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Original Article

Leptin induces partial epithelial-mesenchymal transition in a FAK-ERK dependent pathway in MCF10A mammary non-tumorigenic cells

Villanueva-Duque Alfredo[1], Zuniga Eulogio Miriam Daniela[1], Dena-Beltran Jose[1], Castaneda-Saucedo Eduardo[1], Calixto-Galvez Mercedes[1], Mendoza-Catalán Miguel Angel[2], Ortuno-Pineda Carlos[3], Navarro-Tito Napoleon[1]

[1]Laboratorio de Biología Celular del Cancer, [2]Laboratorio de Biomedicina Molecular, [3]Laboratorio de Acidos Nucleicos y Proteinas, FCQB, Universidad Autónoma de Guerrero, Mexico

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Abstract: Epithelial-mesenchymal transition (EMT) is a biological process involved in different steps of tumor progression and metastasis of breast cancer cells. Epidemiological studies suggest a link between obesity and the progression of breast cancer. Leptin is an adipocyte-secreted hormone which can promote cell migration and invasion as part of EMT in breast cancer cells. We investigated the effect of leptin on expression of EMT markers in MCF10A cells, as well as, the role of FAK and ERK in this process. We found that leptin induces morphological changes from an epithelial phenotype towards a mesenchymal phenotype and promotes cell migration in MCF10A cells. Moreover, leptin induces an increase in vimentin expression, changes in the cellular localization of E-cadherin and increase in FAK and ERK phosphorylation. Furthermore, using FAK and ERK chemical inhibitors we show that leptin regulates EMT markers in a FAK and ERK dependent manner. In conclusion, leptin promotes vimentin expression and cell migration in a FAK and ERK dependent pathway in the non-tumorigenic epithelial cell line MCF10A.

Keywords: Leptin, EMT, cell migration, breast cancer

Introduction

Epithelial-mesenchymal transition (EMT) is a biological process characterized by a cell transdifferentiation from an epithelial phenotype towards a mesenchymal phenotype. EMT is an essential process during embryonic development, wound healing, fibrosis and tumor progression. During EMT, cells of epithelial origin lose their epithelial characteristics and acquire a mesenchymal phenotype characterized by a fibroblastoid morphology, increased migratory and invasive potential, and resistance to anoikis [1-4]. At the molecular level, EMT is accompanied by changes in actin organization, downregulation of E-cadherin, increased expression of N-cadherin, vimentin, Twist, Snail, as well as, an increase in MMPs secretion, and activation of signaling pathways that contribute to the increased migratory and invasive capacities of the cells [2-5].

Leptin is an adipocyte-secreted hormone, which plays a key role in the regulation of food intake and energy expenditure through binding to the leptin receptor (ObR) [6, 7]. However, leptin and the ObR receptor have been reported to be overexpressed in ductal and lobular breast cancer as well as node lymph metastasis, suggesting an important role for both proteins in tumor development and/or progression [8, 9].

Leptin promotes the activation of several signaling molecules including the JAK/STAT canonical signal pathway, PI3K, Rho GTPases, and kinases such as FAK and ERK [10-12]. FAK and ERK kinases are involved in the regulation of cell adhesion, motility, invasion, survival, angiogenesis, and they have recently been associated with EMT [13]. FAK activation occurs canonically when integrins bind to extracellular matrix proteins or through activation of growth factor receptors. FAK undergoes autophosphorylation on Tyr397, generating a high-affinity binding site for proteins containing SH2 domains, such as the Src kinase. The interaction between Src

and FAK promotes Src autophosphorylation and its subsequent activation. Active Src phosphorylates Tyr576 and Tyr577 in the kinase domain of FAK, promoting its maximum catalytic activity. In addition, Src induces FAK phosphorylation at Tyr925, generating a docking site for Grb2, leading to the activation of the MAPK pathway [13, 14]. It has been reported that ERK is involved in EMT and promotes the expression of regulatory factors such as ZEB and Twist [15, 16].

In the present study, we show that leptin promotes a switch from an epithelial to a mesenchymal phenotype in MCF10A mammary epithelial cells, increased cell migration, changes in the subcellular localization of E-cadherin, and an increase in the expression of vimentin, as well as, the activation of FAK and ERK. Together, our results suggest that leptin promotes EMT in a FAK/ERK-dependent manner in MCF10A cells.

Materials and methods

Recombinant human leptin, ERKI (3-(2-Aminoethyl)-5-((4-ethoxyphenyl) methylene)-2, 4-thiazolidinedione) and FAK (PF-573228) inhibitors were obtained from Sigma-Aldrich (St Louis, MO.). Anti-E-cadherin, anti-Vimentin, anti-Actin and anti-FAK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA.). Anti-ERK and anti-p-ERK1/2 were purchased from Millipore (Billerica, MA.). Phosphospecific Ab to FAK was obtained from Invitrogen (Waltham, MA.).

Cell culture

The non-tumorigenic mammary epithelial cell line, MCF10A, was cultured in DMEM/F12 (50:50) medium supplemented with 5% fetal bovine serum (FBS), 10 mg/ml insulin, 0.5 mg/ml hydrocortisone, 20 ng/ml recombinant epidermal growth factor and antibiotics 1% in a humidified atmosphere containing 5% CO_2 at 37°C. For experimental purposes, confluent cultures of these cells were serum-starved for 6 h before treatment with FAK and ERK inhibitors and/or leptin.

Cell stimulation

MCF10A cells were grown in 60-mm dishes containing 4 ml of DMEM/F12 until confluence. Cells were washed with PBS, and treated with inhibitors and/or leptin for the times and concentrations indicated. Cell stimulation was terminated by aspirating the medium, and cells were solubilized in 0.5 ml of ice-cold RIPA buffer, containing 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 $MgCl_2$, 0.1% SDS and 1 mM phenylmethylsulfonyl fluoride (PMSF).

Western blot

Cell lysates were resolved on 10% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes. Membranes were incubated with 1:1,000 dilutions of primary antibodies overnight at 4°C, followed by 2 h incubation with a 1:5,000 dilution of secondary HRP-conjugated antibodies (Millipore) at room temperature. Immunoblots were developed using an ECL chemiluminescent detection reagent (GE Health care). Blot data were analyzed using ImageJ software and compiled from three independent experiments.

Immunofluorescence and F-actin staining

MCF10A cells were seeded on glass coversilps and grown to 70% confluence, then stimulated with or without leptin during 24 h. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton-X100 in PBS for 5 min at room temperature. For IF, cells were blocked with 3% albumin in PBS for 1 h at room temperature. E-cadherin and Vimentin primary antibodies were used at a 1:250 dilution. Anti-rabbit secondary antibody conjugated with Alexa Fluor 488 (Invitrogen) was used at a 1:750 dilution. Immunofluorescence images were acquired with an Olympus BX43 microscope, under 100X objectives. For F-actin staining, cells were incubated with Rhodamine Phalloidin (1:500) for 30 min at room temperature, images were acquired with an Olympus BX43 microscope, under 40× and 100× objectives.

Scratch-wound assays

MCF10A cells were grown to confluence on 60-mm culture dishes. Cells were starved for 6 h in DMEM/F12 without FBS and treated for 2 h with AraC (Cytosine β-D-Arabinofuranoside)

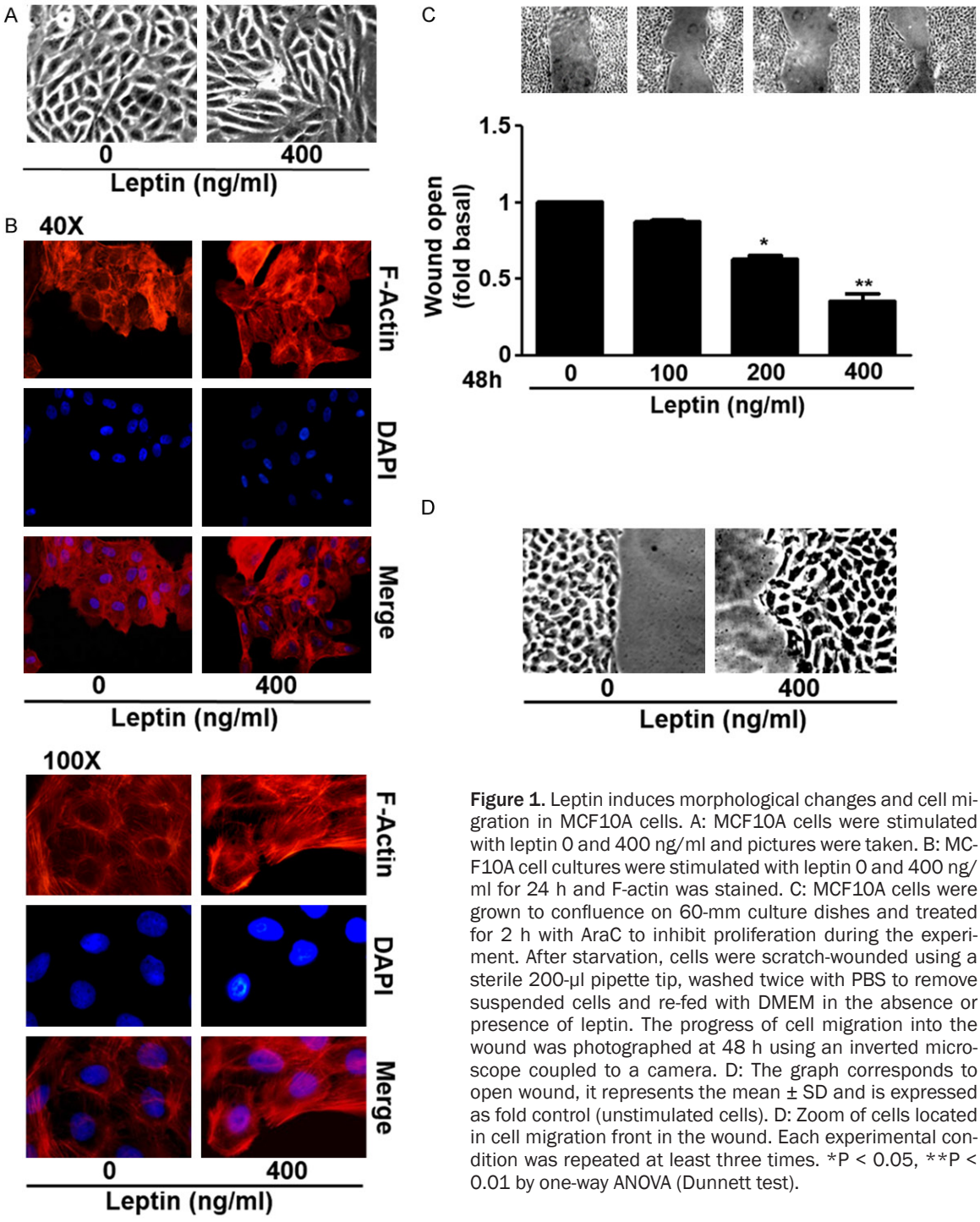

Figure 1. Leptin induces morphological changes and cell migration in MCF10A cells. A: MCF10A cells were stimulated with leptin 0 and 400 ng/ml and pictures were taken. B: MCF10A cell cultures were stimulated with leptin 0 and 400 ng/ml for 24 h and F-actin was stained. C: MCF10A cells were grown to confluence on 60-mm culture dishes and treated for 2 h with AraC to inhibit proliferation during the experiment. After starvation, cells were scratch-wounded using a sterile 200-µl pipette tip, washed twice with PBS to remove suspended cells and re-fed with DMEM in the absence or presence of leptin. The progress of cell migration into the wound was photographed at 48 h using an inverted microscope coupled to a camera. D: The graph corresponds to open wound, it represents the mean ± SD and is expressed as fold control (unstimulated cells). D: Zoom of cells located in cell migration front in the wound. Each experimental condition was repeated at least three times. *P < 0.05, **P < 0.01 by one-way ANOVA (Dunnett test).

to inhibit proliferation. After starvation, cells were scratch-wounded using a sterile 200-µl pipette tip, washed twice with PBS to remove suspended cells and re-fed with DMEM/F12 without FBS, in the absence or presence of inhibitors and/or leptin. The progress of cell migration into the wound was photographed at 48 h using an Olympus BX43 microscope using a 10× objective. The open area was determined by measuring the total area of the wound using the ImageJ software. Each experimental condition was repeated at least three times.

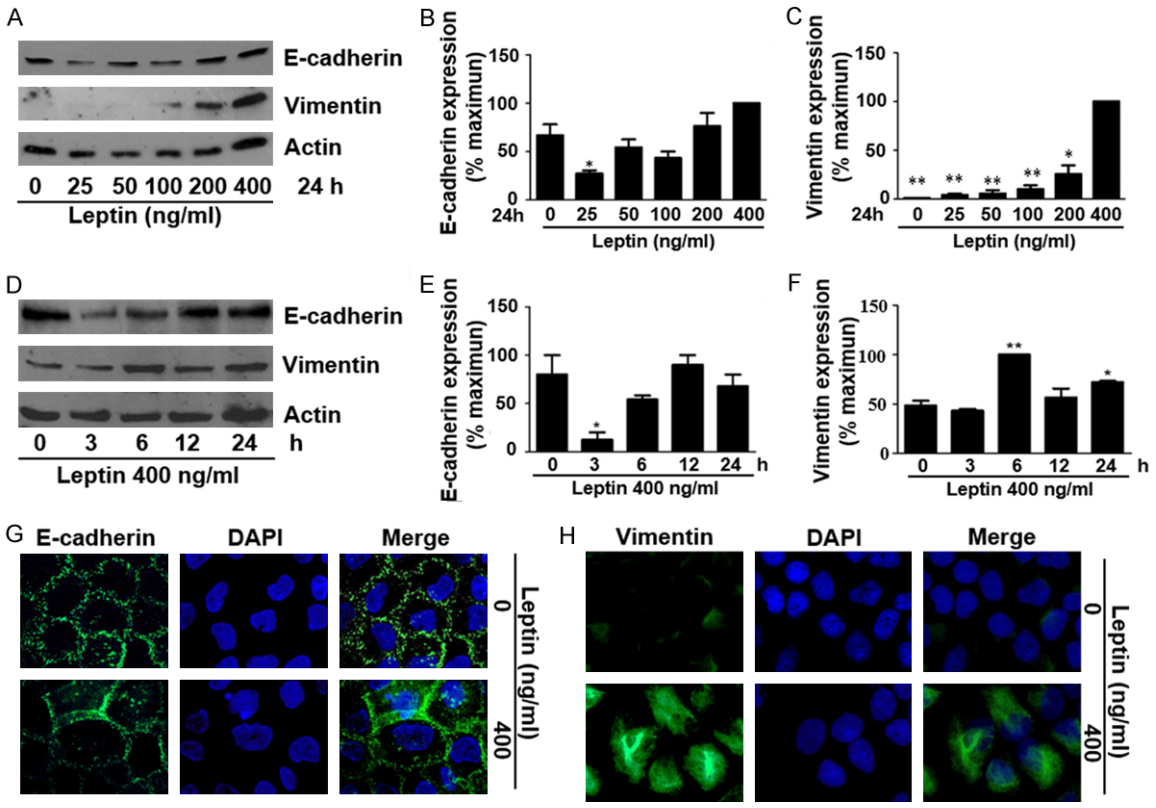

Figure 2. Leptin regulates E-cadherin and vimentin expression and cellular location in MCF10A cells. A: MCF10A cells were treated with leptin 0, 25, 50, 100, 200 and 400 ng/ml for 24 h and lysates were obtained. B, C: The graphs represent the densitometric and statistical analysis of the bands obtained by Western blot. The graphs represent the mean ± SD of at least three independent experiments and are expressed as changes respect to the control (unstimulated cells). D: MCF10A cells were treated with leptin 400 ng/ml for various times 0, 3, 6, 12 and 24 h and lysates were obtained. E-cadherin expression was analyzed by Western blotting using anti-E-cadherin and anti-vimentin Abs. The membranes were re-probed using anti-actin Ab as loading control. E, F: The graphs represent the mean ± SD of at least three independent experiments and are expressed as changes with respect to the maximum percentage. G, H: Cells cultures were grown in coverslips and treated with or without leptin 400 ng/ml for 24 h, fixed and incubated with anti-E-cadherin and incubated with anti-vimentin Abs. Asterisks denote comparisons made to unstimulated cells. *$P < 0.05$, **$P < 0.01$ by one-way ANOVA (Dunnett test).

FAK and ERK inhibition assays

MCF10A cell cultures were pretreated for 1 h with FAK (PF-573228) or ERK (ERKI) inhibitors, then the medium was aspirated, and cell cultures were refed with DMEM/F12 medium with inhibitors and/or leptin for 24 h according to each experiment. Total protein extracts were obtained as described above and vimentin expression was analyzed by WB.

Statistical analysis

Results are expressed as mean ± SD. Data were statistically analyzed using one-way ANOVA and the comparisons were performed using Newman-Keuls and Dunette's multiple comparison test. Statistical probability of $P < 0.05$ was considered significant.

Results

Leptin induces morphological changes and cell migration in MCF10A cells

To determine the effect of leptin on EMT markers, we first evaluated the effect of leptin on cell morphology. As shown in **Figure 1A**, leptin treatment results in a switch from a cuboid epithelial morphology to an enlarged mesenchymal phenotype. Moreover, we observed stress fiber formation in cells that were stimulated with leptin (**Figure 1B**). To investigate whether leptin induces cell migration in MCF10A cells, the cells were treated with 0, 100, 200 or 400 ng/ml of leptin for 48 h, and migration was evaluated by scratch-wound assays. As shown in **Figure 1C**, the treatment with leptin promotes an increase in cell migration, with a max-

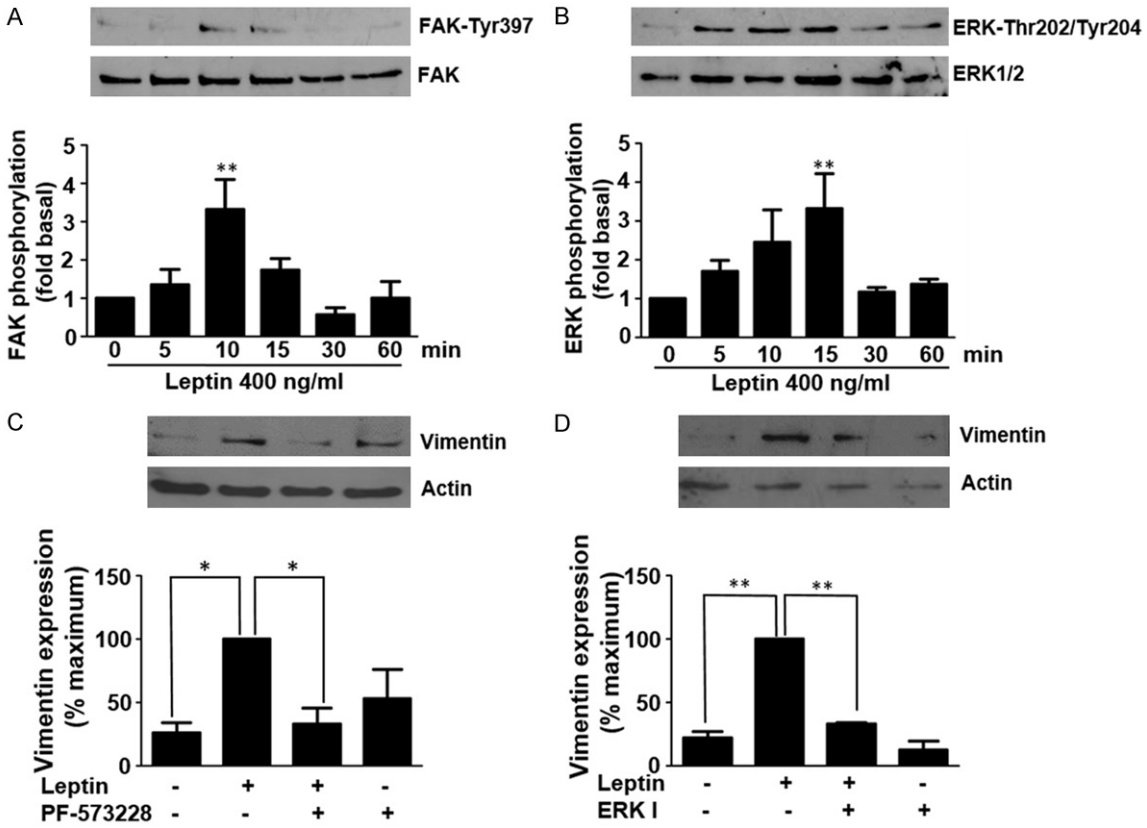

Figure 3. Leptin promotes an increase of vimentin expression in a FAK- and ERK-dependent manner. (A) MCF10A cells were treated with leptin 400 ng/ml for various times and lysates were obtained. FAK activation was analyzed by Western blotting using an anti-p-Tyr397 FAK antibody. The membranes were re-probed using anti-FAK Ab as loading control. (B) MCF10A cells were treated with leptin 400 ng/ml for various times and lysates were obtained. ERK activation was analyzed by Western blotting using an anti-p-Thr202-Tyr204 ERK antibody. The membranes were re-probed using anti-ERK Ab as loading control. (C) Cultures were untreated (-) or treated (+) with PF-573228 10 mM and leptin 400 ng/ml for 24 h and lysed. Lysates were analyzed by Western blot using an anti-vimentin Ab and anti-actin Ab as loading control. (D) Cultures were untreated (-) or treated (+) with ERKI 30 mM and leptin 400 ng/ml for 24 h and lysed. Lysates were analyzed by Western blot with an anti-vimentin Ab and anti-actin Ab as loading control. The graphs represent the mean ± SD of three independent experiments, comparisons were made respect to control cultures (unstimulated). Results shown are representative of at least three independent experiments. *P < 0.05, **P < 0.01 by one-way ANOVA analysis (Dunnett test, B, C; and Neuman-Keuls test, C, D).

imum migration observed at 400 ng/ml of leptin. In addition, we observed that leptin induces collective cell migration, with cells showing a fibroblast-like morphology at the front of the migration group (**Figure 1D**).

Leptin regulates E-cadherin and vimentin expression and cellular location in MCF10A cells

To examine whether leptin induces changes in E-cadherin and vimentin expression, confluent cultures of MCF10A cells were treated with vehicle or with 25, 50, 100, 200 or 400 ng/ml of leptin for 24 h (dose-response assays), or stimulated with 400 ng/ml of leptin for different times (time-course assays). As shown in **Figure 2A**, **2B**, a significant decrease on the levels of E-cadherin was observed when the cells were treated with 25 and 100 ng/ml of leptin. However, when the cells were treated with 50, 200 or 400 ng/ml of leptin, no significant change in E-cadherin expression was observed. Furthermore, leptin treatment induces an increase in vimentin levels in a dose-dependent manner (**Figure 2A**, **2C**).

To evaluate whether the changes in E-cadherin and vimentin expression in response to leptin is time-dependent, we stimulated MCF10A cells with 400 ng/ml of leptin for 0, 3, 6, 12 and 24 h. We found that leptin induces a decrease on E-cadherin levels only at 3 h of stimulation

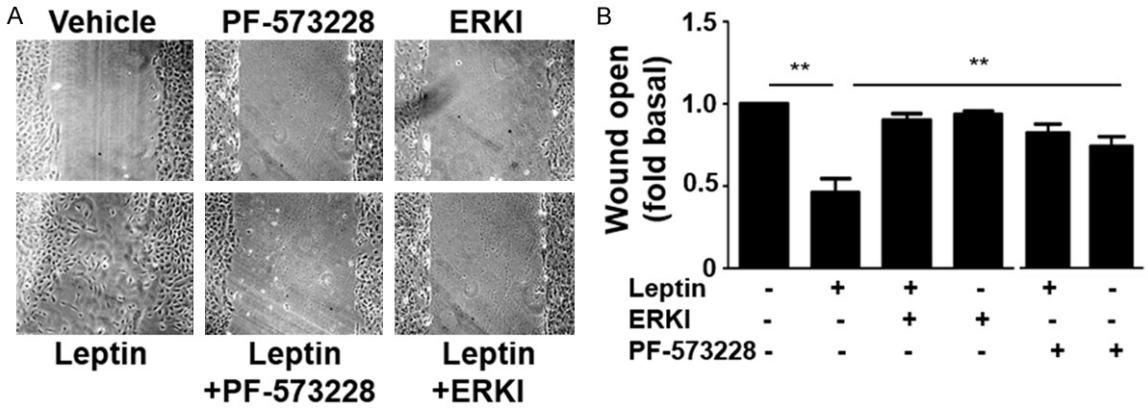

Figure 4. Leptin promotes cell migration in a FAK- and ERK1/2-dependent manner. A: Cell cultures were scratch-wounded and treated with vehicle (Tris HCl pH 7.4), leptin 400 ng/ml, leptin 400 ng/ml + PF-573228 10 mM, PF-573228 10 mM, leptin 400 ng/ml + ERKI 30 mM and, ERKI 30 mM. Pictures were taken at 48 h after wounding. B: The graphs are the cuantification of open wound area and represent the mean ± SD of the percentage of total open area of the experiment. Results shown are representative of at least three independent experiments. Statistic significance: **P < 0.01 by one-way ANOVA analysis (Newman-Keuls test).

(**Figure 2D**, **2E**), whereas increased vimentin levels were observed at 6 and 24 h of treatment (**Figure 2D**, **2F**). Interestingly, by immunofluorescence we notice that in MCF10A cells stimulated with leptin 400 ng/ml for 24 h, there is a subcellular relocalization of E-cadherin from de plasma membrane to the cytoplasm of the cell (**Figure 2G**). Furthermore, we notice an increase in vimentin expression with a perinuclear distribution in cells stimulated with leptin (**Figure 2H**).

Leptin promotes an increase of vimentin expression in a FAK- and ERK-dependent manner

To evaluate the effect of leptin on FAK and ERK phosphorylation, MCF10A cultures were treated with 400 ng/ml of leptin for 0, 5, 10, 15, 30 and 60 min. Leptin treatment induces an increase in FAK phosphorylation (Tyr397), reaching a maximum peak of phosphorylation at 10 min (**Figure 3A**). Similarly, leptin treatment induced an increase on ERK phosphorylation (Thr202/Tyr204) reaching a maximum phosphorylation level at 15 min (**Figure 3B**). To establish the role of FAK and ERK kinases in leptin-induced vimentin expression, MCF10A cells were incubated in the absence or presence of chemical inhibitors for FAK (PF-573228 10 μM) or ERK (ERKI), and stimulated with or without 400 ng/ml of leptin for 24 h. We found that in the presence of FAK or ERK inhibitors, no increase in vimentin expression was observed in response to leptin (**Figure 3C**, **3D**).

Leptin promotes cell migration in a FAK- and ERK1/2-dependent manner

Finally, we evaluated the involvement of FAK and ERK kinases in leptin-induced cell migration. Our results show that in the presence of PF-573228 or ERKI, the leptin-induced increase in cell migration was abolished; suggesting that cell migration induced by leptin is dependent on FAK and ERK kinases (**Figure 4**).

Discussion

Several epidemiological studies have shown a strong relationship between obesity and the development and progression of breast cancer [17]. Recently, it has been shown that high serum concentration of leptin is correlated with the onset and progression of breast cancer [18]. Moreover, leptin promotes EMT in several cancer cell lines in culture [19-21]. It has been reported that leptin induces EMT in MCF-7 and MDA-MB-231 breast cancer cells, promoting the decrease in E-cadherin expression and increase in Fibronectin, N-cadherin, vimentin and Snail [22]. In this study, we evaluated the effect of leptin treatment on EMT markers in the non-tumorigenic mammary epithelial cell line MCF10A. Our results show that leptin promotes a morphological switch from an epithelial to a mesenchymal phenotype, which is accompanied by an increase in stress fiber formation and vimentin expression, as well as an increase in cell migration, suggesting that leptin induces EMT in these cells. However,

leptin-treated MCF10A cells show a collective cell migration behavior and although leptin induces a partial redistribution of the epithelial marker E-cadherin at the plasma membrane, there is no decrease of E-cadherin expression, which may explain the collective cell migration phenotype observed in this study. Our results are in agreement with data reported by other groups, showing that inducers such as collagen IV, arachidonic acid and linoleic acid induce a partial EMT in MCF10A cells, which is characterized by collective cell migration [23-25]. Partial EMT has been associated with the formation of clustered tumor cells that are more resistant to apoptosis and have a higher potential for tumor formation than those that are circulating individually [23], and it has been demonstrated that leptin can induce apoptosis resistance in cancer cells in culture [26, 27]. Recently, it was demonstrated that leptin induces the self-renewal of normal breast stem cells, suggesting that this might be a mechanism that increases the risk of developing breast cancer, as it has been hypothesized that breast stem cells may be the initiators of breast cancer [28]. Taken together, these data support the active role of leptin in breast cancer initiation and progression. On one hand, by increasing stem cell population, and therefore increasing the target cells for carcinogenesis. On the other hand, it induces EMT, which promotes migration and dissemination of breast cancer cells. Finally, leptin may induce resistance to apoptosis. These antecedents and the findings described in our study support the idea that leptin is involved in different steps of carcinogenesis, either by increasing the population of cancer stem cells or promoting EMT, resistance to apoptosis and survival in circulation stimulating metastasis.

FAK and ERK are two kinases involved in cellular events such as cell proliferation and migration. Overexpression or hyperactivation of these proteins has been reported at different stages of tumor progression, including EMT [29-31]. Here, we evaluated the role of FAK and ERK in the regulation of leptin-induced changes in EMT markers. Our data show that leptin induces the activation of both FAK and ERK kinases, and that leptin-induced migration of MCF10A cells is dependent on the activation of these kinases. In other studies, cytosolic kinases such as Src and FAK have been reported to be involved in the control of collective cell movement [32]. In addition, ERK has been shown to play an important role in cell migration in MCF7 cells, in the induction of EMT in thyroid and pancreatic cancer cells [33, 34]. Recently, it has been reported that leptin has the ability to promote migration in MCF7 cells in an ERK, Akt and STAT3 dependent pathway [35]. These data demonstrate that the processes related to partial EMT in our experimental model are related to the kinase activity of FAK and ERK.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Navarro-Tito Napoleon, Laboratorio de Biologia Celular del Cancer, FCQB, UAGro. Av. Lazaro Cardenas s/n Chilpancingo, Gro. 39090, Mexico. Tel: 7474725503; Fax: 747472-5503; E-mail: nnavarro@uagro.mx

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