

Migration and invasion induced by linoleic acid are mediated through fascin in MDA-MB-231 breast cancer cells

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Abstract Epidemiological studies strongly suggest an association between high levels of dietary fat intake and an increased risk of developing breast cancer. Linoleic acid (LA) is an essential omega-6 PUFA and the major fatty acid in occidental diets. In breast cancer cells, LA induces expression of plasminogen activator inhibitor-1, proliferation, migration, and invasion. Fascin is an actin crosslinker globular protein that generates actin bundles made of parallel actin filaments, which mediate formation and stability of microspikes, stress fibers, membrane ruffles, and filopodia. However, the role of fascin in migration and invasion induced by LA in MDA-MB-231 breast cancer cells remains to be studied. We demonstrate here that LA induces an increase of fascin expression in MDA-MB-231 and MCF12A mammary epithelial cells. Particularly, LA induces the formation of filopodia and lamellipodia and the localization of fascin in these actin structures in MDA-MB-231 breast cancer cells. However, LA only induces formation of microspikes and the localization of fascin in these actin structures in mammary non-tumorigenic epithelial cells MCF12A. In addition, LA induces migration, invasion, and matrix metalloproteinase-9 secretion through a fascin-dependent pathway in MDA-MB-231 cells. In summary, our findings demonstrate that fascin is required for migration and invasion induced by LA in MDA-MB-231 breast cancer cells.

Keywords Breast cancer · Linoleic acid · Fascin · Migration · Invasion

Introduction

Breast cancer is the most prevalent cause of cancer-related death among females worldwide [1]. Epidemiological and animal studies propose a relationship between great amounts of dietary fat intake and an elevated risk of developing breast cancer [2–4]. Particularly, free fatty acids (FFAs) are the components of membranes, a source of energy, and the precursors of bioactive components including eicosanoids [5]. In breast cancer cells, FFAs mediate a variety of cell processes including proliferation, migration, and invasion [6–8].

Linoleic acid (LA) is the major fatty acid in occidental diets. It is an essential omega-6 polyunsaturated fatty acid (PUFA), which is able to be converted to arachidonic acid (AA). Moreover, AA is the precursor of eicosanoids that mediate inflammatory responses and play a crucial role in tumor initiation and progression [9, 10]. In breast cancer cells, LA promotes expression of plasminogen activator inhibitor-1, proliferation, migration, and invasion through a process that does not require the conversion from LA to AA [11–14]. In addition, LA promotes an epithelial-mesenchymal-transition like process in mammary non-tumorigenic epithelial cells MCF10A [15].

Fascin is an actin crosslinker globular protein of ~ 55 kDa that has been well conserved throughout animal evolution. It is a member of cytoskeleton proteins that generates actin bundles built of parallel actin filaments, which mediate formation and stability of cellular protrusions including microspikes, stress fibers, membrane ruffles, and filopodia [16–18]. In vertebrates, fascin has three isoforms: (1) Fascin-1, also known as fascin, is extensively present in mesenchymal tissues and nervous system; (2) Fascin-2, which is present in retinal cells; and (3) Fascin-3 is only expressed in testis [19, 20]. Particularly, fascin is expressed in adult human tissues, endothelial cells,

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fibroblasts, glial cells, neurons, smooth muscle cells, and dendritic cells. In contrast, fascin is expressed at very low levels or absent in normal epithelial cells, but it is upregulated in certain human epithelial tumors such as colon, skin, lung, prostate, and breast cancer [21–23].

In the present research, we study whether LA promotes an increase of fascin expression and the role of fascin in migration and invasion induced by LA in MDA-MB-231 breast cancer cells. Our findings show that LA promotes an increase of fascin expression in MDA-MB-231 and MCF12A mammary epithelial cells. Particularly, LA promotes the formation of filopodia and lamellipodia and the localization of fascin in these actin structures in MDA-MB-231 cells. However, LA only induces formation of microspikes and the localization of fascin in these actin structures in mammary non-tumorigenic epithelial cells MCF12A. In addition, LA promotes migration, invasion, and matrix metalloproteinase-9 (MMP-9) secretion through a fascin-dependent pathway in MDA-MB-231 cells.

Materials and methods

Materials

Fascin antibody (Ab), epidermal growth factor (EGF), fascin siRNA, and LA sodium salt were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phalloidin-tetramethylrhodamine B isothiocyanate (TRITC-conjugated phalloidin) and hydrocortisone were obtained from Sigma-Aldrich Co. (St. Louis, MO). BD Matrigel™ basement membrane matrix was obtained from BD Biosciences (Bedford, MA). Actin Ab was kindly provided by Dr. José-Manuel Hernández (Cinvestav-IPN).

Cell culture

The human MDA-MB-231 breast cancer cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 3.7 g/L sodium bicarbonate, and antibiotics. The human non-tumorigenic mammary epithelial cell line MCF12A was cultured in DMEM/F12 medium supplemented with 10% FBS, 10 µg/mL insulin, 0.5 µg/mL hydrocortisone, 20 ng/mL EGF, and antibiotics. Cells were cultured in a humidified atmosphere containing 5% CO₂ and 95% air at 37 °C. For experimental purposes, MDA-MB-231 and MCF12A cells were starved for 24 h in DMEM without FBS and supplements (insulin, hydrocortisone, and EGF) before treatment.

Cell stimulation

Cultures of MDA-MB-231 and MCF12A cells were washed twice with phosphate buffered saline (PBS), equilibrated in DMEM for at least 30 min at 37 °C and then treated with LA. Stimulation was terminated by aspirating the medium (conditioned 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 MgCl₂, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride). Protein content of each sample was determined using the micro-Bradford protein assay.

Western blotting

Equal amounts of protein were separated by SDS-PAGE using 10% separating gels followed by transfer to nitrocellulose membranes. 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, 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 the Western blotting luminol reagent (Santa Cruz Biotechnology). Autoradiograms were scanned, and the labeled bands were quantified using the ImageJ software (NIH, USA).

Interference RNA

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

Scratch-wound assay

Cells were grown to confluence in 35-mm culture dishes and starved. After starvation, cultures were treated for 2 h with 40 µM mitomycin C to inhibit proliferation, and they were scratch-wounded using a sterile 200-µL pipette tip, washed twice with PBS, and refed with DMEM without or with LA for 48 h. Progress of migration into the wound space was photographed using an inverted microscope coupled to a camera. Each experimental condition was repeated three times. Cell migration was evaluated using the ImageJ Software (NIH, USA).

Invasion assay

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). An amount of 30 μL Matrigel BD was added into culture inserts and kept overnight at 37 °C to form a semisolid matrix. MDA-MB-231 cells were plated at 1×10^5 cells per insert in 100 μL serum-free DMEM on top chamber. Lower chamber contained 600 μL DMEM without or with 90 μM LA. Chambers were incubated for 48 h at 37 °C. After incubation, cells and matrigel were removed from the upper side with cotton swabs, and cells on lower surface of membrane were washed and fixed with methanol for 5 min. The number of invaded cells was estimated by staining with 0.1% crystal violet in PBS. Dye was eluted with 500 μL 10% acetic acid and absorbance was measured at 600 nm. Background value was obtained from wells without cells.

Immunofluorescence confocal microscopy

Cells were grown on coverslips, washed with PBS, equilibrated in FBS-free DMEM, and treated with LA. Cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 for 20 min, and blocked for 30 min with 3% bovine serum albumin (BSA) at room temperature. Cells were incubated overnight with anti-fascin Ab, followed by FITC-labeled anti-mouse secondary Ab for 2 h at 4 °C. Next, cells were stained with TRITC-conjugated phalloidin to reveal F-actin. Cells were viewed using a Leica confocal microscope (Model TCS SP2; Leica Microsystems, Wetzlar, Germany). Serial optical sections of 0.8–0.99 μm thick were taken in both xyz and xzy. To prevent interference from the fluorescent probes, images of the same optical section were taken as separate channels and they were analyzed using ImageJ software.

Zymography

Conditioned mediums were collected and concentrated using Amicon® ultra centrifuge filters (Merck Millipore). Equal volume of non-heated conditioned medium samples were mixed with sample buffer (2.5% SDS, 1% sucrose, 4 $\mu\text{g}/\text{ml}$ phenol red) without reducing agents, and loaded to 8% polyacrylamide gels copolymerized with gelatin (1 mg/ml). Gels were rinsed twice with 2.5% Triton X-100, and then incubated in assay buffer (50 mM Tris-HCl pH 7.4, 5 mM CaCl_2) at 37°C for 48 h. Gels were fixed and stained with 0.25% Coomassie Brilliant Blue G-250 in 10% acetic acid

and 30% methanol. Proteolytic activity was detected as clear bands against the background stain of undigested substrate.

Statistical analysis

Results are expressed as the mean \pm SD of at least three independent experiments. Data were statistically analyzed using one-way ANOVA and Dunnett's multiple comparison test. Statistical probability of $P < 0.05$ was considered significant. Asterisks denote comparisons made to control (unstimulated cells).

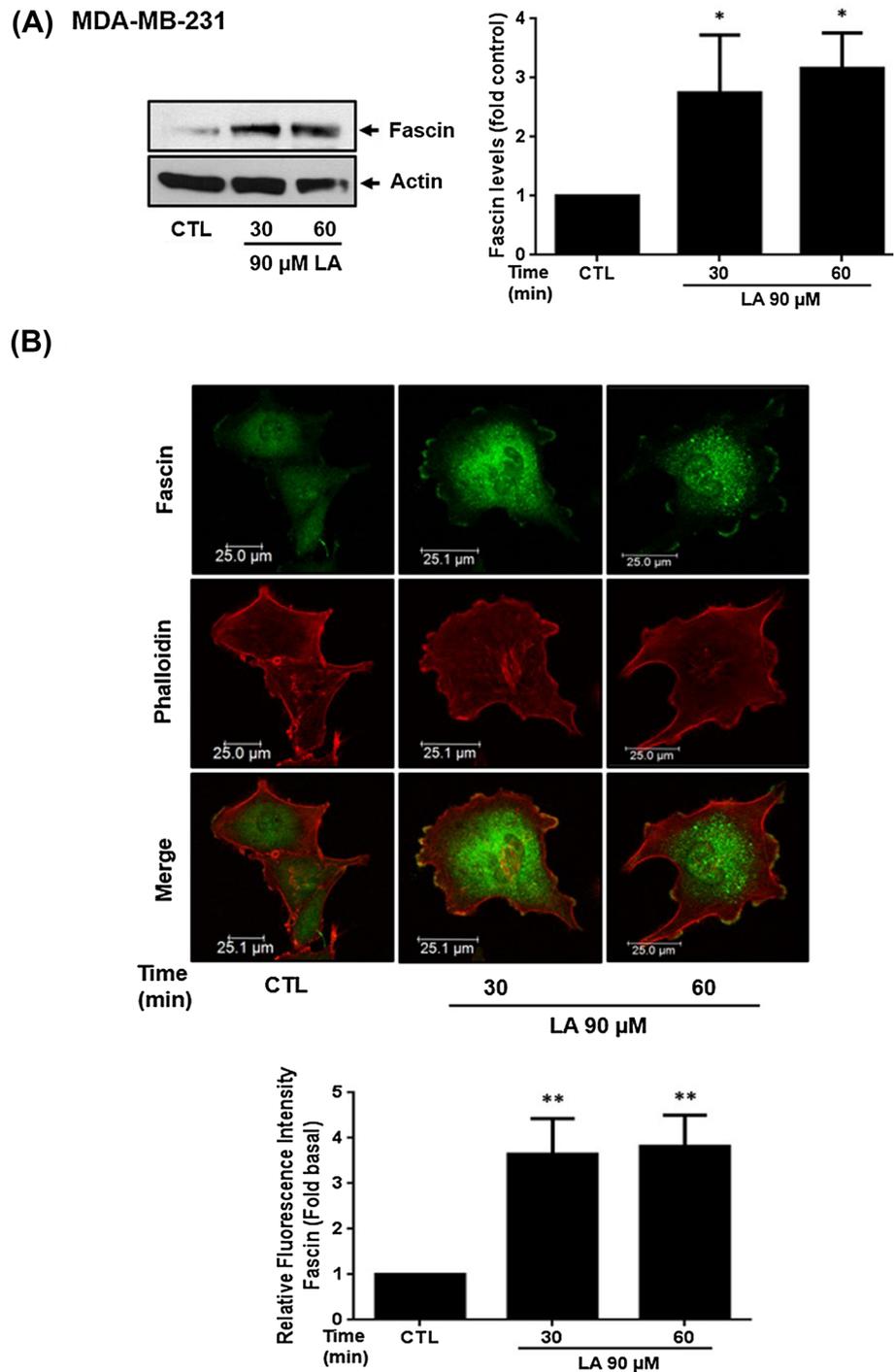
Results

LA induces an increase of fascin expression in mammary epithelial cells

First, we determined whether LA induced an increase of fascin expression levels in MDA-MB-231 breast cancer cells and mammary non-tumorigenic epithelial cells MCF12A. Cultures of MDA-MB-231 and MCF12A cells were stimulated without or with 90 μM LA for 30 and 60 min and lysed. Cell lysates were analyzed by Western blotting with anti-fascin Ab and with anti-actin Ab as loading control. As illustrated in Figs. 1a and 2a (upper panel), MDA-MB-231 cells express fascin and treatment with LA for 30 and 60 min induced an increase of fascin expression levels, whereas MCF12A cells also express fascin, and treatment with LA only induced a little bit of increase of fascin expression levels at 60 min. Western blotting with anti-actin Ab of the same membranes confirmed that similar amounts of protein were recovered after treatment with LA (Figs. 1a, 2a, lower panel).

To further substantiate that LA induced an increase of fascin expression and to determine whether treatment with LA induced relocalization of fascin, we studied fascin expression and its localization by confocal microscopy. MDA-MB-231 and MCF12A cells were cultured on coverslips and treated with 90 μM LA for 30 and 60 min. Cells were fixed and analyzed by immunofluorescence using anti-fascin Ab, whereas F-actin was stained with TRITC-conjugated phalloidin. Our findings showed that LA induced an increase of fascin expression in cytosol and the formation of filopodia and lamellipodia in MDA-MB-231 cells. Moreover, fascin was mainly localized with filopodia and lamellipodia (Fig. 1b). In contrast, treatment with LA induced a little bit of increase of fascin expression in cytosol and the formation of microspikes in MCF12A cells. However, LA did not induce formation of filopodia and lamellipodia and fascin was localized at the edges of cells with microspikes (Fig. 2b).

Fig. 1 LA induces an increase of fascin expression in MDA-MB-231 breast cancer cells (a). MDA-MB-231 cells were stimulated without or with 90 μ M LA for 30 and 60 min and lysed. Cell lysates were analyzed by Western blotting with anti-fascin Ab and with anti-actin Ab as loading control (b). MDA-MB-231 cells cultured on coverslips were treated with 90 μ M LA for 30 and 60 min and fixed. Cells were stained with anti-fascin Ab. F-actin was stained with TRITC-conjugated to phalloidin. Images were obtained by confocal microscopy. Fascin is shown in green and F-actin structures are shown in red. Graphs represent the mean \pm SD of fascin or fascin fluorescence intensities and are expressed as fold of control (unstimulated cells). Comparisons were made with control cells. * $P < 0.05$ and ** $P < 0.01$. (Color figure online)



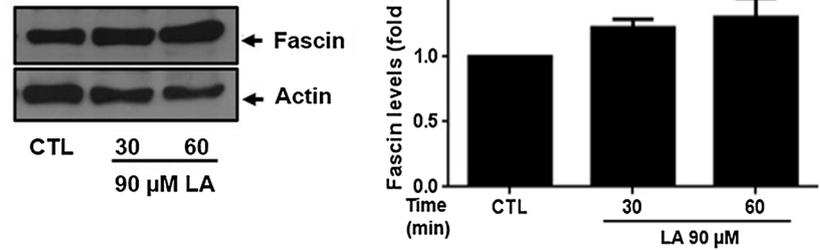
Migration of MDA-MB-231 cells induced by LA requires fascin expression

Since we have previously demonstrated that LA induced migration of MDA-MB-231 cells [14], we studied the role of fascin on migration. As shown in Fig. 3a, in agreement with our previous findings [14], LA induced migration of MDA-MB-231 cells. Next we determined the role of fascin

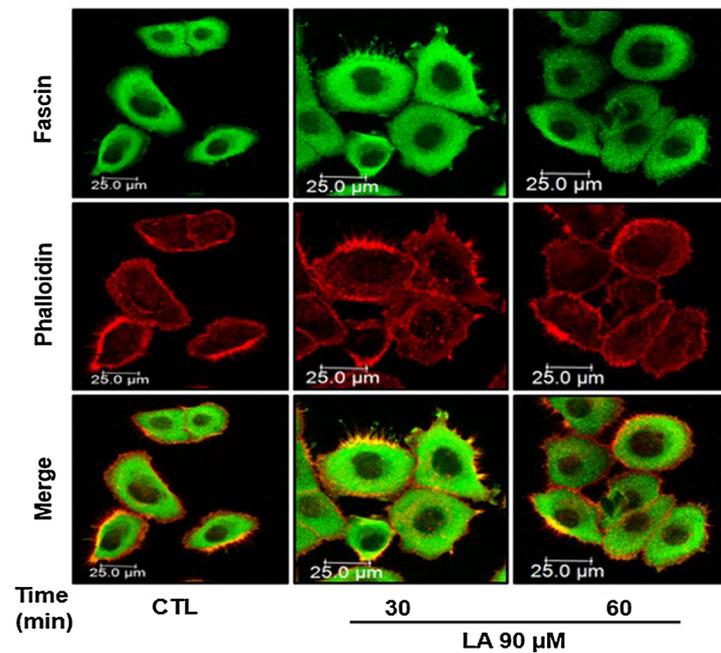
in migration induced by LA. First, we inhibited fascin expression using siRNAs against fascin. Our results showed a clear inhibition of fascin expression (Fig. 3b). Next, cultures of MDA-MB-231 cells, in which fascin expression was knocked down, were scratch-wounded and stimulated with 90 μ M LA for 48 h. Our results showed that migration induced by LA required fascin expression in MDA-MB-231 cells (Fig. 3c).

Fig. 2 LA induces an increase of fascin expression in mammary non-tumorigenic epithelial cells MCF12A (a). MCF12A cells were stimulated without or with 90 μ M LA for 30 and 60 min and lysed. Cell lysates were analyzed by Western blotting with anti-fascin Ab and with anti-actin Ab as loading control (b). MCF12A cells cultured on coverslips were treated with 90 μ M LA for 30 and 60 min and fixed. Cells were stained with anti-fascin Ab. F-actin was stained with TRITC-conjugated to phalloidin. Images were obtained by confocal microscopy. Fascin is shown in green and F-actin structures are shown in red. Graphs represent the mean \pm SD of fascin or fascin fluorescence intensities and are expressed as fold of control. Comparisons were made with control cells. * $P < 0.05$ and *** $P < 0.001$. (Color figure online)

(A) MCF12A



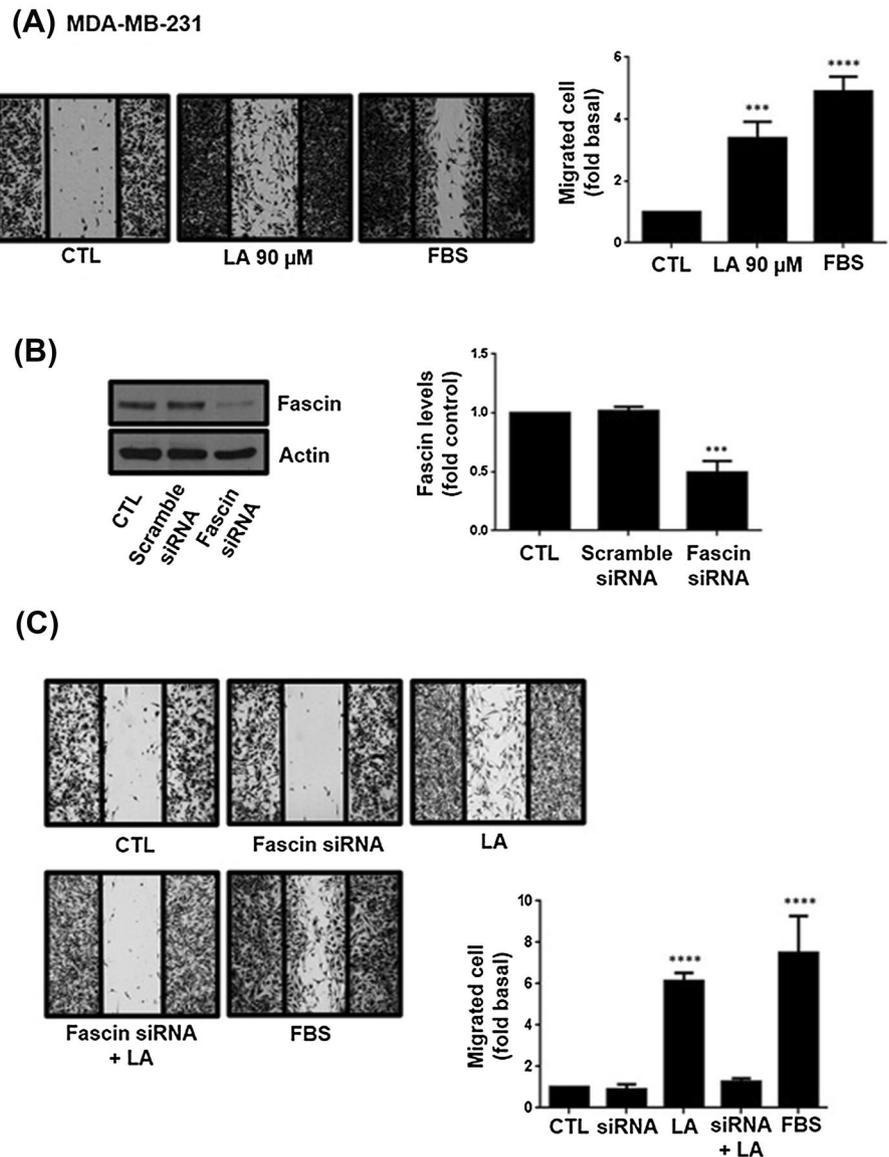
(B)



Cell migration requires formation of focal contacts, whereas paxillin is a protein localized in these structures [24]. We determined whether treatment with LA induced a colocalization of fascin and paxillin. MDA-MB-231 cells cultured on coverslips were stimulated with 90 μ M LA for 60 min. Cells were analyzed by immunofluorescence

with anti-paxillin Ab and anti-fascin Ab. As we mention above, treatment with LA induced the increase of fascin expression, which was colocalized with paxillin, whereas cells adopted a fibroblast-like structure (Fig. 4a). Our findings suggest that LA also induced an increase of paxillin expression.

Fig. 3 LA induces migration through a fascin-dependent pathway in MDA-MB-231 cells (a). Confluent cultures of MDA-MB-231 cells were scratch-wounded and treated with 90 μ M LA for 48 h (b). Cell lysates of MDA-MB-231 cells transfected with fascin siRNAs or scramble siRNAs were analyzed by Western blotting with anti-fascin Ab. Actin was included as loading control (c). Confluent cultures of MDA-MB-231 cells transfected with fascin siRNAs or scramble siRNAs were scratched and treated with 90 μ M LA for 48 h. One control of fetal bovine serum (FBS) was included. Graphs are the mean \pm SD and are expressed as the fold of migrated cells or fascin above control. Comparisons were made with control cells. *** P < 0.001, **** P < 0.0001



Invasion of MDA-MB-231 cells induced by LA requires fascin expression

LA induces invasion of MDA-MB-231 cells [11]. We determined the role of fascin on invasion induced by treatment with LA. We performed invasion assays using MDA-MB-231 cells, in which fascin expression was knocked down using siRNAs against fascin, and treated with 90 μ M LA for 48 h. As illustrated in Fig. 4b, LA induced a clear invasion and it was dependent on fascin expression in MDA-MB-231 cells.

Next, we determined whether LA induced an increase of matrix metalloproteinase-2 (MMP-2, gelatinase A) and MMP-9 (gelatinase B) secretions and the role of fascin on secretion of these gelatinases. Cultures of MDA-MB-231 cells, in which fascin expression was knocked down, were

stimulated with 90 μ M LA for 48 h, conditioned medium was obtained, and cells were lysed. Conditioned medium was subjected to gelatin zymography and cell lysates were analyzed by Western blotting with anti-actin Ab. Since ethanol (EtOH) and PDB stimulate secretion of MMP-2 and MMP-9, respectively [25, 26], positive controls of MMP-2 and MMP-9 secretions were included, which were prepared by treatment of MDA-MB-231 cells with 100 ng/ml PDB or 400 mg/dl EtOH for 24 h. Our results showed that LA induced an increase of MMP-9 secretion, but it did not induce an increase of MMP-2 secretion in MDA-MB-231 cells. Interestingly, inhibition of fascin expression blocked the increase of MMP-9 secretion (Fig. 4c). Western blotting with anti-actin Ab of cell lysates confirmed that similar number of cells was present in the different conditions analyzed (Fig. 4c).

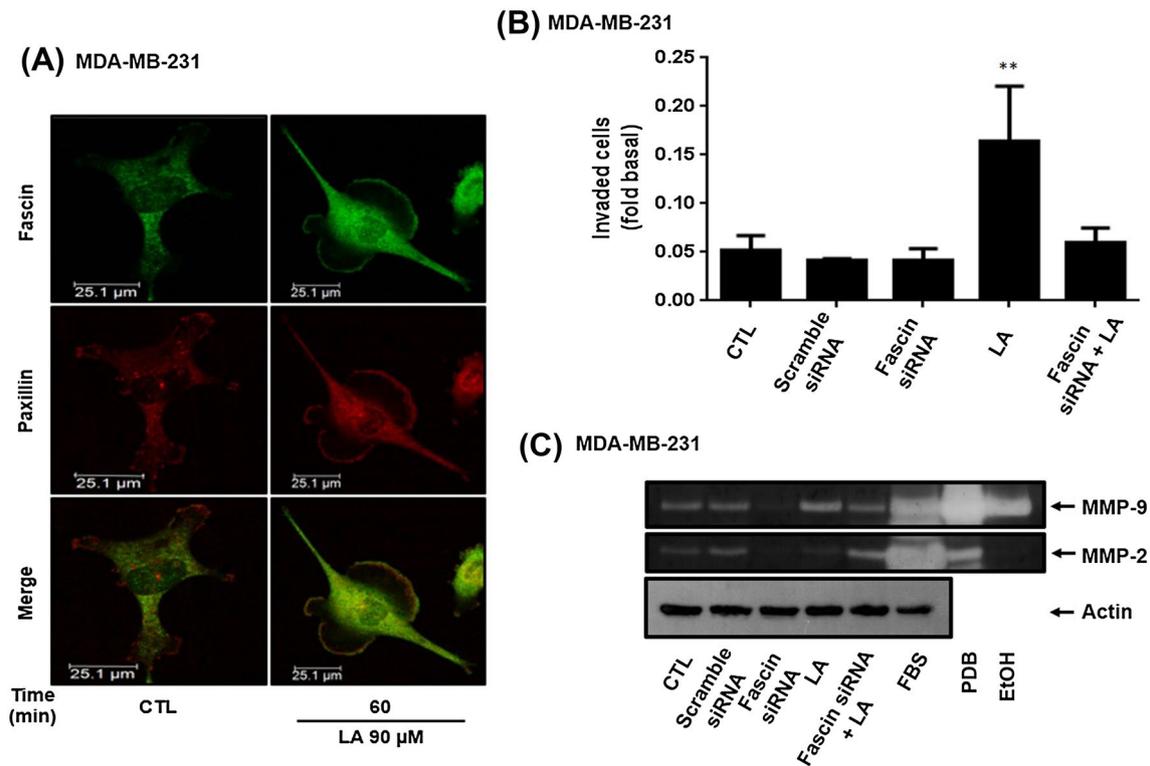


Fig. 4 LA induces invasion through a fascin-dependent pathway in MDA-MB-231 cells (a). MDA-MB-231 cells cultured on coverslips were stimulated with 90 μ M LA for 60 min and fixed. Cells were stained with anti-fascin Ab and anti-paxillin Ab. Images were obtained by confocal microscopy. Fascin is shown in green and paxillin is shown in red (b). Invasion assays were performed using MDA-MB-231 cells transfected with fascin siRNAs or scramble siRNAs and treated with 90 μ M LA for 48 h (c). MDA-MB-231 cells transfected with fascin siRNAs or scramble siRNAs were treated with

90 μ M LA for 48 h, conditioned medium was obtained, and cells were lysed. MMP-2 and MMP-9 secretions were analyzed on conditioned medium using gelatin-substrate gels. Controls of MMP-2 (PDB) and MMP-9 (EtOH) secretions, and one of fetal bovine serum (FBS) were included. Cell lysates were analyzed by Western blotting with anti-actin Ab. Graph is the mean \pm SD and is expressed as the fold of invasion above control. Comparisons were made with control cells. ** $P < 0.01$. (Color figure online)

Discussion

Epidemiological studies demonstrate that a dietary pattern characterized for high fat choices, including n-6 PUFAs, increase the risk of breast cancer. Particularly, LA is the major fatty acid in occidental diets with an intake of 15–20 g/day/person and a plasma concentration of $\sim 275 \mu$ M [27–30]. We demonstrate that LA induces migration and invasion of MDA-MB-231 breast cancer cells [11, 14]. However, the role of LA in the expression and organization of fascin/actin, as well as its role on migration and invasion mediated by treatment with LA in MDA-MB-231 cells, remains to be studied.

Fascin expression has not been reported in normal adult epithelia, but it is expressed in basal layer of epidermis. In contrast, upregulation of fascin expression has been demonstrated in metastatic human carcinomas from different sites [23]. Particularly, elevated levels of fascin correlate with clinically aggressive phenotypes, poor prognosis, and

shorter disease-free in breast cancer patients [31]. Here we demonstrate that LA induces an increase of fascin expression at 30 and 60 min of treatment in MDA-MB-231 cells. In agreement with our findings, MDA-MB-231 cells express basal levels of fascin and treatment with IL-6 and TNF- α induces an increase of fascin transcripts at 30 min of treatment [32]. In addition, we demonstrate that mammary non-tumorigenic epithelial cells MCF12A express basal levels of fascin and treatment with LA induces only an increase of fascin expression at 60 min of treatment. Our findings strongly suggest that mammary epithelial cells also express fascin and therefore additional studies are necessary to determine whether fascin is or not expressed in adult mammary epithelial cells.

Fascin contributes to organization of actin-based structures, including cortical cell protrusions and cytoplasmic microfilaments bundles, which mediate migration, cell interactions, architecture, and intracellular movements [19]. Moreover, fascin is in equilibrium between cytosol and

cytoskeleton, and its function is modulated by a variety of ligands including extracellular matrix components, growth factors, and cytokines [33, 34]. Our findings demonstrate that one fatty acid (LA) induces formation of filopodia and lamellipodia and the localization of fascin with these structures in MDA-MB-231 cells. Particularly, fascin is mainly localized with lamellipodia. In contrast, LA just induces formation of microspikes and the localization of fascin with these structures; however, it does not induce formation of lamellipodia and filopodia in mammary non-tumorigenic epithelial cells MCF12A. We propose that fascin contributes to formation of specific actin/fascin structures in human mammary cells and they are specific of breast cancer and mammary non-tumorigenic epithelial cells. Supporting our proposal, it has been demonstrated that filopodia are rich in metastatic tumor cells and its number correlates with their invasiveness, whereas filopodium-like structures are critical for colonization of secondary tissues or organs for metastatic tumor cells [35, 36].

Invasion/metastasis process involves cell detachment, migration/invasion, intravasation, survival in circulation, extravasation, and proliferation at distal tissues [37]. In breast cancer, fascin expression correlates inversely with the presence of estrogen and progesterone receptors, but it is not related with HER-2 overexpression [31, 38]. We previously demonstrated that LA induces migration and invasion of MDA-MB-231 cells, but it is not able to induce migration of MCF12A cells. Here we demonstrate that migration and invasion induced by LA require fascin expression in MDA-MB-231 cells. We propose that LA plays an important role in migration and invasion in breast cancer through fascin expression. Supporting our proposal, it has been demonstrated that fascin expression promotes downregulation of breast cancer metastasis suppressor-1 (BRMS1) and upregulation of proteins involved in metastasis including urokinase-type plasminogen activator, MMP-2, and MMP-9 in MDA-MB-231 cells [39].

Matrix metalloproteinases (MMPs) are a family of endopeptidases that collectively are able to degrade all extracellular matrix (ECM) components and are implicated in tumor progression, because they promote angiogenesis, tumor cell growth, and degradation of basement membrane (BM) and interstitial matrices [40, 41]. However, MMPs are synthesized and secreted as zymogens that require activation to become proteolytically active, and therefore activation is a critical step in regulation of MMP-dependent proteolytic activity [42, 43]. Particularly, MMP-2 and MMP-9 are highly expressed in malignant tumors, including breast cancer, and are associated with invasion and metastasis because they are able to degrade type IV collagen, which is the main component of BM [43, 44]. We demonstrate here that LA induces secretion of MMP-9 and it is dependent on fascin expression in

MDA-MB-231 cells. Our findings demonstrate a new role of fascin in MMP-9 secretion induced by LA and they support an important role of fascin in migration and invasion of breast cancer cells.

Interestingly, MMP-2 and MMP-9 appear in the supernatant fluid long before an increase in gelatinases mRNA is detected by RT-PCR in Jurkat T and primary T cells transfected with FAK wild-type cDNA. In addition, blocking transcription with actinomycin D does not inhibit the increase of gelatinases secretion, whereas blocking protein secretion with monensin and brefeldin A reduces gelatinases production in supernatant [45]. In addition, we demonstrate that treatment of MCF7 breast cancer cells with type IV collagen induces an increase of MMP-9 secretion, but it does not induce an increase of MMP-9 mRNA at 30 h of treatment [26]. We propose that treatment with LA for 48 h induces secretion of MMP-9, but it does not induce an increase of MMP-9 mRNA in MDA-MB-231 cells.

Fascin is localized in focal adhesion and is considered as an adhesome protein [46, 47]. Moreover, fascin is also localized in paxillin-positive adhesions, whereas the number of mature adhesions is higher in cells depleted of fascin [48]. We demonstrate here that untreated MDA-MB-231 cells do not show colocalization of fascin with paxillin. However, treatment with LA induces an increase of fascin expression, which is accompanied with its colocalization with paxillin. Since reorganization of actin cytoskeleton and focal adhesions is required for migration, invasion, and metastasis and fascin and paxillin play an important role in the formation of these structures, we propose that colocalization of fascin and paxillin induced by LA is required for migration and invasion process in breast cancer cells. Supporting our proposal, fascin and paxillin are expressed in infiltrating duct carcinoma and they correlate with poor prognosis factors [49].

In conclusion, our results demonstrate that fascin mediates migration and invasion induced by LA in MDA-MB-231 breast cancer cells.

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Compliance with ethical standards

Conflict of interest The authors declare that do not have any conflict of interest.

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