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ORIGINAL PAPER

STEAP1 is over-expressed in breast cancer and down-regulated by 17β-estradiol in MCF-7 cells and in the rat mammary gland

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Abstract Six transmembrane epithelial antigen of the prostate 1 (STEAP1) was identified as a prostate-specific cell-surface antigen over-expressed in prostate cancer, and in human cancer cell lines obtained from several other tissues. Its cell surface location in all tumor types analyzed so far, and its absence in most vital organs in humans, turned STEAP1 into a potential target for anti-tumor immunotherapy. This study provides experimental evidence that STEAP1 is also over-expressed in human breast cancer cases, and in normal breast tissue adjacent to breast tumors, where it is localized in the cell membrane of epithelial cells. It is also demonstrated that STEAP1 transcription correlates negatively with estrogen receptor (ER) immunoreactivity, and positively with tumor grading in breast cancer cases. As estrogens are involved in breast cancer onset and progression, the response of STEAP1 to 17β-estradiol (E_2) was investigated in the mammary gland of rats, and in the human breast cancer cell line, MCF-7. These experiments demonstrated that STEAP1 is down-regulated by E_2 in both models. The mechanisms underlying the STEAP1 response to E_2 in vitro were further investigated in MCF-7 cells, and the results obtained suggest an effect mediated by the membrane-bound ERα (mbERα).

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Introduction

Six transmembrane epithelial antigen of the prostate 1 (STEAP1) was first identified as a prostate-specific cell-surface antigen, over-expressed in human prostate cancer, and in the spontaneous transgenic mouse model of prostate cancer, by suppressive subtractive hybridization [1, 2]. It is also expressed in several human cancer cell lines obtained from pancreas, bladder, gastrointestinal tract, testis, ovary, cervix, Ewing sarcoma, and melanomas, with little or no expression in vital organs. Recent data showed that STEAP1 is involved in intercellular communication between adjacent cells in culture, and that it seems to favor tumor development [3]. Its structure prediction, and location at cell–cell junctions, suggests that STEAP1 must be a channel, or a transport protein [1, 4]. Its cell surface location in all tumor types analyzed so far, and its absence in most vital organs in humans, turned STEAP1 into a potential target for anti-tumor immunotherapy. Several studies were conducted to ascertain if STEAP1 peptide epitopes could trigger immune responses against STEAP1 expressing tumor cells, with promising results: STEAP1 peptide epitopes can be used to stimulate cytotoxic T and helper T lymphocytes, which kill STEAP1 expressing tumor cells [4–7]. Moreover, STEAP1 vaccination can extend significantly the survival rate of mice bearing tumors [8]. Therefore, STEAP1 is gaining support as a target for anti-tumor immunotherapy, which may be used against a variety of tumors from different types, and particularly in prostate cancer, where its levels of expression are 5- to 10-fold higher than in tumors from other tissues [1].

Alike prostate cancer in men, breast cancer is the most common cancer type in women, and it is still responsible for high rates of mortality and morbidity, particularly in western countries. Targeted tumor-specific therapies against breast cancer are still awaited with expectancy. Although STEAP1 expression has been found in the MDA-MB-435s, and MCF-7 breast cancer cell lines [1, 4], no studies have focused on the expression of STEAP1 in the mammary gland, thereafter. This study aimed at fulfilling this gap, thereby investigating the expression of STEAP1 in human breast cancer cases, and in the human breast cancer MCF-7 cell line. STEAP1 expression was also studied in the rat mammary gland to evaluate the suitability of this animal model for future studies on STEAP1 function and regulation. Moreover, as estrogens are involved in breast cancer onset and progression, the response of STEAP1 to 17β-estradiol (E_2) was analyzed in vivo and in vitro by real-time PCR.

Results

STEAP1 is expressed in the cell membrane of mammary gland epithelial cells

The presence of STEAP1 transcripts in rat mammary gland, human breast cancer cases, and in the MCF-7 cell line was initially investigated by RT-PCR using STEAP1-specific primers (data not shown) and confirmed by sequencing the amplicons obtained, which were 100% identical to the rat (XM_001067555.1), and human (NM_012449) STEAP1 sequences, available in GenBank.

The cellular localization of STEAP1 protein in normal human breast sections (Fig. 1a), and in breast cancer sections (Fig. 1b, infiltrating ductal carcinoma; Fig. 1c, medullary carcinoma), was analyzed by immunohistochemistry (IHC). Staining was detected in glandular epithelial cells and in fibroblasts. No staining was detected when incubation with the primary antibody was omitted in the procedure (Fig. 1d, normal breast; Fig. 1e, ductal infiltrating carcinoma; and Fig. 1f, medullary carcinoma). Moreover, double labeling experiments in breast cancer sections of infiltrating ductal carcinomas, using the STEAP1 antibody (Fig. 1g), and wheat germ agglutinin, which labels the cell membrane (Fig. 1h), show that STEAP1 localization is restricted to the cell membrane (Fig. 1i).

STEAP1 is over-expressed in breast cancer cases

Tissue sections of normal breast, and breast tumors, were classified for STEAP1 immunoreactivity according to a score scale established for this parameter, which takes into account intensity of the signal and percentage of stained cells (Table 1). In normal breast samples ($n = 2$), STEAP1

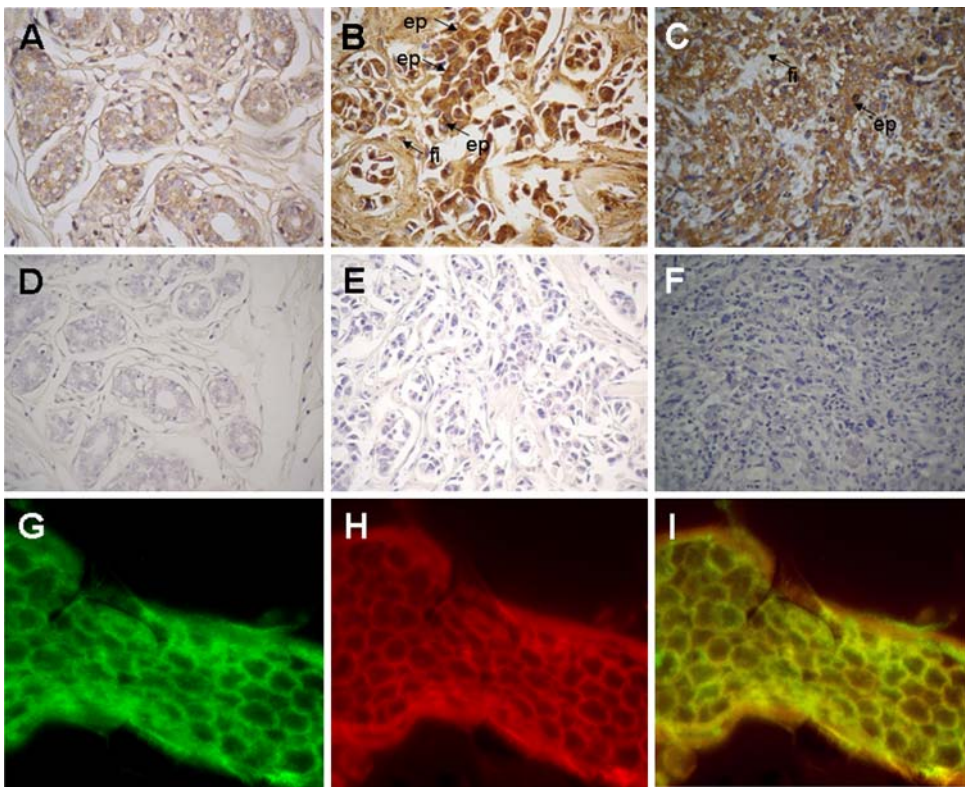

Fig. 1 Immunohistochemical analysis of STEAP1 protein in **a** human normal breast ($n = 7$), **b** infiltrating ductal carcinoma ($n = 49$), and **c** medullary carcinoma ($n = 5$). Slides were incubated with an anti-STEAP1 (1:100) polyclonal antibody (630×) (**a**–**c**); negative controls without anti-STEAP1 polyclonal antibody (400×) were carried out for human normal breast (**d**), in infiltrating ductal carcinoma (**e**), and in medullary carcinoma (**f**). *Arrows* indicate strong staining of epithelial cells (*ep*) and some fibroblasts (*fi*). Dual labeling of STEAP1 (**g**–**i**), and cell membrane using the wheat germ agglutinin, was carried out in infiltrating ductal carcinoma sections: STEAP1 (**g**), cell membrane (**h**); localization of STEAP1 in the cell membrane is shown in the merged figure (**i**)

Table 1 STEAP1 immunoreactivity in normal human breast, benign breast lesions, and in breast cancer cases

Histological diagnoses	Grade	Number of samples	STEAP1 immunoreactivity			*P* value (χ^2 test)
			1	2	3	
Non-malignant tissue[a]		7	71% (5)	29% (2)	0%	<0.01
Normal		2	100% (2)	0%	0%	
Fibroadenoma		5	60% (3)	40% (2)	0%	
Malignant tissue		59	17% (10)	24% (14)	59% (35)	
Infiltrating ductal carcinoma		49	12% (6)	21% (10)	67% (33)	<0.01
	I	22	18% (4)	18% (4)	64% (14)	
	II	22	9% (2)	23% (5)	68% (15)	
	III	5	0%	20% (1)	80% (4)	
Medullary		5	0%	60% (3)	40% (2)	N.A.
	II	3	0%	100% (3)	0%	
	III	2	0%	0%	100% (2)	
In situ ductal carcinoma		5	40% (2)	60% (3)		N.A.
	I	1	100% (1)	0%	0%	
	II	4	25% (1)	75% (3)	0%	

The number of samples in each group are indicated in parentheses. The STEAP immunoreactivity in infiltrating ductal carcinoma is associated with tumor grading

1 low expression, *2* moderate expression, *3* high expression, *N.A.* not applicable

[a] The STEAP immunoreactivity in non-malignant and malignant tissues is significantly different

staining was low. In benign lesions (fibroadenomas, $n = 5$), STEAP1 staining ranged from low (60%) to moderate (40%). In malignant tumors ($n = 59$), STEAP1 staining was low in 17%, moderate in 24%, and high in 59% of the samples analyzed. When normal or benign lesion samples ($n = 7$) were compared to malignant tumors ($n = 59$), STEAP1 was significantly over-expressed in the latter ($P = 0.005$, χ^2 test), indicating a positive correlation with tumor grading in IDC. Examples of STEAP1 staining in normal breast, and in breast cancer samples, are given in Fig. 1a, and 1b and c, respectively, where breast cancer tissue exhibits much stronger staining than normal breast.

Real-time PCR analysis using STEAP1-specific primers (Table 2), and cDNA from normal breast tissue ($n = 5$), infiltrating ductal carcinomas ($n = 42$), and normal tissue adjacent to these tumors ($n = 7$), showed that STEAP1 mRNA is over-expressed in breast tumors compared to normal breast tissue ($P < 0.05$, one-way ANOVA followed

Table 2 Details of specific primers used in conventional or in real-time PCR for amplification of human and rat STEAP1 and β_2M, rat cyc A and human GAPDH

Oligo name	Sequence	Exon location	Concentration[a] (nM)	Amplicon size (bp)
rSTEAP_849[b]	5′ GCT GCT CAA CTG GGC TTA CAA AC 3′	2	200	157
rSTEAP_1006[b]	5′ AGT CGC TCA CAG ATG GGA TAG ATG 3′	3		
hSTEAP_619[b]	5′ GGC GAT CCT ACA GAT ACA AGT TGC 3′	3	50	128
hSTEAP_747[b]	5′ CCA ATC CCA CAA TTC CCA GAG AC 3′	4		
rB2M_24	5′ CCG TGA TCT TTC TGG TGC TTG TC 3′	1	100	149
rB2M_173	5′ CTA TCT GAG GTG GGT GGA ACT GAG 3′	2		
rCycA_393	5′ CAA GAC TGA GTG GCT GGA TGG 3′	4	100	162
rCycA_555	5′ GCC CGC AAG TCA AAG AAA TTA GAG 3′	5		
hGAPDH_74	5′ CGC CCG CAG CCG ACA CAT C 3′	2	300	75
hGAPDH_149	5′ CGC CCA ATA CAA TCC G 3′	3		
hB2M_347	5′ ATG AGT ATG CCT GCC GTG TG 3′	2	300	92
hB2M_439	5′ CAA ACC TCC ATG ATG CTG CTT AC 3′	3		

[a] Final concentration in PCR reactions

[b] Primers used in both conventional and real-time PCR

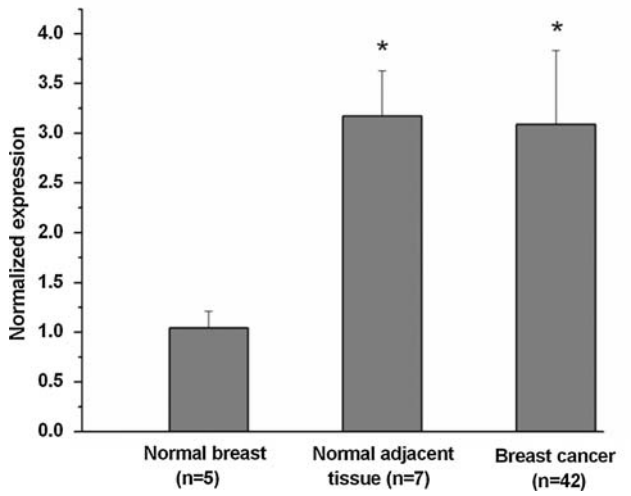

Fig. 2 Comparison of STEAP1 expression in normal breast ($n = 5$), in normal tissue surrounding breast cancer ($n = 7$), and in infiltrating ductal carcinomas ($n = 42$). STEAP1 gene expression was determined by real-time PCR and normalized to hGAPDH and hB2M expression. *$P < 0.05$ (one-way ANOVA followed by Dunnett's test)

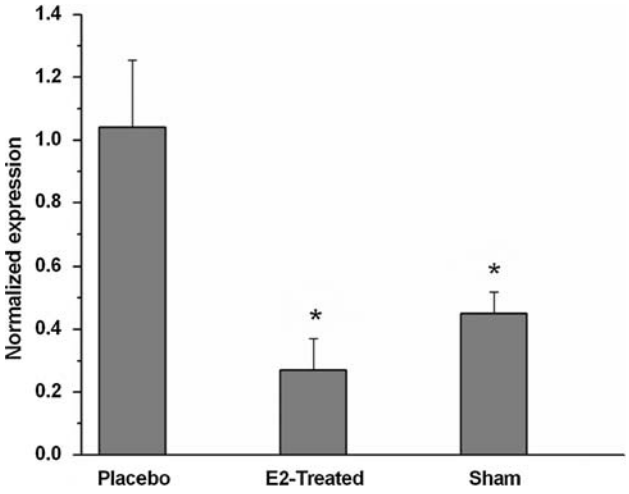

Fig. 3 Comparison of STEAP1 expression in rat mammary gland in ovariectomized rats administered E_2 (E_2-treated) or vehicle alone (placebo) for 7 days. Sham-operated animals were also included in the study. STEAP1 gene expression was determined by real-time PCR and normalized to rCycA and rB2M expression. *$P < 0.01$ (one-way ANOVA followed by Dunnett's test)

by Dunnett's test) (Fig. 2). Remarkably, normal tissue adjacent to breast tumors of the same patient did also overexpress STEAP1 at levels similar to those found in tumors ($P = 0.005$).

To determine if STEAP1 mRNA levels correlate with tumor grading and estrogen receptor (ER) positivity, the χ^2 test (SPSS, version 15) was applied to the histological data collected from these tumors (ER immunoreactivity and grading classification), and the score scale established for STEAP1 mRNA expression described in the section "Materials and methods." A significant negative correlation (Table 3) ($P < 0.01$) between STEAP1 mRNA expression and ER positivity was found. A statistically significant positive correlation ($P < 0.01$) was also found between STEAP1 mRNA expression and the histological grade of tumors.

Table 3 Comparative analysis of STEAP1 mRNA expression with ER immunoreactivity, and histological grade of breast cancer

Variable	Number of samples	Percentage of samples showing the STEAP1 mRNA expression scores			
		1	2	3	*P* value (χ^2 test)
ER					<0.01
Negative	7	0%	0%	100% (7)	
Positive	35	46% (16)	31% (11)	23% (8)	
Grade					<0.01
I	3	67% (2)	33% (1)	0%	
II	19	48% (9)	26% (5)	26% (5)	
III	20	25% (5)	25% (5)	50% (10)	

The number of samples in each group are indicated in parentheses

1 low expression, *2* moderate expression, *3* high expression

17β-estradiol down-regulates STEAP1 expression in the mammary gland

In vivo studies

The response of STEAP1 transcription to E_2 in rat mammary gland was also analyzed by real-time PCR in E_2-treated, placebos and in sham-operated animals. Placebos contained approximately 3.5-fold higher levels of STEAP1 transcripts than E_2-treated ($P = 0.009$) or sham animals. E_2 treatment nearly restored STEAP1 expression to the levels of sham animals, in which half of the expression of placebos ($P = 0.029$) was detected (Fig. 3).

In vitro studies

The response of STEAP1 transcription to E_2 in MCF-7 cells was analyzed by real-time PCR. A dose–response/time-course experiment was carried out using three different concentrations of E_2 (0, 1, and 10 nM), and three incubation times for each hormone dosage (6, 12, and 24 h) (Fig. 4). At every time point, STEAP1 expression was significantly reduced compared to controls ($P < 0.05$), but in a dose-independent manner. Shorter exposure incubations (6 h) were more effective in reducing STEAP1 expression. In order to elucidate the mechanism underlying the response of STEAP1 to E_2, MCF-7 cells were exposed

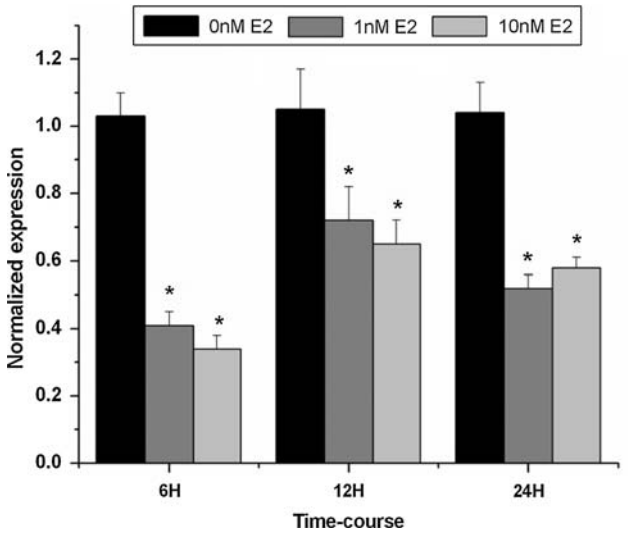

Fig. 4 Analysis of STEAP1 transcription in response to E_2 stimulation, in human MCF-7 cells. MCF-7 cells were exposed to E_2 (0, 1, and 10 nM), and three incubation times for each hormone dosage (6, 12, and 24 h). STEAP1 expression was determined by real-time PCR and normalized to hGAPDH and hB2M expression. *Statistically significant differences between controls and E_2 treated cells ($P < 0.01$; one-way ANOVA followed by Dunnett's test)

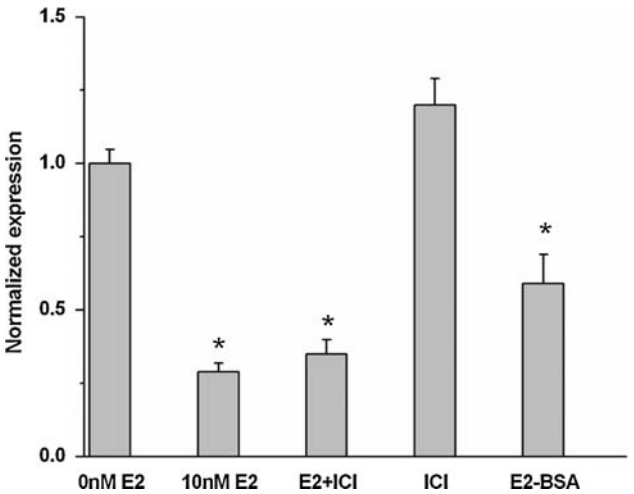

Fig. 5 STEAP1 transcription in response to E_2, ICI 182,780, and E_2-BSA in human MCF-7 cells. STEAP1 gene expression was determined by real-time PCR and normalized to hGAPDH and hB2M expression. *$P < 0.01$ (one-way ANOVA followed by Dunnett's test in relation to 0 nM E_2)

to 10 nM E_2 (alone or combined with ICI 182,780), ICI 182,780 alone, and E_2-BSA, during 6 h. STEAP1 expression was assessed by real-time PCR (Fig. 5). The down-regulatory effect of E_2 on STEAP1 expression was not abrogated in the presence of ICI 182,780, and E_2 conjugated to BSA ($P = 0.01$, Dunnett's test) reproduced the effect of E_2 alone.

Discussion

In this report, we provide experimental evidence that STEAP1 is over-expressed in human breast cancer epithelial cells. It is also expressed in rat mammary gland, and in the human breast cancer cell line MCF-7, which may be used as suitable in vivo and in vitro models to study STEAP1 regulation, respectively.

IHC analysis showed that STEAP1 protein is present in epithelial cells, and in fibroblasts of normal breast, and at much higher levels in breast cancer cases. Its localization, which is restricted to the cell membrane of epithelial cells, suggests that, like in prostate, STEAP1 can also be used as a cell surface antigen for the development of breast cancer immunotherapy. The higher levels of STEAP1 protein found in human breast tumors in comparison with normal breast led us to investigate the levels of STEAP1 transcription in normal breast, breast cancer samples, and in normal epithelial tissue adjacent to breast tumors, obtained from the same patients. A strong and significant indication that STEAP1 transcription is higher in breast cancer samples than in normal breast, concurrent with IHC data, was achieved using this approach. In addition, a positive correlation was found between STEAP1 mRNA expression and tumor grading, suggesting that STEAP1 mRNA expression increases in more aggressive tumors.

Interestingly, there was no difference in the STEAP1 mRNA expression between infiltrative ductal carcinoma and normal epithelial tissue adjacent to these tumors, suggesting that an increase in STEAP1 may anticipate tumor development or invasiveness. Although in this study we have a low number of samples of in situ ductal carcinoma, it seems that STEAP1 immunoreactivity increases in grade II non-invasive carcinomas, and it is actually higher in IDC, which reinforces its possible involvement in tissue invasiveness. Several studies have shown similar results for cytochrome P450 [9–11], IGF-2 [12], and MYC oncogene [13]; that is, the expression of these genes is similar between breast tumors and surrounding normal breast. Also, in normal tissue adjacent to breast cancer, DNA amplifications occur at several loci, including chromosome 7q [14–16], suggesting an early involvement of this loci, where the STEAP1 gene is also located [1] in breast carcinogenesis.

STEAP1 mediates the transfer of small molecules between adjacent PC-3 cells in culture, and monoclonal antibodies against STEAP1 inhibit the growth of human prostate and bladder tumor xenografts in vivo [3]. So, it is liable to speculate that high levels of STEAP1 in breast tumors, and in the surrounding tissue, may provide more suitable conditions for tumor development. However, to better understand the relationship between STEAP1 expression and onset/progression of breast cancer, analyses

of many more samples of benign lesions, and in situ ductal carcinomas, that is, non-invasive breast lesions, are required.

STEAP1 is down-regulated by E_2 both in rat mammary gland and in MCF-7 cells. Suppression of ovarian function, a widely recognized adjuvant therapy for pre-menopausal patients with early breast cancer, despite its well-documented benefits in breast cancer therapy [17], may therefore increase STEAP1 expression. On the other hand, increased STEAP1 in patients subjected to suppression of ovarian function may be useful as a target for T-cell-based immunotherapy, which has already been used with promising results against prostate, colon, bladder, Ewings sarcoma, and melanoma tumor cells [4, 6, 8]. The balance between the beneficial effects of chemical, or chirurgical ablation of ovarian function, and the consequences arising from the resulting increase in STEAP1 levels, which may promote cell proliferation [3], should be addressed in future studies. However, as STEAP1 expression correlates negatively with ER, it is more likely that STEAP1 may become a useful target for tumor immunotherapy in ER-negative tumors.

Finally, we looked at the mechanisms underlying the STEAP1 response to E_2 in MCF-7 cells. The presence of ICI 182,780 did not abrogate the down-regulatory effect of E_2 on STEAP1 expression, which could still be observed when E_2-BSA was used. These data suggest an effect mediated by mbERα, which has been previously described in this cell line [18, 19], and not through nuclear ER. mbERα mediated non-genomic signalling, induces cAMP and ERK1 and ERK2 downstream signalling cascades, which regulate MCF-7 proliferation upon E_2 stimulation [20, 21]. Therefore, it would be of interest to determine if the cAMP or the ERK1/2 signalling pathways can induce STEAP1 transcription.

Methods

Animals, cell lines, and human tissues

Female rats (*Rattus norvegicus*) were housed under a 12 h light, 12 h dark photoperiod, with food and water available ad libitum during the course of the experiment. Animals were handled in compliance with the NIH guidelines and the National and European Union guidelines for the care and handling of laboratory animals (Directive 86\609\EEC).

The Human breast cancer cell line MCF-7 was purchased from the European Collection of Cell Cultures (Ref. 86012803).

Breast tumor cases (infiltrating ductal carcinoma, $n = 42$), normal breast (normal tissue adjacent to benign lesions, $n = 4$, and normal breast purchased from Clontech, $n = 1$), and normal tissue adjacent to breast cancer (composed of mammary epithelium, $n = 7$) were obtained at the Salamanca University Hospital from patients with breast cancer who did not receive preoperative chemotherapy nor any other treatment. All tissue samples were obtained under informed consent of the patients and according to the local ethical committee guidelines. These samples were frozen at −80°C immediately after excision for RNA extraction, or fixed in 10% formalin for histopathology analysis. These samples were graded (I–III) according to tubule formation, mitotic index, and nuclear pleomorfism as previously proposed by Elston and Ellis [22]. ER immunoreactivity was assessed using the following criteria: less than 10% of nuclei immunostained-negative and above 11% of nuclei immunostained-positive.

Human normal breast (2 μm, $n = 1$), infiltrating ductal carcinoma (2 μm, $n = 9$), in situ ductal carcinoma ($n = 2$), and fibroadenomas (2 μm, $n = 5$) tissue sections were obtained from Centro Hospitalar Cova da Beira (CHCB) under informed consent of the patients and according to the local ethical committee guidelines. The Max Array Human Breast Carcinoma tissue slide containing samples of normal breast ($n = 1$) and breast cancer sections of different types, infiltrating ductal carcinoma ($n = 40$), ductal in situ carcinoma ($n = 3$), and medullary carcinoma ($n = 5$) (Zymed, Ref. 75-4043), was purchased from Invitrogen (Hercules, USA) and used in IHC.

Hormonal stimulation experiments

In vivo studies

Adult female rats (±3 months old) were ovariectomized ($n = 10$) under anesthesia (Clorketam1000, Vétoquinol, Lure, France) following standard procedures, and 5 weeks after surgery they were either implanted with an Alzet mini-osmotic pump (Charles River Laboratories, Model 1007D, Durect™, Barcelona, Spain) delivering 400 μg/kg/day of E_2 (Sigma, Saint Louis, USA) ($n = 5$) or vehicle alone (0.5% ethanol:99.5% polypropyleneglycol, Aldrich, Saint Louis, USA, $n = 5$) for 7 days. Sham-operated animals ($n = 5$) were also included in the experiment, but not implanted. After treatment, rats were sacrificed under anesthesia. Mammary glands were excised, frozen in liquid nitrogen, and stored at −80°C, or fixed in 10% formalin for IHC.

In vitro studies

Human breast cancer MCF-7 cells were cultured in D-MEM medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin/

streptomycin). Approximately 4×10^5 cells were seeded in 6 multi-well plates. Before stimulation, cells were grown in phenol red-free and serum-free medium for 2 days. Then, cells were exposed to three different concentrations of E_2 (0, 1, and 10 nM in vehicle; 0.5% ethanol: 99.5% sterile water), and to four incubation times for each hormone dosage (6, 12, and 24 h). After adjusting time exposure to 6 h, and dosage to 10 nM, the assay was repeated using 0 and 10 nM E_2, 10 nM ICI 182,780 (Tocris Cookson, Bristol, U.K.), 10 nM E_2 plus 10 nM ICI 182,780, and 10 nM E_2 conjugated to BSA (E_2-BSA). ICI 182,780 was added 1 h before stimulation with E_2. Assays were carried out in hexaplicate.

Total RNA extraction and cDNA synthesis

Total RNA from rat mammary gland, MCF-7 cells, and human biopsies was extracted in TRI reagent (Sigma) according to the manufacturer's instructions. In order to assess the quantity and integrity of total RNA, its optical density was determined (Pharmacia Biotech, Ultrospec 3000, Denmark), and RNA extracts were inspected by agarose gel electrophoresis. Total RNA (2 μg) was denatured for 5 min at 65°C in a reaction containing 500 μM deoxynucleotide triphosphates (Amersham, Uppsala, Sweden) and 250 ng of random primers (Invitrogen). Reverse transcription was carried out at 37°C for 60 min in a 20 μl reaction containing reverse transcriptase buffer (50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$, and 0.1 M DTT), 10 mM DTT, 60U of RNaseOUT (Invitrogen), and 200U of M-MLV RT (Invitrogen). Reactions were stopped at 75°C for 15 min.

Cloning of STEAP1 cDNA from rat mammary gland, human breast biopsies, and MCF-7 cells

An RT-PCR reaction was carried out using 1 μl of cDNA synthesized from rat mammary gland, human breast cancer samples, and MCF-7 cells in a 25 μl reaction containing 1× Taq DNA polymerase buffer (20 mM Tris–HCl and 50 mM KCl), 500 μM deoxynucleotide triphosphates (Amersham), 3.0 mM of $MgCl_2$ (Promega, Madison, USA), 300 nM of each primer, and 0.125U of Taq DNA polymerase (Promega). One pair of specific primers for rat STEAP1 and one pair of specific primers for human STEAP1 (Table 2) were used to amplify fragments of 157 and 128 bp, respectively. After an initial step at 95°C to denature the cDNA, the cycling conditions used were the following: 35 cycles consisting of denaturation at 95°C for 20 s, annealing at 60 or 64°C (for rat and human STEAP1, respectively) for 30 s, and extension at 72°C for 20 s. All PCR products were cloned in pGEM-T easy vector (Promega) and sequenced (Stabvida, Oeiras, Portugal) to confirm the identity of the amplicons.

Immunohistochemistry

Formalin-fixed, paraffin-embedded human breast cancer and normal breast tissue sections were deparaffinized with xylene and rehydrated using graded ethanol series. Sections were incubated in 0.3% hydrogen peroxide for 5 min to inactivate the endogenous peroxidase. Non-specific protein binding was eliminated by incubation with 5% normal goat serum (Santa Cruz Biotechnology, Santa Cruz, USA) for 1 h at room temperature. Sections were then incubated at room temperature with a rabbit polyclonal primary antibody against STEAP1 (Invitrogen), diluted to 1:100 in PBS containing 1% BSA for 1 h at room temperature, washed with PBS for 5 min, and incubated with a biotinylated goat anti-rabbit IgG, diluted to 1:100 (Sigma) for 1 h. Sections were washed in PBS for 5 min at room temperature, incubated with a Rabbit ExtrAvidin Peroxidase reagent (Sigma) for 30 min, and then washed in PBS for 10 min. Color development was carried out using 0.05% 3,3′diaminobenzidine hydrochloride (DAB, Sigma) and 0.0006% hydrogen peroxide in TBS. Sections were counterstained in Mayer's hematoxylin, dehydrated, cleared, and mounted. As negative controls, sections were incubated in PBS containing 1% BSA omitting the primary antibody.

For dual labeling of breast cancer tissue sections (2 μm) with STEAP1, and wheat germ agglutinin (Invitrogen), which labels the cell membrane, sections were deparaffinized with xylene and rehydrated using graded ethanol series. Sections were then incubated for 10 min with wheat germ agglutinin (5 μg/ml), washed in PBS, and permeabilized with 1% Triton X-100 in PBS for 10 min, and blocked by incubation with 20% FCS in PBS with 0.1% Tween-20 for 60 min at room temperature. Sections were then incubated for 1 h with the STEAP1 antibody as described earlier. After washing, cells were incubated for 60 min at room temperature with Alexa Fluor® 488 goat anti-rabbit IgG conjugate (Molecular Probes; 1 μg/ml). To assess immunostaining specificity, the primary antibody and wheat germ agglutinin were omitted in some coverslips. Fluorescence was analyzed on a Zeiss inverted fluorescence microscope (Carl Zeiss, Germany).

Real-time PCR

Real-time PCR was used to evaluate the response of STEAP1 to E_2 in rat mammary gland and in MCF-7 cells and to compare the levels of STEAP1 transcription in human normal breast and in breast cancer biopsies. Specific primers to rat and human STEAP1 were used to amplify STEAP1 fragments of 157 and 128 bp, respectively. Rat

beta-2-microglobulin (rB2M), rat cyclophilin A (rCycA), human GAPDH (hGAPDH) and human beta-2-microglobulin (hB2M) primers were used as internal controls to normalize STEAP1 expression (Table 2). Real-time PCR efficiency was determined for STEAP1, B_2M, CycA, and GADPH primers: reactions were prepared using serial dilutions (1:1, 1:10, and 1:100) of rat mammary gland, human breast samples, and MCF-7 cells cDNA. Real-time PCR reactions were carried out using 1 µl of cDNA synthesized from rat mammary gland, MCF-7 cells, or human breast samples, in a 20 µl reaction containing 10 µl of SYBR Green Supermix (Biorad) and the appropriate concentration of each primer (indicated in Table 1). After an initial denaturation at 95°C for 5 min, cycling conditions (40 cycles) were the following: 95°C for 10 s, annealing temperature (60°C for rat and 64°C for human) for 30 s and 72°C for 10 s. The amplified PCR fragments were checked by melting curves: reactions were heated from 55 to 95°C with 10 s holds at each temperature (0.05°C/s). Samples were run in triplicate in each real-time PCR assay. Fold differences were calculated following the mathematical model proposed by Pfaffl using the formula $2^{-(\Delta\Delta Ct)}$ [23].

Establishment of score scales for STEAP1 expression and immunoreactivity in human breast tumors

A score scale for STEAP1 mRNA levels in tumors was established according to the following criteria: expression up to 1.5-fold compared to normal breast, low; expression between 1.5- and 2.5-fold, moderate; and expression above 2.5-fold, high.

STEAP1 immunoreactivity was assessed semiquantitatively by IHC. This system was based on the intensity and percentage of stained cells [24]. Briefly, the staining intensity was divided into moderate (score 1) and strong (score 2) and the percentage of stained tumor cells into occasional, less than 1/3 (score 1), up to 2/3 (score 2), and almost all (score 3). A case was scored as negative when no staining was seen in neoplastic cells; low positive indicated that up to 1/3 of the neoplastic cells were moderately stained (score $1 = 1 \times 1$); moderate positive indicated strong staining of up to 2/3 of the neoplastic cells (score 2–$4 = 1 \times 2$ or 2×2), or moderate staining in almost all neoplastic cells (3×1); and high positive was used when almost all the neoplastic cells were strongly stained (score $6 = 3 \times 2$).

Statistical analyses

All experimental data are shown as mean ± SEM. The statistical significance of differences in STEAP mRNA expression among experimental groups was assessed by ANOVA, followed by the Dunnett's test. Significant distributions among the parameters analyzed by IHC were determined using the χ^2 test. Significant differences were considered when $P < 0.05$. The SPSS software (version 15) was used in all statistical analysis.

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