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LH AND FSH PROMOTE MIGRATION AND INVASION PROPERTIES OF A BREAST CANCER CELL LINE THROUGH REGULATORY ACTIONS ON THE ACTIN CYTOSKELETON

Angel Matias Sanchez[1, 3], Marina Ines Flamini[1, 3], Eleonora Russo[1], Elena Casarosa[1], Simone Pacini[2], Mario Petrini[2], Andrea Riccardo Genazzani[1], Tommaso Simoncini[1]

[1]Molecular and Cellular Gynecological Endocrinology Laboratory (MCGEL), Department of Clinical and Experimental Medicine, University of Pisa, Pisa, 56100, Italy.

[2]Division of Hematology, Department of Clinical and Experimental Medicine, University of Pisa, Pisa, 56100, Italy.

[3]Institute of Medicine and Experimental Biology of Cuyo (IMBECU), CCT-CONICET Mendoza, National University of Cuyo, Parque General San Martin s/n, CP:5500 Mendoza, Argentina.

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Address correspondences and reprint requests to:

Tommaso Simoncini, M.D., Ph.D.,
Department of Clinical and Experimental Medicine,
Division of Obstetrics and Gynecology, University of Pisa,
Via Roma, 57, 56100, Pisa, Italy.
Phone: +39.050.553412
Fax: +39.050.553410
E-mail: tommaso.simoncini@med.unipi.it

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Precis: LH/FSH activate FAK and Moesin, enhancing motility in BC cells. This may have clinical implications for drugs used to modulate gonadotrophins in BC patients.

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Abstract

Reproductive hormones influence breast cancer development and progression. While the actions of sex steroids in this setting are established, tentative evidence suggests that follicle-stimulating hormone (FSH) and luteinizing hormone (LH) may also play a role, yet this remains elusive. We here identify that T-47D breast cancer cells express functional receptors for FSH and LH, and that these hormones regulate breast cancer cell motility and invasion through the control of the actin cytoskeleton and the formation of cortical actin aggregates and focal adhesion complexes. Such actions are mediated by the cytoskeletal controllers Moesin and focal adhesion kinase (FAK). Moesin is recruited rapidly by FSH and LH through a signaling cascade requiring the G protein $G\alpha_{13}$ and the Rho-associated kinase, ROCK-2. FSH and LH activate FAK via a $G\alpha_i/\beta$ and c-Src-dependent signaling cascade. Both cascades involve signaling to phosphatidylinositol-3 kinase and Akt. FSH and LH receptors and the related signaling intermediates are necessary for the actions of gonadotrophins on breast cancer cell cytoskeletal rearrangement, migration and invasion. These findings provide original information on the actions of gonadotrophins on breast cancer cells and may have clinical implications for the use of drugs that modulate gonadotrophins in breast cancer patients.

Introduction

Breast cancer (BC) is the most frequent malignancy in Western countries, affecting approximately one out of eight women, and one-fifth die for the disease (Janssens, Russo, Russo et al., 2007). Most breast cancers are hormone-dependent, express receptors for estrogen and are affected by therapies that decrease the levels of these hormones or interfere with their receptors, such as aromatase inhibitors or tamoxifen. Although the available evidence is scanty and contradictory, there are indications that gonadotrophins may play a regulatory role in the breast as much as in breast cancer (Huhtaniemi, 2010).

Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are glycoprotein hormones synthesized and secreted by pituitary gonadotrophs in response to gonadotrophin-releasing hormone (GnRH) (Wildt, Hausler, Marshall et al., 1981,Knobil, 1981). GnRH agonists, that suppress the release of LH and FSH (and therefore block estradiol production in the ovaries), are effective in the treatment of breast cancer in fertile women (Robertson and Blamey, 2003). In parallel, a few studies suggest that women exposed to gonadotrophins for ovulation induction (Pappo, Lerner-Geva, Halevy et al., 2008) or to drugs that raise gonadotrophins, such as clomiphene citrate (Lerner-Geva, Keinan-Boker, Blumstein et al., 2006,Orgeas, Sanner, Hall et al., 2009), may incur in mildly elevated increases in breast cancer risk. It is difficult to discriminate from this clinical data any possible effects of gonadotrophins from those of estrogens, that are likely prevalent. However, the hypothetical concept that gonadotrophins might regulate breast cancer would be particularly relevant in postmenopausal women, when elevated FSH and LH along with the highest breast cancer incidence rate are found (Bray, McCarron and Parkin, 2004).

Actions of gonadotrophins outside the gonads have been identified in a variety of tissues (Rao and Lei, 2007), including the breast (Jiang, Russo and Russo, 2002,Jiang, Russo and Russo, 2002). Breast cancers (Meduri, Charnaux, Loosfelt et al., 1997,Meduri, Charnaux, Spyratos et al., 2003) and breast cancer cell lines (Bodek, Rahman, Zaleska et al., 2003) express the HCG/LH receptor, and gonadotrophins have been proposed to enhance breast cancer tumorigenesis, however this is supported by weak evidence and hence debated (Huhtaniemi, 2010). Recent reports suggest that gonadotrophin receptors may be important in

tumor-associated endothelial cells in a wide variety of cancers, including breast cancer (Radu, Pichon, Camparo et al., 2010).

Local progression and distant metastasis are the main reason for morbidity and mortality in women affected by breast cancer (Janssens et al., 2007). While different therapeutic interventions against cancer cell proliferation are established, strategies to counteract cancer cell invasion or dissemination are not yet available. Recent evidence highlights that hormone-sensitive cancers, including breast and endometrial cancer, can be driven to invade the surrounding environment by sex steroids through the recruitment of signaling pathways that regulate cell movement and interaction with the extracellular environment (Fu, Flamini, Sanchez et al., 2008,Giretti, Fu, De Rosa et al., 2008,Flamini, Sanchez, Goglia et al., 2009). Activation of dynamic remodeling of the actin cytoskeleton and of cell membrane adhesive properties is central for these actions and selected intermediates have been identified as targets of sex steroids. For instance, estrogen-induced threonine^{558} phosphorylation of the actin-binding protein Moesin enhances breast cancer cell movement and invasion by promoting the formation of molecular bridges between actin, integrins, and focal adhesion complexes at specialized cell membrane sites such as ruffles and pseudopodia (Giretti et al., 2008). Moreover, in the presence of estrogens, the focal adhesion complex regulator, focal adhesion kinase (FAK), is phosphorylated at tyrosine^{397} and localizes to the plasma membrane where it endows cells with higher motility through increased adhesion to the extracellular matrix (Fu, Goglia, Sanchez et al., 2010,Sanchez, Flamini, Baldacci et al., 2010).

In the present paper we investigated whether FSH and LH affect the motility or invasion of breast cancer cells. We here describe that FSH and LH act through their receptors in T-47D breast cancer cells by modulating Moesin and FAK, turning into cell membrane remodeling and enhanced horizontal motility and invasion of three-dimensional matrices, and we characterize the relevant signaling cascades.

Materials and methods

Cell cultures and treatments

The human breast carcinoma cell lines T-47D, MCF-7 and MDA-MB-231 were obtained from the American Type Culture Collection. T-47D cells were grown in RPMI 1640 supplemented with L-glutamine (2 mM), 10% fetal bovine serum. Before treatments, the breast cancer cells lines were kept 24 hours in medium containing steroid-deprived FBS. Whenever an inhibitor was used, the compound was added 30 minutes before starting the treatment. LH (Luveris 75 IU) and FSH (GONAL-f 75 IU) were from Serono; 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo (3,4-d) pyrimidine (PP2) was from Calbiochem (EMD Biosciences, Germany); Pertussis Toxin (PTX), Y-27632, PD98059 and Wortmannin (WM) were from Sigma-Aldrich (Saint-Louis, MO). Experiments showing selectivity of action of some of the compounds are shown in Fig. 1A-D, supplemental data online).

Immunoblottings

Cell lysates were separated by SDS-PAGE. Antibodies used were: FAK, p-FAK (Y397) and Moesin (clone 38, Transduction Laboratories, Lexington, KY); Thr^{558}-p-Moesin (sc-12895), p-FAK-Tyr^{397} (sc-11765), LHR (H-50), FSHR (N-20), Gαi1 (R4), Gβ1 (C-16), Gα13 (A-20), c-Src (sc-5266), ACTIN (sc-1615) (Santa Cruz Biotechnology, Santa Cruz, CA), ERK1/2 (p44/p42) (05-1152, Millipore), Thr^{34}-P-Akt (07-789, Upstate, Lake Placid, NY), Akt (9272), Phospho-p44/42 MAPK (ERK1/2, Thr^{202}/Tyr^{204}, E10) and Phospho-Src (Tyr416) (Cell Signaling Technology Danvers, MA). Primary and secondary Abs were incubated with standard technique. Immunodetection was accomplished with enhanced chemiluminescence. Chemiluminescence was acquired with a quantitative digital imaging system (Quantity One, BioRad, Hercules, CA). The blots were quantified using the Image J software. All western blot experiments reported in the manuscript are representative images of at least three repeats, generating similar results.

Kinase assays

T-47D cells were harvested in 20 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, 0.5% IGEPAL and 0.1 mg/mL PMSF. Equal amounts of cell lysates were immunoprecipitated vs. ROCK-2 (C-20, Santa Cruz). The IPs were washed three times with buffer containing 20 mM Tris-HCl, 10 mM EDTA, 150 mM NaCl, 0.1%

IGEPAL and 0.1 mg/mL PMSF. For ROCK-2 activity assay, two additional washes were performed in kinase assay buffer (20 mM MOPS, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM DTT) and the samples were therefore resuspended in this buffer. 5 mg of de-phosphorylated myelin basic protein (Upstate) together with 500 mM ATP and 75 mM $MgCl_2$ were added to each sample and the reaction was started at 30°C for 20 min. The reaction was stopped on ice and by resuspending the samples in Laemmli Buffer. The samples were separated with SDS-PAGE and Western analysis was performed using antibodies recognizing Thr^{98}-P-myelin basic protein (05-429, Upstate).

Quantitative analysis of cell membrane morphology and thickness and of actin fibers remodeling

The remodeling of actin fibers and the morphological changes of the membrane were quantified by assessing the intensity of actin fluorescence using the Leica QWin software (Leica Microsystems, Germany). This analysis was performed by randomly measuring fluorescence using boxes of a fixed size including the extra- and intra-cellular space across the membrane, by recording the intensity of the signal in a spatial-related fashion. In other words, the program provides a graph where the pixel fluorescence intensity is linearly recorded. By visually selecting the inner and outer parts of the membrane in each microphotograph we were able to quantify the thickness of the membrane as sampled in each box. In addition, the measure of the mean fluorescent intensity in the areas identified as membrane space or intracellular space within the box was used to quantify the amount of cytosolic vs. membrane-associated actin. Using these boxes we sampled five areas per each cell, and we repeated this on 40 different cells per experimental condition.

Cell immunofluorescence

T-47D cells were grown on coverslips. Cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton for 5 min. Blocking was performed with PBS containing 3% bovine serum albumin for 30 min. Cells were incubated with antibodies against Tyr^{397}-phospho-FAK (Transduction Laboratories, Lexington, KY), Vinculin (sc-7648), and Thr^{558}-p-Moesin (sc-12895) (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4º C followed by incubation with a fluorescein-conjugated goat anti-rabbit/mouse secondary antibody (1:200; Vector Laboratories). Then cells were incubated with Texas Red-phalloidin to stain actin fibers (Sigma) for 30 min. After washing, the nuclei were counterstained with

or 4'-6-diamidino-2-phenylindole (DAPI) (Sigma) and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Immunofluorescence was visualized using an Olympus BX41 microscope and recorded with a high-resolution DP70 Olympus digital camera.

Transfection experiments

The dominant negative constructs for $G\alpha_{i1}$ ($G\alpha_{i1}$ G202T) and $G\alpha_{13}$ ($G\alpha_{13}$ Q226L/D294N) were from the Guthrie cDNA Resource Center (www.cdna.org). The inserts were cloned in pcDNA3.1+. The plasmids (10 μg) were transfected into T-47D cells using Lipofectamine (Invitrogen, Carlsbad, CA). Parallel cells were transfected with empty pcDNA3.1+ plasmid. Cells (60-70% confluent) were treated 24 h after transfection.

Gene silencing with RNA interference

A synthetic small interfering RNA targeting Focal Adhesion Kinase (siRNA SMARTpool FAK), ROCK2 (siRNA SMARTpool ROCK-2) and c-Src (siRNA SMARTpool SRC, Dharmacon, USA); and two SureSilencing shRNA Plasmid Human LHCGR and FSHR (Cat KH01310G and KH07073G, SuperArray Bioscience Corporation) were used at the final concentration of 50-75 nM to silence FAK, ROCK-2, c-Src and LHR and FSHR according to the manufacturer's instructions. T-47D breast cancer cells were treated 48 hours after siRNA transfection. The efficacy of gene silencing was checked with western analysis and found to be optimal at 48 hours. Control experiments demonstrating selectivity and efficacy of silencing of the different targets can be found in supplemental data online, Fig 2A-D and Fig. 2F-G.

Moesin silencing with antisense oligonucleotides

Validated antisense phosphorothioate oligonucleotides (S-modified) (PONs) complementary to the 1-15 position of the human Moesin gene-coding region were obtained. The sequence was 5'-TACGGGTTTTGCTAG-3' for Moesin antisense PON. The complementary sense PON was used as control (5'-CTAGCAAAACCCGTA-3'). PONs transfections were performed on sub-confluent T-47D breast cancer cells. PONs were resuspended in serum-free medium with 2% Lipofectin (Invitrogen) and added to the culture medium for 48 hours at the final concentration of 50-75 nM. Moesin silencing was assessed through

protein analysis up to 48 hours following transfection. Control experiments demonstrating selectivity and efficacy of Moesin silencing can be found in relevant Figures or in supplemental data online, Fig 2E.

Cell migration assay

Cell migration was assayed with razor scrape assays. Briefly, a razor blade was pressed through the confluent T-47D breast cancer cell monolayer into the plastic plate to mark the starting line. T-47D cells were swept away on one side of that line. Cells were washed, and 2.0 mL of RPMI 1640 containing steroid-deprived FBS and gelatin (1 mg/mL) were added. Cytosine β-D-arabinofuranoside hydrochloride (Sigma) (10 μM), a selective inhibitor of DNA synthesis which does not inhibit RNA synthesis was used 1 hour before the test substance was added to prevent cell proliferation. Absence of cell proliferation and viability of the cells were checked in preliminary experiments with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tests. Migration was monitored for 48 hours. Cells were digitally imaged and migration distance was measured by using phase-contrast microscopy.

Cell invasion assay

Cell invasion were assayed using the BD BioCoat^{TM} Growth Factor Reduced (GFR) Matrigel^{TM} Invasion Chamber (BD Bioscience, USA). In brief, after rehydrating the GFR Matrigel inserts, the test substance was added to the wells. An equal number of Control Inserts (no GFR Matrigel coating) were prepared as control. 0.5 mL of T-47D cell suspension (2.5 x 10^4 cells/mL) was added to the inside of the inserts. The chambers were incubated for 48 h at 37°C, 5% CO_2 atmosphere. After incubation, the non-invading cells were removed from the upper surface of the membrane using cotton-tipped swabs. Then the cells on the lower surface of the membrane were stained with Diff-Quick stain. The invading cells were observed and photographed under the microscope at 100 X magnification. Cells were counted in the central field of triplicate membranes.

Quantitative RT-PCR

Total RNA was extracted using an RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) as indicated by the manufacturer's protocol. 500 ng of each RNA sample was retrotranscribed with iScript cDNA Synthesis Kit

(Bio-Rad Laboratories, Hercules, CA USA) and a 5-fold dilution of cDNAs was analyzed by quantitative Real Time PCR (qRT-PCR) using an iCycler-iQ5 Optical System (Bio-Rad) utilizing Sso Fast Evagreen Supermix (Bio-Rad). All samples were run in duplicate. Primers were designed from coding sequences published in Gene Bank with the help of Beacon Designer Software (Premier Biosoft International, Palo Alto, CA-USA). Primers used were: forward 5'-ACGACACTGACTTCACTGGAG-3' and reverse 5'-GCAATTAGCCTCTGAATGGACTC-3' for LHCGR (NM_000233.3); forward 5'-AGAGCAAGGTGACAGAGATTCC-3' and reverse 5'-GGTTGATGTAGAGCAGGTTGTTG-3' for FSHR (NM_000145); Relative quantitative analysis was performed following 2-ΔΔCt Livak method (Livak and Schmittgen, 2001). Normalization was performed with the housekeeping gene HPRT (NM_013556) using primers forward 5'-AGACTTTGCTTTCCTTGGTCAGG-3' and reverse 5'-GTCTGGCTTATATCCAACACTTCG-3'.

Measurement of cAMP

Cells were plated at a density of 0.5×10^6 per well in 24-well plates and transfected with or without the specific siRNA vs. LHR and FSHR. 48 hours after transfection, cells were treated for different times, with a range of concentrations of LH and/or FSH (5, 25 and 50 mIU/ml). At the end of the incubation, cells were lysed, and cAMP levels were evaluated by Direct Cyclic AMP Enzyme Immunoassay Kit (Assay Designs, Enzo Life Sciences International). The assay was performed according to the manufacturer's instructions. The sensitivity of the assay was 0.041 pM/mL, with a linear measuring range between 1-80 pM/mL. Absorbance values were measured at 405 nm using a microplate reader (Thermo Scientific Multiscan EX).

Immunohistochemistry (IHC)

Human breast cancer tumors were fixed in 10% buffered formalin and paraffin-embedded. Sections were treated with 2 µg/ml anti-LHR and FSHR (Santa Cruz Biotecnology) antibodies overnight at 4°C and detected with a biotin-conjugated secondary antibody (Vector Laboratories, CA, USA). ABC reagent (Vector) and DAB peroxidase substrate kit (Vector) were used to visualize specific staining. Citrate buffer high-temperature antigen retrieval was required for all antibodies.

Statistical analysis

All values were expressed as mean ± SD. Statistical analyses and graphics were done using InStat from Graph Pad Prism Software. Statistical differences between mean values were determined by ANOVA, followed by the Fisher's protected least significance difference (PLSD).

Results

T-47D cells express functional LH and FSH receptors

T-47D (ER+/PR+) cells actively express LH receptor (LHR) and FSH receptor (FSHR) proteins and RNAs (Fig. 1A-C). Human ovary tissue lysates used as positive controls confirmed specificity of the antibodies (Fig. 3A, supplemental data online). Expression of FSHR and LHR was also found in MCF-7 (ER+/PR+) and MDA-MB-231 (ER-/PR-) breast cancer cell lines (Fig. 3B, supplemental data online). In addition, FSH and LH receptors were identified in human breast cancer specimens with immunohistochemistry (Fig. 4, supplemental data online). Transfection of LHR or FSHR small interfering RNAs (siRNAs) reduced the expression of the corresponding receptor's protein (Fig. 1A-B) and mRNA (Fig. 1C).

LH and FSH receptor activation turns into cAMP production in target cells. To show that FSH and LH receptors in T-47D cells are functional, we measured cAMP levels upon administration of gonadotrophins. Both LH and FSH increased intracellular cAMP levels in a time- and concentration-related manner (Fig. 1D-E). Ablation of LHR or FSHR with siRNAs blocked cAMP production during LH and FSH administration (Fig. 1D-E).

LH and FSH rapidly induce FAK and Moesin phosphorylation through FSH and LH receptors

Treatment with follicular phase levels of LH and FSH (5 mIU/ml) of T-47D breast cancer cells rapidly increased Thr^{558} Moesin phosphorylation, which corresponds to activation (Simoncini, Scorticati, Mannella et al., 2006,Fu, Giretti, Baldacci et al., 2008,Sanchez, Flamini, Fu et al., 2009). This phenomenon was time-dependent and transient, being maximal after 10–20 minutes and receding to baseline after 60 minutes (Fig. 2A-B). In parallel, treatment with LH and FSH (5 mIU/ml) rapidly increased focal adhesion kinase (FAK) phosphorylation on Tyr^{397} with a similar temporal pattern. Phosphorylation of Moesin and FAK was related to LH and FSH concentration, however, when the concentrations of either FSH or LH were raised over 50 mIU/ml, the phosphorylation levels of FAK and moesin went down (Fig. 2C-D). No increase in the levels of Moesin or FAK phosphorylation was found in the absence of treatment (Fig. 5A, supplemental data online). The increase in phosphorylation of Moesin or FAK associated with the administration of 5 mIU/mL of FSH

or LH was somewhat comparable to that obtained with 1nM 17β-estradiol (E2), corresponding to a late-follicular phase level of this hormone (Fig. 5 B-C, supplemental data online).

Treatment with LH and FSH for 20 minutes (both 5 mIU/ml) resulted in a rapid change in actin organization, with a remodeling of the actin cytoskeleton toward the cell membrane resulting in a thickening of the membrane (Fig. 2E).

Silencing of LHR or FSHR determined a significant decrease in Moesin and FAK phosphorylation during exposure to LH and FSH (5 mIU/ml) (Fig. 3A-C), without modifications of Moesin or FAK expression. To confirm that the administration of siRNAs does not turn into non-specific effects, cells were also transfected with scrambled (inactive) siRNAs, with no functional consequence (Fig. 3A-B).
Moreover, LH still increased the amounts of Thr^{558}-p-Moesin and Tyr^{397}-p-FAK in cells where siRNAs toward FSHR was present, and FSH did the same in cells exposed to LHR siRNAs (Fig. 3C).

A 20-min exposure of T-47D cells to LH or FSH (5 mIU/ml) determined a shift in the localization of Thr^{558}-p-Moesin and Tyr^{397}-p-FAK toward the membrane, where cortical actin and vinculin complexes were formed (Fig. 3D-E). LHR and FSHR silencing resulted in lack of actin remodeling and blockade of focal adhesion complexes formation, along with absent Moesin or FAK phosphorylation in response to LH and FSH (Fig. 3D-E).

LHR and FSHR activate Moesin and FAK through a G protein-dependent signaling pathway

The G proteins $G\alpha_i$ and $G\beta_1$ are a key step in signaling to FAK (Sanchez et al., 2010). Blockade of these proteins with dominant negative constructs or siRNAs resulted in impaired phosphorylation of FAK $tyrosine^{397}$ in the presence of LH or FSH, but not of Moesin (Fig. 4A). On the opposite, blockade of $G\alpha_{13}$, which is involved in the signaling to Moesin (Sanchez, Flamini, Genazzani et al., 2013), with a dominant negative construct, impaired Moesin phosphorylation by LH and FSH, but not FAK phosphorylation (Fig. 4B).

In agreement with the previous set of experiments, LH and FSH (5 mIU/ml) induced a quick membrane co-localization of LHR and FSHR with $G\alpha_i$ and $G\alpha_{13}$ (Fig. 4C-F). This finding supports the concept that FSHR and LHR in T-47D cells are functional and that they work as G protein-coupled receptors as expected.

Intracellular events linking activation of LHR and FSHR to Moesin and FAK

Moesin and FAK activation in breast cancer cells requires c-Src signaling to the phosphatidylinositol-3-OH-kinase (PI3K)/Akt pathway. Akt activation then leads to Rho-associated kinase (ROCK)-mediated activation of Moesin. Hence, we assessed if this signaling module is also triggered by FSHR and LHR in T-47D cells. LH and FSH (5 mIU/ml) administration to T-47D cells resulted in c-Src and Akt phosphorylation (Fig. 5A-B). In addition, ROCK-2 was functionally activated in the presence of LH or FSH as shown by enhanced Thr-phosphorylation of the bait protein myelin basic protein (MBP) by ROCK-2 immunoprecipitates (IPs) (Fig. 5C).

Consistently with the previous findings, blockade of phosphatidylinositol 3-OH kinase (PI3K) with wortmannin (WM), of Rho-associated kinase (ROCK-2) with Y-27632 (Y) or of G proteins with pertussis toxin (PTX) abolished LH/FSH-dependent Moesin phosphorylation (Fig. 5D-E). Inhibition of ERK1/2 with PD98059 (PD) or of c-Src with PP2 was ineffective (Fig. 5D-E). Efficacy of PP2 and of PD98059 in inhibiting the activation of c-Src and of ERK1/2 was confirmed (Fig. 1A-D, supplemental data online). FAK phosphorylation induced by either LH or FSH was prevented by PTX, PP2 and WM, but not by PD (Fig. 5D-E). Interestingly, the ROCK inhibitor Y-27632 prevented FAK phosphorylation induced by FSH treatment, whereas it did not block FAK phosphorylation induced by LH (Fig. 5D-E).

Consistent results were found using the same set of inhibitors when Thr^{558}-p-Moesin or Tyr^{397}-p-FAK localization to the plasma membrane in the presence of LH or FSH was tested (Fig. 6A-D, supplemental data online).

To check whether the set of signaling events that we found in T-47D cells could extend to other breast cancer cell types, we repeated the key experiments in MCF-7 cells, where we could replicate FSH and LH activation of Moesin and FAK, and the same pattern of signaling (Fig. 7A-B, supplemental data online).

Effects of LH and FSH on breast cancer cell horizontal migration

We next studied whether the signaling actions of LH and FSH to cytoskeletal regulation translate into functional modulation of breast cancer cell motility. In order to distinguish cell migration from proliferation, we pre-treated breast cancer cells with cytosine b-D-arabinofuranoside hydrochloride (ARA-C, 10 µM), a selective inhibitor of DNA synthesis that does not inhibit RNA synthesis. Both LH and FSH (5 mIU/ml) promoted breast cancer cell horizontal migration (Fig. 6A-B). The extent of migration associated with this concentration of gonadotrophins was roughly comparable to that obtained with 1 nM E2 (Fig. 8, supplemental data online). Silencing LHR or FSHR with siRNAs significantly reduced T-47D cell migration (Fig. 6A-B).

Silencing FAK with small interfering RNAs and Moesin with antisense phosphorothioate oligonucleotides both resulted in decreased cell motility during exposure to gonadotrophins (Fig. 6A–B). Similar inhibitory effects were found in cells treated with siRNAs for ROCK-2 or c-Src and in the presence of the PI3K inhibitor, wortmannin (Fig. 6A-B). In parallel, no inhibitory effect was found when PD98059 was added to either LH or FSH (Fig. 6A-B).

LH and FSH effects on T-47D cell invasion of three-dimensional matrices

To test the impact of the LH/FSH-dependent signaling to Moesin or FAK on breast cancer cell invasion we performed three-dimensional invasion assays using Matrigel. ARA-C-pretreated cells showed enhanced invasion of the matrix in the presence of LH or FSH (both 5 mIU/mL, Fig. 7A-B), which was comparable in range with that obtained with 1 nM E2 (Fig. 9, supplemental data online). Inactivation of LHR, FSHR, c-Src, FAK, ROCK-2 and Moesin all blocked the effect of LH and FSH (Fig. 7A-B).

Discussion

The key finding of this work is that LH and FSH act directly on breast cancer cells, increasing motility and invasion. This is achieved through the recruitment of functional LH and FSH receptors that are expressed in these cells. LH and FSH receptors signal to the actin regulators Moesin and FAK through two distinct signaling cascades, enhancing actin remodeling and the turnover of focal adhesion complexes.

Previous publications suggest that LH and FSH may promote cell movement in cancer cells, such as ovarian (Mertens-Walker, Bolitho, Baxter et al., 2010) and endometrial cancer cells (Noci, Pillozzi, Lastraioli et al., 2008). The identification of actions of gonadotrophins related to breast cancer progression may stimulate investigating whether therapies decreasing LH or FSH could counteract the progression of this disease.

Drugs that decrease the synthesis and release of FSH and LH (gonadotrophin-releasing hormone [GnRH] agonists and antagonists) are widely available. These drugs are used to decrease the circulating amounts of sex steroids in fertile females or males as adjuvant treatment of hormone sensitive-tumors, such as breast or prostate cancer (Huhtaniemi, White, McArdle et al., 2009,Chengalvala, Pelletier and Kopf, 2003). However, their possible use in the management of cancers in castrated or menopausal individuals, where they would turn into decreased gonadotrophin levels, has never been studied, as the rationale for this is currently missing. Similarly, clinical trials investigating gonadotrophin suppression for the treatment of rare, gonadotrophin-sensitive cancers, such as ovarian granulosa cell tumors, have been scanty and contradictory (Fishman, Kudelka, Tresukosol et al., 1996,Maxwell, Soisson and Miles, 1994). Thus, it is important to further characterize the biology of gonadotrophins and of their receptors in the setting of cancer. This concept has been recently reinforced by the finding of a strong expression of FSHR by tumor-specific vascular endothelial cells in a wide variety of cancers, including breast cancer (Radu, Pichon, Camparo et al.). Expression of gonadotrophin receptors in breast cancers is however still very debated. A recent manuscript reported that on over 1500 different breast cancer samples, LH receptor expression was absent in over 60% of the samples and detectable but at very low levels in the remaining 40% (Kuijper, Ruigrok-Ritstier, Verhoef-Post et al., 2009), raising doubts on the possible relevance of such a low expression in the clinical setting. Our results are relevant to T-47D cells, but we were also able to show expression and

functional responses in MCF-7 and MDA-MB-231 breast cancer cells, hence suggesting that FSHR and LHR signaling to cytoskeletal controllers may also extend to other breast cancer cell lines. On the other end, a number of publications have been produced to back up the epidemiological evidence of low morbidity of breast cancers during pregnancy. This work has concentrated on the recognized effects of LHR in the presence of the pregnancy-associated gonadotrophin HCG (human chorionic gonadotrophin), showing how HCG administration to breast cancer cell lines reduces proliferation and induces apoptosis (Srivastava, Russo, Mgbonyebi et al., 1998). While HCG is different from LH or FSH and it is produced only during pregnancy, this work supports the principle that gonadotrophins may also regulate directly breast cells. At the same time, mechanistic differences in LH receptor recruitment by HCG vs. LH may explain the discrepancy between our results and those from these studies.

Our manuscript describes actions of FSH and LH receptors that alter breast cancer cell motility. The regulation of the actin-binding protein, Moesin, and of the focal adhesion modulator, FAK, may justify functional effects on breast cancer progression. Indeed, Moesin expression and sub-cellular distribution are deranged in metastatic breast cancers (Giretti et al., 2008). A Moesin-related protein, ezrin (Tsukita and Yonemura, 1999), is over-expressed in endometrial (Chen, Fadiel, Feng et al., 2001) and breast carcinomas (Elliott, Meens, SenGupta et al., 2005), being related to the presence of tumor metastasis. Moesin resides at the nexus of multiple pathways regulating cell attachment with the extracellular matrix and with nearby cells, cell motility and metastatic potential as well as cell survival. These functions are orchestrated by Moesin, which supports the formation of cortical actin complexes that help the formation of molecular bridges between the actin cytoskeleton, integrins and focal adhesion complexes within membrane ruffles and pseudopodia (Pollard and Borisy, 2003).

Over-expression of FAK has also been related to metastatic behavior of various tumors, including breast (Lark, Livasy, Dressler et al., 2005), lung (Fong, Liu, Huang et al., 2009), and ovarian cancer (Hu, Meng and Fang, 2008). On the opposite, silencing of FAK in breast cancer results in cell senescence and in loss of invasive ability (Pylayeva, Gillen, Gerald et al., 2009). FAK is involved in the turnover of adhesion sites, which is crucial for cell adhesion, proliferation, migration, invasion and survival (Mitra and Schlaepfer,

2006). FAK is a non-receptor tyrosine kinase that recruits Src family kinases and PI3K via auto-phosphorylation (Thamilselvan, Craig and Basson, 2007). Phosphorylation of FAK on Tyr^{397} creates a high affinity binding site for the c-Src homology 2 (SH2) domain of several proteins including c-Src kinase (McLean, Carragher, Avizienyte et al., 2005). The association of c-Src with FAK leads to a conformational change and to the activation of c-Src. This leads to recruitment and/or phosphorylation of a number of other proteins involved in motility and invasion, as much as in cell growth and survival (McLean et al., 2005).

Thus, identification of Moesin and FAK as targets of gonadotrophins may offer mechanistic insights into the possible role of these hormones on breast cancer progression.

The re-structuring of cortical actin and focal adhesion complexes induced by gonadotrophins in breast cancer cells is triggered by a FSHR/LHR signaling to $G\alpha_{13}$ and ROCK-2 to Moesin and via a parallel pathway involving $G\alpha_i/G_\beta$ and c-Src to FAK. These cascades require phosphatidylinositol-3 kinase, which seems to play a central role for the activation of both Moesin and FAK by gonadotrophins. Relevant to this point, we have recently identified that the G-protein coupled prolactin receptor is also able to stimulate breast cancer cell motility by controlling c-Src and FAK (da Silva, do Amaral, Gabrielli et al., 2015). It is also interesting the fact that within a physiological range of concentrations, a bell-shaped curve of FAK and moesin activation by gonadotrophins is found, with higher amounts resulting in less or no activation compared to low-intermediate amounts. This may point to a complex set of regulatory events, possibly plaid though differential activation of G proteins. Overall, some of these molecular events may thus offer potential for specific interference with suitable pharmacological or biological tools, which could be of interest for new strategies to alter the metastatic potential of hormone-sensitive cancers.

In conclusion, the present results suggest that within the actions of LH and FSH receptors outside the gonads, rapid signaling to the actin cytoskeleton through the $G\alpha_i/G\beta$/c-Src/PI3K/FAK and $G\alpha_{13}$/ROCK-2/Moesin cascade are relevant for the generation of LH- and FSH-dependent breast cancer cell movement and invasion. Through these cascades, gonadotrophins lead to rapid changes of cell membrane morphology; with a rearrangement of the actin cytoskeleton and the formation of focal adhesion complexes at sites where

structures related with cell movement are formed. The identification of these actions provides original information on the effects of LH and FSH on breast cancer and might potentially be useful to develop new tools to interfere with the ability to diffuse locally or at distant sites of breast tumors.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure Legends

Figure 1. T-47D breast cancer cells express functional LH and FSH receptors. (A-B) T-47D breast cancer cells were transfected with siRNA *vs.* LH Receptor and FSH Receptor (siRNAs LHR and FSHR) or with vehicle, and protein analysis for FSHR or LHR and actin were performed on cell lysates with or without treatment for 20 min with LH and FSH. Bar graphs indicate the mean intensity ± SD of the bands assessed using three separate experiments. **(C)** RNA extracted from breast cancer cells was tested with Real-Time PCR to monitor the presence of LHR and FSHR coding RNA sequences, as well as the efficacy of siRNA interference. (**D-E)** cAMP production was measured in T-47D cell lysates. The results are expressed as the mean ± SD of the measurements. * = $p < 0.05$ vs. control.

Figure 2. LH and FSH induce Moesin and FAK phosphorylation and a dynamic actin remodeling in T-47D cells through FSH and LH receptors. Protein extracts show the time- **(A-B)** and concentration- **(C-D**) dependent FAK/Moesin phosphorylation in T-47D breast cancer cells after treatment with the LH or FSH. Total cell amount of wild-type FAK, Moesin or Tyr^{397}-phosphorylated FAK (p-FAK) and Thr^{558}-phosphorylated Moesin (p-Moesin) are shown with western blot. **E)** Actin fibers were stained with phalloidin linked to Texas Red (red labeling) and nuclei were counterstained with DAPI (blue labeling). The white boxes on the cells indicate a sample cellular area that is analyzed in the colored graphs. In these graphs, the longitudinal axis represents the gray level and the horizontal axis indicates pixels. Blue, red and yellow areas indicate the extracellular, plasma membrane and cytoplasmic fractions, respectively. Mean intensity ratio of actin staining in the membrane/cytoplasm in the same experiment. The results are derived from the sampling of five areas of the cell membrane of 40 different random cells. Areas of minimum and maximum cell membrane thickness were always included. The results are expressed as the mean ± SD of the measurements. * = $p < 0.05$ vs. control.

Figure 3. LH and FSH signal to FAK and Moesin through LH and FSH receptors. (A-C) T-47D breast cancer cells were transfected with siRNA *vs.* LH Receptor and FSH Receptor (siRNAs LHR and FSHR) or with vehicle or scrambled siRNAs, and protein analysis for FSHR, LHR, FAK, phospho-FAK^{397}, Moesin,

phospho-Moesin^{558}, actin were performed on cell lysates after treatment for 20 min with 5 mIU/mL LH or FSH. **(D-E)** T-47D breast cancer cells were transfected with siRNA *vs.* LH Receptor and FSH Receptor (siRNAs LHR and FSHR) or with vehicle. Cells received a vehicle or a 20 min treatment with 5 mIU/mL of either FSH or LH. Cells were stained for phospho-Moesin^{558}, phospho-Tyr^{397} focal adhesion kinase (p-FAK), actin or vinculin and nuclei were counterstained with DAPI. Red arrows indicate actin aggregates at the cell membrane; yellow arrows indicate areas where phosphorylated Moesin or FAK are concentrated at the cell membrane. Representative images are shown.

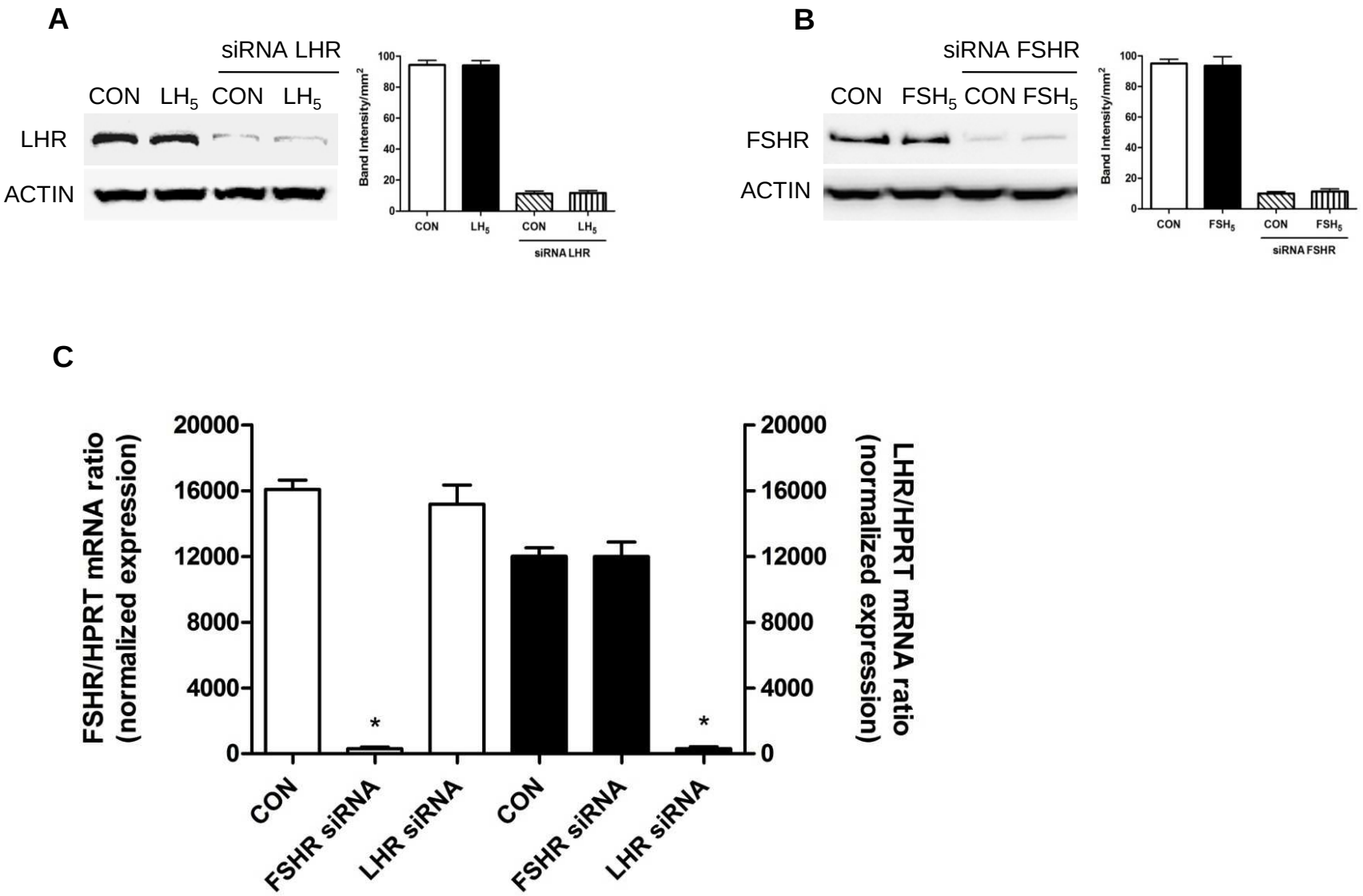
Figure 4. LHR and FSHR signaling to Moesin and FAK. (A-B) Breast cancer cells were exposed for 20 min to 5 mIU/mL of either LH or FSH after transfection with dominant negative $G\alpha_{13}$ or $G\alpha_i$ constructs or siRNAs vs. $G\beta_1$. Wild-type FAK, phospho-FAK^{397}, Moesin, phospho-Thr^{558}Moesin, $G\alpha_{13}$, $G\alpha_i$ and $G\beta_1$ were assayed in cell extracts. **(C-F)** Cells were incubated in the presence of 5 mIU/mL of either LH or FSH for 20 min in baseline conditions or after LHR or FSHR silencing with siRNAs. Co-localization of LHR and FSHR with $G\alpha_i$ and/or $G\alpha_{13}$ was measured with immunofluorescence after staining of LHR and FSHR with FITC, $G\alpha_i$ and $G\alpha_{13}$ with Texas Red and the nuclei were counterstained with DAPI. Small inserts show magnified details of the plasma membrane where co-localized FSHR or LHR with the related G proteins are visible as double-stained areas.

Figure 5. LHR and FSHR signaling to Moesin and FAK. (A-B) Cells were treated with LH or FSH (both 5 mIU/ml) for 20 min in the presence or absence of PP2 or WM. Total c-Src and Akt, and phosphorylated c-Src or Akt amounts are shown. (**C)** ROCK-2 was immunoprecipitated with a specific Ab and the IPs were used to phosphorylate the bait protein, myelin basic protein (MBP). ROCK-2 kinase activity is shown as the amount of phosphorylated MBP (P-MBP). **(D-E)** T-47D cells were exposed for 20 min to 5 mIU/ml LH and FSH, in the presence or absence of the MAPK inhibitor PD98059 (PD; 5 mM), PI3 kinase inhibitor wortmannin (WM; 30 nM), c-Src inhibitor, PP2 (0,2 µM), the G protein inhibitor PTX (100 ng/ml) or of ROCK inhibitor, Y-27632 (Y - 10 µM). Western analysis for the indicated targets was performed.

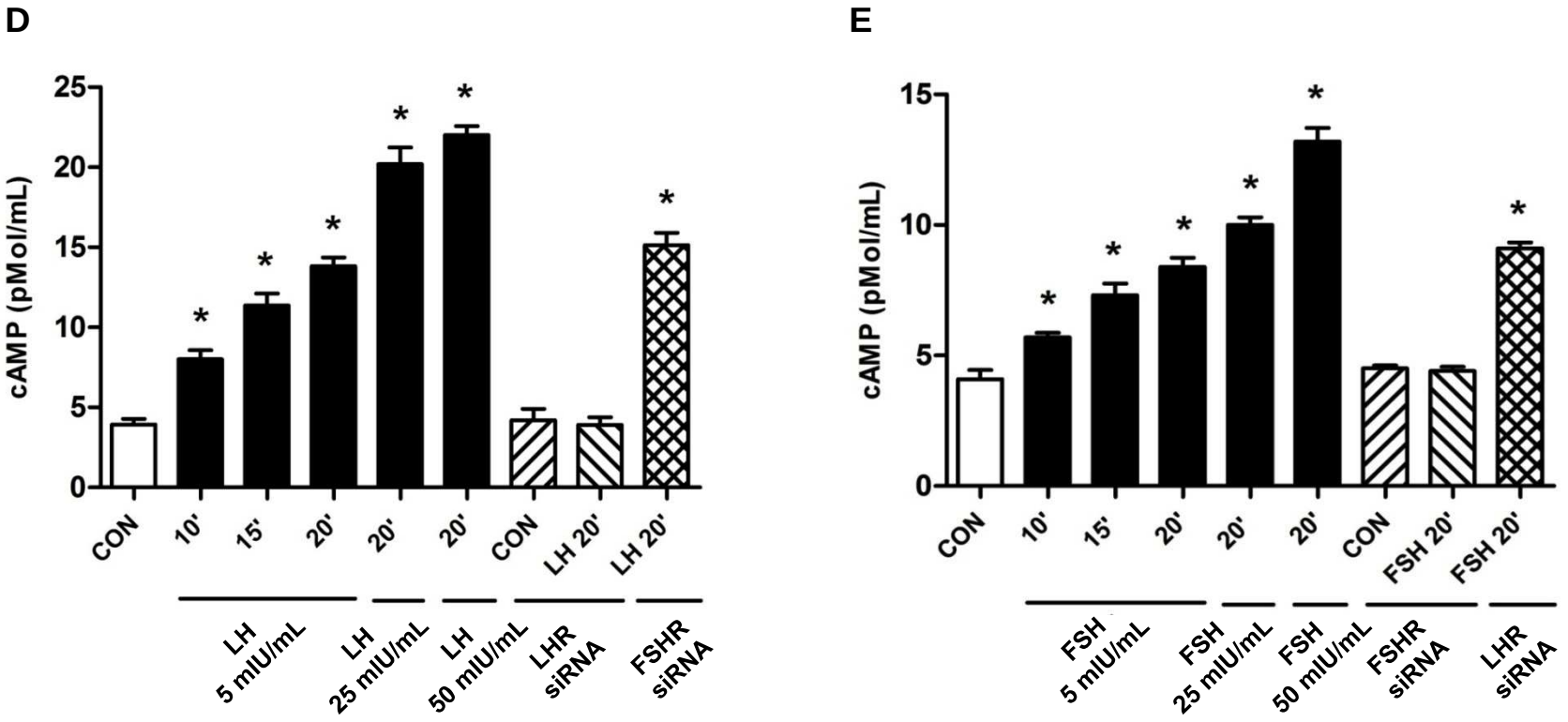
Figure 6. LH and FSH enhance T-47D cell horizontal migration. Cytosine β-D-arabinofuranoside hydrochloride (10 μM), a selective inhibitor of DNA (but not of RNA) synthesis was used 1 hour before treatment to arrest cell proliferation. Breast cancer cells were scraped out of the cell culture dish with a razor blade at the beginning of the experiment and then treated with LH or FSH (both 5 mIU/ml) for 48 hours, in the presence or absence of the PI3 kinase inhibitor wortmannin (WM; 30 nM); of the MAPK inhibitor PD98059 (PD; 5 mM) or after silencing of LHR, FSHR, c-Src, ROCK-2, FAK with specific siRNAs or of Moesin with antisense PONs (Sense PONs for Moesin served as control, data not shown). Horizontal cell migration was measured as the mean ± SD of migration distance from the starting line. **(A)** Shows sample images of horizontal migration in the different conditions, the black lines indicate the mean migration distances. **(B)** Shows the quantitative analysis in terms of mean migration length ± SD. Dates are expressed as the mean ± SD of three separate experiments. * = $P \leq 0.05$ vs. control.

Figure 7. LH and FSH enhance T-47D cell invasion. T-47D breast cancer cells were seeded on top of a Matrigel invasion chamber and then treated with cytosine β-D-arabinofuranoside hydrochloride (10 μM) to arrest cell proliferation. The cells were then treated for 24 hours with LH and FSH (both 5 mIU/ml), after silencing of LHR, FSHR, c-Src, FAK, ROCK-2 with specific siRNAs or of Moesin with antisense PONs (Sense PONs for Moesin served as control, data not shown). Invading cells were photographed at 100 × magnification and counted in the central field of triplicate membranes. **(A)** Shows sample images of invasion in the different conditions. **(B)** Indicates the mean number of invading cells ± SD from three separate experiments. * = $P \leq 0.05$ vs. Con.

Sanchez et al. Fig. 1



Sanchez et al. Fig. 1



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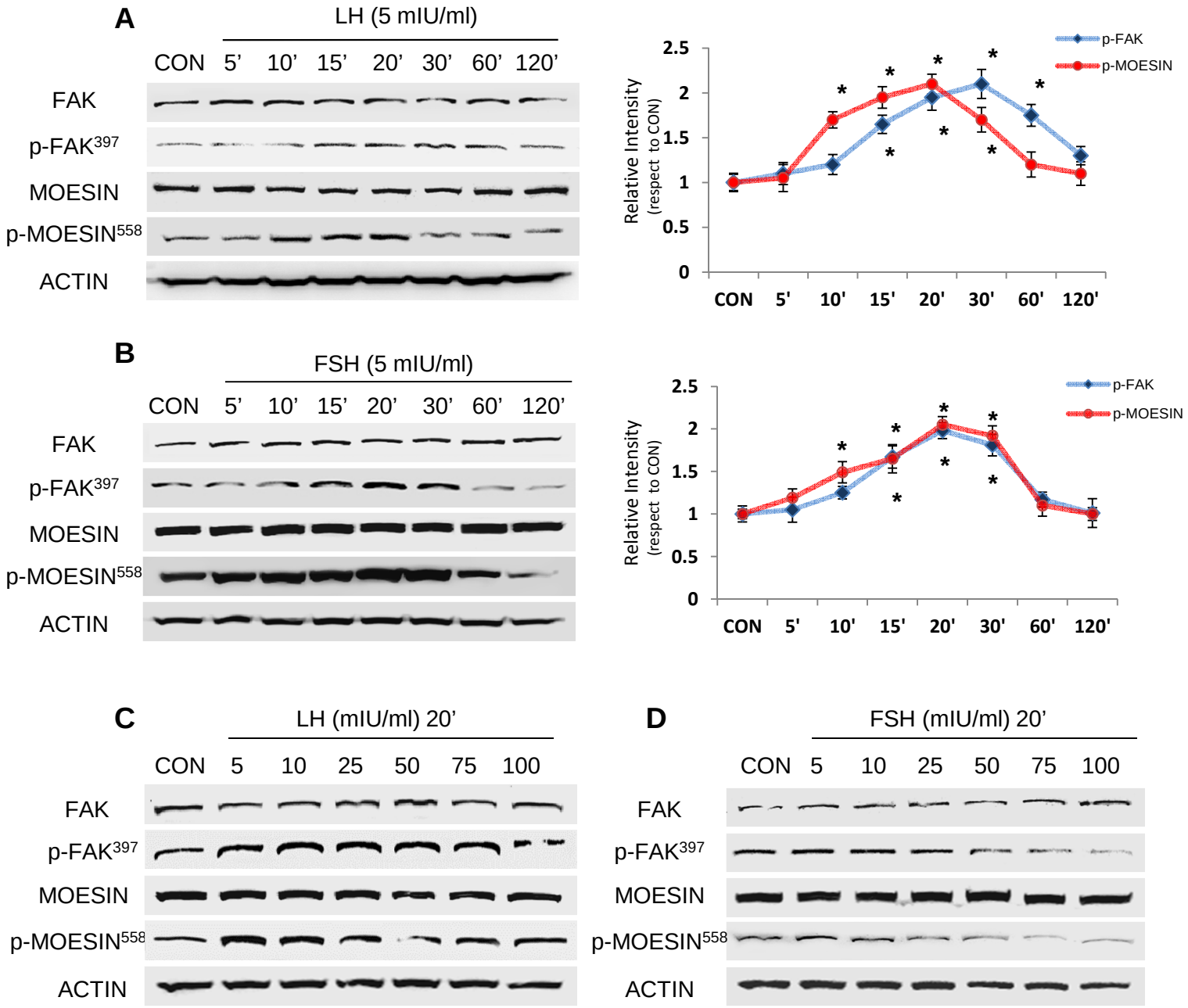

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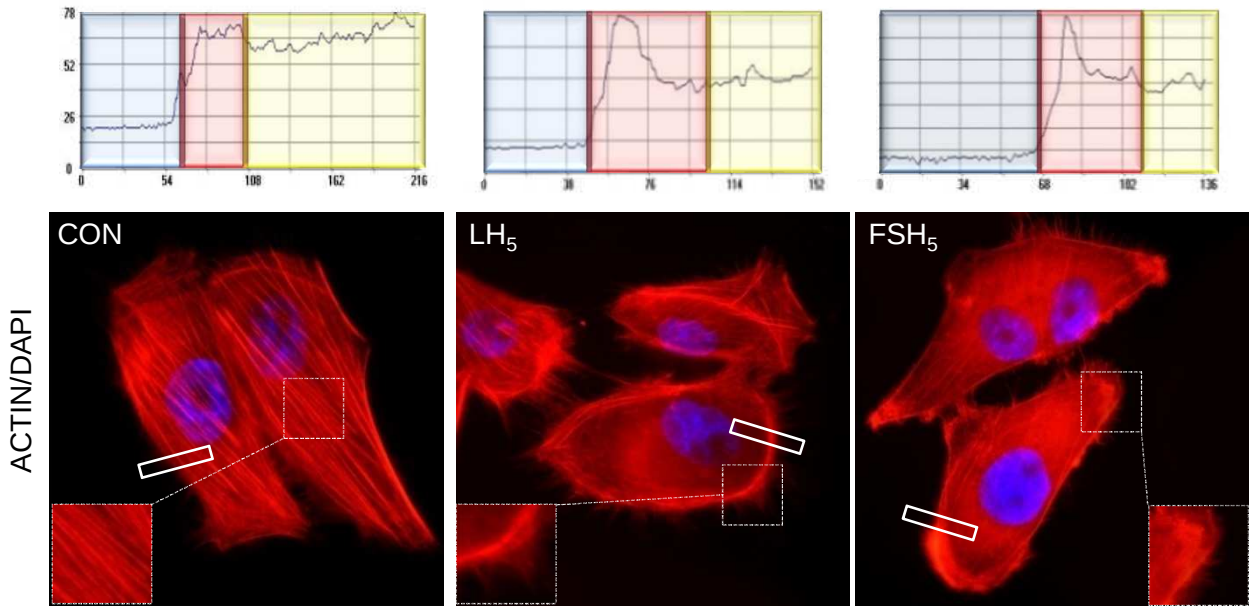
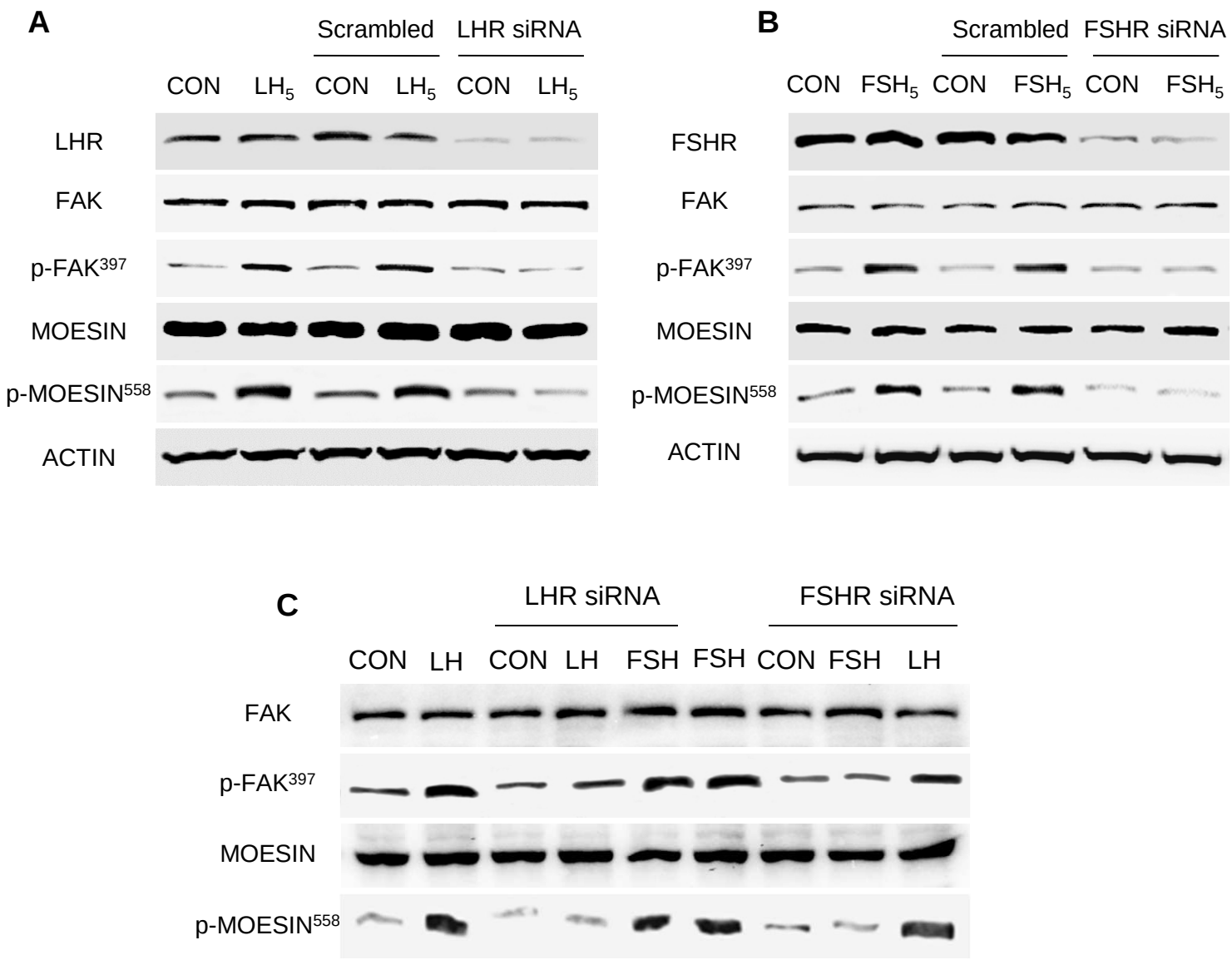
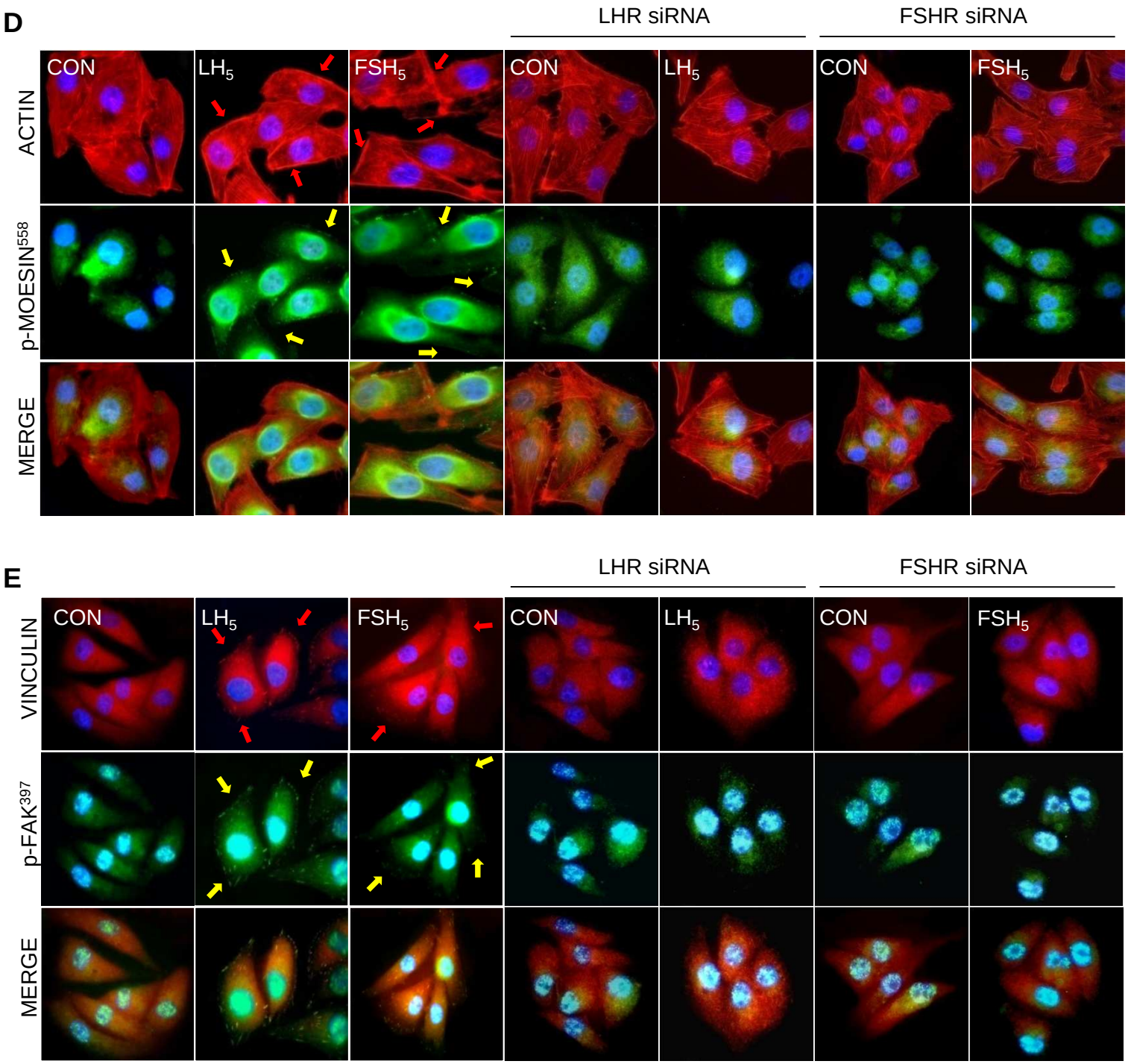


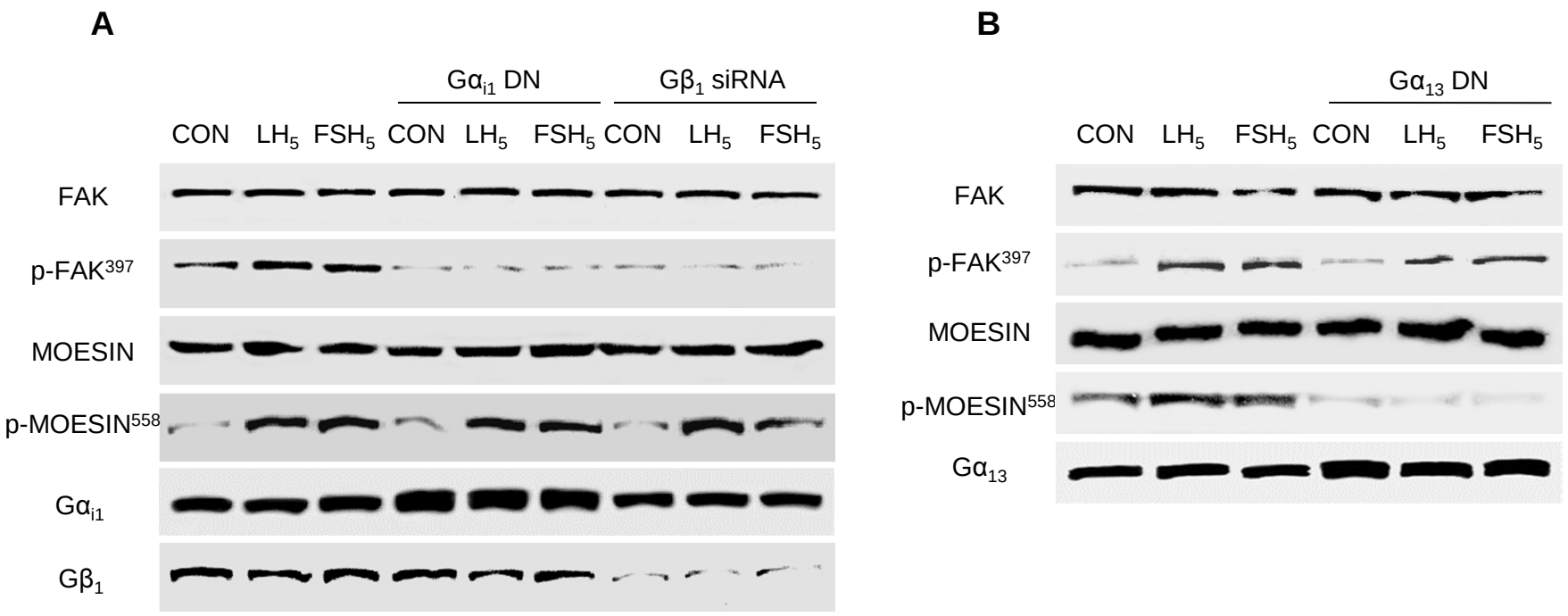
	Mean Membrane Thickness (pixel±SD)	Membrane/ Cytosol Intensity Ratio
CON	18±3,9	1,09
LH_5	56±5,9	2,06*
FSH_5	45±6,2	1,78*

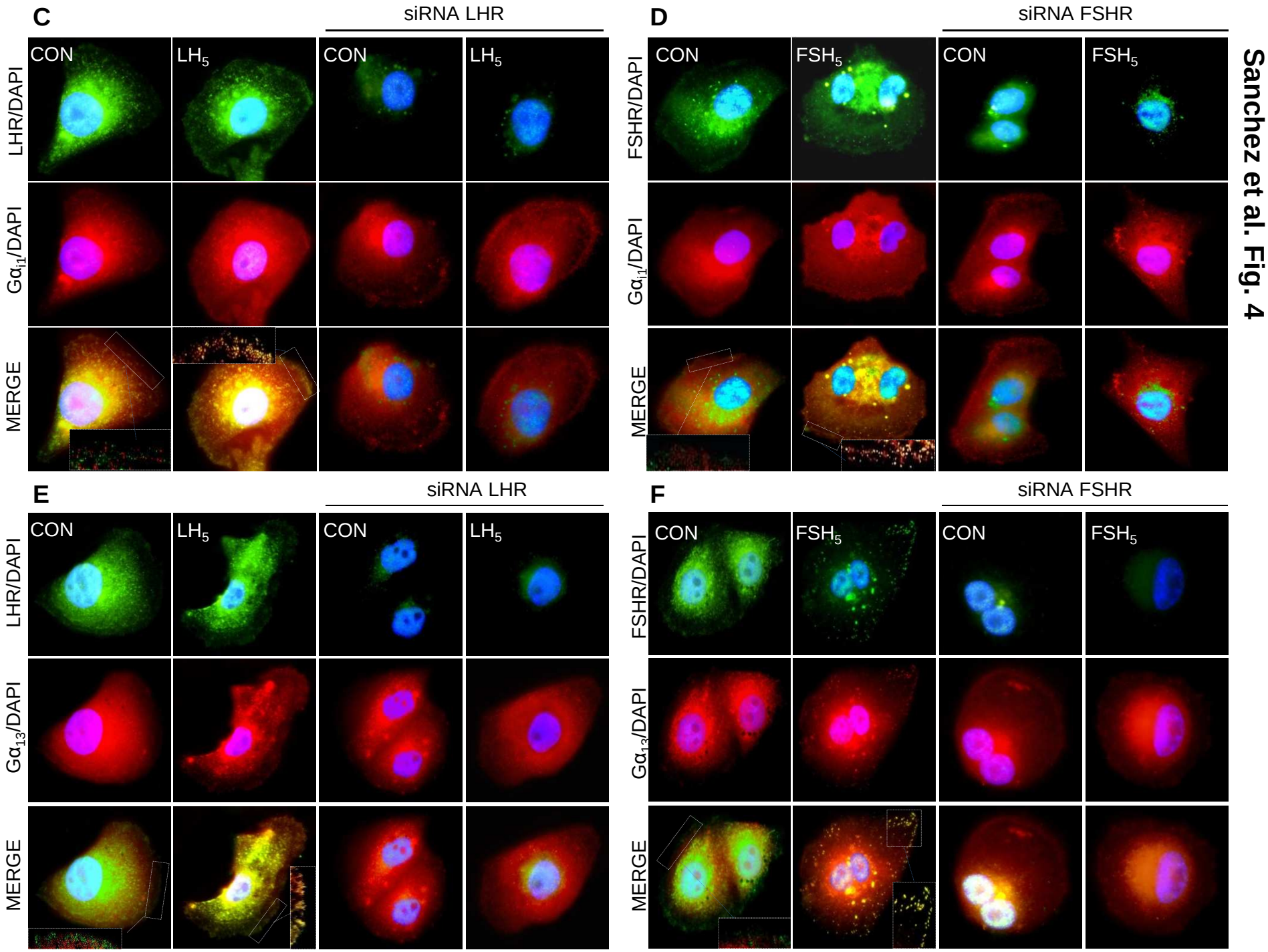
Sanchez et al. Fig. 3





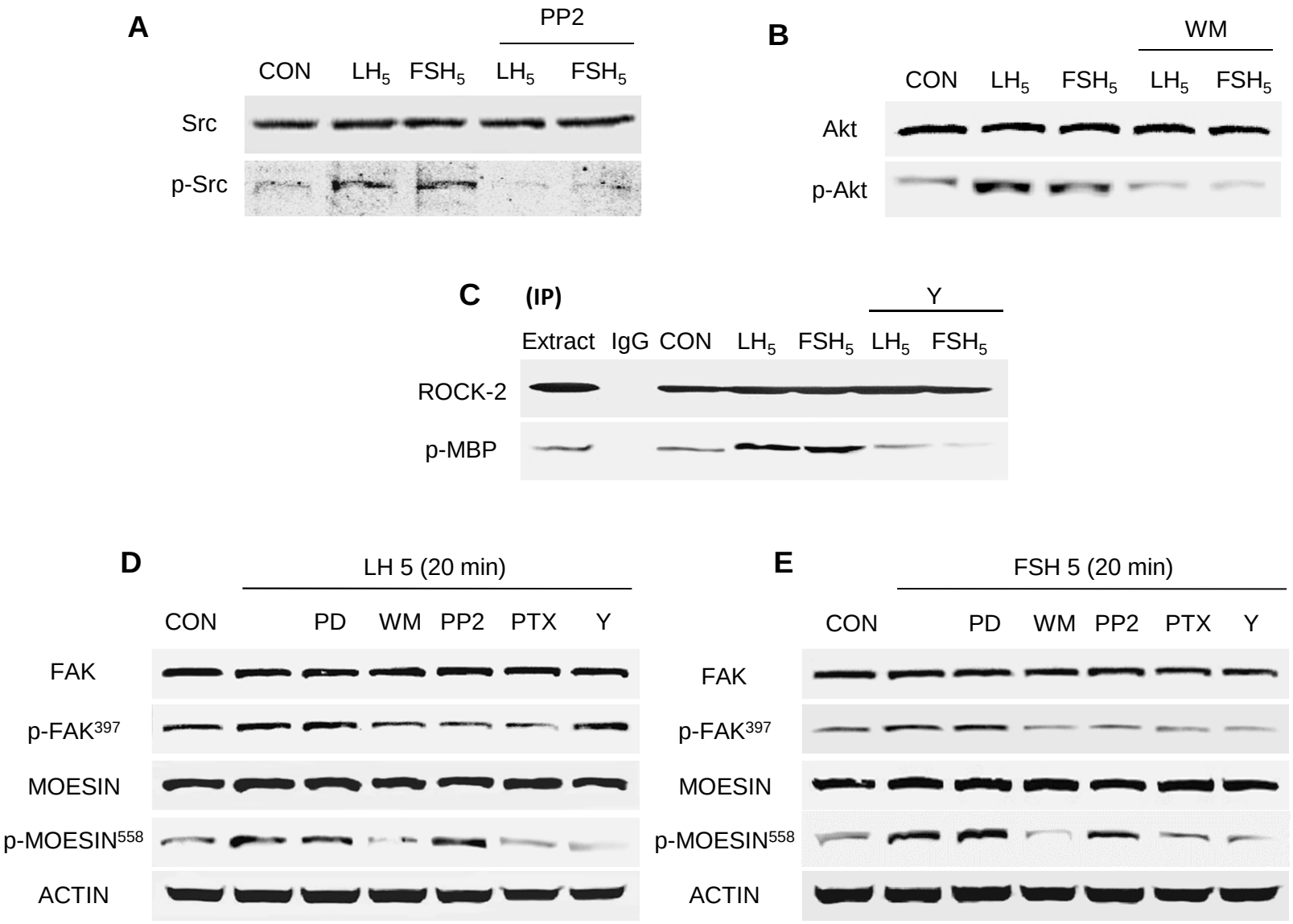
Sanchez et al. Fig. 4



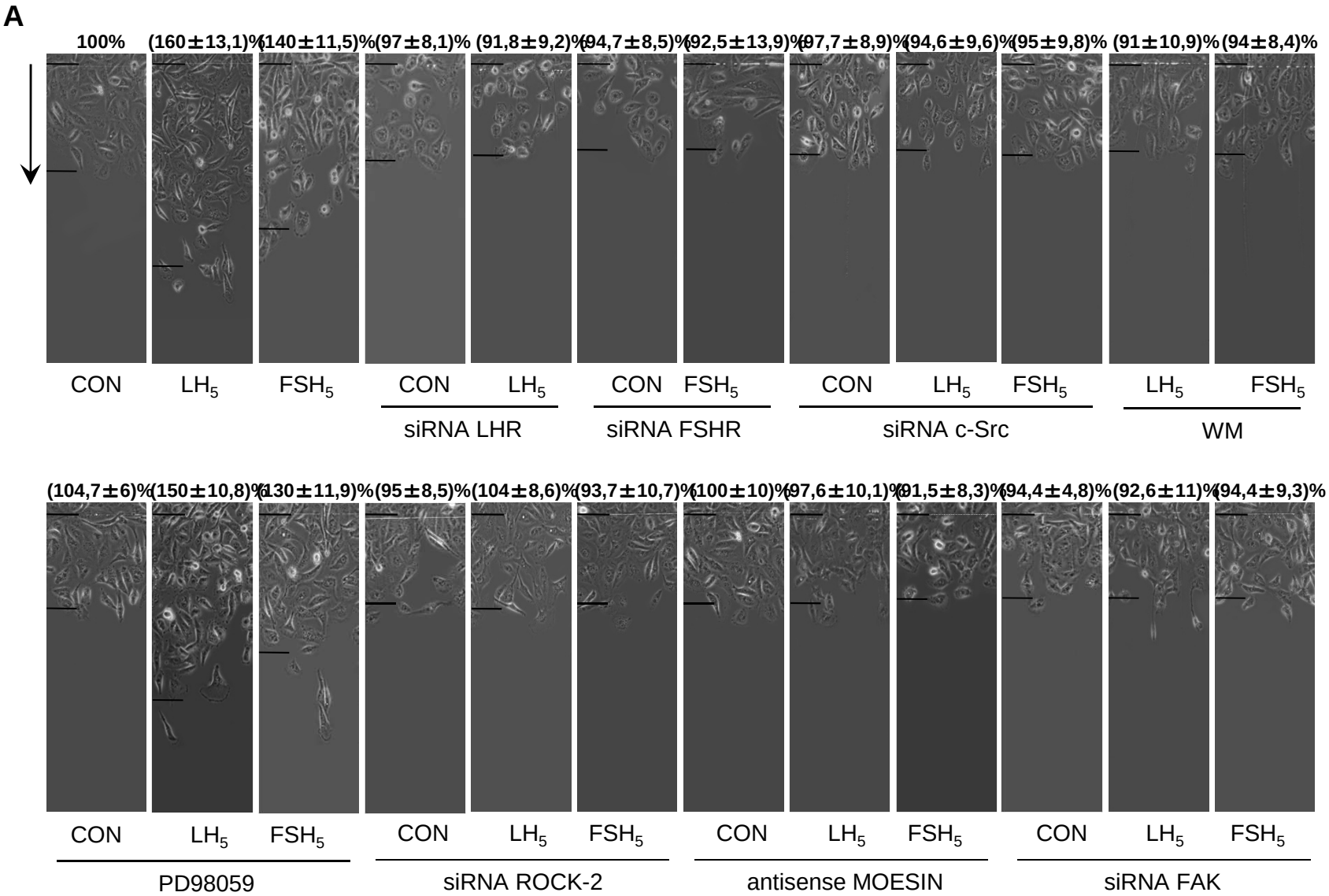


Sanchez et al. Fig. 4

Sanchez et al. Fig. 5

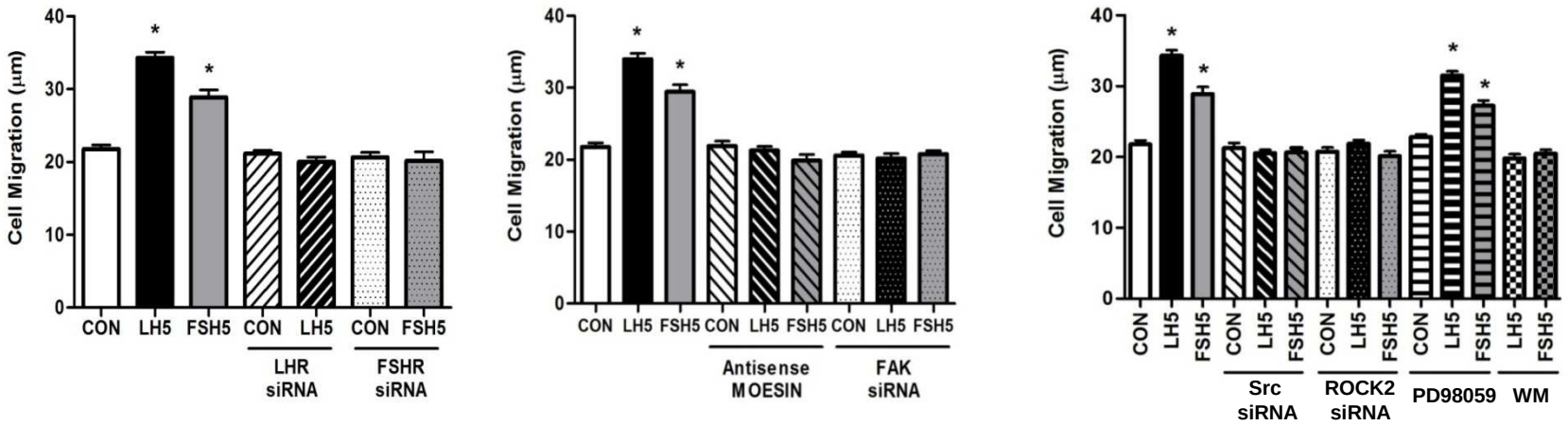


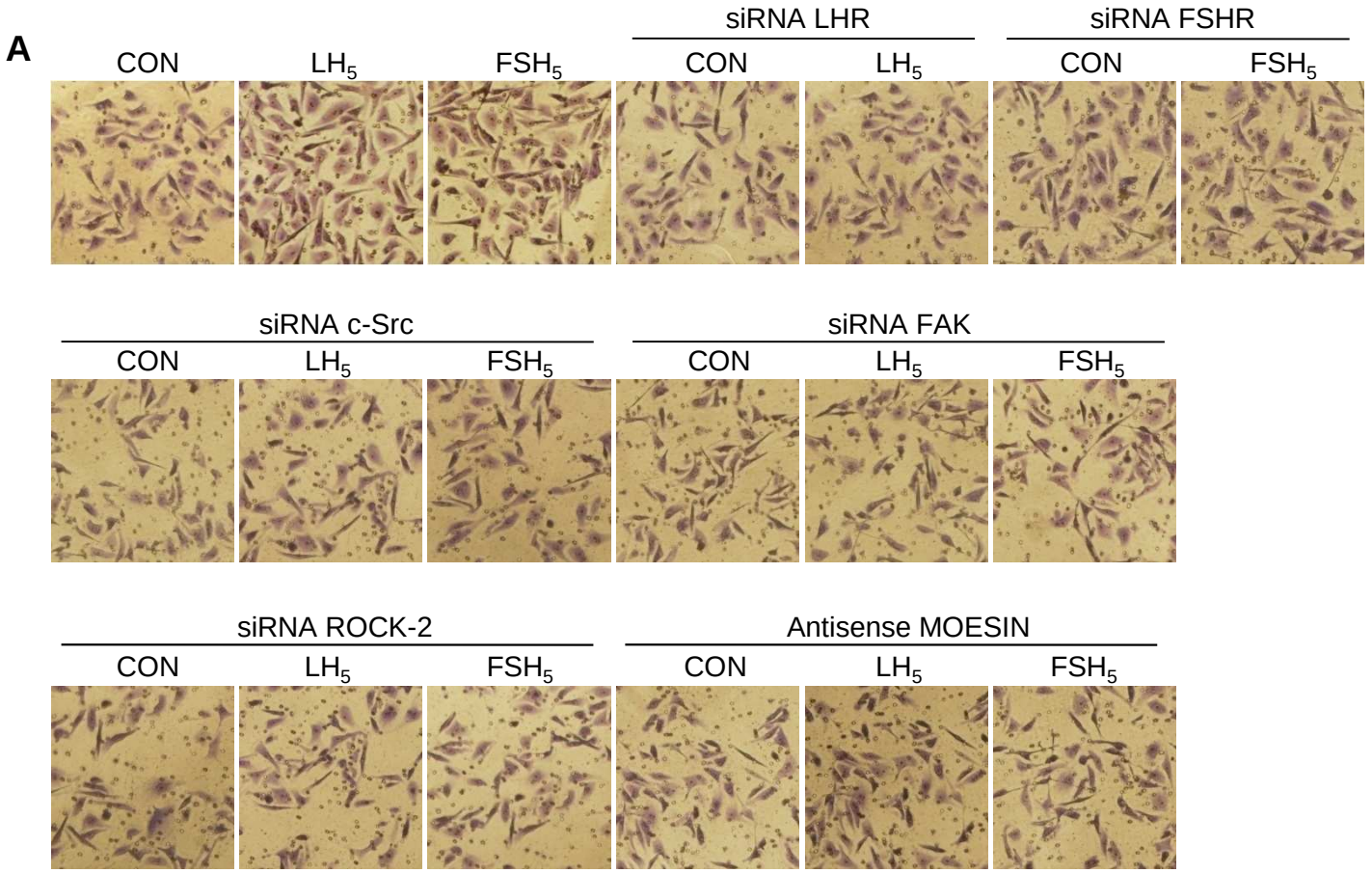
Sanchez et al. Fig. 6

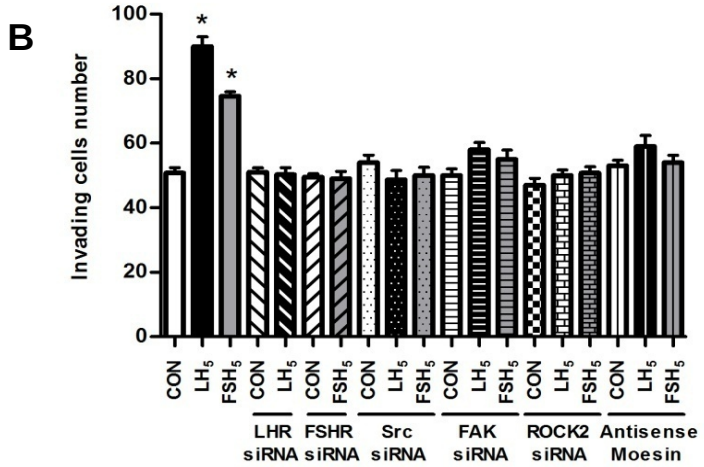


Sanchez et al. Fig. 6

B







Sanchez et al. Fig. 7