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Role of LOXs and COX-2 on FAK activation and cell migration induced by linoleic acid in MDA-MB-231 breast cancer cells

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Abstract
Background Epidemiological studies and animal models suggest a link between high levels of dietary fat intake and an increased risk of developing breast cancer. Particularly, free fatty acids (FFAs) are involved in several processes, including proliferation, migration and invasion, in breast cancer cells. Linoleic acid (LA) is a dietary n-6 polyunsaturated fatty acid that is known to induce proliferation and invasion in breast cancer cells. So far, however, the contribution of LA to focal adhesion kinase (FAK) activation and cell migration in breast cancer cells has not been studied.
Results Here, we show that LA promotes FAK and Src activation, as well as cell migration, in MDA-MB-231 breast cancer cells. FAK activation and cell migration require Src, Gi/Go, COX-2 and LOXs activities, whereas both are independent of Δ6 desaturase activity. In addition, we show that cell migration requires FAK activity, whereas FAK activation requires Src activity, thus suggesting a reciprocal catalytic activation mechanism of FAK and Src.
Conclusions In summary, our findings show that LA induces FAK activation and cell migration in MDA-MB-231 breast cancer cells.

1 Introduction

Epidemiological studies and animal models suggest an association between high levels of dietary fat intake and an increased risk of developing breast cancer [1, 2]. Free fatty acids (FFAs) bind to nuclear peroxisome proliferator-activated receptors (PPARs) and mediate gene expression related to glucose and lipid metabolism. In the mammary gland FFAs are utilized as a source of energy and for milk lipid synthesis, whereas in breast cancer cells FFAs induce cellular processes independent of PPARs including proliferation, migration and invasion [3–5]. The major polyunsaturated fatty acid (PUFA) in most diets is linoleic acid (LA), which is an omega-6 essential fatty acid that contributes to the development of chronic diseases [6]. LA is metabolized through a variety of pathways including the conversion to arachidonic acid (AA), which is the precursor of a variety of eicosanoids such as prostaglandins (PGs). Inflammatory diseases increase the risk of developing various types of cancer, and a hallmark of cancer-related inflammation includes the presence in tumor tissue of inflammatory cells and inflammatory mediators such as chemokines, cytokines and PGs [7, 8]. In breast cancer cells, LA induces the expression of plasminogen activator inhibitor-1, cell proliferation and invasion, whereas it induces an epithelial-mesenchymal-transition (EMT)-like process in non-tumorigenic MCF10A mammary epithelial cells [9–12].

Focal adhesions are the sites where integrins mediate adhesion links to the actin cytoskeleton and the extracellular matrix.

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The components of focal adhesions are diverse and include scaffolding proteins, GTPases and kinases [13, 14]. Focal adhesion kinase (FAK) is a 125 kDa protein tyrosine kinase localized to focal adhesions that can be activated by multiple agonists [15, 16]. It has been implicated in the regulation of cell spreading, differentiation, proliferation, apoptosis, migration, invasion, survival and angiogenesis [17–19]. The activation of FAK is mediated by autophosphorylation at tyrosine-397 (Tyr-397), which creates a binding site for Src kinase and other downstream signaling effectors. Subsequently, Src mediates the phosphorylation of FAK at Tyr-576 and Tyr-577, which are localized in the activation domain. These latter phosphorylations are important for a maximal activation of FAK and its downstream signaling events [19, 20]. Moreover, the Src-FAK complex can regulate the phosphorylation of other proteins involved in the regulation of cell migration, including p130Cas and paxillin [15, 21, 22].

In the present study we investigated whether LA induces FAK activation and cell migration, as also the role of LOXs and COX-2 in these processes. Our results show, for the first time, that LA promotes FAK activation and cell migration through a Src, Gi/Go, COX-2 and LOXs activity-dependent pathway in MDA-MB-231 breast cancer cells. In addition, we show that cell migration requires FAK activity, whereas FAK activation requires Src activity, thus suggesting a reciprocal catalytic activation mechanism of FAK and Src.

2 Materials and methods

2.1 Materials

LA sodium salt (99 % purity, Lot 079 K5203) and *Bordetella pertussis* toxin (PTX) were obtained from Sigma (St. Louis MO). FAK antibody (Ab) C-20, Src Ab N-16 and SC-26196 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phosphospecific Abs to Tyr-397 and Tyr-577 of FAK (anti-p-FAK and p-FAK-Tyr[577]), as well as phosphospecific Ab to Tyr-418 of Src (anti-p-Src) were obtained from Invitrogen (Camarillo, CA). PP2, PP3, U73122, nordihydroguaiaretic acid (NDGA), 5-bromo-2-(4-fluorophenyl)-3-(4-(methylsulfonyl)phenyl) thiophene (DuP-697) and N-{(2S,4R)-4-(Biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl}-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]acrylamide,HCl (cPLA2α inhibitor) were obtained from Calbiochem-Novabiochem (San Diego, CA).

2.2 Cell culture

MDA-MB-231 and ZR-75 breast cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 3.7 g/l sodium bicarbonate, 5 % fetal bovine serum (FBS) and antibiotics, in a humidified atmosphere containing 5 % CO_2 and 95 % air at 37 °C. For experimental purposes, cells were serum-starved for 12 h before treatment.

2.3 Cell stimulation

Confluent cultures were washed twice with phosphate buffered saline (PBS), equilibrated in DMEM at 37 °C for at least 30 min and then treated with inhibitors and/or LA. LA stock solution (90 mM) preparation and stimulation were performed as described previously [10]. Briefly, LA sodium salt was dissolved in methanol, and the resulting LA stock solution was added to the culture dishes in concentrations and times as indicated. For Western blot assays, the stimulation was terminated by aspirating the medium, after which the cells were solubilized in 0.5 ml ice-cold RIPA buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EGT4, 1 mM sodium orthovanadate, 100 mM NaF, 10 mM sodium pyrophosphate, 10 % glycerol, 1 % Triton X-100, 1 % sodium deoxycholate, 1.5 mM $MgCl_2$, 0.1 % SDS and 1 mM PMSF). The protein content of each sample was determined by the micro Bradford protein assay [23].

2.4 Western blotting

Equal amounts of protein were separated by SDS-PAGE using 8 % and 10 % separating gels followed by transfer to nitrocellulose membranes. After transfer, membranes were blocked using 5 % non-fat dried milk in PBS pH 7.2/0.1 % Tween 20 (wash buffer), and incubated overnight at 4 °C with primary Ab. Next, the membranes were washed three times with wash buffer and incubated with secondary Ab (horseradish peroxidase-conjugated) (1:5000) for 2 h at room temperature. After washing three times with wash buffer, immunoreactive bands were visualized using an ECL detection reagent. Autoradiograms were scanned and the labeled bands were quantified using the Image J software (NIH, USA).

2.5 Scratch-wound assay

Cells were grown to confluence in 35-mm culture dishes, starved for 12 h in DMEM, and treated for 2 h with mitomycin C (40 μM) 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 in the absence or 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.

2.6 Chemotactic migration assay (Boyden chamber method)

Cell migration assays were performed in 24-well plates containing 12 cell culture inserts with 8 μm pore size (Costar, Corning, Inc). Cells were starved for 12 h in DMEM, treated for 2 h with 40 μM mitomycin C and then treated with inhibitors. Cells were re-suspended in DMEM and seeded into the upper chamber at 1×10^5 cells/well. DMEM, inhibitors and/or LA were added to the lower chamber at the indicated concentrations, and both the upper and lower chambers contained DMEM. After 48 h of incubation at 37 °C, non-migrated cells were removed from the upper side of the membrane with cotton swabs, and cells on the lower surface of the membrane were fixed in cold methanol for 5 min. Subsequently, the membrane was stained with 0.1 % crystal violet in PBS. The dye was eluted with 200 μl of 10 % acetic acid, and the absorbance at 600 nm was measured. Background values were obtained from wells without cells.

2.7 Over-expression of FRNK

To exogenously express FRNK (pFLAG-CMV-2-FRNK) in MDA-MB-231 cells, cell cultures were transfected with 8 μg of plasmid using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions. Following transfection, cells were incubated with complete medium for 24 h. Cells were serum-starved for 12 h and subsequently migration assays were performed.

2.8 Statistical analysis

Results were expressed as mean±S.D. Data were statistically analyzed using one-way ANOVA and Knewman-Keuls's multiple comparison tests. A statistical probability of $P<$ 0.05 was considered significant.

3 Results

3.1 LA induces FAK activation in breast cancer cells

Previously, we demonstrated that AA promotes FAK activation and cell migration in breast cancer cells [24]. Here, we examined whether LA induces FAK activation through its phosphorylation at Tyr-397 [19]. To this end, MDA-MB-231 cells were treated for 5 min with increasing concentrations of LA and lysed. Cell lysates were analyzed by Western blotting with anti-p-FAK Ab. As shown in Fig. 1a (upper panel), treatment of cells with LA induced an increase in FAK phosphorylation at Tyr-397 (p-FAK), reaching at maximum at 90 and 120 μM LA. Moreover, LA induced an increase in p-FAK in a time-dependent manner, reaching its maximum between 5 and 10 min of treatment (Fig. 1b, upper panel). Anti-FAK and

anti-actin Ab were used as loading controls (Fig. 1a and b, middle and lower panel).

To further substantiate the notion that LA induces FAK activation, we examined whether LA promotes FAK phosphorylation at Tyr-577 (p-FAK-Tyr577). To this end, MDA-MB-231 cells were treated with 90 μM LA for various times and lysed. The cell lysates were analyzed by Western blotting with anti-p-FAK-Tyr577 Ab. Our findings show that LA induces an increase in FAK phosphorylation at Tyr-577 (p-FAK-Tyr577) in a time-dependent manner (Fig. 1c).

In addition, we examined whether LA induces p-FAK in Zr-75 breast cancer cells. Cultures of these cells were stimulated with 90 μM LA for various times and subsequently lysed. Cell lysates were analyzed by Western blotting with anti-p-FAK Ab. In agreement with our previous results, stimulation of ZR-75 cells with LA induced an increase of p-FAK in a time-dependent manner (Fig. 1d).

3.2 LA induces cell migration

Next we determined whether LA induces cell migration. Therefore, MDA-MB-231 cells were cultured to confluence and treated with 40 μM mitomycin C for 2 h. Cell cultures were scratch-wounded and treated with increasing concentrations of LA for 48 h. As shown in Fig. 2a, cells treated with LA showed a significantly increased migration pattern toward the middle of the scratch compared to untreated cells, reaching a maximum at 90 μM LA.

Next, the role of FAK on cell migration induced by LA was analyzed. For this purpose, FAK function was inhibited using a dominant-negative FAK construct, FRNK (FAK-related non-kinase), consisting of amino acids 668-1053 of wild type FAK [16, 25]. MDA-MB-231 cells were transfected with the FRNK construct and lysed. Cell lysates were analyzed by Western blotting with anti-FAK Ab, which recognizes the C-terminus of FAK and is recommended for detection of FAK and FRNK. Our results show that cells transfected with the FRNK construct expressed FRNK, whereas cells transfected with an empty control vector and cells without transfection did not express FRNK (Fig. 2b). In order to determine the role of FAK on cell migration, MDA-MB-231 cells transfected with the FRNK construct were treated with 40 μM mitomycin C for 2 h. Next, cell cultures were scratch-wounded and treated with 90 μM LA for 48 h. Our results show that FAK activity inhibition completely inhibits cell migration induced by LA (Fig. 2c).

3.3 Role of Src family members on cell migration induced by LA

In order to determine the contribution of Src family members on FAK activation and cell migration induced by LA, we first investigated whether LA regulates Src activation

Fig. 1 LA induces FAK activation in breast cancer cells. Panel **a**. MDA-MB-231 cells were treated with increasing concentrations of linoleic acid (LA) for 5 min and lysed. Panel **b**, **c** and **d**. MDA-MB-231 and Zr-75 cells were treated with 90 μM LA for various times and lysed. Lysates were analyzed by Western blotting with anti-p-FAK Ab or with anti-p-FAK-Tyr[577]. The membranes were analyzed further by Western blotting with anti-FAK and anti-actin Ab as loading controls. The graphs represent the mean±S.D. and are expressed as the fold p-FAK or p-FAK-Tyr[577] above unstimulated cells value. Asterisks denote comparisons made to unstimulated cells. *$P<0.05$, **$P<0.01$
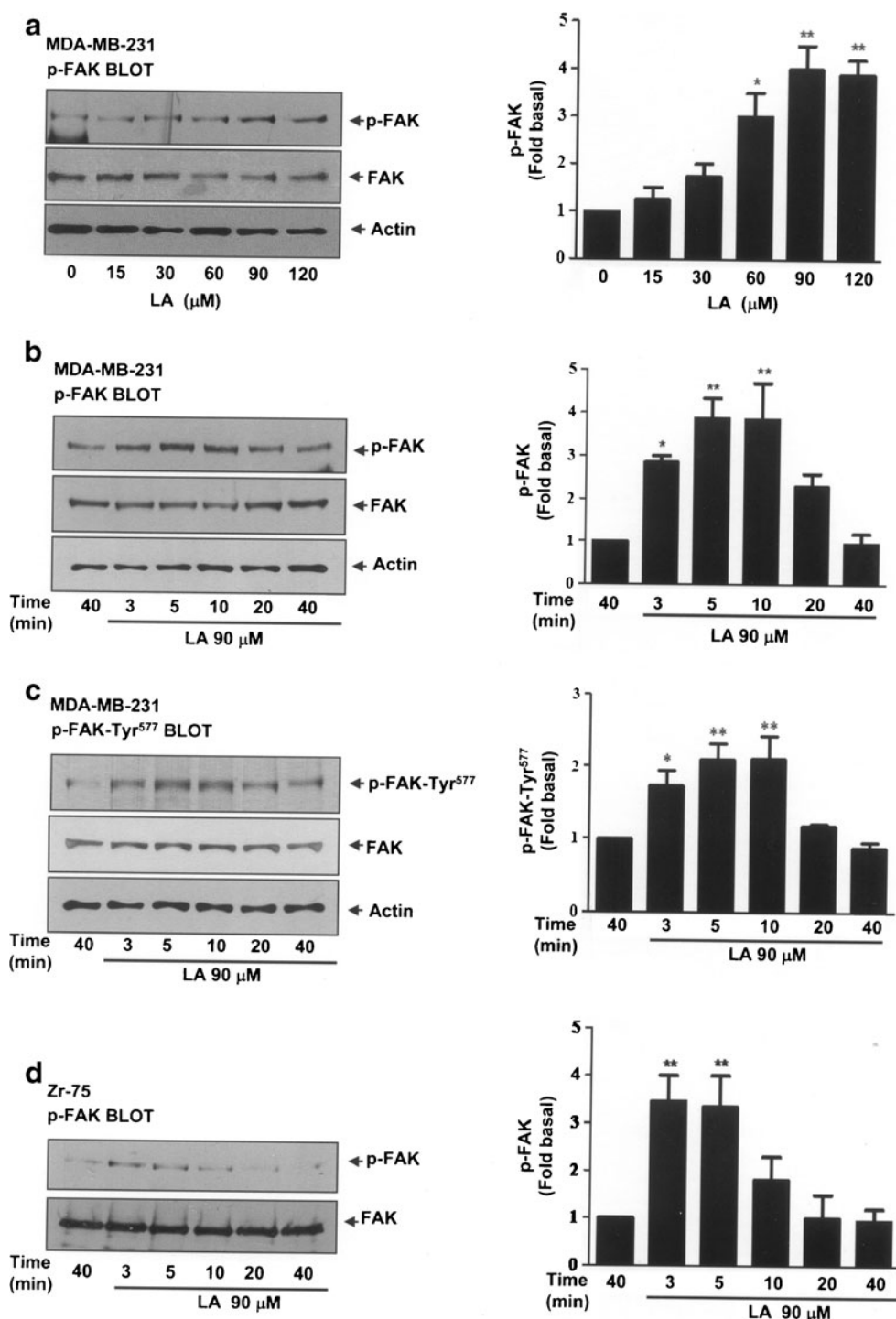

through its phosphorylation at Tyr-418 [26]. Therefore, MDA-MB-231 cells were stimulated with 90 μM LA for various times and lysed. Cell lysates were analyzed by Western blotting with anti-p-Src Ab. As illustrated in Fig. 3a, some Src phosphorylation at Tyr-418 (p-Src) was detected in unstimulated cells. Subsequent treatment with LA induced an increase in p-Src in a time-dependent manner, which reached a maximum between 3 and 5 min and declined toward base-line levels at 10 min of treatment.

Next, we studied the role of Src family kinases on FAK activation and cell migration induced by LA. To this end, MDA-MB-231 cells were treated for 30 min with 10 μM PP2, a selective inhibitor of Src family members [27], or with 10 μM PP3, which is a structurally related but inactive analog of PP2, and then stimulated with 90 μM LA for 5 min. Cells were lysed and FAK activation was determined by Western blotting with anti-p-FAK Ab. As illustrated in Fig. 3b and c, treatment of cells with PP2 completely

Fig. 2 LA induces cell migration in MDA-MB-231 cells. Panel **a**. MDA-MB-231 cells were grown in 35 mm dishes, and pretreated with 40 μM mitomycin C for 2 h. Cell cultures were scratch-wounded and treated for 48 h with serum-free DMEM containing increasing concentrations of LA. Pictures were taken at 48 h after wounding. Panel **b**. MDA-MB-231 cells were transfected with a FRNK construct or with an empty vector (Mock). Cells were lysed and FRNK expression was analyzed by Western blotting using anti-FAK Ab. FAK and FRNK are indicated by arrows. Panel **c**. Cultures of MDA-MB-231 cells (control) and cells transfected with FRNK or with empty vector were pretreated with 40 μM mitomycin C for 2 h. Cell cultures were scratch-wounded and treated for 48 h with serum-free DMEM containing 90 μM LA. Pictures were taken at 48 h after wounding. The results shown are representative of at least three independent experiments
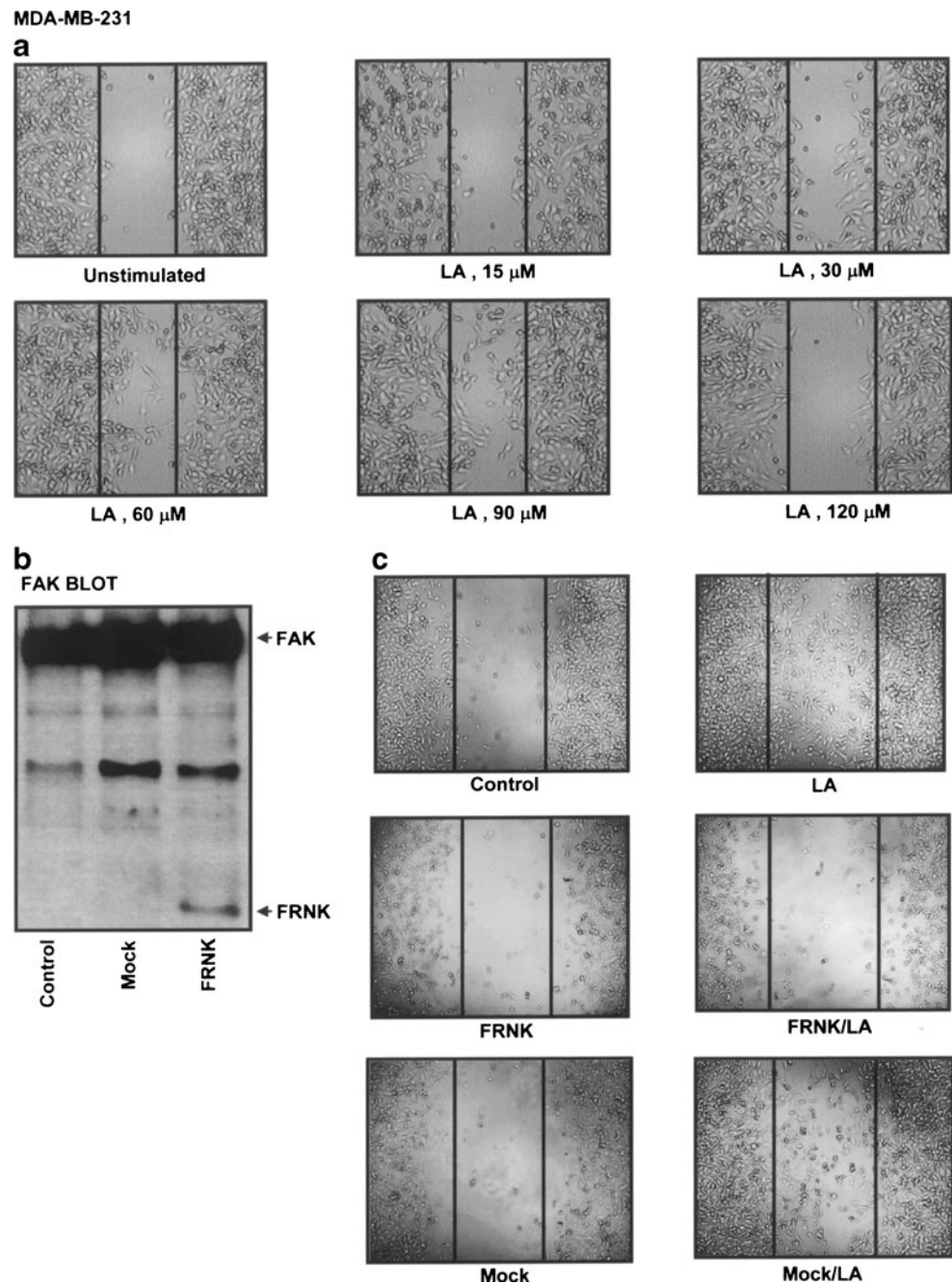
inhibited p-FAK, whereas treatment with PP3 did not interfere with p-FAK induced by LA.

We next examined the contribution of Src family members on cell migration induced by LA. For this end, MDA-MB-231 cells were treated with 40 μM mitomycin C for 2 h and then with 10 μM PP2 or 10 μM PP3 for 30 min. Cell cultures were scratch-wounded and stimulated with 90 μM LA for 48 h. Our results show that treatment of cells with PP2 completely inhibited cell migration, whereas treatment with PP3 did not inhibit cell migration induced by LA (Fig. 3d).

To further substantiate our results, cell migration assays were performed by using the Boyden Chamber method. In agreement with our previous results, treatment of cells with PP3 did not inhibit cell migration, whereas treatment with PP2 inhibitor completely inhibited cell migration induced by LA (Fig. 3e).

3.4 Role of LOXs and COX-2 on cell migration induced by LA

AA is known to induce FAK activation and cell migration via LOXs activity in breast cancer cells [24]. Here, we studied the role of COX-2 and LOXs on FAK activation and cell migration induced by LA. First, MDA-MB-231
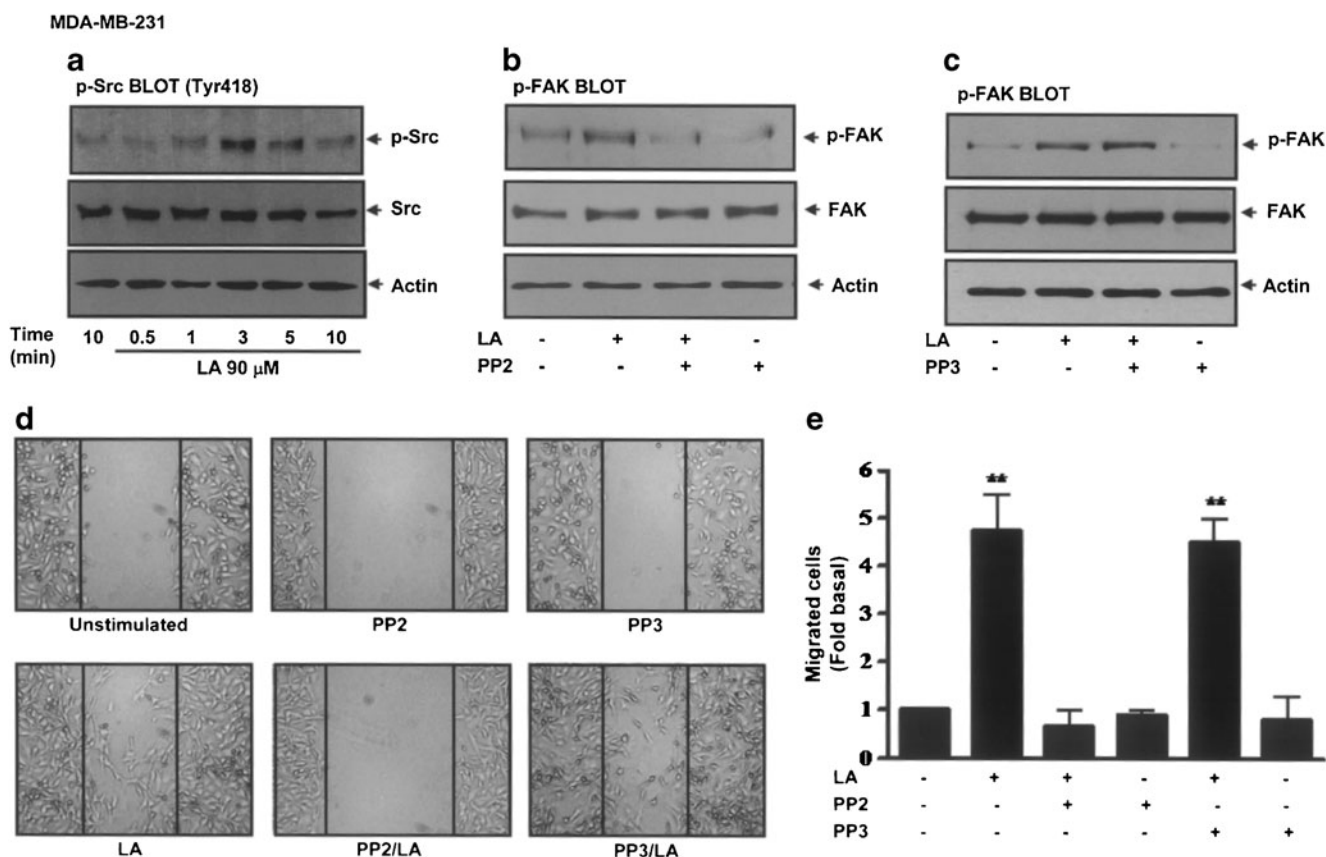
Fig. 3 LA induces FAK activation and cell migration through a Src family members-dependent pathway. Panel **a**. MDA-MB-231 cells were treated with 90 μM LA for various times and lysed. Lysates were analyzed by Western blotting with anti-p-Src Ab. The membranes were analyzed further by Western blotting with anti-Src and anti-actin Ab as loading controls. Panel **b** and **c**. MDA-MB-231 cells were treated without (−) or with (+) 10 μM PP2 or 10 μM PP3 for 30 min, stimulated with 90 μM LA for 5 min and lysed. Lysates were analyzed by Western blotting with anti-p-FAK Ab. The membranes were analyzed further by Western blotting with anti-FAK and anti-actin Ab as loading controls. Panel **d**. MDA-MB-231 cells were grown in 35 mm dishes, pretreated with 40 μM mitomycin C for 2 h and for 30 min with 10 μM PP2 or 10 μM PP3. Cell cultures were scratch-wounded and treated for 48 h with serum-free DMEM containing 90 μM LA. Pictures were taken at 48 h after wounding. Panel **e**. MDA-MB-231 cells were pretreated with 40 μM mitomycin C for 2 h and without (−) or with (+) 10 μM PP2 or 10 μM PP3 for 30 min and cell migration assays were performed by using the Boyden chamber method without (−) or with (+) treatment with 90 μM LA for 48 h. The graph represents the mean±S.D. and is expressed as fold migration above unstimulated cells value. Asterisks denote comparisons made to unstimulated cells. **$P<0.01$

cells were treated for 24 h with 10 μM NDGA or 5 μM DuP-697, which are selective inhibitors of LOXs and COX-2, respectively [28, 29], and stimulated with 90 μM LA for 5 min. Next, cells were lysed and FAK activation was analyzed by Western blotting with anti-p-FAK Ab. Our findings show that inhibition of LOXs and COX-2 activities partly inhibited p-FAK induced by LA (Fig. 4a).

In order to subsequently determine the role of LOXs and COX-2 on cell migration, MDA-MB-231 cells were treated with 10 μM NDGA or 5 μM DuP-697 for 24 h and then with 40 μM mitomycin C for 2 h. Cells were stimulated with 90 μM LA for 48 h and migration was analyzed by using the scratch wound assay and the Boyden chamber method. As shown in Fig. 4b and c, inhibition of LOXs activity completely inhibited cell migration, whereas COX-2 inhibition partly inhibited cell migration induced by LA.

It has been well-established that the calcium-dependent cytosolic group IVA PLA2α (cPLA2α) is a critical enzyme involved in AA release, and is the only enzyme that releases AA directly in a single step reaction [30]. Here, we determined whether p-FAK induced by LA requires cPLA2α activity. To this end, MDA-MB-231 cells were treated with 1 μM cPLA2αI (cPLA2α inhibitor) for 30 min and then stimulated with 90 μM LA for 5 min. Cells were lysed and analyzed by Western blotting with anti-p-FAK Ab. Our results show that treatment of cells with cPLA2αI did not inhibit the increase in p-FAK induced by LA (Fig. 5a).

Alternatively, it is known that phospholipase C (PLC) releases AA from the phospholipid-bound form [31]. Here, we studied whether p-FAK induced by LA requires PLC activity. For this, MDA-MB-231 cells were treated with 2 μM U73122 (PLC inhibitor) for 24 h, and then stimulated
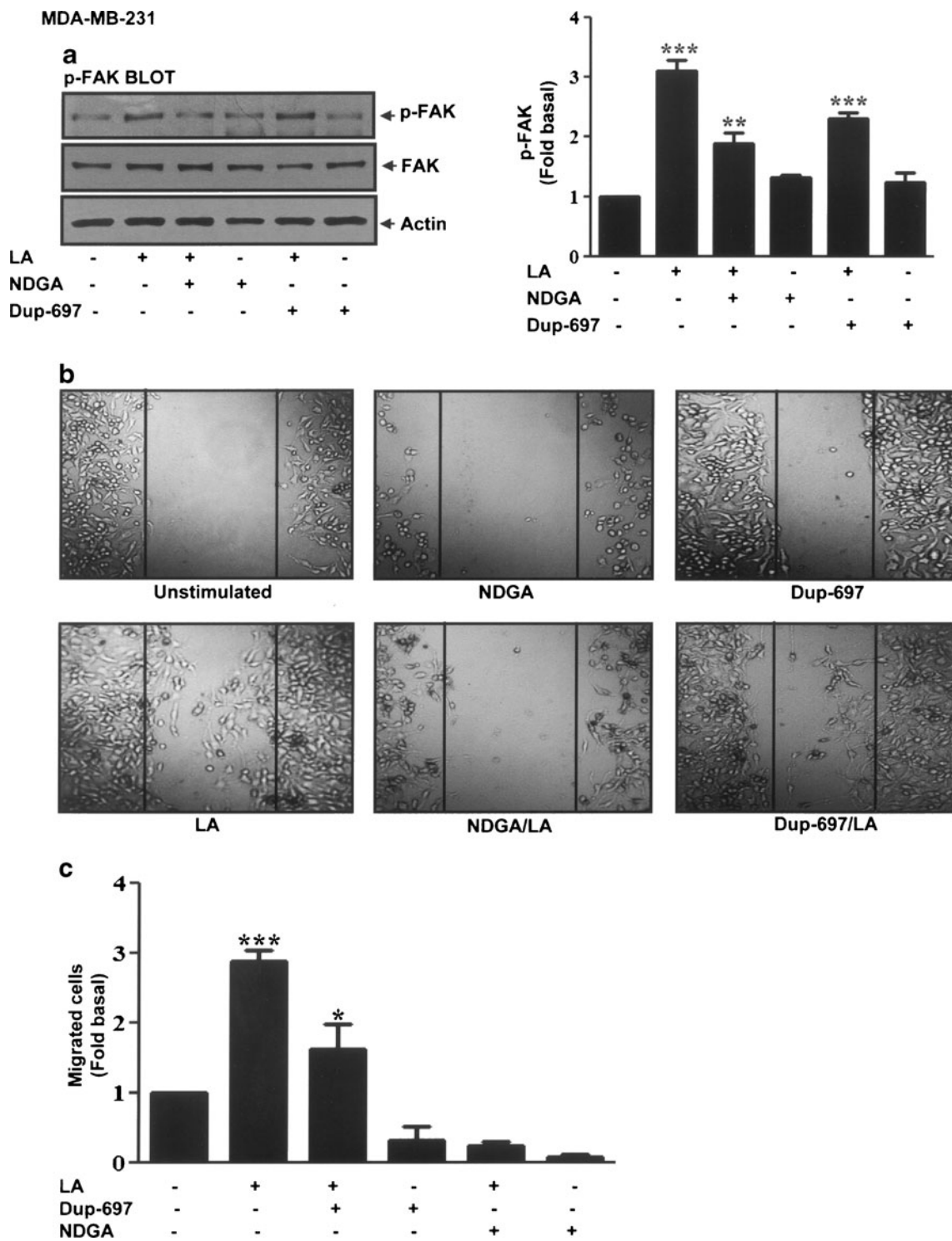
Fig. 4 Role of COX-2 and LOXs on FAK activation and cell migration. Panel **a**. MDA-MB-231 cells were treated for 24 h without (−) or with (+) 10 μM NDGA or 5 μM DuP-697, stimulated with 90 μM LA for 5 min and lysed. Cell lysates were analyzed by Western blotting with anti-p-FAK Ab. The membranes were analyzed further by Western blotting with anti-FAK and anti-actin Ab as loading controls. Panel **b** and **c**. MDA-MB-231 cells were treated without (−) or with (+) 10 μM NDGA or 5 μM DuP-697 for 24 h and with 40 μM mitomycin C for 2 h. Cell migration assays were performed by using the scratch wound assay (Panel **b**) or Boyden chamber method (Panel **c**) without (−) or with (+) treatment with 90 μM LA for 48 h. The graphs represent the mean±S.D. and are expressed as the fold p-FAK (Panel **a**) or migration (Panel **c**) above unstimulated cells value. Asterisks denote comparisons made to unstimulated cells *$P<0.05$, **$P<0.01$, ***$P<0.001$
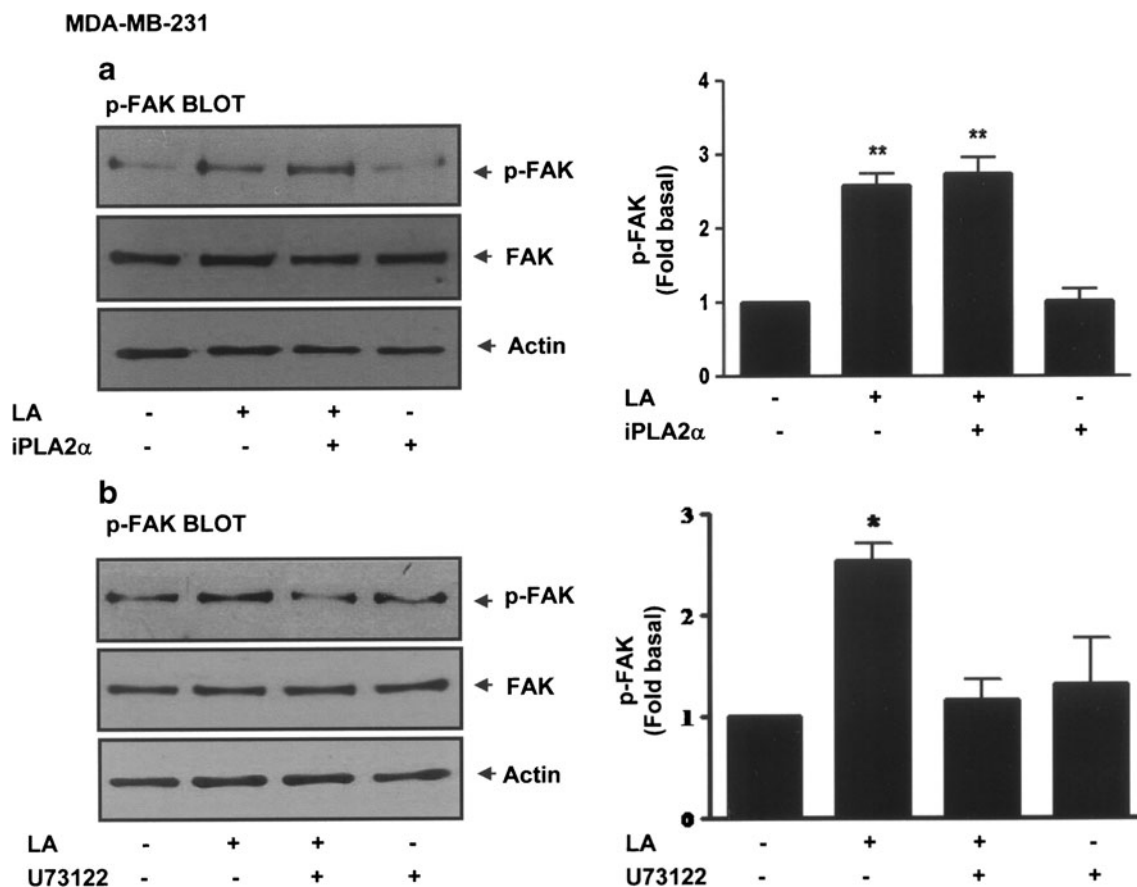
MDA-MB-231

Fig. 5 FAK activation induced by LA requires PLC activity. MDA-MB-231 cells were treated without (−) or with (+) 1 μM cPLA2αI for 30 min (Panel **a**) or with 2 μM U73122 for 24 h (Panel **b**), stimulated with 90 μM LA for 5 min and lysed. Cell lysates were analyzed by Western blotting with anti-p-FAK Ab. The membranes were analyzed further by Western blotting with anti-FAK and anti-actin Ab as loading controls. The graphs represent the mean±S.D. and are expressed as the fold p-FAK above unstimulated cells value. Asterisks denote comparisons made to unstimulated cells. *$P<0.05$, **$P<0.01$

with 90 μM LA for 5 min. Next, cells were lysed and analyzed by Western blotting with anti-p-FAK Ab. As shown in Fig. 5b, treatment of cells with the PLC inhibitor completely inhibited p-FAK induced by LA.

3.5 FAK activation and cell migration induced by LA do not require Δ6 desaturase activity and are mediated by a G-protein-dependent pathway

AA is synthesized from LA through a sequential series of enzymatic conversions, where Δ6 desaturation, which is mediated by Δ6 desaturase, is the rate-limiting step [32, 33]. Here, we studied the role of Δ6 activity on FAK activation and cell migration induced by LA. To this end, cells were treated for 3 h with 2 μM sc-26196, which is a specific inhibitor of Δ6 desaturase [34, 35], stimulated with 90 μM LA, and then FAK activation and cell migration were assessed. FAK activation was analyzed by Western blotting of cell lysates using the anti-p-FAK Ab, and cell migration was studied by using the scratch-wound assay and the Boyden chamber method. Our results show that treatment

with sc-26196 inhibitor did not inhibit FAK activation and cell migration induced by LA (Fig. 6a, b and c).
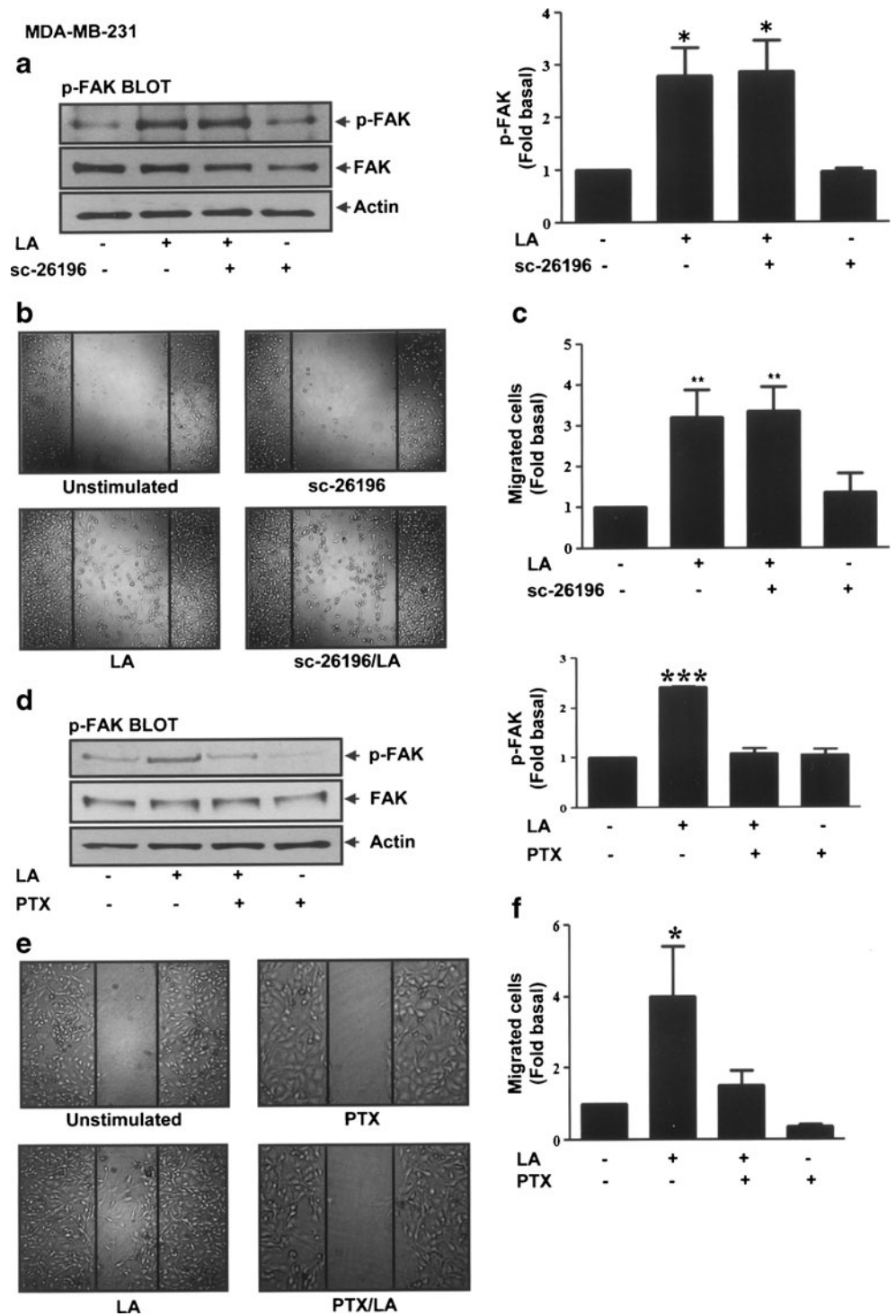
Eicosanoids (prostaglandins and leukotrienes) produced by AA metabolism are released from the cells and exert their biological effects through interaction with G protein-coupled receptors (GPCRs) [36]. Here, we studied the role of Gi/Go proteins on FAK activation and cell migration induced by LA. MDA-MB-231 cells were treated for 12 h with 100 ng/ml PTX, an inhibitor of Gi/Go proteins [37], stimulated with 90 μM LA and then FAK activation and cell migration were assessed. Our results show that treatment of cells with PTX completely inhibited p-FAK and cell migration induced by LA (Fig. 6d, e and f).

4 Discussion

Ample studies have demonstrated that a dietary pattern characterized by high-fat choices, including saturated fatty acids, mono-unsaturated fatty acids, n-3 PUFA and n-6 PUFA, is associated with an increased risk of breast cancer [2, 38].

Particularly, LA intake in the majority of the Western world is estimated to be 15–20 g per day per person, with a plasma concentration of ~275 μM [39, 40]. Moreover, LA has been linked to the development of cancer in animal models and to the induction of an EMT-like process in MCF10A cells [1, 10]. However, the contribution of LA to tumor progression through the regulation of cell migration in breast cancer cells has so far not been studied in detail.

Cancer metastasis involves cell detachment, migration, invasion, extravasation and proliferation at distal sites. FAK is a signal molecule involved in tumorigenesis and metastasis regulating cell migration, survival, proliferation, spreading, invasion and angionesis [18]. In the present study we show that LA induces FAK activation as well as cell migration through a FAK-dependent pathway in MDA-MB-231 breast cancer cells. In agreement with these findings, we

previously showed that AA and oleic acid (OA) induce FAK activation and cell migration of breast cancer cells [24, 41], whereas LA induces growth and metastasis of MDA-MB-231 and MDA-MB-435 breast cancer cells in nude mice [42]. Here, we hypothesize that LA induces FAK activation and cell migration by the activation of two GPCRs, i.e., FFAR1 and GPR120, because these receptors are expressed in MDA-MB-231 breast cancer cells and are activated by medium and long chain FFAs, such as LA [4, 24]. This hypothesis still remains to be tested, but in support of our hypothesis, LA has been found to induce an increase in cellular Ca^{2+} concentration and proliferation via FFAR1 in breast cancer cells [4, 43].

Src family kinases play an important role in a variety of cellular functions including cell cycle progression, growth, survival and migration [44]. Particularly, breast tumors and breast cancer cell lines exhibit an increase in Src kinase activity [45, 46]. Moreover, we showed that OA and AA induce Src family member activation and cell migration through a Src family member-dependent pathway in breast cancer cells [24, 41, 47]. In line with this notion, we here demonstrate that LA induces FAK and Src activation, as well as cell migration, via Src family kinase activity in MDA-MB-231 cells.

A model of reciprocal catalytic activation of FAK and Src has been proposed, where Src family kinases associated with FAK, phosphorylated at Tyr-397, phosphorylate FAK at additional tyrosine residues, including Tyr-576 and Tyr-577, with a maximal induction of FAK kinase activity. Maximal FAK kinase activity promotes intermolecular phosphorylation between FAK molecules at Tyr-397 leading to signal amplification [20, 48, 49]. In line with this model, our current findings show that LA induces p-FAK and p-FAK-Tyr^{577}, and that p-FAK is dependent on Src family kinase activity. These findings are in agreement with previous reports from our laboratory showing that OA and AA also induce p-FAK through a mechanism of reciprocal catalytic activation of FAK and Src family members [24, 41]. We here propose that FFAs mediate FAK activation through a mechanism of reciprocal catalytic activation of FAK and Src family members in breast cancer cells.

AA is a PUFA and an essential fatty acid that is obtained through dietary sources, or is derived from LA. Free AA is enzymatically metabolized via three major pathways including LOXs, cyclooxygenases (COXs) and cytochrome P450 (CYP450) epoxygenases. The LOXs pathway produces several hydroeicosatetraenoic acids (HETEs) that lead to the formation of leukotrienes (LKs), lipoxins (LOs) and hepoxillins (HOs) [50]. The COXs pathway is mediated by COX-1 and COX-2, which produce prostaglandins (PGs), prostacyclin D_2 (PGD$_2$), prostacyclin E_2 (PGE$_2$) prostacyclin PGF$_{2\alpha}$ (PGF$_{2\alpha}$), prostacyclin I_2 (PGI$_2$) and thromboxane A_2 (TXA$_2$) [51]. AA and its metabolites are involved in

several biological processes including chemotaxis, inflammation, angiogenesis and apoptosis. Particularly, AA promotes adhesion to type IV collagen via 15-(S)-LOX activity in MDA-MB-435 cells, whereas AA induces FAK activation and cell migration through a LOXs-dependent pathway in MDA-MB-231 cells [24, 28]. Moreover, OA promotes FAK activation and cell migration through a PLC- and LOX-dependent pathway in MDA-MB-231 cells [41]. Here, we show that FAK activation and cell migration induced by LA require LOXs and COX-2 activities in MDA-MB-231 cells. These findings strongly suggest that eicosanoids produced by LOXs and COX-2 mediate FAK activation and cell migration in MDA-MB-231 cells. In support of our results, it has been demonstrated by others that LA stimulates proliferation through a COX and LOX activity-dependent pathway in MDA-MB-231 cells [9, 52].

CYP450 enzymes constitute a superfamily of 57 isoenzymes that catalyze the metabolism of a large number of compounds including steroids, vitamins, fatty acids, PGs and LKs [53, 54]. Particularly, several CYP450 epoxygenases, including CYP450 2 W1 and CYP450 4 F11, are highly expressed in breast cancer cells and metabolize AA to biologically active eicosanoids termed cis-epoxyeicosatrienoic acids [55–57]. Since, our findings show that FAK activation and cell migration induced by LA are partly dependent on LOXs and COX-2 activity, we propose that CYP450 epoxygenasas may play a role in FAK activation and cell migration. This option, however, remains to be investigated.

In mammalian cells, AA is esterified into phospholipids of the cell membranes, and it is the central eicosanoid precursor. The critical enzyme involved in AA release is the calcium-dependent cPLA2α, but other enzymes have also been shown to be involved, including PLC and phospholipase D (PLD) [58–60]. Our results show that FAK activation induced by LA is independent on cPLA2α activity, whereas it requires PLC activity. These findings suggest that LA induces AA release through a PLC activity-dependent pathway. The role of PLD on FAK activation, however, still remains to be studied. Also, an alternative pathway has been described that releases AA from specific compartments including mitochondria, where the rate-limiting enzyme is ACSL4, which provides arachydonoyl-CoA to a mithochondrial acyl-CoA thioesterase that releases AA and directs this fatty acid to the LOXs for its oxidation to eicosanoids [61]. In highly aggressive breast cancer cell lines, ACSL4 is over-expressed and appears to play a pivotal role in proliferation, invasion and migration [62, 63]. The role of this pathway on FAK activation and migration, induced by LA in MDA-MB-231 cells, remains to be studied.

LOXs constitute a family of dioxygenases including 5-, 12-, and 15-LOX-1 and -2, and their main products are 5(S)-, 12(S)- and 15(S)-HETE, respectively [50]. Particularly, up-regulation of 12(S)-LOX and exposition to

12-(S)-HETE have been shown to induce malignant growth and migration in colorectal cancer cells, whereas 15-LOX-1 has been shown to mediate the invasion of intra-metastatic lymphatic vessels and propagates lymph node metastasis of human mammary carcinoma xenografts in mice [64]. Moreover, 12(S)-HETE has been shown to induce the formation of focal adhesion plaques via a PTX sensitive pathway, leading to enhanced adhesion to fibronectin in murine B16 amelanotic cells [65]. The action of 12(S)-HETE on target cells is mediated by the activation of GPCRs coupled to the heterotrimeric GTP-binding proteins Gi/Go [37]. Our findings show that FAK activation and cell migration require LOXs activity and Gi/Go proteins. We propose that treatment of cells with LA induces the production of LOXs metabolites, such as 12(S)-HETE which, subsequenty, induce the activation of GPCRs coupled with Gi/Go and signal transduction pathways that mediate FAK activation and cell migration in MDA-MB-231 cells.

COX-1 is constitutively expressed in many tissues and cell types, and generates PGs that control normal physiological functions such as platelet aggregation. COX-2 expression is induced by a variety of growth factors, pro-inflammatory stimuli, tumor promoters and mitogens, and is over-expressed in a variety of cancers including primary breast cancer and breast cancer cell lines [66–68]. COX-2 mainly produces PGE_2 and over-expression of COX-2 and PGE_2 are associated with an unfavorable prognosis [68, 69], whereas an elevated COX-2 expression correlates with distant metastasis in patients with breast cancer [70]. E-type prostanoid receptors (EP1-EP4) are GPCRs that mediate the actions of PGE_2. Particularly, EP3 modulates adenyl cyclase activity through Gi/Gs activation and through G-protein-Rho interactions [71]. We demonstrate that FAK activation and cell migration induced by LA require COX-2 activity, and that treatment with PTX inhibits FAK activation and cell migration. We propose that LA induces COX-2 activation and the production of COX-2 metabolites, which induce EP3 activation and then FAK activation and cell migration. This notion, however, remains to be investigated.

AA is synthesized from LA by a sequential series of enzymatic conversions. Specifically, LA is converted to γ-LA by the $\Delta 6$ desaturase, after which γ-LA is elongated to dihomo-γ-LA by four enzymes, collectively referred to as elongase, after which dihomo-γ-LA is converted to AA by the $\Delta 5$ desaturase [32, 33]. We show that FAK activation and cell migration induced by LA do not require $\Delta 6$ desaturase activity. Therefore, our findings strongly suggest that FAK activation and cell migration do not require the conversion of LA to AA. In support of our proposal, we demonstrated previously that LA induces an EMT-like process through a $\Delta 6$ desaturase-independent pathway in MCF10A cells [10].

In conclusion, we propose that FFAs play an important role in the invasion of breast cancer cells via the induction of FAK activation and cell migration. In support of this proposal, it has been shown that increased expression of FAK correlates with increased invasion and migration of breast tumors, and that tumor cells over-expressing FAK show a tendency to invade surrounding tissues and to metastasize [48, 72, 73]. In addition, we here show that LA induces a new signal transduction pathway, where FAK activation and cell migration induced by LA require Src, Gi/Go, COX-2 and LOXs activities in MDA-MB-231 breast cancer cells. We also show that cell migration requires FAK activity, whereas FAK activation requires Src activity, suggesting a reciprocal catalytic activation mechanism of FAK and Src. These findings, for the first time, indicate that LA may play an important role during the invasion process in breast cancer.

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