

Linoleic acid induces migration and invasion through FFAR4- and PI3K-/Akt-dependent pathway in MDA-MB-231 breast cancer cells

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Abstract An increased risk of developing breast cancer has been associated with high levels of dietary fat intake. Linoleic acid (LA) is an essential fatty acid and the major ω -6 polyunsaturated fatty acid in occidental diets, which is able to induce inappropriate inflammatory responses that contribute to several chronic diseases including cancer. In breast cancer cells, LA induces migration. However, the signal transduction pathways that mediate migration and whether LA induces invasion in MDA-MB-231 breast cancer cells have not been studied in detail. We demonstrate here that LA induces Akt2 activation, invasion, an increase in NF κ B–DNA binding activity, miR34a upregulation and miR9 downregulation in MDA-MB-231 cells. Moreover, Akt2 activation requires EGFR and PI3K activity, whereas migration and invasion are dependent on FFAR4, EGFR and PI3K/Akt activity. Our findings demonstrate, for the first time, that LA induces migration and invasion through an EGFR-/PI3K-/Akt-dependent pathway in MDA-MB-231 breast cancer cells.

Keywords Breast cancer · Linoleic acid · FFAR4 · PI3K/Akt

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Introduction

An increased risk of developing breast cancer has been associated high levels of dietary fat intake [1, 2]. Free fatty acids (FFAs) are an energy source for the body, structural units of cell membranes and precursors of biologically active biomolecules including eicosanoids [3]. In breast cancer cells, FFAs induce activation of signal transduction pathways that mediate several processes including proliferation, migration and invasion [4, 5].

Free fatty acid receptor 1 (FFAR1, GPR40) and FFAR4 (GPR120) are G protein-coupled receptors (GPCRs) activated by medium- and long-chain FFAs, such as linoleic acid (LA) [6, 7]. FFAR4 is expressed in intestine and breast cancer cells and is coupled with G_{q/11} proteins [8–10]. LA is an essential fatty acid and the major ω -6 polyunsaturated fatty acid (PUFA) in occidental diets, which induces activation of signal transduction pathways that mediate migration of breast cancer cells and an epithelial–mesenchymal-transition-like (EMT) process in mammary non-tumorigenic epithelial cells MCF10A [11–14].

The phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B) signaling pathway plays a central role in a variety of cell processes including growth, proliferation, motility and survival in both normal and tumor cells [15, 16]. The PI3Ks are a family of lipid kinases that generate phosphoinositol lipids that act as second messengers in a number of signaling pathways, including activation of PDK1 and Akt. The Akt family of serine–threonine kinases consists of three members, namely Akt1, Akt2 and Akt3, which share a similar domain structure. Akt maximal activation requires phosphorylation at threonine (Thr)-308, Thr-309 and Thr-305, as well as phosphorylation at serine (Ser)-473, Ser-474 and Ser-472 in Akt1, Akt2 and Akt3, respectively [15, 16]. PI3K catalyzes the synthesis of

membrane phospholipid PI(3,4,5)P3, which recruits Akt to plasma membrane, and it is phosphorylated. Activated Akt is transferred to several subcellular locations and phosphorylates its targets [17, 18].

The microRNAs are small, noncoding RNAs of approximately 18–25 nucleotides in length and the main class of small RNAs, which are transcribed from individual genes or intragenically from spliced portions of protein-coding genes. In the target mRNA, the major determinant for miRNA binding to RNAm is a sequence of 6–8 nucleotides localized in the 3' end of mRNA (seed sequence) [19, 20]. MicroRNAs mediate post-transcriptional regulation of gene expression in a variety of cell processes. Particularly, expression of microRNAs in breast cancer has an important role in the pathophysiology of disease, because microRNAs facilitate invasion, metastasis, EMT and maintenance of breast cancer stem cells [21, 22].

In the present study, we demonstrate that LA induces Akt2 activation, invasion, an increase in NF κ B–DNA binding activity, miR34a upregulation and miR9 downregulation in MDA-MB-231 breast cancer cells. Akt2 activation requires epidermal growth factor receptor (EGFR) and PI3K activity, whereas migration and invasion are dependent on FFAR4, EGFR and PI3K/Akt activity.

Materials and methods

Materials

LA sodium salt, A6730 and epidermal growth factor (EGF) were from Sigma (St. Louis MO). AH7614 was from TOCRIS (Minneapolis, MN). Wortmannin and AG1478 were from Calbiochem–Novabiochem (San Diego, CA). [γ - 32 P] ATP was from PerkinElmer (Boston, MA). LY294002 and Akt2 antibody (Ab) F-7 were from Santa Cruz Biotechnology (Santa Cruz, CA). FFAR4 Ab was from OriGene (Rockville, MD). Phosphospecific Abs to Thr-308 244F9 (anti-p-Akt-Thr) and Ser-473 of Akt (anti-p-Akt-Ser) were from Cell Signaling (Danvers, MA). Actin Ab was provided for Dr. Manuel Hernandez (Cinvestav-IPN).

Cell culture

MDA-MB-231 breast cancer cells were cultured in DMEM supplemented with 3.7 g/l sodium bicarbonate, 5% fetal bovine serum (FBS) and antibiotics. Non-tumorigenic epithelial cells MCF12A were cultured in DMEM/F12 (1:1) supplemented with 5% FBS, 10 μ g/ml insulin, 0.5 μ g/ml hydrocortisone, 20 ng/ml EGF and antibiotics. Cells were cultured in a humidified atmosphere containing 5% CO $_2$ and 95% air at 37 °C. For experimental purposes,

MDA-MB-231 and MCF12A cells were starved for 24 and 4 h, respectively, in DMEM without FBS and supplements, before treatment with inhibitors and/or LA.

Cell stimulation

Stimulation was performed as described previously [12].

Immunoprecipitation and Western blotting

Immunoprecipitation (IP) and Western blotting were performed as described previously [23].

Interference RNA

FFAR4 expression was silenced in MDA-MB-231 cells by using the Silencer siRNA kit from Santa Cruz Biotechnology, according the manufacturer's guidelines. One control of scramble siRNAs was included.

Silencing of FFAR4 with shRNA

Lentiviral shRNA vectors from Santa Cruz Biotechnology (Santa Cruz, CA) targeting human FFAR4 were utilized for generation of stable knockdown in MDA-MB-231 cells, according the manufacturer's guidelines. Transfected cells were selected by their resistance to puromycin (5 μ g/ml).

Scratch-wounded assay

Cells were grown to confluence on 35-mm culture dishes, and they were treated for 2 h with 40 μ M mitomycin C to inhibit proliferation. Cultures were scratch-wounded using a sterile 200- μ l pipette tip, washed twice with PBS and re-fed with DMEM without or with inhibitors and/or LA. The progress of migration into the wound space was photographed at 48 h using an inverted microscope coupled to a camera. Cell migration was evaluated by using the ImageJ software (NIH, USA).

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described previously [10]. For EMSA assays, double-stranded oligonucleotide containing specific binding sites for NF κ B 5'-AGCTAAGG GACTTCCGCTGGGGACTTCCAGG-3' was used as probe [24]. An amount of 20 pmol of annealed oligonucleotide was labeled with [γ - 32 P] ATP using T4 polynucleotide kinase. The 32 P-labeled oligonucleotide probe (~1 ng) was incubated with 5 μ g nuclear extract in a reaction mixture containing 1 μ g of poly (dI-dC), 0.25 M HEPES, pH 7.5, 0.6 M KCl, 50 mM MgCl $_2$, 1 mM EDTA,

7.5 mM DTT and 9% glycerol for 20 min at 4 °C. One hundred-fold excess of unlabeled specific (NFκB) and non-specific probes were used as competitors. The samples were fractionated on a 6% polyacrylamide gel in 0.5X Tris borate–EDTA buffer. Gels were dried and analyzed by autoradiography.

Invasion assays

Invasion assays were performed by the modified Boyden chamber method in 24-well plates containing 12 cell culture inserts with 8-μm pore size (Costar, Corning Inc). Briefly, 30 μl BD matrigel was added into culture inserts and kept overnight at 37 °C. Cells were plated at 1×10^5 cells per insert in 100 μl FBS-free DMEM on the top chamber, whereas lower chamber contained 600 μl DMEM without or with 90 μM LA. Chambers were incubated for 48 h at 37 °C and cells, and matrigel was removed with cotton swabs. Cells on the lower surface of membrane were washed and fixed with cold methanol for 5 min. The number of invaded cells was estimated by staining of membranes with 0.1% crystal violet in PBS. The dye was eluted with 500 μl 10% acetic acid, and the absorbance at 600 nm was measured. Background value was obtained from wells without cells.

Isolation of miRNAs and quantitative real-time PCR (RT-qPCR)

Total RNA was obtained by using TRIzol reagent (Invitrogen) from MDA-MB-231 cells untreated and treated with LA. First-strand cDNA synthesis was performed using the miRCURY LNA Universal RT microRNA PCR kit (Exiqon) and 500 ng of total RNA as template. Reactions were incubated at 42 °C for 60 min, followed by 95 °C for 5 min. The cDNA was diluted 40× in nuclease-free water, and 4 μl was assayed in 10 μl PCR using primers for miR9, miR21 and miR34a (Exiqon), according to the protocol for the miRCURY LNA ExiLent SYBR Green (Exiqon). miR16 was used as endogenous control. Real-time PCR cycle conditions were an incubation of mix at 95 °C for 10 min followed for 45 cycles of 10 s at 95 °C and 1 min at 60 °C. Results were analyzed by using the $2^{-\Delta\Delta Ct}$ method and normalized to miR16 data.

Statistical analysis

Results were expressed as mean \pm SD of at least three independent experiments. Data were statistically analyzed using one-way ANOVA and Knewman–Keuls multiple comparison tests. Asterisks denote comparisons made to unstimulated cells, and a statistical probability of $P < 0.05$ was considered significant.

Results

LA induces Akt2 activation in MDA-MB-231 breast cancer cells

We studied whether LA induces Akt2 activation by using Akt2 phosphorylation at Thr-309 and Ser-474 [17]. MDA-MB-231 cells were treated with 90 μM LA for various times and lysed. Lysates were immunoprecipitated with Akt2 Ab, and complexes were analyzed by Western blotting with anti-p-Akt-Thr Ab and anti-p-Akt-Ser Ab, which recognize the phosphorylation on Thr-309 and Ser-474 of Akt2, respectively. As shown in Fig. 1a, b (upper panel), LA induced an increase on Akt2 phosphorylation at Thr-309 (p-Akt2-Thr) and Ser-474 (p-Akt2-Ser) in a time-dependent manner. Western blotting with anti-Akt2 Ab was used as loading control (Fig. 1a, b, lower panel).

To further substantiate that LA induces Akt2 activation, we determined Akt2 phosphorylation at Ser-474 in the presence of an Akt1/2 inhibitor (A6730) [25]. MDA-MB-231 cells were treated with A6730 and stimulated with 90 μM LA for 5 min and lysed. Lysates were analyzed by IP with anti-Akt2 Ab and Western blotting with anti-p-Akt-Ser Ab. Our findings showed that inhibition of Akt1/2 activity inhibited Akt2 phosphorylation at Ser-474 (activation) induced by LA (Fig. 1c).

Akt2 activation induced by LA requires PI3K and EGFR activity

We determined the role of PI3K and EGFR in Akt2 activation. MDA-MB-231 cells were treated for 1 h with 60 nM wortmannin (Wort), an inhibitor of PI3K activity [26], and for 30 min with 500 nM AG1478, an inhibitor of EGFR activity [27], and they were stimulated with 90 μM LA for 5 min and lysed. Lysates were analyzed by IP with anti-Akt2 Ab and Western blotting with anti-p-Akt-Ser Ab. As shown in Fig. 1c, treatment with Wort and AG1478 inhibited Akt2 phosphorylation at Ser-474 induced by LA.

FFAR4 mediates migration and invasiveness induced by LA

To determine whether FFAR4 mediates migration, we inhibited FFAR4 expression by using FFAR4 shRNA lentiviral particles. Our results showed a significant inhibition of FFAR4 expression using the specific FFAR4 shRNA (Fig. 2a). Next, MDA-MB-231 cells transfected with shRNAs were cultured to confluence and treated with 12 μM mitomycin C for 2 h. Cell cultures were scratch-wounded and treated with 90 μM LA for 48 h. As shown in Fig. 2b, inhibition of FFAR4 expression inhibited migration induced by LA.

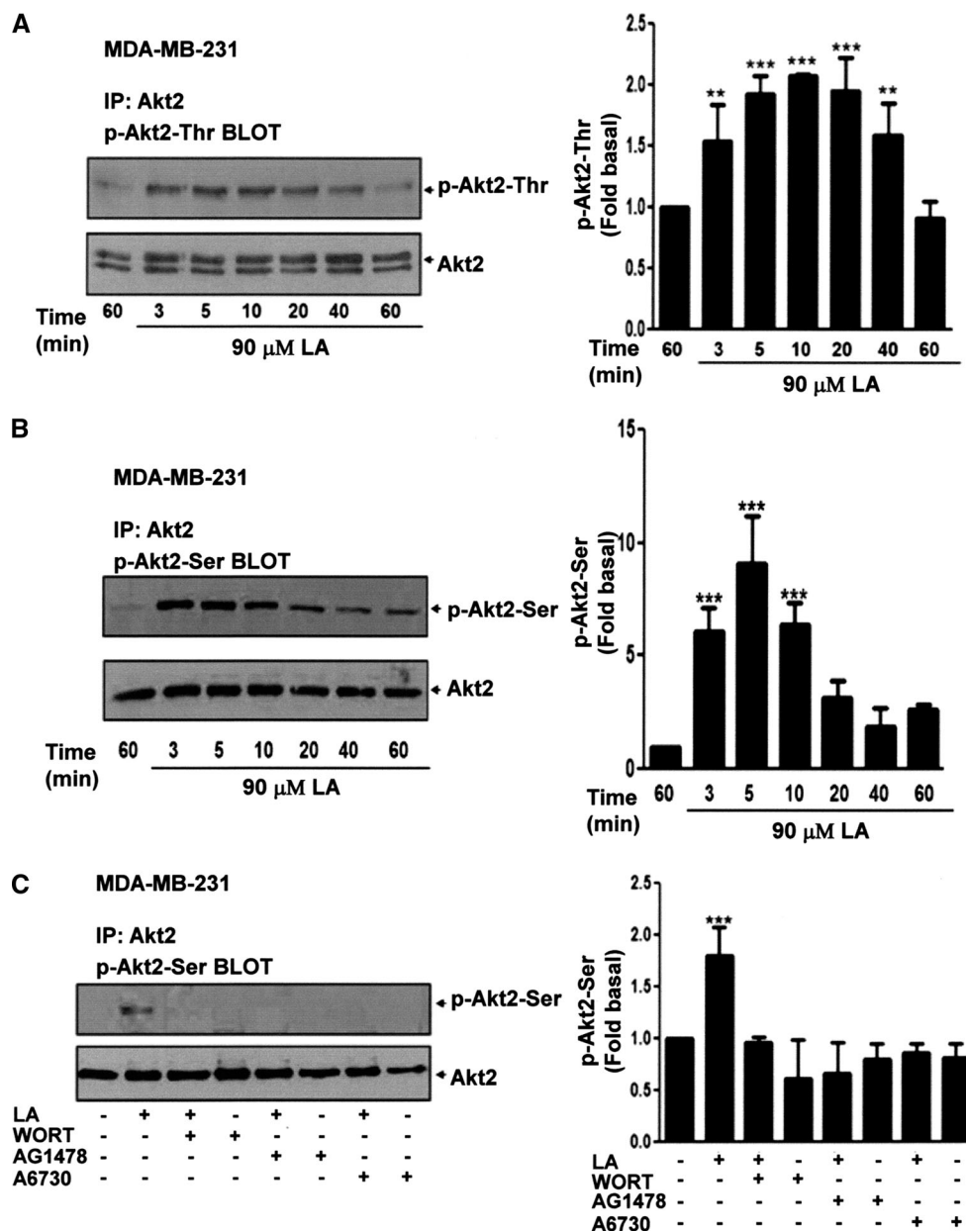


Fig. 1 Linoleic acid induces Akt2 activation in MDA-MB-231 breast cancer cells. **a, b** MDA-MB-231 cells were treated with 90 μM linoleic acid (LA) for various times and lysed. **c** MDA-MB-231 cells were treated without (–) or with (+) 500 nM AG1478, 60 nM wortmannin (Wort) or 2 μM A6730, stimulated with 90 μM LA for 5 min and lysed. Lysates were immunoprecipitated (IP) with Akt2 Ab

followed by Western blotting with anti-p-Akt-Thr Ab or anti-p-Akt-Ser Ab as indicated. Membranes were analyzed further by Western blotting with anti-Akt2 Ab. *Graphs* represent the mean ± SD and are expressed as the fold of p-Akt-Thr or p-Akt2-Ser above unstimulated cells. ** $P < 0.01$; *** $P < 0.001$

Since our findings showed a partial inhibition of FFAR4 expression by using the specific FFAR4 shRNA, we inhibit FFAR4 activity by using an antagonist of FFAR4 (AH7614) [28]. Cultures of MDA-MB-231 cells were treated with 12 μM mitomycin C for 2 h and with 20 μM AH7614 for 1 h. Cultures were scratch-wounded and stimulated with 90 μM LA for 48 h. Our findings showed that treatment with AH7614 inhibited migration induced by LA (Fig. 2c).

Next, we determined whether LA induces invasion and the role of FFAR4 in MDA-MB-231 cells. Invasions assays were performed by using the Boyden chamber method and MDA-MB-231 cells stimulated with 90 μM LA for 48 h. Our results showed that treatment with LA induced a clear invasion of MDA-MB-231 cells (Fig. 3a). To determine the role of FFAR4 in cell invasion, FFAR4 activity was inhibited by treatment with AH7614 or FFAR4 expression was blocked by using siRNA against FFAR4, and then,

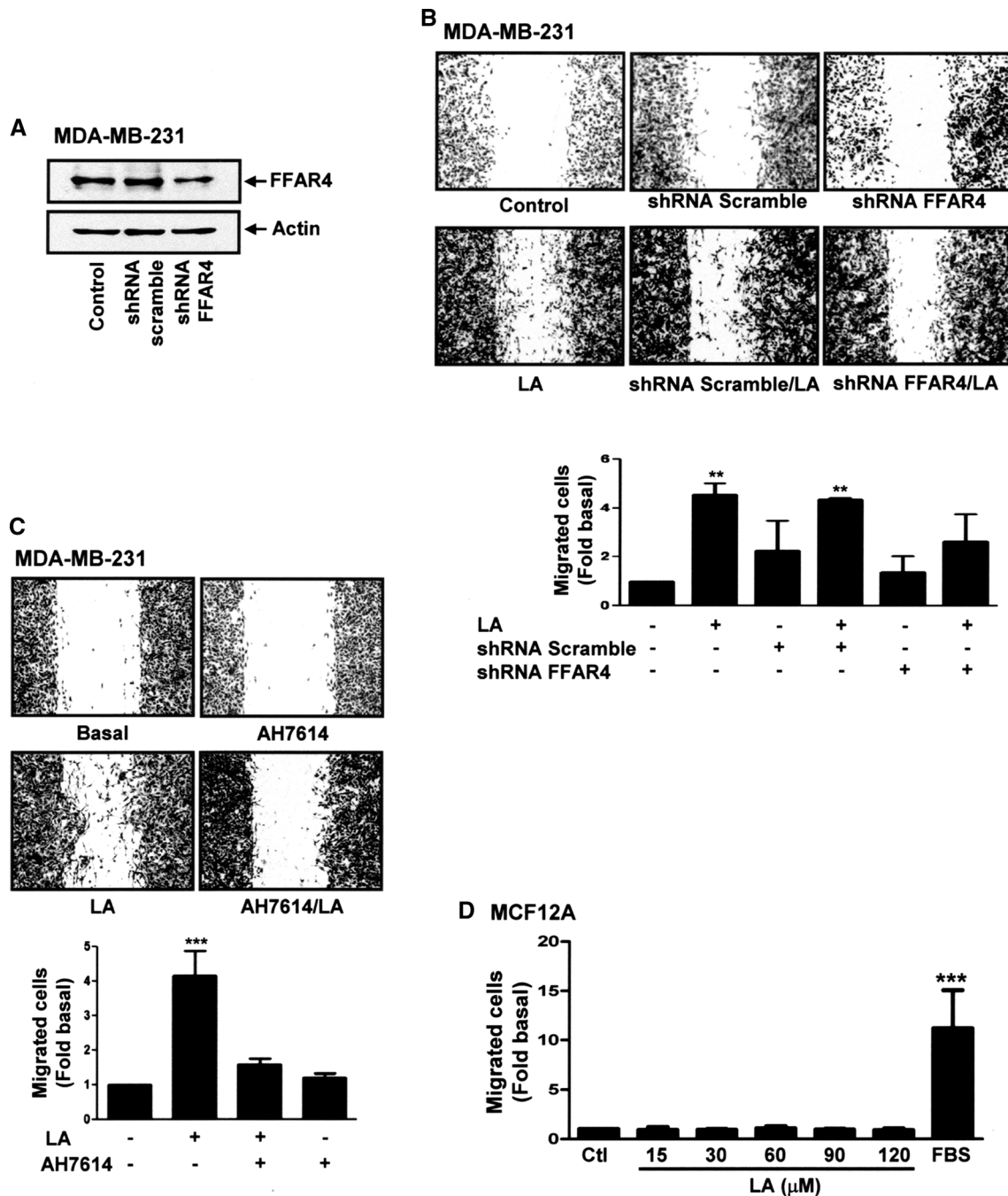


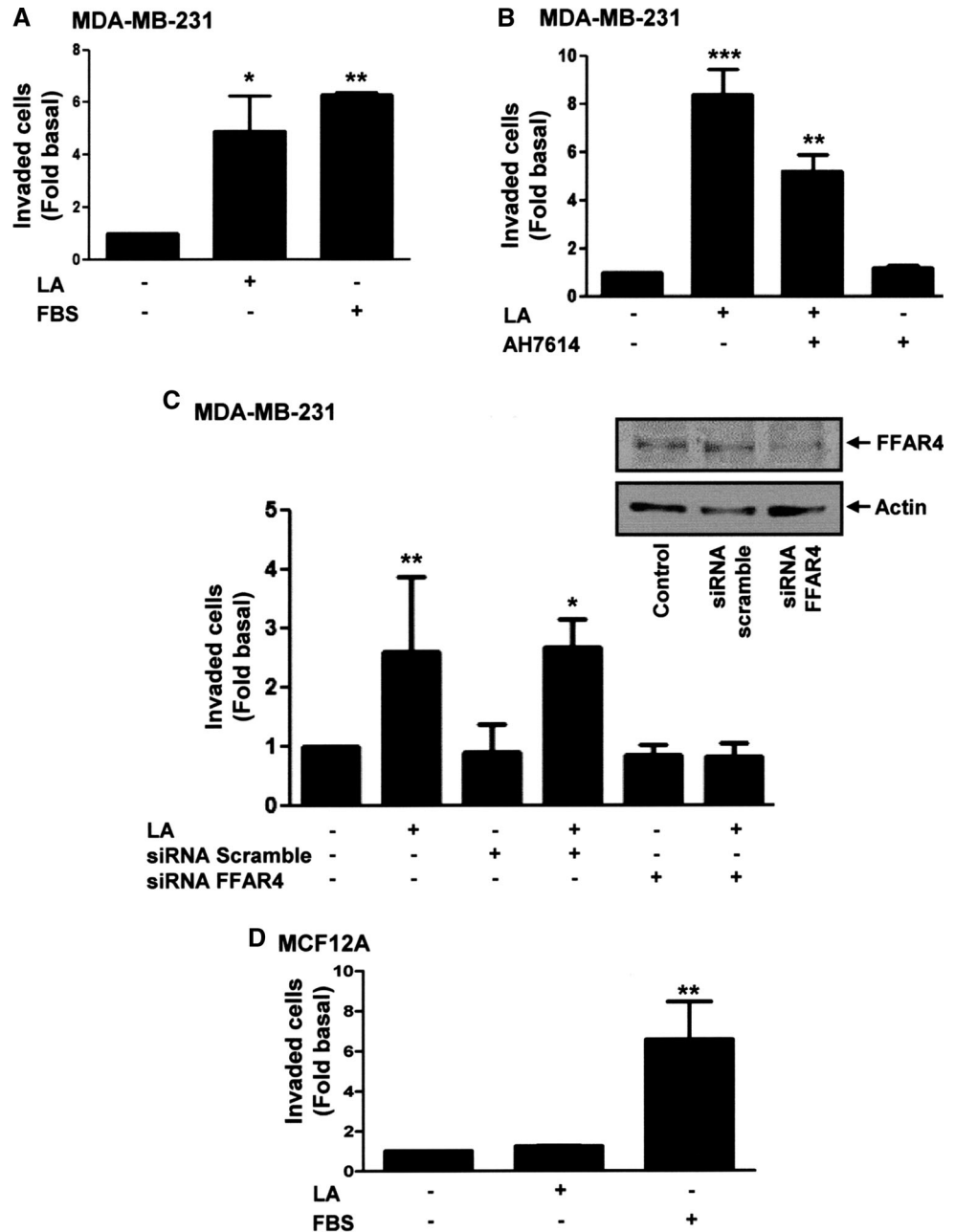
Fig. 2 FFAR4 mediates cell migration induced by LA. **a** MDA-MB-231 cells were transfected with lentiviral particles bearing specific FFAR4 shRNA or a scramble shRNA. FFAR4 expression was analyzed by Western blotting with anti-FFAR4 Ab. Anti-actin Ab was included as loading control. **b** Cultures of MDA-MB-231 cells transfected with FFAR4 shRNA or scramble shRNA were scratch-wounded and treated with 90 μ M LA for 48 h. **c** Cultures of MDA-

MDA-MB-231 cells were treated with 90 μ M LA for 48 h. As shown in Fig. 3b, c, MDA-MB-231 cells treated with LA showed a clear invasion, and inhibition of FFAR4 expression inhibited migration; however, inhibition of FFAR4 activity inhibits partly the invasion induced by LA.

MB-231 cells were treated with 20 μ M AH7614 for 1 h, and they were scratch-wounded and stimulated with 90 μ M LA for 48 h. **d** Cultures of MCF12A cells were scratch-wounded and stimulated with 90 μ M LA for 48 h. *Graphs* represent the mean \pm SD and are expressed as the fold of migrated cells above unstimulated cells. ** $P < 0.01$; *** $P < 0.001$

In order to determinate whether LA is able to induce migration and invasion in mammary non-tumorigenic epithelial cells. We performed cell migration assays with mammary non-tumorigenic epithelial cells MCF12 stimulated with increasing concentrations of LA for 48 h.

Fig. 3 FFAR4 mediates cell invasion induced by LA. **a**, **d** Invasion assays were performed with MDA-MB-231 and MCF12A cells treated with 90 μ M LA for 48 h. One control of invasion was included (FBS). **b** Invasion assays were performed with MDA-MB-231 cells treated with 20 μ M AH7614 for 1 h and stimulated with 90 μ M LA for 48 h. **c** MDA-MB-231 cells were transfected with FFAR4-specific or scramble siRNA. FFAR4 expression was analyzed by Western blotting with anti-FFAR4 Ab (*Inset*). Invasion assays were performed with MDA-MB-231 cells transfected with FFAR4 siRNA or scramble siRNA and treated with 90 μ M LA for 48 h. *Graphs* represent the mean \pm SD and are expressed as the fold of invaded cells above unstimulated cells. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$



Moreover, invasion assays were performed with MCF12A cells stimulated with 90 μ M LA for 48 h. As shown in Figs. 2 and 3d, LA did not induce migration and invasion of MCF12A cells.

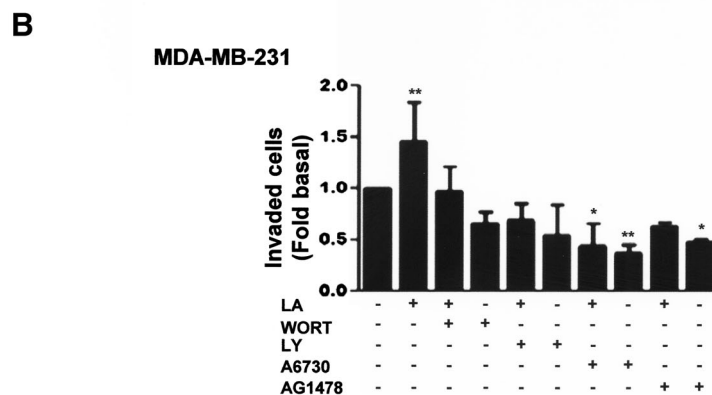
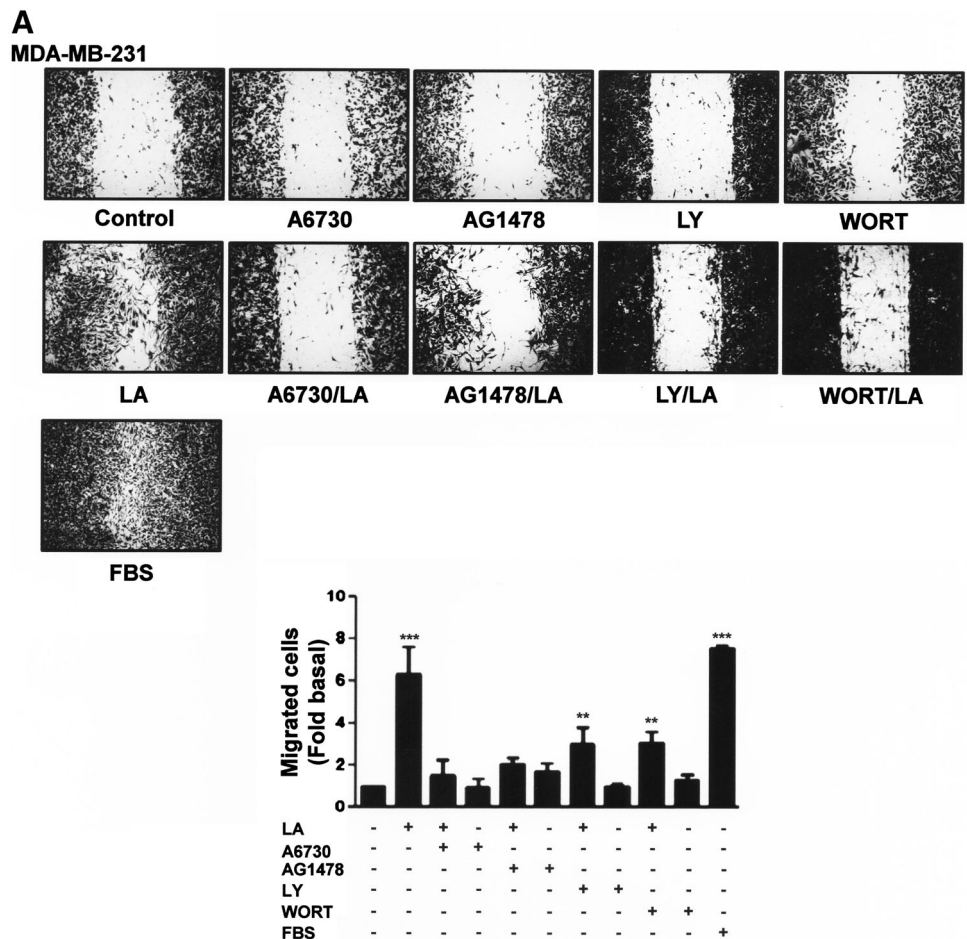
Role of EGFR and PI3K/Akt in cell migration and invasion induced by LA

The role of EGFR and PI3K/Akt in cell migration induced by LA was studied. MDA-MB-231 cells were treated for 30 min with 500 nM AG1478 and for 1 h with 2 μ M A6730, 60 nM Wort or 2 μ M LY294002. Wort and

LY294002 are inhibitors of PI3K activity [26]. Cell cultures were scratch-wounded and stimulated with 90 μ M LA for 48 h. Our findings demonstrated that inhibition of EGFR and Akt1/2 activity completely inhibited migration, whereas treatment with PI3K inhibitors partly inhibited migration induced by LA (Fig. 4a).

To determine the role of EGFR and PI3K/Akt in cell invasion, MDA-MB-231 cells were treated with 2 μ M LY294002, 60 nM Wort, 2 μ M A6730 and 500 nM AG1478 and invasions assays were performed by using the Boyden chamber method and treatment with 90 μ M LA for 48 h. As illustrated in Fig. 4b, inhibition of EGFR, PI3K

Fig. 4 Role of EGFR, PI3K and Akt on cell migration and invasion induced by LA. **a** Cultures of MDA-MB-231 cells were treated without (-) or with (+) 500 nM AG1478, 60 nM Wort, 10 μM LY294002 (LY) or 2 μM A6730, scratch-wounded and treated with 90 μM LA for 48 h. **b** MDA-MB-231 cells were treated with 500 nM AG1478, 60 nM Wort, 10 μM LY or 2 μM A6730 and invasion assays were performed by treatment with 90 μM LA for 48 h. *Graphs* represent the mean ± SD and are expressed as the fold of migrated or invaded cells above unstimulated cells. * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001



and Akt1/2 activity completely inhibited migration induced by LA.

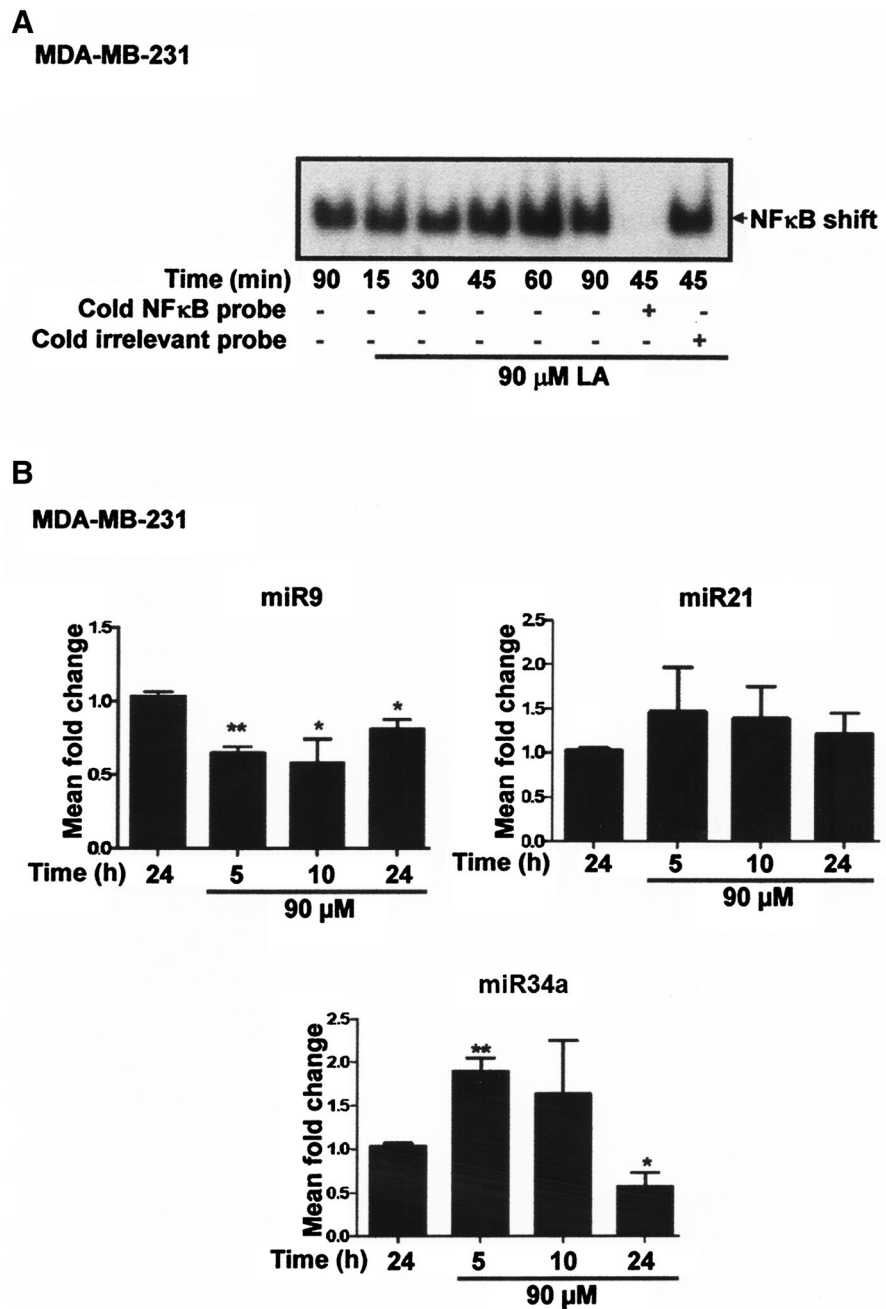
LA induces NFκB–DNA binding activity and changes in miR34a/miR9 expression

We determined whether LA induces NFκB–DNA binding activity. EMSAs were performed by using nuclear extracts from MDA-MB-231 cells stimulated with 90 μM LA for various times and an oligonucleotide probe containing canonical NFκB binding sites. As illustrated in Fig. 5a,

treatment with LA induced an increase in NFκB–DNA binding activity. Specificity of these complexes was demonstrated by inhibition of binding with a specific cold competitor, whereas an irrelevant competitor did not inhibit NFκB–DNA complex formation.

Next, we determined whether treatment with LA induced changes on miR9, miR21 and miR34a expression. MDA-MB-231 cells were stimulated with 90 μM LA for various times, total RNA was obtained, and miRNAs expression was analyzed by RT-qPCR. Our findings showed that LA induced an increase in miR34a expression

Fig. 5 LA induces NF κ B–DNA binding activity and changes on miR9 and miR34a expression. **a** MDA-MB-231 cells were treated with 90 μ M LA for various times, and nuclear extracts were obtained. NF κ B–DNA binding activity was analyzed by EMSA. Autoradiogram shown is representative. **b** MDA-MB-231 cells were stimulated with 90 μ M LA for various times, and total RNA was obtained. Expression of miRNAs was determined by RT-qPCR. Graph represents the mean \pm SD of three independent experiments. miR16 was used for normalization (endogenous control). * $P < 0.05$; ** $P < 0.01$



and downregulation of miR9, whereas miR21 expression was not affected in MDA-MB-231 cells (Fig. 5b).

Discussion

Epidemiological and experimental studies have demonstrated a strong correlation between a high dietary fat intake and the risk of developing breast cancer [1, 2]. Particularly, LA is a fatty acid of vegetable oils that promotes a variety of biological processes in breast cancer cells, including proliferation and invasion, whereas it mediates tumor growth

and metastasis in nude mice [13, 29, 30]. However, the signal transduction pathways that mediate migration induced by LA and whether LA induces invasion in breast cancer cells have not been studied.

FFAR4 is a GPCR activated by medium- and long-chain FFAs that mediate several physiological processes including intestine incretin hormone secretion, food preference, anti-inflammation and adipogenesis [8, 31, 32]. Moreover, FFAR4 is expressed in MDA-MB-231 and MCF7 breast cancer cells, whereas stimulation of these cells with medium-chain FFAs including oleic acid (OA), arachidonic acid (AA) and LA induces migration [12, 23, 33]. Here we

demonstrate that LA mediates migration and invasion through a FFAR4-dependent pathway in MDA-MB-231 cells. Since inhibition of FFAR4 expression and activity inhibits migration induced by LA, we propose that migration is mainly mediated by FFAR4 in MDA-MB-231 cells. Interestingly, our findings show that inhibition of FFAR4 expression inhibits completely invasion; however, treatment with FFAR4 antagonist partly inhibits invasion induced by LA. We propose that in intact cells FFAR1 and FFAR4 cooperate to activate the signal transduction pathways to mediate invasion; however, inhibition of FFAR4 expression blocks the capacity of FFAR1 to induce activation of signal transduction pathways that mediate invasion induced by LA in MDA-MB-231 cells. It remains to be investigated, as well as the role of FFAR1 in migration, because FFAR1 is able to be activated by medium-chain FFAs, including LA. We propose that FFAR4 and LA play an important role in the invasion process of breast cancer. Supporting our proposal, FFAR4 is able to act like a tumor-promoting receptor because it promotes migration and angiogenesis through a PI3K-/Akt-NFκB-dependent pathway in colorectal carcinoma cells [34].

Akt family members mediate migration, proliferation, survival and protein synthesis; however, they have been also involved in breast cancer [35]. In mouse models of oncogene-induced mammary tumorigenesis, Akt1 is involved in tumor development, whereas Akt2 mediates tumor invasion and metastasis [17, 36]. Here we demonstrate that LA induces Akt2 activation via PI3K activity, as well as migration and invasion through a PI3K- and Akt-dependent pathway in MDA-MB-231 cells. In agreement with these findings, we demonstrate that AA induces migration and invasion through an Akt-dependent pathway in MDA-MB-231 cells [37]. We propose that LA induces migration and invasion through Akt2 activation in MDA-MB-231 cells. Supporting our proposal, overexpression of Akt2 duplicates the invasive effects of PI3K transfected in breast cancer cells, whereas inhibition of Akt2 expression blocks the invasion induced by either HER-2 overexpression or PI3K activation [38]. It remains to be investigated whether Akt1 and/or Akt2 mediates migration and invasion induced by LA, because we used a compound that inhibits Akt1 and Akt2 activity.

Growth factor receptors, including insulin-like growth factor receptor (IGF-IR) and EGFR family, are able to induce Akt activation [18]. Particularly, EGFR transactivation, mediated by GPCRs, occurs via activation of metalloproteinases (MMPs) and subsequent release of EGF-like ligands, such as HB-EGF, from growth factors precursors in the plasma membrane [39]. In EGFR transactivation, Src kinase activity mediates MMPs activation and it is able to phosphorylate the intracytoplasmic tails of EGFR. Therefore, it is suggested that Src kinases are mediators upstream

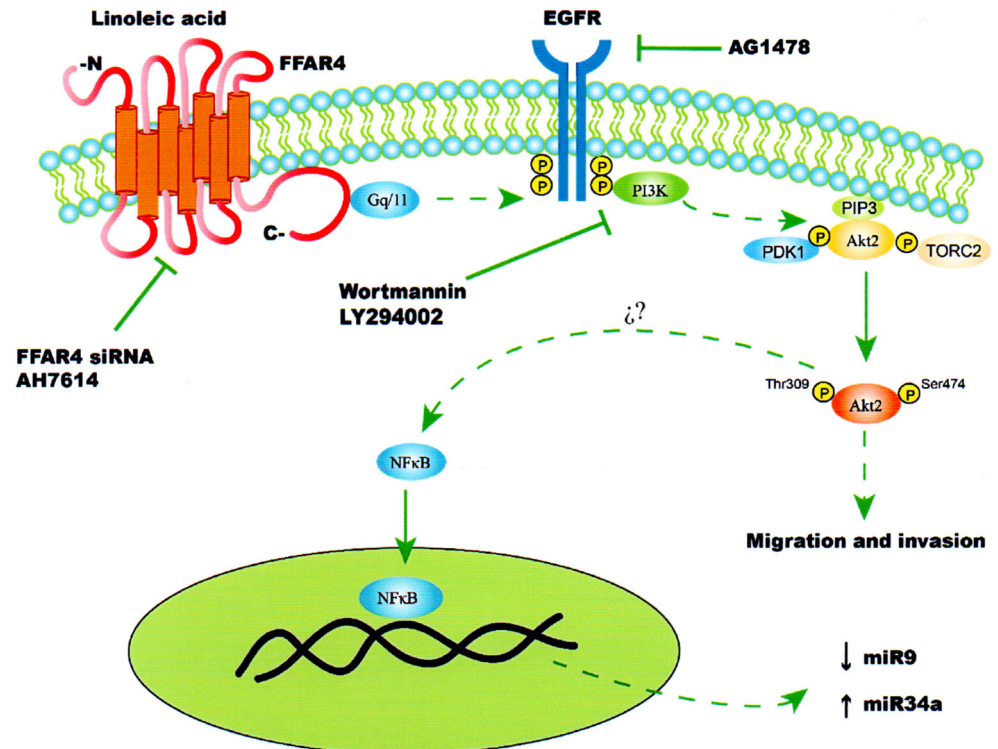
and downstream of EGFR transactivation [39]. Here, we demonstrate that EGFR activity is required for Akt2 activation, migration and invasion induced by LA. Taken together our findings, we propose that FFAR4 activation induced with LA promotes migration and invasion through EGFR transactivation and that activated EGFR mediates PI3K/Akt activation and they mediate migration and invasion of MDA-MB-231 cells. Supporting our proposal, we demonstrate that LA induces Src activation and migration through a Src-dependent pathway [12], whereas OA and AA induce invasion through an EGFR-dependent pathway in MDA-MB-231 cells [5, 37]. Our findings also demonstrate that LA is not able to induce migration and invasion of non-tumorigenic epithelial cells MCF12A.

The transcription factor NFκB is broadly associated with development and progression of several cancers. Particularly, NFκB is related to metastasis and angiogenesis, because it regulates expression of tumor-promoting genes including TNF, IL-1, iNOS and MMP-9 [40]. Akt controls activity of NFκB in PTEN-null/inactive prostate cancer cells via interaction with and stimulation of IKK. Moreover, in a model of prostate cancer progression in TRAMP mice has been demonstrated a differential protein expression of PI3K, Akt phosphorylated at Ser-473, IKK kinase activity and NFκB-DNA binding activity [41, 42]. Our findings show that LA induces an increase in NFκB-DNA binding activity and Akt2 activation in MDA-MB-231 cells. We propose that LA induces expression of genes implicated in the invasion/metastasis process through a NFκB-dependent pathway and that NFκB activation is mediated by Akt2 in MDA-MB-231 cells.

The miRNAs control a wide range of physiological and pathological processes including development, differentiation, proliferation, cancer initiation and metastasis [43]. Our findings demonstrate that LA induces miR9 downregulation and miR34a upregulation, whereas it does not modify miR21 expression levels in MDA-MB-231 cells. In agreement with our findings, simvastatin promotes Akt phosphorylation with a decrease in miR9 expression in bone marrow-derived stem cells, whereas miR9 expression is reduced and inversely correlated with activated leukocyte cell adhesion molecule (ALCAM) mRNA, which is associated with advanced tumor stage, in gastric cancer (GC) tissues and cell lines [44, 45]. In contrast to our findings, inhibition of miR34a expression increases phosphorylation of PI3K and Akt in human gastric cancer cells SGC-7901 [46, 47]. We propose that miR9 mediates Akt phosphorylation, whereas miR34a does not mediate Akt activation induced by LA in MDA-MB-231 cells.

In addition, knockdown of miR34a suppresses proliferation in MCF7 cells and is suggested that miR34a overexpression may be an acquired feature during carcinogenesis and support proliferation in breast tumors, whereas breast

Fig. 6 Model of migration/invasion induced by LA in MDA-MB-231 breast cancer cells



cancer patients at advanced tumor stages have more total RNA and miR34a in their blood than patients at early tumor stages [48, 49]. In contrast, Snai2 upregulates expression of carbonic anhydrase isoenzyme 9 and downregulates miR34a expression in hypoxic MCF7 cell-derived mammospheres and it is suggested that it plays an important role in hypoxic breast cancer stem cell niche [50]. Moreover, primary and mature miR34a is suppressed by treatment with p53RNAi or the dominant-negative p53 mutant in MCF7 cells and tumors from nude mice treated with miR34a are significantly smaller compared with control mice [51]. We propose that changes on miR9 and miR34a expression are specific of cell types and ligands, including LA, and they mediate specific cell responses that modulate cancer processes.

Conclusions

We conclude that LA induces migration and invasion through a FFAR4-, EGFR- and PI3K-/Akt-dependent pathway in MDA-MB-231 breast cancer cells. In addition, LA induces Akt2 activation, an increase in NFκB–DNA binding activity, upregulation of miR34a and downregulation of miR9 expression. Akt2 activation requires PI3K and EGFR activity. These findings are depicted in Fig. 6.

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Compliance with ethical standards

Conflict of interest Authors declare that they have no conflict of interest.

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