GPR30 expression and function in breast cancer cells are induced through a cis-acting element targeted by ETS factors

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Abstract. The capacity that G protein-coupled receptor 30 (GPR30) has demonstrated for triggering estrogen-dependent signaling pathways has attracted the interest of breast cancer researchers; however, the reported expression profiles and functions of GPR30 in breast cancer are inconsistent. The main purpose of the present investigation was to identify transcriptional mechanisms underlying the expression of GPR30 that allow a better understanding of its role in breast cancer progression. In the cell lines used as different polarity models in the present study, it was determined immunologically that GPR30 is expressed in normal mammary gland cells and that this expression decreased considerably during breast cancer development, where cell identity is lost. However, it was also determined that, in spite of low GPR30 expression levels in breast cancer cells with little differentiation, this membrane estrogen receptor (ER) is able to increase cell viability and suppress migration in cells that have acquired metastatic capacity. In addition, through transient expression assays in breast cancer cells, it was revealed that a transcriptional mechanism dependent on protein kinase A and susceptible to retinoic acid in ER-positive cells induces GPR30 expression through a cis-regulatory element for E26 transformation-specific transcription factors, located between -631 and -625 bp from the GPR30 translation start codon. Overall, these results suggested that in vitro transcriptional regulation of GPR30 expression in breast cancer cells may serve a relevant role in the conservation of an epithelial phenotype, and also may be important to avoid the transition to metastasis.

Introduction

With the current advances in determining the mechanisms underlying estrogen-dependent cell proliferation in breast cancer, it has been possible to treat patients with in situ (or even invasive) ductal carcinoma, which are estrogen receptor (ER)α positive, through hormone therapy with antagonist molecules, such as tamoxifen or fulvestrant (1). Although these treatments are partially responsible for an ~40% decrease in the mortality rate over the last three decades (2), one-third of patients with breast cancer are not positive for hormone nuclear receptors and, therefore, do not respond favorably to such treatments. These treatments may be detrimental for certain intrinsic molecular subtypes (3). In the last 20 years, signal transduction through G protein-coupled receptor 30 (GPR30) has been considered a mechanism involved in the resistance to endocrine therapy, owing to its ability to trigger signaling pathways induced by antiestrogens (4). Additionally, it has been reported that GPR30 serves a regulatory role in several cellular processes, such as migration (5), proliferation (6) and cell survival (7) in breast cancer.

Although GPR30 was initially classified as an orphan receptor, subsequent biochemical studies demonstrated that this membrane receptor is strongly stimulated by estrogens, as well as by other compounds, including insecticides (i.e., DDT), phytoestrogens (i.e., genistein), xenoestrogens (i.e., bisphenol A) and antagonistic modulators of ERα and ERβ (i.e., fulvestrant and tamoxifen) (8,9). In addition, selective binding compounds for GPR30, such as the agonists G1 [1-(4-[6-bromobenzo-(1,3)-dioxol-5-y]-3a,4,5,9b-tetrahydro-3H-cycloptena-[c]-quinolin-8-y]-ethanone] and G6 [4-(6-bromo-benzo-[1,3]-dioxol-5-yl)-isopropyl-3a,4,5,9b-tetrahydro-3H-cycloptena-(c)-quinoline], and the antagonist G15 [4-(6-bromo-benzo-[1,3]-dioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cycloptena-(c)-quinoline], have been synthesized to monitor the specific biological activity of GPR30 (10-12).

Previous studies on GPR30 function have reported the involvement of this receptor in the induction of the epidermal growth factor receptor/ERK signaling pathway that promotes tamoxifen resistance (13), as well as in the transactivation of genes linked to proliferation and migration in breast cancer, such as connective tissue growth factor and N-acylsphingosine.
amidohydrolase 1 (14,15). There is also evidence that GPR30 expression in breast cancer can be regulated by a positive feedback loop with hypoxia-inducible factor 1 (HIF-1) (16). Recently, it was also reported that the activity of both proteins, GPR30 and HIF-1, is linked to a complex crosstalk process with the Notch1 signaling pathway, which in turn induces epithelial-mesenchymal transition (EMT) (17). However, the data reported thus far from GPR30 expression and function studies during the course of breast cancer is contradictory; for example, GPR30 expression has been reported to be down-regulated during breast cancer development, and variously reported to function as a suppressor or inducer of proliferation or migration (14,18-26). Considering the importance of the expression profile of GPR30 for the integral understanding of its role in the progress of breast cancer, the present study aimed to determine the transcriptional mechanisms that regulate GPR30 expression in cellular models of different breast cancer subtypes (metastatic and non-metastatic).

Materials and methods

Cell cultures. The non-metastatic MCF-7 [HTB-22; American Type Culture Collection (ATCC)] and metastatic MDA-MB-231 (HTB-26; ATCC) breast cancer cell lines, as well as the normal breast cell line MCF-10A (CRL-10317; ATCC), were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), Leibovitz's L-15 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and Mammary Epithelial Cells Growth Medium (Lonza Walkersville, Inc.) supplemented with cholerae toxin (Sigma-Aldrich; Merck KGaA), respectively. All cultures were incubated at 37°C in a 5% CO2 atmosphere. All cell lines were tested for mycoplasma and cellular authentication by commercial PCR Mycoplasma Detection Set (Takara Bio, Inc.), according with to manufacturer’s instructions, and capillary electrophoresis in a 3500 Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.), respectively.

Western blotting. For GPR30 immunoblotting, total protein was isolated from 1.5x106 cells of MCF-10A, MCF-7, MDA-MB-231 by lysis with RIPA buffer (1 M Tris/HCl pH 7.4; 6.5 M EDTA pH 8.0; 5 M NaCl; 0.4% deoxycholate; 0.8% IGEPAL-CA-630), PMSF and Complete Mini protease inhibitor cocktail tablets (Roche Diagnostics). For the ETS translocation variant (ETV1), ETV4 and ETV5 immunoblots, nuclear extracts were obtained from MCF-10A, MCF-7 and MDA-MB-231 metastatic cell lines, as well as the normal breast cell line MCF-10A (CRL-10317; ATCC), subjected to a 24 h starvation period and were incubated after this period of 16 h and stimulated afterwards with either 10 µM or 20 µM G15 (Azano Biotech) or 10 µM G1 (Azano Biotech) (Fig. S1) or 10−M retinoic acid (RA; Sigma-Aldrich; Merck KGaA) or vehicle (DMSO) incorporated into the corresponding growth medium. Cells were subsequently fixed at 0, 24, 48 and 72 h with 1% formaldehdye for 15 min, washed with water three times and left to dry at room temperature. The cells were stained with crystal violet for 20 min, washed three times with water and allowed to dry at room temperature. Finally, 10% acetic acid was added for 20 min. The absorbance of each well was determined at 590 nm using a Synergy HT microplate reader (BioTek Instruments, Inc.).

Cell viability assay. MCF-7 and MDA-MB-231 cells were seeded into 96-well culture plates at a density of 7.5x103 cells/well. The adhered cells were subjected to a starvation period of 16 h and stimulated afterwards with either 10 µM or 20 µM G15 (Azano Biotech) or 10 µM G1 (Azano Biotech) for total extracts. Cell viability assay. MCF-7 and MDA-MB-231 cells were seeded into 96-well culture plates at a density of 7.5x103 cells/well. The adhered cells were subjected to a starvation period of 16 h and stimulated afterwards with either 10 µM or 20 µM G15 (Azano Biotech) or 10 µM G1 (Azano Biotech) for total extracts. Cells were subsequently fixed at 0, 24, 48 and 72 h with 1% formaldehyde for 15 min, washed with water three times and left to dry at room temperature. The cells were stained with crystal violet for 20 min, washed three times with water and allowed to dry at room temperature. Finally, 10% acetic acid was added for 20 min. The absorbance of each well was determined at 590 nm using a Synergy HT microplate reader (BioTek Instruments, Inc.).

Migration assay. A migration assay was performed on the MCF-7 (Fig. S2) and MDA-MB-231 metastatic cell lines. Cells were seeded in 6-well culture plates with a 7.5x103 cell density and grown in Leibovitz’s L-15 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS, until they reached confluence. The cell monolayer was carefully wounded using a 100 µl pipette tip and the detached cells were removed by washing with PBS. The wounded monolayer cultures were subjected to a 24 h starvation period and were incubated afterwards for 48 h in serum-free medium containing 5 µM G1 (Azano Biotech) or 5 µM G1 + 10 µM G15 (Azano Biotech). Images of the wounds were capture at 0, 24 and 48 h using an Axiovert 25 microscope (Carl Zeiss AG) with a PC-Achromat 10x/0.25 Ph1 objective (Carl Zeiss AG) and a Powershot A580 digital photographic camera (Canon, Inc.) equipped with a DC150 camera adapter (Leica Microsystems GmbH). The wound areas were analyzed with the Icy bioimage analysis software v2.0.3.0 (http://icy.bioimageanalysis.org).

GPR30 5’ flanking region cloning. MCF-7 and MDA-MB-231 cells seeded and grown to 75% confluence in 100 mm plates and genomic DNA was isolated according to the procedure described by Sambrook et al (28). A 1,987 bp DNA fragment
from both genetic materials was amplified by PCR using the following thermocycling conditions: Initial denaturation of 94°C for 30 sec; followed by 30 cycles of 94°C for 30 sec, 50.4°C for 1 min, and 68°C for 4 min, followed by a final extension at 68°C for 7 min. PCR was carried out with an Expand Long Template PCR system (Roche Diagnostics) and the pair of specific primers described in Table I. The amplification product was separated by 10% agarose gel electrophoresis, visualized by ethidium bromide staining, and excised from the gel and purified by the manufacturer’s instructions. The recombinant plasmid was propagated in JM109 Escherichia coli (Promega Corporation) and purified with a Wizard Plus SV Miniprep system (Promega Corporation). To obtain a reliable sequence of the cloned fragment, three different recombinant plasmids of each cell line were sequenced with a Big Dye Terminator System and an ABI PRISM 77 automatic sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Sequence analysis. The nucleotide sequences obtained from the fragment of the 5′ flanking region of GPR30 cloned from the MCF-7 and MDA-MB-231 cell lines was analyzed using BLAST (National Center for Biotechnology Information; http://blast.ncbi.nlm.nih.gov). Both sequences were compared with each other and with the chromosome 7 sequence obtained from the human genome database (https://www.ncbi.nlm.nih.gov/nuccore/18643712). In addition, the binding sites for transcription factors were scanned with the prediction program in the JASPAR website (http://jaspar.genereg.net).

Reporter gene constructs. The recombinant plasmid harboring the human GPR30 promoter from MCF-7 cells was used as template to amplify five segments of different lengths (0.2, 0.5, 1.0, 1.5 and 2.0 kbp), using specific forward and reverse primers with BglII and HindIII restriction sites, respectively. Each amplicon was purified using a GeneClean III system (Promega Corporation), previously digested with the same restriction enzymes, and ligated with T4 DNA Ligase (Promega Corporation) into a pGEM-T vector (Promega Corporation) according to the manufacturer’s instructions. The recombinant plasmid was propagated in JM109 Escherichia coli (Promega Corporation) and purified with a Wizard Plus SV Miniprep system (Promega Corporation). To obtain a reliable sequence of the cloned fragment, three different recombinant plasmids of each cell line were sequenced with a Big Dye Terminator System and an ABI PRISM 77 automatic sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Stimulation assays with RA. MCF-7 cells were cultured in 96-well plates at a density of 1.5x10⁴ cells/well, and transfected with pGPR0.5 or pGPR1.0, aforementioned, and subsequently synchronized for 24 h with DMEM without phenol red (Sigma-Aldrich; Merck KGaA), supplemented with inactivated FBS (HyClone; GE Healthcare Life Sciences) and stimulated with increasing RA concentrations in DMEM (0, 10⁻¹², 10⁻¹¹, 10⁻¹⁰, 10⁻⁹, 10⁻⁸, 10⁻⁷ and 10⁻⁶ M) for 48 h. Reporter gene activity was measured using the Dual-Luciferase Reporter assay system in a Sirius L Single Tube Luminometer (Titertek-Berthold).

Reverse transcription (RT)-PCR assays. MCF-7 cells were seeded at a density of 7.5x10⁴ cells in 60 mm dishes with DMEM supplemented with 10% FBS and incubated at 37°C in a 5% CO₂ humid atmosphere for 24 h. Subsequently, the cells were washed with PBS and synchronized for 24 h with free-serum DMEM medium without phenol red. Later the cells were treated with RA (10⁻⁹ M) or vehicle (DMEM) for 48 h. At the end of the stimulation, cells were washed with cold PBS and lysed with 1 ml of Trizol® (Invitrogen; Thermo Fisher Scientific, Inc.). The cell lysate was mixed vigorously with 0.2 ml of chloroform (MP Biomedicals,
(LLC) for 30 sec and centrifuged for 40 min at 14,000 rpm and 4°C. The aqueous phase was recovered and mixed in 1 ml of isopropanol (Amresco, LLC) to precipitate RNA, followed by centrifugation at 14,000 rpm and 4°C for 1 h. The RNA pellet was washed with 75% ethanol and centrifuged at 14,000 rpm and 4°C for 12 min. Finally, the total RNA was resuspended in water treated with diethyl pyrocarbonate (Thermo Fisher Scientific, Inc.). cDNA was synthesized using the Phusion RT-PCR kit (Finnzymes; Thermo Fisher Scientific, Inc.), and PCR assays were performed using the cDNA as template and specific oligonucleotides (Table I) for amplification of the transcripts corresponding to GPR30, ETV1, ETV4, ETV5 and housekeeping gene GAPDH. PCR was carried out with Clone Amp HiFi PCR mix (Clontech Laboratories, Inc.) with the following thermocycling conditions: Initial denaturation of 94°C for 30 sec; followed by 30 cycles of 94°C for 30 sec, 58°C for 45 sec and 73°C for 1 min, followed by a final extension period of 5 min at 74°C. The amplification products were resolved in a 10% polyacrylamide gel, stained with ethidium bromide (0.5 µg/ml) and visualized in a UV transilluminator.

**Statistical analysis.** The results are expressed as the mean ± standard deviation. Comparisons between >2 groups were made using ANOVA followed by Tukey's post hoc test, or by Dunnett's post hoc test for comparisons between treatments and the vehicle control. Statistical analyzes were performed using the SigmaPlot 12.0 (Systat Software, Inc.) software for...
Windows. Each assay was performed in triplicate. P<0.05 was considered to indicate a statistically significant difference.

Results

GPR30 regulates cell viability and migration of breast cancer cells. The assays carried out for the immunodetection of GPR30 revealed a 44 kDa band corresponding to the molecular weight of this receptor (29) in the extracts of non-malignant epithelial cells (MCF-10A) and in dedifferentiated malignant cells (Fig. 1). Densitometric evaluation of the bands obtained indicated that after the control cell line (ovarian cancer cell line OVCAR3), the non-tumorigenic mammary gland cell line MCF-10A exhibited highest relative expression level of GPR30 (7.3%), followed by the non-metastatic breast cancer cell line MCF-7 (2.4%) and the metastatic MDA-MB-231 line (1.2%).

The effects of the specific GPR30 antagonist, G15, on MCF-7 and MDA-MB-231 viability was evaluated at 0, 24, 48 and 72 h (Fig. 2). The viability of MCF-7 cells treated with G15 was significantly reduced at 72 h compared with the vehicle-treated control group (Fig. 2A). MCF-7 cells that were cultured for 72 h in the absence of G15 increased their absorbance 4.8-fold compared with the 0 h time point, whereas G15-treated cells increased their absorbance 2.8-fold with a 10 µM concentration and 2.4-fold with a 20 µM concentration. These data indicated that in cell cultures treated for 72 h with 10 and 20 µM G15, cell viability decreased regarding untreated cells 40 and 49%, respectively. However, although an increase in viability in the MDA-MB-231 cell cultures was also observed with and without G15 treatment, there was a significant decrease in viability in cells treated with 20 µM G15 for 72 h compared with vehicle-treated cells at the same time point (Fig. 2B). The absorbance obtained in untreated-cells after 72 h of culture was 2.6-fold greater compared with cells at 0 h, whereas the increase in absorbance in cells treated with 10 and 20 µM G15 was 2.3 and 1.7-fold compared with the respective 0 h time point. These results indicated that MDA-MB-231 cell viability decreased 31% when cells were treated with 20 µM G15 compared with control cells at 72 h.

In the cell viability assays, untreated MCF-7 cell viability was increased 4.8-fold at 72 h compared with the 0 h time point, whereas the viability of cells treated with the GPR30 agonist (10 µM G1) increased only 2.6-fold over the same time period (Fig. S1A). In the case of MDA-MB-231 cells, viability increased 1.9-fold after 72 h without treatment, whereas the G1-treated cells increased only 1.35-fold after the same time elapsed (Fig. S1B). These data demonstrated that the viability of MCF-7 and MDA-MB-231 cells decreased significantly by 74 and 64%, respectively, after 72 h of treatment with G1, compared with the respective control at the same time point.

The role of GPR30 in cell migration was examined by wound-healing assays with the metastatic MDA-MB-231 cell line (Fig. 2C and D), since the MCF-7 cell line showed no significant changes in its migration (Fig. S2). In untreated MDA-MB-231 cells, the wound area was reduced by 41% after 24 h and 56% after 48 h. Conversely, in G1-treated cells the wound area reduced only by 13% at 24 h and 22% at 48 h. To confirm that the suppressive effect of G1 on migration was through GPR30, the effect of its antagonist, G15, on migration impaired by G1 was also assayed. For this, a G15 concentration twice as high as the minimum G1 concentration that was required to observe an effect on migration was used, since the affinity of the antagonist for the receptor is three times lower compared with that of G1 (10). The cells treated simultaneously with both G1 and G15, diminish the wound area 23% at 24 h and 33% at 48 h. This means that G1-treated cells lost 39% of its migratory capacity with respect to untreated cells, while cells treated with both agonist and antagonist recovered 20% of its migratory capacity regarding cells treated only G1.

Structural analysis of the 5' flanking region of the GPR30 encoding gene. Through structural and predictive analysis of the 1,987 bp sequence cloned from the 5' flanking region of the gene encoding the GPR30 receptor, it was possible to characterize the GPR30 promoter fragment in three regions. A proximal region ranging from -1 to -1,009 bp from TSC with a heterogeneous sequence, and two distal regions ranging from -1,010 to -1,512 bp and -1,513 to -1,987 bp from the TSC, which have a high GC content (70%) and a high AT content (72%), respectively (Fig. 3). The sequence alignment of the GPR30 promoter cloned from both MCF-7 and MDA-MB-231, with the chromosome 7 sequence did not reveal any mismatch (data not shown). In addition, 14 putative cis-regulatory elements with a similarity greater than 98% in relation to consensus sequences for forkhead box P1 (FOXP1), ERR1, NGRE, brain-specific homeobox (BSX) and member of the ETS, SOX and hypoxia-inducible factors (HIF) families, were found by JASPAR program. Given its high nucleotide heterogeneity, the distribution of these possible sites of transcriptional regulation are concentrated in the proximal region, whereas owing to the low nucleotide heterogeneity of distal regions, it was only possible to find three sites in one of them (Fig. 3).
Transcriptional regulation pattern of the GPR30 promoter in breast cancer. The transient expression of the five reporter constructs governed by different length versions of the GPR30 promoter truncated at the 5’ end (Fig. 3), revealed that even without the background activity generated by the empty vector, the transcriptional regulation pattern of the promoter turned out to be very similar in all three mammary gland cell lines. All reporter constructs transfected in the three cell lines, except pGPR1.0 in MCF-10A, produced a similar change pattern in luciferase activity. The maximum activity of GPR30 promoter was found in pGPR0.5 being this 1.6-fold higher in MCF-10A compared with MCF-7, and approximately 8.8-fold higher compared with MDA-MB-231 (Fig. 4). Using the luciferase activity induced solely by the initial 200 bp (pGPR0.2) of the GPR30 promoter induced solely by the initial 200 bp (pGPR0.2) of the GPR30 promoter in each cell line as the basal promoter activity, it was demonstrated that in MCF-10A cells the activity of pGPR0.5 significantly increased 93%, whereas activity with pGPR2.0 significantly decreased by 22% of basal activity of promoter. In the case of MCF-7, luciferase activity with pGPR1.5 and pGPR0.5 significantly increased 80 and 29% of basal activity, respectively. Conversely, the reporter activity of pGPR2.0 and pGPR1.0 in the same cell line, significantly decreased 44 and 50% of basal activity, respectively. Similarly, luciferase activity in MDA-MB-231 also significantly increase 98 and 71% of basal activity with pGPR1.5 and pGPR0.5, respectively. Although the activity of the reporter gene with pGPR2.0 and pGPR1.0 in MDA-MB-231 had a similar pattern of change as was observed in MCF-7, such changes were not significant (Fig. 4). Altogether, the changes observed in the three cell lines, suggest that the GPR30 promoter fragments ranging from 2.0 to 1.5 kbp and 1.0 to 0.5 kbp could contain transcription suppressing elements, while fragments ranging from 1.5 to 1.0 kbp and 0.5 to 0.2 kbp could host transcription activating elements.

A cis-regulatory element that resembles the polyomavirus enhancer activator 3 homolog (PEA3) subfamily consensus sequence, activates the GPR30 promoter. Bearing in mind the role of the sequence between -1,009 to -511 bp contained in the construct pGPR1.0 in the suppression of the transcription, functional analysis of the putative cis-regulatory elements for negative regulation factors located in this region was conducted. This was done by the transient expression of the ETSµ, NGREµ, ERRµ and SOXµ reporter
None of the mutations exhibited increased luciferase activity, as expected; however, the ETSµ mutation significantly decreased the reporter gene activity by 24% in MCF-10A, 65.8% in MCF-7 and 46% in MDA-MB-231 compared with the wild-type construct pGPR1.0 (Fig. 5A). Although with the site-directed mutations in the ERRµ, NGREµ and SOXµ constructs also is observed a decrease in the reporter gene activity in any of transfected cell lines, these changes were not statistically significant. The functionality of a putative ETS cis-regulatory element was confirmed by comparing the activity of pGPR1.0 and the construct containing an ETS factor consensus elements (ETSc) in the MDA-MB-231 cell line (Fig. 5B). As expected, the luciferase activity obtained with ETSc was 1.27-fold greater compared with the pGPR1.0 construct containing the wild-type GPR30 promoter.
In addition, to gather additional evidence regarding the participation of any of the factors of the ETS family in the positive regulation of the GPR30 promoter observed with the ETS element studied, the common phosphorylation pathways of such factors were inhibited to verify if GPR30 promoter activity decreases. For this, transient expression assays performed with pGPR1.0 (with ETS sites) and pGPR0.5 (without an ETS site) on MDA-MB-231 cells, treated with SB203580 (p38 inhibitor), H89 (PKA inhibitor) and PD98059 (ERK1/2 inhibitor) demonstrated statistically significant changes only with the 1.0 kbp GPR30 promoter in presence of H89 and PD98059, reducing its activity to 0.59 and 0.79-fold, respectively (Fig. 5C). Similar assays using the pGPR0.5 construct had no significant effect on the activity of the reporter gene, since the ETS factors of the PEA3 subfamily require the phosphorylation of PKA and ERK to perform their transcriptional activity (30-32), and given that the functional ETS element identified is located in the region affected by these inhibitors (pGPR1.0); the results obtained from assays with protein kinases inhibitors point to ETV1, ETV4 and ETV5 as the factors with the greatest possibility of interacting with this element.

The activity of the factors of the PEA3 subfamily have been closely related to the development of breast cancer (33-37), but the expression of each of them may be committed to specific phenotypes. To confirm which of these factors may be active in the cellular models in the present study, and possibly involved in the activation of the GPR30 promoter through the inducing ETS element, their expressions were verified qualitatively by immunoblotting assays (38). ETV5 has been previously reported as a factor expressed by MCF-7 (39). Therefore, the immunoblotting with anti-ETV5 was carried out using a commercial MCF-7 nuclear extract as positive control. This assay confirmed the expression of a 72 kDa protein that correspond to the molecular weight of ETV5 factor (40) in the nuclear extracts of all mammary gland cell lines used in the previous experiments and in the nuclear extract of positive control as expected. Conversely, a 66 kDa protein corresponding to the molecular weight of ETV4 was detected with anti-ETV4 only in the positive control extract (K-562) (40). In addition, a 62 kDa protein corresponding to the molecular weight of ETV1 (40) was also detected with anti-ETV1 in the nuclear extracts of the MCF-7 cell line and the positive control (mouse brain) (Fig. 5D). These results suggested that ETV5 and ETV1 may be able to transactivate GPR30 expression through the ETS element found as functional in pGPR0.1.

RA suppresses the activity of the GPR30 promoter and its ETS site located at -630 bp. Since RA suppresses proliferation in ER-positive breast cancer (41,42) and regulates the expression of several factors of the ETS family (43), dose-response assays with this metabolite were carried out on the MCF-7 cell line transfected with pGPR0.5 (without ETS site) and pGPR1.0 (with ETS site). In the case of the pGPR0.5 construct, the reporter gene activity exhibited a statistically significant suppression of 28.1% only when it was exposed to a final concentration of 10^{-6} M. By contrast, with the pGPR1.0 construction containing the inducer element for ETS factors, the activity of the promoter decreased significantly from 10^{-11} M concentration and continued significantly decreasing up to 60% with the highest concentration (Fig. 6A). In addition, the transient expression assays in RA-treated and untreated MCF-7 cells with the transfected pGPR1.0, ETSc or ETSµ constructs, exhibited a statistically significant decrease of ~50% in luciferase activity promoted by pGPR1.0 and ETSc.
when the cells are treated with RA, while with ETSµ no effect was observed (Fig. 6B).

The RT-PCR assays of GPR30 and its possible transcriptional regulators, performed with total RNA isolated from MCF-7 cells exhibited a notable decrease in amplicon production of GPR30, ETV5, ETV4 and ETV1 transcripts in RA-treated cells compared with the vehicle-treated cells (Fig. 6C). Similarly, viability assays using RA-treated MCF-7 cells showed no significant changes in absorbance during different culture times, whereas the absorbance of vehicle-treated MCF-7 cells increased 21% at 48 h and 67% at 72 h of cultivation. No significant differences in viability were identified in RA-treated cells at any time point compared with the respective 0 h control, which indicated a complete depletion of viability in MCF-7 cells associated with RA (Fig. 6D).

**Discussion**

A few years after the identification of GPR30 as an orphan receptor in 1996 (44), the expression and function of this membrane receptor in breast cancer was intensively studied. However, although it has been reported that the expression of GPR30 in the mammary gland is null (45) or restricted to myoepithelial cells (46), Scaling *et al* (47) have provided evidence that suggests the involvement of GPR30 in regulation of the in vitro proliferation of breast tissue epithelial cells. Conversely, data reported by other research groups regarding the role of GPR30 in breast cancer not allowed to infer its therapeutic importance (6,48-50). The lack of a well-defined expression pattern of GPR30 in the course of breast cancer hinders the correct interpretation of evidence concerning the role that this receptor may serve in the development of this malignancy (7,51-53). In the present study, the GPR30 immunoblotting assays showed that in the most differentiated mammary gland cell line (MCF-10A) there is a greater expression of GPR30 compared with expression levels in the cellular models of breast cancer that have a partially (MCF-7) or completely (MDA-MB-231) lost cell polarity. In turn, the difference observed between the GPR30 expression in MCF-7 and MDA-MB-231, helps to explain why the GPR30-dependent induction on proliferation deduced from viability assays with G15 in MCF-7 cells is more evident than in MDA-MB-231 cells. It is important to mention that the effect caused by G1
on the viability of the two models of breast cancer was not considered for the interpretation of the GPR30 activity, since it has been recently reported that this agonist suppresses proliferation through an GPR30-independent pathway (54). Overall, these results, together with the gradual loss observed in transcriptional regulation of the GPR30 promoter in breast cancer cell lines, indicate that mammary gland epithelial cells such as MCF-10A have GPR30 activity and that this can be extinguished in the course of breast cancer as a result of gene expression reprogramming that the malignant cells undergo during their dedifferentiation. In this regard, several research groups have reported that this can occur with GPR30 in breast cancer through epigenetic mechanisms that act at the promoter level (49,51,55).

However, the viability and migration assays of the present study show that despite the role epigenetic silencing may be playing in the expression of GPR30, poorly differentiated breast cancer cells, such as MDA-MB-231, still retain responsiveness to both the agonist and the GPR30 antagonist. Contrasting the results obtained from the viability assays with those of migration, it is interesting to note that agonist and antagonist of GPR30 promote contrary effect in the migration, which indicated that G1 may reduce migratory ability through GPR30 and that G15 may be able to at least partially block this suppressive effect. These results also suggested that GPR30 induced proliferation through a different pathway to the one with which it suppresses migration, which could help to explain how the antagonism proposed by some authors between proliferation and metastasis could happen (56). It was not possible to evaluate the effects of G1 and G15 on the migration of MCF-7 because this cell line showed no ability to migrate from untreated cells, which had been previously reported by other authors (57). It was also verified that the doses used in the different assays of viability and migration did not have toxic effects that would alter the results obtained (data not shown). Therefore, since the results indicated that GPR30 expression and function are subject to changes in cell differentiation in the course of breast cancer, it is possible to better interpret why there are some studies that report the expression of GPR30 as a risk factor for the use of hormonal

Figure 6. GPR30 promoter and the ETS *cis*-element located -630 bp from TSC is suppressed by RA in MCF-7 differentiated breast cancer cells. (A) Analysis of reporter gene activity of pGPR0.5 and pGPR1.0 in transient expression assays performed in MCF-7 cells exposed to a RA dose-response curve. "*P<0.01, "**P<0.001 vs. Vehicle. (B) Assessment of reporter gene activity of the pGPR1.0, ETSc and ETSµ constructs in RA-treated and untreated MCF-7 cells. "*P<0.01, "**P<0.001 vs. Vehicle. (C) Effects of RA treatment (10^-8 M) on mRNA expression of GPR30 and PEA3 subfamily factors in MCF-7 cells were determined by reverse transcription-PCR. (D) Viability of MCF-7 cells in the absence (vehicle control) and presence of RA. "*P<0.01, "**P<0.001 vs. 0 h. Data are presented as the mean ± SD of three independent experiments in triplicate. ETS, E26 transformation-specific; GPR30, G protein-coupled receptor 30; PEA3, polyomavirus enhancer activator 3 homolog; RA, retinoic acid; TSC, translation start codon.
therapy (13,58) and other studies that postulate this receptor as a favorable prognostic factor for disease-free survival of patients with ER-positive breast cancer (50,51,59).

Confirmation of GPR30 biological activity at different differentiation stages in breast cancer led to the investigation of the mechanisms that regulate GPR30 expression at the transcriptional level. The present study aimed to determine if there are different transcriptional mechanisms of GPR30 that match with its performance in the different phenotypes studied, since the studies in this regard are scarce and the majority have focused on establishing the role of epigenetic regulation in GPR30 expression (16,51,55,60,61). The predictive analysis of cis-regulatory elements performed on the 5' flanking region of the GPR30 gene obtained of the two malignant cell lines found only three putative sites in DR1 (GC 70%) and none in DR2 (AT 72%) probably due to its low heterogeneity, whereas in the proximal region of greatest heterogeneity 11 putative sites were identified for diverse transcription factors previously related to breast cancer. According to the data obtained from the transient expression assays carried out with pGPR0.2, the basal activity in MDA-MB-231 cells resulted in six-fold lower than MCF-7 and eleven-fold lower than MCF-10A. These results support the notion that GPR30 could be downregulated by EMT epigenetic reprogramming that malignant cells may undergo (51,55,61). However, this same epigenetic reprogramming could at the same time be indirectly involved in the positive regulation of GPR30 expression, since a specific cis-regulatory element for members of the ETS family found between -631 and -625 bp of the TSC in the promoter of GPR30 exhibited the greatest activity in MCF-7 and MDA-MB-231 breast cancer cells compared with activity in non-malignant mammary gland cells (MCF-10A), suggesting an incorporation of transcriptional regulators that were absent in the program of gene expression.

To determine possible transcription factors of the ETS family that may be involved in the transactivation of GPR30 through cis-regulatory element located between -631 and -625 bp from TSC, those repressor factors of this family, such as Ets-2 repressor factor, ETV6, ETV7, ETS-like gene 3, and E74-like factor 1, were identified through previous reports on their predominant function (62,63). Subsequently, taking as inclusion criterion their relationship with the progression of breast cancer, only the activators ETS1 (64), ETS2 (65) and the PEA3 subfamily (66) remained as possible interactors with the ETS binding site of the GPR30 promoter. To complete this analysis, transient expression assays were conducted with the reporter constructs pGPR1.0 and pGPR0.5 in untreated MDA-MB-231 cells and in cells treated with inhibitors of the PKA and ERK phosphorylation pathways, including SB203580 (p38), H89 (PKA), PD98059 (ERK1/2); exposure to H89 and PD98059 resulted in six-fold lower than MCF-7 and eleven-fold lower than MCF-10A. These results support the notion that GPR30 could be downregulated by EMT epigenetic reprogramming that malignant cells may undergo (51,55,61). However, this same epigenetic reprogramming could at the same time be indirectly involved in the positive regulation of GPR30 expression, since a specific cis-regulatory element for members of the ETS family found between -631 and -625 bp of the TSC in the promoter of GPR30 exhibited the greatest activity in MCF-7 and MDA-MB-231 breast cancer cells compared with activity in non-malignant mammary gland cells (MCF-10A), suggesting an incorporation of transcriptional regulators that were absent in the program of gene expression.

In conclusion, the results suggested a possible mechanism by which GPR30-dependent cell viability in non-invasive and ER-positive breast cancer cells may be downregulated by RA, but with a risk of triggering dedifferentiation. This indicated that specific activation of GPR30 may contribute to the treatment of non-invasive breast cancer tumors, preventing the transition to a more aggressive phenotype or may intervene by reversing the invasiveness of more aggressive tumors for the use of a more conventional treatment. Given the implications that the mechanism proposed in the present study may have for the prognosis and optimization of treatments for breast cancer at specific stages, it is also important to consider that the evidence reported here comes only from in vitro assays. Therefore, it is required that in subsequent studies, the functional ETS site between -631 and -625 bp from the TSC in the GPR30 promoter, as well as its possible transfactors, be included in assays that have a closer approach to the in vivo conditions, such as RNA interference or CRISPR.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon request.

Authors’ contributions

DSB, MAPS and AO conceived and designed the experiments. DSB, MAPS and PCG performed the experiments. DSB, MAPS and AO analyzed the data. MAPS, ZS and EB substantial contributed to the conception and drafting of work and revising it critically for important intellectual content. DSB, MAPS and AO wrote the paper. All authors read and approved the manuscript and agreed to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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