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Histamine H4 receptor agonists induce epithelial-mesenchymal transition events and enhance mammosphere formation via Src and TGF-β signaling in breast cancer cells

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Abbreviations

A83-01: A83

BCSCs: breast cancer stem-like cells

CLOB: Clobenpropit

DDA: Dendrogenin A. 5α-hydroxy-6β-[2-(1H-imidazol-4-yl)-ethylamino]-cholestan-3β-ol)

EMT: epithelial-mesenchymal transition

FBS: fetal bovine serum

GPCR: G-protein coupled receptors

IFI: indirect immunofluorescence

J77: JNJ7777120

JNJ28: JNJ28610244

HA: histamine

H4R: histamine H4 receptor

NSCLC: non-small cells lung carcinoma

PP2: 4-Amino-3-(4-chlorophenyl)-1-(t-butyl)-1H-pyrazolo[3,4-d]pyrimidine

α-SMA: α-Smooth muscle actin

TGF-β1: Transforming growth factor-β1

TβRI: Transforming Growth Factor β Type I receptor

VUF: VUF8430

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Abstract

Epithelial-mesenchymal transition (EMT) contributes to cell invasion and metastasis during the progression of epithelial cancers. Though preclinical evidence suggests a role for histamine H4 receptor (H4R) in breast cancer growth, its function in the EMT is less known. In this study we proposed to investigate the effects of H4R ligands on EMT and mammosphere formation as a surrogate assay for cancer stem cells in breast cancer cells with different invasive phenotype. We also investigated the participation of Src and TGF-β signaling in these events.

Breast cancer cells were treated with the H4R agonists Clobenpropit, VUF8430 and JNJ28610244 and the H4R antagonist JNJ7777120. Immunodetection studies showed cytoplasmic E-cadherin, cytoplasmic and nuclear beta-catenin, nuclear Slug and an increase in vimentin and α-smooth muscle actin expression. There was also an enhancement in cell migration and invasion assessed by transwell units. All these effects were prevented by JNJ7777120. Moreover, H4R agonists induced an increase in phospho-Src levels detected by Western blot. Results revealed the involvement of phospho-Src in EMT events. Upon treatment with H4R agonists there was an increase in phospho-ERK1/2 and TGF-β1 levels by Western blot, in Smad2/3 positive nuclei by indirect immunofluorescence, and in tumor spheres formation by the mammosphere assay. Notably, the selective TGF-β1 kinase/activin receptor-like kinase inhibitor A83-01 blocked these effects. Moreover, cells derived from mammospheres exhibited higher Slug expression and enhanced migratory behavior.

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Collectively, findings support the interaction between H4R and TGF-β receptor signaling in the enhancement of EMT features and mammosphere formation and point out intracellular TGF-β1 as a potential mediator of these events.

Key words: Epithelial-mesenchymal transition, Histamine H4 receptor; breast cancer; TGF-β1.

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1. Introduction

Breast cancer is a heterogeneous disease mainly involved in women death worldwide being metastasis rather than the primary tumor a relevant player in breast cancer mortality [1]. Epithelial-mesenchymal transition (EMT) is a physiologic program associated with organogenesis and tissue remodeling. However, accumulating evidence shows that EMT is improperly activated and contributes to cell invasion and metastasis during the progression of epithelial cancers. This critical program comprises a sequence of cellular events including morphological changes, loss of epithelial markers (E-cadherin, desmoplakin, Muc-1, cytokeratin-18, occludins, claudins, and ZO-1) and acquisition of mesenchymal markers (N-cadherin, vimentin, fibronectin, vitronectin, α-smooth muscle actin, and FSP1) [2]. Moreover, EMT induces the expression of transcription factors as Slug, Snail, ZEB and Twist, which in turn regulate the expression of other genes that may confer invasive and metastatic properties to cancer cells [3,4]. Histamine (HA) is a pleiotropic amine that exerts its physio-pathologic actions through the activation of four different receptors H1, H2, H3, H4 (H1R-H4R). These receptors belong to the seven membrane G protein coupled receptors (GPCR) family and diverse signaling pathways are described to be activated by HA or HA receptors ligands stimulation [5]. HA receptors are present in several neoplasms and malignant cell lines [6]. A great body of research supports the relevant role of HA in cancer biology through the modulation of cell growth and tumor microenvironment. Interestingly, the biological responses observed in each case are dependent on HA concentration, HA receptor involved, signaling pathways activated, tissue or cell type and tumor microenvironment as recently reviewed [6]. Moreover**,** high intracellular HA content and release together

with the expression of HA receptors modulating cell growth uphold a potential role of HA as an autocrine growth factor in different tumor tissues and cell lines [6]. Remarkably, several reports reveal that conjugation of 5,6*α*-epoxycholesterol with HA produces Dendrogenin A (DDA**)** which functions as a tumor suppressor metabolite in mammalian [7,8]. Deregulation of DDA biosynthesis in cancer might contribute to endogenous HA action on tumor cell growth since DDA concentration drastically decreases in tumor tissues when compared to healthy tissues.

During the last decade, pharmacology research on H4R (the last HA receptor discovered) has become important in the context of immune and inflammatory disorders. H4R is expressed in cells of the immune system and in different tissues [5]. Particularly, H4R is present in normal and neoplastic mammary tissues and cell lines. H4R expression is higher in carcinomas than in benign lesions being also associated with invasive and aggressive tumors [9]. It was reported that H4R agonists inhibit MCF-7 and MDA-MB-231 breast cancer cells proliferation [10]. *In vivo* studies employing MDA-MB-231 xenotransplanted tumors revealed that the H4R agonists exert an inhibitory action on tumor growth even though the H4R agonist JNJ28610244 produces a significant increase in lung metastases [11]. In agreement, we have previously reported that the H4R agonist VUF8430 or low doses of HA may enhance the activity of metalloproteases and the migratory capacity of MDA-MB-231 cells [12].

The transforming growth factor-β (TGF-β) is a multifunctional cytokine linked to cell growth and differentiation, embryogenesis, immunity, tissue remodeling, fibrosis, and cancer. A dual role for this cytokine has been long discussed in cancer. It is known as a tumor suppressor agent at the beginning of carcinogenesis meanwhile it then assists cancer progression and metastasis by promoting neo-angiogenesis, escape of

immunosurveillance and EMT [13-15]. TGF-β binds and activates cell surface receptor serine/threonine kinase complexes, including TGF-β receptor type II and TGF-β receptor type I. Consequently, Smad family members 2 and 3 are phosphorylated, form a stable complex with Smad family member 4, move to the nucleus, and regulate target genes transcription [13]. In addition, there are non-Smad pathways (MAPK, PI3K/AKT and Rho family GTPases) induced by TGF-β which supplement the canonical via (Smad) driving the cellular responses [16].

Breast cancer stem cells (BCSCs), a small set of cells with properties of stem-like cells, are involved in the heterogeneous population present in the tumor bulk of breast cancer. BCSCs exhibit the phenotype CD44+/CD24−/low, form mammospheres in a serum-free medium and non-adherent condition and initiate tumors in immunocompromised mice [17]. Accumulating evidence shows that EMT is involved in BCSCs generation [18,19].

In this study, we proposed to improve the knowledge about the effects of histamine H4R ligands on EMT induction and mammosphere formation in breast cancer cells and investigate the participation of Src and TGF-β signaling in these events as well.

2. Materials and Methods

2.1 Cell culture

MCF-7 and MDA-MB-231 mammary carcinoma cells were purchased from American Type Culture Collection (ATCC, USA). Cells were subcultured according to the ATCC protocol within 2 months of resuscitation and used between passages 5 and 20. Cell cultures were mycoplasma tested monthly using DAPI as a direct DNA stain.

MCF-7 and MDA-MB-231 cell lines exhibit distinct invasive properties. MCF-7 cells represent the luminal breast cancers; they are non-invasive and show a cobblestone morphology and low migratory capacity. MDA-MB-231 cells represent the triple negative breast cancer (TNBC). They are highly invasive and show a more elongated morphology. Both cell lines express the four histamine (H1-H4) receptor subtypes [9]. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco, CA, USA), 0.3 g/L L-glutamine and 40 mg/L gentamicin at 37ºC in a humidified atmosphere with 5% CO2. Twenty-four hours after plating cells were treated with the following selective H4R ligands: Clobenpropit (CLOB; H3R antagonist/H4R agonist; 3-(1H-imidazol-5-yl)propyl N'-[(4-chlorophenyl)methyl]carbamimidothioate;dihydrobromide; Tocris Bioscience, UK), VUF 8430 (VUF; H4R agonist; 2-(diaminomethylideneamino)ethyl carbamimidothioate;dihydrobromide; Sigma-Aldrich, St Louis, MO, USA), JNJ28610244 (JNJ28, H4R agonist; (Z)-(5-methyl-1H-indol-2-yl)-(1-methyl-piperidin-4-yl)-methanone oxime, Janssen Research & Development, San Diego, USA) and JNJ7777120 (J77; H4R antagonist; (5-chloro-1H-indol-2-yl)-(4-methylpiperazin-1-yl)methanone; Sigma-Aldrich, St Louis, MO, USA). Since the H4R agonists exert an inhibitory effect in MCF-7 and MDA-MB-231 cell proliferation [10], we performed all our experiments using both breast tumor cells after 5 days of treatment to assure that we were working with the surviving cells. The H4R antagonist J77, the selective inhibitor of Src-family tyrosine kinases PP2 (4-Amino-3-(4-chlorophenyl)-1-(t-butyl)-1H-pyrazolo[3,4-d] pyrimidine, Sigma-Aldrich, St Louis, MO, USA) and the selective TGFβ kinase/activin receptor-like kinase inhibitor A83-01 (A83, 3-(6-methylpyridin-2-yl)-N-phenyl-4-quinolin-4-ylpyrazole-1-carbothioamide, Santa Cruz Biotechnology, TX, USA) were added to cell cultures 30

min before H4R agonists for combined treatments. We selected the concentrations of H4R ligands and inhibitors for this study in agreement with our previous reports and other researchers' work [10-12,20]. These concentrations are mentioned in legends.

2.2 Immunoblotting

Immunoblots were carried out as previously described [20]. Briefly, 30-50 µg of protein were separated on a 12% SDS-PAGE gel. Blue Plus® IV Protein Marker (10-180 kDa), DM131-01, from TransGen Biotech Co, Beijing, China was used. Gels were blotted onto a polyvinylidenedifluoride membrane, blocked and finally probed with anti E-cadherin (1:400, Santa Cruz Biotechnology, TX, USA; sc 8426; lot #H0417), anti vimentin (1:500, Invitrogen, CA, USA, 18-0052; lot 1484697A), anti α-SMA (1:500, Abcam, Cambridge, UK; ab5694), anti N-cadherin (1/500, Invitrogen, NY, USA; #33-3900), anti β-catenin (1:400, Santa Cruz Biotechnology, sc 7963; lot B2217), anti Slug (1:200, Santa Cruz Biotechnology, TX, USA ;sc 166476; lot #G1317), anti phospho-Src (1:250, Cell Signaling, MA, USA; #2101), anti Src (1:250, Cell Signaling, MA, USA; #2108), anti TGF-β1 (1:250, Abcam, Cambridge UK, ab27969; lot #GR3193029-1), anti phospho-ERK 1/2 (1:500, Santa Cruz Biotechnology Inc., TX, USA; sc-7383), anti ERK1/2 (1:500, Santa Cruz Biotechnology Inc., TX, USA; sc- 514302),anti β-actin (1:2000, Sigma-Aldrich, St Louis, MO, USA ; A5441; lot #014M4759) or α-tubulin (1:2000, Sigma-Aldrich, St Louis, MO, USA; T8203; lot #059M4863V) antibodies overnight and with anti-mouse IgG (whole molecule) horseradish peroxidase-conjugated (1:3000, Sigma-Aldrich, St Louis, MO, USA; A9044; lot #052M4826) antibody or anti-rabbit IgG (H+L)-HRP conjugate (1:2000, Bio-Rad, CA, USA; 1706515) for 1 h at room temperature. The immunoreactive bands were detected by enhanced chemiluminescence (ECL,

Amersham Biosciences, Argentina) and analyzed by densitometry using the software ImageJ 1.42q (NIH, USA).

2.3 Immunofluorescence studies

Cells grown on cover glasses were fixed, permeabilized, blocked in PBS with 1% bovine serum albumin (BSA) and incubated overnight at 4ºC with anti E-cadherin (1:100, Santa Cruz Biotechnology, TX, USA; sc 8426; lot #H0417), anti β-catenin (1:100, Santa Cruz Biotechnology, TX, USA; sc 7963; lot #B2217), anti Slug (1:100, Santa Cruz Biotechnology, TX, USA; sc 166476; lot #G1317), anti Slug (1:100, Invitrogen, Thermofisher Scientific, MA, USA; PA1-86737), anti phospho-Smad 2,3 (1:100, Cell Signaling, MA, USA; #8828) or anti CD44 HCAM (1:100, Santa Cruz Biotechnology, TX, USA; sc 7297, lot #H0416) antibodies and for 1 h at room temperature with Alexa Fluor 488 dye conjugated anti-mouse IgG (H+L) (1:2000, Thermofisher Scientific, MA, USA; A-11017; lot #1812170) or Alexa Fluor 546 dye conjugated anti-mouse IgG (H+L) (1:2000, Thermofisher Scientific, MA, USA; A-11003) or anti rabbit IgG (whole molecule)-FITC (1:1000, Sigma-Aldrich, St Louis, MO, USA; F9887, lot 079K 4804) antibody plus DAPI (0.1 µg/ml) to stain nuclei. Immunoreactivity was visualized by an immunofluorescence microscope (Olympus Fluo View FV1000). Immunofluorescence studies were also performed using mammospheres.

2.4 Migration and invasion assays

Migration studies were performed as previously described [20]. Briefly, using a 24-well transwell unit with polyethylene terephthalate membranes showing a pore size of 8.0 µm (BD Falcon, Basel, Switzerland). MCF-7 (3.10^4) or MDA-MB-231 (5.10^3) cells in serum-

free RPMI were loaded into the upper compartment. The lower chamber contained treatments in 10% FBS/RPMI medium. After 20 h (MDA-MB-231) or 40 h (MCF-7) at 37ºC non-migrated cells on the upper surface of membranes were gently scrubbed with a cotton swab. Migrated cells were fixed in 4% formaldehyde/PBS and stained with 0.5% crystal violet. Total migrated cells were counted with an Olympus Bx50y microscope and photos were taken with an Olympus DP73 camera. Results were expressed as the number of migrated cells compared to control. For invasion assays, transwell units were previously coated with Matrigel® (BD Biosciences, MA, USA)/RPMI (1:2) and MCF-7 (1.10^5) or MDA-MB-231 (1.10^4) cells were loaded in the upper chamber. Cells that invaded through Matrigel were fixed, stained and counted as previously described for migration studies.

2.5 Mammosphere formation assay

Mammary tumor cells were seeded and treated with H4R ligands during 5 days and then harvested. Next MCF-7 (10^4) and MDA-MB-231 cells (10^3) were plated into each well of the ultralow attachment 12-well plate in RPMI 1640 serum-free medium, supplemented with B27 (1:50, Invitrogen, CA, USA), 20 ng/ml basic fibroblast growth factor (Sigma-Aldrich, St Louis, MO, USA), 20 ng/ml human epidermal growth factor (Sigma-Aldrich, St Louis, MO, USA), 4 µg/ml heparin, and 15 µg/ml gentamicin. After 7-9 days the number of mammospheres/well were determined under a Carl Zeiss inverted microscope and photographs were acquired with Ampscope capture software.

2.6 Statistical analysis

One-way ANOVA and Bonferroni post-test were used to analyze data employing the GraphPad Prism Version 7.0 software (GraphPad Software Inc, Philadelphia, USA). For data with two sets that were normally distributed, an unpaired one-tailed t-test was conducted to determine significance. In all cases, P values less than 0.05 were considered statistically significant.

3. Results

3.1 Effects of H4R ligands on EMT molecular markers in breast cancer cells

To understand the role of H4R ligands on the EMT induction in MCF-7 and MDA-MB-231 breast cancer cells we employed three H4R agonists (CLOB, VUF or JNJ28) and the H4R selective antagonist J77 to evaluate the subcellular localization of E-cadherin, β-catenin, vimentin and Slug by indirect immunofluorescence (IFI). We also evaluated the expression of the mesenchymal markers vimentin, N-cadherin and α-smooth muscle actin (α-SMA) by Western blot.

In MCF-7 cells treated with VUF or JNJ28, there was no change in the expression of the epithelial marker E-cadherin and in β-catenin expression by Western blot (Figure 1A). Moreover, we could not detect the mesenchymal markers vimentin, N-cadherin and α-SMA in control and treated MCF-7 cells (Figure 1A). IFI studies revealed that E-cadherin left the cell membrane to be mainly localized in cytoplasm. In addition**,** nuclear beta-catenin and Slug and a weak cytoplasmic stain for vimentin were detected by IFI upon VUF treatment (Figure 1B). There was also a significant increase in the number of Slug positive nuclei when cells received VUF (Figure 1C). In MDA-MB-231 cells, E-cadherin was not present as previously reported [20,21]. Notably, the H4 agonist VUF augmented

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vimentin and α-SMA expression in a significant way while N-cadherin could not be detected in treated and untreated cells (Figure 1D). We also observed a change in the subcellular localization of β-catenin protein by IFI. We observed nuclear β-catenin and Slug nuclear localization in VUF-treated MDA-MB-231 cells (Figure 1E). Data revealed a significant increase in the number of Slug positive nuclei (Figure 1F). Remarkably, the H4R antagonist J77 blocked all the EMT events induced by H4R agonists in both cell lines (Figure 1).

3.2 H4R ligands modify the migratory and invasive properties of breast cancer cells

EMT is characterized by the activation of a series of events that lead to various degrees of epithelial plasticity and to the activation of migratory programs [3,22].

To explore cell invasion and migration we performed invasion and migration assays employing migration chambers with or without Matrigel, respectively. There was a significant increase in the number of MCF-7 migrated cells upon CLOB or VUF treatment compared to non-treated cells (Figure 2A). However, this increase was not observed when cells received the H4R antagonist J77 previously. Figure 2B shows representative photos. Invasion assays also revealed that the H4R agonists VUF and JNJ28 significantly enhanced the invasive capability of MCF-7 cells (Figure 2C). Similar results were observed in MDA-MB-231 cells (Figure 2D-2F).

The expression of CD44 in tumor cells is associated to a more aggressive and invasive behavior. It is known that EMT promotes CD44 expression being Slug positively correlated with this expression [23]. In order to explore this association, we performed IFI studies to detect CD44 and the EMT transcription factor Slug simultaneously in both

cell lines treated with the H4R agonist. Findings showed that MCF-7 cells expressed this positive correlation upon VUF treatment. In addition, there was a significant increase in MDA-MB-231 cells expressing Slug. However, we could not verify the correlation between Slug and CD44 due these cells were almost 100% positive for CD44 independently of VUF treatment. This result is consistent with the more invasive traits that MDA-MB-231 cells exhibit. Representative photos are shown in Figure 2G while numerical data are seen in Table 1.

3.3 Src phosphorylation mediates EMT changes and cell migration induced by H4R agonists

Src is a non-receptor tyrosine kinase protein implicated in cell adhesion and migration through the interaction with different signaling proteins. We have previously reported that high doses of histamine may prevent radiation-induced mesenchymal changes and cell migration in breast cancer cells through Src inhibition [20]. To investigate whether this kinase was involved in H4R agonist-induced signaling in MCF-7 and MDA-MB-231 cells we evaluated Src phosphorylation by Western blot. Noticeably, H4R stimulation with JNJ28 led to a significant increase in phospho-Src levels while the H4R antagonist J77 prevented this increase in both cell lines. The selective Src inhibitor PP2 also hindered the effect of H4R agonists on Src phosphorylation (Figure 3A).

We then examined the participation of phospho-Src in some molecular and functional EMT related events, like Slug subcellular localization and cell migration, in MCF-7 and MDA-MB-231 cells. After five days we observed that PP2 could block the increase in Slug positive nuclei and reduce the number of migrated cells upon VUF or JNJ28 treatment in both cell lines (Figure 3B, 3C). Our findings support the relevance of Src

activation/phosphorylation in the mesenchymal changes and in cell migration induced by H4R agonists.

3.4 Participation of TGF-β signaling in EMT changes induced by H4R agonists

In the last decade, GPCR transactivation of Protein Serine/Threonine Kinase receptors like the Transforming Growth Factor β Type I receptor (TβRI) has been described [24]. To explore this point, we checked phospho-Smad2/3 and phospho-ERK1/2 as representative molecules of the Smad and non-Smad activated TβRI signaling in MCF-7 and MDA-MB-231 cells respectively. Results are shown in Figure 4. The H4R agonist VUF or JNJ28 significantly increased the number of phospho-Smad2/3 positive nuclei in both cells lines. Notably, this effect was blocked when tumor cells were previously treated with the selective inhibitor of TβRI ALK5 kinase, type I activin A83-01 (A83) (Figure 4A). In addition, the H4R agonists improved ERK1/2 phosphorylation while A83 blocked this effect (Figure 4B). Our findings suggest a possible interaction between H4R and TβRI signaling pathways.

Since TGF-β is a key inducer of EMT in a variety of tumor cell lines [25,26], we investigated intracellular and extracellular TGF-β1 levels at the time that we could observe mesenchymal changes induced by H4R agonists in MCF-7 and MDA-MB-231 cells. Results indicated a significant increase in TGF-β1 intracellular levels by Western blot in both cell lines. Interestingly, A83 pre-treatment significantly blocked this increase (Figure 5A). Moreover, there was a significant enhancement in TGF-β1 content in conditioned media from MCF-7 and MDA-MB-231 cell cultures treated with H4R agonists (Figure 5B). To clarify whether TGF-β1 levels might influence the biological responses related to EMT we performed IFI and cell migration studies adding A83

before VUF or JNJ28 treatment. IFI studies revealed that A83 could prevent the Slug nuclear localization and the rise in Slug positive nuclei induced by H4R agonists in MCF-7 and MDA-MB-231 cells as well (Figure 5C). We also studied the migratory behavior of these tumor cells after five days of treatment. The significant increase in the number of MCF-7 and MDA-MB-231 migrated cells upon VUF or JNJ28 treatment was reduced when cells were pretreated with A83 (Figure 5D). Taken together these findings suggest that the intracellular TGF-β1 might be an important player in the EMT events and migration induced by H4R agonists in breast cancer cells.

3.5 Effect of H4R ligands on mammosphere formation

BCSCs like normal mammary stem cells exhibit the ability to form mammospheres in serum-free medium and non-attachment conditions [17]. In order to evaluate the effect of H4R ligands on mammosphere generation we performed mammosphere cultures. Results indicated a significant increase in the number of mammospheres in MCF-7 and MDA-MB-231 cells treated with the H4 agonists VUF or JNJ28 while the H4R antagonist J77 blocked the effect (Figure 6A). Interestingly, we could observe a substantial reduction in tumor spheres generation when we added A83 alone or in combination with the H4R agonist in cell cultures. We propose the participation of the endogenous TGF-β1 in mammosphere formation since the experimental condition for this assay requires cells to be cultured in serum-free medium. Moreover, intracellular TGF-β1 might participate in EMT events and tumor sphere formation via an autocrine loop since TGF-β1 levels induced by H4R agonists were reduced by A83 as shown in Figure 5A. Evidence supports that epithelial cells may acquire stem cell traits when they undergo EMT [18,27]. In order to explore a potential link between the gain of mammosphere-

forming ability and EMT we performed IFI studies to detect Slug in intact mammospheres and migration studies with cells derived from mammospheres. MCF-7 cells treated with H4R agonists formed tumor spheres exhibiting Slug green fluorescent stain in almost all cells while cells derived from mammospheres exhibited higher migratory properties. Meanwhile non-treated cells generated tumor spheres showing cells with very low green fluorescent stain for Slug and poor migratory properties (Figure 6B, 6C). Even though it was impossible to keep MDA-MB-231 cells in intact spheres like MCF-7 for IFI studies (probably due to E-cadherin absence in the cell membrane), we could observe that almost all cells derived from spheres were positive for Slug. Moreover, their migratory properties were also significantly increased (Figure 6B, 6C). Collectively, data support the relevance of endogenous TGF-β1 in tumor sphere formation and exposed the potential connection between EMT and the mammosphere-forming ability.

4. Discussion

The highly conserved cellular program called EMT is activated during tumor progression, metastasis, drug resistance and tumor recurrence [28]. EMT endows epithelial cells to acquire mesenchymal characteristics to migrate, invade and spread providing changes in the adhesion, motility and ability of tumor cells to degrade the extracellular matrix, as well as resistance to apoptosis during tumor progression and metastasis [2]. Signaling pathways activated by different growth factors and cytokines among other factors are master regulators of EMT and converge on the induction of transcription factors as Snail, Slug, Zeb, Twist. These EMT-transcription factors

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downregulate the expression of epithelial markers like E-cadherin while upregulate the expression of mesenchymal markers in order to endow cells with changes in the structural proteins of the cytosketelon and cell membrane to migrate and invade [3].

We have previously reported that HA may dose-dependently modulate gelatinolytic activities and cell migration in pancreatic and mammary tumor cells disclosing an inhibitory action over 10 µM or a stimulatory effect with doses lower than 1µM [12,29]. Interestingly, the H4R agonist VUF 8430 reproduced the stimulatory action in the triple negative breast cancer (TNBC) cell line MDA-MB-231 [12].

In the present work, we proposed to further investigate the effects of the H4R ligands on EMT and mammosphere formation in MCF-7 and MDA-MB-231, breast cancer cells with different invasive phenotypes, and also evaluate the potential participation of Src and TβRI signaling in these events.

Several reports show that HA and HA receptor ligands may regulate cell-cell adhesion and cadherin expression in different normal and neoplastic cells tightly dependent on the cell/tissue and the HA receptor subtype involved [30-32]. Our results showed cytoplasmic E-cadherin and cytoplasmic/nuclear β-catenin in breast cancer cells treated with H4R agonists. The loss of the epithelial marker E-cadherin from the cell membrane and changes in the subcellular localization, from membrane to cytoplasm, lead to the disruption of intercellular contacts so that epithelial cells separate and are able to migrate. The dissociation of the E-cadherin /β-catenin complex is defined as the cause-effect relationship of the EMT process since β-catenin is a linking protein between E-cadherin and the cytoskeleton catenins [2,33,34]. In addition, accumulating evidence shows that cytoplasmic β-catenin may translocate into the nucleus and act as a transcription factor regulating the expression of EMT related genes like SLUG, SNAIL,

ZEB1, TWIST and cell adhesion molecules genes as CD44 [3,35]. Our findings revealed that H4R agonists led to cytoplasmic and nuclear β-catenin together with a significant rise in the number of Slug positive nuclei and migrated cells in both cell lines. However, we could only observe a significant increase in CD44 positive cells in MCF-7 but not in MDA-MB-231, probably due to their different invasive phenotype. In agreement with our results, Uchino and coworkers demonstrated that the non-invasive cells MCF-7 become aggressive and acquire an invasive phenotype showing nuclear β-catenin and CD44 upregulation when they were subjected to a sequential invasion process [36].

Vimentin, alpha-smooth muscle actin (α-SMA) and N-cadherin are described as acquired mesenchymal markers in cells ongoing EMT [37]. Vimentin is a cytoskeletal protein present in mesenchymal normal cells that participates in attachment, migration, and cell signaling. Its upregulation or its *de novo* synthesis is a mechanism associated with EMT in tumors [38,39]. In the present study H4R agonists increased the expression of vimentin in MDA-MB-231 cells while promoted its appearance in MCF-7 cells. α-SMA, a cytoskeletal marker is usually present in smooth muscle actin, myofibroblasts and blood vessels. However, a high expression of α-SMA has been reported in tumor cells linked to invasive and metastatic behavior and poor prognosis [40]. Our results show a significant enhancement of α-SMA expression induced by the H4R agonist VUF in MDA-MB-231 cells but this protein could not be detected in treated and untreated MCF-7 cells. In agreement with our data, evidence supports the presence of α-SMA in the more invasive and aggressive breast cancer, the basal-like breast cancer phenotype [41]. N-Cadherin is a mediator in cell-cell adhesion in epithelial tissues with different roles during normal development and tumor progression. The replacement of E-cadherin by

N-cadherin leads to weaker cell-cell adhesions between adjacent cells. Though this switch is a typical EMT event related to cancer [42], we could not detect N-cadherin expression, either in control MCF-7 and MDA-MB-231 cells as reported [21,43] or in cells treated with the H4R agonists. In summary, we observed the loss of E-cadherin in cell membrane without changes in its expression level, an increase in Slug and β-catenin nuclear localization and a weak cytoplasmic vimentin expression in MCF-7 cells treated with H4R agonists. Taken together these findings showed that treated MCF-7 cells exhibit some mesenchymal attributes usually acquired by epithelial cells undergoing EMT. Emerging evidence shows that EMT program is not a binary switch to move cells from a full epithelial to a full mesenchymal state [44,45]. Cells undergoing EMT may retain certain epithelial markers while exhibit some mesenchymal markers; this is described as partial EMT. At present, it is known that most tumor cells do not go through a full EMT. As regards MDA-MB-231, control cells show vimentin and α-SMA expression and important Slug and β-catenin nuclear localization which were significantly increased by the H4R agonist treatment, indicating an enhancement of mesenchymal features. It is well known that the structural changes acquired by epithelial cells through EMT contribute to motility and invasiveness [44]. Notably consistent, our data reveal that H4R agonists also increased cell migration and invasion in both cancer cell lines. Unlike our results, other authors have reported a disruption of EMT by H4R agonists in cholangiocarcinoma cells [46] and in human non-small cell lung cancer (NSCLC) [47].

Histamine H4R is a Gi/G0-coupled GPCR that signals increasing intracellular Ca^{2+} and activates kinases such as ERK, PI3K and P38 [5]. Additionally, direct and indirect mechanisms of Src family kinases activation by GPCR have been described [48]. We

have previously reported that histamine in high concentration may prevent the radio-induced EMT and migration through the inhibition of Src phosphorylation in MCF-7 and MDA-MB-231 cells [20]. In this study, we confirm the participation of activated Src in EMT events and migration of both cell lines treated with H4R agonists.

Diverse signaling pathways are involved in EMT induction [49]. Among them, TGF-β canonical and non-canonical pathways play an important role in normal and neoplastic human mammary epithelial cells [50]. We hypothesized that GPCR transactivation of TGF-beta receptor might contribute to H4R agonist-induced EMT. In this regard, we found that H4R agonists significantly increased the number of P-Smad2/3 positive nuclei and also enhanced ERK1/2 phosphorylation in MCF-7 and MDA-MB-231 cells. Besides, the selective TGF-β kinase/activin receptor-like kinase inhibitor A83 prevents these increases and the mesenchymal changes induced by H4R agonists in both cell lines.

We propose a potential interaction between H4R and TGF-β signaling. In line with our results, it has been reported the crosstalk between G-coupled receptor proteinase activated receptor 2 (PAR2) and TGF-β signaling, via Smad2/3 and P38 MAPK, to enhance migration/invasion and metastasis in advanced pancreatic adenocarcinoma [51]. Based on our findings, Src activation/phosphorylation might be upstream or downstream TGF-β signaling; we need to investigate it.

TGF-β1 is considered a key player for EMT and for the maintenance of the mesenchymal and stem cell state in the breast [18,50,52]. In a previous study, we demonstrated that conditioned media from fibroblasts treated with histamine in high concentration (20 μM) decrease TGF-β1 levels in MDA-MB-231 cells and block EMT events supporting the TGF-β1 contribution to EMT-related changes induction via a paracrine or autocrine loop [21]. In this study, we found that H4R stimulation led to a

significant increase in intra and extra cellular TGF-β1 levels at the time we could observe the mesenchymal changes in MCF-7 and MDA-MB-231 cells. Our results are opposite to those reported by Cai *et al*. who described that H4R stimulation in NSCLC impedes EMT progress by inhibiting the TGF-β1 signaling pathway and by reducing levels of several important EMT transcription factors such as ZEB1, Snail and Slug [47]. However, Cai and coworkers have recently demonstrated that genetic variations in the H4R gene may affect H4R function and so attenuate the anti-EMT effect of this receptor in NSCLC as well as the outcome of these patients [53]. Particularly in breast cancer, data from a case-control study performed in Chinese Han populations support the idea that the genetic variations of histamine H4R may also affect the EMT process and metastasis when considering the association between H4R polymorphisms and clinicopathological characteristics of breast cancer [54]. Genetic dysfunctions of H4R should be considered and investigated in MDA-MB-231 and MCF-7 cells.

BCSCs are present in different breast tumors and cell lines and their involvement in radio- and chemo-resistance, tumor recurrence and cell spreading has been extensively commented [18,55]. BCSCs have been identified by the expression of cell-surface markers like CD44+/CD24-/low and ALDH+. Cells with these phenotypes are able of generating a tumor in serial dilution tumorigenesis assays in immunodeficient mice, the golden standard for evaluating CSC properties. In addition, BCSCs cells form mammospheres in non-adherent culture. Given the ability of mammosphere cells to induce tumors in immunocompromised mice [56,57], mammosphere culture, as a surrogate assay, has been used to enrich for BCSCs [17].

Our data demonstrated that H4R agonists enhanced MCF-7 and MDA-MB-231 mammospheres formation, supporting the participation of TGF-β1 possibly via an

autocrine loop. In agreement with our findings, Scheel and colleagues described the TGF-β pathway as a relevant signaling pathway involved in the activation of the EMT program and functioning in an autocrine fashion to maintain the mesenchymal state in the breast [52].

The relationship between cell adhesion, EMT, and the acquisition of stem cell properties has been recently discussed [58]. The loss of E-cadherin by EMT induction in breast cancer cells is associated with increased CD44 expression and stemness [18]. In agreement with these reports, we could observe an enhancement in the simultaneous expression of CD44 and the EMT transcription factor Slug in MCF-7 cells treated with H4R agonists. Although we could not verify this association in the TNBC cell line MDA-MB-231, a meta-analysis recently conducted with breast tumor biopsies revealed that the most aggressive breast cancers like the basal-type exhibit elevated levels of CD44 expression associated with EMT, cancer stem cell gene profiles and poor prognosis [23]. In addition, our results show that H4R agonists enhanced the expression of Slug in mammosphere cells and also increased the migratory properties of cells derived from tumor spheres suggesting a potential link between EMT and the mammosphere-forming ability, an attribute of BCSC. In line with this, it was reported a complex connection between stemness, cellular plasticity, and EMT mediated by the transcription factor Slug in breast cancer [59].

In summary, despite the fact that MCF-7 and MDA-MB-231 cells exhibit different invasive characteristics our findings indicate that H4R agonists may induce EMT events, increase the migratory and invasive properties and enhance mammosphere formation in both cell lines. Our results also point out the participation of Src kinase and TGF-β

signaling in these events and suggest that intracellular levels of TGF-β1 might maintain the mesenchymal state through an autocrine loop.

The role of the H4 receptor and its ligands in breast tumor progression and metastasis is the subject of ongoing studies with controversial results in the last decade. It has been reported that the *in vitro* stimulation of the H4R exerts an inhibitory action on breast cancer cell proliferation while the *in vivo* treatment with the H4R agonist JNJ28 reduces tumor growth but increases lung metastases in immune-deficient nude mice xenotransplanted with MDA-MB-231 tumor [10,11]. In the light of our results, it might be possible that JNJ28 treatment leads to an enrichment of BCSCs in surviving cells of the tumor bulk via EMT promotion, which might explain the increased lung metastases reported by authors. Meanwhile, Sterle and colleagues studied the immunomodulatory function of H4R in breast cancer employing a syngeneic murine model of TNBC and reported that mice lacking H4R (H4R knockout) present reduced tumor size and decreased number of lung metastases compared to wild type mice [60]. Thus, the role of H4R in tumor biology is complex and needs to be further explored.

In order to improve the survival of cancer patients, researchers must face enormous challenges in their fields to find out pharmacological and therapeutic approaches targeting specific molecules or steps that govern the primary tumor growth and prevent the dissemination of cancer cells as well.

Additional Information

Conflict of interest

The authors declare no conflict of interest

Authors' contributions

TEG: Investigation, Visualization. MATD: Investigation. NAM: formal analysis, Validation. GPC: Conceptualization, Methodology, Writing-Original draft preparation. GAM: Conceptualization, Supervision, Writing-Reviewing and Editing.

All authors read and approved the final manuscript.

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LEGENDS

Figure 1

Effects of H4 histamine receptor ligands on EMT molecular markers in breast cancer cells. Epithelial and mesenchymal markers were evaluated by Western blot and IFI in MCF-7 (A, B, C) and MDA-MB-231 cells (D, E, F) after 5 days of treatment with 10 µM VUF8430 (VUF), 10 µM JNJ28610244 (JNJ28) and 10 µM J7777120 (J77). A) Immunoblots for E-cadherin, β-catenin, vimentin N-cadherin and alpha-smooth muscle actin (α-SMA). A representative Western blot is shown for each protein. β-actin and α-Tubulin were used as load control and for normalization. Bar chart: mean protein relative expression to control ± SEM of three independent experiments. One-way ANOVA. P *ns*. B) Indirect immunofluorescence (IFI) studies were performed to evaluate subcellular localization of E-cadherin, β-catenin, vimentin and Slug in MCF-7 cells. Cells were fixed and stained with the specific primary antibody and then with Alexa Fluor 488 dye conjugated anti-mouse IgG (H+L) or anti rabbit IgG (whole molecule)-FITC antibody as appropriate. Nuclei were counterstained with DAPI. Representative photographs are

shown. Bar: 20µm. C) Bar chart shows the percentage of Slug positive nuclei in MCF-7 cells. At least three hundred cells were counted to determine the percentage of Slug positive nuclei. Data represent the mean ± SEM of three independent experiments, run in duplicate. One-way ANOVA and Bonferroni post-test, *P< 0.05. D) Immunodetection of vimentin, N-cadherin and α-SMA in MDA-MB-231 cells. A representative Western blot is shown for each protein. Bar chart: mean protein relative expression to control ± SEM of three independent experiments. β-actin and α-Tubulin were used as load control and for normalization. One-way ANOVA and Bonferroni post-test, *P < 0.05, **P < 0.01, ***P < 0.001. E) Subcellular localization of Slug and β-catenin by IFI in MDA-MB-231 cells. Cells were fixed and stained with the specific primary antibody and the appropriate secondary antibody. Nuclei were counterstained with DAPI. Representative photos are shown. Bar: 20 µM. F) Bar chart shows the percentage of Slug positive nuclei in MDA-MB-231 cells. At least three hundred cells were counted to determine the percentage of Slug positive nuclei. Data represent the mean ± SEM of three independent experiments, run in duplicate. One-way ANOVA and Bonferroni post-test, *P < 0.05, ***P < 0.001.

Figure 2

Effects of H4 histamine receptor ligands on cell migration and invasion in MCF-7 and MDA-MB-231 cells. Co-staining of CD44 and Slug in breast cancer cells. Cell migration was evaluated using transwell units after 5 days of treatment with the H4R ligand Clobenpropit (CLOB, 10 µM) or VUF8430 (VUF, 10 µM). Breast tumor cells in serum-free RPMI medium were seeded in the upper compartment. The lower chamber contained RPMI plus 10% FBS. After 40 h (MCF-7) or 20 h (MDA-MB-231) migrated cells were fixed and stained. *MCF-7:* A, B, C. A) Bar chart represents the total number of

migrated cells (mean ± SEM) from three independent experiments, run in duplicate. One-way ANOVA and Bonferroni post-test, *P < 0.05; **P < 0.01. B) Representative photographs of migration studies are shown. Bar: 20 µm. C) Cell invasion was evaluated using transwell units coated with Matrigel®. Bar chart represents the total number of invading cells (mean ± SEM) from three independent experiments, run in duplicate. One-way ANOVA and Bonferroni post-test, *P < 0.05, **P < 0.01, ***P <0.001 and ****P < 0.0001. *MDA-MB-231:* D, E, F. D) Bar chart represents the total number of migrated cells (mean ± SEM) from three independent experiments, run in duplicate. One-way ANOVA and Bonferroni post-test, ***P < 0.01, ****P < 0.0001. E) Representative photographs are shown. Bar: 20 µm. F) Cell invasion was evaluated using transwell units coated with Matrigel®. Bar chart represents the total number of invading cells (mean ± SEM) from three independent experiments, run in duplicate. One-way ANOVA and Bonferroni post-test, * <0.05 and **p < 0.01. G) CD44 and Slug were detected simultaneously by indirect immunofluorescence (IFI) in breast tumor cells after 5 days of treatment with VUF8430 (VUF, 10 µM). Cells were incubated with the primary antibodies during 24 h and then with Alexa Fluor 546 dye conjugated anti-mouse IgG (H+L) and anti-rabbit IgG (whole molecule)-FITC antibodies during 1 h. Representative photos are shown. Bar: 20µm.

Figure 3

H4 histamine receptor agonists induce EMT events via Src phosphorylation. A) Immunodetection of phospho-Src (P-Src) and total Src (T-Src) levels in breast cancer cells. MCF-7 and MDA-MB-231 cells were serum-starved during 24 h. Then cells were stimulated with 10 µM VUF8430 (VUF) and 10 µM JNJ28610244 (JNJ28) during 15 min.

The H4R antagonist JNJ7777120 (J77, 10 µM) and the selective Src inhibitor PP2 (1 µM) were added 30 min before H4R agonists for combined treatment. A representative Western blot is shown for each cell line. β-actin was used as load control and for normalization. Bar chart on the right: mean protein relative expression to control ± SEM of three independent experiments. One-way ANOVA and Bonferroni post-test, *P < 0.05, **P < 0.01, ***P < 0.001. B) P-Src mediates Slug nuclear localization in breast tumor cells. MCF-7 and MDA-MB-231 cells were treated with 10 µM JNJ28610244 (JNJ28) during 5 days. The selective Src inhibitor PP2 (1 µM) was added 30 min before JNJ28 for combined treatment. Slug immunodetection was performed by indirect immunofluorescence (IFI). Nuclei were counterstained with DAPI. Bar: 20µm. Representative photos for each cell line are shown on the left. Bar charts on the right show the mean percentage of Slug positive nuclei from three independent IFI experiments, run in duplicate. At least three hundred cells were counted to determine the percentage of Slug positive nuclei. Data represent the mean ± SEM. One-way ANOVA and Bonferroni post-test, **P < 0.01, ***P < 0.001. C) Activation/phosphorylation of Src mediates MCF-7 and MDA-MB-231 cell migration. Cell migration was evaluated using transwell units after 5 days of treatment with the H4R ligand VUF8430 (VUF, 10 µM). The selective Src inhibitor PP2 (1 µM) was added 30 min before VUF for combined treatment. Breast tumor cells in serum-free RPMI medium were seeded in the upper compartment. The lower chamber contained RPMI plus 10% FBS. After 40 h (MCF-7) or 20 h (MDA-MB-231) migrated cells were fixed and stained. Bar charts represent the total number of migrated cells (mean ± SEM) from three independent experiments, run in duplicate. One-way ANOVA and Bonferroni post-test, **P < 0.01; ***P < 0.001.

Figure 4

TGF-β signaling is induced by H4 histamine receptor agonists in breast cancer cells. A) Detection of Phospho-Smad2/3 (P-Smad) by indirect immunofluorescence (IFI) in breast tumor cells. MCF-7 and MDA-MB-231 cells were serum starved during 24 h and then stimulated with 10 µM VUF8430 (VUF) and 10 µM JNJ28610244 (JNJ28) during 1 h. For combined treatments, the selective TGF-β kinase/activin receptor-like kinase inhibitor A83-01 (A83, 10 µM) was added 30 min before VUF or JNJ28. Nuclei were counterstained with DAPI. Bar charts on the left show the percentage of p-Smad2/3 positive nuclei from three independent IFI experiments, run in duplicate. At least three hundred cells were counted to determine the percentage of p-Smad positive nuclei. Data represent the mean ± SEM. One-way ANOVA and Bonferroni post-test, *P < 0.05, **P < 0.01, ***P < 0.001. Representative photos for each cell line are shown on the right. Bar: 20µm. B) Immunodetection of ERK1/2 MAPK in breast tumor cells. MCF-7 and MDA-MB-231 cells were serum starved during 24 h and then stimulated with 10 µM VUF8430 (VUF) and 10 µM JNJ28610244 (JNJ28) during 15 min. For combined treatments, the selective inhibitor A83 (10 µM) was added 30 min before VUF or JNJ28. A representative immunoblotting of phospho-ERK1/2 (P-ERK) and total ERK1/2 (T-ERK1/2) in each cell line is shown. α-Tubulin was used as load control and for normalization. Bar chart: mean protein relative expression to control ± SEM of three independent experiments. One-way ANOVA and Bonferroni post-test, *P < 0.05, **P < 0.01, ***P < 0.001.

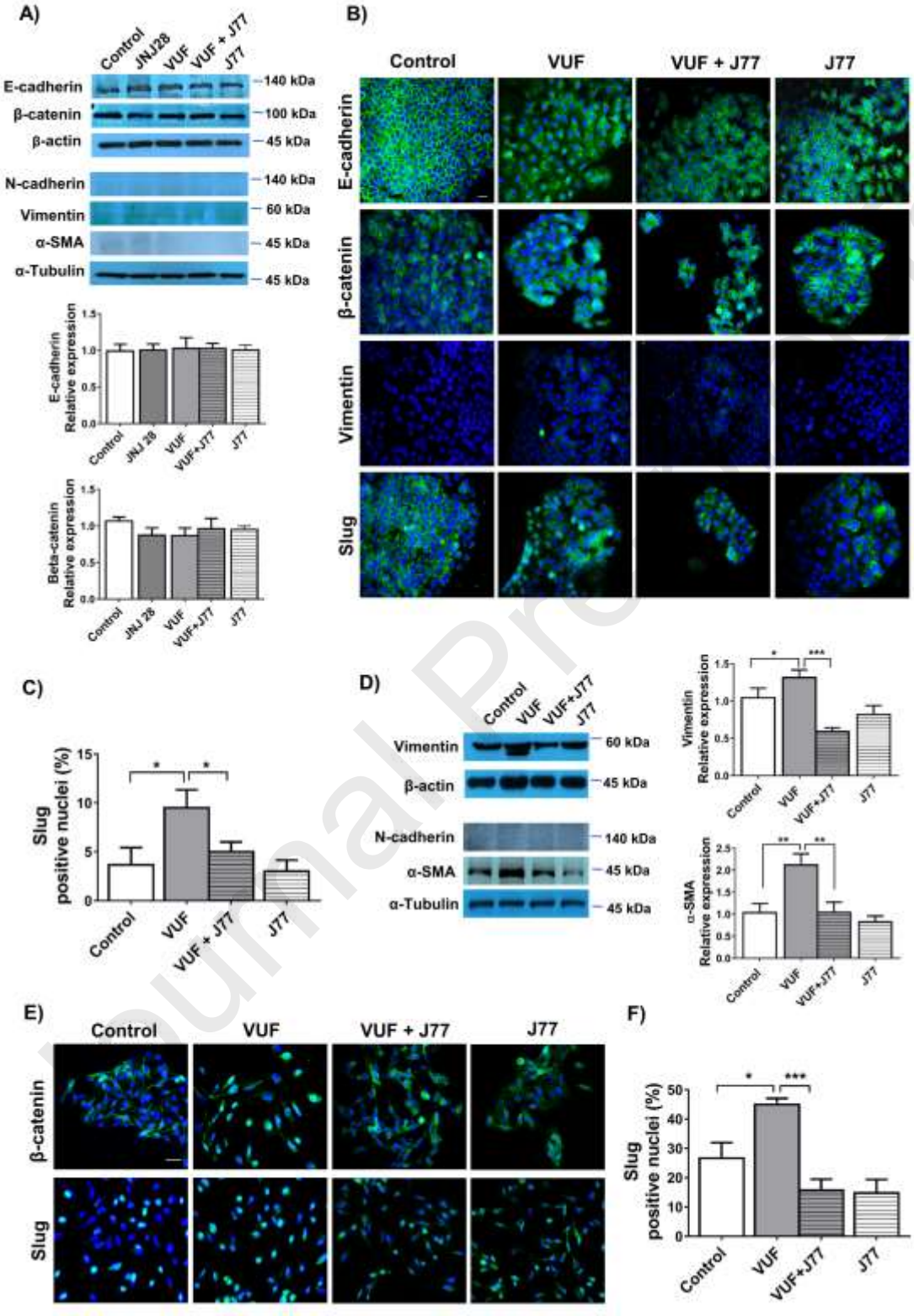
Figure 5

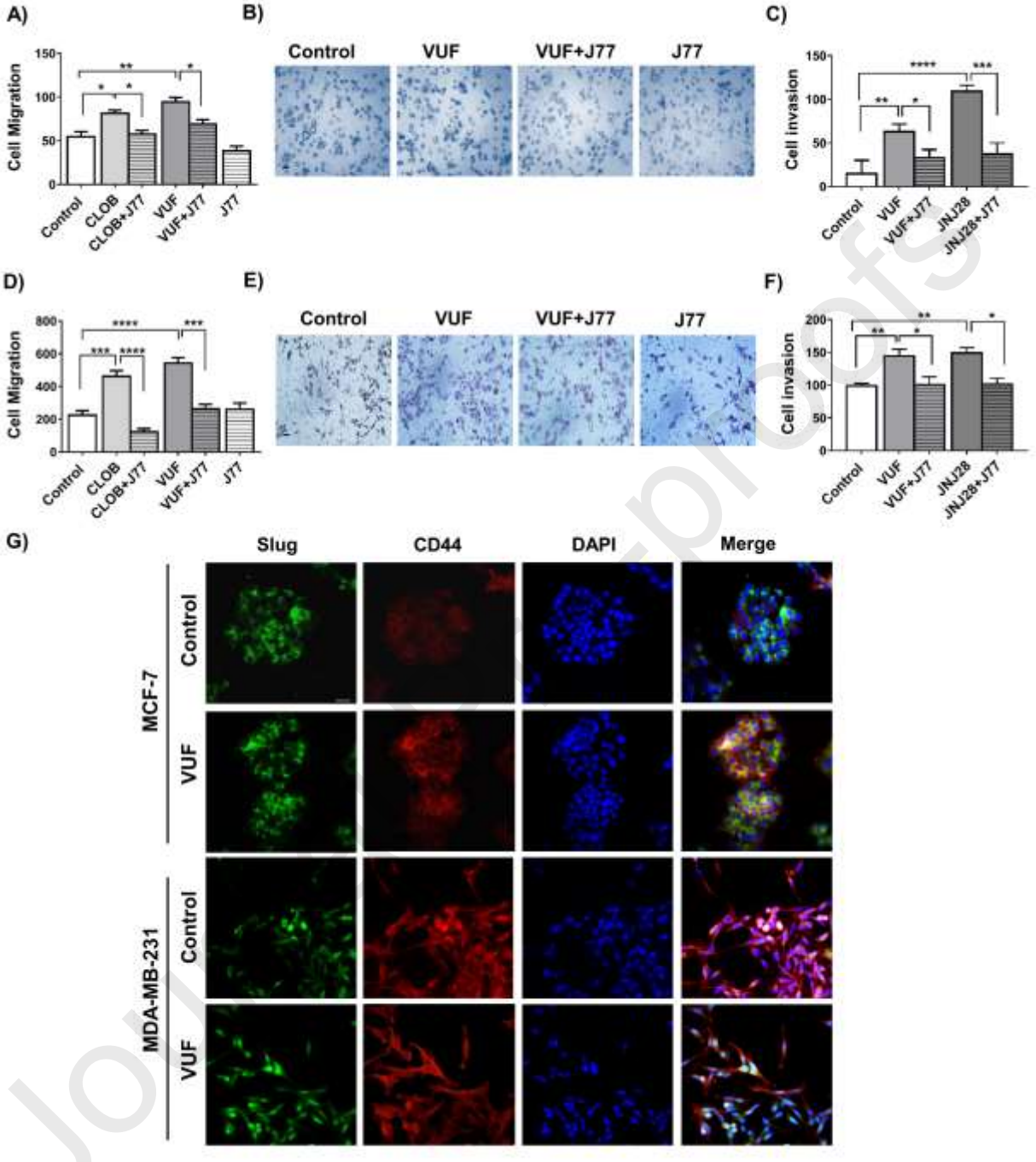
H4 histamine receptor agonists modulate TGF-β1 levels and increase Slug nuclear localization and migration in breast cancer cells. A) Intracellular TGF-β1 detection in breast tumor cells. MCF-7 and MDA-MB-231 (MDA) cells were treated with 10 µM VUF8430 (VUF) and 10 µM JNJ28610244 (JNJ28) during 5 days. The selective TGF-β kinase/activin receptor-like kinase inhibitor A83-01 (A83, 10 µM) was added 30 min before JNJ28 for combined treatments. A representative Western blot is shown for each cell line. α-Tubulin was used as load control and for normalization. Full length 44 kDa form was detected. Bar chart: mean protein relative expression to control ± SEM of three independent experiments. One-way ANOVA and Bonferroni post-test, *P < 0.05, **P < 0.01. B) Immunodetection of TGF-β1 in conditioned media from cell cultures. MCF-7 and MDA-MB-231 (MDA) cells were treated with 10 µM Clobenpropit (CLOB), 10 µM VUF8430 (VUF) and 10 µM JNJ28610244 (JNJ28) during 5 days. A83 (10 µM) was added 30 min before JNJ28 for combined treatments. Then, media were replaced by serum free-RPMI and after 8 h the conditioned media from cell cultures were collected and cleared by centrifugation. Cells from cell cultures were harvested and processed for α-Tubulin immunoblotting to be used as load control and for normalization. 25 kDa dimeric form was detected. C) Slug cellular localization was evaluated by IFI in breast tumor cells. MCF-7 and MDA-MB-231 were treated with 10 µM JNJ28610244 (JNJ28) during 5 days. A83 (10 µM) was added 30 min before JNJ28 for combined treatment. Nuclei were counterstained with DAPI. Representative photos are shown. Bar: 20µm. Bar charts on the right show the percentage of Slug positive nuclei from three independent IFI experiments, run in duplicate. At least three hundred cells were counted to determine the percentage of Slug positive nuclei. Data represent the mean ± SEM. One-way ANOVA and Bonferroni post-test, *P < 0.05, **P < 0.01. D) Cell migration was

evaluated after 5 days of treatment with 10 µM VUF8430 (VUF) and 10 µM JNJ28610244 (JNJ28). For combined treatments, A83 (10 µM) was added 30 min before VUF or JNJ28. Bar chart represents the total number of MCF-7 or MDA-MB-231 (MDA) migrated cells (mean ± SEM) from three independent experiments, run in duplicate. One-way ANOVA and Bonferroni post-test, *P < 0.05, **P< 0.01.

Figure 6

H4 Histamine receptor agonists promote mammosphere formation in MCF-7 and MDA-MB-231 cells and enhance Slug fluorescence stain and migration of mammosphere cells. A) Mammosphere formation was evaluated in MCF-7 and MDA-MB-231 (MDA) cells after 5 days of treatment with the H4R agonists VUF8430 (VUF, 10 µM) and JNJ28610244 (JNJ28, 10 µM) combined or not with the H4R antagonist J7777120 (J77, 10 µM) or the selective TGF-β kinase/activin receptor-like kinase inhibitor A83-01 (A83, 10 µM). Representative photoghaphs are shown. Bar: 50µm. Bar charts show the total number of mammospheres/well (mean ± SEM) from three independent experiments, run in duplicate. One-way ANOVA and Bonferroni post-test, *P < 0.05, **P < 0.01 and ***P< 0.001. B) Indirect immunofluorescence (IFI) studies for Slug detection were performed using intact mammospheres. Representative photos of the immunodetection of Slug in MCF-7 (Bar: 50µm) and MDA cells (Bar: 20µm) are shown. C) MCF-7 and MDA-MB-231 mammospheres were collected by gentle centrifugation (1,000 × g) after 7–10 days and were dissociated enzymatically. Cells were used for cell migration assay as previously described. Bar charts represent the total number of MCF-7 and MDA-MB-231 migrated cells as mean ± SEM from three independent experiments, run in duplicate, **P < 0.01 *vs* Control, test *t*.


A)
Control
JNJ28
VUF
VUF + J77
J77
E-cadherin
β-catenin
β-actin
N-cadherin
Vimentin
α-SMA
α-Tubulin
140 kDa
100 kDa
45 kDa
140 kDa
60 kDa
45 kDa
45 kDa
E-cadherin Relative expression
Beta-catenin Relative expression
B)
Control
VUF
VUF + J77
J77
E-cadherin
β-catenin
Vimentin
Slug
C)
Slug positive nuclei (%)
Control
VUF
VUF + J77
J77
D)
Control
VUF
VUF+J77
J77
Vimentin
β-actin
N-cadherin
α-SMA
α-Tubulin
60 kDa
45 kDa
140 kDa
45 kDa
45 kDa
Vimentin Relative expression
α-SMA Relative expression
E)
Control
VUF
VUF + J77
J77
β-catenin
Slug
F)
Slug positive nuclei (%)
Control
VUF
VUF+J77
J77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Cell Migration
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CLOB
CLOB+J77
VUF
VUF+J77
J77
B)
Control
VUF
VUF+J77
J77
C)
Cell invasion
Control
VUF
VUF+J77
JNJ28
JNJ28+J77
D)
Cell Migration
Control
CLOB
CLOB+J77
VUF
VUF+J77
J77
E)
Control
VUF
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J77
F)
Cell invasion
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JNJ28
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CD44
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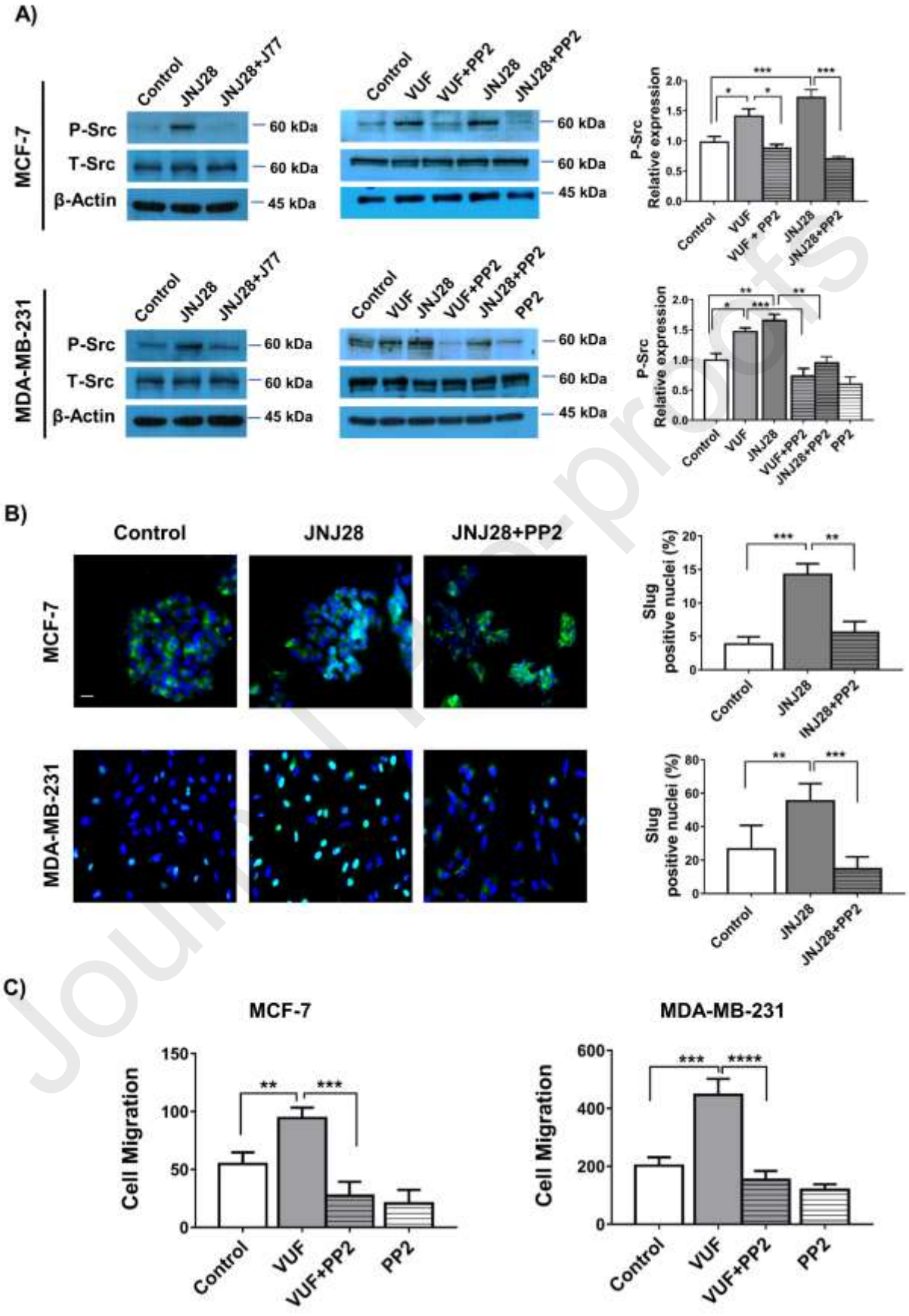

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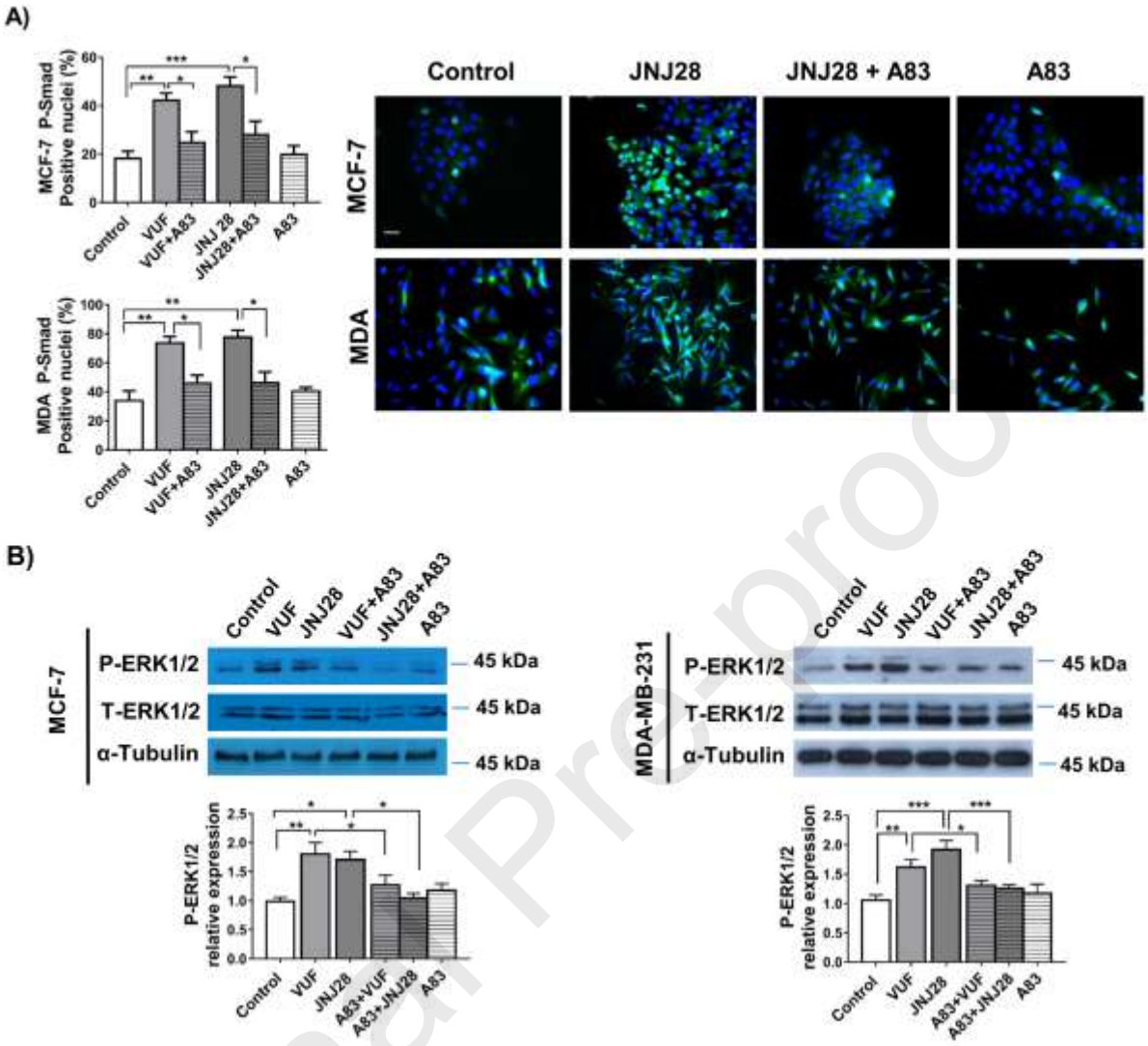

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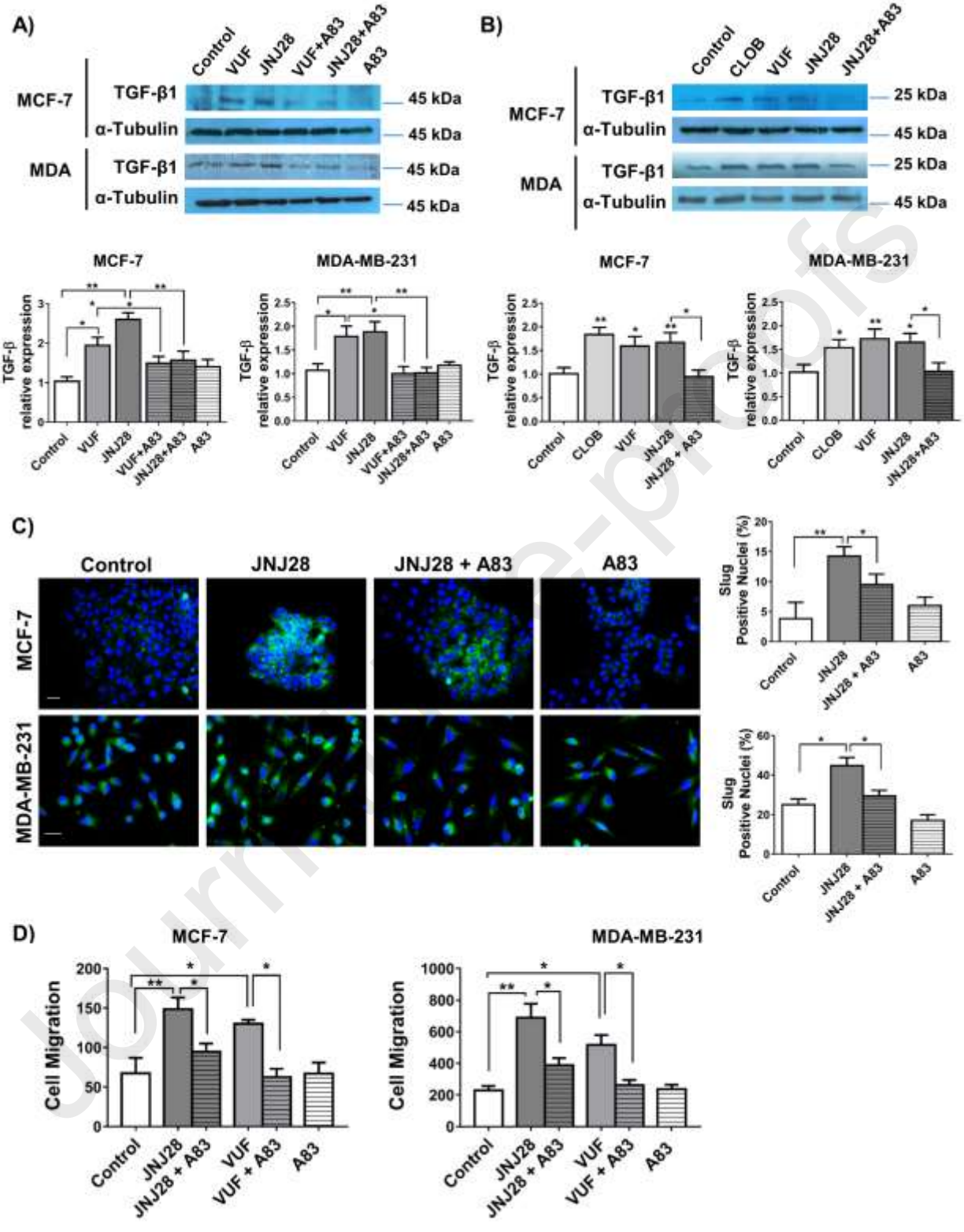
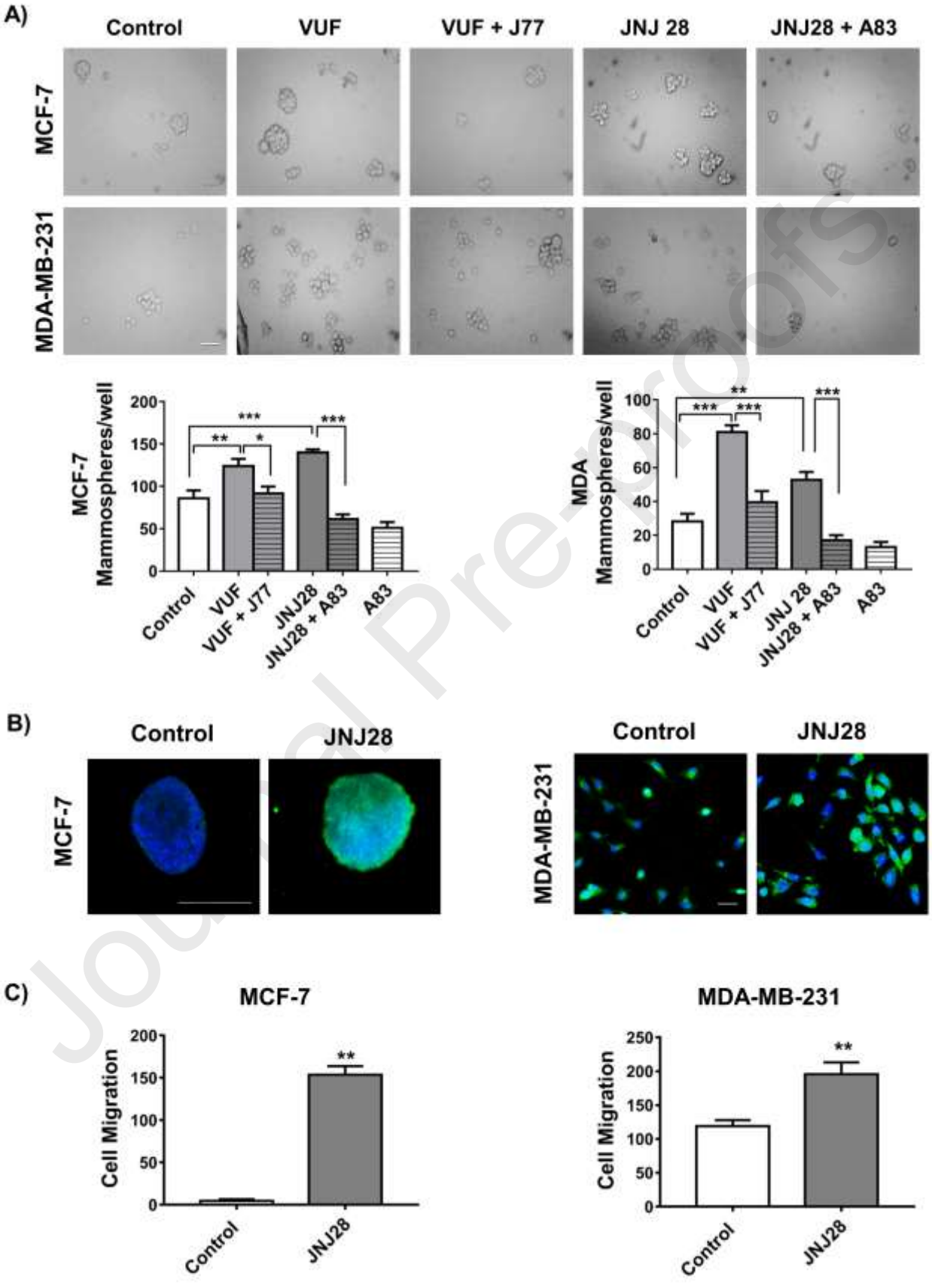

A)
Control
VUF
JNJ28
VUF+A83
JNJ28+A83
A83
MCF-7
TGF-β1
α-Tubulin
MDA
45 kDa
B)
Control
CLOB
VUF
JNJ28
JNJ28+A83
25 kDa
MCF-7
MDA-MB-231
TGF-β relative expression
C)
Control
JNJ28
JNJ28 + A83
A83
Slug Positive Nuclei (%)
D)
Cell Migration
VUF + A83


A)
Control
VUF
VUF + J77
JNJ 28
JNJ28 + A83
MCF-7
MDA-MB-231
MCF-7 Mammospheres/well
MDA Mammospheres/well
Control
VUF
VUF + J77
JNJ28
JNJ28 + A83
A83
B)
Control
JNJ28
MCF-7
MDA-MB-231
C)
MCF-7
MDA-MB-231
Cell Migration
Control
JNJ28

	MCF-7		MDA-MB-231	
	Control	VUF	Control	VUF
Slug	4 ± 2	13 ± 3	23 ± 3	42 ± 2
CD44	45 ± 4	67 ± 8	93 ± 2	95 ± 2
Co-staining	3 ± 2	12 ± 2**	20 ± 3	39 ± 2**

Table 1. Immunodetection of Slug and CD44 in breast cancer cells. Co-staining of Slug and CD44 was evaluated by indirect immunofluorescence (IFI) in MCF-7 and MDA-MB-231 cells after five days of treatment with the H4R agonist VUF (10 µM). Cells were incubated with the primary antibodies during 24 h and then with Alexa Fluor 546 dye conjugated anti-mouse IgG (H+L and anti rabbit IgG (whole molecule)-FITC antibodies during 1 h. Nuclei were counterstained with DAPI. At least three hundred cells were counted to determine the percentage of CD44 and Slug with or without merge. Table shows the mean percentage of positive cells for CD44 and Slug ± SEM of three independent experiments, run in duplicate. Test t, **p < 0.01 *vs* Control.

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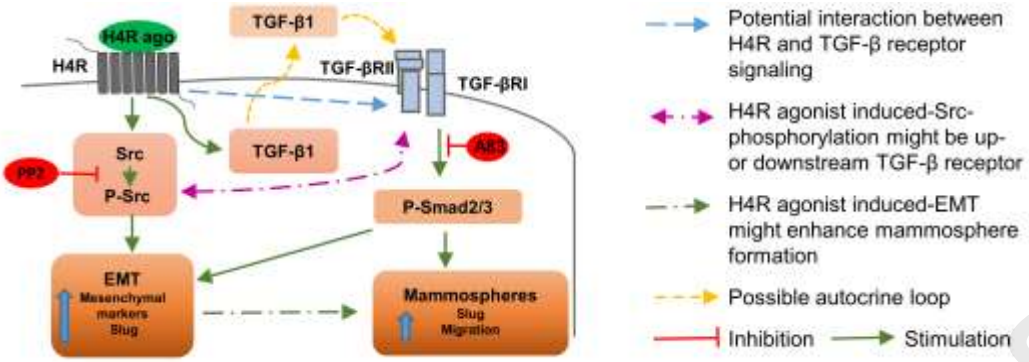

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Authors' contributions

TEG: Investigation, Visualization. MATD: Investigation. NAM: formal analysis, Validation. GPC: Conceptualization, Methodology, Writing-Original draft preparation. GAM: Conceptualization, Supervision, Writing-Reviewing and Editing.

All authors read and approved the final manuscript.

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