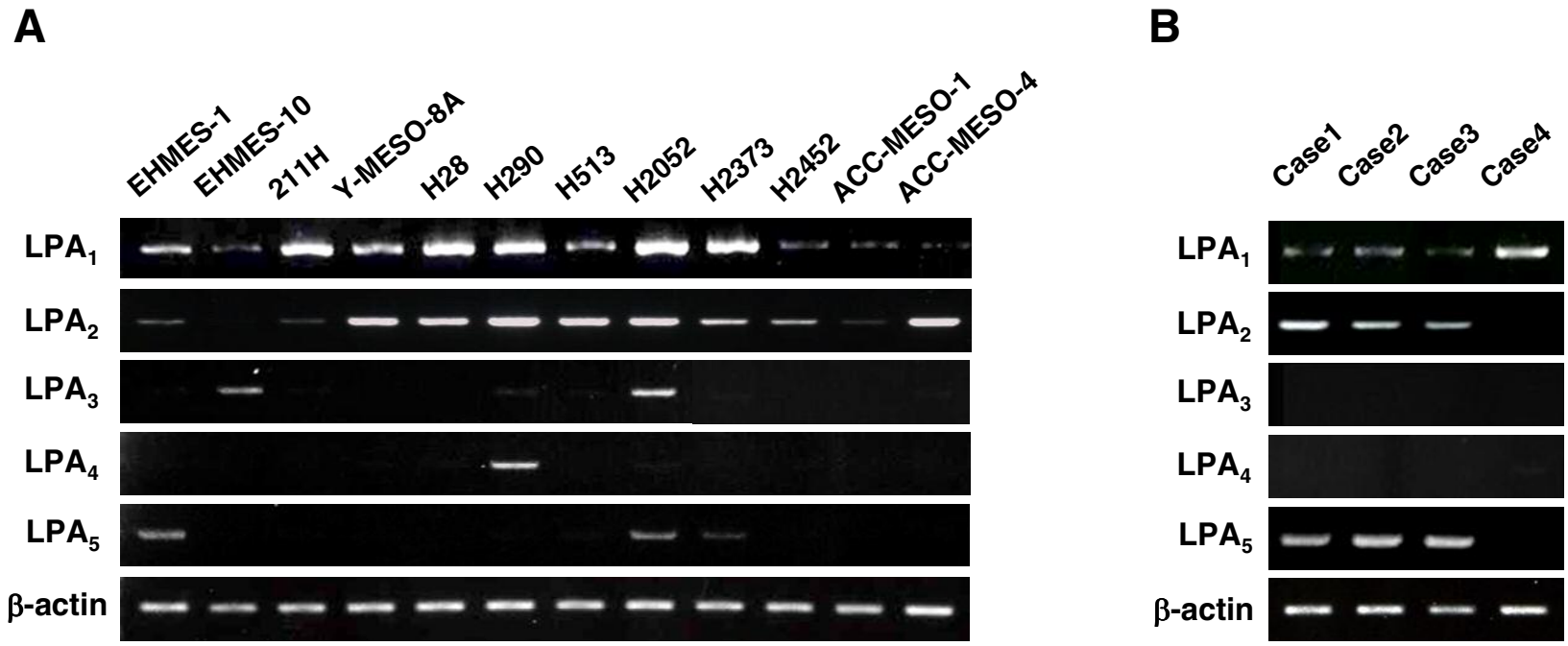

Figure 2.

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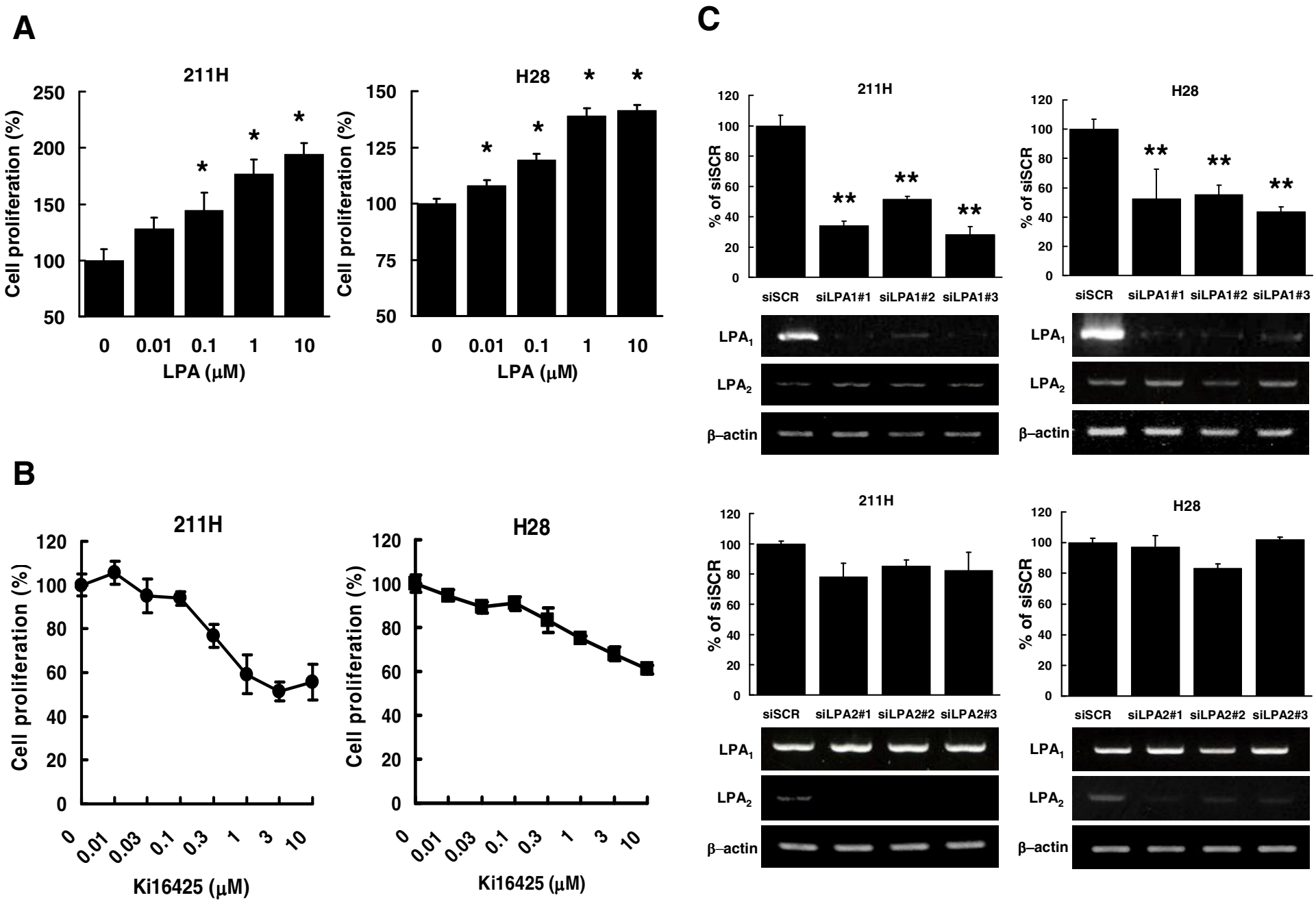

Figure 3.

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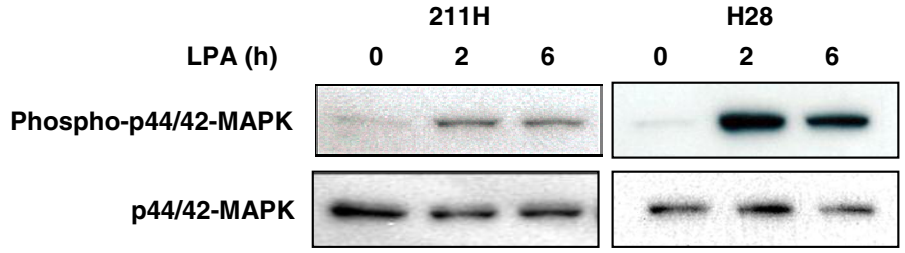
A



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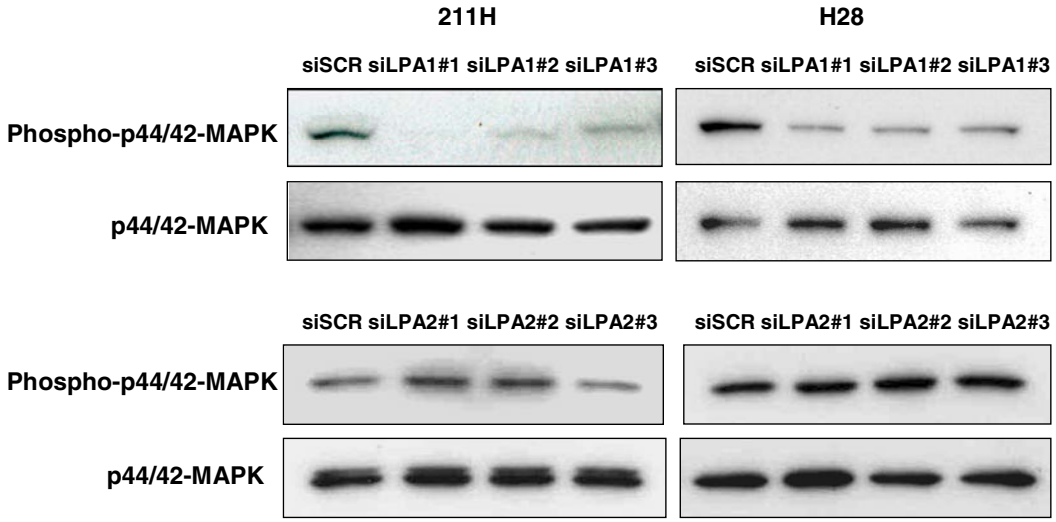

Figure 4.

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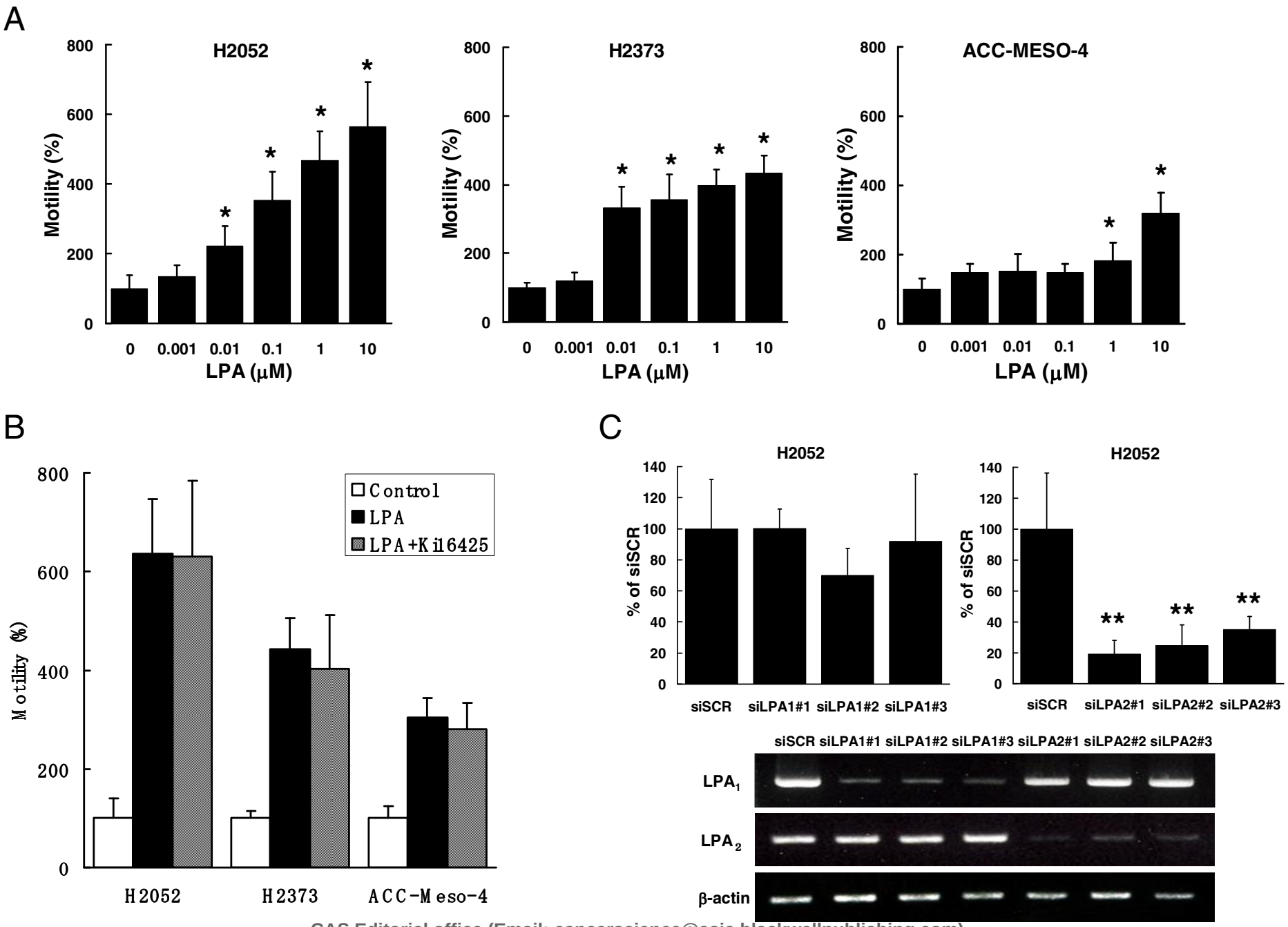

Figure 5.

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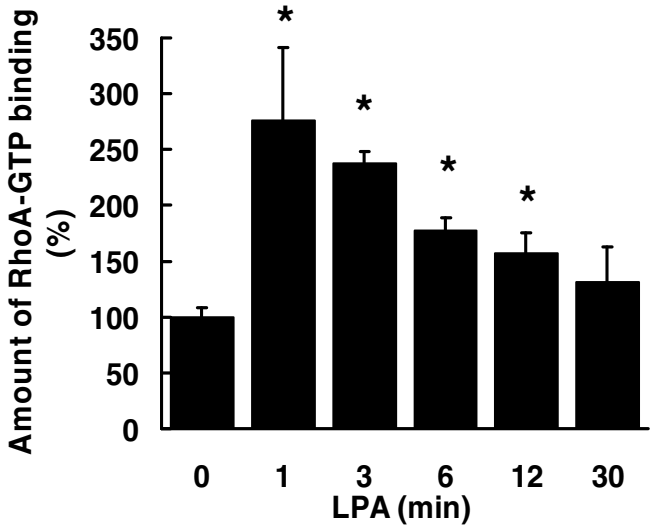
A



B