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Title: The β_2-Adrenergic Agonist Salbutamol Inhibits Migration, Invasion and Metastasis of the Human Breast Cancer MDA-MB-231 Cell Line

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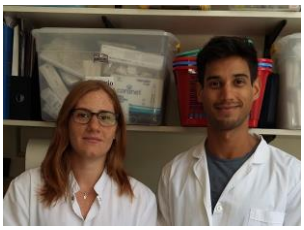

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Abstract:

Background: Breast cancer is the most diagnosed and the major cause of cancer death in women worldwide. Metastasis is the main cause of these deaths. The metastatic cascade involves multiple steps and it has been described that adrenergic receptors can modulate this process at multiple levels. However, β-adrenergic action in breast cancer is controversial. We have previously shown that β-adrenergic agonists inhibit cell proliferation and tumor growth of numerous breast cancer models. **Objective:** The purpose of the present investigation was to evaluate adrenergic effect in parameters related to tumor progression (migration, invasion and metastases) in two human breast cancer cell lines. **Method:** Migration was assessed in IBH-6 and MDA-MB-231 cells by time-lapse videomicroscopy and modified Boyden chambers. Invasion was evaluated by Transwells coated with Matrigel and expression of pro-metastatic genes was determined by RT-qPCR. Experimental metastases studies were performed by injection of the cells in the tail vein of NSG immuno-deficient mice. **Results:** In both cell lines, salbutamol (β_2-agonist) and propranolol (β-blocker) significantly diminished cell migration while epinephrine exerted opposite effects. Moreover, salbutamol inhibited invasion of both breast cancer cell lines and enhanced adhesion to extracellular matrix. Salbutamol treatment was also able to decrease the expression of pro-metastatic genes in MDA-MB-231 cells. Finally, this compound decreased the number and size of MDA-MB-231 lung experimental metastases in NSG immuno-deficient mice. No effect on the establishment of IBH-6 metastases was observed. **Conclusion:** Our results suggest that salbutamol could be an effective adjuvant drug for the treatment of metastatic breast cancer.

Keywords: Breast cancer, metastasis, β-adrenoceptors, salbutamol, MDA-MB-231, IBH-6.

1. INTRODUCTION

Breast cancer is the most diagnosed cancer and the major cause of cancer death in women worldwide [1]. The treatments for the different subtypes of breast cancer are well established. For example, endocrine therapy for hormone-dependent tumors and anti-human epidermal growth factor receptor 2 (HER-2) therapies for tumors overexpressing this receptor. Triple negative tumors (TNBC) have the worst outcome among breast cancer subtypes, lack specific treatment and present high recurrence rates. De novo and acquired resistance is a great concern in every case [2-4]. Immortalized cell lines remain powerful tools and give valuable information that can benefit treatment [5]. IBH-6 [6, 7] and MDA-MB-231 [8] are cell lines developed from breast cancer lesions that represent different breast cancer subtypes. The former are estrogen and progesterone receptor positive and HER-2 negative and arose from a primary tumor [7]. IBH-6 cells are able to grow in nude mice without exogenous hormone supply and to invade adjacent tissues but not to metastasize [6]. MDA-MB-231 cells are highly metastatic and usually considered as triple negative, although they have been reclassified within the claudin-low molecular subtype [9]. Metastases are the main cause of cancer deaths. The metastatic process involves multiple steps: The first one is local invasion, followed by intravasation into the circulatory system [10], systemic dissemination, extravasation in secondary organs, settlement into latency, reactivation and outgrowth [11]. It has been shown that, in general, luminal breast cancer tumors are associated with bone metastasis,

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whereas basal-like and HER2-enriched molecular subtypes are associated with brain and lung relapse [12]. As the majority of breast cancer deaths occur in least developed countries, new effective and low-cost therapies are a health priority [13].

β-adrenoceptors (β-AR) are G protein–coupled receptors that have been described in the 1990s in breast cancer cell lines [14] and tumors [15]. Since then, the effect of their stimulation in breast cancer progression has been controversial. Some authors have associated them with an increase in cell proliferation and tumor growth [16], while our group and others to a decrease in these parameters [17, 18]. Also, it has been described that norepinephrine acting through β-AR inhibits the migration of human ES-2 ovarian carcinoma [19] and human MDA-MB-231 breast cancer cells [20]. However, the effect seems to be dependent on cellular context because there are various reports showing that, on the contrary, activation of these receptors increases migration, invasion and metastasis of ovarian and breast tumors [21-25]. In line with this, propranolol, a β-blockers used to treat hypertension, have been proposed as candidate for drug repurposing in metastatic disease [26-29]. On the other hand, the β_2-adrenergic specific agonist, salbutamol has shown promising results in our laboratory in terms of cell proliferation and tumor growth in several murine and human breast cancer pre-clinical models [18]. In the present manuscript, we take a step forward to determine the action of β-adrenergic drugs on several parameters related to cancer progression both *in vitro* and *in vivo* and we describe that salbutamol-induced β_2-AR stimulation decreases migration, invasion and metastasis of human breast cancer cells.

2. MATERIALS AND METHODS

2.1 Breast cancer cell lines and culture conditions

IBH-6 human breast cancer cell line was originally isolated from a primary human breast carcinoma [7]. MDA-MB-231 human breast cells were recently obtained from ATCC (Manassas, VA). Cells were maintained in Dulbecco´s Minimum Essential/Ham F12 (1:1) medium supplemented with heat-inactivated 10% fetal bovine serum (FBS), bovine or recombinant human insulin (2 µg/ml), 2 mM glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were grown on plastic, incubated at 37°C in 5% CO2 and subcultured at approximately 80% confluence using 0.25 % trypsin-0.025% EDTA.

2.2 Migration experiments

Time-lapse videomicroscopy migration experiments were carried out as previously described [30]. In brief, a cell suspension of $7x10^4$ MDA-MB-231 or $9x10^4$ IBH-6 cells was mixed with a carbonate-buffered collagen type I solution (1.63 mg/ml collagen) containing minimal essential medium. The suspension was filled into self-constructed migration chambers and after polymerization of collagen, migration of cells was recorded by time-lapse videomicroscopy for 15 h at 37ºC. The paths of 30 randomly selected cells were digitized by computer assisted cell tracking. The migratory activity was calculated as the proportion of cells (in percentage) which was locomotory active. The following adrenergic compounds were added to the collagen lattices and were used in subsequent experiments: norepinephrine, epinephrine, salbutamol, propranolol.

For migration assays in modified Boyden chambers, a suspension of $1.2x10^4$ cells in 50 µl serum-free medium was seeded on the upper wells. Cells were allowed to migrate for 6 h through an 8 µm pore polycarbonate filter towards a chemotactic stimulus (medium with 2.5% FBS) on the lower chamber wells. Adrenergic compounds were added to both sides of the chamber. The filter was carefully removed and cells on the upper side were scraped off. Cells attached to the lower side of the filter were fixed in 2% paraformaldehyde, stained with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) and photographed. Images of 3 representative visual fields were analyzed using CellProfiller software (www.cellprofiller.com).

2.3 Invasion assay

Tissue culture Transwell inserts of 8 µm pore size were coated with growth factor reduced Matrigel for 1h at 37°C and then dried for 6 h at room temperature under hood (14 µg and 4 µg per insert of Matrigel were used for MDA-MB-231 and IBH-6, respectively). Matrix was re-hydrated and placed in 24-well plates containing 10% FBS medium prior to the addition of $5x10^4$ cells in serum-free medium to the upper well. Adrenergic compounds were added to both upper and lower wells. After 24 h of incubation, inserts were washed with phosphate saline buffer (PBS), fixed with ice-cold methanol and stained with 0.05% crystal violet. Cells on the upper side of the membrane were scraped off with a cotton swab and the cells on the underside were counted under a bright-field microscope.

2.4 Adhesion assay

96-well tissue culture plates were coated with 10 µg of Matrigel or 9 µg of collagen type I per well in PBS. After reconstitution of the matrix, 50 µl of medium with 4% FBS and adrenergic compounds at 2X concentration were added to each well. Cells were harvested using TrypLE™ Express and seeded ($1x10^4$/50µl) in serum-free medium. MDA-MB-231 cells and IBH-6 cells were allowed to adhere to each matrix at 37°C for 1 h and 1.5 h respectively. Wells were carefully washed with PBS to remove non-adherent cells, fixed with ice-cold methanol and stained with 0.05% crystal violet. Each well was photographed under a bright-field microscope at 40 X magnification. Cell number and average cell area were quantified automatically with ImageJ [31].

2.5 Quantitative Real Time PCR

RNA was obtained using TRI reagent and cDNA was synthesized from 1µg of total RNA using MMLV reverse transcriptase, according to the manufacturer's instructions. For qPCR, FastStart Universal SYBR Green Master Mix was used in a CFX96 Touch™ Real-Time PCR Detection System. Primers:
GAPDH
5'-TGCACCACCAACTGCTTAGC-3'
5'- GGCATGGACTGTGGTCATGAG-3',

β2-AR
5'-GCCATTGCCTCTTCCATCGT-3'
5'- AAGCGGCCCTCAGATTTGTC-3',
MMP-9
5'-GCCTGCAACGTGAACATCTTCG-3'
5'-AGAGAATCGCCAGTACTTCCCATCC-3',
IL-8
5'-CCAGGAAGAAACCACCGGA-3'
5'-GAAATCAGGAAGGCTGCCAAG-3',
VEGF
5'-CCTTGCTGCTCTACCTCCAC-3'
5'-CCATGAACTTCACCACTTCG-3',
E-Cadherin
5'-GACCAAGTGACCACCTTAGA-3'
5'-CTCCGAAGAAACAGCAAGAGC-3´[32],
N-Cadherin
5'-GAATGACAACAGACCTGAGTTC-3'
5'-CGATTCTGTACCTCAACATCC-3',
Vimentin
5'-TCCCTGAACCTGAGGGAAACT-3'
5'-AGGTCATCGTGATGCTGAGA-3'.

For all experiments, gene expression was normalized to GAPDH housekeeping expression using the $2^{-\Delta\Delta ct}$ method.

2.6 *In vivo* experiments

Eight-week-old NOD-scid/IL2Rgamma^{null} mice (NSG mice) were obtained from The Jackson Laboratory and bred at IBYME's animal facility under pathogen free conditions, in ventilated racks, with a 12h light-dark cycle. Tumor cells were injected via the lateral tail vein ($5x10^5$) for experimental metastasis studies. Animals were randomized and received daily subcutaneous injections of salbutamol (1.2mg/kg/day) or saline for control groups. At day 30, lungs were fixed in Bouin's Solution and macroscopic metastatic nodules were counted under a dissection microscope.

2.7 Immunohistochemistry

Tissue sections (5 μm) were deparaffinized and inhibition of the endogenous peroxidase was performed with H_2O_2 in ethanol. Antigen retrieval was carried out in a thermal bath at 90ºC with 10 mM citrate buffer pH 6. The sections were thoroughly washed with PBS and incubated overnight with the primary antibody diluted in PBS (the proliferation marker protein Ki67 1/500, phospho-cAMP Responsive Element-Binding, pCREB 1/400, pan-cytokeratins, pan-CK 1/100). Then, the tissue sections were washed, incubated with the appropriate secondary antibody and the ABC kit and signal was detected using 3,3P-diaminobenzidine chromogen solution according to manufacturer's protocol. All tissue sections were counterstained with hematoxylin, dehydrated, and mounted with DPX.

2.8 Statistical analysis

Data are presented as mean ± SD and all statistical analysis were carried out with Graphpad Prism 6 software. Statistical analysis was performed by unpaired two-tailed Student's t test or ANOVA followed by Tukey-Kramer or Dunnet–Kramer test [33]. The normality was tested by D'Agostino-Pearson´s test and the homogeneity of the variance by Bartlett´s test. A p value less than 0.05 was considered statistically significant.

2.9 Materials

Culture medium, and all the compounds used for cell culture were from Invitrogen, Thermo Fisher Scientific, (Waltham, MA, USA). Fetal Bovine Serum was from Invitrogen or NATOCOR, Córdoba, Argentina. The poly-carbonate filters were Nucleopore membranes, Neuroprobe (MD, USA). DAPI was acquired from Sigma-Aldrich (St. Louis, MO, USA). Transwells and Matrigel were from BD Biosciences (San Jose, CA, USA). Collagen for videomi-croscopy was from Invitrogen and for cell adhesion from BD Biosciences. TRI reagent was acquired from MRC (Cincinnati, OH, USA) and MMLV reverse transcriptase from Promega, (Madison, WI, USA). FastStart Universal SYBR Green Master Mix was purchased from ROCHE (Mannheim, Germany) and the CFX96 Touch™ Real-Time PCR Detection System, from BIORAD (Hercules, CA, USA). Anti-Ki67 antibody (ab15580) was from Abcam; anti-pCREB (Ser133), from Cell Signaling and anti-cytokeratin Pan Type I/II (AE1/AE3), from Thermo Fisher Scientific. ABC kit and diaminobenzidine chromogen solution were from Vector Lab (Burlingame, CA) and DPX Mountant for histology (a mixture of distyrene, a plasticizer, and xylene), as well as norepinephrine, epinephrine, salbutamol, propranolol were from Sigma-Aldrich.

3. RESULTS

Adrenergic effect on cell proliferation and tumor growth has already been studied in the two cell lines used in this investigation. Salbutamol, particularly, has proved to be a potent inhibitor of these parameters. However, the most important features of tumor progression must be addressed in experimental models before performing any clinical evaluation. As a first step for studying tumor progression, cell migration was measured using time-lapse videomicroscopy in IBH-6 and MDA-MB-231 cell lines. We chose concentrations that have already proved effective *in vitro* [18, 20]. Specifically, propranolol was used 10X with respect to salbutamol in order to compete for β_2-ARs when used in combination [18]. Both cell lines showed an extremely high spontaneous migration, ranging between 60-80% of moving cells. Interestingly, both the β_2-agonist salbutamol (Fig. 1A and C) and the β-blocker propranolol (Fig. 1B and D) significantly diminished cell locomotion. Epinephrine and norepinephrine significantly inhibited migration of MDA-MB-231 cells (Fig 1B). On the contrary, in IBH-6 cells epinephrine enhanced this parameter (Fig 1D). Additionally, modified Boyden Chamber assays were used to evaluate cell migration towards a chemotactic stimulus in the presence of adrenergic drugs. Again, salbutamol and propranolol inhibited cell migration (Fig. 1E for MDA-MB-231, Fig. 1F for IBH-6 cells). Moreover, when used in combination, propranolol was not able to reverse the effect of the agonist. In MDA-MB-231 cells, epinephrine showed the same inhibitory effect (at 1 μM concentration) as in cell-tracking experiments but no action

on IBH-6 cells was detected. Epinephrine is a ligand for all ARs, α and β, so its effects are highly dependent on AR relative concentration. Therefore, β_2-AR expression was assessed by RT-qPCR in MDA-MB-231 and IBH-6 (Fig. 1G). The expression of β_2-AR is approximately 20 times higher in MDA-MB-231 than in IBH-6 cells and more than 200 times higher than in MCF-7 cells, which were used as control.

Another important feature of cancer progression is invasion. We assessed adrenergic modulation of this parameter *in vitro* using Transwell chambers coated with the reconstituted basement membrane Matrigel. Salbutamol treatment significantly inhibited invasion in both cell lines (Fig. 2A, B and C). In MDA-MB-231 propranolol was able to reverse the effect of the agonist. However, in IBH-6 cells, as for migration, propranolol not only did not reverse the action of salbutamol but also exerted the same inhibitory effect.

The action of adrenergic compounds on cell adhesion and spreading onto Matrigel and collagen matrices was then evaluated. Epinephrine and salbutamol significantly enhanced cell adhesion to Matrigel although not to collagen in MDA-MB-231 cells (Fig. 2D). As a measurement of cell spreading, cell area was quantified. In this cell line, cell area over Matrigel matrix was also very significantly increased by both epinephrine and salbutamol, being the effect of the β_2-agonist partially reversed by propranolol (Fig. 2E). On collagen, this parameter was also increased by salbutamol and a tendency to produce the same effect was seen for epinephrine. For IBH-6, no change was observed in the number of adherent cells after adrenergic stimulus, however, epinephrine decreased the cell area onto collagen matrix (Fig. 2F and G).

The expression of several genes involved in cell migration, invasion and metastatic spread was then investigated by RT-qPCR in MDA-MB-231 cells treated with epinephrine or salbutamol. Treatment with salbutamol for 24 h significantly inhibited the expression of the mesenchymal differentiation marker vimentin (Fig. 3A) and stimulated E-cadherin expression, a marker of epithelial differentiation, (Fig. 3B) with no change in N-cadherin expression (Fig. 3C). Furthermore, epinephrine was also able to increase E-cadherin expression levels. Interestingly, both adrenergic compounds significantly diminished the expression levels of the matrix metalloprotease 9 (MMP-9, Fig. 3D), the chemokine interleukin-8 (IL-8, Fig. 3E) and the vascular endothelial growth factor (VEGF, Fig. 3F); all of them factors directly related to the invasive and metastatic potential of a cancer cell.

Our previous results show that salbutamol, at the dose used herein, is able to diminish tumor growth in both MDA-MB-231 and IBH-6 cells [18]. As tumor size is an important factor that affects the number of spontaneous metastasis, we assessed the effect of salbutamol on the ability of both breast cancer cell lines to establish experimental lung metastases, which are independent of tumor size. MDA-MB-231 and IBH-6 cells were inoculated intravenously to NSG mice and after 30 days of salbutamol treatment, lungs were inspected. The β_2-adrenergic agonist significantly decreased the total number of lung macroscopic metastases of MDA-MB-231 cells (Fig. 4A). Furthermore, it produced a reduction in the overall size of these metastases, as seen in the quantification of metastatic nodules larger than 0.5 mm (Fig. 4B). Although a small number of mice was used, salbutamol treatment had no effect either on the number or the size of IBH-6 experimental metastases (Fig. 4D and E). Representative histological images of lungs from mice inoculated with MDA-MB-231 and IBH-6 cells are shown in panels C and F. Next, we studied by immunohistochemistry, the expression of pan-cytokeratine (Pan-CK), the proliferation marker Ki67 and pCREB in MDA-MB-231 lung metastatic foci. These foci were positive for pan-CK staining confirming that these cell cumuli came from epithelial origin (Fig. 5A). Metastases from lungs of salbutamol-treated mice presented significantly lower Ki67 staining compared to those from the control group (Fig. 5B) possibly accounting for the observed reduction in size. Additionally, to confirm active β_2-AR signaling, we quantified the number of positive cells for pCREB in metastatic foci. As expected, given that β_2-adrenergic activation increases cAMP cellular levels, metastases in the lungs of salbutamol-treated mice showed an increased number of pCREB positive cells compared to control (Fig. 5C).

4. DISCUSSION

β-ARs, particularly β_2-AR, are emerging as promising targets for the treatment of breast cancer [26-28]. Several studies have assessed the effect of β-AR ligands (both agonists and antagonists) in breast cancer development and progression [16-18, 27, 28, 34]. However, almost none of them studied the action of salbutamol, a short acting β_2-AR selective agonist used for the treatment of asthma and chronic obstructive pulmonary disease. Here we report that salbutamol inhibits processes intimately related to cancer progression and metastasis in pre-clinical breast cancer models.

Our results show that in MDA-MB-231 cells, salbutamol and epinephrine inhibit migration and enhance adhesion to extracellular matrix. MDA-MB-231 are typically spindle shaped mesenchymal-like cells and after salbutamol treatment they acquire a cobblestone-like morphology with more apparent cell-cell contacts, characteristic of epithelial cells. This is in agreement with the enhancement found in E-Cadherin expression. The first type of morphology is generally associated with increased motility whereas the latter with a less migratory phenotype which is consistent with our results. Accordingly, it has been described that, in this cell line, norepinephrine decreases cell locomotion and increases adhesion to vascular endothelium [20]. Salbutamol also inhibited migration in IBH-6 cells, but on the contrary, epinephrine enhanced this parameter and in line with our previous statement, cell area of IBH-6 cells was decreased in collagen after epinephrine treatment. Likewise, it has been shown that the migration of several breast and non-breast cancer cell lines is stimulated by catecholamines [22, 30], this effect being blocked by β-AR antagonists [30, 35, 36]. Taken together, the three subtypes of α_2-AR are expressed at similar levels in IBH-6 and MDA-MB-231 cells as already reported by binding assays [37], however, β_2-AR expression is much higher in MDA-

MB-231. The β_2-AR concentration in this cell line has been previously described as the highest of several cell lines tested by binding assays [14]. In a previous report, we have shown that β_2-AR expression levels define the phenotype of human breast tumor and non-tumor cell lines [34]. Epinephrine binds to both α_2 and β-AR and these receptors have antagonistic actions in cAMP production. The data presented herein could be then interpreted as epinephrine increasing migration in IBH-6 cells acting mainly through the α_2-AR, while inhibiting this parameter on MDA-MB-231 cells through the β_2-AR. It has been recently described that activation of cAMP signaling inhibits the migration of this cancer cell line [38].

The metastatic process requires that cancer cells acquire the capacity to detach from the primary tumor and to invade surrounding tissue. Tumor angiogenesis is also a prerequisite for breast cancer progression [10]. Salbutamol proved to be able to enhance adhesion and inhibit invasion of breast cancer cell lines. In agreement with this, we found a decrease in MMP9 and vimentin expression levels and an increase in E-cadherin mRNA in MDA-MB-231 cells. MMP-9 is implicated in aberrant extracellular matrix remodeling associated with tumor angiogenesis, invasion and metastasis [39]. Partial or total loss of the E-cadherin is associated to changes or loss of differentiation, acquisition of an invasive phenotype, increased tumor grade, metastatic behavior and poor prognosis [39]. Vimentin is overexpressed in several tumors including breast cancer, and its expression correlates with increased tumor growth, invasion and worse outcome [40]. We found no change in N-cadherin expression, suggesting that salbutamol treatment does not induce a cadherin switch. It has already been reported that E-cadherin re-expression in MDA-MB-435 breast cancer cells did not modify the endogenous levels of N-cadherin [41]. Also, salbutamol decreased expression levels of VEGF and IL-8, two well-known pro-angiogenic factors. VEGF production has also been described to be inhibited by the β_2-agonist terbutaline and norepinephrine in MDA-MB-231 cells [42]. IL-8, also known as CXCL8, is a pro-inflammatory chemokine that binds to CXCR1 and CXCR2, two GPCRs. Both receptors are overexpressed in some cancer cells and have been associated with cell proliferation, invasion and angiogenesis [43]. It has been described that IL-8 is significantly upregulated in highly metastatic cells as compared to less metastatic ones [44].

Opposing to our results, formoterol, a long acting β_2-AR agonist, has been recently associated with enhanced invasion in a highly metastatic (HM) MDA-MB-231 derived cell line [21, 23]. According to the authors, this effect was due to a novel positive feedforward cAMP/Ca^{2+} loop [23]. Interestingly, and in line with our work, salbutamol was not able to induce Ca^{2+} release in HM cells and moreover, this loop was absent in parental MDA-MB-231 cells. In fact, in another study, norepinephrine inhibited the invasion of MDA-MB-231 cells via β_2-AR [45]. Still more, indacaterol, an ultra-long acting β_2-AR also inhibited this parameter on human fibrosarcoma HT-1080 cells [46]. Taken together, these results suggest that β_2-AR agonist-induced signaling, and therefore its biologic effect, is highly dependent not only on the agonist itself but on the cell line used.

We have previously shown that both salbutamol and propranolol exert inhibitory effects on MDA-MB-231 tumor growth in immuno-deficient mice [18]. Here we described that these two compounds have the same effect on other parameters. Propranolol has recently been described as having, unlike other β-blockers, a similar agonist effect as salbutamol in ERK1/2 signaling in human embryonic kidney (HEK) 293S cells stably transfected with human β_2-AR, this effect being reversed by the pure β_2-AR antagonist ICI 118551. This behavior is compatible with that of a partial agonist [47]. In non-tumor MCF-10A breast cells, we have found two distinct β_2-AR populations, one localized in raft microdomains stimulating a Gs/cAMP/Epac/adhesion-signaling module, and another population outside rafts inhibiting cell proliferation by a Gs/cAMP/PKA-dependent signaling pathway [48]. It is not astonishing that propranolol is able to reverse salbutamol effect on cell proliferation [18] and not in cell migration if different subsets of β_2-AR are involved in each process. An alternative explanation for the coincident action of salbutamol and propranolol is that long-term administration of the agonist could induce desensitization of these receptors [49], acting therefore in an antagonistic-like manner. Unfortunately, there is no consensus regarding β-blocker action in breast cancer. Propranolol has been associated in retrospective studies to improved outcome as reviewed [27-29, 50]. In TNBC in particular, perioperative use of propranolol was associated with reduced breast cancer recurrence and metastasis [51]. Moreover, a brain-seeking metastatic variable of MDA-MB-231 cells pretreated with propranolol alone *in vitro*, generated less brain metastases than non-treated cells *in vivo* [51]. On the other hand, others found no association between propranolol use and improved outcome [26, 52]. In fact, some β-blockers were associated with increased recurrence rates in a large Danish cohort [53].

Finally, salbutamol was able to diminish the number and size of experimental lung metastases of MDA-MB-231 cells in NSG mice. Also, the proliferation index of these metastases was decreased in salbutamol-treated mice, associated to an enhancement in CREB phosphorylation, suggesting the classical cAMP signaling pathway. It was reported that tumors belonging to the claudin-low molecular subtype overexpress β_2-AR compared with other tumors [54]. MDA-MB-231 claudin-low cells have proved to be particularly sensitive to β-adrenergic agonists [18] and herein. Hence, this subtype of breast cancer could be highly responsive to β_2-AR agonists and/or antagonists. To confirm this hypothesis, as salbutamol (also known as albuterol) is a very widely β_2-AR agonist used worldwide for the treatment of asthma and chronic obstructive pulmonary disease [55], an analysis of clinical outcome in salbutamol-treated breast cancer patients (with emphasis in the triple negative tumors) could be easily performed in the future. This kind of study has been successfully carried out for propranolol [56].

5. STANDARD PROTOCOL ON APPROVALS, & ANIMAL PROTECTION:

Animal care and manipulation were in agreement with the Guide for the Care and Use of Laboratory Animals [57, 58], the United Kingdom Co-ordinating Committee on Cancer Research guidelines for research with laboratory animals [59] and approved by the Institution´s Veterinary and Committee for welfare of the animals.

CONCLUSION

Salbutamol is an adrenergic compound with minimal side-effects that has proved to be an inhibitor of tumor growth and metastatic progression in human breast cancer pre-clinical models and therefore it could result useful for the treatment of some tumor subtypes. The repurposing of this drug could be of great value, mainly in developing countries because of its low cost.

LIST OF ABBREVIATIONS

β-AR: β-adrenoceptor
cAMP: cyclic adenosine 3',5'-monophosphate
CXCL-8: interleukin-8
FBS: fetal bovine serum
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
HER-2: human epidermal growth factor receptor 2
IL-8: interleukin 8
MMP9: matrix metalloproteinase 9
NSG mice: NOD/LtSz-scid/IL-2R gamma null mice
Pan-CK: pan-cytokeratine
RT-qPCR: quantitative real time PCR
VEGF: vascular endothelial growth factor
TNBC: triple negative breast cancer

CONFLICT OF INTEREST

The authors declare they have not conflicts of interest.

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REFERENCES

[1] Torre, L. A.; Bray, F.; Siegel, R. L.; Ferlay, J.; Lortet-Tieulent, J.; Jemal, A., Global cancer statistics, 2012. CA Cancer J Clin 2015, 65 (2), 87-108.
[2] Jamdade, V. S.; Sethi, N.; Mundhe, N. A.; Kumar, P.; Lahkar, M.; Sinha, N., Therapeutic targets of triple-negative breast cancer: a review. Br J Pharmacol 2015, 172 (17), 4228-37.
[3] Kangaspeska, S.; Hultsch, S.; Jaiswal, A.; Edgren, H.; Mpindi, J. P.; Eldfors, S.; Bruck, O.; Aittokallio, T.; Kallioniemi, O., Systematic drug screening reveals specific vulnerabilities and co-resistance patterns in endocrine-resistant breast cancer. BMC Cancer 2016, 16 (1), 378.
[4] Mendes, D.; Alves, C.; Afonso, N.; Cardoso, F.; Passos-Coelho, J. L.; Costa, L.; Andrade, S.; Batel-Marques, F., The benefit of HER2-targeted therapies on overall survival of patients with metastatic HER2-positive breast cancer--a systematic review. Breast Cancer Res 2015, 17, 140.
[5] Holliday, D. L.; Speirs, V., Choosing the right cell line for breast cancer research. Breast Cancer Res 2011, 13 (4), 215.
[6] Bruzzone, A.; Vanzulli, S. I.; Soldati, R.; Giulianelli, S.; Lanari, C.; Luthy, I. A., Novel human breast cancer cell lines IBH-4, IBH-6, and IBH-7 growing in nude mice. J Cell Physiol 2009, 219 (2), 477-84.
[7] Vazquez, S. M.; Mladovan, A.; Garbovesky, C.; Baldi, A.; Luthy, I. A., Three novel hormone-responsive cell lines derived from primary human breast carcinomas: functional characterization. J Cell Physiol 2004, 199 (3), 460-469.
[8] Cailleau, R.; Young, R.; Olive, M.; Reeves, W. J., Jr., Breast tumor cell lines from pleural effusions. J Natl.Cancer Inst. 1974, 53 (3), 661-674.
[9] Prat, A.; Perou, C. M., Deconstructing the molecular portraits of breast cancer. Mol Oncol 2011, 5 (1), 5-23.
[10] Massague, J.; Obenauf, A. C., Metastatic colonization by circulating tumour cells. Nature 2016, 529 (7586), 298-306.
[11] Obenauf, A. C.; Massague, J., Surviving at a distance: organ specific metastasis. Trends Cancer 2015, 1 (1), 76-91.
[12] Harrell, J. C.; Prat, A.; Parker, J. S.; Fan, C.; He, X.; Carey, L.; Anders, C.; Ewend, M.; Perou, C. M., Genomic analysis identifies unique signatures predictive of brain, lung, and liver relapse. Breast Cancer Res Treat 2012, 132 (2), 523-35.
[13] Coates, A. S.; Winer, E. P.; Goldhirsch, A.; Gelber, R. D.; Gnant, M.; Piccart-Gebhart, M.; Thurlimann, B.; Senn, H. J., Tailoring therapies - improving the management of early breast cancer: St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2015. Ann Oncol 2015, 26, 1533-1546.
[14] Vandewalle, B.; Revillion, F.; Lefebvre, J., Functional beta-adrenergic receptors in breast cancer cells. J Cancer Res. Clin. Oncol. 1990, 116 (3), 303-306.
[15] Marchetti, B.; Spinola, P. G.; Plante, M.; Poyet, P.; Follea, N.; Pelletier, G.; Labrie, F., Beta-adrenergic receptors in DMBA-induced rat mammary tumors: correlation with progesterone receptor and tumor growth. Breast Cancer Res.Treat. 1989, 13 (3), 251-263.
[16] Cakir, Y.; Plummer, H. K., III; Tithof, P. K.; Schuller, H. M., Beta-adrenergic and arachidonic acid-mediated growth regulation of human breast cancer cell lines. Int.J.Oncol. 2002, 21 (1), 153-157.
[17] Carie, A. E.; Sebti, S. M., A chemical biology approach identifies a beta-2 adrenergic receptor agonist that causes human tumor regression by blocking the Raf-1/Mek-1/Erk1/2 pathway. Oncogene 2007, 26 (26), 3777-3788.
[18] Perez Pinero, C.; Bruzzone, A.; Sarappa, M. G.; Castillo, L. F.; Luthy, I. A., Involvement of alpha2- and beta2-adrenoceptors on breast cancer cell proliferation and tumour growth regulation. Br J Pharmacol 2012, 166 (2), 721-36.
[19] Bastian, P.; Balcarek, A.; Altanis, C.; Strell, C.; Niggemann, B.; Zaenker, K. S.; Entschladen, F., The inhibitory effect of norepinephrine on the migration of ES-2 ovarian carcinoma cells involves a Rap1-dependent pathway. Cancer Lett 2009, 274 (2), 218-24.
[20] Strell, C.; Niggemann, B.; Voss, M. J.; Powe, D. G.; Zanker, K. S.; Entschladen, F., Norepinephrine Promotes the beta1-Integrin-Mediated Adhesion of MDA-MB-231 Cells to Vascular Endothelium by the Induction of a GROalpha Release. Mol Cancer Res 2012, 10 (2), 197-207.
[21] Creed, S. J.; Le, C. P.; Hassan, M.; Pon, C. K.; Albold, S.; Chan, K. T.; Berginski, M. E.; Huang, Z.; Bear, J. E.; Lane, J. R.; Halls, M. L.; Ferrari, D.; Nowell, C. J.; Sloan, E. K., beta2-adrenoceptor signaling

regulates invadopodia formation to enhance tumor cell invasion. Breast Cancer Res 2015, 17 (1), 145.
[22] Drell, T. L.; Joseph, J.; Lang, K.; Niggemann, B.; Zaenker, K. S.; Entschladen, F., Effects of neurotransmitters on the chemokinesis and chemotaxis of MDA-MB-468 human breast carcinoma cells. Breast Cancer Res.Treat. 2003, 80 (1), 63-70.
[23] Pon, C. K.; Lane, J. R.; Sloan, E. K.; Halls, M. L., The beta2-adrenoceptor activates a positive cAMP-calcium feedforward loop to drive breast cancer cell invasion. FASEB J 2015.
[24] Sloan, E. K.; Priceman, S. J.; Cox, B. F.; Yu, S.; Pimentel, M. A.; Tangkanangnukul, V.; Arevalo, J. M.; Morizono, K.; Karanikolas, B. D.; Wu, L.; Sood, A. K.; Cole, S. W., The sympathetic nervous system induces a metastatic switch in primary breast cancer. Cancer Res 2010, 70 (18), 7042-52.
[25] Nagaraja, A. S.; Dorniak, P. L.; Sadaoui, N. C.; Kang, Y.; Lin, T.; Armaiz-Pena, G.; Wu, S. Y.; Rupaimoole, R.; Allen, J. K.; Gharpure, K. M.; Pradeep, S.; Zand, B.; Previs, R. A.; Hansen, J. M.; Ivan, C.; Rodriguez-Aguayo, C.; Yang, P.; Lopez-Berestein, G.; Lutgendorf, S. K.; Cole, S. W.; Sood, A. K., Sustained adrenergic signaling leads to increased metastasis in ovarian cancer via increased PGE2 synthesis. Oncogene 2016, 35 (18), 2390-7.
[26] Cardwell, C. R.; Coleman, H. G.; Murray, L. J.; Entschladen, F.; Powe, D. G., Beta-blocker usage and breast cancer survival: a nested case-control study within a UK clinical practice research datalink cohort. Int J Epidemiol 2013, 42 (6), 1852-61.
[27] Childers, W. K.; Hollenbeak, C. S.; Cheriyath, P., beta-Blockers Reduce Breast Cancer Recurrence and Breast Cancer Death: A Meta-Analysis. Clin Breast Cancer 2015, 15 (6), 426-31.
[28] Entschladen, F.; Thyssen, D. A.; Drell, D. W., Re-Use of Established Drugs for Anti-Metastatic Indications. Cells 2016, 5 (1).
[29] Powe, D. G.; Entschladen, F., Targeted therapies: Using beta-blockers to inhibit breast cancer progression. Nat Rev Clin Oncol 2011, 8 (9), 511-2.
[30] Masur, K.; Niggemann, B.; Zanker, K. S.; Entschladen, F., Norepinephrine-induced migration of SW 480 colon carcinoma cells is inhibited by beta-blockers. Cancer Res 2001, 61 (7), 2866-9.
[31] Schneider, C. A.; Rasband, W. S.; Eliceiri, K. W., NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012, 9 (7), 671-5.
[32] Marin-Briggiler, C. I.; Veiga, M. F.; Matos, M. L.; Echeverria, M. F.; Furlong, L. I.; Vazquez-Levin, M. H., Expression of epithelial cadherin in the human male reproductive tract and gametes and evidence of its participation in fertilization. Mol Hum Reprod 2008, 14 (10), 561-71.
[33] Dowdy, S.; Wearden, S., Statistics for research. John Wiley & Sons, Inc: New York, 1983.
[34] Gargiulo, L.; Copsel, S.; Rivero, E. M.; Gales, C.; Senard, J. M.; Luthy, I. A.; Davio, C.; Bruzzone, A., Differential beta(2)-adrenergic receptor expression defines the phenotype of non-tumorigenic and malignant human breast cell lines. Oncotarget 2014, 5 (20), 10058-69.
[35] Lang, K.; Drell, T. L. t.; Lindecke, A.; Niggemann, B.; Kaltschmidt, C.; Zaenker, K. S.; Entschladen, F., Induction of a metastatogenic tumor cell type by neurotransmitters and its pharmacological inhibition by established drugs. Int J Cancer 2004, 112 (2), 231-8.
[36] Palm, D.; Lang, K.; Niggemann, B.; Drell, T. L. t.; Masur, K.; Zaenker, K. S.; Entschladen, F., The norepinephrine-driven metastasis development of PC-3 human prostate cancer cells in BALB/c nude mice is inhibited by beta-blockers. Int J Cancer 2006, 118 (11), 2744-9.
[37] Vazquez, S. M.; Mladovan, A. G.; Perez, C.; Bruzzone, A.; Baldi, A.; Luthy, I. A., Human breast cell lines exhibit functional alpha(2)-adrenoceptors. Cancer Chemother. Pharmacol. 2006, 58, 50-61.
[38] Dong, H.; Claffey, K. P.; Brocke, S.; Epstein, P. M., Inhibition of breast cancer cell migration by activation of cAMP signaling. Breast Cancer Res Treat 2015, 152 (1), 17-28.
[39] Hanahan, D.; Weinberg, R. A., Hallmarks of cancer: the next generation. Cell 2011, 144 (5), 646-74.
[40] Satelli, A.; Li, S., Vimentin in cancer and its potential as a molecular target for cancer therapy. Cell Mol Life Sci 2011, 68 (18), 3033-46.
[41] Agiostratidou, G.; Hulit, J.; Phillips, G. R.; Hazan, R. B., Differential cadherin expression: potential markers for epithelial to mesenchymal transformation during tumor progression. J Mammary Gland Biol Neoplasia 2007, 12 (2-3), 127-33.
[42] Madden, K. S.; Szpunar, M. J.; Brown, E. B., beta-Adrenergic receptors (beta-AR) regulate VEGF and IL-6 production by divergent pathways in high beta-AR-expressing breast cancer cell lines. Breast Cancer Res Treat 2011, 130 (3), 747-58.
[43] Roeser, J. C.; Leach, S. D.; McAllister, F., Emerging strategies for cancer immunoprevention. Oncogene 2015, 34 (50), 6029-39.
[44] Singh, B.; Berry, J. A.; Vincent, L. E.; Lucci, A., Involvement of IL-8 in COX-2-mediated bone metastases from breast cancer. J.Surg.Res. 2006, 134 (1), 44-51.
[45] Wang, L. P.; Jin, J.; Lv, F. F.; Cao, J.; Zhang, J.; Wang, B. Y.; Shao, Z. M.; Hu, X. C.; Wang, Z. H., Norepinephrine attenuates CXCR4 expression and the corresponding invasion of MDA-MB-231 breast cancer cells via beta2-adrenergic receptors. European review for medical and pharmacological sciences 2015, 19 (7), 1170-81.
[46] Lee, S. U.; Ahn, K. S.; Sung, M. H.; Park, J. W.; Ryu, H. W.; Lee, H. J.; Hong, S. T.; Oh, S. R., Indacaterol inhibits tumor cell invasiveness and MMP-9 expression by suppressing IKK/NF-kappaB activation. Molecules and cells 2014, 37 (8), 585-91.
[47] van der Westhuizen, E. T.; Breton, B.; Christopoulos, A.; Bouvier, M., Quantification of ligand bias for clinically relevant beta2-adrenergic receptor ligands: implications for drug taxonomy. Mol Pharmacol 2014, 85 (3), 492-509.
[48] Bruzzone, A.; Sauliere, A.; Finana, F.; Senard, J. M.; Luthy, I.; Gales, C., Dosage-dependent regulation of cell proliferation and adhesion through dual beta2-adrenergic receptor/cAMP signals. FASEB J 2014, 28 (3), 1342-54.
[49] Mukherjee, C.; Lefkowitz, R. J., Desensitization of beta-adrenergic receptors by beta-adrenergic agonists in a cell-free system: resensitization by guanosine 5'-(beta, gamma-imino)triphosphate and other purine nucleotides. Proc Natl Acad Sci U S A 1976, 73 (5), 1494-8.
[50] Raimondi, S.; Botteri, E.; Munzone, E.; Cipolla, C.; Rotmensz, N.; DeCensi, A.; Gandini, S., Use of beta-blockers, angiotensin-converting enzyme inhibitors and angiotensin receptor blockers and breast cancer survival: Systematic review and meta-analysis. Int J Cancer 2016, 139 (1), 212-9.
[51] Choy, C.; Raytis, J. L.; Smith, D. D.; Duenas, M.; Neman, J.; Jandial, R.; Lew, M. W., Inhibition of beta2-adrenergic receptor reduces triple-negative breast cancer brain metastases: The potential benefit of perioperative beta-blockade. Oncol Rep 2016, 35 (6), 3135-42.
[52] Numbere, B.; Fleming, K. M.; Walker, A.; Card, T. R., Adrenergic blockers and the risk for common solid cancers: a case-control study. Eur J Cancer Prev 2015.
[53] Sorensen, G. V.; Ganz, P. A.; Cole, S. W.; Pedersen, L. A.; Sorensen, H. T.; Cronin-Fenton, D. P.; Garne, J. P.; Christiansen, P. M.; Lash, T. L.; Ahern, T. P., Use of beta-blockers, angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, and risk of breast cancer recurrence: a Danish nationwide prospective cohort study. J Clin Oncol 2013, 31 (18), 2265-72.
[54] Prat, A.; Parker, J. S.; Karginova, O.; Fan, C.; Livasy, C.; Herschkowitz, J. I.; He, X.; Perou, C. M., Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res 2010, 12 (5), R68.
[55] Patel, M.; Thomson, N. C., (R)-salbutamol in the treatment of asthma and chronic obstructive airways disease. Expert Opin Pharmacother 2011, 12 (7), 1133-41.
[56] Powe, D. G.; Voss, M. J.; Zanker, K. S.; Habashy, H. O.; Green, A. R.; Ellis, I. O.; Entschladen, F., Beta-blocker drug therapy reduces secondary cancer formation in breast cancer and improves cancer specific survival. Oncotarget 2010, 1 (7), 628-38.
[57] Institute of Laboratory Animal Resources Commission on Life, S.; National Research, C., Guide for the Care and Use of Laboratory Animals. National Academy Press Washington, D.C. , 1996.
[58] Institute of Laboratory Animal Resources Commission on Life, S. N. R., Council, Guide for the Care and Use of Laboratory Animals: Eighth Edition. National Academy Press: Washington, D.C. , 2010.
[59] United Kingdom Co-ordinating Committee on Cancer Research, U., United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) Guidelines for the Welfare of Animals in Experimental Neoplasia (Second Edition). Br J Cancer 1998, 77 (1), 1-10.

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FIGURES AND LEGENDS

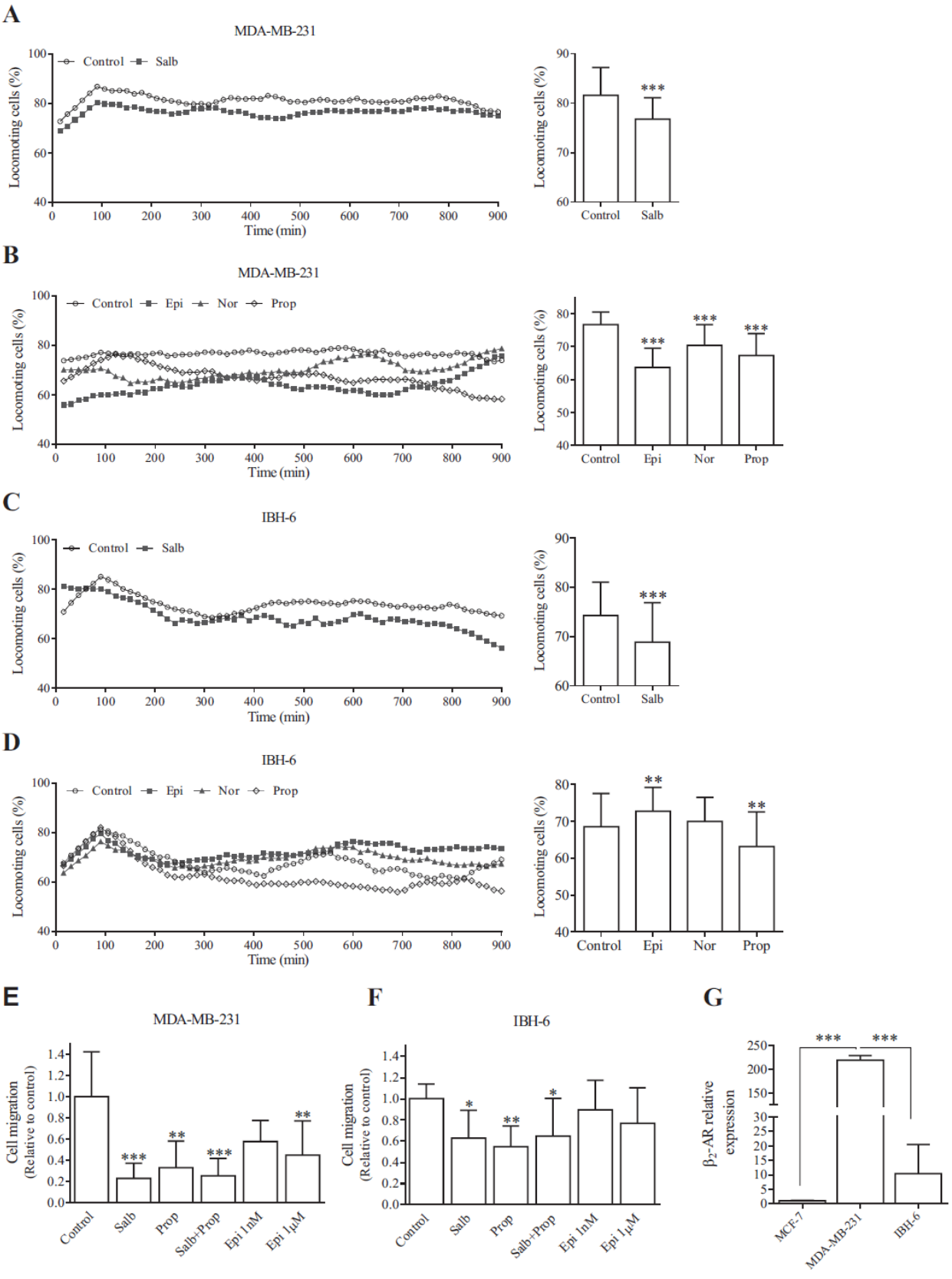
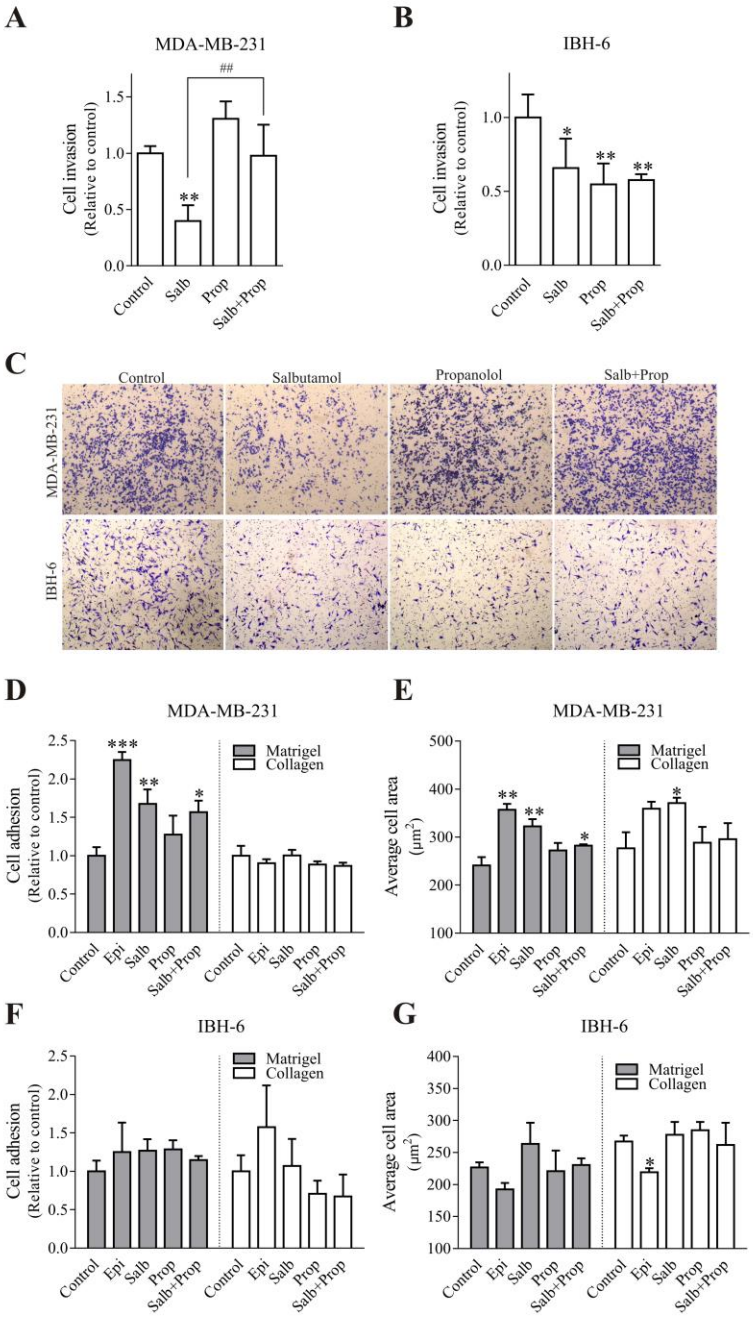


Figure 1. Effect of adrenergic compounds on breast cancer cell migration. A, B, C and D: Time-lapse videomicroscopy cell tracking experiments. MDA-MB-231 (panels A and B) and IBH-6 (panels C and D) cells were incubated with the β_2-AR agonist salbutamol (Salb, 1 µM), the natural adrenergic agonists epinephrine (Epi, 10 µM) or norepinephrine (Nor, 10 µM) and the β-AR antagonist propranolol (Prop, 10 µM) in three-dimensional collagen lattices and were recorded overnight at 37ºC. The graphs on the left side show the time course of migratory activity. Graphs on the right side show the mean number (in percentage) of locomoting cells throughout the whole experiment (90 cells were analyzed per sample). E and F: Boyden chamber migration assay. Panel E for MDA-MB-231 and F for IBH-6 cells. Salb 1 µM, Prop 10 µM. G: Quantification of β_2-adrenoceptors by Real Time qPCR, as described in Materials and Methods. Data is presented as the fold change compared to MCF-7 cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ with respect to control, ANOVA followed by Dunnett–Kramer test. All the experiments were performed three times with similar results.


A
MDA-MB-231
Cell invasion (Relative to control)
##
**
Control
Salb
Prop
Salb+Prop
B
IBH-6
Cell invasion (Relative to control)
*
**
**
Control
Salb
Prop
Salb+Prop
C
Control
Salbutamol
Propanolol
Salb+Prop
MDA-MB-231
IBH-6
D
MDA-MB-231
Cell adhesion (Relative to control)
***
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*
Matrigel
Collagen
Control
Epi
Salb
Prop
Salb+Prop
E
MDA-MB-231
Average cell area (μm²)
**
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*
Matrigel
Collagen
F
IBH-6
Cell adhesion (Relative to control)
Matrigel
Collagen
G
IBH-6
Average cell area (μm²)
*
Matrigel
Collagen

Figure 2. Effect of adrenergic compounds on breast cancer cell invasion and adhesion. A, B and C: Cell invasion was assessed by Transwell chambers coated with Matrigel, as described in Materials and Methods, in MDA-MB-231 (panels A and C) and IBH-6 cells (panels B and C). Cells were incubated with salbutamol (1µM Salb), propranolol (10 µM Prop), the combination of both or in the absence of adrenergic compounds (Control). Panel C shows representative images of invasive cells at 40X magnification. D, E, F and G: Cell adhesion assays. MDA-MB-231 (panels D and E) and IBH-6 cells (panels F and G) were allowed to attach to Matrigel or collagen for 1 h and 1.5 h, respectively, in the presence or absence (control) of 1 µM epinephrine (Epi), 1 µM Salb or 10µM Prop. The total number of adherent cells (panel D and F) and the average cell area in μm^2 (panels E and G) were automatically quantified using ImageJ. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ with respect to control, ## $p < 0.01$ with respect to salbutamol by ANOVA followed by Dunnett–Kramer or Tukey-Kramer (panel A) test. Invasion experiments were performed twice, while adhesion ones three times.

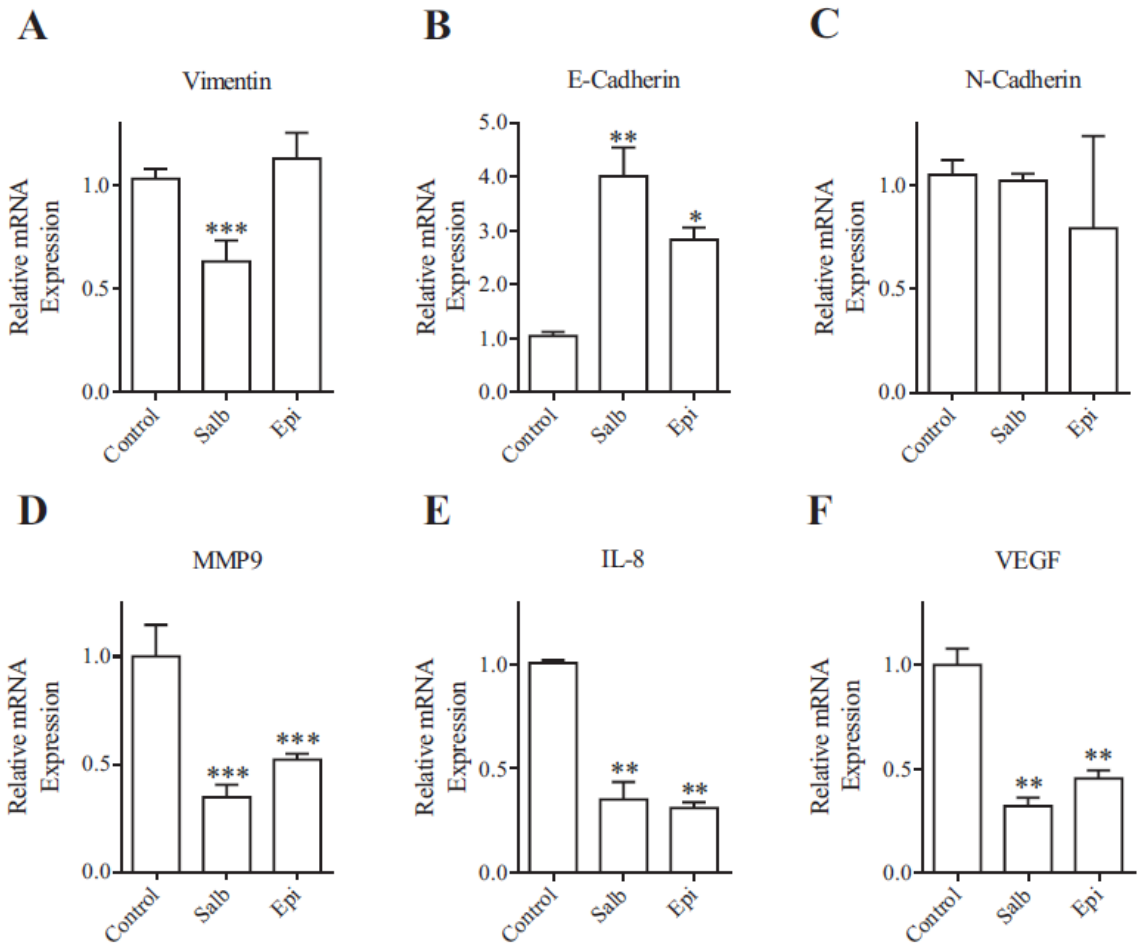

Figure 3: Effect of salbutamol and epinephrine on the expression of pro-metastatic genes in MDA-MB-231 cells. Gene expression of vimentin (panel A), epithelial cadherin (E-Cadherin, panel B), neural cadherin (N-Cadherin, panel C), matrix metalloprotease 9 (MMP-9, panel D), interleukin 8 (IL-8, panel E) and vascular endothelial growth factor (VEGF, panel F) was quantified by RT-qPCR in MDA-MB-231 cells incubated with salbutamol (Salb, 1 μM) or epinephrine (Epi, 1 μM). Data is shown as the fold change of expression with respect to control. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ with respect to control, ANOVA followed by Dunnett–Kramer test. All the experiments were performed twice.

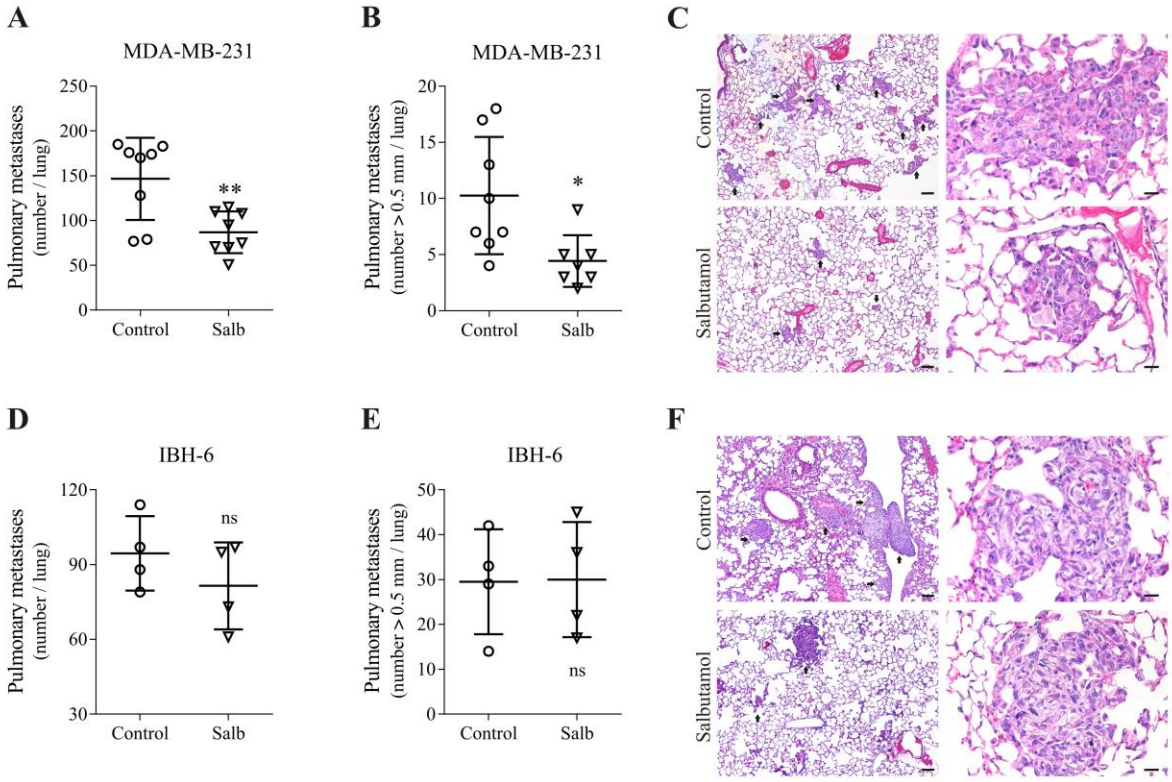

Figure 4. Effect of salbutamol on breast cancer experimental metastases. MDA-MB-231 (panels A, B and C, 8 mice per group) or IBH-6 cells (panels D, E and F, 4 mice per group) were injected via the lateral tail vein to NSG mice and, after 30 days of daily subcutaneous injections of salbutamol (1.2 mg/kg/day) or saline, lungs were inspected. A and D: total number of lung macroscopic metastases, B and E: number of macroscopic metastases with diameter larger than 0.5 mm. C and F: Representative H&E pictures of lungs at 100 X (left images) and 400 X (right images) magnification. Bar = 100 μm in the left pictures, 20 μm in the right ones, black arrows point to metastatic foci. *$p < 0.05$, **$p < 0.01$ with respect to control, Student t test. Two independent experiments were performed.

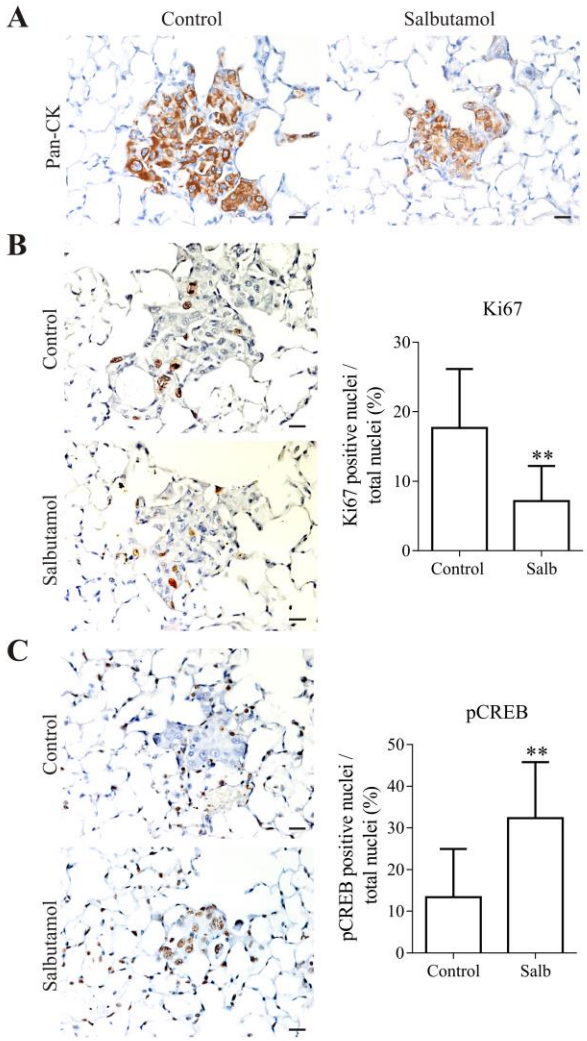

Figure 5. Immunohistochemistry of MDA-MB-231 lung metastases. Lungs from salbutamol (Salb) and saline (control) treated NSG mice were stained for pan-cytokeratins (pan-CK, panel A), the proliferation marker Ki67 (panel B) and phospho-CREB (pCREB, panel C). Number of positive nuclei (for Ki67) or cells (for pCREB) were quantified within metastatic foci. Bar = 20 μm, 400 X magnification. *$p < 0.05$, **$p < 0.01$ with respect to control, Student t test. Two independent experiments were performed.