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## Bioactive lipids lysophosphatidic acid and sphingosine 1-phosphate mediate breast cancer cell biological functions through distinct mechanisms

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### Abstract

Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are structurally related bioactive lipids with growth factor like activities. LPA and S1P are naturally produced *in vivo* by blood platelets upon platelet aggregation and at least *in vitro* by fibroblasts, adipocytes and multiple types of tumor cells. Breast cancer cells respond to LPA and S1P. However, their specific actions on breast cancer cell biological functions remain unclear. We therefore conducted an *in vitro* side by side study of these two lipids on breast cancer cells. LPA mediates human breast cancer MDA-BO2 cell proliferation, migration and invasion through activation of a G $\alpha$ i/ERK1/2-dependent signalling pathway, whereas activation of G $\alpha$ i/PI3K predominates upon S1P stimulation. In MDA-BO2 cells, LPA but not S1P activities were dependent on active type 1 insuline-like growth factor and epithelial growth factor receptors. LPA and S1P act directly on endothelial cells to induce angiogenesis. We demonstrate that LPA and S1P have indirect angiogenic properties as judged by induced secretion of angiogenic factors by breast cancer cells primed with these lysophospholipids. S1P, but not LPA, controlled the expression of VEGF-A by breast cancer cells, while LPA, but not S1P, controlled the expression of GM-CSF, Gro- $\alpha$ , MCP-1, IL-6. According to the secretion of these paracrine osteoclastic factors, LPA, but not S1P, stimulate breast cancer cell-induced osteoclastogenesis. These findings suggest that *in vivo*, LPA and S1P can coordinate

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their action on tumor and surrounding cells to induce breast cancer progression both at primary and bone metastatic sites.

### Keywords

Lysophosphatidic acid; Sphingosine 1-phosphate; Breast cancer cells; Proliferation; Migration; Invasion; Angiogenesis; Osteoclastogenesis

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### Introduction

Recent studies indicate that the bioactive lipids lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) present in tumor microenvironment, must be considered as key components during cancer progression and metastatic dissemination (1,2). We have shown that LPA promotes the growth of tumor xenographs and the formation of bone metastases in animals (1). The blockade of LPA signaling by genetic and pharmacological approaches is effective in inhibiting both the formation and the progression of bone metastases (3). A highly specific monoclonal antibody against S1P successfully reduces angiogenesis and the growth of multiple human cancer cell types in animal models (2). LPA and S1P have been shown to exhibit similar growth factor-like activities including the stimulation of cell proliferation, survival, contraction, migration, invasion and matrix assembly (4). They were also described as regulators of angiogenesis, wound healing, and modulators of the immune and cardiovascular systems (5). In the context of breast cancer, the specific mechanisms mobilized by these lysophospholipids are largely unknown because of a lack of a side by side study using the same cell line.

Tumor cells can produce LPA and S1P through the mobilization of specific enzymes such as Autotaxin and Sphingosine kinase 1, respectively (6,7). Consequently, LPA and S1P can act as autocrine and paracrine factors. Platelets are the main source of LPA and S1P in the organism (8,9). They also have the unique property of generating high amount of both LPA and S1P upon aggregation (8,9). LPA and S1P act on a class of structurally related cell surface receptors, which possess high sequence homologies with the endothelial differentiation gene 1 (EDG1) (10). Two distinct subgroups have been defined based on their ligand specificity. S1P<sub>1,2,3,4,5</sub> respond specifically to S1P whereas LPA<sub>1,2,3</sub> are specifically activated by LPA (10). In addition, four additional cell surface receptors unrelated to the members of the EDG family and the transcription factor PPAR $\gamma$  also transduced LPA signals (11–15). LPA and S1P receptors are widely expressed in human tissues (5). Cancer cells frequently express multiple types of LPA and S1P receptors. This suggests specific repertoires of both lysolipid receptors might drive specific biological responses in presence of LPA and S1P in tumor microenvironment.

Direct experimental data are missing to elucidate the role of LPA and S1P in breast cancer growth and metastasis dissemination. In this study we conducted an original side by side comparison of the role of LPA and S1P on breast cancer cell biological functions *in vitro*. Our results identified overlapping and specific cell signaling activation pathways stimulated by LPA and S1P. These results suggest that the combined activities of LPA and S1P

integrate into the mechanisms involving cytokine and growth factor secretion controlling breast cancer development and the formation of metastases.

## Materials and Methods

### Cell culture

Human breast cancer cells lines, Hs 578T, MCF-7, MDA-MB-231, MDA-MB-435S, SK-BR-3, T-47D, and ZR-75-1 cells were obtained from the American Type Culture Collection. MDA-MB-231/BO2 (MDA-BO2) cells, MDA-BO2 stables transfectants (Sbl 9, Sbl 10 and siLPA<sub>1</sub> 122, siLPA<sub>1</sub> 123, siLPA<sub>1</sub> 132) were described elsewhere (16,17). All cell lines with the exception of SK-BR-3 cells, were routinely cultured in DMEM/NUT.MIX f-12 W/GLUT-1 medium (Life Technologies, Cergy pontoise, France) supplemented with 10% (v/v) fetal bovine serum (FBS, Bio-Media, Issy les moulineaux, France) and 1% penicillin/streptomycin (Life Technologies, Cergy pontoise, France) at 37°C in a 5% CO<sub>2</sub> incubator. SK-BR-3 cells were grown in complete McCoy's 5a medium (Life Technologies, Cergy Pontoise, rance).

### Reverse transcription and quantitative PCR

Total RNA from Human breast cancer cell lines was extracted using Total RNA Isolation System (Promega,, Charbonnieres, France). cDNA was synthesized using Moloney murine leukemia virus-1 (Promega, Charbonnieres, France). Primers for human S1P1, S1P2, S1P3, LPA<sub>1</sub> and LPA<sub>2</sub> were described previously (18). Primers for GAPDH were described before (19). LPA<sub>3</sub> primers were designed from the Lpa3 gene (National Center for Biotechnology Information [NCBI] accession number AF127138) using nucleotides 738–756 as the forward primer and nucleotides 994–973 as the reverse primer. PCR reactions were run using a program consisting of 40 cycles of 95°C for 15 s, 53°C for 30 s, and 72°C for 20 s with a preincubation of 95°C for 2 min. Products from standard PCR were separated by electrophoresis on a 2% agarose gel and then visualized with ethidium bromide under ultraviolet light. Human LPA<sub>1</sub>, LPA<sub>2</sub>, and LPA<sub>3</sub> mRNAs were quantified by real-time PCR using the Master SYBR Green I kit (Roche Diagnostics, Meylan, France). Fluorescence was monitored and analyzed in a Light Cycler (Roche Diagnostics, Meylan, France). The fluorescence data were quantitatively analyzed by using serial dilution of control samples included in each reaction to produce a standard curve. GAPDH mRNA expression was analyzed in parallel to confirm the use of equal amounts of cDNAs in each reaction. Results are expressed as the percentage of gene expression in each cell line compared with that in the parental MDA-BO2 cells.

### Cell proliferation assays

Experiments were carried out as previously described (1). Briefly, quiescent tumor cells ( $5 \times 10^3$  cells per well) cultured in 96-well tissue culture plates (BD Biosciences, Franklin Lakes, USA) in medium containing 0.1% (w/v) BSA fatty acid-free (Sigma-Aldrich, Saint Quentin fallavier, France) treated overnight in the presence or absence of increasing concentrations of 1-oleoyl LPA or S1P, and then pulsed with [<sup>3</sup>H]-thymidine (Sigma-aldrich, Saint Quentin Fallavier, France) for the last 8 hours.

### Cell migration and invasion assays

Migration assays were carried out using Bio-Coat cell migration chambers (Becton Dickinson, Franklin Lakes, USA) as described previously (20). Quiescent tumor cells and HUVEC cell culture were incubated in DMEM with or without Wortmannin ( $10^{-7}$  M), PTX (100ng/mL), chelerythrine chloride ( $10^{-6}$  M), PP2 ( $10^{-7}$  M), or PD98059 ( $10^{-5}$  M) (Sigma-Aldrich, Saint Quentin fallavier, France) 3h before stimulation with LPA or S1P (1  $\mu$ M) in serum-free medium (DMEM) containing 0.1% (wt/vol) fatty acid free BSA. After a 6h incubation at 37°C in a 5% CO<sub>2</sub> incubator, cells that had migrated through the filter pores (8 mm pore size) and attached on the below surface of the filter, were fixed and stained. The filter membranes were mounted on glass slides and the cells were counted under microscope. Cell invasion experiments were performed using Bio-Coat cell migration chambers (Becton Dickinson Franklin Lakes, USA) as described above. Filters were previously coated with the basement membrane Matrigel (0.3 mg/ml, Becton Dickinson, Franklin Lakes, USA). After incubation for 48h at 37°C in a 5% CO<sub>2</sub> incubator, cells that had migrated through the filters were counted as described above for the migration assay.

### ELISA measurements of IL-6 and IL-8 MCP-1, GM-CSF, Gro- $\alpha$ , and VEGF-A in culture media

Conditioned media of cell lines either untreated, or treated with LPA or S1P were collected and analyzed for human cytokine production by ELISA following the manufacturer's instructions (Bender MedSystems, Vienna, Austria). Concentrations of IL-6, IL-8, MCP-1, GM-CSF, GRO $\alpha$ , VEGF-A were expressed in pg/ml per 10<sup>6</sup> cells.

### Osteoclastogenesis assays in vitro

Bone marrow cells from mice were collected and seeded in a 48-well tissue culture plate at a density of  $2.5 \times 10^3$  cells per well in  $\alpha$ -MEM medium (Life Technologies, Cergy pontoise, France). This medium was supplemented with the conditioned media of MDA-BO2 cells which had been stimulated for 48h with LPA, S1P (1  $\mu$ M), FBS, macrophage-CSF (PreproTech, Rocky Hill, NJ), or receptor-activated nuclear receptor factor B ligand (RANK-L, generous gift of Pr. Jurdic, CNRS, Lyon, France). After incubation for 6 days, differentiated osteoclasts were enumerated under a light microscope to quantify multinucleation (more than three nuclei) and TRAP activity (Tartrate-resistant acid phosphatase). Results were expressed as the number of osteoclasts per mm<sup>2</sup>.

### Statistical analysis.

Data were analyzed with the Stat-View 5.0 software using unpaired Student's *t* test. *P* values less than 0.05 were considered statistically significant.

## Results

### Human breast cancer cells express LPA and S1P receptors.

To evaluate the role of LPA and S1P on breast cancer cell activities, we first quantified the expression levels of the receptors specific for LPA (LPA<sub>1</sub>, LPA<sub>2</sub>, LPA<sub>3</sub>) and S1P (S1P<sub>1</sub>, S1P<sub>2</sub>, S1P<sub>3</sub>) in a panel of human breast cancer cell lines (MDA-MB-231, MDA-BO2, MDA-

MB-435s, MCF-7, T47D, ZR751, Hs578Ts, SKBr3) (Fig. 1). Expression level of each target gene was normalized to GAPDH mRNA expression. Upon cautions taken due to potential errors in samples tested due to the use of a single housekeeping gene, our real-time PCR experiments indicated that the expression levels of LPA receptors vary strongly among these cell lines (Fig. 1A). In contrast, the receptors for S1P were more homogeneously expressed (Fig. 1B). However, we detected elevated expression of S1P<sub>1</sub> in MDA-MB-231, MCF-7, and Hs578Ts cell lines. In agreement with previous observations, no expression of LPA receptor was detected in SKBr3 cells (1,21). Interestingly, these cells express S1P receptor mRNA with levels less than 90% compared to other cell lines.

### LPA and S1P control breast cancer cell proliferation.

We have previously observed that all breast cancer cell lines used in this study that express LPA receptors responded to LPA stimulation in a dose-dependent manner and further demonstrated that LPA is highly mitogenic on breast cancer cells both *in vitro* (Fig. 2A) and *in vivo* (1). To analyse the effect of S1P on cell proliferation, we stimulated our series of human breast cancer cells with increasing concentrations of S1P. All the cells that expressed S1P receptors responded to S1P stimulation in a dose-dependent manner (Fig. 2B). SKBr3 cells did not respond to S1P stimulation. MDA-BO2 cells which express both lysolipid receptors were more sensitive to S1P than LPA stimulation, as shown by a significant 1.8 fold increase of H<sup>3</sup>-thymidine incorporation using equimolar concentrations of lysolipids (Fig. 2C). We have previously shown that LPA-induced MDA-BO2 cell proliferation was dependent on activation of the MAP Kinase ERK1/2 (3). We found that S1P-induced H<sup>3</sup>-thymidine incorporation was totally blocked when cells were treated with inhibitors for PI3K, G<sub>i</sub> protein, MEK1, PKC and Src family kinases (Fig. 2C). Previous reports have shown that anti-IGF-II and anti-IGFR1 antibodies partially inhibited proliferation of breast cancer cells to LPA and S1P (19). Additionally, LPA and S1P were shown to transactivate EGFR in cancer cells (22,23). We found that LPA-induced but not S1P-mediated proliferation of MDA-BO2 cells was totally inhibited by anti-IGFR1 and anti-EGFR antibodies (Fig. 2D).

### LPA and S1P control breast cancer cell migration.

Motility is a hallmark of metastatic cancer cells. S1P and LPA have been shown to control the migration of many cell types including immune cells (24), endothelial cells (25) and cancer cells (26). To analyze the effect of LPA and S1P on the motility of breast cancer cells, we used transwell migration chambers wherein MDA-BO2 cells were challenged to migrate throughout a membrane with 8 μm pores. Cells were incubated in the absence or presence of either a positive or a negative gradient of lysolipid concentration. Each lysolipid enhanced the migration of MDA-BO2 cells when added to the lower chamber (Fig. 3). In contrast to S1P, LPA induced cell migration when added in the upper chamber only, or in both the upper and the lower chambers (Fig. 3). Then, we challenged our series of cell lines with increasing concentrations of S1P or LPA added in the lower chamber. We observed that S1P and LPA induced migration of all cell lines that express cognates receptors (Fig. 4A, 4B). In contrast to S1P, the action of LPA followed a bell-shape curve of concentrations with a maximum activity at 10<sup>-7</sup> M and a complete inhibition of cell migration at 10<sup>-5</sup> M. Recent reports indicate that LPA receptor LPA<sub>1</sub> was the motility factor for neoplastic

and non-neoplastic cells in response to LPA (26). Therefore, we silenced the expression of LPA<sub>1</sub> in MDA-BO2 cells using a RNA interference strategy (siRNA) (3), and tested the ability of these cells to migrate in response to LPA. We observed that silencing of LPA<sub>1</sub> expression almost totally abolished the LPA-induced migration of MDA-BO2 cells (Fig. 4C). To determine the signalling pathways involved in LPA- and S1P-induced breast cancer cell migration we challenged MDA-BO2 cells to migrate in response to the lysolipids in the presence of specific inhibitors or anti-growth factor receptor blocking antibodies. We observed that S1P activity was totally inhibited with inhibitors Wortmannin and PTX (Fig. 4D). Interestingly, migratory activity of LPA on MDA-BO2 cells was partially (66%) inhibited with Wortmannin and totally blocked with inhibitors of ERK1/2, PKC, Src family kinases, G<sub>αI</sub> and blocking antibodies against IGF1 and EGF receptors (Fig. 4C and 4D).

#### **LPA and S1P control tumor cell invasion in vitro.**

Invasion activity is crucial for tumor cells to escape from their primary site and to disseminate to secondary target tissues (27). To analyze the effects of LPA and S1P on invasion of breast cancer cells, we used transwell migration chambers with membranes coated with matrigel which mimics an artificial basal membrane. All cell lines that express LPA and S1P receptors responded to LPA and S1P, respectively (Fig. 5A). Treatments with specific blockers of G<sub>αI</sub> and PI3K totally inhibited S1P activity on cell invasion (Fig. 5B). Treatments of all breast cancer cell lines with inhibitors of ERK1/2, PKC, Src family kinases, G<sub>αI</sub> or PI3K totally inhibited LPA activity. Also, cocubation of breast cancer cells with anti-IGFR1 or anti-EGFR antibodies totally inhibited cell invasion induced by LPA but not S1P (Fig. 5C).

#### **LPA and S1P control angiogenesis through direct and indirect tumor cell-induced stimulation of endothelial cells (HUVEC).**

It is known for more than a decade that the angiogenesis switch is a prerequisite for tumors to expand over a volume of 5 mm<sup>3</sup> and to achieve the dissemination of distant metastases (28). Many reports indicate that LPA and S1P act directly on endothelial cell to stimulate proliferation, survival and migration (25). Here, we observed that S1P induced an 80% folds increase of migration (Fig. 6A) and proliferation (data not shown) of human vascular endothelial cells (HUVEC) than equimolar concentrations of LPA. We have shown recently that, in the context of bone metastasis formation, LPA acts on tumor cells to induce the secretion of paracrine factors that stimulate osteoclasts and bone destruction (1). Therefore, we sought to determine whether LPA and S1P induced the secretion of paracrine factors by breast cancer cells that stimulate endothelial cells. We primed our series of breast cancer cell lines with LPA or S1P for two days. Then, cell-conditioned media were collected and used to stimulate HUVEC migration. We observed that culture media collected from all cell lines, excepted SKBr3 cells, sustained HUVEC migration (Fig. 6A).

#### **S1P, but not LPA, stimulates the secretion of VEGF-A by breast cancer cells.**

It has been shown that LPA induces the release of vascular endothelial growth factor (VEGF) from ovarian cancer cells (29). Therefore, we ask to know whether LPA and S1P induced the secretion of VEGF-A by breast cancer cells. Except for SKBr3 cells, all cell lines tested here, secreted high levels of VEGF-A at steady-state (Fig. 6B). Consistent with

our previous observation on MDA-BO2 cells (3), LPA did not promote the secretion of VEGF-A on this series of breast cancer cell lines. In contrast to LPA, S1P increased the secretion of VEGF-A by these cells from 3 to 6 folds (Fig. 6B). These results suggested that LPA and S1P might drive tumor-induced angiogenesis through distinct pathways involving a S1P-dependent and LPA-independent secretion of VEGF-A.

### **LPA, but not S1P, stimulates breast cancer cell osteoclastic activity.**

Bone is a frequent metastatic site for many cancers including breast cancers (30). We have recently shown that LPA, derived from activated platelets, plays a central role in the progression of bone metastases by controlling both tumor growth and tumor cell-induced osteoclast activity (1). Because platelets produce both LPA and S1P during platelet aggregation (8,31,32) we investigated the potential activity of S1P on breast cancer cells to stimulate osteoclast formation. LPA induced the secretion of pro-osteoclastic cytokines and growth factors, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), growth-related oncogene (Gro- $\alpha$ ), and macrophage chemoattractant protein 1 (MCP-1) by MDA-BO2 cells (3). We observed that S1P also enhanced the secretion of IL-8 (Fig. 7A). However, in contrast to LPA, S1P did not promote the secretion of IL-6, GM-CSF, GRO- $\alpha$  and MCP-1 by MDA-BO2 cells (Fig. 7A) and did not induce osteoclast formation *in vitro* (Fig. 7B).

## **Discussion**

LPA and S1P act on two distinct, but structurally related, seven-transmembrane spanning domain receptors coupled to G proteins (5). LPA and S1P receptors share similar downstream signalling pathways suggesting potential redundant activities of each receptor subtype (5). In agreement with previous reports, we observed that breast cancer cells express both LPA and S1P receptors. The role of S1P receptors in breast cancers is largely unknown since gene expression analyses for these receptors at the primary site have not been conducted yet. Expression of LPA<sub>2</sub> was consistently found elevated at the primary site of different cancers, including ductal breast carcinoma (33). This suggests that LPA<sub>2</sub> might play a preferential role in carcinogenesis. However, direct proofs are still needed as genes encoding either LPA<sub>1</sub>, LPA<sub>2</sub>, or LPA<sub>4</sub> support the transformation of MEF cells overexpressing c-Myc and Tbx2, indicating that these genes behave as oncogenes(34). Cell motility is controlled by both LPA and S1P. LPA, but not S1P, exerted a chemokinetic activity on tumor cells. In the context of primary tumor growth this suggests that LPA might be a metastatic promoting factor. However, we found that LPA activity, but not S1P, followed a bell-shape curve which suggested that high local concentrations of LPA *in vivo* might preferentially drive tumor cells to proliferate rather than to induce migration. This assumption was supported by the increase of xenograft tumor growth of MDA-BO2 cells previously sensitized to LPA consequently to over-expression of LPA<sub>1</sub> after cell transfection (3).

EGFR is known as a central transducer of a wide range of different agonists that mobilized G protein-coupled receptors, receptor with tyrosine kinase activity, and sensing receptors (35). Activation of LPA receptor, LPA<sub>1</sub>, induces gastric cancer cell migration through

SPHK1/S1P formation and transactivation of EGFR (23). Similar “criss-cross” signal transduction was reported between EGF and S1P receptors to mediate estrogen-dependent breast cancer cell proliferation (22). Strikingly, we observed that biological activities of LPA, but not S1P, on MDA-BO2 breast cancer cells were dependent on EGFR and IGF1R1 transactivations. G $\alpha$ i/ERK1/2 and G $\alpha$ i/PI3K signalling pathways control specifically LPA and S1P biological activities in these cells, respectively. In Cos7 and mammary epithelial cells, transactivation of EGFR is required for LPA- and IGF1-induced phosphorylation of ERK1/2 (36,37). As observed in MDA-BO2 cells, S1P-induced migration of vascular smooth muscle cells is mediated through a pathway dependent on PI3K but not on EGFR (38). Specific activation of G $\alpha$ i/ERK1/2 and G $\alpha$ i/PI3K pathways might explain discrepancies in the central role of receptors for EGF and IGF1 as signal transducers for LPA and S1P. This suggests that, in the context of cancer development, LPA and S1P could have overlapped synergistic activities through the mobilization of specific signalling pathways to mediate tumor growth and distant metastasis formation.

LPA and S1P are known to exhibit pro-angiogenic activities, as they induce the proliferation and migration of endothelial cells (25). Besides these direct angiogenesis activities, we found that LPA and S1P also elicited indirect tumor cell-induced angiogenesis. S1P activity correlated with its capacity to stimulate the secretion of VEGF-A by breast cancer cell lines. LPA also induced indirect tumor cell-driven angiogenesis. However, in contrast to what was shown in ovarian cancer cells (29), LPA did not enhance the secretion of VEGF-A from our series of breast cancer cell lines. VEGF-A is highly expressed at steady state by the cell lines used in this study in normoxic cell culture conditions. LPA-induced expression of VEGF-A was shown to be dependent on HIF1 $\alpha$  expression which occurs when cells are placed under hypoxic conditions (39). Thus, our observation does not exclude that *in vivo* LPA might stimulate the secretion of VEGF-A upon hypoxia. Additionally, the indirect tumor cell-driven angiogenesis elicited by LPA is likely to be achieved through enhanced secretion of cytokines and growth factors from tumor cells. These include IL-6 and IL-8 which are known to promote angiogenesis (40,41).

IL-6 and IL-8 are also known to mediate the differentiation of mature osteoclasts from circulating precursors and their miss-regulation in breast cancers were associated with increased formation of bone metastasis (42,43). We showed previously that LPA exhibited an indirect action on osteoclasts by inducing the secretion of pro-osteoclastic cytokines (IL-6, IL-8, GM-CSF, Gro- $\alpha$  and MCP-1) (1,3). Interestingly, S1P also enhanced the secretion of IL-6 on MDA-BO2 cells, but not of the other pro-osteoclastic cytokines. As a marked difference with LPA, S1P was not effective in eliciting a full osteoclast activity by these cells *in vitro* (Fig. 7B). This observation suggests that, at the bone metastatic site, S1P might sustain the action of LPA on tumor cell proliferation, but not on bone destruction.

Altogether, our data provide new insights on the role of LPA and S1P during breast cancer cell progression and indicate that LPA and S1P exhibit additive and complementary activities to mediate tumor growth, to the escape of the tumor cells from their primary site, and to support bone metastasis formation. Together, these new findings suggest that LPA and S1P are prime targets for the development of new therapies for cancer patients.



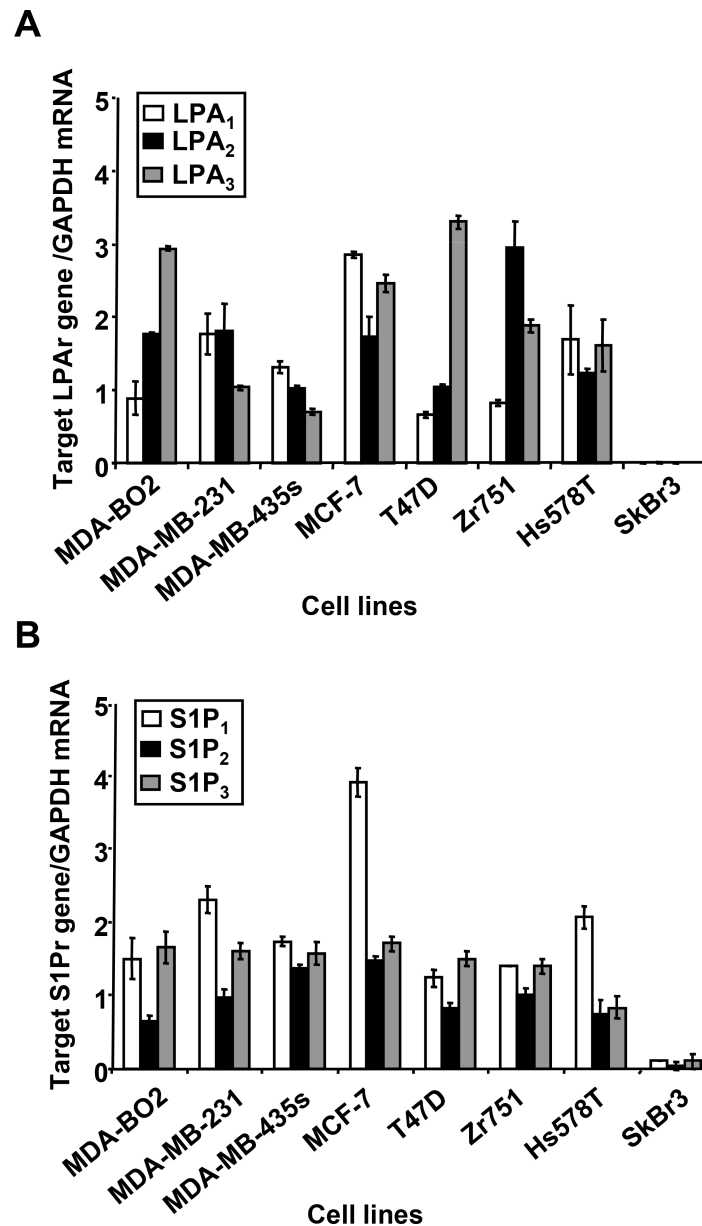
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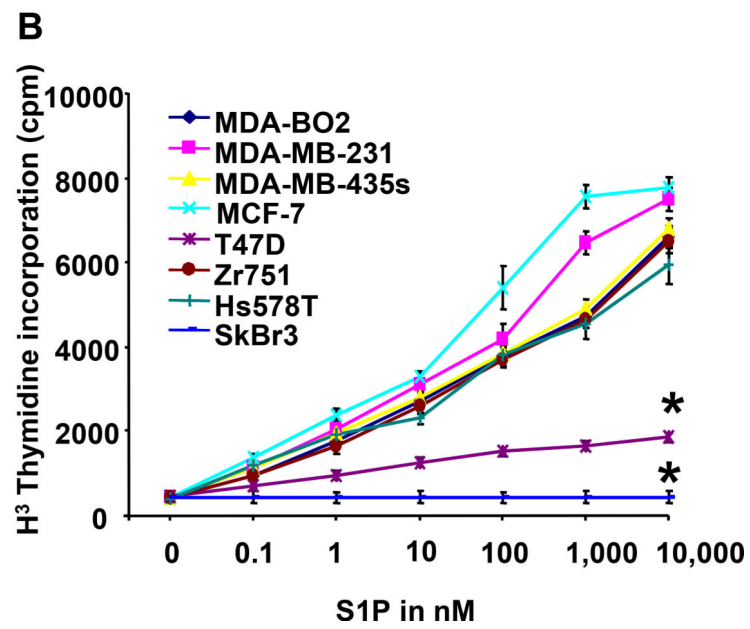
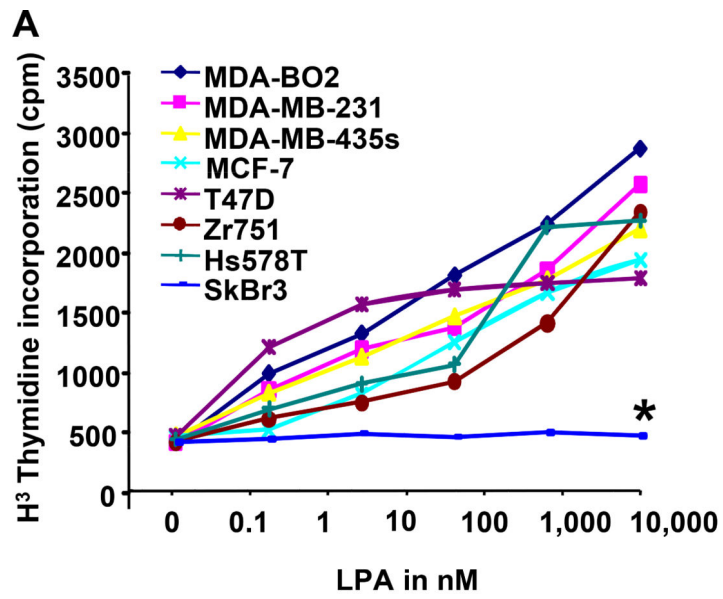
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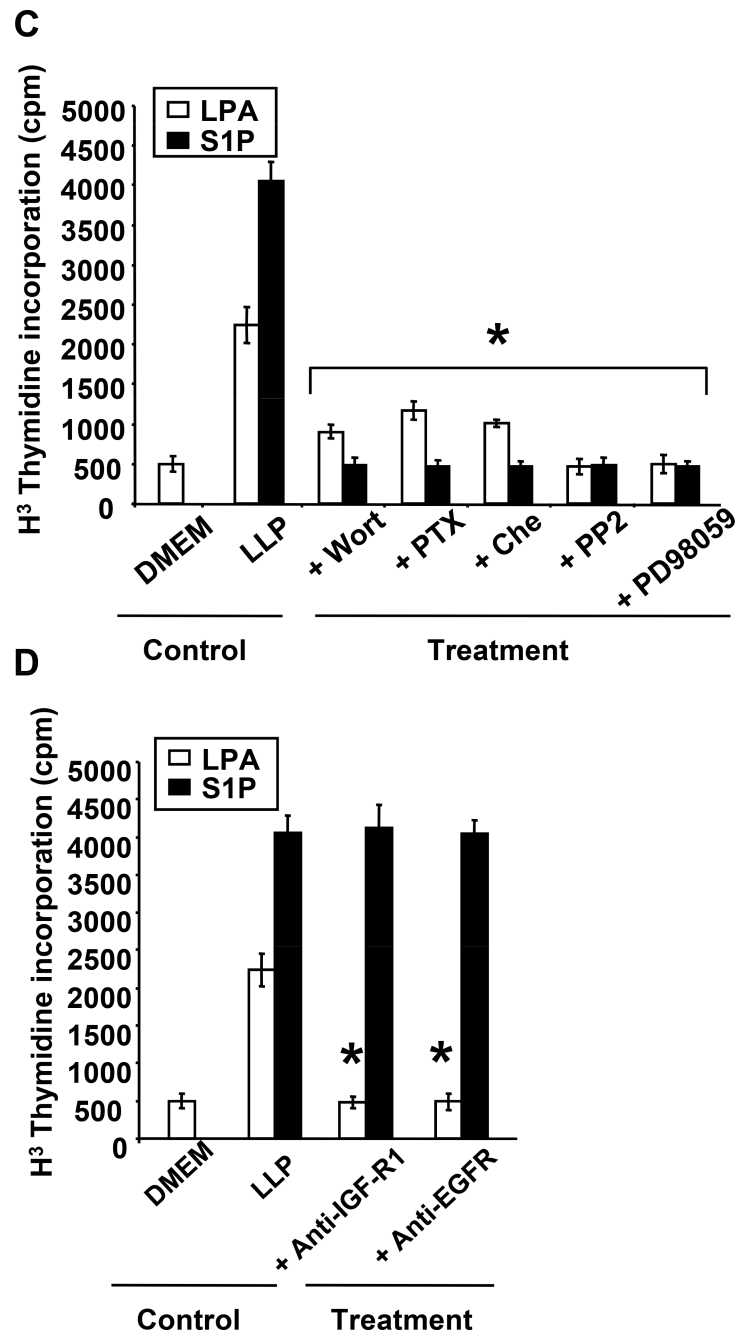


**Fig. 1–. Expression of LPA and S1P receptors in human breast cancer cell lines.**

The level of target lysolipid receptor mRNA in breast cancer cell lines was measured using quantitative real-time PCR. Results are expressed as a relative value to GAPDH mRNA.

**A.** Expression levels of LPA receptors (LPA<sub>1</sub>, LPA<sub>2</sub>, LPA<sub>3</sub>). **B.** Expression levels of S1P receptors (S1P<sub>1</sub>, S1P<sub>2</sub>, S1P<sub>3</sub>). Cell lines are MDA-MB-231, MDA-BO2, MDA-MB-435S, MCF-7, T47D, Zr751, Hs578T and SkBr3. Bars represent means  $\pm$  SD of at least three experiments.





**Fig. 2-- LPA and S1P induce breast cancer cell proliferation through distinct signaling pathways.** **A.** Mitogenic activity of LPA and **B.** S1P were analyzed on quiescent tumor cell lines treated overnight in the presence of increasing concentrations of either lysolipid (0 to  $10^{-5}$  M) and pulsed with [<sup>3</sup>H]-thymidine for the last 8 hours of lysolipid treatment. \*,  $P < 0.001$  compared to MDA-BO2 cell line. **C.** Effect of inhibitors PI3K (Wortmannin,  $10^{-7}$  M),  $G_{\alpha_i}$  (PTX, 100ng/mL), PKC (chelerythrine chloride,  $10^{-6}$  M), Src kinase (PP2,  $10^{-7}$  M), or ERK1/2 phosphorylation (PD98059,  $10^{-5}$  M) on the proliferation of MDA-BO2 cells stimulated with either lysolipids (LLP), LPA or S1P ( $10^{-7}$  M). **D.** Effect of anti-IGF-R1 and EGFR antibodies (1 $\mu$ g/ml) on the mitogenic activity of LPA and S1P ( $10^{-7}$  M) on MDA-BO2 cells. Data are

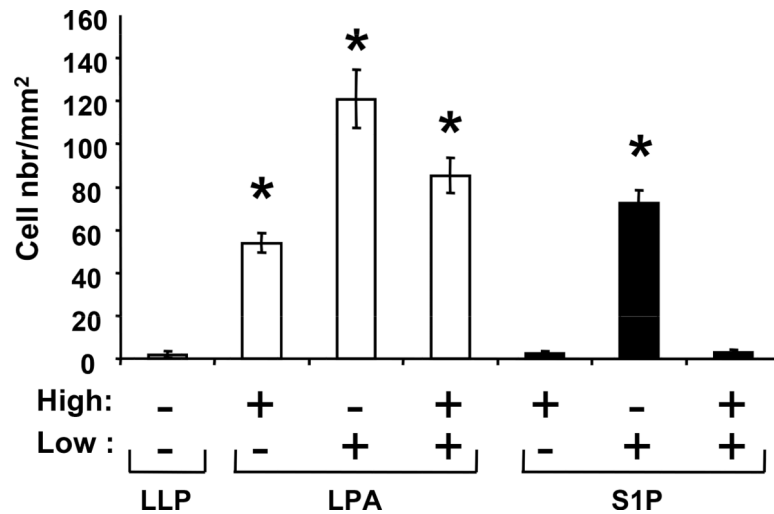
expressed as specific H<sup>3</sup>Thymidine incorporation and represent means  $\pm$  SD of at least three independent experiments. \*,  $P < 0.001$  compared to corresponding lysolipid treatment.

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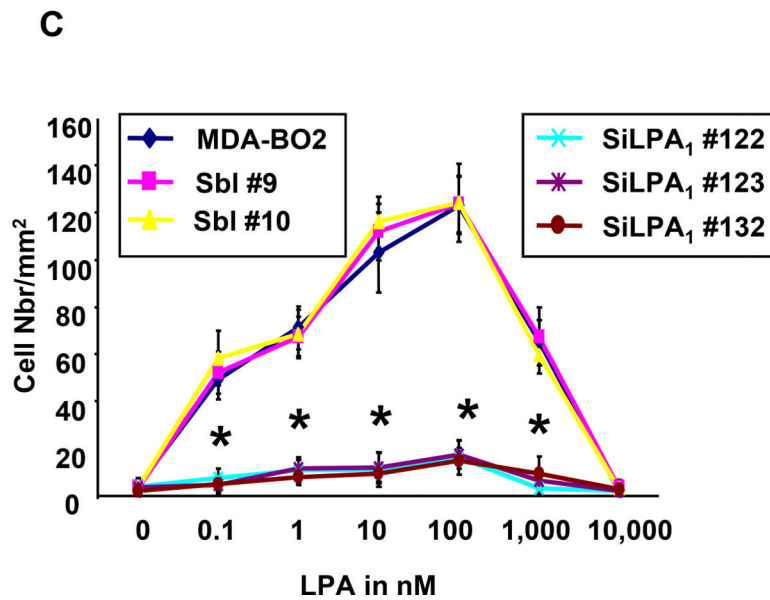
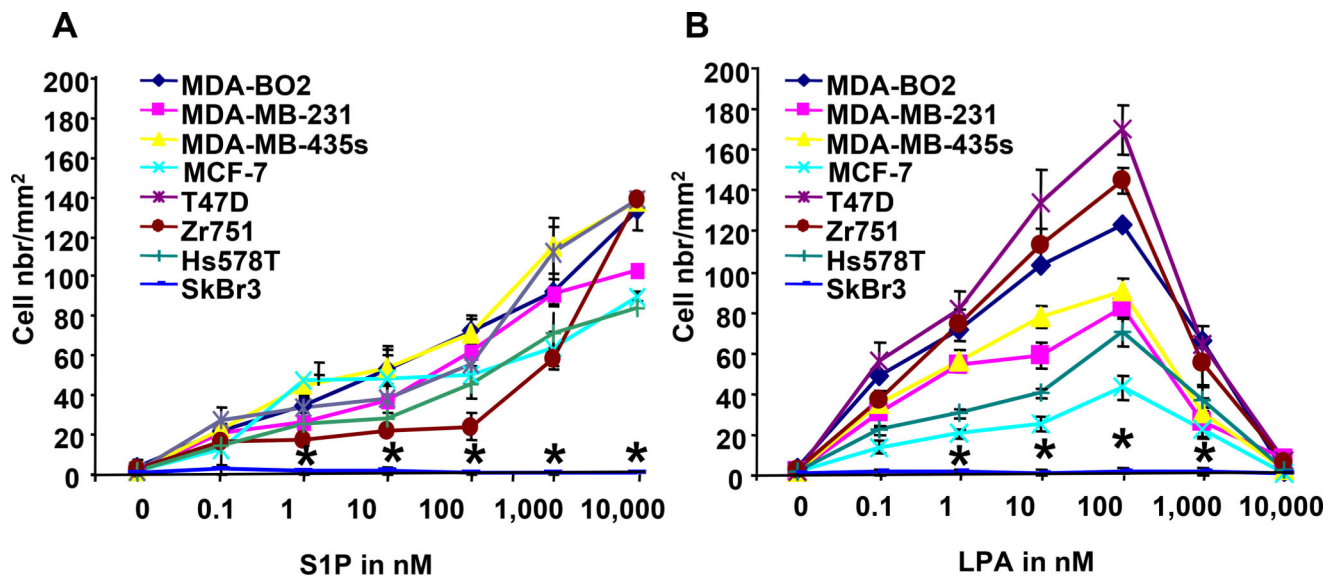
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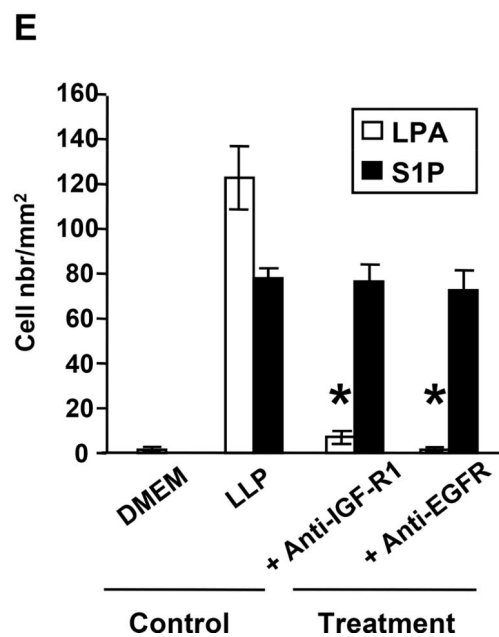
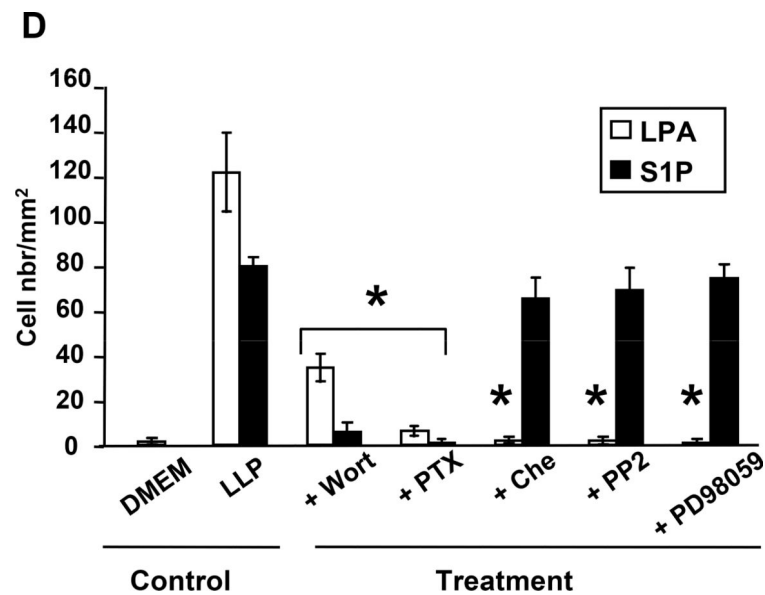
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**Fig. 3-. LPA and S1P exhibit differential migratory activities on breast cancer cells.** MDA-BO2 cells were seeded on higher (High) and/or lower (Low) chambers of transwell migration chambers. No lysolipid (LLP) or S1P ( $10^{-7}$ M) or LPA ( $10^{-7}$ M) were added on indicated chambers. LPA exhibits both chemotactic and chemokinetic activities, whereas S1P acts only as a chemo-attractant factor on MDA-BO2 cells. Cells that have passed through the 8  $\mu$ m membrane were enumerated after 6 hours of treatment. Data are expressed as number of cells/mm<sup>2</sup>. Bars represent means  $\pm$  SD of at least three experiments. \*,  $P < 0.001$  compared to control condition in absence of lysolipid.



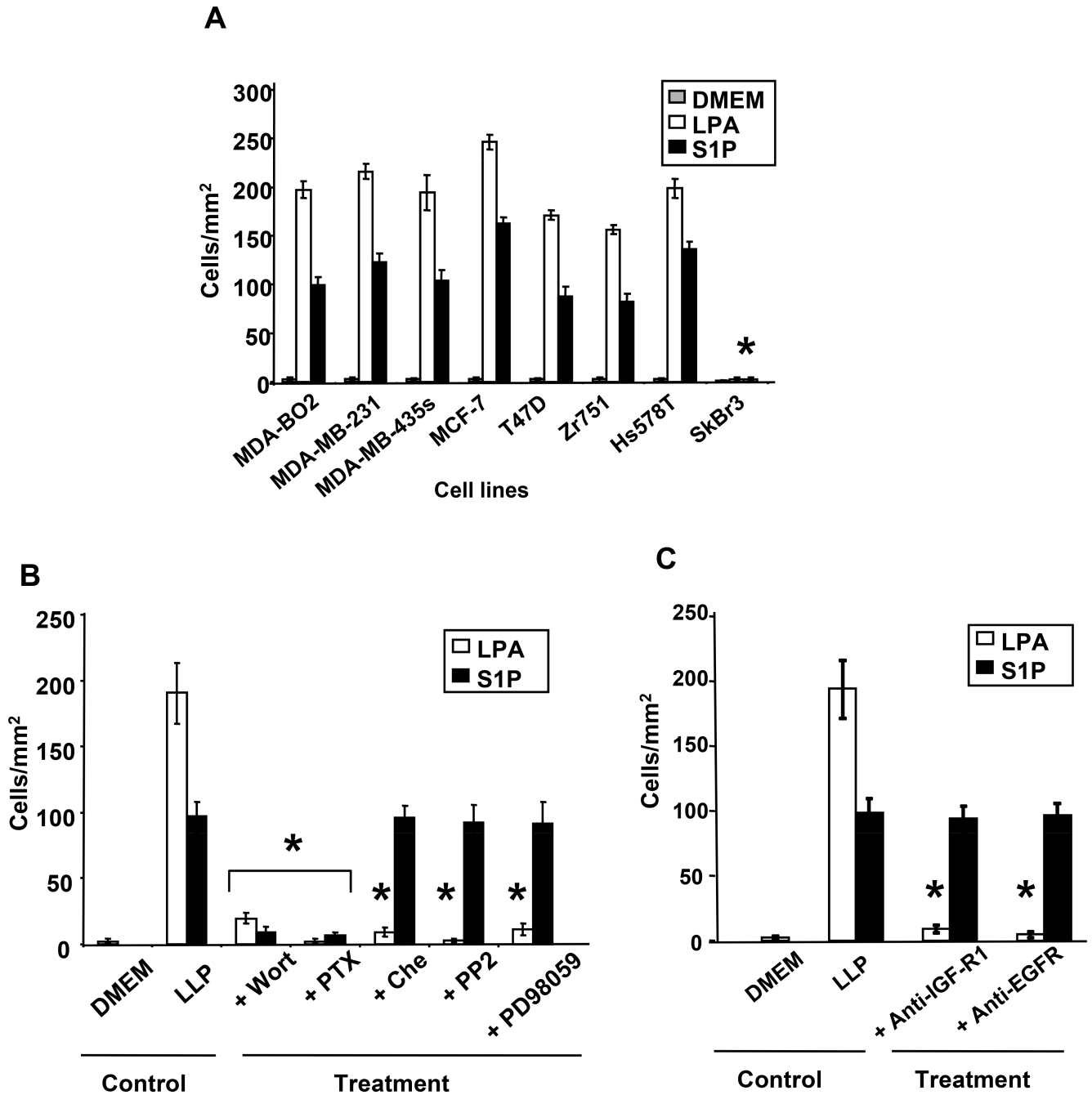




**Fig. 4-. LPA and S1P induce migration of breast cancer cells through specific signaling pathways.**

**A** and **B**. LPA and S1P stimulate dose-dependently the migration of human breast cancer cells that express cognate lysolipid receptors. Increased concentrations (0 to  $10^{-5}$  M) of S1P (**A**) or LPA (**B**) were added in the lower compartments of transwell plate migration chambers. Indicated cell lines were seeded on the higher chamber and challenged to migrate through the filter membrane for 6h. While S1P exhibits a continuous chemotactic activity throughout the range of concentrations, LPA action follows a belt shape curve, with a pic

of maximum activity at  $10^{-7}$ M. \*,  $P<0.001$  compared to MDA-BO2 cells. **C.** Silencing of  $LPA_1$  expression inhibits LPA-induced migration of MDA-BO2 cells. Three independent Si $LPA_1$  transfectants, two scramble-SiRNA control clones (Sb1) and MDA-BO2 parental cells (3) were incubated with increased concentrations of LPA as described above. \*,  $P<0.001$  compared to MDA-BO2 cells. **D.** Effect of inhibition of signaling pathways on migration of MDA-BO2 cells stimulated with LPA or S1P ( $10^{-7}$ M). Inhibitors used were identical as described in Fig. 2B. **E.** Effect of anti-IGFR1 and EGFR antibodies ( $1\mu\text{g/ml}$ ) on the migration of MDA-BO2 cells induced by LPA and S1P ( $10^{-7}$  M). Data are expressed in cell number/ $\text{mm}^2$ . Bars represent means  $\pm$  SD of at least three experiments. \*,  $P<0.001$  compared to corresponding lysolipid treatment.



**Fig. 5-. LPA and S1P stimulate invasion of human breast cancer cells through specific signaling pathways.**

Transwell migration chambers were previously coated with Matrigel. **A.** Cells were incubated in absence (DMEM) or presence of S1P ( $10^{-7}$ M) or LPA ( $10^{-7}$ M) added in the lower chamber. After 48h hours of lysolipid treatment cells passed thought the matrigel barrier were enumerated. \*,  $P < 0.001$  compared to MDA-BO2 cells. **B.** MDA-BO2 cells pretreated with Wortmannin ( $10^{-7}$ M), PTX (100ng/mL), chelerythrine chloride ( $10^{-6}$ M), PP2 ( $10^{-7}$ M), or PD98059 ( $10^{-5}$ M) were seeded on the higher chamber and challenged for 48 hours, to invade and migrate through the filter membrane precoated with matrigel, upon

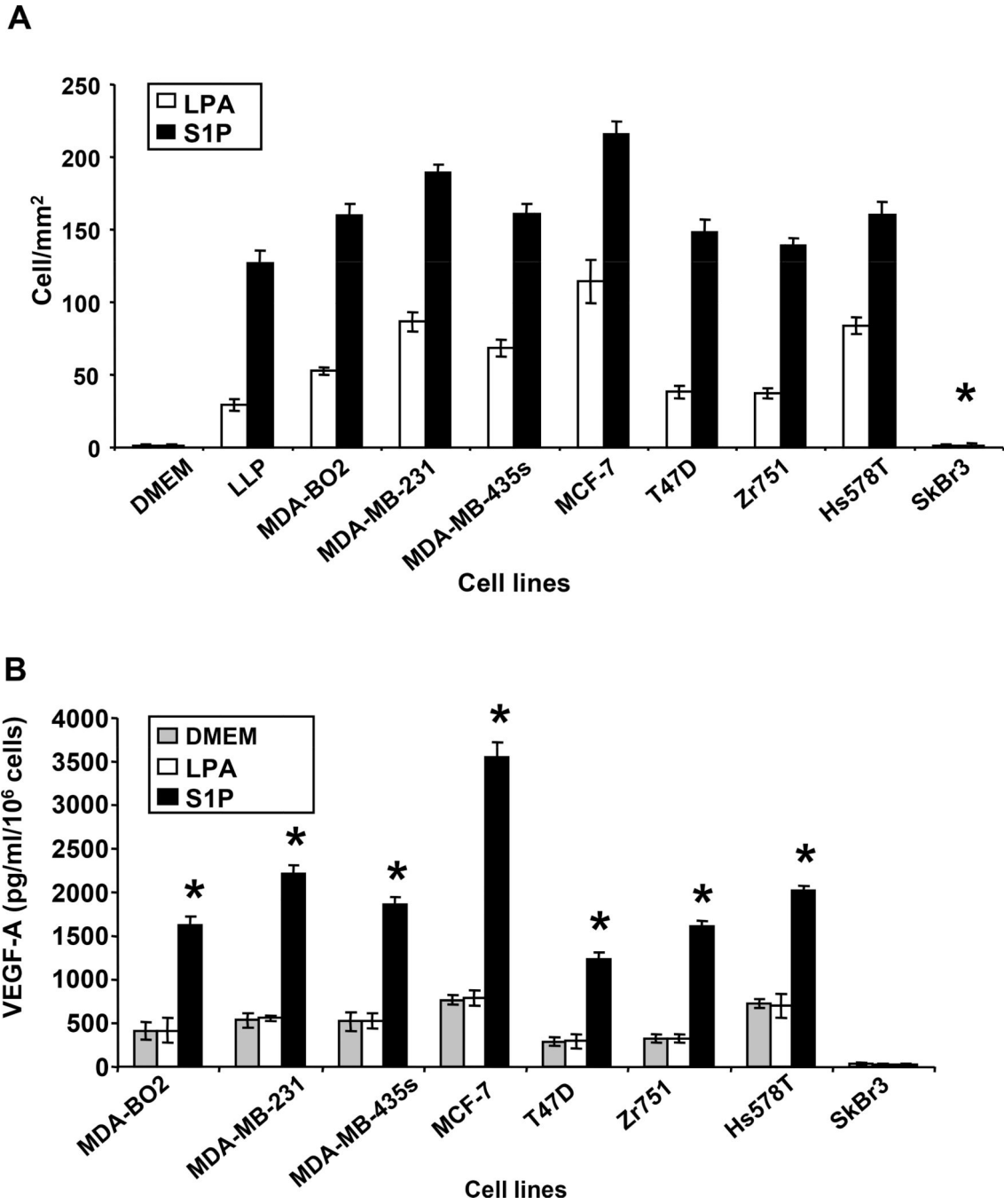
stimulation with lysolipids (LPL) added in the lower chamber. **C.** Effect of anti-IGFR1 and EGFR antibodies (1 $\mu$ g/ml) on invasion of MDA-BO2 cells induced by LPA and S1P (10<sup>-7</sup> M). Data are expressed in cell number/mm<sup>2</sup>. Bars represent means  $\pm$  SD of at least three experiments. \*,  $P < 0.001$  compared to corresponding lysolipid treatment.

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**Fig. 6-. Commune induction of tumor cell-mediated angiogenesis by LPA and S1P and specific secretion of VEGF-A by S1P.**  
**A.** Conditioned media from human breast cancer cell lines primed with LPA or S1P ( $10^{-6}M$ ) for 48h, were tested for their capacity to induce HUVEC cell migration. HUVEC cells were seeded in the higher chamber of transwell migration chambers and challenged to migrate through the filter membrane for 6h. Data are expressed as the number of cells per  $mm^2$ .  
 \*,  $P < 0.001$  compared to MDA-BO2 cell conditioned medium **B.** The secretion of VEGF-A was quantified by ELISA from conditioned media collected from indicated breast cancer

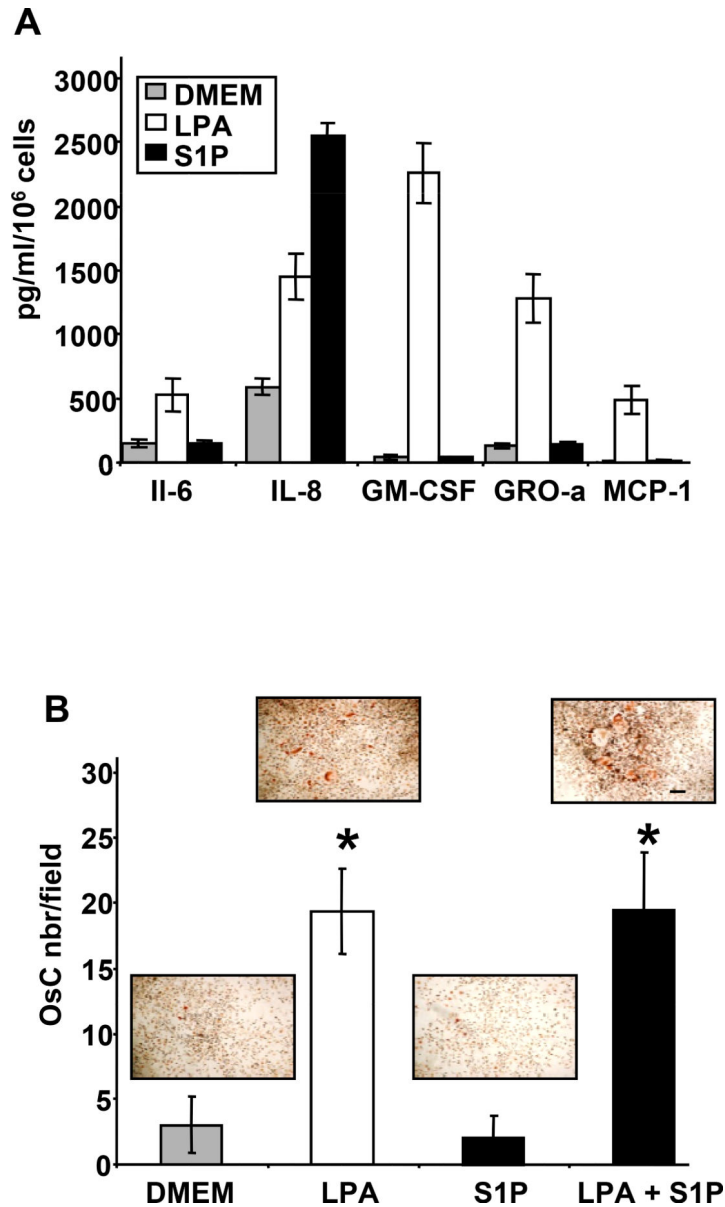
cell lines stimulated with LPA ( $10^{-7}$  M) or S1P ( $10^{-7}$  M) for 48h. Data are expressed in pg/ml/ $10^6$  cells. All bars and curves represent means  $\pm$  SD of at least three experiments. \*,  $P < 0.001$  compared to DMEM treated cells.

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**Fig. 7-. LPA but not S1P induces pro-osteoclastogenic activity of MDA-BO2 cells.**  
**A.** pro-osteoclastogenic factors (IL-6, IL-8, GM-CSF, GRO-a, MCP-1) were quantified by ELISA from conditioned media generated from MDA-BO2 cells stimulated with LPA (10<sup>-6</sup>M) or S1P (10<sup>-6</sup>M). **B.** LPA but not S1P, stimulates tumor cell-induced formation of mature osteoclasts. Bone marrow cells were cultured in the presence of conditioned media collected from MDA-BO2 cells treated with LPA (10<sup>-6</sup>M) or S1P (10<sup>-6</sup>M), or with a combination of LPA and S1P (10<sup>-6</sup>M). Mature osteoclasts, which are TRAP positive multinucleated cells, were enumerated under microscope. Data are expressed as the number of osteoclasts per field. Bars represent means ± SD of at least three experiments. Insets: representative picture of osteoclasts differentiated in each condition. Size ladder 100 µm. \*, P<0.001 compared to DMEM treated cells.