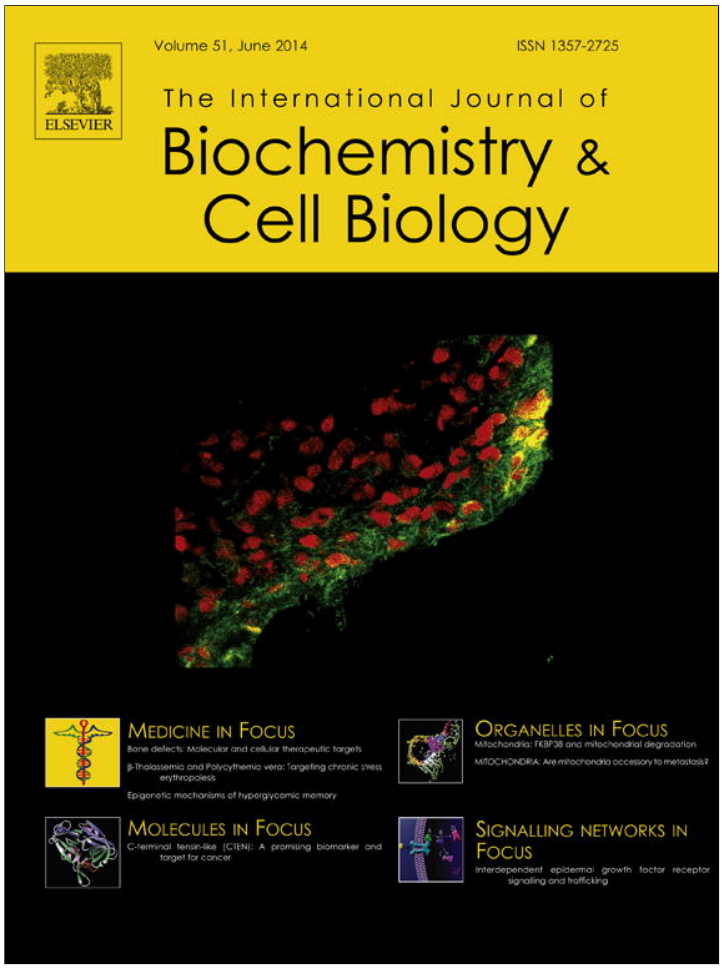

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Fibroblasts induce epithelial to mesenchymal transition in breast tumor cells which is prevented by fibroblasts treatment with histamine in high concentration

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ABSTRACT

Epithelial to mesenchymal transition (EMT) of cancer cells is an essential process in cancer progression. Cancer cells that undergone EMT loose cell–cell contacts, acquire mesenchymal properties and develop migratory and invasive abilities.

In previous studies we have demonstrated that histamine may modify the invasive phenotype of pancreatic and mammary tumor cells. In this work we proposed to investigate whether histamine may also influence the interaction between tumor cells and normal fibroblasts. The potential activation of normal CCD-1059Sk fibroblasts by histamine and EMT phenotypic changes induced in MCF-7 and MDA-MB-231 breast tumor cells by the conditioned media (CM) derived from fibroblasts were evaluated. Initially, we determined the presence of H1, H2 and H4 histamine receptors and matrix metalloproteinase 2 (MMP2) mRNA in CCD-1059Sk fibroblasts. MMP2 gelatinolytic activity, cell migration and alpha-smooth muscle actin expression were increased in fibroblasts by low doses (<1 μM) and decreased by high doses (20 μM) of histamine. MCF-7 cells cultured with CM from fibroblasts exhibited spindle-shaped morphology, cell spreading and cytoplasmic expression of β-catenin but there was no change in MMP2 activity and cell migration. MDA-MB-231 cells cultured with CM from fibroblasts showed a more elongated phenotype, cell spreading, cytoplasmic β-catenin, increased MMP2 activity and endogenous TGF-β1 expression, and enhanced cell migration and invasion. Notably, all these features were reversed when mammary tumor cells were cultured with CM from fibroblasts treated with 20 μM histamine. In conclusion, high doses of histamine may prevent the activation of fibroblasts and also avert the EMT related changes induced in epithelial tumor cells by fibroblasts CM.

1. Introduction

Epithelial to mesenchymal transition (EMT), a critical normal process during embryogenesis, organ development and wound healing, is also present in fibrosis and cancer (Kalluri and Weinberg, 2009). During EMT epithelial cells can undergo impressive phenotypic changes that reflect their "transformation" to mesenchymal cells. Epithelial cells lose their apico-basal polarity and their cell–cell contacts. Down-regulation of the epithelial marker E-cadherin usually highlights the beginning of EMT (Baranwal and Alahari, 2009). Cytoplasmic and nuclear accumulation of β-catenin and enhancement in the expression of EMT related genes (Slug, Snail, Twist) are observed in epithelial cells during this process (Andrews et al., 2012). Moreover, the expression of mesenchymal markers like N-cadherin, vimentin and alpha-smooth muscle actin (α-SMA) as well as the secretion and activity of MMP2 and MMP9 are augmented during EMT (Kalluri and Weinberg, 2009).

Metastasis is the major cause for cancer related mortalities. It depends on the ability of tumor cells to change their epithelial phenotype to a migratory and invasive or mesenchymal-like phenotype. Carcinomas arising from epithelial tissues represent 90% of human neoplasias and the inappropriate activation of EMT in epithelial cells allows benign tumors to progress into invasive and metastatic cancers (Acloque et al., 2009; Gavert and Ben-Ze'ev, 2008). It is recognized that the induction of EMT requires cells to be

Abbreviations: CM, conditioned media; CM(−), unconditioned media; CM(+), CM from CCD-1059Sk fibroblasts; CM(20μM HA), CM from fibroblasts treated with 20 μM histamine; ECM, extracellular matrix; EMT, epithelial to mesenchymal transition; HA, histamine; MMP, matrix metalloproteinase; α-SMA, alpha-smooth muscle actin.

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competent for undergoing EMT and an EMT-permissive microenvironment to exist. Interactions of malignant cells with tumor stroma are crucial to increase the malignancy of neoplastic cells and change the phenotype of normal fibroblasts in activated fibroblasts. In turn, activated fibroblasts secrete a variety of factors that influence neighboring cells in a paracrine manner to promote tumor growth and invasion (Kalluri and Zeisberg, 2006; Räsänen and Vaheri, 2010; Xouri and Christian, 2010).

Histamine is a biogenic amine whose actions mainly comprise allergic and inflammatory responses through the activation of four G protein-coupled receptors (H1, H2, H3 and H4). However during the last decades accumulating evidence supports histamine actions in proliferation of normal and tumor cells with different responses depending on histamine receptor subtype activated and cells or tissues where receptors are expressed. A stimulatory effect on cell growth is commonly observed in epithelial cells at doses lower than 1 μM while histamine over 10 μM reduces cell proliferation (Cricco et al., 2006a, 2008; Francis et al., 2009; Massari et al., 2011; Medina et al., 2006; Rivera et al., 2000). In previous studies we have demonstrated that histamine may also modify the invasive phenotype of pancreatic and mammary tumor cells modulating the expression and activity of MMPs and cell migration in a dose-dependent manner (Cricco et al., 2006b, 2011; Genre et al., 2009).

In this work we proposed to investigate whether histamine may also influence the interaction between tumor cells and normal fibroblasts. To better understand the role of histamine in tumor biology we evaluated the potential activation of normal fibroblasts by histamine and the EMT phenotypic changes induced in breast tumor cells by conditioned media (CM) derived from histamine-treated fibroblasts. An extensive knowledge of multiple interactions between tumor and stromal cells in human cancer will help to delineate more effective strategies for therapeutic intervention.

2. Materials and methods

2.1. Cell culture

CCD-1059Sk fibroblasts derived from normal skin of human mammary gland (ATCC CRL-2072) and the breast cancer cells MCF-7 (ATCC HTB-22) and MDA-MB-231 (ATCC HTB-26) were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS, Gibco, CA, USA), 0.3 g/l L-glutamine and 40 mg/l gentamicine. 1 mM sodium pyruvate was added to fibroblasts cultures. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Treatments

Histamine dihydrocloride and histamine H1 receptor agonist 2-[3-(trifluoromethyl)phenyl]histamine dimaleate were from Sigma–Aldrich (St Louis, MO, USA). Histamine H2 receptor agonist Amthamine dihydrobromide, histamine H3 receptor agonist R-(α)-methyl-histamine dihydrobromide and histamine H4 receptor agonist Clobenpropit dihydrobromide were from Tocris (MO, USA). H1, H2 and H3 histamine receptor agonists are specific (Leschke et al., 1995; Eriks et al., 1992; Leurs et al., 1995). Clobenpropit is an H4 histamine receptor agonist and H3 histamine receptor antagonist (Liu et al., 2001).

2.3. Generation of conditioned media

Fibroblasts grown up to 60% confluence in RPMI supplemented with 1 mM sodium pyruvate and 10% FBS. Subsequently fibroblasts were incubated with histamine or not for 24 h at 37 °C, 5% CO_2. Then media were discarded, fibroblasts were rinsed with phosphate buffered saline (PBS) and FBS free RPMI was added to cultures. Fibroblasts were incubated for additional 24 h and then media were collected, centrifuged at 13,000 × *g*, 4 °C for 5 min and frozen at −70 °C for further use. Treatments did not modify the number of fibroblasts during the experiment, as evaluated by counting adherent cells at the end of the experiment.

Conditioned media [CM(+) and CM(20 μM HA)] were prepared by mixing media collected from fibroblasts (not treated or treated with histamine respectively) and fresh RPMI in a ratio 1:2. The mixture was supplemented with 10% FBS.

CM(−) was prepared trying to resemble CM(+) and CM(20 μM HA) preparation as much as possible by incubating RPMI at 37 °C, 5% CO_2 for 24 h and posterior centrifugation at 13,000 × *g*, 4 °C for 5 min. This medium was frozen at −70 °C and then diluted in a ratio 1:2 with RPMI and supplemented with 10% FBS to be used as CM(−).

2.4. Cell-scatter assay

2×10^3 MDA-MB-231 or MCF-7 cells were seeded onto six-well plates, allowed to adhere overnight and then switched to CM(−), CM(+) or CM(20 μM HA) for 7 days. Media were changed twice during incubation time. Cell scattering was evaluated microscopically following staining with 1% toluidine blue solution.

2.5. Immunocytochemical/Immunofluorescence analysis

Fibroblasts were grown onto glass coverslips and treated with different doses of histamine for 24 h. Tumor cells were seeded onto glass coverslips and incubated with CM(−), CM(+) or CM(20 μM HA) for 24 h.

Immunocytochemistry: cells were fixed with 3.7% formaldehyde and permeabilized with 0.1% Triton X-100 in 1% FBS/PBS. Endogenous peroxidase activity was blocked with 3% H_2O_2. Cells were incubated overnight at 4 °C with rabbit anti MMP2 and anti MMP9 antibodies (1:100, Calbiochem, La Jolla, CA, USA). Immunoreactivity was detected by using peroxidase-conjugated anti-rabbit IgG and visualized by diaminobenzidine staining (Sigma). Finally, cells were counterstained by immersion in hematoxylin. Light microscopy (Axiolab Karl Zeiss, Göttingen, Germany) was performed.

Immunofluorescence: cells were fixed, permeabilized, blocked in PBS with 1% bovine seroalbumine and incubated overnight at 4 °C with anti E-cadherin (1:50, Santa Cruz Biotechnologies, CA, USA), anti β-catenin (1:100, Invitrogen, NY, USA), anti α-SMA (1:50, Abcam, Cambridge, UK) antibodies and for 1 hr at room temperature with Alexa Fluor 488 dye conjugated anti-mouse (1:400, Invitrogen) or FITC-conjugated mouse anti-rabbit (1:100, Sigma) antibodies. Cells were stained with 0.25 μg/ml propidium iodide or 0.1 μg/ml DAPI to visualize nuclei. Coverslips were mounted with FluorSave Reagent (Calbiochem), and immunoreactivity was visualized by a laser confocal microscope (Olympus Fluo View FV1000). Signal specificity was controlled by replacing the first antibody with PBS or Mouse Isotype Control (purified normal mouse immunoglobulin, Invitrogen, Camarillo, USA) in the case of rabbit or mouse antibody respectively.

2.6. Gelatin zymography

Cells were seeded and treated for 24 h. Then media were replaced by fresh serum-free RPMI. After 24 h supernatants were collected, mixed with non-reducing buffer and electrophoresed on 7% sodium dodecyl sulfate-polyacrilamide gels with 0.1% gelatin. Gelatinolytic activity was visualized by staining zymograms with Coomassie Brilliant Blue G250 (Sigma) and destaining in acetic acid–methanol–H_2O (1:3:6). Densitometric analyses were performed using the software ImageJ 1.42q (NIH, USA).

2.7. Migration/Invasion assay

Cell migration was investigated using a 24-well transwell unit with polyethylene terephthalate membranes having a pore size of 8.0 μm (BD Falcon, Basel, Switzerland). Equal number of serum-starved fibroblasts (1×10^3), MCF-7 (3×10^4) or MDA-MB-231 (1×10^4) cells was loaded into the upper compartment in serum-free medium. In the case of fibroblasts 1 mM sodium pyruvate was added. The lower chamber contained the appropriate medium (as indicated in figure legends) plus 1% FBS. After 20 h at 37 °C non-migrated cells on the upper surface of membranes were gently scrubbed with a cotton swab. Cells migrated were fixed in 3.7% formaldehyde and stained with 0.5% crystal violet. Six fields were randomly counted at 200× and the average number of cells was informed.

To assess the invasive potential of breast cancer cells, the same protocol as above described was used with matrigel-coated transwells. Briefly, Matrigel (BD Biosciences, MA, USA) was diluted to 500 μg/ml with cold RPMI, applied to the upper surface of the filter (5 μg/filter), and dried at room temperature. Equal number of serum-starved MCF-7 (1×10^5) or MDA-MB-231 (5×10^4) cells was loaded into the upper chamber in serum-free medium. After 24 h at 37 °C the cells that invaded through Matrigel and reached to the reverse side were fixed, stained and counted under a microscope in six random fields at 200×. Duplicate wells were used per condition in each independent experiment.

2.8. Immunoblot analysis

MCF-7 and MDA-MB-231 cells were seeded to be at 70% confluence at the time of changing the media by CM(−), CM(+) or CM(20 μM HA). After 24 h cells were processed as previously described (Medina et al., 2006). Briefly, proteins (50 μg) were fractioned on sodium dodecyl sulfate-polyacrilamide gels (12%) and blotted onto polyvinylidene difluoride membranes. Membranes were blocked and probed with α-SMA (1:100, Abcam, Cambridge, UK), anti E-cadherin (1:200, Santa Cruz Biotechnologies, CA, USA), anti N-cadherin (1:500, Invitrogen, NY, USA), anti β-catenin (1:500, Invitrogen, NY, USA), anti TGF-β (1:1000, Abcam, Cambridge, UK), anti Slug (1:100, Santa Cruz Biotechnologies, CA, USA), anti α-tubulin and β-actin (1:2000, Sigma, St Louis, MO, USA) antibodies overnight and with anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibodies (1:2000, Sigma) for 1 h at room temperature. The immunoreactive bands were detected by enhanced chemiluminescence (ECL, Amersham Biosciences Argentina SS, Argentina). Densitometric analyses were performed using the software ImageJ 1.42q (NIH, USA).

2.9. Total RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted using Trizol according to the instructions of the manufacturer (Invitrogen, Carlsbad, CA, USA). Two micrograms of total RNA were added to the reverse transcription (RT) reaction mixture in the presence of oligodT, dNTPs and MMLV-RT enzyme (Promega, WI, USA). Samples were incubated at 42 °C for 60 min and at 96 °C for 5 min in a thermocycler (Perkin Elmer, GeneAmp PCR System 2400). The resulting cDNA was amplified by PCR in the presence of each specific primer, dNTPs and Green GoTaq® polymerase (Promega) in time and temperature conditions as appropriate.

PCR primers and conditions for histamine receptors H2, H3 (one set of primers), H4 and β-actin were as in Medina et al. (2006); for MMP2 as in Cricco et al. (2011) and for MMP9 (one set of primers) as in Ming et al. (2005). Primers were designed for H1 receptor (H1R), H3 receptor (H3R, additional set of primers), MMP9 (additional set of primers) and Slug with PrimerQuestSMR, IDT and verified with Primer-BLAST (NCBI). H1R-F: TCATCAACTTCTACCTGCCCAC, H1R-R: AGACTCCTTCCCTGGTTTCTTG, amplified fragment 179 bp. H3R-F: CCTCCGCACCCAGAACAACTT, H3R-R: AGCCGTGATGAGGAAGTACCA, amplified fragment 417 bp. MMP9-F: GATGCGTGGAGAGTCGAAAT, MMP9-R: CACCAAACTGGATGACGATG, amplified fragment 337 bp. Slug-F: TTTCTGGGCTGGCCAAACATAAGC, Slug-R: TGCAAATGCTCTGTTGCAGTGAGG, amplified fragment 251 bp. Incubation was as follows: H1R: 5 min 95 °C, 30 cycles of 45 s 95 °C, 45 s 57 °C, 50 s 72 °C; H3R: 5 min 95 °C, 35 cycles of 45 s 95 °C, 45 s 55 °C, 50 s 72 °C; MMP9: 5 min 95 °C, 30 cycles of 45 s 95 °C, 45 s 62 °C, 50 s 72 °C; Slug: 5 min 95 °C, 30 cycles of 45 s 95 °C, 45 s 55 °C, 50 s 72 °C. Reactions were terminated by final elongation step of 7 min at 72 °C. MDA-MB-231 cells were used as positive controls for histamine receptors (Medina et al., 2006), and PANC-1 cells for MMP2 and MMP9. RT experiments in which control reaction mixtures were prepared replacing reverse transcriptase with water to test for DNA contamination were performed and PCR reactions were carried out including RT control reaction mixtures as negative controls. PCR products were subjected to gel electrophoresis and detected by gel documentation system LumiBis DNR (Bio-Imaging Systems, Jerusalem, Israel).

2.10. Statistical analysis

Each experiment was repeated at least three times. Results were expressed as means ± SEM. Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software Inc., PA, USA). One way ANOVA and Bonferroni's post test were employed. *P* values lower than 0.05 were considered as significant.

3. Results

3.1. Expression of histamine receptors and histamine actions in CCD-1059Sk fibroblast cell line

We first investigated the presence of histamine receptors in the fibroblast cell line CCD-1059Sk derived from skin of human mammary gland since they were not reported in the literature at the moment. mRNA for histamine receptors were evaluated by RT-PCR. Results are shown in Fig. 1A. CCD-1059Sk fibroblasts express mRNA for H1, H2 and H4 histamine receptors since only one product for the messenger of each receptor was observed accordingly to the size reported for the primers used in this study (H1R: 179 bp; H2R: 496 bp and H4R: 512 bp). Two sets of primers were used to assess H3R mRNA expression with negative results.

Activated fibroblasts are major players in matrix remodeling during various steps in cancer development and metastasis. They are characterized by the augmented expression of α-SMA and fibroblast activation protein (FAP) and by an increase in migration as well as in their ability for remodeling ECM and secreting cytokines among other cellular events (Xouri and Christian, 2010). In attempt to assess whether histamine might drive CCD-1059Sk fibroblasts to an activated state we employed different doses of histamine to evaluate MMPs gelatinolytic activities and cell migration. Initially, we determined that CCD-1059Sk cells expressed MMP2 mRNA and we confirmed MMP2 protein expression by immunocytochemistry (Fig. 1B). MMP9 expression could not be detected either by RT-PCR (using two sets of primers) or by immunocytochemistry. The enzymatic activity of MMP2 was then examined by gelatin zimography (Fig. 1C). Two lytic bands (72 and 66 kDa) corresponding to pro-MMP2 and activated MMP2 respectively were observed. Results showed a significant increase in MMP2 activity in CCD-1059Sk fibroblasts treated with 0.1 μM histamine which was mainly reproduced by the H1 agonist 2-[3-(trifluoromethyl)phenyl]histamine

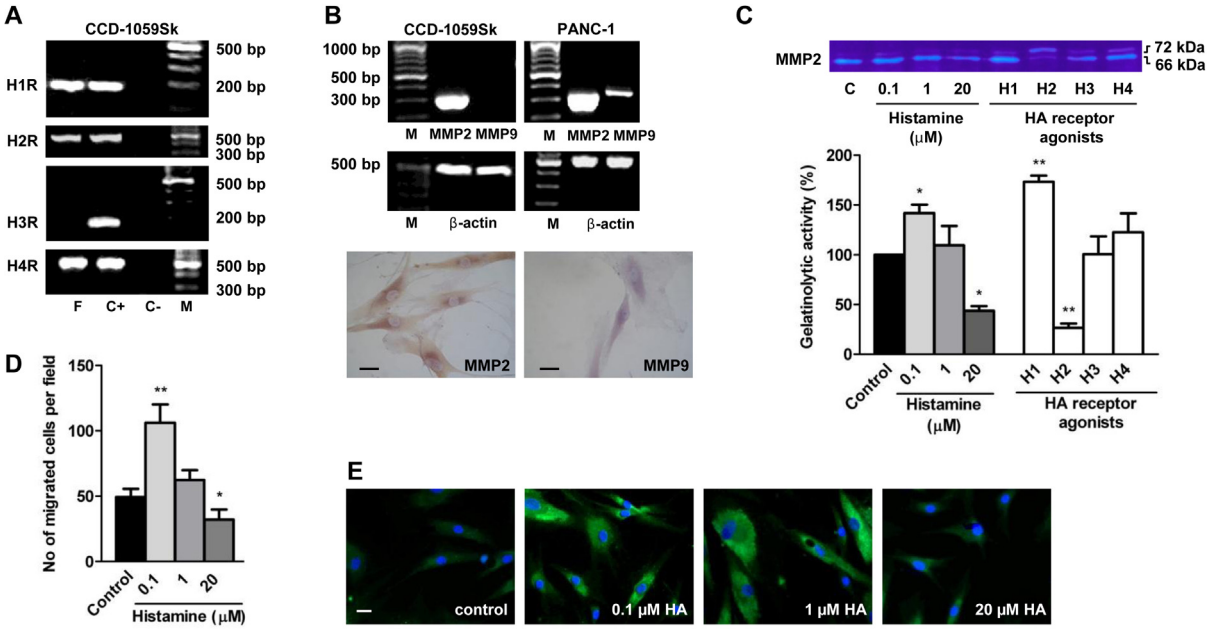

Fig. 1. CCD-1059Sk human normal fibroblast cell line: histamine receptors expression and histamine actions. Fibroblasts were grown in RPMI with 10% FBS. (A) *Expression of histamine receptors by RT-PCR.* PCR products were electrophoresed and photographed. M: DNA ladder molecular size marker. Product sizes: H1 histamine receptor (H1R): 179 bp; H2 histamine receptor (H2R): 496 bp; H3 histamine receptor (H3R): 194 bp; H4 histamine receptor (H4R): 512 bp. Fibroblasts: F; positive control (C+): MDA-MB-231 cells; negative control (C−): PCR reactions were performed with RT control reaction mixtures replacing reverse transcriptase with water. (B) *Expression of matrix metalloproteinases (MMPs).* MMPs mRNA were evaluated by RT-PCR. MMP2: 306 bp; MMP9: 337 bp. Immunocytochemistry for MMPs: Fibroblasts were fixed and probed with anti MMP2 and MMP9 antibodies. PANC-1 cells were used as positive control. Pictures were taken at 630× magnification. (C) *Gelatinolytic activity of MMP2.* Fibroblasts were treated with different doses of histamine (HA) or HA receptors agonists (H1: 10 μM 2-[3-(trifluoromethyl)phenyl]histamine, H2: 10 μM Amthamine, H3: 0.1 μM R-(α)-methyl-histamine, H4: 10 μM Clobenpropit) during 24 h. Media were changed by serum free-RPMI and supernatants were employed to assess the enzymatic activity 24 h later. A representative gel is shown. Activity of activated MMP2 lytic bands (66 kDa) for each treatment was determined by densitometry and normalized to control values. Bars represent the mean ± SEM of four independent experiments run in duplicate, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control. (D) *Cell migration*: Fibroblasts in serum free-medium with 1 mM sodium pyruvate were loaded into the upper chamber while RPMI with 1 mM sodium pyruvate, 1% FBS and different doses of histamine were added to the lower chamber. After 20 h fibroblasts on the lower surface of the upper chamber were fixed, stained and photographed. Cells from six randomly chosen fields were counted under a microscope at 200× magnification. Bar chart: mean ± SEM of three independent experiments run in duplicate, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control. (E) *Immunofluorescence for α-smooth muscle actin (α-SMA).* The expression of α-SMA was examined after 24 h in fibroblasts treated with different doses of HA. Nuclei were counterstained with DAPI. Pictures were taken at 400× magnification. (B and E) Scale bar: 20 μm.

dimaleate. A minor increase was produced by the H4 agonist/H3 antagonist Clobenpropit though it was not significant. In contrast, 20 μM histamine and the H2 agonist significantly reduced MMP2 activity. Furthermore, when cell migration was evaluated the number of migrated fibroblasts augmented significantly with 0.1 μM histamine while 20 μM histamine considerably reduced fibroblasts migration (Fig. 1D). We also evaluated the expression of α-SMA by immunofluorescence and determined an increase with histamine doses lower than 1 μM whereas fibroblasts treated with 20 μM histamine showed an expression similar to controls. Taken together our data indicate that histamine may dose-dependently modulate the activation of CCD-1059Sk fibroblasts.

Though experimental data do not allow us to discard the expression of H3R in CCD-1059Sk fibroblasts, this fact does not interfere with the conclusions about the direct action of histamine on fibroblasts and about its ability to modify the conditioned media.

3.2. Conditioned media from CCD-1059Sk fibroblasts induce morphological changes in breast tumor cells

MDA-MB-231 and MCF-7 cells express histamine receptors in cell membrane. *In vitro*, histamine has a direct effect on these tumor cell lines affecting cell proliferation, differentiation and death in a dose dependent way (Medina et al., 2006, 2011). We have also demonstrated that histamine may modify some EMT-related processes in MDA-MB-231 tumor cells modulating the expression and activity of metalloproteinases and cell migration in a dose-dependent manner *via* different receptors (Cricco et al., 2011; Genre et al., 2009).

In vivo, histamine might act on both types of cells, fibroblasts and tumor cells. To better understand the role of histamine in tumor biology we next evaluated EMT related processes induced in breast tumor cells by conditioned media (CM) derived from histamine-treated fibroblasts. As our previous findings showed that histamine over 10 μM inhibits proliferation and some events of the EMT process in tumor cells and since 20 μM histamine could prevent events tightly related to activation of fibroblasts, we selected this histamine concentration to treat fibroblasts and obtain the CM to further culture MDA-MB-231 and MCF-7 cells. A thorough comprehension of interactions within tumor microenvironment can help to delineate more effective strategies for therapeutic intervention. Thus it might be particularly attractive to target those pathways that regulate tumor cell proliferation, dedifferentiation and/or survival and simultaneously control EMT.

In order to investigate the effect of CM from CCD-1059Sk fibroblasts on morphology of breast tumor cells we observed the cells with an optical microscope (Fig. 2). After 7 days, MDA-MB-231 cells cultured with the CM from CCD-1059Sk fibroblasts [CM(+)] displayed a more elongated appearance with decreased cell–cell interactions when compared to cells cultured with unconditioned media [CM(−)]; spreading of cell colonies was also observed. MCF-7 cells cultured with CM(−) grew as discrete colonies with tight cell–cell junctions. However in the presence of CM(+) some cells within the colony detached from their neighboring cells and exhibited an altered morphology characteristic of actively spreading cells. Interestingly, all these features were not observed when both mammary cell lines were cultured with CM

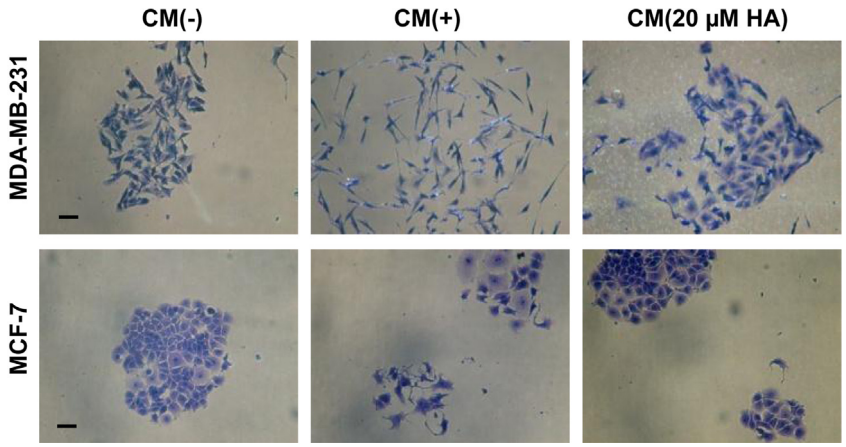

Fig. 2. Phenotypical changes induced in mammary tumor cells by fibroblasts conditioned media. MDA-MB-231 and MCF-7 cells were cultured for 7 days with unconditioned media [CM(−)] or CM obtained from fibroblasts treated with 20 μM histamine [CM(20 μM HA)] or not [CM(+)]. Cells were fixed, stained and photographed. Pictures were taken at 400× magnification. Scale bar: 20 μm.

from fibroblasts treated with 20 μM histamine [CM(20 μM HA)] (Fig. 2).

3.3. The expression of the molecular EMT markers in breast tumor cells is modified by conditioned media from CCD-1059Sk fibroblasts

We conducted further experiments to investigate the potential activation of EMT program in MDA-MB-231 and MCF-7 cells employing CM from fibroblasts. Epithelial and mesenchymal markers were evaluated by immunofluorescence and immunoblotting (Fig. 3).

MDA-MB-231 cells cultured with CM(−) showed a very low expression of E-cadherin and a basal level of β-catenin and α-SMA by immunoblot. Accordingly, immunofluorescence staining showed only a few cells with a low membrane expression of E-cadherin while β-catenin was mainly localized in the cell membrane. After 24 h of culturing MDA-MB-231 cells with CM(+), the level of E-cadherin was very low but a significant increase in the mesenchymal marker α-SMA could be detected. In addition, β-catenin was mostly observed in the cytoplasm by confocal microscopy. Notably, CM(20 μM HA) induced an up-regulation of E-cadherin expression and counteracted the enhancement of the expression induced by CM(+). Meanwhile β-catenin was mainly localized in the cell membrane (Fig. 3A and B).

MCF-7 cells showed no expression of α-SMA protein while no significant differences in E-cadherin total levels could be detected with CM(−), CM(+) or CM(20 μM HA). Immunofluorescence studies revealed that the membrane expression of β-catenin in MCF-7 cells treated with CM(−) was changed to a cytoplasmic localization with CM(+). Remarkably the employment of CM(20 μM HA) reduced β-catenin level, being this protein localized in the cell membrane again (Fig. 3A and B).

The expression of N-cadherin, other mesenchymal marker, was not detected in breast cancer cells cultured with CM from fibroblasts (Fig. 3B).

Since Slug is described as an E-cadherin repressor in epithelial cells (de Herreros et al., 2010), we next decided to examine Slug expression by RT-PCR and immunoblot. We detected Slug in MDA-MB-231 cells with an increase in the expression in CM(+) treated cells (Fig. 3C). Interestingly, a very low expression of mRNA and protein was observed in the presence of CM(20 μM HA). Additionally, MCF-7 exhibited very low or undetectable levels of Slug mRNA and protein. No differences were observed when MCF-7 cells were cultured with or without CM (Fig. 3C).

3.4. Effects of conditioned media from normal fibroblasts on gelatinolytic activity, cell migration and invasion of breast tumor cells

During EMT epithelial cells can undergo phenotypic but also biochemical changes that allow them to acquire a fibroblastoid-like phenotype, to raise extracellular matrix components production and to enhance migratory capacity and invasiveness.

To evaluate the modulation of functional EMT markers by CM from fibroblasts in MDA-MB-231 and MCF-7 cells we determined the gelatinolytic activity of MMPs and the migratory and invasive capacity. Data indicated that CM(+) enhanced MMP2 activity (66 kDa) in MDA-MB-231 cells when compared to CM(−). This increase was partly abrogated by the employment of CM(20 μM HA). We also assessed MMP2 activity in MCF-7 cells. Only one lytic band was observed (66 kDa) and no differences were observed when MCF-7 cells were cultured with or without CM (Fig. 4A). Moreover, cell migration studies revealed that CM(+) enhanced the migratory capacity of MDA-MB-231 cells while CM(20 μM HA) hindered this effect. Only a few number of MCF-7 cells migrated through the membrane of the transwell units and no statistic differences were observed (Fig. 4B). Similar results were obtained for invasion assays (Fig. 4C).

3.5. The expression of TGF-β1 in breast tumor cells is modified by conditioned media from CCD-1059Sk fibroblasts

The transforming growth factor-β (TGF-β) is a family of pleiotropic regulatory cytokines (TGF-β1, β2, β3) produced and secreted by different cell types and tissues. TGF-β has been described as a potent inducer of EMT in cancer (Wendt et al., 2009).

In view of some reports that describe the induction of an autocrine loop of TGF-β1 in relation to EMT in breast cancer cells (Gregory et al., 2011; Vendrell et al., 2012) we investigated the expression of TGF-β1 in tumor cells (Fig. 4D). After 24 h there was a significant increase in the expression of TGF-β1 in MDA-MB-231 cells treated with CM(+) compared to CM(−). Meanwhile CM(20 μM HA) significantly reduced TGF-β1 intracellular levels. In MCF-7 cells the expression of TGF-β1 was low and no differences were observed in the presence of CM(−), CM(+) or CM(20 μM HA) after 24 h.

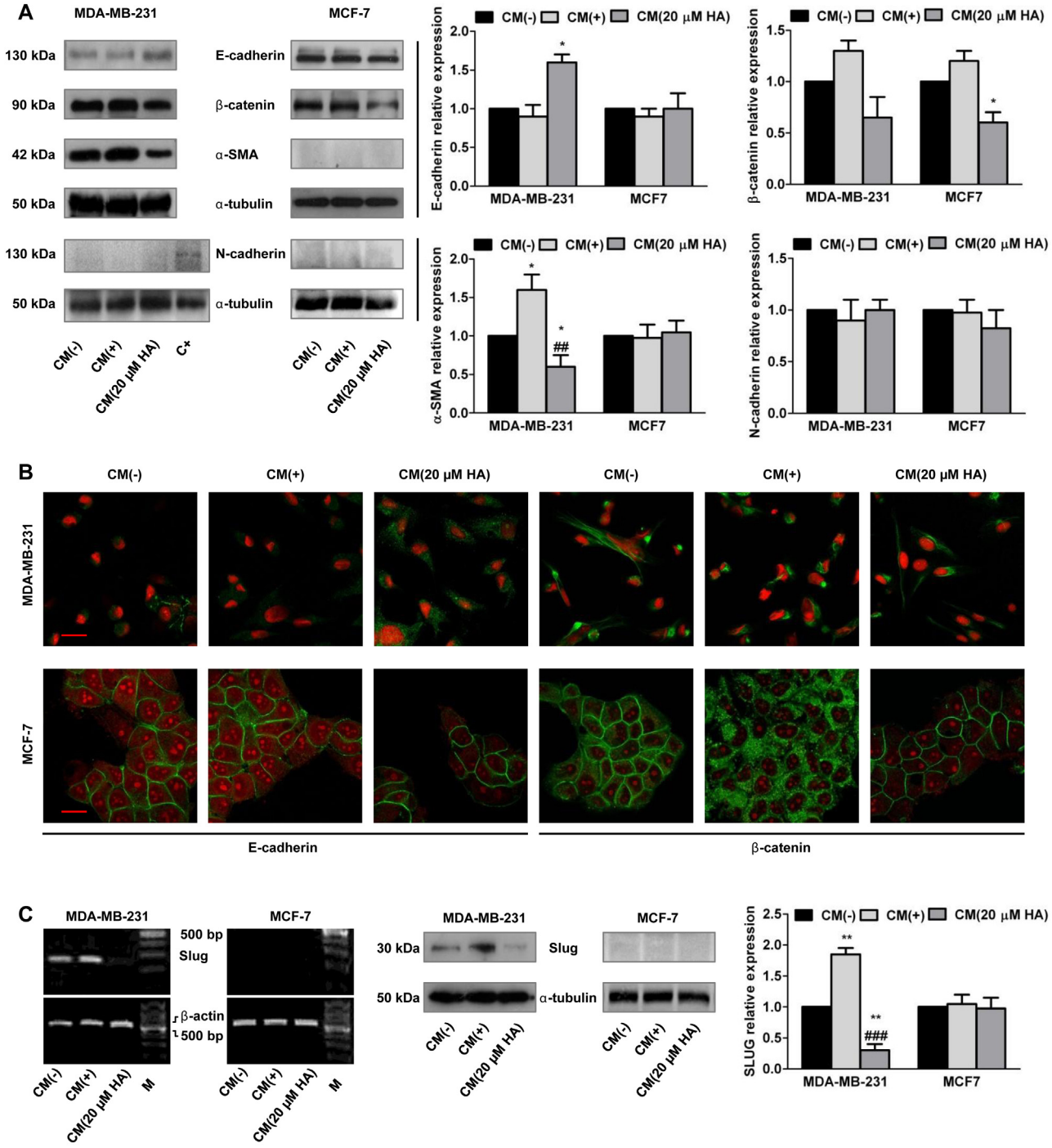

Fig. 3. Effects of conditioned media from fibroblasts on the expression of epithelial and mesenchymal markers in mammary tumor cells. MDA-MB-231 and MCF-7 cells were cultured with CM from fibroblasts treated during 24 h with CM(−), CM(+) or CM(20 μM HA). (A) *Immunoblots for E-cadherin, β-catenin, α-SMA and N-cadherin*. Cell lysates were electrophoresed and probed with the specific antibodies. α-tubulin was used as load control and for normalization. A representative Western blot is shown for each cell line. Bar chart: mean protein relative expression to CM(−) ± SD of independent experiments ($n = 4$ for MDA-MB-231; $n = 3$ for MCF-7), $^{*}P < 0.05$ *vs.* CM(−), and $^{\neq\neq}P < 0.01$ *vs.* CM(+). (B) *Immunofluorescence for E-cadherin and β-catenin*. MDA-MB-231 and MCF-7 cells were seeded on glass coverslips and treated. After 24 h cells were probed with the specific antibodies. Pictures were taken at 600× magnification using a confocal microscope. Scale bar: 20 μm. (C) *Slug expression. RT-PCR*: PCR products were electrophoresed and photographed. β-actin was used as load control. A representative gel is shown for each cell line. M: DNA ladder molecular size marker. Slug: 251 bp; β-actin: 521 bp. *Immunoblot for Slug*: Bar chart shows the mean protein relative espression to CM(−) ± SD of three independent experiments, $^{**}P < 0.01$ *vs.* CM(−); $^{\neq\neq\neq}P < 0.001$ *vs.* CM(+).

4. Discussion

Tumor microenvironment also called tumor stroma is a structural network that comprises the extracellular matrix (ECM), endothelial and inflammatory cells and also fibroblasts. Nowadays, it is well accepted that tumor stromal environment discloses not only a supportive role. Heterotypic signals between stromal and tumor cells due to a direct cell-to-cell contact or by soluble factors influence the tumor microenvironment and are essential for tumor growth and progression to metastasis (Calorini and Bianchini, 2010; Strell et al., 2012; Ungefroren et al., 2011). Among host cells, fibroblasts are a major component of solid tumors and they

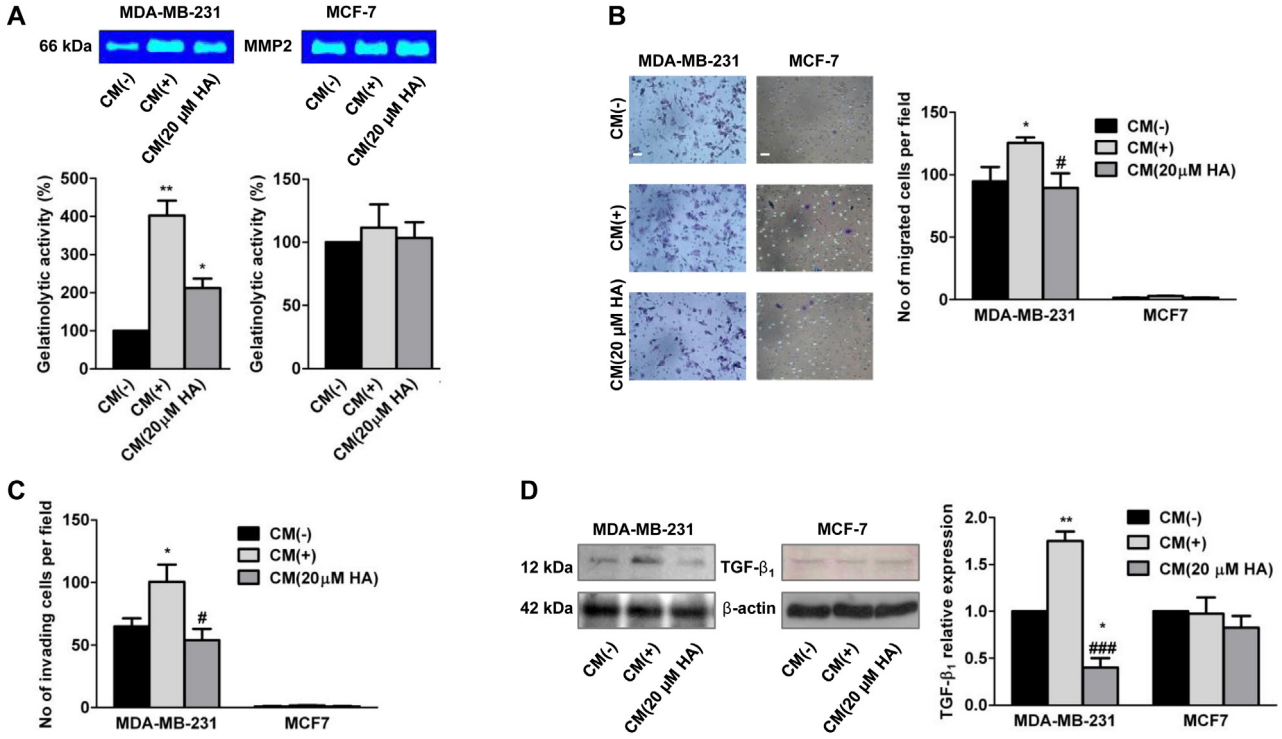

Fig. 4. Action of conditioned media from fibroblasts on the gelatinolytic activity, migration, invasion and intracellular expresion of TGF-β1 in mammary tumor cells. (A) *Gelatinolytic activity*. MDA-MB-231 and MCF-7 cells were cultured with unconditioned media [CM(−)] or with CM from fibroblasts treated with 20 μM histamine [CM(20 μM HA)] or not [CM(+)]. After 24 h media were replaced by fresh serum free media. Enzymatic activity of MMP2 was assessed by gelatin zimography. A representative gel is shown for each cell line. Bars represent the mean ± SEM of four independent experiments run in duplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* CM(−). (B) *Cell migration*. MDA-MB-231 and MCF-7 cells in serum free medium were loaded into the upper chamber while CM(−), CM(+) or CM(20 μM HA) with 1% FBS were added to the lower chamber. After 20 h migrated cells were fixed, stained and photographed. Six random fields were counted. Bar chart: mean ± SEM of three independent experiments run in duplicate, $^{*}P < 0.05$ *vs.* CM(−) and $^{\neq}P < 0.05$ *vs.* CM(+). Pictures were taken at 400× magnification. Scale bar: 20 μm. (C) *Cell invasion*: Similar protocol to cell migration with the upper chamber coated with Matrigel was used to evaluate cell invasion after 24 h. Bar chart: mean ± SEM of three independent experiments run in duplicate, $^{*}P < 0.05$ *vs.* CM(−) and $\neq P < 0.05$ *vs.* CM(+). (D) *Intracellular expression of TGF-β1*. MDA-MB-231 and MCF-7 cells were cultured with CM(−), CM(+) or CM(20 μM HA) for 24 h. Total cellular lysates were electrophoresed and probed with the specific antibodies. β-actin was used for load control and for normalization. A representative Western blot is shown for each cell line. Bar chart: mean protein relative expression to CM(−) ± SD of 3 independent experiments.$^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* CM(−); $^{\neq\neq\neq}P < 0.001$ *vs.* CM(+).

are characterized for remaining as "constantly activated". Fibroblasts activated by different stimuli promote tumor cell growth and increase angiogenesis, invasion and metastasis (Räsänen and Vaheri, 2010; Xouri and Christian, 2010). In this work we tested the action of CM from histamine-treated CCD-1059Sk fibroblasts on breast tumor cells regarding EMT to clarify the role of histamine in tumor microenvironment. MCF-7 and MDA-MB-231 cell lines were chosen for the experiments as representative of the two most frequent subtypes of human breast cancer cells, the luminal and the basal-like respectively, which exhibit distinctive biological characteristics such as morphology, different pattern of epithelial and mesenchymal markers expression and invasive potential. Luminal cells appear more differentiated (cobblestone-shaped) while basal cells have a more mesenchymal-like appearance (spindle-shaped) and a higher invasive potential. Plasticity of MDA-MB-231 and MCF-7 cells concerning EMT is reported in literature (Cannito et al., 2008; Liu and Feng, 2010; Theys et al., 2011). Thus, initially the possibility existed for a positive regulation of mesenchymal markers in both cell lines incubated with CM from fibroblasts.

In view of our aim, we firstly evaluated histamine receptors in CCD-1059Sk fibroblasts derived from normal breast tissue, and detected mRNA for histamine H1, H2 and H4 receptors by RT-PCR. Other authors also describe the presence of H1, H2 and H4 receptors in fibroblasts from human lung and skin involved in cell proliferation and migration and related to wound healing and inflammatory and fibrotic disorders (Garbuzenko et al., 2004; Ikawa et al., 2008; Kohyama et al., 2010; Kunzmann et al., 2007). It is known that fibroblasts produce and secrete collagen, fibronectin and laminin to the ECM and regulate the ECM turnover by producing proteolytic enzymes as MMPs which affect cell morphology, proliferation, survival and cell death (Bhowmick et al., 2004; Xouri and Christian, 2010). We demonstrated that CCD-1059Sk fibroblasts express mRNA, protein and activity of MMP2. Histamine differentially modulates MMP2 activity with an increase at 0.1 μM mainly through H1 receptor and a significant reduction at 20 μM *via* H2 receptor. Several reports demonstrate that histamine may modify the production of distinct MMPs in human nasal and synovial fibroblasts and also influence the mRNA and protein expression of MMPs in epithelial normal and tumor cells (Asano et al., 2004; Cricco et al., 2011; Doyle and Haas, 2009; Genre et al., 2009; Gschwandtner et al., 2008; Tetlow and Woolley, 2004). In this work, we also demonstrated that migration of fibroblasts is histamine dose-dependently modulated, being enhanced by low concentrations while inhibited by high doses of histamine. Accordingly, a great deal of evidence supports histamine action on the migratory ability of human lung fibroblasts in fibrosis and also immune cells to the inflammatory spots in tissues (Gschwandtner et al., 2011; Kohyama et al., 2010; Truta-Feles et al., 2010). Besides, we evaluated the expression of α-SMA and determined an increase with histamine doses lower than 1 μM whereas fibroblasts treated with 20 μM histamine showed an expression similar to controls. Thus, our current data showed for the first time that CCD-1059Sk fibroblasts express H1, H2 and H4 histamine receptors and 20 μM histamine may prevent the activation of CCD-1059Sk fibroblasts.

EMT has been defined as a driving force in tumor progression, supporting cancer cells to leave behind their native land

and colonize distant locations. Distinctive molecular hallmarks of EMT comprise down-regulation of epithelial cadherins and up-regulation of mesenchymal genes related to motility and remodeling of extracellular matrix (Foroni et al., 2012).

E-cadherin is a glycoprotein involved in adherens junctions and acts as a repressor of tumor progression in maintaining intact cell–cell contacts and preventing cell mobility, invasion and metastatic dissemination. This protein may be regulated by changes in subcellular distribution, translational or transcriptional events and by protein degradation (Baranwal and Alahari, 2009). E-cadherin is also described as a negative regulator of β-catenin, an essential component of junctional complexes linking E-cadherin to the actin filaments (Wells et al., 2008). During EMT the cadherin–catenin complex is disrupted and this action results in increased cytoplasmic β-catenin. Hence this protein enters into the nucleus modulating target gene expression. Genes as Snail, Slug and Twist are induced (Conacci-Sorrell et al., 2003; Jamora et al., 2003) while others like E-cadherin and occludin that encode the cell–cell junctional apparatus are repressed (Thiery et al., 2009).

In the current work EMT related changes induced by CM(+) in MDA-MB-231 cells could easily be discerned at both morphological and molecular levels. CM(+) induced a more elongated morphology in MDA-MB-231 cells after 7 days in culture. There was a very low expression of E-cadherin in cells cultured with or without CM(+) for 24 h in agreement with other reports (Sehrawat and Singh, 2011) while an increase in the typical mesenchymal marker α-SMA was observed. Accordingly, the expression of α-SMA is mainly described for EMT in breast cancer cells, being detected in breast tumors of the basal phenotype (Sarrió et al., 2008) and in basal-like tumor cells as MDA-MB-231 cells (Neve et al., 2006).

Different reports signal MDA-MB-231 cells as positive or negative for the expression of N-cadherin (Nieman et al., 1999; Wang et al., 2002). We could not detect N-cadherin expression either in control or in treated cells.

β-Catenin is known as a key suppressor of tumor cell migration and invasion when it is a component of the stable adherens junction at cell membrane (Schmalhofer et al., 2009). In MDA-MB-231 cells treated with CM(+) we could observe cytoplasmic and perinuclear β-catenin, enhanced MMP2 activity and increased cell migration, invasion and Slug expression. In this regard, associations of nuclear and cytoplasmic β-catenin with nuclear Slug expression and loss of membrane E-cadherin expression were also reported (Prasad et al., 2009). Considering our results we may conclude that CM from fibroblasts [CM(+)] enhanced traits related to oncogenic EMT in MDA-MB-231 cells. Notably, CM(20 μM HA) reversed all these features and led to a significant up-regulation of E-cadherin. Moreover, an inverse trend between E-cadherin and Slug expression was observed in MDA-MB-231 cells with CM(20 μM HA), supporting the role of the transcriptional factor Slug as a repressor of E-cadherin (Bolós et al., 2003; Hajra et al., 2002). Several studies confirmed that the phenotype of breast cancer cells may be determined by targeting the EMT regulator Slug (Liang et al., 2013; Storci et al., 2008).

MCF-7 cells showed some phenotypical changes related to EMT in the presence of CM(+) which were not evident when epithelial cells were cultured with CM(20 μM HA). After 7 days we observed spindle-shaped cells and colony scattering in MCF-7 cells treated with CM(+). A down-regulation or changes in subcellular distribution of E-cadherin might be involved in these events. However we could not determine either a change in E-cadherin total level or a differential subcellular distribution after 24 h. Though MCF-7 cells are negative for N-cadherin, the expression may be modified when cells acquire a mesenchymal-like phenotype (Gao et al., 2010). Mesenchymal markers as α-SMA and N-cadherin could not be detected after 24 h incubation with CM(+), although cytoplasmic β-catenin was observed in cells with CM(+) after 24 h. Additionally, no changes in Slug protein expression were apparent with the different treatments, while cell migration (and invasion) or modulation of MMP2 activity was not observed. Other members of Snail family could have some influence on the expression of β-catenin and need to be investigated.

Interestingly, MCF-7 cells treated with CM(20 μM HA) for 7 days did not display mesenchymal morphology. Moreover, CM(20 μM HA) reduced the expression of β-catenin and localized this protein in the membrane after 24 h. Similar findings in E-cadherin and β-catenin levels and subcellular localization were observed in relation to the *in vitro* aggressive phenotype in tamoxifen-resistant MCF-7 cells (Hiscox et al., 2006) and in clones of MCF-7 cells with more migratory and invasive capabilities than parental cells (Uchino et al., 2010).

Different regulation of characteristics consistent with increased EMT in basal and luminal-like breast tumor cells interacting with stromal cells has been reported in relation with different patterns of expression of EMT genes. The basal-like cells, in contrast to luminal-like cells, showed up-regulation of genes modulating EMT-related pathways and also TGF-β1 and Twist genes, as well as an increase in cell migration (Camp et al., 2011). Furthermore, TGF-β1 autocrine loops are frequently described in cancer in relation to EMT (Gregory et al., 2011; Vendrell et al., 2012; Xu et al., 2012). MCF-7 cells in co-culture with human adipose-derived mesenchymal stem cells display EMT features in a time-dependent manner with modifications in markers after 72 h. EMT changes are related to TGF-β1 secreted by mesenchymal cells and an autocrine TGF-β1 signaling is also initiated after 8 days (Xu et al., 2012). Our experiments showed different responses for each cell line after 24 h incubation with CM(+): MDA-MB-231 increased mesenchymal traits while MCF-7 only changed β-catenin expression and localization. The results for MCF-7 cells suggest a possible action of CM(+) after a prolonged incubation of 7 days (as evidenced by changes in morphology and cell scattering) but the determination of the expression of EMT related genes that are involved remains to be carried out. Cells that respond to TGF-β1 stimulus during EMT show concomitant loss of adherens and tight junctions, down-regulation of E-Cadherin expression, and increase in mesenchymal cell markers (Chaudhury and Howe, 2009). The highly invasive MDA-MB-231 cells express higher levels of TGF-β1 and TGF-β receptors than the least invasive MCF-7 (Arteaga et al., 1998; Wang and Lui, 2012). As other authors we found a higher expression of TGF-β1 in MDA-MB-231 cells compared to MCF-7 cells when cultured with unconditioned medium CM(−).

Snail, Slug, Zeb and Twist proteins act as transcriptional repressors and regulators of TGF-β1-induced EMT (Fuxe and Karlsson, 2012). TGF-β1 signaling proteins interact with these factors and repress promoters of genes encoding E-cadherin and occludin while active others like β-catenin (Xu et al., 2009). In the present work, the pattern expression of TGFβ1 in MDA-MB-231 cells treated with CM(−), CM(+) or CM(20 μM HA) was similar to the pattern of Slug protein expression. It has also been reported that TGF-β1 may regulate MMPs and TIMPS in MDA-MB-231 cells (Wang and Lui, 2012). In concordance, current experiments show that MDA-MB-231 cells treated with CM(+) exhibit the highest increase in MMP2 gelatinolytic activity. Remarkably, in MDA-MB-231 treated with CM(20 μM HA) there was a significant reduction in the intracellular TGF-β1 level together with a significant decrease in the expression of Slug and also in the enzymatic activity of MMP2. In contrast MCF-7 cells expressed low levels of TGF-β1 that were not modified by any CM after 24 h. It is possible that the morphological changes observed in MCF-7 cells after 7 days are related to a late up-regulation of TGF-β1 levels as reported by Xu et al. (2012).

In diverse physiological and tissue contexts, cells that have undergone EMT can regain epithelial characteristics, thus leading to

a mesenchymal–epithelial transition, MET (Kalluri and Weinberg, 2009). Our data denoted that high doses of histamine altered fibroblast–tumor cell interaction not only hindering the acquisition of a more mesenchymal phenotype in the basal-like breast tumor cell line mainly, but even endowing the cells with further epithelial characteristics (increase in E-cadherin, decrease in α-SMA and Slug expression). In the current work we demonstrated that CM from normal fibroblasts may differentially strengthen some events involved in EMT process in breast cancer cells with different malignant behavior. Notably, 20 μM histamine prevents the activation of fibroblasts and the EMT related changes induced in tumor cells by CM from fibroblasts. It is well documented that activated fibroblasts secrete a variety of soluble growth factors and chemokines (TGF-β among others) which act in a paracrine way on different cell types driving tumor growth and progression (Su et al., 2012; Zavadil and Bottinger, 2005). In this regard, histamine may be modulating the secretion of any soluble factor from fibroblasts. Further studies need to be conducted in order to identify them.

The affinity of histamine binding to different histamine receptors varies significantly, ranging from 5 to 40 nM for the H3 and H4 receptors and from 2 to 40 μM for the H1 and H2 receptors; thus the effects of histamine upon receptor stimulation can be very complex (Shahid et al., 2009). *In vivo*, due to its potent pharmacological activity even at very low concentrations, the synthesis, transport, storage, release and degradation of endogenous histamine is tightly regulated. High concentrations (μM) can be locally achieved upon liberation from histamine-storing cells such us mast cells, basophils, macrophages, enterochromaffin-like cells and histaminergic neurons. Infiltrating mast cells and macrophages are abundant components of adenocarcinomas, particularly breast cancers. They accumulate in stroma in response to numerous chemoattractants and they play a dual role in tumor biology by secreting, upon different stimuli, a plethora of factors that may induce tumor cell growth or death, angiogenesis, matrix remodeling and immunosuppression (Cook and Hagemann, 2013; Theoharides and Conti, 2004). Histamine is only one of them, and in light of our results endogenous histamine may also influence fibroblast–tumor cell interaction.

Interactions between tumor and stromal cells are crucial for cancer development and metastasis but they are still far from being fully elucidated. Actual treatments for breast cancer (surgery, radiotherapy, chemotherapy, antibody therapy and endocrine therapy) target the tumor cells but overlook the stromal cells in the surrounding microenvironment. It is a life-size challenge for cancer researchers to recognize drugs which inhibit tumor cell proliferation and also disrupt tumor–stroma interactions by modulating paracrine factors.

Throughout the last decade histamine has been used as an immunomodulator in clinical trials phase II and III for melanoma, metastatic renal cell–carcinoma and acute myeloid leukemia, being well tolerated and with non side-effects (Berry et al., 2011; Brune et al., 2006; Perz and Ho, 2008; Yang and Perry, 2011). Notably, the doses of histamine used in these treatments were predicted to reach high (μM) concentrations (Brune et al., 2006).

Therefore, our current data together with our previous reports about histamine and the inhibitory effect on tumor cell proliferation (Cricco et al., 2006a, 2008; Medina et al., 2006) lead us to suggest histamine as a potential agent to be considered in the investigation for new combined treatments in conventional or targeted therapies for basal-like breast tumors.

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