Estrogen receptors regulate galectin-3 in androgen-independent DU-145 prostate cancer cells

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Abstract. The aim of the present study was to investigate the role of estrogen receptor (ER) α and ER β , and galectin-3 (GAL-3) in migration and invasion of androgen-independent DU-145 prostate cancer cells, and to examine the regulation of the expression of GAL-3 by the activation of these receptors. Wound healing and cell invasion assays were performed using the control (basal level of cellular function) and treated DU-145 cells. At 24 h of treatment, 17\beta-estradiol (E2), the ERα-selective agonist, 4,4',4"-(4-propyl-(1H)-pyrazo le-1,3,5-trivl)trisphenol (PPT), or the ERβ-selective agonist, 2,3-bis(4-hydroxyphenyl)-propionitrile (diarylprepionitrile; DPN), increased the migration and invasion of the DU-145 cells. Pre-treatment with the ERa- and ER\beta-selective antagonists blocked these effects, indicating that ER α and ER β are upstream receptors regulating these processes. Western blot analysis and immunofluorescence staining for the detection of the GAL-3 were performed using the control and treated DU-145 cells. Treatment of the DU-145 cells with E2, PPT or DPN for 24 h increased the expression of the GAL-3 compared to the control. Furthermore, a specific inhibitor of GAL-3 (VA03) inhibited the migration and invasion of DU-145 cells, indicating the involvement of the complex ER α /GAL-3 and ER β /GAL-3 in the regulation of these processes. On the whole, the present study demonstrates that the activation of both ERs increases the expression and signaling of GAL-3, and promotes the migration and invasion of DU-145 cells. The findings of the present study provide novel insight into

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the signatures and molecular mechanisms of ER α and ER β in DU-145 cells.

Introduction

Radical prostatectomy or radiotherapy with or without androgen deprivation therapy are the current treatments for localized prostate cancer (1). However, disease recurrence and the emergence of castration-resistant prostate cancer (CRPC) are frequent occurrences following therapy, and treatment options for these remain insufficient; in addition, the survival rate for patients with advanced disease remains low (2), highlighting the importance of basic research required for this disease.

The overexpression of galectins (GALs), carbohydrate binding proteins, with affinity for N-acetylglucosamine, their roles in cancer progression (3) and their potential as therapeutic targets have been demonstrated in various tumor types over the past 10 years (4). The role of GAL in prostate cancer has previously been described (5,6). These previous studies have mainly focused on GAL-1 and GAL-3, although the importance of GAL-4, GAL-7, GAL-8 and GAL-9 has also been highlighted in this disease (7). The expression of GAL-3 at the mRNA and protein level in the tumor decreases during prostate cancer progression (8-17). However, cytoplasmic overexpression in tumors cells has also been shown to be positively associated with disease progression (11), suggesting the dual role of GAL-3 in prostate cancer cells, depending on its subcellular localization (18).

In vitro studies have revealed that GAL-3 inhibits the apoptosis of prostate cancer cells (19-21), and induces T-cell apoptosis (22) and tumor cell adhesion to endothelial cells (23,24). It has also been demonstrated that in androgen-independent (PC-3 cells) and androgen-dependent prostate cancer cells (LNCaP) transfected with GAL-3 (LNCaP-GAL-3 cells), GAL-3 induces proliferation, migration and invasion (13,25). These findings were corroborated *in vivo* using tumor xenograft mouse models, in which GAL-3 inhibition with pharmacological or RNA interference (RNAi) strategies impaired tumor growth (13,25) angiogenesis (22) and metastasis (21,22,26).

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LNCaP cells do not express GAL-3, whereas the androgen-independent prostate cancer cells, DU-145 and PC-3, highly express GAL-3 (21,25). As previously demonstrated, the overexpression of GAL-3 in LNCaP cells (21,25) or the knockdown of GAL-3 in PC-3 cells does not alter the expression level of the androgen receptor (AR) (21); similarly, it has been demonstrated that the overexpression of AR in PC-3 cells has no regulatory effect on the expression of GAL-3 (21).

The molecular regulatory mechanisms responsible for the expression of GAL-3 in tumor cells are not yet clear (27,28). The expression of GAL-3, at both the transcriptional and translational level, can be regulated by various stimuli (27,28). It has been suggested that promoter methylation is not the only factor regulating the expression of GAL-3 (29). The expression of GAL-3 is increased by transcription factors, such as the RUNX protein family, homeodomain-interacting protein kinase 2, cAMP-response element-binding protein, the NF-kB transcription factor, hypoxia-inducible factor- 1α , and inflammatory cytokines and the Ras/MAPK pathway (27,28). Several of these transcription factors and signaling pathways are activated by estrogen receptors (ERs) or interact with ERs (30,31). The regulatory effects of ERs on the expression of GAL-3 remain to be explored in prostate cancer cells.

The authors have previously demonstrated the presence of the ERs, ER α (ESR1) and ER β (ESR2), in the androgen-independent PC-3 and DU-145 prostate cancer cells, and these receptors are mostly located outside the cell nucleus (32,33). The activation of ER α and ER β can activate rapid cell signaling pathways in these cells, including an increase in the phosphorylation of ERK1/2 in PC-3 and DU-145 cells (32,33), and SRC and AKT in PC-3 cells (34,35). It is noteworthy that the expression of ER α (unpublished data, Fig. S1) and ER β (33) is higher in DU-145 than in PNT1A and PC-3 cells, so the present study focused on DU-145 cells.

The present study aimed to examine the roles of ER α , ER β and GAL-3 in the migration and invasion of DU-145 cells, and to determine the regulatory effects of the activation of these receptors on the expression of the GAL-3.

Materials and methods

Cells and cell culture. The human post-pubertal prostate epithelial cell line, PNT1A, was obtained from Public Health England Culture Collections (lot 11B010; cat. no. 95012614). The DU-145 (derived from brain metastasis) and PC-3 (derived from bone metastasis) cell lines were obtained from ATCC (DU-145 cells, lot7000 9869, cat. no. HTB-81; and PC-3 cells, lot BCRJ:0269, cat. no. CRL-1435; deposited at the Rio de Janeiro Cell Bank). Mycoplasma testing was carried out for all cell lines used. The PNT1A, DU-145 and PC-3 cells were cultured as previously described (32-36). The culture medium was replaced by serum free medium for 24 h before the assays. All experimental procedures (cell culture, western blot analysis, immunofluorescence, wound healing, cell invasion and cell viability analyses, and statistical analysis) were described, submitted, analyzed and approved by the Research Ethics Committee at the Paulista School of Medicine (EPM), Federal University of São Paulo (UNIFESP; no. 3527220917). Western blot analysis for the detection of GAL-3 and $ER\alpha$ (ESR1). The PNT1A and PC-3 (used in some experiments) and the DU-145 cells were incubated in the absence (control, untreated cells) or presence of 17 β -estradiol (E2, 10 nM; MilliporeSigma), the ER α -selective agonist, 4,4', 4"-(4-propyl-(1H)-pyrazole-1,3,5-triyl)trisphenol (PPT; 10 nM, MilliporeSigma) or the ER β -selective agonist, 2,3-bis(4-hydroxyphenyl)-propionitrile (diarylprepionitrile; DPN; 10 nM, MilliporeSigma) for 30 min, 1, 2 and 4 h at 37°C. At these concentrations, the agonists are highly selective, as previously reported (32,37,38).

Total cell lysates (20 or 50 µg of protein/lane), SDS/PAGE and western blot analysis were performed as previously described (33,39). The protein concentration was determined using the Bio-Rad protein assay, using bovine serum albumin as standard (Bio Rad Laboratories, Inc.). Briefly, rabbit polyclonal antibody raised against a peptide mapping at the carboxyterminal of ER α of mouse origin, similar to human ERa [MC-20, sc-542, Santa Cruz Biotechnology, Inc.; diluted at 1:200 in Tris-buffered saline containing 0.2% Tween-20 (TBS-T) (Sigma Chemical Co.) and 10% non-fat dry milk (Nestle), pH 7.6, overnight at 4°C] and anti-GAL-3 [hybridoma M3/38.1.2.8 HL.2, TIB-166[™], ATCC; donated by Professor Roger Chammas, Center for Translational Research in Oncology, Instituto do Cancer do Estado de São Paulo, São Paulo, SP, Brazil; diluted at 1:100 in phosphate-buffered saline containing 0.1% Tween-20 (PBS-T) (Sigma Chemical Co.) and 5% non-fat dry milk (Nestle), pH 7.2, for 1 h at room temperature], were used. Proteins were visualized by enhanced chemiluminescence reagent (ECL, GE Healthcare), after incubation for 1 h at room temperature, with the appropriate HRP-conjugated secondary antibody (GE Healthcare) diluted in TBS-T at 1:3,000 or in PBS-T 1:3,500. The band intensities of ERα, GAL-3, β-tubulin and GAPDH from individual experiments were quantified using the densitometric analysis of linear-range autoradiograms, using an Epson Expression 1680 scanner (Epson America, Inc.) and the quick Scan 2000 WIN software (Helena Laboratories Co.). β-tubulin or GAPDH were used as protein loading controls. The results were normalized to the respective expression of β -tubulin or GAPDH, expressed in relation to the control (C=1) or in arbitrary unit and plotted (mean \pm SEM) from three to six independent experiments. The blots are representative of three to six independent experiments.

Immunofluorescence staining for the detection of the GAL-3. The DU-145 cells were incubated in the absence (control) or presence of E2, 10 nM); PPT (10 nM) or DPN (10 nM) for 2, 4 and 24 h at 37°C. The cells were also untreated or pre-treated with the ER α -selective antagonist, 1,3-bis(4-hydrox- yphenyl)-4-methyl-5-[4-(2-piper-idinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP; 10 nM, MilliporeSigma) and the ER β -selective antagonist, 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a] pyrimidin-3-yl]phenol (PHTPP; 10 nM, Tocris Bioscience) for 30 min at 37°C. Incubation was continued in the absence or presence of E2 (10 nM), PPT (10 nM) or DPN (10 nM) for 2 and 4 h at 37°C, as previously described (33). Subsequently, the DU-145 cells were washed with ice-cold PBS, fixed in 2% formalin (formaldehyde EM grade, Electron Microscopy Sciences) for

20 min at room temperature, and washed with PBS containing 0.1 M glycine (Sigma Chemical Co). The immunofluorescence assays were performed as previously described (32,33). Briefly, rat monoclonal antibody raised against GAL-3, at 1:50 dilution, in PBS containing 0.01% saponin (Sigma Chemical Co.) and 1% BSA (Sigma Chemical Co.), for 1 h at room temperature. The cells were also incubated with Alexa Fluor 594-labeled secondary antibody (anti-rat; 1:300; Molecular Probes[®], Invitrogen; Thermo Fisher Scientific, Inc.). Nuclear staining was performed with DAPI (4',6-diamidino-2-phenylindole, Sigma Chemical Co.). Negative controls were performed in the absence of primary antibodies. The immunostaining of GAL-3 was visualized under a confocal microscope Leica Microsystems TCSSP8 (Leica Microsystems GmbH). Images of five random microscope fields containing ~20 cells were captured, in duplicate, in each assay (three independent experiments) and analyzed using LAS-X software version: 3.7.0.20979 (Leica Microsystems CMS GmbH). Images are representative of two to four independent experiments performed in duplicate. The fluorescence intensity of whole cell was obtained and analyzed using ImageJ software 1.53t (National Institutes of Health) from the control and treated cells and expressed in arbitrary units.

Wound healing assay. The DU-145 cells in culture medium without serum containing a blocking DNA replication mitomycin C (10 μ g/l; MilliporeSigma) to avoid cell proliferation, were wounded using 200 μ l sterile pipette tips as previously described (40,41). The DU-145 cells were incubated in the absence (control, basal level of cellular function) or presence of E2 (10 nM), PPT (10 nM) DPN (10 nM) for 24 h at 37°C. The cells were also untreated or pre-treated with MPP (10 nM), PHTTP (10 nM), simultaneously with MPP (10 nM) and PHTPP (10 nM), or with the inhibitor of GAL-3, 1,2,3-triazole-galactosyl arylsulfadimethoxine [VA03; donated by Professor Vanessa Leiria Campo Barão de Mauá University Center (CBM), Ribeirão Preto, SP, Brazil. 200 μ M] (42) for 30 min at 37°C. Incubation was continued in the absence or presence of E2 (10 nM), PPT (10 nM) or DPN (10 nM) for 24 h at 37°C. Wound healing analysis was performed as previously described (40,41). For measuring the closure of the wound in the control and treated cells, images of the same area of the wound were obtained at 0 and 24 h. Images were captured using an inverted optical microscope Axio Observer Z1 (Zeiss Nikon Eclipse, Zeiss GmbH) and ZEN 3.3 blue edition software (eiss Nikon Eclipse, Zeiss GmbH). The areas that were occupied by migrating cells after 24 h of incubation (control and treated cells) were calculated by subtracting the background levels at 0 h. The experiments were quantified using ImageJ software 1.53t (National Institutes of Health). The results were expressed in relation to the control (C=100%) and plotted (mean \pm SEM) from three to five independent experiments, in duplicate. Images are representative of three to five independent experiments performed in duplicate.

Cell invasion assay. The DU-145 cells in culture medium without serum were seeded in Thincert[®] chambers (Greiner Bio-One) with polyethylene terephthalate membranes (8 μ m pore size) pre-coated with 50 μ l of phenol red-free Matrigel (1:10, Corning, Inc.). These chambers were placed in 24-well

plates containing culture medium with 10% of fetal bovine serum in the lower chamber. The DU-145 cells in the upper chamber were incubated in the absence (control) or presence of E2 (10 nM), PPT (10 nM) or DPN (10 nM) for 24 h at 37°C. The cells were also untreated or pre-treated with MPP (10 nM), PHTPP (10 nM), simultaneously with MPP (10 nM) and PHTPP (10 nM), or VA03 (200 µM) for 30 min at 37°C (42). Incubation was continued in the absence or presence of E2 (10 nM), PPT (10 nM) or DPN (10 nM), for 24 h at 37°C. Cell invasion analysis was performed as previously described (35,41). Briefly, the membranes were washed thoroughly with 10 mM PBS (Sigma Chemical Co.), fixed in 4% paraformaldehyde (Electron Microscopy Science) for 30 min, and stained with 0.2% crystal violet (Merck KGaA) for 10 min (35,41). Non-invading cells from the membrane upper surface were removed using a sterile cotton swab. The membranes containing the invaded cells (under the surface of membrane), were photographed. Images of three random microscope fields, in duplicate, were captured using an inverted optical microscope (Nikon Eclipse, Nikon Corporation). The areas of invaded cells were analyzed using Micrometrics SE Premium 4 software (Nikon Eclipse, Nikon Corporation). The experiments were quantified using ImageJ software1.53t (National Institutes of Health). The results were expressed in relation to the control (C=100%) and plotted (mean ± SEM) from three to six independent experiments, in duplicate. Images are representative of three to six independent experiments performed in duplicate.

MTT cell viability assay. The DU-145 cells were incubated in the absence (control) or presence of VA03 (20 and 200 μ M) for 24 h at 37°C. Cell viability assay was performed using MTT assay (Thermo Fisher Scientific Inc.) as previously described (44). The cells were washed with ice-cold PBS, replaced with 100 μ l of fresh culture medium containing MTT (2.4 mM), and incubated for 2 h at 37°C. The medium was removed, and the formazan product was dissolved in DMSO (100 μ l to each well) at room temperature for 10 min with intermittent shaking. Each sample was mixed again and the absorbance at 595 nm was read using the ELx800 absorbance microplate reader (Biotek ELX800, BioTek Instruments, Inc.). Each assay was repeated at least three times in triplicate. The negative control was supplemented with 100 µl DMSO (Sigma Chemical Co.; without cells). Each sample from the control and treated cells was subtracted from the negative control, and the results were plotted as the mean \pm SEM.

Statistical analysis. Data are expressed as the mean \pm SEM. Statistical analysis was performed using one-way ANOVA followed by the Newman-Keuls test or Tukey's post hoc test for multiple comparisons. P-values <0.05 were considered to indicate statistically significant differences.

Results

The activation of ER α and ER β promotes the migration and invasion of DU-145 cells. The present study analyzed various cellular characteristics of tumor development *in vitro*, using DU-145 cells. At 24 h of treatment E2 (10 nM), the ER α -selective agonist, PPT (10 nM), or the ER β -selective agonist, DPN (10 nM), increased the migration of the DU-145



Figure 1. Effects of E2, the ER α -selective agonist, PPT, and the ER β -selective agonist, DPN, on the migration of the DU-145 cells. (A) Cells, in the same culture plate in different wells, were wounded and then incubated in the absence (C, control) or presence of E2 (10 nM), ER α -selective agonist PPT (10 nM) or ER β -selective agonist DPN (B) for 24 h at 37°C. (B) Cells were also untreated or pre-treated with the ER α -selective antagonist MPP (10 nM), ER β -selective antagonist PHTPP (10 nM) or with both antagonists, MPP (10 nM) and PHTPP (10 nM) for 30 min. Incubation was continued in the presence of E2 (10 nM) for 24 h at 37°C. Wound healing assay was performed as described in the Materials and methods. The results are expressed in relation to the control (C=100%) and plotted (mean ± SEM) from four to five independent experiments, in duplicate (bar graphs). Images (x100 magnification) are representative of four to five independent experiment in duplicate. *P<0.05, significantly different from the control; #P<0.05, significantly different from the MPP + E2, PHTPP + E2, or MPP + PHTPP + E2 groups (determined using ANOVA and Tukey's post hoc test). E2, 17 β -estradiol; ER, estrogen receptor; PPT, 4,4',4"-(4-propyl- (1H)-pyrazole-1,3,5-triyl)trisphenol; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile; MPP, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride; PHTPP, 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol.



Figure 2. Effects of E2, the ER α -selective agonist, PPT, and the ER β -selective agonist, DPN, on the invasion of the DU-145 cells. Cells in culture medium without serum were seeded in ThincertR chambers with polyethylene terephthalate membranes pre-coated with phenol red-free Matrigel. These chambers were placed in 24-well plates containing culture medium with 10% FBS in the lower chamber. Cells in upper chambers of the same culture plate were incubated in the absence (C, control) and the presence of E2 (10 nM), ER α -selective agonist PPT (10 nM) or ER β -selective agonist DPN (10 nM) for 24 h at 37°C. Cell invasion assay was performed as described in the Materials and methods. The results are expressed in relation to control (C=1) and plotted (mean ± SEM) from five to six independent experiments, in duplicate (bar graphs). Images (x200 magnification) are representative of five to six independent experiments performed in duplicate. *P<0.05, significantly different from the control (determined using ANOVA and Tukey's post hoc test). E2, 17 β -estradiol; ER, estrogen receptor; PPT, 4,4',4"-(4-propyl-(1H)-pyrazole-1,3,5-triyl)trisphenol; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile.

cells (2.0-, 1.5- and 1.5-fold, respectively) compared to the control (Fig. 1A), suggesting the involvement of the ERs, ER α and ER β , in this process. Of note, the increase in DU-145 cell migration induced by E2 (10 nM) at 24 h was blocked by the ER α -selective antagonist (MPP, 10 nM), the ER β -selective antagonist (PHTPP, 10 nM) or simultaneous pre-treatment with both MPP (10 nM) and PHTPP (10 nM) (Fig. 1B). Pre-treatment with MPP, PHTPP or both MPP and PHTPP, in the absence of the E2, yielded results similar to those of the control (data not shown).

Treatment with E2 (10 nM), PPT (10 nM) or DPN (10 nM) for 24 h led to an enhancement of the invasion of the DU-145 cells (5-, 3,5- and 4-fold, respectively) (Fig. 2). The increase in DU-145 cell invasion induced by E2 was blocked by simultaneous pre-treatment with both MPP and PHTPP (Fig. S2A), suggesting that ER α and ER β may play a role in the DU-145 cells invasion. To confirm the involvement of these receptors, the DU-145 cells were also untreated or pre-treated with MPP (10 nM) or PHTPP (10 nM) and the incubation was continued in the absence or presence of PPT (10 nM) or DPN (10 nM). The increase in DU-145 cell invasion induced by PPT or DPN was blocked, respectively, by MPP or PHTPP (Fig. S2B and C), confirming that ER α and ER β are upstream receptors regulating this process. Pre-treatment with MPP, PHTPP or simultaneous pre-treatment with both antagonists, in the absence of E2, PPT or DPN, yielded results similar to those of the control (Fig. 1).

The activation of ERa and ER β for 24 h increases the expression of GAL-3 in DU-145 cells. GAL-3 was detected as a single protein band of 31 kDa in the total cell extracts of the PNT1A, PC-3 and DU-145 cells (Fig. 3). The expression of GAL-3 was higher in the DU-145 and PNT1A cells than in

the PC-3 cells (Fig. 3), suggesting that the AR is not involved in the regulation of GAL-3. No difference was observed in the expression of the GAPDH among the three cells, used as protein loading control (Fig. 3). Thus, the androgen-independent prostate cancer cells, DU-145, were used in the analyses of the regulatory effects of the activation of the ERs on the expression of GAL-3.

Treatment of the DU-145 cells with E2 (10 nM) for 4 h or with PPT (10 nM) for 1 and 2 h increased the expression of GAL-3 compared to the control DU-145 cells (untreated cells) (Fig. 4A and B). On the other hand, treatment of the DU-145 cells with DPN (10 nM) for 30 min, 1, 2 and 4 h did not have any marked effects on the expression of GAL-3 compared to the control (Fig. 4C). No difference was observed in the expression of β -tubulin under any of these conditions, used as the protein loading control (Fig. 4).

The localization and expression of GAL-3 were determined using immunofluorescence assays. In the control DU-145 cells (Figs. 5-7 and S3), the immunostaining of GAL-3 was predominantly found in the cytoplasm, although immunostaining in some nuclei was also observed. Treatment of these cells with E2 (10 nM) for 4 h (Figs. 5 and S4) or PPT (10 nM) for 2 and 4 h (Figs. 6 and S4) increased the expression of GAL-3 in the cytoplasm and nuclei. On the other hand, treatment of the DU-145 cells with DPN (10 nM) for 2 and 4 h did not have any marked effects on the expression of GAL-3 compared to the control (Fig. S3).

The effects of treatment of the DU-145 cells with E2 (10 nM) for 2 h were blocked by pre-treatment with MPP (10 nM) and partially blocked by PHTPP (10 nM) (Figs. 5 and S4). The expression of GAL-3 induced by 2 or 4 h of treatment with PPT (10 nM) was blocked by pre-treatment with MPP (10 nM)



Figure 3. Expression of GAL-3 in PNT1A, DU-145 and PC-3 cells. Western blot analysis was performed for the detection of GAL-3 in PNT1A, DU-145 and PC-3 cells, using 50 μ g of protein/lane and antibody specific for GAL-3 (top row) or antibody that recognizes GAPDH (bottom row). The protein sizes of GAL-3 and GAPDH are shown on the right. The data shown are representative of six independent experiments. Results of the densitometric analysis of the western blots were normalized to the respective expression of GAPDH, expressed in arbitrary units and plotted (mean ± SEM) from four independent experiments (bar graph). ^bP>0.05, significantly different from PNT1A cells; ^eP>0.05, significantly different from PC-3 cells (determined using ANOVA and the Newman-Keuls test, n=6). GAL-3, galectin-3.

(Figs. 6 and S4). Treatment with MPP or PHTPP alone did not have any marked effects on the expression of the GAL-3, and the effects were similar to those of the control (data not shown).

It is important to mention that at 24 h of treatment with E2, PPT or DPN, the expression of GAL-3 increased compared to the control (Fig. 7). The analysis of these findings using ImageJ software revealed that treatment with E2, PPT and DPN for 24 h increased the fluorescence intensity of GAL-3 by 25, 39 and 28%, respectively in the whole DU-145 cells compared to the control (Fig. S4). No immunostaining was observed in the negative control, performed in the absence of primary antibodies for GAL-3 (Figs. 5-7 and S2, inserts).

GAL-3 is involved in the migration and invasion of DU-145 cells. To explore the involvement of GAL-3 in the migration and invasion of DU-145 cells, VA03 (a specific inhibitor of GAL-3) was used at 200 μ M (Figs. 8 and 9). Pre-treatment with VA03 inhibited the migration of the DU-145 cells induced by E2 (10 nM) (Fig. 8), PPT (10 nM) or DPN (10 nM) (data not shown). Pre-treatment with VA03 inhibited the invasion of the DU-145 cells induced by E2 (10 nM) or DPN (10 nM) (data not shown). Pre-treatment with VA03 inhibited the invasion of the DU-145 cells induced by E2 (10 nM), PPT (10 nM) or DPN (10 nM) (Fig. 9), indicating the involvement of the complex ER α /GAL-3 and ER β /GAL-3 in the regulation of the migration and invasion of DU-145 cells. Treatment with VA03 alone did not have any marked effects on the migration or invasion of DU-145 cells (Figs. 8 and 9). In addition, treatment with VA03 (20 and 200 μ M) for 24 h did not have any notable effects

on the number and viability of the DU-145 cells compared to the control cells (Fig. S5).

Discussion

The expression of the ERs, ER α and ER β , changes in the different stages of prostate cancer and conflicting findings on the expression, regulation and roles of these receptors in prostate cancer development have been found (45-48). It is recognized that there is wide variability in the sensitivity and specificity of ER β antibodies, which may contribute to the uncertainties surrounding its molecular action and tissue expression. Nelson et al (49) published a study advising about which antibodies are acceptable against ER^β. Using the antibody previously reported (49), the authors previously demonstrated the presence of ERB in PNT1A, PC-3 and DU-145 cells (33). The expression of ERa also was shown in these cells (32,33, Fig. S1). Taken together, these results confirm that the expression of ER α and ER β is higher in the DU-145 cells than in the PNT1A and PC-3 cells, suggesting that distinct androgen-independent mechanisms are involved in the regulation of these receptors. These mechanisms remain to be explored.

The activation of ER α and ER β promoted an increase in the migration and invasion of the DU-145 cells. In the PC-3 cells, the activation of ER β and ER α increased the invasion and anchorage-independent growth of these cells (40,50). The activation of ER β by DPN has also been shown to promote the survival and migration of the CPEC cell line (cells expressing prostate-specific antigens), established from patients with prostate cancer (51). Furthermore, the expression of the ER β 5 (ERß splice variant) in PC-3 cells increased the cell migration, and the expression of ER β 2 and ER β 5 increased the invasion, but did not affect the proliferation of the cells (52). It is important to emphasize that $ER\beta$ splice variants do not bind ligands (53), although dimers may be observed with ER β (ER β 1). ER α / β heterodimers formation was observed in DU-145 cells (33). Taken together, these results support an oncogenic role for ER α and ER β in DU-145 cells.

In the present study, the activation of ER α by PPT, but not ER β by DPN, for 2 h increased the expression of the GAL-3 in DU-145 cells. However, at 24 h of treatment, DPN also increased the expression of GAL-3 compared to the control (basal level of cellular function). Taken together, these results indicate that ER α and ER β are involved in the regulation of the expression of the GAL-3. The promoter region of the human LGALS3 gene contains several regulatory elements: Five putative Sp1 binding sites (GC boxes), five cAMP-dependent response element motifs, four AP-1- and one AP-4-like sites, two NF-kB-like sites, one sis-inducible element and a consensus basic helix-loop-helix core sequence (27,54). Several of these transcription factors interact with ERs (30) and induce the genomic signaling. Furthermore, ERs also activate two major pathways regulating cell proliferation and survival, SRC/MAPK and PI3K/AKT pathways (rapid or non-genomic signaling) (31). Indeed, in the DU-145 cells, the activation of ER α and ER β can activate rapid cell signaling pathways in these cells, including an increase in the phosphorylation of ERK1/2 (33). Thus, the transcriptional regulation (genomic activity) combined with direct activation



Figure 4. Effects of E2, the ER α -selective agonist, PPT, and the ER β -selective agonist, DPN, on the expression of GAL-3 in DU-145 cells. Cells were incubated in the absence (control, C) or presence of (A) E2 (10 nM), (B) ER α -selective agonist PPT (10 nM) or (C) ER β -selective agonist DPN (10 nM) for different periods of time at 37°C. Western blot analysis for the detection of the GAL-3 in PNT1A, DU-145 and PC-3 cells was performed as described in the Materials and methods, using 20 μ g of protein/lane and antibody specific for GAL-3 (top row) or antibody that recognizes β -tubulin (bottom row). The protein sizes of GAL-3 and β -tubulin proteins are shown at the right. The data shown are representative of three to five independent experiments. Results of the densitometric analysis of the western blots were normalized to the respective expression of β -tubulin, expressed in relation to the control (C=1) and plotted (mean \pm SEM) from three to five independent experiments (bar graphs). *P<0.05, significantly different from the control (determined using ANOVA and Tukey's post hoc test). E2, 17 β -estradiol; ER, estrogen receptor; PPT, 4,4',4"-(4-propyl-(1H)-pyrazole-1,3,5-triyl)trisphenol; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile.

of signaling cascades (non-genomic activity) by ERs may be involved in the expression of the GAL-3 in DU-145 cells. These mechanisms remain to be explored in DU-145 and other prostate cancer cells.

It is important to mention that E2 and progesterone have also been shown to induce the upregulation of GAL-3 expression in RL95-2 epithelial cells from the human endometrium, which in turn decreases the apoptotic rate of these cells (55). Furthermore, in a preliminary study, ER α and GAL-3 were shown as markers of aggressiveness and prognosis in prolactinoma (56). In the present study, in the DU-145 cells (control), the immunostaining of GAL-3 was predominantly found in the cytoplasm, although immunostaining in some nuclei was also observed. Treatment of these cells for 24 h with E2, PPT or DPN increased the expression of the GAL-3 in the cytoplasm and nuclei. Several cytosolic molecules were identified, as GAL-3 ligands and these proteins are involved in the regulation of cell proliferation, differentiation, survival and death (27). In addition, GAL-3 interacts with nuclear factors to regulate the expression of multiple genes related to tumor plasticity (57). For example, GAL-3 interacts with the factor activator protein



Figure 5. Effects of treatment with E2 for 4 h on the expression and localization of the GAL-3 in DU-145 cells. Cells were incubated in the absence (control, C) or presence of E2 (10 nM) for 4 h at 37°C. Cells were also untreated or pre-treated with the ER α -selective antagonist, MPP, (10 nM) or the ER β -selective antagonist, PHTPP (10 nM), for 30 min. Incubation was continued in the absence or presence of E2 (10 nM) for 4 h at 37°C. Immunostaining for GAL-3 (red) was detected as described in the Materials and methods. Nuclei were stained with DAPI (blue). Negative control was performed in the absence of primary antibody (insert). Scale bars, 20 μ m. Images are representative of two independent experiments. E2, 17 β -estradiol; GAL-3, galectin-3; ER, estrogen receptor; MPP, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride; PHTPP, 4-[2-phenyl-5,7-bis(trifluorome thyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol.

1 (AP1); this GAL-3-AP1 complex binds to the matrix metalloproteinase-1 promoter and mediates its transcription, which facilitates the migration and invasion of melanoma cells (58). Herein, to explore the involvement of GAL-3 in migration and invasion of the DU-145 cells induced by activation of ERs, VA03 (a specific inhibitor of GAL-3) was used. Pre-treatment



Figure 6. Effects of the ER α -selective agonist, PPT, for 2 and 4 h on the expression and localization of GAL-3 in DU-145 cells. Cells were incubated in the absence (control, C) or presence of ER α -selective agonist PPT (10 nM) for 2 h and 4 h at 37°C. Cells were also untreated or pre-treated with ER α -selective antagonist MPP (10 nM) for 30 min. Incubation was continued in the absence or presence of PPT (10 nM) for 4 h at 37°C. Immunostaining for GAL-3 (red) was detected as described in the Materials and methods. Nuclei were stained with DAPI (blue). Negative control was performed in the absence of primary antibody (insert). Scale bars, 20 μ m. Images are representative of four independent experiments. ER, estrogen receptor; PPT, 4,4',4''-(4-propyl-(1H)-pyrazole-1,3,5-triyl)trisphenol; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile; GAL-3, galectin-3; MPP, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy) phenol]-1H-pyrazole dihydrochloride.



Figure 7. Effects of treatment with E2, the ER α -selective agonist, PPT, or the ER β -selective agonist, DPN, for 24 h on the expression and localization of the GAL-3 in DU-145 cells. Cells were incubated in the absence (control, C) or presence of E2 (10 nM), ER α -selective agonist PPT (10 nM) or ER β -selective agonist DPN (10 nM) for 24 h at 37°C. Immunostaining for GAL-3 (red) was detected as described in the Materials and methods. Nuclei were stained with DAPI (blue). Negative control was performed in the absence of primary antibody (insert). Scale bars, 20 μ m. Images are representative of two independent experiments. E2, 17 β -estradiol; ER, estrogen receptor; GAL-3, galectin-3; PPT, 4,4',4"-(4-propyl-(1H)-pyrazole-1,3,5-triyl)trisphenol; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile.



Figure 8. Effects of the specific inhibitor of GAL-3 (VA03) on the migration of DU-145 cells induced by E2. Cells, in the same culture plate in different wells, were wounded and then incubated in the absence (C, control) or presence of E2 (10 nM) for 24 h at 37°C. Cells were also untreated or pretreated with a specific inhibitor of GAL-3 (VA03, 200 μ M) for 30 min. Incubation was continued in the absence or presence of E2 for 24 h at 37°C. Wound healing assay was performed as described in the Materials and methods. Results are expressed in relation to control (C=100%) and plotted (mean ± SEM) from three independent experiments, in duplicate (bar graph). Images (x100 magnification) are representative of four different experiments. *P<0.05, significantly different from the VA03 + E2 group (determined using ANOVA and Tukey's post hoc test). E2, 17β-estradiol; GAL-3, galectin-3.

with VA03 inhibited the migration and invasion of the DU-145 cells induced by the activation of ERs, indicating that the complex ER α /GAL-3 and ER β /GAL-3 plays a role in these processes.

The regulation of the expression of GAL-3 by ERs in other prostate cancer cell lines and in different stages of the prostate cancer remains to be explored. It is important to mention that in PC-3 cells, the activation of ERs induces an increase of the active non-phosphorylated β -catenin, and these proteins are involved in the proliferation, migration, invasion and colony formation of these cells (34,40). It has been shown that β -catenin can co-localize with GAL-3 in other cancer cells (59). Taken together, these results indicate that the process is more complex that should be addressed in near future using different prostate cancer cell lines and prostate cancer tissues.

GAL-3 inhibitors have shown promising results in preclinical studies (22,60). Notably, TFD100, a GAL3-binding glycopeptide, has been shown to block GAL3-induced T-cell apoptosis, and to impair angiogenesis and metastasis in xeno-graft models (22). In addition, G3-C12-modified copolymers (targeting GAL-3) have been shown to improve the antitumor activity of 5-fluorouracil in prostate cancer xenograft mouse models (60), and modified citrus pectin, a natural dietary

fiber soluble polysaccharide, that plays a role as an antagonist of extracellular GAL-3 (61), sensitizes prostate cancer cells to radiotherapy, and reduces their migratory and invasive capabilities (62).

Overall, although GAL-3 levels in the tumor are decreased during prostate cancer progression, the cytoplasmic overexpression of this protein has been reported during progression, and *in vitro* data and preclinical xenograft models have shown that strategies targeting GAL-3 may be effective in impairing prostate cancer progression *in vivo*. However, several important challenges need to be addressed before anti-GAL-based strategies can be translated to clinical settings.

In conclusion, the activation of both ERs increases the expression and signaling of the GAL-3, and induces the migration and invasion of DU-145 cells. The findings of the present study provide novel insight into the signatures and molecular mechanisms of ER α and ER β in DU-145 cells.

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Figure 9. Effects of a specific inhibitor of GAL-3 (VA03) on the invasion of DU-145 cells induced by E2, PPT and DPN. Cells in culture medium without serum were seeded in ThincertR chambers with polyethylene terephthalate membranes pre-coated with phenol red-free Matrigel. These chambers were placed in 24-well plates containing culture medium with 10% FBS in the lower chamber. Cells in the upper chambers of the same culture plate were incubated in the absence (C, control) or presence of (A) E2 (10 nM), (B) the ER α -selective agonist, PPT (10 nM), or (C) the ER β -selective agonist, DPN (10 nM), for 24 h at 37°C. Cells were also untreated or pre-treated with a specific inhibitor of GAL-3 (VA03, 200 μ M) for 30 min. Incubation was continued in the absence or presence of (A) E2, (B) PPT or (C) DPN for 24 h at 37°C. Cell invasion analysis was performed as described in the Materials and methods. Results are expressed in relation to the control (C=1) and plotted (mean ± SEM) from three to four independent experiments, in duplicate (bar graphs). Images (x200 magnification) are representative of three to four different experiments. *P<0.05, significantly different from the control; #P<0.05, significantly different from the control; #P<0.05, significantly different from the vA03 + E2; VA03 + PPT, VA03 + DPN and VA03 groups (determined using ANOVA and Tukey's post hoc test). GAL-3, galectin-3; E2, 17 β -estradiol; ER, estrogen receptor; PPT, 4,4',4''-(4-propyl-(1H)-pyrazole-1,3,5-triv)]trisphenol; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DSS conceived and designed the study, collected the data, performed the analysis and wrote the manuscript. CM and RPC contributed to data analysis. CMV conceived and designed the study, contributed data or analysis tools and performed the analyses. VLC performed the synthesis of the galectin-3 inhibitor. CSP conceived and designed the study, and performed the revision of the manuscript. DSS and CSP confirm the authenticity of all the raw data. All authors have read and approved the submitted version of the manuscript.

Ethics approval and consent to participate

All experimental procedures were approved by the Research Ethical Committee at EPM-UNIFESP (#3527220917).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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