



Role of phospholipase D in migration and invasion induced by linoleic acid in breast cancer cells

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Abstract

Linoleic acid (LA) is an essential and omega-6 polyunsaturated fatty acid that mediates a variety of biological processes, including migration and invasion in breast cancer cells. Phospholipase D (PLD) catalyses the hydrolysis of phosphatidylcholine to produce phosphatidic acid and choline. Increases of expression and activity of PLD are reported in several human cancers, including gastric, colorectal, renal, stomach, lung and breast. In this article, we demonstrate that LA induces an increase of PLD activity in MDA-MB-231 breast cancer cells. Particularly, PLD1 and/or PLD2 mediate migration and invasion induced by LA. Moreover, LA induces increases in number and size of spheroids via PLD activity. FFAR1 also mediates migration and invasion, whereas PLD activation induced by LA requires the activities of FFAR1, FFAR4 and EGFR in MDA-MB-231 cells. In summary, PLD plays a pivotal role in migration and invasion induced by LA in MDA-MB-231 breast cancer cells.

Keywords Breast cancer · Linoleic acid · PLD · Migration · Invasion · Spheroids · FFAR1 · FFAR4

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Introduction

Free fatty acids (FFAs) are sources of energy for the body; however, they are also ligands for nuclear peroxisome proliferator-activated receptors, which regulate expression of genes involved in glucose and lipid metabolism [1, 2]. The FFAs induce the activation of signal transduction pathways, which mediate several biological processes including migration and invasion in breast cancer cells [3, 4]. Linoleic acid (LA) is an essential and omega-6 polyunsaturated fatty acid, which is the major fatty acid in occidental diets with an intake of 15–20 g/day/person and a plasma concentration of ~275 μM [5, 6]. Particularly, LA mediates a variety of cell processes in breast cancer cells, including the expression of plasminogen activator inhibitor-1, migration and invasion, whereas it induces an epithelial-to-mesenchymal transition-like process in mammary non-tumourigenic epithelial cells, MCF10A [4, 7–9].

Free fatty acid receptors, 1 (FFAR1) and FFAR4, are G protein-coupled receptors (GPCRs) activated by medium- and long-chain fatty acids, including LA. In contrast, these receptors are not activated by short-chain fatty acids [10, 11]. FFAR1 and FFAR4 are expressed in pancreatic β cells, adipose tissue, breast cancer cell lines (MDA-MB-231 and

MCF-7) and mammary non-tumorigenic epithelial cells, MCF10A [10, 12, 13]. Particularly, oleic acid induces proliferation via FFAR1 activation, whereas LA induces migration and invasion via FFAR4 activation in MDA-MB-231 cells [4, 14, 15].

Phospholipase D (PLD) is an enzyme that catalyses the hydrolysis of phosphatidylcholine, the major membrane phospholipid, to produce phosphatidic acid (PA) and choline [16]. PA is a second messenger that can be metabolised to other lipid metabolites, such as lyso-PA and diacylglycerol [17, 18]. Two mammalian isoforms of PLD have been described, namely PLD1 and PLD2, which are almost ubiquitous and shared about 50% homology including two highly conserved HDK motifs that define its catalytic activity [19–21]. Particularly, PLD1 and PLD2 are activated by a wide variety of GPCRs and receptor tyrosine kinases (RTKs) in response to a wide range of mitogens. Activation of PLD results in an increase of local concentrations of PA and its involvement in many cellular biological processes, including proliferation, survival, chemotaxis, vesicular trafficking, secretion, migration and invasion [22–25]. Moreover, increases of expression and activity of PLD are reported in several types of human cancers, including gastric, colorectal, renal, stomach, lung and breast [17, 26, 27].

Previously, we demonstrated that LA induces migration and invasion of MDA-MB-231 breast cancer cells. However, the role of PLD in migration and invasion induced by LA remains to be studied. In this article, we demonstrate that LA induces an increase of PLD activity in MDA-MB-231 breast cancer cells. Particularly, PLD1 and/or PLD2 mediate migration and invasion induced by LA. Moreover, LA induces increases in number and size of spheroids via PLD activity. FFAR1 also mediates migration and invasion, whereas PLD activation induced by LA requires the activity of FFAR1, FFAR4 and EGFR in MDA-MB-231 cells.

Materials and methods

Materials

LA sodium salt, DC260126, phorbol 12,13-dibutyrate (PDB), 1-butanol (BtOH) and *tert*-butanol (*tert*-BtOH) were from Sigma (St. Louis MO). Platelet-derived growth factor (PDGF) and interleukin 1 β (IL1 β) were from Prepro Tech (Rocky Hill, NJ). AH7614 was from TOCRIS (Minneapolis, MN). Phosphospecific antibody (Ab) to phosphorylated tyrosines PY20 (anti-P-Tyr), VU0155069 and CAY10594 were from Santa Cruz Biotechnology (Santa Cruz, CA). AG1478 was from Calbiochem-Novabiochem (San Diego, CA). Epidermal growth factor receptor (EGFR) Ab was from Cell Signalling Technology (Beverly, MA). Actin Ab was provided for PhD to Manuel Hernandez (Cell Biology

Department, Cinvestav-IPN). Basement membrane matrix (BD Matrigel) was from BD Biosciences (Bedford, MA, USA). Type I collagen was from Costar, Corning Inc (NY, USA).

Cell culture

MDA-MB-231 and MCF-7 breast cancer cells were procured from ATCC, and they were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 3.7 g/L sodium bicarbonate, 5% foetal bovine serum (FBS) and antibiotics, in a humidified atmosphere containing 5% CO₂ and 95% air at 37 °C. For experimental purposes, cells were serum-starved for 16 h before treatment with inhibitors and/or LA.

Cell viability assay

After starvation, MDA-MB-231 cells were washed twice with phosphate-buffered saline (PBS). Cells were equilibrated in DMEM without FBS for 30 min at 37 °C, and incubated with inhibitors used in this study for 48 h. After incubation, cells were washed with PBS and reagent WST-1 (Sigma–Aldrich) was added, and then cells were incubated for 2 h at 37 °C. The absorbance was quantified with a microplate reader at 450 nm. Each experiment was repeated three times.

Cell stimulation

Confluent cultures were washed twice with PBS, equilibrated in DMEM at 37 °C for 30 min, and then treated with inhibitors and/or LA for various times. Stimulation was terminated by aspirating the medium, and cells were solubilized in 0.5 mL of ice-cold RIPA buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM sodium orthovanadate, 100 mM NaF, 10 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1% sodium deoxycholate, 1.5 mM MgCl, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride).

PLD activity assay

Cells were cultured for 72 h, serum-starved for 16 h, washed with PBS, and lysed by three freeze–thaw cycles in 150 μ l ice-cold assay solution (10 mM Tris-HCl pH 8, 10 mM NaCl, 3.2 mM NaF, 0.1% IGEPAL, 0.3% sodium deoxycholate). Next, 50 μ l of lysate was incubated for 1 h at 37 °C with choline oxidase (Phospholipase D Activity Assay Kit, Sigma–Aldrich), and activity was estimated by absorbance at 570 nm. A standard curve was generated with different concentrations of choline. All results were normalised with respect to the protein concentration of the lysate.

Immunoprecipitation and Western blotting

Immunoprecipitation (IP) and Western blotting was performed as described previously [9, 28].

Scratch-wound assay

Cells were grown to confluence in 35-mm culture dishes, starved for 16 h in DMEM without FBS, and treated for 2 h with 12 μM mitomycin C to inhibit proliferation during the experiment. Cultures were scratch-wounded using a sterile 200- μl pipette tip, washed twice with PBS and re-fed with DMEM without FBS in the absence or the presence of inhibitors and/or LA. The progress of cell migration into the wound space was photographed at 48 h using an inverted microscope coupled to a camera. Each experiment was repeated three times. Cell migration was evaluated using the ImageJ software (NIH, USA).

Chemotactic and invasion assays

Chemotactic and invasion assays were performed by the modified Boyden chamber method in 24-well plates containing 12-cell culture inserts of 8 μm pore size (Costar, Corning Inc). For invasion assays, 50 μl matrigel (3 mg/mL) was added into culture inserts and kept for 2 h at 37 $^{\circ}\text{C}$. Cells were plated at 1×10^5 cells per insert in 100 μl FBS-free DMEM on the top chamber, whereas the lower chamber containing 600 μl DMEM with 90 μM LA and chambers were incubated for 48 h at 37 $^{\circ}\text{C}$ in a 5% CO_2 atmosphere. After incubation, cells and matrigel were removed with cotton swabs, and cells on the lower surface of membrane were washed and fixed with 4% (vol/vol) paraformaldehyde in PBS for 15 min. The number of migrated or invaded cells was estimated by staining of membranes with 0.1% crystal violet in PBS. Membranes were washed three times with PBS, photographed and the resulting dye was eluted with 500 μl 30% acetic acid, and then the absorbance at 600 nm was measured. Background value was obtained from wells without cells.

Spheroid formation assay on low-attachment

Confluent cultures of MDA-MB-231 cells (80%) were harvested with trypsin and gently pipetted to form a single cell suspension. Trypsin was inactivated by addition of FBS-containing medium and the cells were collected by centrifugation. The cells were quantified and seeded at 5,000 cells per well in six-well ultra-low attachment cluster dishes (Costar, Inc). Spheroids were permitted to grow in DMEM with 1% FBS for 5 days, and then spheroids were untreated or treated with 90 μM LA and/or 0.3% BtOH for other 5 days. Spheroid number and area were quantified using an inverted

microscope coupled to a camera. The number of spheroids was determined by using the ImageJ software (NIH, USA).

Spheroid formation assay on matrigel and type I collagen

Confluent cultures of MDA-MB-231 cells (80%) were harvested with trypsin and gently pipetted to form a single cell suspension. Trypsin was inactivated by addition of FBS-containing medium and cells were collected by centrifugation and quantified.

A volume of 500 μl of matrigel (3 mg/mL) or 500 μl type I collagen (250 $\mu\text{g}/\text{mL}$) were spread with a pipette tip on the bottom of a 24-well chamber slide. A suspension of MDA-MB-231 single-cells was laid on top of the matrix with a density of 3,000 cells per well and then incubated for 24 h. After incubation, cells were untreated or treated with 90 μM LA and/or 0.3% BtOH and spheroids were permitted to grow in DMEM with 0.1% FBS for 15 days. LA and BtOH were renewed every third day. Spheroid number and area were quantified using an inverted microscope coupled to a camera. The number of spheroids was determined by using the ImageJ software (NIH, USA).

Statistical analysis

Experiments were performed at least three times and results are expressed as mean \pm SEM. Data were statistically analysed using one-way ANOVA and Knewman-Keuls's multiple comparison tests. A statistical probability of $P < 0.05$ was considered significant.

Results

LA induces PLD activation in MDA-MB-231 breast cancer cells

We studied whether LA induced activation of PLD. MDA-MB-231 breast cancer cells were treated with 90 μM LA for various times and PLD activity was determined. Our findings showed that LA induced PLD activation at 5 and 60 min of stimulation in MDA-MB-231 cells (Fig. 1a).

Since PDGF, IL1 β and PDB induce PLD activation [29, 30], we further substantiate that LA induced the PLD activation by stimulation of MDA-MB-231 cells with 20 ng/ml PDGF, 10 ng/ml IL1 β , 1 μM PDB for 30 min or 90 μM LA for 5 min, and then PLD activity was determined. Our findings demonstrated that LA, PDGF, IL1 β and PDB induced activation of PLD in MDA-MB-231 cells (Fig. 1b).

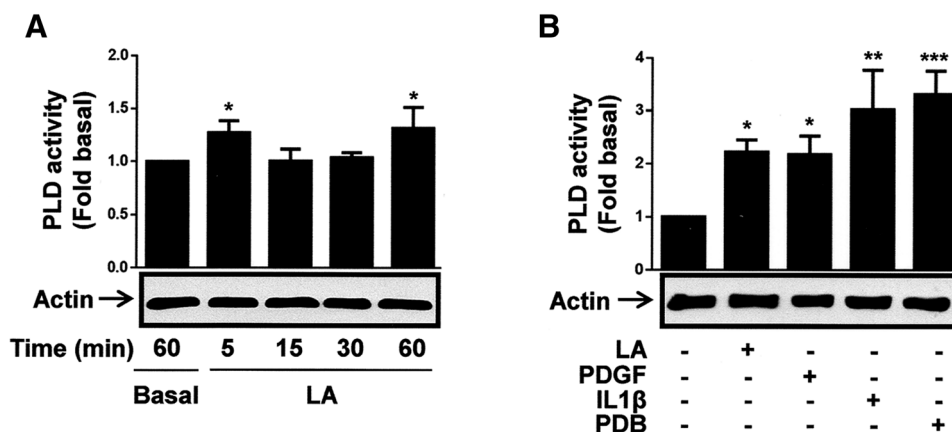


Fig. 1 LA-induced PLD activation in MDA-MB-231 breast cancer cells. **a** MDA-MB-231 cells were stimulated with 90 μ M LA for various times, and PLD activity was determined. **b** MDA-MB-231 cells were treated with 90 μ M LA for 5 min, 20 ng/ml PDGF, 10 ng/ml IL1 β and 1 μ M PDB for 30 min, and PLD activity was determined.

LA induces migration and invasion through a PLD-dependent pathway in MDA-MB-231 cells

Since LA induces migration and invasion in breast cancer cells [4, 9], we studied the role of PLD in migration and invasion induced by LA in MDA-MB-231 cells. We first determined whether inhibitors used in these studies were cytotoxic for cells. The MDA-MB-231 cells were treated with different inhibitors to concentrations used in these studies, and viability assays were performed. Our findings showed that inhibitors were not toxic for MDA-MB-231 cells (Fig. 2a).

Next, we studied the role of PLD in migration. Since PLD is able to utilise primary alcohols for a transphosphatidylation reaction to generate phosphatidylalcohols in place of PA, the incubation with BtOH has been used like a PLD inhibitor [31]. Cultures of MDA-MB-231 cells were scratch-wounded and untreated or treated with 0.3% BtOH, 0.3% *tert*-BtOH and 90 μ M LA for 48 h. The *tert*-BtOH was included as a negative control of BtOH. As illustrated in Fig. 2b, treatment with BtOH inhibited migration, whereas *tert*-BtOH did not inhibit migration induced by LA in MDA-MB-231 cells.

To further substantiate our findings, we performed cell migration assays using the Boyden chamber method and MDA-MB-231 cells untreated or treated with 0.3% BtOH and stimulated without or with 90 μ M LA for 48 h. Our findings showed that treatment with BtOH inhibited migration induced by LA in MDA-MB-231 cells (Fig. 2c).

Next, we studied the role of PLD in the invasion process induced by LA. Invasion assays were performed using the Boyden chamber method and MDA-MB-231 cells untreated

or treated with 0.3% BtOH and stimulated without or with 90 μ M LA for 48 h. Our findings demonstrated that treatment with BtOH inhibited invasion induced by LA in MDA-MB-231 cells (Fig. 2d).

Roles of PLD1 and PLD2 in migration and invasion induced by LA

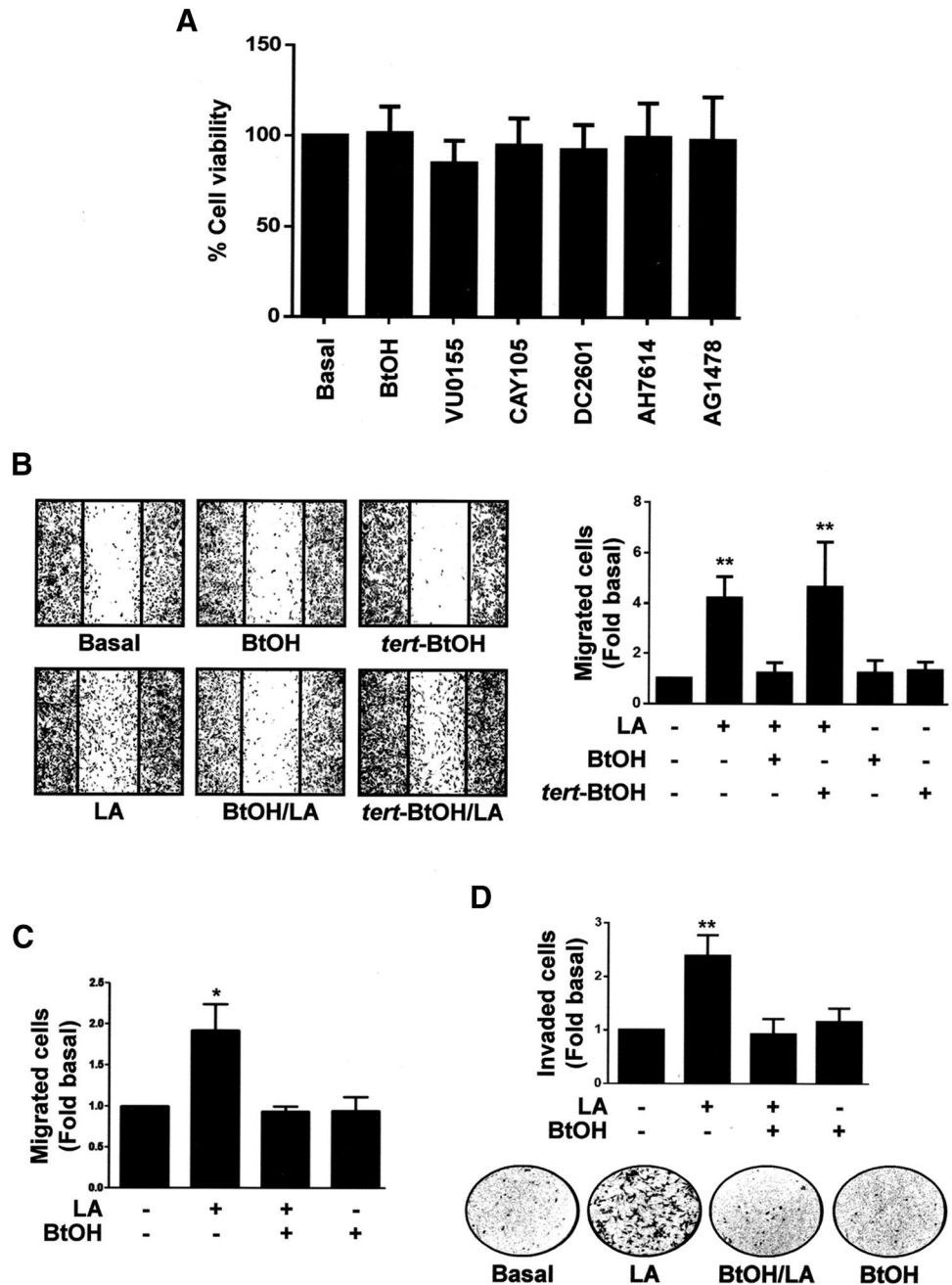
We determined whether PLD1 and/or PLD2 mediate migration and invasion induced by LA in MDA-MB-231 cells. Migration assays were performed by using scratch-wound assays and Boyden chamber method with MDA-MB-231 cells untreated or treated for 2 h with 5 μ M VU0155069 and 1 μ M CAY10594, which are specific inhibitors of PLD1 and PLD2 respectively [32, 33], and stimulated without or with 90 μ M LA for 48 h. Our findings demonstrated that inhibition of PLD1 and PLD2 activity inhibited migration induced by LA in MDA-MB-231 cells (Fig. 3a, b).

Invasion assays were performed by the Boyden chamber method and MDA-MB-231 cells untreated or treated for 2 h with 5 μ M VU0155069 and 1 μ M CAY10594, and stimulated without or with 90 μ M LA for 48 h. Our results showed that treatment with VU0155069 completely inhibited invasion, whereas CAY10594 partly inhibited invasion induced by LA in MDA-MB-231 cells (Fig. 3c).

LA induces migration through a PLD-dependent activity in MCF-7 breast cancer cells

To further substantiate the role of PLD in migration induced by LA in breast cancer cells, we studied the role of PLD in migration induced by LA in another breast cancer cell line (MCF-7). First, migration assays were

Fig. 2 LA-induced migration and invasion through a PLD-dependent pathway. **a** MDA-MB-231 cells were treated with 0.3% BtOH, 5 μ M VU0155069, 1 μ M CAY10594, 3 μ M DC260126, 20 μ M AH7614 and 500 nM AG1478 for 48 h and cytotoxicity assays were performed. Graph represents % cell viability of three independent experiments. **b** Cultures of MDA-MB-231 cells were untreated or treated with 0.3% BtOH or 0.3% *tert*-BtOH for 2 h, scratch-wounded and stimulated with 90 μ M LA for 48 h. **c** Migration assays were performed by Boyden chamber method and MDA-MB-231 cells untreated or treated with 0.3% BtOH for 2 h and stimulated with 90 μ M LA for 48 h. **d** Invasion assays were performed by Boyden chamber method and MDA-MB-231 cells untreated or treated with 0.3% BtOH for 2 h and stimulated with 90 μ M LA for 48 h. Images were taken at 48 h. Graphs represent the mean \pm SEM and are expressed as the fold of migrated or invaded cells above unstimulated cells. Asterisks denote comparisons made to unstimulated cells (Basal). * P <0.05, ** P <0.01



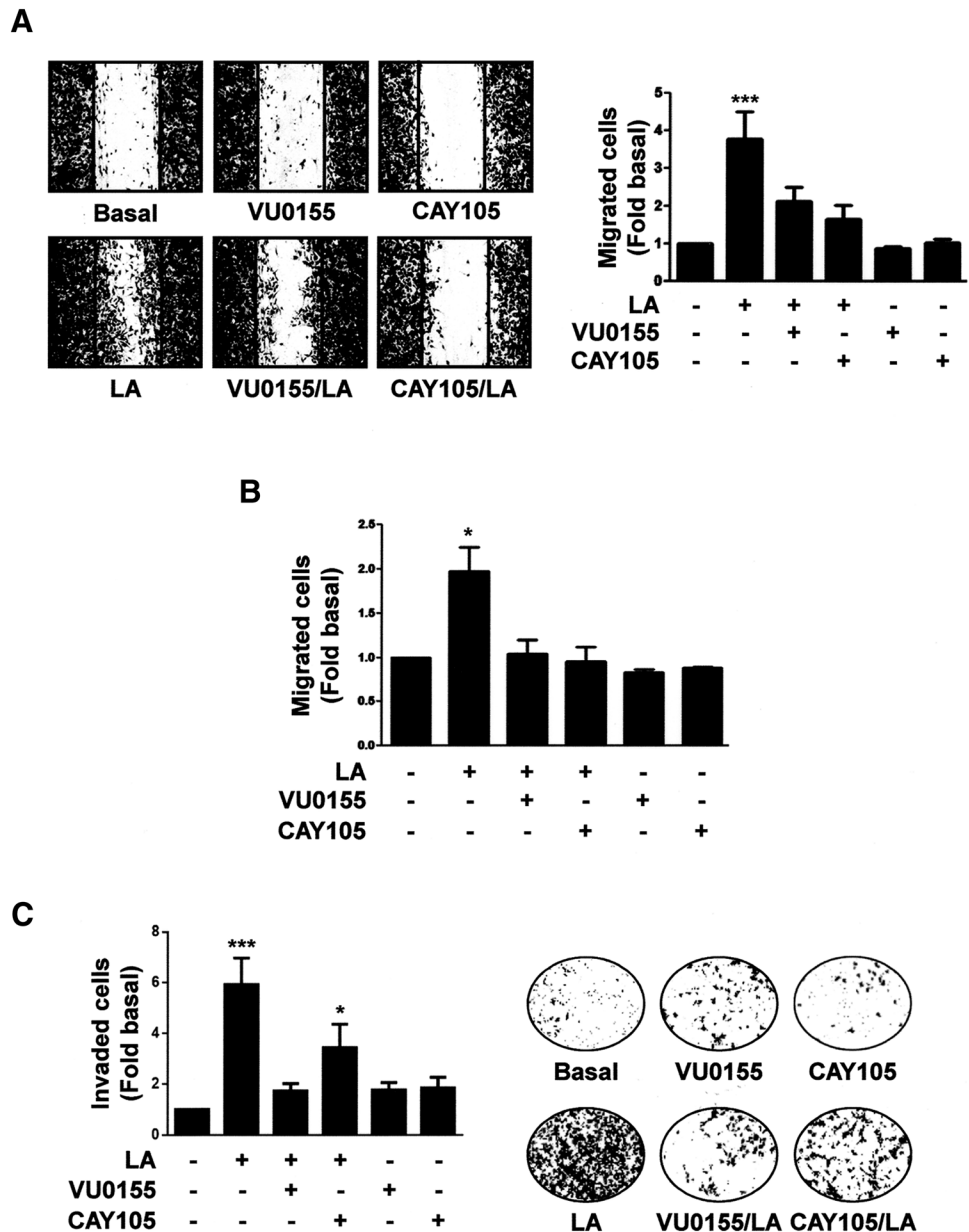
performed by scratch-wound assays and MCF-7 cells treated with 90 μ M LA for various times. Our findings demonstrated that LA induced migration at 72 h of treatment in MCF-7 breast cancer cells (Fig. 4a).

To determine the role of PLD in migration, MCF-7 cells were untreated or treated with 0.3% BtOH and stimulated without or with 90 μ M LA for 72 h. Our findings demonstrated that treatment with BtOH inhibited migration induced by LA in MCF-7 cells (Fig. 4b).

LA induces spheroid formation through a PLD-dependent pathway

The three-dimensional cell culture systems mimic better the *in vivo* conditions [34]. We studied the role of PLD in spheroid formation induced by LA. First, we determined whether stimulation of MDA-MB-231 cells with 90 μ M LA for 5 days induced spheroids formation and the role of PLD in low-attachment. Our findings demonstrated that treatment

Fig. 3 Roles of PLD1 and PLD2 in migration and invasion induced by LA. **a** and **b**. Migration assays were performed by scratch-wound assays and Boyden chamber method with MDA-MB-231 cells untreated or treated with 5 μ M VU0155069 or 1 μ M CAY10594 for 2 h and stimulated with 90 μ M LA for 48 h. **c**. Invasion assays were performed by Boyden chamber method, and MDA-MB-231 cells untreated or treated with 5 μ M VU0155069 or 1 μ M CAY10594 for 2 h, and stimulated with 90 μ M LA for 48 h. Images were taken at 48 h. Graphs represent the mean \pm SEM and are expressed as the fold of migrated or invaded cells above unstimulated cells (basal). Asterisks denote comparisons with unstimulated cells. * $P < 0.05$, *** $P < 0.001$



of MDA-MB-231 cells with LA induced an increase in number of spheroids, and treatment with BtOH inhibited their formation in low-attachment (Fig. 5a). Moreover, treatment of MCF-7 cells with 90 μ M LA for 5 days also induced an increase in number of spheroids in low-attachment (Fig. 1S, Panel A).

To further substantiate our findings, we determined whether treatment of MDA-MB-231 cells with 90 μ M LA for 15 days induced an increase in the number of spheroids on matrigel and type I collagen and the role of PLD. Our findings demonstrated that stimulation of MDA-MB-231 cells with LA induced increases in number and relative area of spheroids in matrigel and type I collagen, and treatment with BtOH inhibited their formation (Figs. 5b, c, 6a, b). In

addition, treatment of MCF-7 cells with 90 μ M LA for 15 days also induced increases in the number and relative area of spheroids in matrigel (Fig. 1S, Panel B).

Roles of FFAR1 and FFAR4 in PLD activation

Since LA induces migration and invasion via FFAR4 in MDA-MB-231 cells [4], we studied the role of FFAR1 in migration and invasion induced by LA. Migration, and invasion assays were performed with MDA-MB-231 cells untreated or treated for 1 h with 3 μ M DC260126, which is a specific inhibitor of FFAR1 [35], and stimulated without or with 90 μ M LA for 48 h. Our findings demonstrated that treatment with DC260126 partly inhibited

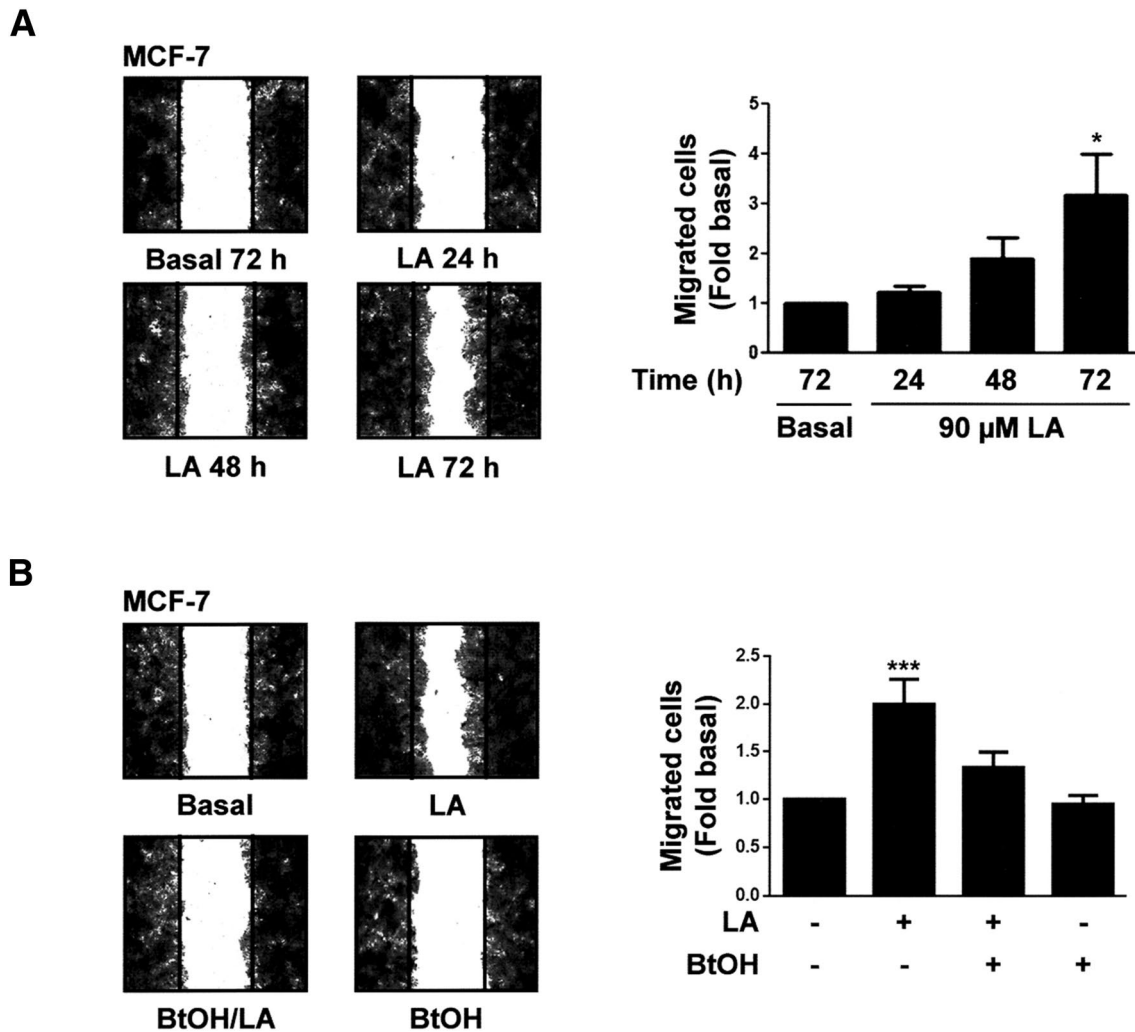


Fig. 4 LA-induced migration via PLD activity in MCF-7 breast cancer cells. **a** Cultures of MCF-7 cells were scratch-wounded and treated with 90 μ M LA for various times. **b** Cultures of MCF-7 cells were untreated or treated with 0.3% BtOH for 2 h, scratch-wounded

and stimulated without or with 90 μ M LA for 72 h. Pictures were taken at 72 h. Graphs represent the mean \pm SEM and are expressed as the fold of migrated cells above unstimulated cells (Basal). Asterisks denote comparisons with unstimulated cells. * P < 0.05, *** P < 0.001

migration, whereas it inhibited the invasion induced by LA in MDA-MB-231 cells (Fig. 7a, b).

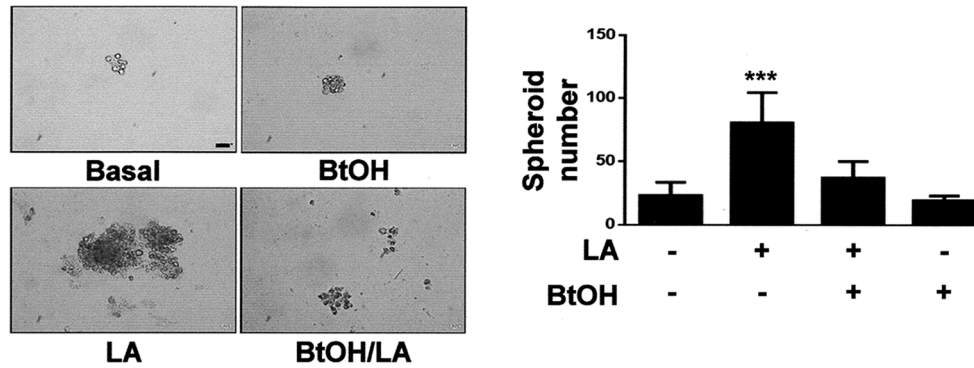
Next, we determined the roles of FFAR1 and FFAR4 in PLD activation induced by LA. MDA-MB-231 cells were untreated or treated for 1 h with 3 μ M DC260126 and 20 μ M AH7614, which are specific inhibitors of FFAR1 and FFAR4, respectively [35, 36], and stimulated without or with 90 μ M LA for 5 min, and then PLD activity was analysed. Our findings demonstrated that treatment with DC260126 or AH7614 partly inhibited the PLD activity induced by LA in MDA-MB-231 cells. However, treatment with both DC260126 and AH7614 completely inhibited the PLD activity induced by LA in MDA-MB-231 cells (Fig. 7c).

Role of EGFR in PLD activation

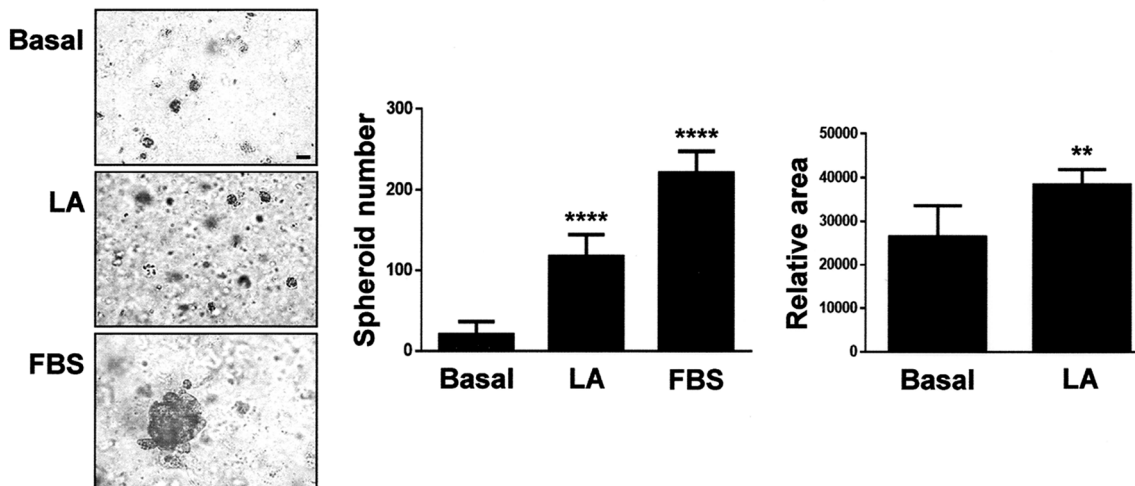
LA induces migration and invasion via EGFR activity in MDA-MB-231 cells [4]. We determined the role of EGFR in PLD activation induced by LA. PLD activity assays were performed with MDA-MB-231 cells untreated or treated for 30 min with 500 nM AG1478, an inhibitor of EGFR [37], and stimulated without or with 90 μ M LA for 5 min. Our results showed that PLD activation induced by LA required the activity of EGFR (Fig. 7d).

In order to support our findings, we determined whether LA induced EGFR activation, given by its tyrosine phosphorylation [38]. MDA-MB-231 cells were treated with 90 μ M LA for various times and lysed. Lysates were

A Low-attachment



B Matrigel



C Matrigel

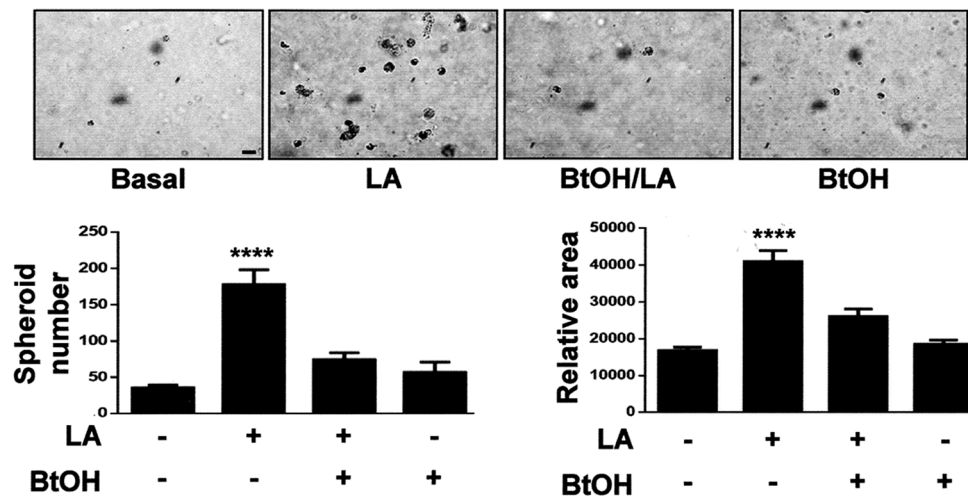


Fig. 5 LA-induces increases in spheroids number and size through a PLD-dependent pathway. **a** MDA-MB-231 cells were untreated or treated with 0.3% BtOH and stimulated with 90 μ M LA for 5 days on low-attachment, and spheroid formation was evaluated. **b** and **c**. MDA-MB-231 cells were untreated or treated with 0.3% BtOH and stimulated with 90 μ M LA for 15 days on matrigel, and spheroid

formation was evaluated. One positive control was included (FBS). Images were acquired and analysed for number and/or size. Representative images are shown. Graphs represent the mean \pm SEM of four independent experiments. Scale bar=300 μ m. Asterisks denote comparisons made to unstimulated cells (Basal). ** P <0.01, *** P <0.001, **** P <0.0001

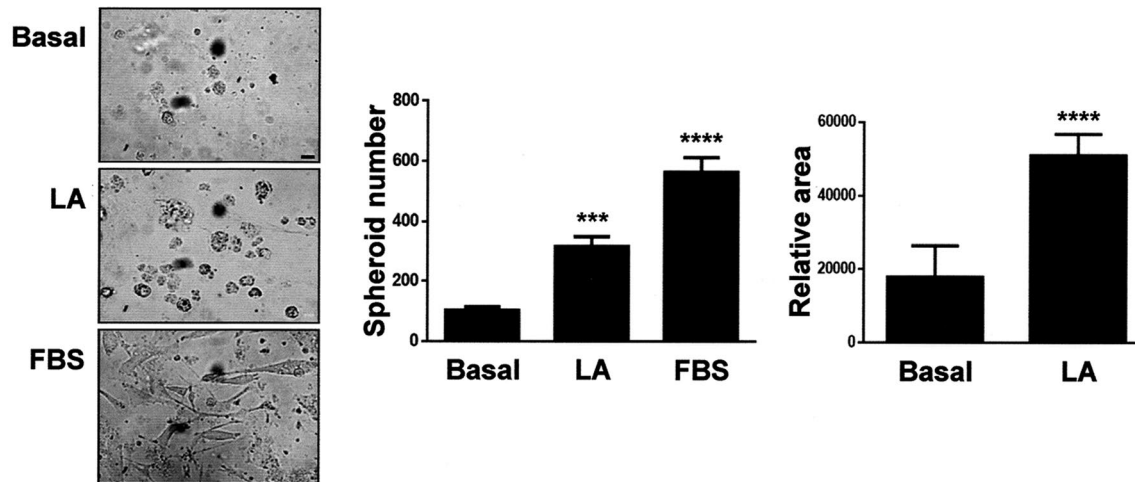
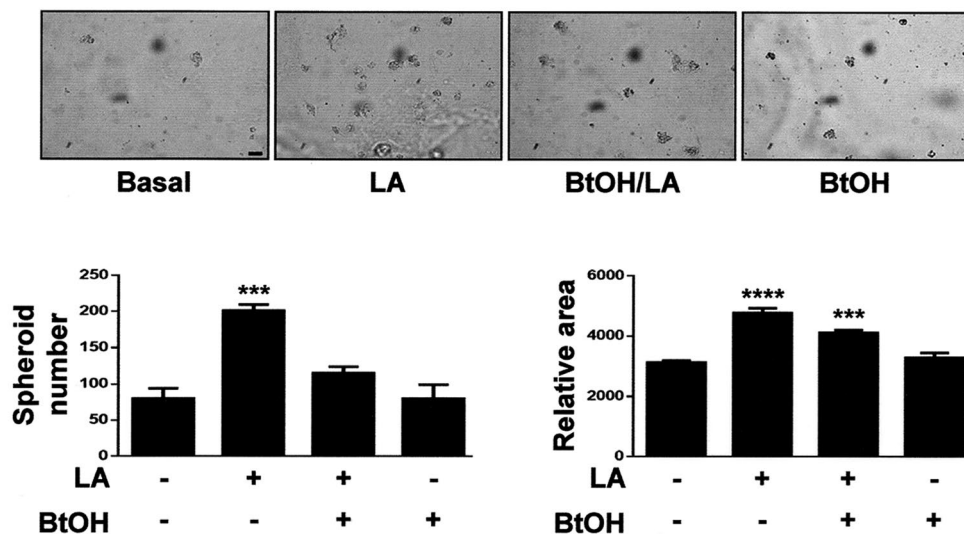
A Type I collagen**B Type I collagen**

Fig. 6 LA-induced increases in spheroids number and size on type I collagen through a PLD-dependent pathway. **a** MDA-MB-231 cells were untreated or treated with 90 μ M LA for 15 days on type I collagen, and spheroid formation was evaluated. One positive control was included (FBS). **b** MDA-MB-231 cells were untreated or treated with 0.3% BtOH and stimulated with 90 μ M LA for 15 days on type I col-

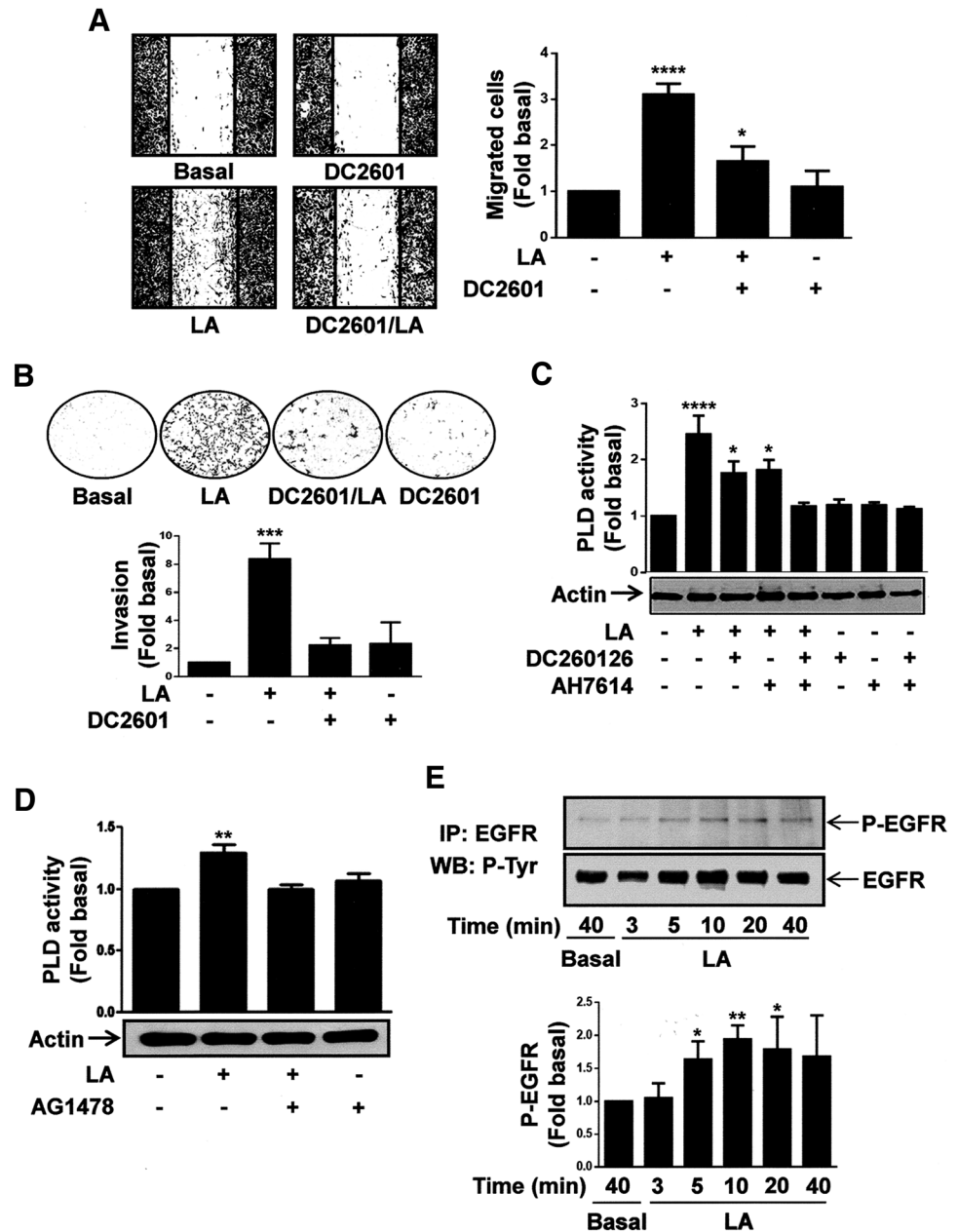
lagen, and spheroid formation was evaluated. Images were acquired and analysed for number and size. Representative images are shown. Graphs represent the mean \pm SEM of four independent experiments. Scale bar=300 μ m. Asterisks denote comparisons made to unstimulated cells (Basal). *** P <0.001, **** P <0.0001

immunoprecipitated with anti-EGFR Ab, and complexes were analysed by Western blotting with anti-P-Tyr Ab. As illustrated in Fig. 7e, LA induced EGFR tyrosine phosphorylation in MDA-MB-231 cells.

Discussion

A strong correlation between a high dietary fat intake and the risk of developing breast cancer has been described previously [39–41]. Particularly, LA is a fatty acid and a

Fig. 7 Roles of FFAR1/FFAR4 and EGFR in migration/invasion and PLD activity induced by LA. **a** and **b**. Migration and invasion assays were performed by scratch-wound assays and Boyden chamber method with MDA-MB-231 cells untreated or treated with 3 μ M DC260126 for 1 h and stimulated with 90 μ M LA for 48 h. **c** and **d**. MDA-MB-231 cells were untreated or treated with 3 μ M DC260126 and 20 μ M AH7614 for 1 h or 500 nM AG1478 for 30 min and stimulated with 90 μ M LA for 5 min, and PLD activity was determined. Actin was included as loading control. **e**. MDA-MB-231 cells were treated with 90 μ M LA for various times and lysed. Lysates were immunoprecipitated (IP) with EGFR Ab followed by Western blotting with anti-P-Tyr Ab. Membranes were analysed further by Western blotting with anti-EGFR Ab. Graphs represent the mean \pm SEM and are expressed as the fold of migrated, invaded, PLD activity or P-EGFR above unstimulated cells. Asterisks denote comparisons with unstimulated cells (Basal). * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001



component of vegetable oils, which activates signal transduction pathways that mediate a variety of biological processes including migration and invasion in breast cancer cells, whereas it mediates tumour growth and metastasis in nude mice [4, 7, 9, 42]. However, the capacity of LA to form spheroids and the roles of PLD in spheroids' formation, migration and invasion induced by LA in breast cancer cells have not been studied.

PLD plays a pivotal role in cancer, because the expression and total PLD activity are increased in several human cancers including gastric, colorectal, renal, stomach and breast cancer, and PLD levels correlate with breast cancer grade [17, 26, 27]. We demonstrate here that LA induces a transient PLD activation in MDA-MB-231 cells. Moreover, LA induce migration via PLD activity in MDA-MB-231 and MCF-7 cells, whereas it also mediates invasion via PLD activity in MDA-MB-231 cells.

In agreement with our findings, angiotensin II induces a transient PLD activation in human adrenocortical carcinoma NCI H295R cells, whereas EGF induces also PLD2 activation with two peaks of maximal activity in COS-7 cells expressing PLD2 [43, 44]. Our findings strongly suggest that different ligands induce PLD activation with different kinetics of activity in different cell types. In addition, depriving of serum induces PLD activation and migration through a PLD-dependent pathway in breast cancer cells [45].

PLD1 and PLD2 are expressed differentially and play specific roles in cancer. Particularly, PLD1 is required for secretion of metalloproteinase-9 (MMP-9) and MMP-2 in colorectal cancer cells and glioma cells, respectively [30, 46]. PLD2 activation induces an increase in FAK phosphorylation, Akt activation and invasion in EL4 lymphoma cells, whereas inactivation of PLD2 inhibits metastasis [47]. We demonstrate here that migration induced by LA requires PLD1 and PLD2 activities in MDA-MB-231 cells. Since EGF and PDGF induce PA production, which induces activation of Ras-ERK/PI3K-NF κ B signalling pathway and then PLD1 expression, which promote invasion in SK-BR3 breast cancer cells [29]. We propose that LA induces production of PA through PLD2 activation and then activation of a signalling pathway that mediates expression and activation of PLD1, which mediates migration in MDA-MB-231 cells. Supporting our proposal, activation of Wnt pathway induces an increase of PLD1/PLD2 expression, which mediate proliferation and invasion in cancer cells [48].

In addition, we demonstrate that invasion induced by LA requires PLD1 activity in MDA-MB-231 cells. Interestingly, the invasion induced by LA is also partly dependent on PLD2 activity. In agreement with our findings, EGF induces invasion through a PLD2-dependent pathway in MDA-MB-231 cells, whereas invasion induced by PDGF requires PLD1 activity in SK-BR3 breast cancer cells [29, 49]. Taken together these findings, we propose that different ligands mediate invasion through activation of PLD1 and/or PLD2 in breast cancer cells.

In order to mimic *in vivo* environment, we used spheroid formation assays because it is a 3D culture and is considered a more reliable biological assay [34, 50]. We demonstrate here that LA induces increases in the number and size of spheroids under different conditions, including low-attachment, matrigel and type I collagen, and it requires PLD activity in MDA-MB-231 cells. The 3D tumour spheroids are self-assembled cultures of tumour cells where cell–cell interactions predominate over

cell–substrate interactions. It is considered that spheroids resemble avascular tumour nodules, micro-metastasis and intervascular regions of large solid tumours, because they have similar morphological features, microenvironment, volume growth kinetics, and gradients of nutrient distribution, oxygen concentration and cell proliferation [51, 52]. We propose that LA through PLD activation plays a pivotal role in the formation of tumour nodules and micro-metastasis. Supporting our proposal, PLD mediates invasion, tumour growth and metastasis in a human breast cancer xenograph model [49]. Moreover, androgen-insensitive prostate cancer cells, DU145 and PC-3, have a higher PLD activity than androgen-sensitive prostate cancer cells LNCaP, whereas inhibition of PLD activity reduces prostate cancer cell proliferation and colony formation in prostate cancer cell lines and patient-derived prostate cancer cells [53, 54].

Previously, we demonstrated that LA induces migration and invasion via FFAR4 in MDA-MB-231 cells [4, 9]. Moreover, LA induces an elevation of cytosolic Ca²⁺ via FFAR1 and Gi/Go in MCF-7 cells, whereas oleic acid induces proliferation via FFAR1 and Gi/Go in MDA-MB-231 cells [15, 55]. In addition, FFAR4 is expressed in intestine cells and is coupled with Gq/11 [10, 56]. We demonstrate that LA induces PLD activation via FFAR1 and FFAR4 in MDA-MB-231 cells. LA also induces invasion via FFAR1, whereas migration is partly dependent on FFAR1. We propose that LA induces activations of FFAR1 and FFAR4 which activate Gi/Go and/or Gq/11, and then PLD activations which mediate migration and invasion in MDA-MB-231 cells.

GPCRs induce EGFR transactivation via activation of metalloproteinases and then release of EGF-like ligands, such as HB-EGF, from growth factor precursors in plasma membrane [57]. Furthermore, LA induces migration and invasion via FFAR1 and/or FFAR4 and that LA induces migration and invasion through an EGFR-receptor-dependent pathway in MDA-MB-231 cells [4]. Since we demonstrate here that LA induces PLD activation via EGFR, and that LA induces EGFR tyrosine phosphorylation (activation) in MDA-MB-231 cells, we propose that LA induces migration and invasion through activation of FFAR1 and FFAR4 and transactivation of EGFR in MDA-MB-231 cells.

Combining together our above findings, we propose that stimulation of breast cancer cells with LA induces the activation of FFAR1/FFAR4, which promotes EGFR transactivation, and then EGFR signalling promotes PLD1/PLD2 activation, which mediates migration and invasion (Fig. 8).

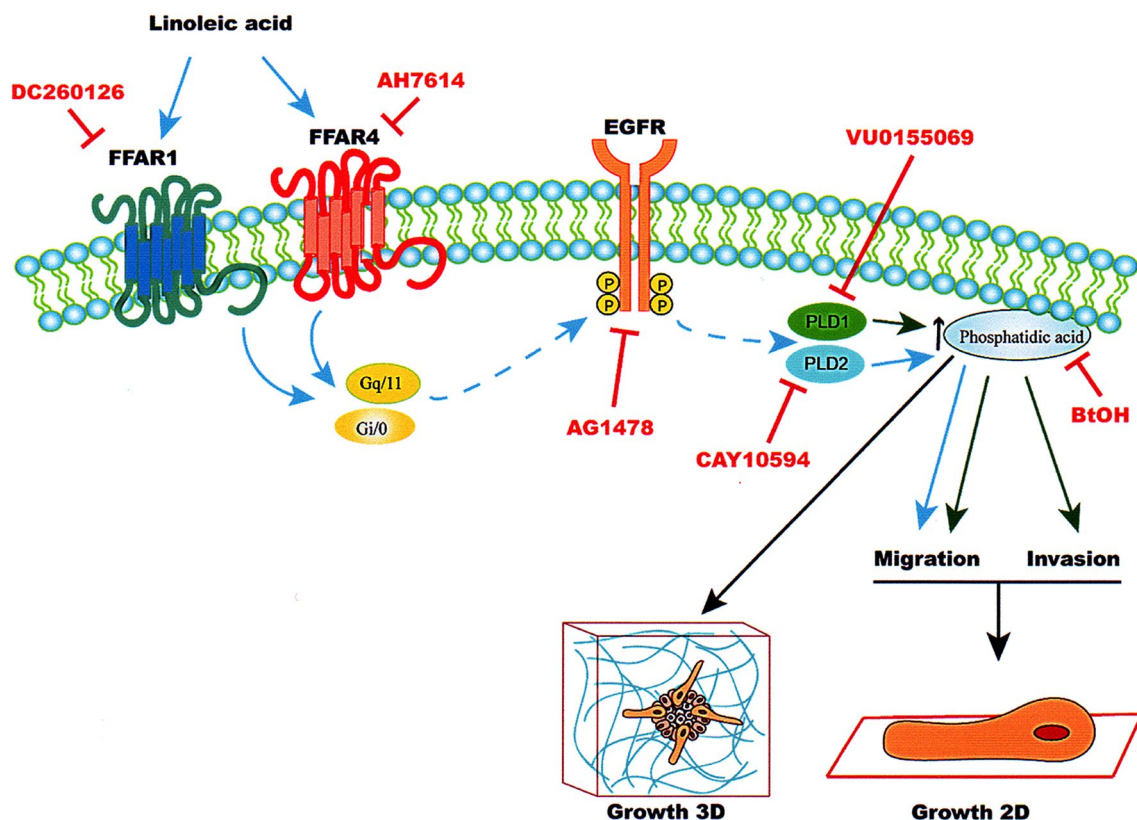


Fig. 8 Model showing roles of PLD1 and PLD2 in migration/invasion induced by LA in MDA-MB-231 breast cancer cells

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Compliance with ethical standards

Conflict of interest Authors declare that there is not conflict of interest.

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