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Alpha$_2$-adrenoceptor agonists trigger prolactin signaling in breast cancer cells

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ABSTRACT

Breast cancer is the most frequent malignancy among women worldwide. We have described the expression of α_2-adrenoceptors in breast cancer cell lines, associated with increased cell proliferation and tumor growth. A mitogenic autocrine/paracrine loop of prolactin (Prl) has been described in breast cancer cells. We hypothesized that the α_2-adrenergic enhancement of proliferation could be mediated, at least in part, by this Prl loop.
In both T47D and MCF-7 cell lines, the incubation with the α_2-adrenergic agonist dexmedetomidine significantly increased Prl release into the culture medium (measured by the Nb2 bioassay), this effect being reversed by the α_2-adrenergic antagonist rauwolscine. No change in Prl receptors (PrlR) was observed by RT-qPCR in these cell lines. In IBH-6 cells a decrease in Prl secretion was observed at the lower dexmedetomidine concentration.
The signaling pathways involved in ovine Prl (oPrl) and dexmedetomidine action were also assessed. Both compounds significantly activated STAT5 and ERK in all three cell lines. In T47D and MCF-7 cell lines also AKT was activated by both Prl and dexmedetomidine. We therefore describe the STAT5 phosphorylation by an α_2-adrenergic agonist, dexmedetomidine.
In T47D cells, the α_2-adrenergic stimulation of cell proliferation is probably mediated, at least in part, by the Prl autocrine/paracrine loop, because this effect is abrogated by the specific PrlR antagonist Δ1–9-G129R-hPrl. The implication of Prl loop describes a novel mechanism of action of this GPCR.

1. Introduction

Breast cancer is the most frequent malignancy among women worldwide, with 1.67 million of new cancer cases diagnosed (25.2% of all cancers in women) and 522,000 deaths (14.7% of all deaths by cancer) as assessed by GLOBOCAN 2012. This disease is the most common cancer and is still the most frequent cause of cancer death in women in less developed regions while it is now the second cause in some developed regions [1,2]. Breast cancer is a heterogeneous disease, as highlighted by the description of different molecular subtypes based on their gene expression profile. Both MCF-7 [3] and T47D [4] are paradigmatic luminal cell lines (estrogen receptor-α positive, progesterone receptor positive, and no overexpression of HER-2) [5]. IBH-6 cells, developed in our laboratory from a primary breast cancer sample, also belonging to this subtype [6].

α_2-Adrenoceptors (α_2-AR, subdivided in α_{2A}, α_{2B}, and α_{2C}-AR) are G protein–coupled receptors (GPCR) which classically couple to Gi inhibiting adenylyl cyclase activity and therefore intracellular cAMP levels. G protein activation causes its dissociation, releasing the βγ-subunit that may promote an alternative signaling through activation of mitogen-activated protein kinases (MAPKs) and ion channels. Adrenoceptors also signal through regulatory proteins (mainly G protein–coupled receptor kinases (GRKs)) and scaffolding proteins (β-arrestins), in a G protein–independent manner [7]. Recently, it has been described that the activation of ERK1/2 pathway by the α_2-AR agonists like dexmedetomidine and clonidine promotes the proliferation and migration capacity of triple negative MDA-MB-231 breast cancer cells [8]. Our group has described the expression of α_2-adrenoceptors in several benign and malignant cell lines. Their stimulation with specific agonists is associated with increased cell proliferation [9] and tumor growth in experimental models of breast cancer. Moreover, the α_2-adrenergic antagonist rauwolscine behaves as an inverse agonist,

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inhibiting cell proliferation and tumor growth below control levels [10,11,12].

The synthesis and secretion of prolactin (Prl) as well as the expression of Prl receptors (PrlR) have been described in various breast cancer cells, suggesting the existence of a stimulatory autocrine/paracrine Prl loop [13]. Although the latter was initially described to promote breast cancer cell proliferation [14], this conclusion was not confirmed by others [15]. However, Prl stimulation of breast cell proliferation has been confirmed by many authors, as reviewed [16]. Recently, it was suggested that Prl levels measured <10 years before diagnosis are associated with postmenopausal breast cancer risk, especially for luminal tumors and metastatic disease [17]. Moreover, the PrlR has been implicated in resistance to chemotherapy [18]. Otherwise, the levels of PrlR expression have been shown to be an independent favorable prognostic marker in breast cancer [19], while Prl stimulation prevented the growth of triple negative MDA-MB-231 breast cancer cells [20]. On the other hand, in a group of patients with PrlR-positive metastatic breast cancer or metastatic castration-resistant prostate cancer, no effect was found when a humanized monoclonal antibody that binds to and inhibits the Prl was administered [21]. Nevertheless, this was a phase I study with a limited number of patients [22].

Prl interacts with a specific, single-pass transmembrane PrlR which exists as different isoforms (named long, intermediate and short) as a result of alternative splicing or posttranslational modifications, as reviewed [23]. Prolactin binding to preformed PrlR homodimers triggers conformational changes within the receptor cytoplasmic tail leading to activation (phosphorylation) of the receptor-associated JAK-2 tyrosine kinase. Activated JAK-2 phosphorylates several tyrosine residues in the intracellular domain of the long and the intermediate PrlR isoforms which subsequently serve as docking sites for signaling proteins including the transcription factor STAT5. Once phosphorylated by JAK-2, STAT5 dimers translocate to the nucleus to activate transcription of PrlR target genes. Phosphorylated tyrosines in the PrlR also serve as docking sites for adaptor proteins like Src homology 2 (Shc), growth factor receptor-bound protein 2 (Grb2) and Son of Sevenless (SOS) that couple the receptor to the mitogen activated protein kinase (MAPK) cascade [24]. c-Src mediated activation of ERK1/2 and AKT by Prl has been described in T47D and MCF-7 human breast cancer cells, with a close correlation with cell proliferation and invasion [25,26].

In the present work, we investigated whether the α_2-adrenergic enhancement of cell proliferation could be mediated, at least in part, by the autocrine/paracrine loop of Prl in breast cancer cells. To asses this hypothesis both components of the loop were analyzed in three luminal breast cancer cell lines T47D, MCF-7 and IBH-6. The luminal breast cancer subtype was chosen because the two former cell lines are paradigmatic in the study of the paracrine/autocrine Prl loop. Our data show that in some luminal cell lines, the α_2-adrenergic stimulation of cell proliferation is mediated, at least in part, by the Prl autocrine/paracrine loop.

2. Materials and methods

2.1. Reagents

Fetal calf serum (FCS), horse serum (HS), culture media, antibiotics and trypsin were purchased from Invitrogen Thermo Fisher Scientific (Waltham, MA, USA). Dexmedetomidine (Precedex) was from Abbott Laboratories. Rauwolscine-HCl was purchased from Sigma-Aldrich (St. Louis, MO, USA). NIDDK ovine prolactin (oPrl) was from NIDDK-NIH, Bethesda, USA. Methyl [^{3}H]-thymidine (NET 027E; specific activity: 20 Ci/mmol) was from Dupont-New England Nuclear (Boston, MA, USA). Liquid scintillation cocktail was Optiphase 'Hisafe' 3 (PerkinElmer Health Sciences, Groningen The Netherlands). Antibodies against pERK (Tyr 204, sc-7383), ERK (sc-94), p-STAT5 (Tyr 694, sc-101806), and STAT5 (sc-835) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while anti-p-AKT (Ser473, #4058) and anti-AKT (#4685) were from Cell Signaling. For immunofluorescence the antibodies used were the anti-α_{2A} (A-271) antibody from Sigma-Aldrich, a rabbit polyclonal antibody raised against amino acids 218-235 of human, mouse, rat, and pig α_{2A} adrenergic receptor localized within the third intracellular loop; anti-α_{2B} (H-96, sc-10723) from Santa Cruz Biotechnology, a polyclonal rabbit IgG raised against an epitope corresponding to amino acids 202-297 mapping to an internal region of α_{2B}-AR of human origin and antihuman α_{2C} (C-20, sc-1480) goat polyclonal antibody also from Santa Cruz Biotechnology, an affinity-purified goat polyclonal antibody raised against a peptide mapping at the COOH terminus of the α_{2C}-adrenergic receptor of human origin and cross-reacts with rat and mouse (per data sheets from the providers). Secondary antibodies were from Amersham (GE Healthcare Argentina S.A., Buenos Aires, Argentina). Vectashield H-1000 was from Vector Laboratories (Burlingame, CA, USA). TRI reagent was from Molecular Research Centre, Inc. (Cincinnati, OH, USA), oligo-dT primers, M-MLV reverse transcriptase were acquired from Promega (Madison, WI, USA) and FastStart SYBR Green Master Mix was from Roche (Mannheim, Germany). All the other reagents, including glutamine, DNAse, luminol and p-coumaric acid were from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture

The breast cancer MCF-7 and T47D (recently acquired at the ATCC) and IBH-6 cell lines (developed in our laboratory) [6], were cultured as already described [9]. Cells were maintained in DMEM:F12 (1:1) medium supplemented with heat-inactivated 10% FCS, 2 mM glutamine, 2 μg/ml insulin, 100 UI/ml penicillin, 100 μg/ml streptomycin and 15 mM HEPES. Cells were incubated at 37 °C in 5% CO_2 and sub-cultured at 80% confluence using 0.25% trypsin–0.025% EDTA.

The lactogen-dependent rat T-cell lymphoma Nb2 cell line was maintained in culture with DMEM:F12 (1:1) medium supplemented with heat-inactivated 10% FCS, 10% horse serum (HS), 2 mM glutamine, 2 μg/ml bovine insulin, 100 UI/ml penicillin, 100 μg/ml streptomycin and 15 mM HEPES. These non-adherent cells were incubated at 37 °C in 5% CO_2 and passed at high density.

2.3. Breast cancer cell proliferation assays

The human T47D cells were seeded at 10,000 cells per well in 2% charcoal-stripped FCS and maintained for 24 h. Phenol red-free medium was used to avoid pro-estrogenic actions, [27]. Then the cells were treated (with daily medium changes) with the α_2- adrenergic agonist dexmedetomidine (2 μM), with 2 μg/ml (87 nM) ovine (oPrl) and/or the PrlR antagonist Δ1–9-G129R-hPrl in 2 log molar excess (10 μM) for 72 h. [^{3}H]-Thymidine at 0.2 μCi/well was added with the last change of medium. After 24 h, cells were harvested in a Nunc Cell Harvester 8 (Nunc, Rochester, NY, USA), and filters were counted in a Tri-Carb 2800TR PerkinElmer liquid scintillation Analyzer.

2.4. Nb2 bioassay

The Nb2 bioassay was originally developed and validated for serum Prl and GH determinations [28,29,30,31]. The conditions were modified to measure Prl in conditioned medium (CM). To obtain the CM, breast cancer cells were incubated in 10% FCS medium up to 80% confluence. The medium was changed to FCS- and insulin-free medium and then the cells were treated with or without the adrenergic compounds for 48 h. After centrifugation, the CM was frozen at −20 °C until used.

The Nb2 cells were arrested in culture medium DMEM/F12, without FCS but with 10% HS, 24 h prior to the bioassay and then 10,000 cells/well were seeded in 96 well plates. The bioassay was performed with 40 μl of CM in 200 μl final volume of medium with 0% FCS, 10% HS in quadruplicates with a standard curve for each assay (oPrl concentration: 100 fg/ml-1 μg/ml). The incubation lasted 72 h, with 0.20 μCi/well [^{3}H]-thymidine during the last 24 h. The cells were harvested in a Nunc Cell Harvester 8 (Nunc, Rochester, NY, USA), and filters were

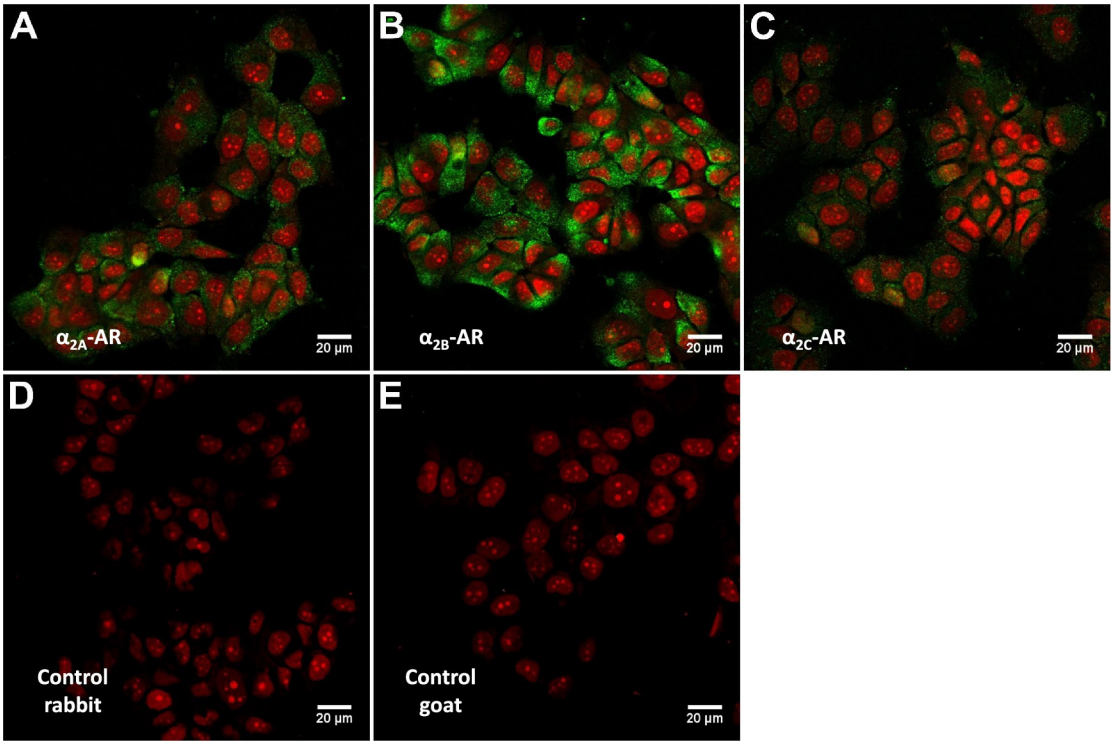

Fig. 1. α_2-Adrenoceptor expression in human T47D cells. Immunofluorescence of the different subtypes of α_2-adrenoceptors in these cells. The expression of the three α_2-adrenoceptor subtypes was analyzed by immunofluorescence using subtype-specific antibodies. A: α_{2A}-adrenoceptor, B: α_{2B}-adrenoceptor and C: α_{2C}-adrenoceptor. Nuclei appear in red (propidium iodide). No staining was observed in negative controls (treated without primary antibodies), which were used to set confocal conditions (panels D and E). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

counted in a liquid scintillation counter. Classical standard curves had a coefficient correlation of 0.9–0.95.

2.5. Immunofluorescence

T47D cells were cultured on cover glasses in 6-well plates in complete medium for 24 h. Cells were washed with PBS, fixed in 70% ethanol for 40 min at −20 °C and blocked in a PBS/BSA 2.5% solution for 1 h at room temperature. Primary antibodies at 1:50 concentration were added and incubated overnight at 4 °C in blocking solution. Primary antibody was omitted in negative controls. Cover glasses were washed and incubated with secondary fluorescein isothiocyanate (FITC) conjugated antibodies. Nuclei were counterstained with propidium iodide. Staining was analyzed under a Nikon C1 Confocal Microscope using the EZ-C1 2.20 software.

2.6. Quantitative real-time PCR

Total RNA from MCF-7 and T47D cells treated in serum-free medium for 24 h with the adrenergic compounds was isolated using TRI reagent according to manufacturer's instructions. RNA concentration was measured with a NanoDrop 1000 spectrophotometer and cDNA was synthesized from 1 μg of total RNA using oligo-dT primers and M-MLV reverse transcriptase following manufacturer's instructions. RT-qPCR was performed in a CFX96 thermal cycler (BioRad) using FastStart SYBR Green Master Mix. Expression levels were normalized to those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Relative gene expression was calculated by the $^{\Delta\Delta}C_T$ method. The following primers were used: α_{2A}-AR: forward 5′-TTCACCCTCTTCGCCTGGTA-3′ reverse 5′-GTGAG CCATGCCCTTGTAGT-3′; α_{2B}-AR: forward 5′- CGGGAGTGGCAGACA GAATC-3′ reverse 5′- GCCGGTGAAATGTCGAAACG-3′; α_{2C}-AR: forward 5′-CGCTCAACCCGGTCATCTAC-3′; reverse 5′-TGCGAGTCACTGCCT GAAG-3′; GAPDH: forward 5′-TGCACCACCAACTGCTTAGC-3′ reverse 5′-GGCATGGACTGTGGTCATGAG-3′; hPrlR: forward 5′-CTGGGACA GATGGAGGACTT-3′ reverse 5′-GGGCCACCGGTTATGTAGT-3′.

Table 1
Relative expression levels of α_2-adrenoceptors in breast cancer MCF-7, T47D and IBH-6 cell lines.

	MCF-7	T47D	IBH-6
α_{2A}-AR	1	57.02 ± 4.45	2.43 ± 0.91
α_{2B}-AR	1.61 ± 0.62	3.92 ± 0.54	0.93 ± 1.03
α_{2C}-AR	192.33 ± 88.79	456.83 ± 70.83	0.64 ± 0.64

The expression of the three α_2-adrenoceptor subtypes was analyzed by RT-qPCR using the primers and conditions described in Materials and methods. The results are expressed as the mean ± SD (n = 3) of fold-change with respect to α_{2A}-AR expression in MCF-7 cells.

2.7. Protein extracts and Western blot

ERK activation was determined as already described [12] and AKT and STAT5 in a similar way. Cells were seeded in 6 well plates in 10% FCS supplemented medium. The medium was changed again to serum-free medium and after 1–2 h, the compounds were added (or not in control cells). The incubation was performed for 10 min and then the protein extraction buffer (RIPA: 10 mM Tris-HCl, 150 mM NaCl, 2 mM sodium vanadate, 1% sodium deoxycholate, 0.1% SDS, 1% Igepal, pH = 7.5) was added after washing the cells with phosphate saline buffer.

Cell extracts (100 μg total protein/lane) were separated on 12% discontinuous polyacrylamide gels (SDS-PAGE). The proteins were dissolved in sample buffer (6 mM Tris-HCl, 2% SDS, 0.002% bromophenol blue, 20% glycerol, 5% mercaptoethanol, pH = 6.8) and boiled for 5 min. After electrophoresis, they were blotted onto a 0.2 mm nitrocellulose membrane and blocked 1 h at room temperature in 5% skim milk in 0.1% TBST (10 mM Tris-HCl, 100 mM NaCl, 0.1% vv-1 Tween 20, pH = 7.5). For analysis of AKT activation, the skim milk was replaced by 5% BSA. Phospho-specific antibodies were used as follows: p-STAT5 (1:500), p-AKT (1:500 except for IBH-6 extracts, 1:1,000) p-ERK1/2

(1:1,000). The membranes were stripped of IgG and re-probed with antibodies anti-STAT5a/b (1:500) or anti pan-AKT (1:500) or anti-ERK 1 (1:1,000) for protein loading normalization. Blots were probed with donkey anti-rabbit and sheep anti-mouse IgG horseradish peroxidase linked whole antibody; 1:2,000 dilutions in 5% skim milk 0.1% TTBS. The luminescent signal was generated with an ECL Western blotting detection solution (2.2 mg luminol, 0.33 mg p-coumaric acid, 15 ml hydrogen peroxide 30%, 333 μl Tris-HCl 1.5 M, pH 8.8, 4.6 ml distilled water), and the blots were exposed to an autoradiographic film (Curix RP1, Agfa, Buenos Aires, Argentina) for 10 s to 5 min. Band intensity was quantified only in unsaturated films. Quantification was performed with ImageJ software.

2.8. Statistical analysis

Data are presented as mean ± SEM. Statistical analysis for the effect of agonists was performed by ANOVA followed by Dunnett-Kramer test [32] using Graphpad Prism 5 software. A p value < 0.05 was considered statistically significant.

3. Results

α_2-ARs have been described by our group in several benign and malignant breast cell lines [9]. However, their expression in T47D cells has never been reported. In Fig. 1 we show using immunofluorescence that the three α_2-AR subtypes are expressed in this cell line. Table 1 shows the comparative expression of the different α_2-AR subtypes as assessed by RT-qPCR. In MCF-7 and T47D cells the most expressed subtype is clearly the α_{2C}, although every receptor subtype is expressed in both cell lines. In IBH-6 cells the expression is much lower.

In order to evaluate whether the incubation of breast cancer cells with α_2-adrenergic compounds had any impact on the Prl loop, Prl concentration was assessed in CM using the Nb2 bioassay. This is a very sensitive assay that has the advantage of measuring the concentration of biologically active lactogenic hormones. As adsorbed FCS did enhance

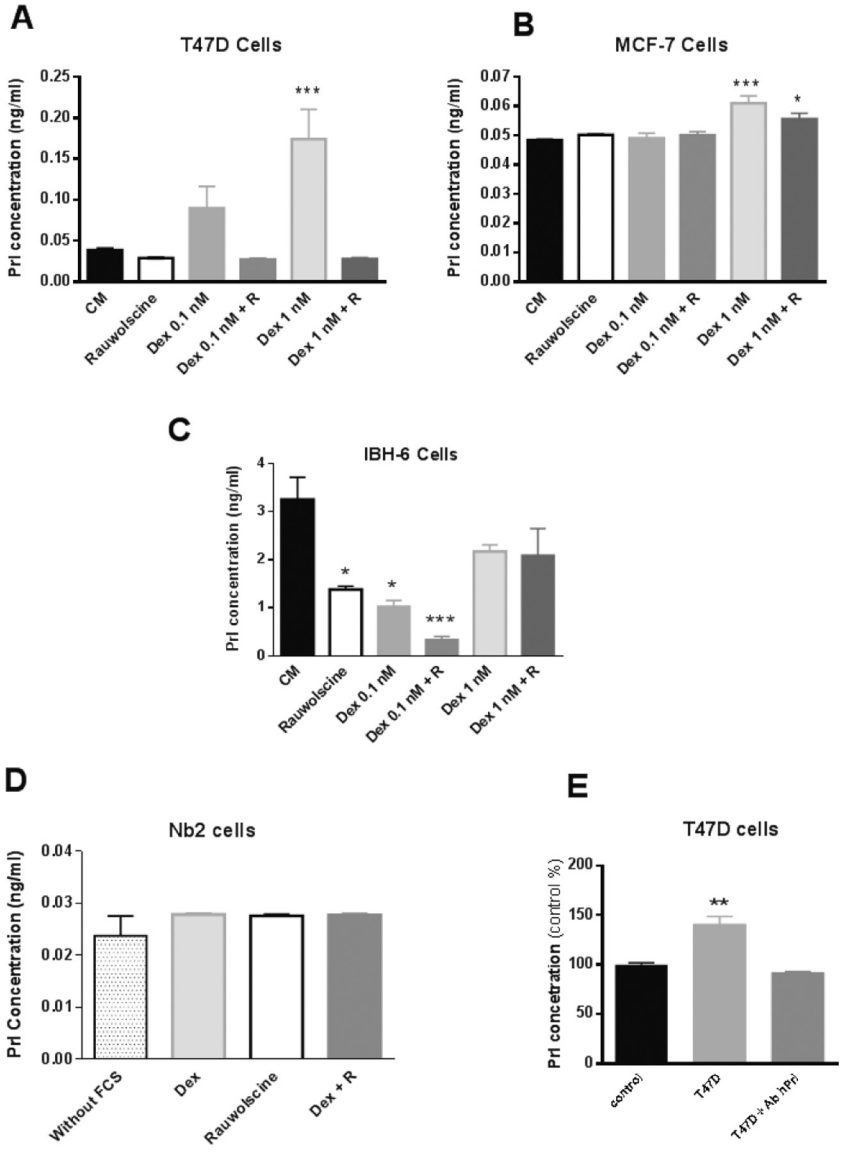

Fig. 2. α_2-Adrenergic compounds alter Prl secretion in T47D (panel A), MCF-7 (panel B) and IBH-6 (panel C) cells. Prl concentration in the conditioned media was measured by the Nb2 bioassay as stated in Materials and methods. The cells were incubated in DMEM/F12 medium in the absence of FCS with dexmedetomidine (Dex) at concentration 0.1 or 1 nM in the presence or absence of 1 μM rauwolscine (R), as indicated. The controls were the conditioned medium in the absence of any additive (CM) and rauwolscine alone. Panel D shows that Dex and R do not alter Nb2 cell proliferation. In panel E, hPrl neutralizing antibody was incubated with T47D CM to validate the specificity of the Nb2 bioassay. * $p < 0.05$ and ** $p < 0.01$ with respect to control conditioned medium (indicated as CM) as analyzed by ANOVA followed by Dunnett-Kramer test. The experiment was performed three times with similar results.

Nb2 cell proliferation at concentrations as low as 2% (data not shown), all the CM were prepared using serum-free media. Fig. 2 shows active Prl concentration in CM from the luminal breast cancer cell lines T47D (panel A), MCF-7 (panel B) and IBH-6 (panel C). Prolactin levels in CM of non-stimulated T47D and MCF-7 cells were similar (~0.05 ng/ml). The incubation of T47D cells with the α_2-adrenergic agonist dexmedetomidine resulted in dose-dependent increase of Prl release to the culture medium that was significant for 1 nM. The increment of Prl levels produced by dexmedetomidine was less spectacular in MCF-7 cells (1.2-fold) compared to T47D (approximately 4-fold) but it was also significant at 1 nM. This effect was fully reversed by the α_2-adrenergic antagonist rauwolscine in T47D, and partially in MCF-7 cells. Rauwolscine alone had no effect on Prl secretion in these cell lines. The picture was totally different for IBH-6 cells. In non-stimulated cells, Prl release in the medium was much higher (~60 fold) than in MCF-7 and T47D cells. In contrast to the latter cell lines, the α_2-adrenergic agonist decreased Prl concentrations. Furthermore, not only the antagonist rauwolscine had the same effect than the agonist, but it even potentiated its action instead of reversing it. As a control, panel D shows that direct incubation of Nb2 cells with the α_2-adrenergic compounds did not change cell proliferation and hence, the measurement of active Prl concentration. Panel E demonstrates that the lactogenic hormone secreted by T47D was Prl, since the addition of a neutralizing antibody against Prl abrogated Nb2 cell proliferation. Supplementary Fig. 1 shows the effect of adrenergic stimulation of two additional cell lines.

α_2-Adrenergic effect in the expression of PrlR, the other component of the autocrine/paracrine Prl loop, was then assessed by real-time qPCR in both cell lines whose Prl production was increased. As can be seen in Fig. 3, dexmedetomidine and/or rauwolscine treatment had no significant effect on PrlR expression, either in T47D (panel A) or in MCF-7 (panel B). As shown in panel C, the expression of the PrlR in IBH-6 was not detected, in this case by classical RT-PCR.

The signaling pathways involved in oPrl and dexmedetomidine action were assessed in MCF-7 (Fig. 4), IBH-6 (Fig. 5) and T47D cells (Fig. 6). In each figure, panels A show representative Western blots of phosphorylated and total STAT5, AKT and ERK, and panels B to D show the quantification (mean ± S.E.M) of pSTAT5, pAKT and pERK1/2 relative to total STAT5, AKT and ERK, respectively. As clearly shown in these figures, Prl caused rapid (within 10 min) and marked increase in phosphorylation of STAT5 and ERK1/2 in all cell lines. The incubation of all three cell lines in the presence of dexmedetomidine caused a similar increase in activation of these pathways. The phosphorylation of AKT by Prl and dexmedetomidine was observed in T47D and MCF-7 cells. Moreover, when the cells were incubated in the presence of both oPrl and dexmedetomidine, no additive or synergic effect was evidenced.

We formerly reported that α_2-AR enhanced the proliferation of several breast cancer cell lines [9]. Based on the results presented in this study, we hypothesized that this effect could be mediated, at least in part, by stimulation of the autocrine/paracrine Prl loop. To address this issue, we used T47D cells. As shown in Fig. 7, dexmedetomidine significantly enhanced cell proliferation as potently as oPrl. These data confirmed the proliferative effect of α_2-AR stimulation on breast cancer cells, which had never been assessed in this particular cell model. When the specific PrlR antagonist Δ1–9-G129R-hPrl [33,34] was added in the presence of oPrl, the proliferative effect of the latter was suppressed, as expected. Importantly, this antagonist also reversed cell proliferation enhancement induced by dexmedetomidine treatment, supporting that the effects of the latter involve the autocrine/paracrine Prl loop.

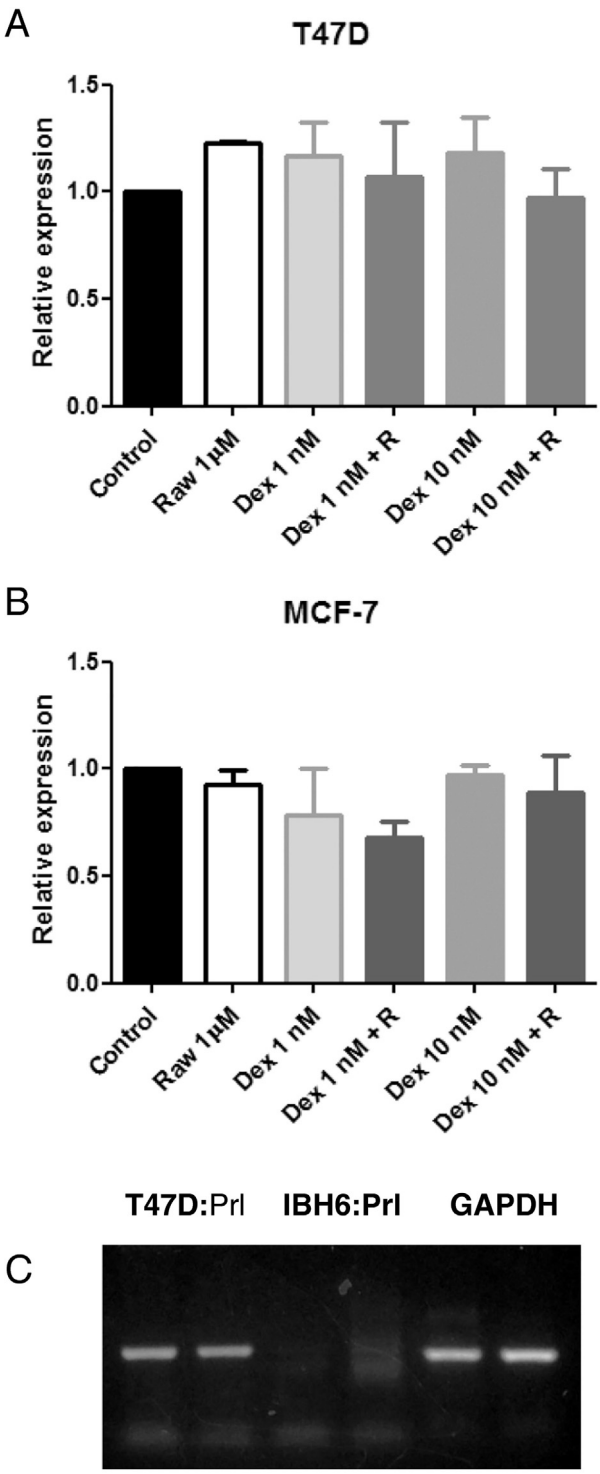

Fig. 3. α_2-Adrenergic stimulation or inhibition does not alter PrlR expression in T47D (panel A) and MCF-7 (panel B) cell lines. PrlR expression was measured as described in Materials and methods by quantitative real-time PCR. The bars are the mean ± S.E.M. of 3 independent assays. There were no significant differences as analyzed by ANOVA followed by Dunnett-Kramer test. Panel C: comparative RT-PCR of T47D and IBH-6 cell lines. This panel shows duplicates of each cell line.

4. Discussion

We have previously reported that α_2-AR stimulation enhanced cell proliferation [9] and tumor growth [10,11] in preclinical models of breast cancer. *In vivo* tumor growth was associated with changes in the fibrillar collagen microstructure, without changing its content [35] and with pre-synaptic α_2-AR which can mediate an autoinhibition of sympathetic transmission [36]. The effect of α_2-AR expression on breast cancer is still uncertain. Some authors found, for example, a positive association between the expression of α_{2A}-AR and an increased risk of

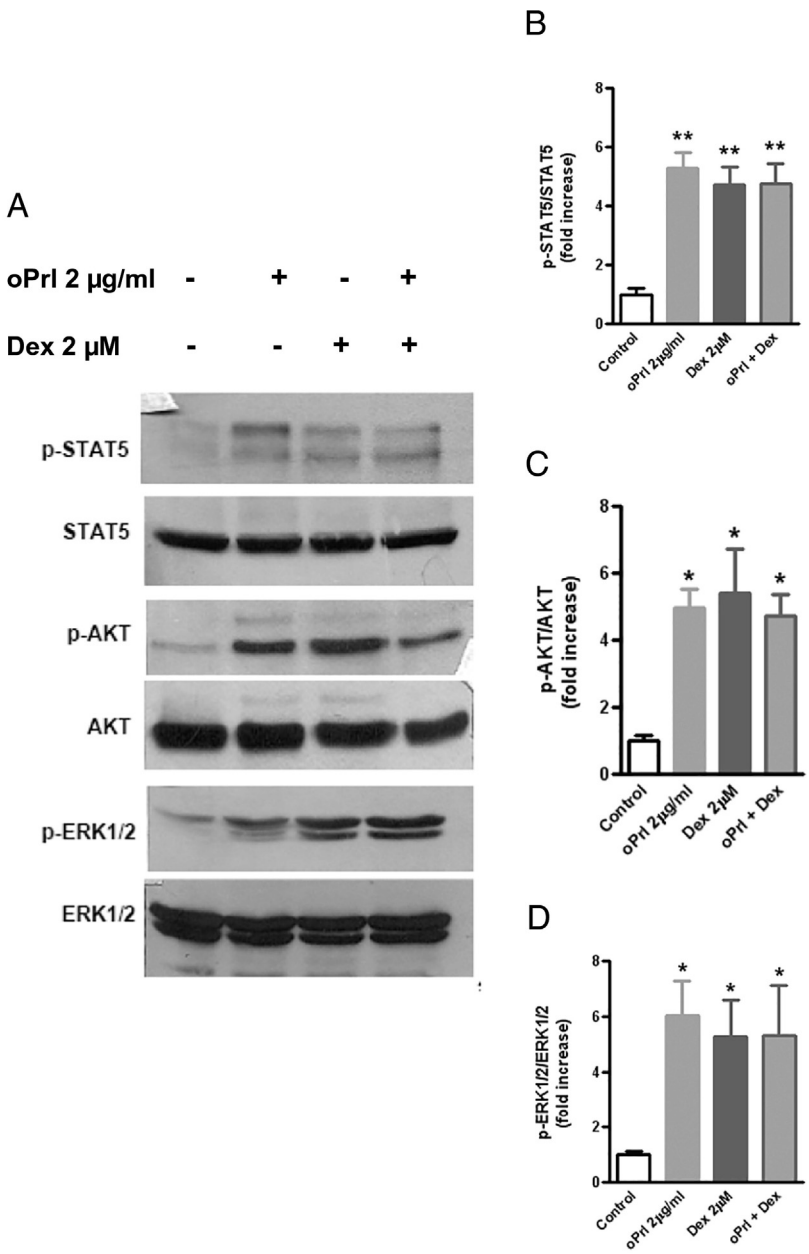

Fig. 4. Activation of signaling pathways in MCF-7 cells. The cells were incubated during 10 min in the presence or absence of ovine prolactin (oPrl) and of dexmedetomidine. Panel A: representative Western blots of the assays. Panel B: the bands were quantified and mean ± S.E.M. of the ratio pSTAT5/STAT5 for 10 independent assays is shown. Panel C: the mean of the ratio pAKT/AKT of 4 independent experiments. Panel D: mean of the ratio pERK/ERK in 6 independent assays. * $p < 0.05$ and ** $p < 0.01$ with respect to control as analyzed by ANOVA followed by Dunnett-Kramer test.

relapse [37] while others found no association at all [38]. In the latter report, however, α_{2A}-AR expression was significantly associated with low HER-2 expression. Also, a positive trend between α_{2A}-AR expression and ER status was found [38]. Strong cytoplasmic α_{2C}-AR staining was significantly more prevalent in high histologic grade tumors of postmenopausal patients. Its expression showed an inverse association with hormonal status [39]. In the present study, we show that the cellular mechanism underlying α_2-AR agonist-induced breast cancer cell proliferation involves the Prl autocrine/paracrine signaling loop.

Prl secretion to the culture medium was analyzed using a modified version of the very sensitive Nb2 cells bioassay [31]. Using this bioassay, others reported Prl concentrations in T47D and MCF-7 conditioned media similar to those observed in this work [40]. Prolactin production by IBH-6 cells had never been assessed; here we show that these cells secrete very high amounts of Prl - nearly 2 orders of magnitude higher than the other cell lines. The reason for such a difference is at present unknown but may involve different Prl gene promoter usage (see below).

Two patterns of Prl expression were observed in response to α_2-adrenergic agonist dexmedetomidine stimulation, stimulatory on MCF-7 and T47D cells, and inhibitory on IBH-6 cells. We performed similar assays for two additional cell lines generated in-house, namely IBH-4 and IBH-7. The first one exhibited an IBH-6 like pattern while the latter shared the same pattern as T47D and MCF-7 cell lines (Supplementary Fig. 1). Human mammary cells use different promoters to transcribe Prl: MCF-7, T47D and SK-BR-3 cells use the proximal (pituitary) promoter, BT-474 and benign MCF-10A, the superdistal (extrapituitary) promoter, and MDA-MB-231, both [41,42]. *In vitro* studies involving EGF-stimulated Prl production in SK-BR-3 cells [43] and *in vivo* studies deciphering the role of autocrine Prl in epithelial differentiation during

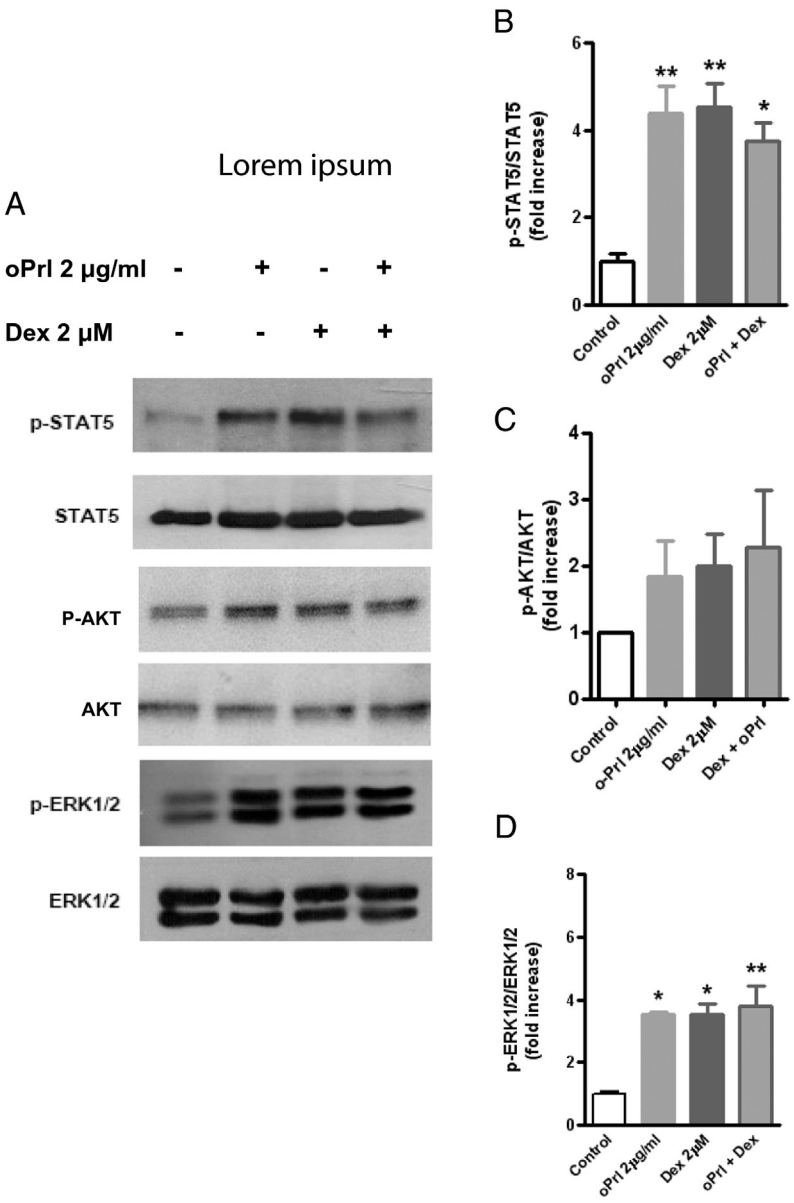

Fig. 5. Activation of signaling pathways in IBH-6 cells. The cells were incubated during 10 min in the presence or absence of ovine prolactin (oPrl) and of dexmedetomidine. Panel A: representative Western blots of the assays. Panel B: the bands were quantified and mean ± S.E.M. of the ratio pSTAT5/STAT5 for 12 independent assays is shown. Panel C: the mean of the ratio pAKT/AKT of 3 independent experiments. Panel D: mean of the ratio pERK/ERK in 11 independent assays. * $p < 0.05$ and ** $p < 0.01$ with respect to control as analyzed by ANOVA followed by Dunnett-Kramer test.

pregnancy [44] pointed to the role of the PI3K/AKT pathway in the regulation of the proximal hPrl pituitary promoter *via* AP-1 sites. Signaling studies performed in the investigation showed that IHB-6 cells differed from the two other cell lines by the lower sensitivity of AKT pathway to α2-adrenergic stimulation. Therefore, it could be speculated that the activation (or not) of this kinase influences the promoter usage with consequence on the pattern of Prl expression.

We may speculate that the IBH-6 cells use the superdistal promoter which could explain the differentially regulated Prl secretion. Further investigations are needed to support this hypothesis and to elucidate the molecular mechanisms underlying these cell-line dependent paradoxical effects of α2-AR agonists.

In IBH-6 cells, α_2-adrenergic antagonist unexpectedly exerted similar effect than agonists on Prl secretion. GPCR exert their functions through interactions with different G proteins and other effectors as production of second messengers, phosphorylation cascades, ion channels and transcriptional regulation [45]. Some ligands of these receptors differentially activate distinct subsets of these effectors, a phenomenon known as biased signaling. One possibility for the similar inhibition of Prl secretion in IBH-6 cells is that in this particular cellular context, rauwolscine could act as a partial or biased agonist. Another possibility is that some agonists as well as the antagonist rauwolscine and yohimbine have been described as antagonists of 5-HT_{2B} serotonin receptors in the rat stomach fundus [46]. Hypothetically, the coincidence of action by agonist and antagonist could be due either to a partial agonism of rauwolscine or to a coincident action on serotonin receptors. In a previous publication from our laboratory [47], a significant enhancement of cell migration was found for 1 µM dexmedetomidine in MCF-7 cells. As Prl has also been described to enhance this parameter in these cells [48], it is tempting to speculate that the α_2-adrenergic effect on cell migration could be mediated by Prl.

The effects of the α_2-adrenergic agonist dexmedetomidine on canonical PrlR signaling cascades were analyzed in comparison to oPrl stimulation, which is well documented in classical breast cancer cell lines [25,49,

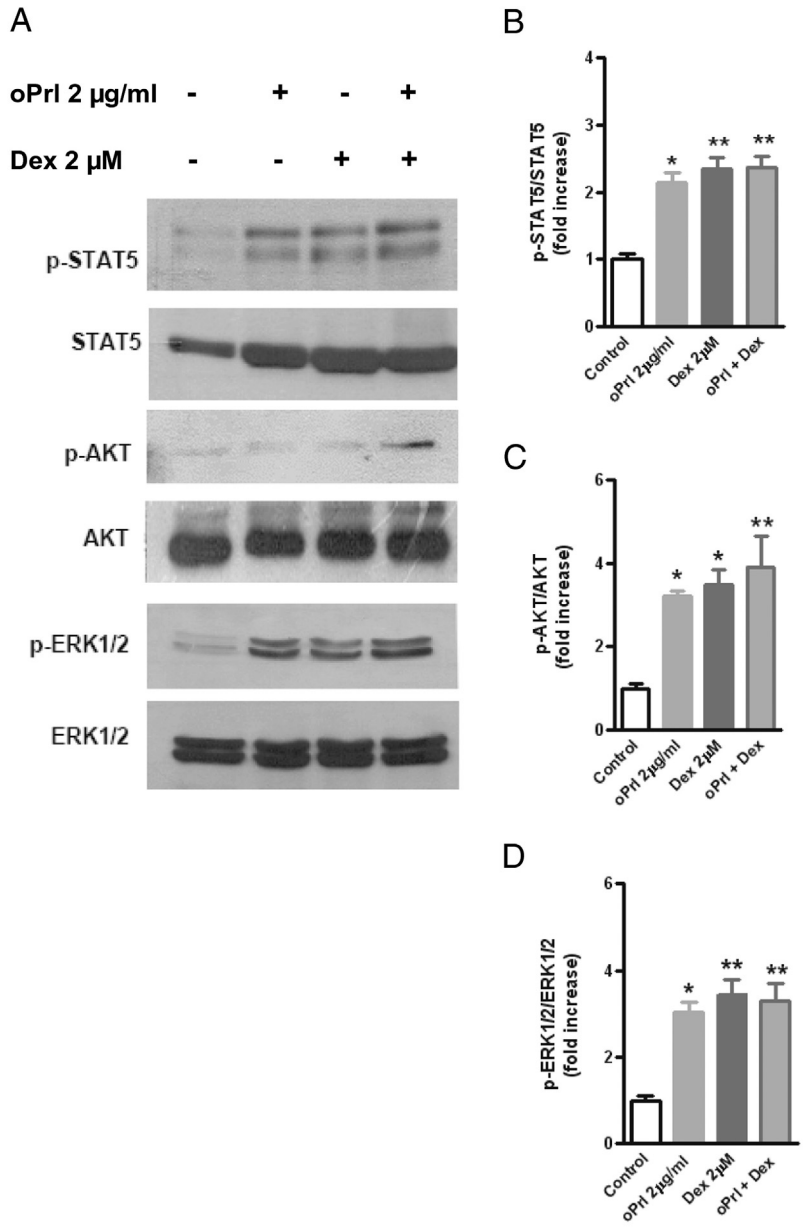

Fig. 6. Activation of signaling pathways in T47D cells. The cells were incubated during 10 min in the presence or absence of ovine prolactin (oPrl) and of dexmedetomidine. Panel A: representative Western blots of the assays. Panel B: the bands were quantified and mean ± S.E.M. of the ratio pSTAT5/STAT5 for 5 independent assays is shown. Panel C: the mean of the ratio pAKT/AKT of 9 independent experiments. Panel D: mean of the ratio pERK/ERK in 8 independent assays. * $p < 0.05$ and ** $p < 0.01$ with respect to control as analyzed by ANOVA followed by Dunnett-Kramer test.

50]. In MCF-7 and T47D cells the incubation with either of the compounds caused a significant activation of STAT5, ERK1/2 and AKT. As mentioned above, AKT activation was modest and not significant in IBH-6 cells. These pathways have all been connected to cell proliferation or survival [51,52,53]. The activation of these pathways was similar for the lactogenic and the adrenergic compounds, and as expected, perfectly correlated with their effects on cell proliferation as shown using T47D cells. This result raised the hypothesis that the effect of dexmedetomidine on canonical PrlR signaling cascades could be mediated by the amplification of the autocrine Prl loop. Two arguments are in favor of such a mechanism in T47D cells. First, 48 h adrenergic stimulation produced an important rise in Prl release in these cells. Of note, no concomitant increase in PrlR expression was observed. Second, the increase in cell proliferation induced by both oPrl and dexmedetomidine after 72 h stimulation was reversed by the specific PrlR antagonist Δ1–9-G129R-hPrl. Since PrlR antagonists were shown to inhibit all signaling pathways triggered by the PrlR [33,54], this result demonstrates that the autocrine/paracrine Prl loop plays an important role in the mitogenic effect of the α_2-adrenergic agonist in the luminal T47D breast cancer cell line. Such a mechanism may also apply to MCF7 cells. However, since a decrease of Prl secretion was observed in IHB-6 cells under adrenergic stimulation, other mechanisms should co-exist.

A direct, Prl-independent effect of adrenergic agonists on the activation of ERK, STAT5 and AKT pathways is supported by short term (10 min) signaling experiments. Indeed, a contribution of Prl/PrlR signaling to this rapid effect of adrenergic agonists would imply the release of pre-synthesized Prl, which is unlikely in extra-pituitary sites [55]. In PC12 cells (derived from a rat pheochromocytoma) stably transfected with human α_2-AR genes, the agonists of these receptors activated ERK1/2 and AKT and this effect was shown to be mediated by Src [56]. In MCF-7 and T47D cells activation of ERK1/2 and AKT by Prl has also been described to occur through Src [25]. Furthermore, it has been

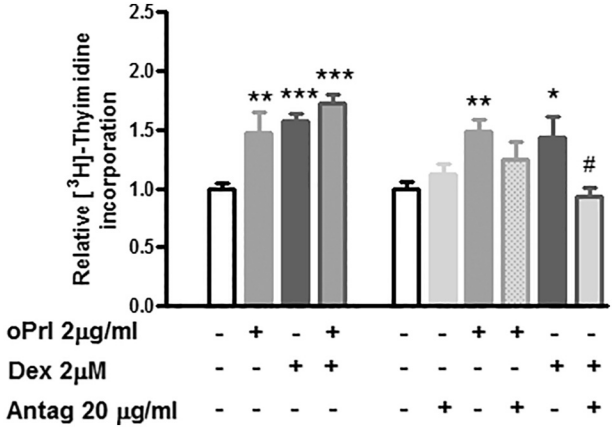

Fig. 7. The stimulation of cell proliferation by α_2-adrenergic stimulation in T47D cells is completely abrogated by the PrlR antagonist. Cell proliferation measured by [^{3}H]-thymidine incorporation after 72 h incubation. Antag is the PrlR antagonist Δ1–9-G129R-hPrl. This experiment was performed four times with similar results. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ with respect to control and # $p < 0.05$ with respect to Dex, as analyzed by ANOVA followed by Dunnett-Kramer test.

described that c-Src is a key node in STAT5 activation by Prl both in normal and malignant breast cells [50]. Therefore, the similar activation of STAT5, AKT and ERK1/2 by Prl and dexmedetomidine could be explained by Src mediation in every case.

As a conclusion, the present investigation describes that in some luminal breast cancer cell lines, the α_2-adrenergic stimulation of cell proliferation could be mediated, at least in part, by the Prl autocrine/paracrine loop. This stimulation of the Prl loop describes a novel mechanism of action of this GPCR. As both prolactin and catecholamines are released during stress, the implication of Prl loop in α_2-adrenergic action could potentiate stress effect in breast cancer. Importantly, our findings stress that various cell lines behave distinctly, highlighting the necessity to assess these effects using different cell lines.

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Author contributions

LF Castillo and EM Rivero performed the experiments, V. Goffin supervised the experiments performed in Paris and IA Lüthy the rest of the experiments. The design of the experiments, analysis and interpretation of the data were shared by every author. IA Lüthy drafted the manuscript, which was corrected and accepted by all authors.

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