Title

1   **The G-protein-coupled estrogen receptor, a gene co-expressed with ERα in breast tumors, is**

2   **regulated by estrogen-ERα signalling in ERα positive breast cancer cells.**

3

4   Uttariya Pal[1@], Mohan Manjegowda[1@], Neha Singh[2], Snigdha Saikia[1], Betty S. Philip[3], Deep Jyoti

5   Kalita[4], Avdhesh Kumar Rai[5], Anupam Sarma[6], Vandana Raphael[3], Deepak Modi[2], Amal Chandra

6   Kataki[7], Anil Mukund Limaye[1]*

7

8   [1]Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati

9   781039, Assam, India

10   [2]Molecular and Cellular Biology Laboratory, ICMR-National Institute for Research in Reproductive

11   and Child Health, Indian Council of Medical Research (ICMR), JM Street, Parel, Mumbai 400012,

12   India

13   [3]Department of Pathology, North Eastern Indira Gandhi Regional Institute of Health & Medical

14   Sciences, Shillong 793018, Meghalaya, India

15   [4]Department of Surgical Oncology, Dr. Bhubaneshwar Borooah Cancer Institute, Guwahati 781016,

16   Assam, India.

17   [5]DBT Centre for Molecular Biology and Cancer Research, Dr. Bhubaneshwar Borooah Cancer

18   Institute, Guwahati 781016, Assam, India

19   [6]Department of Oncopathology, Dr. Bhubaneshwar Borooah Cancer Institute, Guwahati 781016,

20   Assam, India

21   [7]Department of Gynecologic Oncology, Dr. Bhubaneshwar Borooah Cancer Institute, Guwahati

22   781016, Assam, India

23

24   [@]Co-first authors: Uttariya Pal, Mohan Manjegowda

25   *Corresponding author

26   Dr. Anil Mukund Limaye

27   Department of Biosciences and Bioengineering

28   Indian Institute of Technology Guwahati

29   Guwahati 781039,

30   Assam, India

31   Email: amul@iitg.ac.in

32   Phone: 0361-2582218

33   Fax: 0361-2582249

34

Abstract

Purpose: The purpose of this study was to assess the relationship between GPER, a seven transmembrane G-protein coupled estrogen receptor, and ERα in breast tumors, and to make inroads into the mechanistic basis and clinical significance.

Methods: TCGA-BRCA data was mined to examine the relationship between GPER and ERα expression. GPER mRNA, and protein expression were analyzed in ERα-positive or -negative breast tumors from two cohorts using immunohistochemistry, western blotting, or RT-qPCR. The Kaplan-Meier Plotter was employed for survival analysis. The influence of estrogen *in vivo* was studied by examining GPER expression levels in estrus or diestrus mouse mammary tissues, and the impact of 17β-estradiol (E2) administration in juvenile or adult mice. The effect of E2, or propylpyrazoletriol (PPT, an ERα agonist) stimulation on GPER expression was studied in MCF-7 and T47D cells, with or without tamoxifen or ERα knockdown. ERα-binding to the GPER locus was explored by analysing ChIP-seq data (ERP000380), in silico prediction of estrogen response elements, and chromatin immunoprecipitation assay.

Results: Clinical data revealed significant positive association between GPER and ERα expression in breast tumors. The median GPER expression in ERα-positive tumors was significantly higher than ERα-negative tumors. High GPER expression was significantly associated with longer overall survival of patients with ERα-positive tumors. *In vivo* experiments showed a positive effect of E2 on GPER expression. E2 induced GPER expression in MCF-7 and T47D cells; an effect mimicked by PPT. Tamoxifen or ERα-knockdown blocked the induction of GPER. Estrogen-mediated induction was associated with increased ERα occupancy in the upstream region of GPER.

Conclusion: GPER expression is positively associated with ERα in breast tumors, and a transcriptional target of the estrogen-ERα signalling axis. More in-depth studies are required to establish the significance of GPER-ERα co-expression, and their interplay in breast tumor development, progression, and treatment.

INTRODUCTION

The estrogen receptor α (ERα) is a decisive variable that governs the mode of breast cancer treatment. It influences breast cancer prognosis and tumor phenotype. ERα expression is associated with indolence and favourable prognosis, whereas, its absence portends the opposite. Its expression in breast tumors also predicts response to endocrine therapy [1–3].

2

73

74   The genetic or molecular determinants of the aforementioned differences, and their
75   relationship with ERα expression, is poorly understood. Arguably, the involvement of genes within
76   the ERα co-expression network may hold the key [4]. On this supposition, Carmeci et al, employed
77   differential screening of MCF-7 (ERα-positive), and MDA-MB-231 (ERα-negative) cDNA libraries
78   to identify genes associated with ERα; only to find GPCR-Br, which was then an orphan G-protein-
79   coupled receptor (GPCR), as an abundantly expressed marker in the former, but not in the latter. The
80   study also demonstrated GPCR-Br and ERα co-expression in other breast cancer cell lines, and
81   primary breast tumors [4]. Subsequent work from several laboratories showed that 17β-estradiol (E2)
82   binds GPCR-Br [5, 6], to produce short-term non-genomic [5, 7], as well as long-term genomic
83   effects [8, 9] of estrogen. Hence, it was re-christened as a G-protein-coupled estrogen receptor
84   (GPER).

85

86   GPER signal transduction conforms to the typical GPCR signalling paradigm involving G_α,
87   and $G_{\beta\gamma}$ subunits. The highlight of GPER signalling is the activation of EGFR/ERK/MAPK pathway
88   via the HB-EGF ectodomain shedding [7], which not only induces proliferation [10], thereby
89   mimicking EGF like effects, but also enables cross-talk with ERα via Ser118 phosphorylation [11]
90   [12]. Tamoxifen is a GPER agonist [6]. Thus, GPER has gained traction in the field due to the
91   following reasons: a) it serves as a non-canonical membrane estrogen receptor [5–7], b) it explains
92   the EGF-like effects of estrogen [7], and c) it subserves tamoxifen resistance [13–15]. The
93   acknowledgement of its clinical relevance is reflected in the plethora of publications addressing
94   GPER expression in breast and other endocrine cancers, and its association with standard clinico- or
95   histo-pathological markers [16, 17], patient survival [18, 19], endocrine resistance [15, 20], or
96   metastasis [10].

97

98   The clinical import of ERα and GPER co-expression is of value, but warrants due attention in
99   the face of the existing knowledge gaps. The co-expression, or association of GPER with ERα in
100 breast tissue specimen was examined by several investigators. However, while some have reported
101 positive association [16, 17, 21], others have reported negative [19, 22], or no association [15, 23, 24].
102 Hence, there is a need for independent assessment. Furthermore, the mechanistic basis, or significance
103 of co-expression is unknown. Hormonal regulation of GPER, particularly by estrogen has been
104 touched upon in several publications [13, 21]. However, the data are confusing, and do not address the
105 role of hormone receptors, particulary the ERα.

106

107 The present study examined, and confirmed, positive association between ERα and GPER
108 expression in breast tumor specimen from three independent cohorts. Higher expression in estrus,

3

109   compared to diestrus mouse mammary tissues, and E2-driven, ERα-dependent modulation of GPER

110   in human breast cancer cell lines revealed GPER as a transcriptional target of estrogen.

111

112 **MATERIALS AND METHODS**

113

114 **Plasticwares and reagents**

115

116 Cell culture plasticware was purchased from Eppendorf (Hamburg, Germany). Dulbecco's
117 modified eagle's medium (DMEM) and Roswell Park Memorial Institute (RPMI) 1640 medium were
118 purchased from HiMedia (Mumbai, India). Fetal bovine serum (FBS) was purchased from Invitrogen
119 Corporation (Massachusetts, USA). Charcoal stripped fetal bovine serum (csFBS), Trypsin-EDTA,
120 antibiotics, and Dulbecco's phosphate-buffered saline (DPBS) were purchased from HiMedia
121 (Mumbai, India). Anti-H3 antibody (Cat No. BB-AB0055) and Normal rabbit IgG antibody (Cat No.
122 AB0001) was from BioBharati LifeScience (Kolkata, India). ERα (D8H8) antibody (Cat No. 8644S)
123 and anti-rabbit secondary antibody (Cat. No. 7074S) were from Cell Signaling Technology (MA,
124 USA). 17β-estradiol (E2, Cat No. E8875), 4-hydroxytamoxifen (TAM, Cat No. H7904),
125 Propylpyrazoletriol (PPT, Cat No. H6036) and paraformaldehyde were purchased from Sigma (MO,
126 USA). All other reagents, salts and buffers were purchased from Merck or SRL (Mumbai, India).

127

128 **TCGA-BRCA cohort**

129

130 Publicly available breast cancer mRNA expression data from The Cancer Genome Atlas
131 (TCGA), hereafter refered to as TCGA-BRCA data, were collected from the UCSC Xena Browser
132 (xenabrowser.net). This data set contains genome-wide mRNA expression values in the form of
133 log2(RPKM+1) on 114 normal breast tissues and 1097 primary breast tumors.

134

135 **Analysis of GPER expression in tumors from TCGA-BRCA cohort**

136

137 As per the histogram shown in supplementary data 1b, ERα mRNA expression showed
138 bimodal distribution in the primary breast tumors. The ERα mRNA expression data was modelled as a
139 mixture of two Gaussian populations, which led to the estimation of mean ERα mRNA expression in
140 ERα-low and ERα-high groups. Each tumor was classified as ERα-low or ERα-high if ERα mRNA
141 expression was within two standard deviations (sd) of the estimated respective population means.
142 The Shapiro-Wilk test revealed that ERα mRNA expression was not normally distributed in both the
143 subgroups. Hence, Mann-Whitney U test was employed to compare median GPER mRNA expression
144 in ERα-low and ERα-high tumors. Additionally, we considered the immunohistochemical data to
145 classify primary breast tumors as ERα-positive or ER-negative, followed by comparison of the
146 median GPER mRNA expression using the Mann-Whitney U test. We applied the Chi-squared test of
147 significance to examine the association of GPER expression with ERα, PR status, molecular subtype,
148 and stage.

149

150   **Survival analysis**

151

152   Survival was analyzed using the Kaplan-Meier Plotter online (www.kmplot.com) [25]. The

153   JetSet best probe "210640_s_at" for GPER was used for the analysis. Using the "auto select best cut-

154   off" option, tumor samples were divided into GPER-high and GPER-low groups. The effect of GPER

155   expression on overall survival (OS) of patients with ERα-positive or ERα-negative tumors, was

156   graphically represented as Kaplan-Meier plots.

157

158   **Breast tumor samples**

159

160   GPER protein expression was examined in a sample of 65 archival paraffin embedded breast

161   tumors, from the Department of Pathology, North Eastern Indira Gandhi Regional Institute of Health

162   and Medical Sciences (NEIGRIHMS), Shillong, India. These samples were from a cohort of breast

163   cancer patients registered in NEIGRIHMS from 2007 to 2016. The mean age of the cohort was 47

164   years (range: 26-86 years). Additionally, breast tumors were also collected from patients registered at

165   Dr. Bhubaneswar Borooah Cancer Institute (BBCI), Guwahati, India from December 2018 to October

166   2019, after obtaining patients' informed consent, which were used for analysis of GPER mRNA and

167   protein expression. The mean age of the cohort was 47.3 years (range: 26-82 years). ER, PR, and

168   HER-2 expression data were available for the samples. The studies were approved by ethics

169   committees of both centers.

170

171   **Animals and treatments**

172

173   Cyclicity of 8-10 weeks old female C57BL/6J mice was monitored by examination of vaginal

174   swabs stained with Giemsa (Cat No. SO11, Himedia, Mumbai, India). The animals in estrus (n = 11)

175   or diestrus stages (n = 8) of the reproductive cycle were euthanized before collecting the mammary

176   tissues for analysing Gper mRNA and protein using RT-qPCR and immunofluorescence.

177

178   To study the effects of estrogen, 5 weeks and 8 weeks old C57BL/6J mice were administered

179   with E2 (Cat No. E-2758, Sigma, MO, USA), which was dissolved in ethanol and diluted in corn oil

180   (Cat No. C8267, Sigma, MO, USA) at a dose of 1µg/day/mouse subcutaneously for 5 consecutive

181   days. Controls were administered equal amounts of ethanol in corn oil. On 6th day afternoon, the

182   animals were euthanized and mammary tissues were collected. The study was replicated thrice with 3

183   animals in each group. The study was approved by institutional animal ethics committee (IAEC

184   Project No. 1/16).

185

Immunohistochemistry

Sections of 4 μm thickness were cut from the paraffin-embedded tissues and mounted on silane-coated microscopic slides. They were deparaffinised with xylene after an overnight incubation in a hot air oven set at 37°C. The sections were rehydrated with decreasing concentrations of ethanol in water, before microwaving for 10 min at 450 W, followed by 15 min at 600 W in citrate buffer (10 mM, pH 6) for antigen retrieval. The slides were allowed to cool and then rinsed once with PBS. Immunohistochemical staining was done using Super Sensitive™ IHC detection system from BioGenex Laboratories (CA, USA) as per the manufacturer's instructions. Briefly, endogenous peroxidase activity was quenched by peroxide block for 10 min followed by three PBS washes of 5 min each. Sections were then probed with primary antibody [26] for 1 h at a dilution of 1:50. Sections were then incubated with super enhancer (20 min), polymer-HRP (30 min) and DAB substrate (10 min). After each step, the excess reagent was removed by three PBS washes of 5 min each. DAB staining was stopped by rinsing the slides in running tap-water. Sections were counter-stained with Harris hematoxylin and dehydrated by incubating in increasing concentrations of ethanol followed by xylene. Slides were air dried and mounted using DPX mountant, and observed under the microscope.

Modified semi-quantitative scoring of IHC for GPER was done only for the cytoplasmic compartment. The staining intensity was classified as negative (-), very weak (1+), weak (2+) and strongly positive (3+). The H-score was calculated by multiplying the intensity score with the percentage of positive cells with a score ranging from 0 to 300. Thus, the H-score took into consideration both the staining intensity and the percentage of cells. In every batch of slides, normal tissue within the biopsy was taken as internal control and slide with no primary antibody was taken as negative control. In the present study, though occasional tumor samples showed membrane and nuclear positivity, GPER staining was predominantly cytoplasmic. The H-score of the normal breast tissue was less than 100 in 29/35 cases and mostly ranged from 80-90. Samples with H-score > 40 were considered as GPER-positive.

Specificity of staining was confirmed by a peptide blocking experiment involving 4 slides of serial sections. They were incubated with anti-GPER antibody (+ve control), anti-GPER antibody pre-incubated with the peptide (peptide blocked antibody), anti-GPER antibody with peptide resuspension buffer (resuspension buffer control), or no primary antibody (-ve control) for 1 h before proceeding with secondary antibody incubation and chemiluminescence detection. As shown in supplementary data 2, absence of staining in samples treated with the antibody pre-incubated with the peptide indicated the specificity of staining.

Immunofluorescence

223

224   Mammary tissues were harvested and washed in PBS. They were stretched on glass slides, air

225 dried and fixed in 4% paraformaldehyde. Paraffin embedded mouse mammary tissue sections of 5 μm

226 thickness were deparaffinized and hydrated in grades of methanol. Epitopes were exposed in antigen

227 retrieval buffer (Tris-EDTA, pH-9.0) for 35 minutes at 95$^\mathbf{o}$C. Slides were incubated in Gper antibody

228 (Cat No. sc-48524-R, Santa Cruz Biotechnology, Texas, USA) using 1:100 dilution at 4$^\mathbf{o}$C, overnight.

229 The slides were then washed in 1X PBS for 30 minutes followed by incubation with biotinylated

230 secondary antibody for 40 min at room temperature. Signal was amplified by streptavidin-conjugated

231 FITC (Cat No. FTC9, Bangalore Genei, Bangalore, India, Dilution 1:100) at RT for an hour. Nuclei

232 were counterstained with DAPI (Sigma-Aldrich, Missouri, USA) and mounted in VECTASHIELD

233 Antifade media (Cat No. H-1200-10, Vector Laboratories, CA, USA). Slide edges were sealed with

234 nail polish, and imaged with an oil immersion Plan-Apochromat 63X/ 1.4 NA objective using a

235 confocal laser scanning microscope (Zeiss, Jena, Germany) at IIT Bombay.

236

237 **Cell culture**

238

239   MCF-7 and T47D breast cancer cell lines were procured from the National Centre for Cell

240 Science, Pune, India. Phenol red-containing DMEM or RPMI-1640, supplemented with 10% heat-

241 inactivated FBS, 100 μg/ml streptomycin and 100 units/ml penicillin (M1 medium) was used for

242 routine culture of MCF-7, or T47D cells, respectively. Cells were cultured under humidified

243 conditions of 5% CO_2 at 37°C. At 70–80% confluency, cells were trypsinized and subcultured, or

244 seeded for experiments.

245

246 **Treatment of cell lines**

247

248   2×10^5 MCF-7 or T47D cells were seeded in 6-well plates, in M1 medium. After 48 h of

249 incubation, the monolayer was washed with DPBS, and fed with phenol red-free DMEM (for MCF-7)

250 or RPMI-1640 (for T47D), supplemented with 10% heat-inactivated csFBS, 100 units/ml penicillin,

251 and 100 μg/ml streptomycin (M2 medium). After 24 h, the cells were washed with DPBS, and treated

252 in M2 medium, with indicated concentrations of E2, PPT, or tamoxifen, alone or in combinations, for

253 indicated period of time. Control cells were treated with 0.1% ethanol in M2 medium. The treatment

254 medium was replenished every 48 h.

255

256 **siRNA transfection**

257

258   2×10^5 MCF-7 or T47D cells were seeded in 6-well plates and incubated for 24 h in M1

259   medium. Cells were transfected with ERα-specific (Cat No. 4392420, Thermo Scientific, PA, USA)

260   or scrambled siRNA (Cat No. AM4611, Thermo Scientific, PA, USA) using Lipofectamine

261   RNAiMAX (Cat No. 13778075, Thermo Scientific, PA, USA) for 48 h according to the

262   manufacturer's instructions.

263

264   **RNA extraction, cDNA synthesis and RT-qPCR**

265

266   The mRNA expression of GPER variants in breast tumor samples, and treated or untreated

267   MCF-7 or T47D cells was analyzed by RT-qPCR. Total RNA was extracted and reverse-transcribed

268   as described earlier [27] and cDNA equivalent to 20 ng of total RNA was amplified with gene specific

269   primers (table 1). RT-qPCR reactions were carried out in AriaMX (Agilent, CA, USA). Reactions

270   were set up in 1X PowerUP SYBR Green master mix (Cat No. A25743, Thermo Scientific, PA,

271   USA). ROX dye served as passive reference. RPL35a served as an internal control.

272

273   For the analysis of Gper mRNA expression in mouse mammary tissues, total RNA was

274   extracted with TRIzol (Invitrogen, Massachusetts, USA), as per manufacturer's instructions,

275   precipitated in isopropanol, and washed with 70% alcohol, dried, and dissolved in 20 µl of DEPC

276   water. Total RNA was treated with DNase I (Bangalore Genei, Bangalore, India) as described

277   previously [28] and cleaned using RNeasy mini kit (Qiagen, Hilden, Germany). RNA was quantified

278   by nano-spectrophotometer (Eppendorf, Hamburg, Germany). 1 µg of RNA was reverse transcribed

279   using High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Massachusetts, USA) as

280   mentioned previously [29]. The cDNA was diluted 10 times, and RT-qPCR reaction was run using 2

281   µl of diluted cDNA in duplicates against Gper primer (table 1). RT-qPCR was performed using iQ

282   SYBR green super mix in the CFX-96 thermal cycler (Bio-Rad, CA, USA). Amplification was carried

283   out for 35 cycles where each cycle involved initial denaturation 95°C for 30 sec, primer annealing for

284   30 sec, and extension at 72°C for 30 sec. The fluorescence emitted was collected for 30 sec in the

285   extension step of each cycle. The homogeneity of the PCR amplicons was verified using the melt

286   curve method. All PCR amplifications were carried out in duplicate, for all biological replicates. Gene

287   expression was normalized to 18S rRNA and estimated using Pfaffl method [30].

288

289   **Western blotting**

290   Total protein was isolated, quantified and subjected to western blotting with peptide affinity

291   purified antibody to analyze GPER protein expression in breast tumor samples and cell lines as

292   described previously [26]. Histone (H3) served as the internal control.

293

ChIP-seq analysis

Sequence Read Archive (SRA) was searched for ERα-related ChIP-seq studies in MCF-7 cells. Subset of files from the project ERP000380 was selected. The raw data quality was assessed in galaxy web based platform [31]. The quality of all the input read files were assessed by FASTQC [32] with the default settings. The quality scores were converted to Sanger quality type by FASTQ Groomer [33]. Reads were mapped to reference human genome (hg19) using "Map with Bowtie for Illumina" tool [34]. Reads mapping to multiple locations were discarded and unmapped reads in the output SAM file were filtered using "Filter SAM or BAM, output SAM or BAM" tool [35]. The genomic regions enriched by reads were identified by MACS (Model-based analysis of ChIP-Seq) tool [36]. The peaks were visualized in UCSC genome browser [37] after converting Wig files to bigWig files using "Wig/BedGraph-to-bigWig" tool.

Chromatin immunoprecipitation (ChIP) assay

The ChIP protocol used in this study was as described earlier [29]. Briefly, cells were fixed with 0.75% (v/v) formaldehyde at room temperature for 10 mins followed by addition of 125 mM glycine to inhibit the cross-linking reaction. Cells were washed and lysed with 1 ml of ChIP lysis buffer. The lysates were sonicated, to shear the DNA at an amplitude of 30% for 35 cycles, each cycle with a 10-sec pulse on and 25-sec pulse off. Sonicated samples were centrifuged at 14000 rpm for 10 mins at 4^0C, and the supernatant containing the sheared chromatin was pre-cleared with Protein G plus-Agarose beads (Cat No. IP04-1.5ML, Merck Millipore, Burlington, USA), which were pre-coated with bovine serum albumin (BSA, Cat No. PG-2330, Puregene, CA, USA) and herring sperm DNA (Cat No. D7290, Sigma, Missouri, USA). 5% of the pre-cleared chromatin samples were kept aside as input. The remaining portion of the chromatin was incubated with normal rabbit IgG antibody or ERα antibody for 4 h. Protein-antibody complexes were immunoprecipitated by incubating with 20 μl of pre-coated Protein G plus-Agarose beads followed by centrifugation. After washing the beads extensively, the DNA was eluted in elution buffer, column purified and used as a template in PCR reactions with primers to amplify a specific region of GPER (test) or TFF1 (pS2) (positive control, [29]) locus.

Statistical analysis

Two-group, non-normal data were analyzed by Mann-Whitney U test to examine significant differences in median. Else, Welch two-samples t-tests were used. Chi-squared tests, or Fisher's exact tests were used as indicated to test for significance in the association between GPER expression with histo- or clinicopathological variables. Multiple group data were analysed by one-way ANOVA,

10

331 followed by Tukey's HSD post-hoc test. Statistical tests were carried out in R, considering 5% level

332 of significance (p < 0.05).

333

334 **RESULTS**

335

336   The inconsistency in the literature on the association between ERα and GPER, motivated us

337 to examine their correlation in primary breast tumors (n = 1097) from the TCGA-BRCA cohort. There

338 was a significant correlation between the two variables (Pearson's r = 0.266, p < 0.0001,

339 supplementary data 1a). However, the existence of two subgroups of breast tumors, namely ERα-low

340 and ERα-high, was apparent as illustrated in supplementary data 1b. We determined the mean ERα

341 mRNA expression in these subgroups by mixture modelling. ERα-low (n = 286), and ERα-high (n =

342 790) tumors were considered as those that had ERα mRNA expression levels within two standard

343 deviations on either side of their respective means (Fig 1a). There was a significant difference in the

344 median GPER mRNA levels in the subgroups; the ERα-high tumors expressing higher median levels

345 (Fig 1b). Median GPER mRNA expression was also significantly different in immunohistochemically

346 classified ERα-positive, and ERα-negative breast tumors of the TCGA-BRCA cohort (Fig 1c). Table

347 2 shows that GPER-high tumors were more frequent in ERα-, or PR-positive breast tumors, as

348 compared to the negative ones. A Chi-squared test revealed that GPER mRNA expression was

349 significantly associated with ER, or PR positivity (p < 0.0001).

350

351   We also employed immunohistochemistry to examine GPER protein expression in archival

352 paraffin embedded breast tumor specimens from NEIGRIHMS cohort using a polyclonal antibody

353 raised against the N-terminal peptide of GPER [26]. The specificity of the antibody was demonstrated

354 by a peptide blocking experiment (supplementary data 2). Representative images showing varied

355 intensities of GPER staining in tumors with different expression levels of ER, PR and HER2 are

356 provided as a supplement (supplementary data 3). H-score of 40 was used as a cut-off to segregate the

357 GPER-positive (H > 40) from the GPER-negative tumors (H<=40). 44 out of 65 (68%) of the tumors

358 were GPER-positive, whereas the remaining (32%) were GPER-negative (table 3). A Chi-squared test

359 showed significant association between GPER expression and ERα. The tumors were also classified

360 based on TNM stage, Bloom Richardson grade, lymphovascular invasion, tumor type and size, margin

361 type, lymph node status, and molecular subtype. 21 out of 44 (47%) GPER-positive tumors, and 4 out

362 of 21 (19%) GPER-negative tumors were positive for lymphovascular invasion, suggesting a positive

363 association between GPER expression and lymphovascular invasion (table 4, p = 0.031). None of the

364 other clinicopathological parameters was significantly associated with GPER expression in this study

365 (table 4).

366

11

367   We applied western blotting technique to study GPER protein levels in
368   immunohistochemically confirmed ERα-positive or ERα-negative breast tumors received from BBCI
369   cohort. A representative western blot in Fig 2a shows higher GPER protein levels in ERα-positive
370   breast tumors. GPER protein is encoded by three annotated transcript variants, referred to as GPER-
371   v2 (NM_001505.2), -v3 (NM_001039966.1), and -v4 (NM_001098201.1). These transcripts have the
372   same coding sequence and a 3'-UTR, but possess different 5'-UTRs. A previous work from our
373   laboratory showed that the aforementioned variants are expressed in breast cancer cell lines; MCF-7
374   cells expressing elevated levels compared to MDA-MB-231 [27]. Recently we reported another
375   variant of GPER, referred to as GPER-v5 [38]. We examined the expression of these transcript
376   variants in immunohistochemically confirmed ERα-positive (n = 25) and ERα-negative (n = 25)
377   breast tumors of the BBCI cohort. As shown in Fig 2b, GPER-v2, -v3 and -v4 were significantly
378   elevated in ERα-positive breast tumors compared to the negative ones. We were unable to detect the
379   expression of GPER-v5 in these samples.

380

381   Previous studies have shown that primary breast tumors within the TCGA-BRCA cohort
382   exhibit significantly lower levels of GPER mRNA expression than normal breast tissues [27]. Higher
383   GPER expression in breast tumors were shown to have a positive effect on patient survival [27]. The
384   aforementioned results motivated us to examine the effect of GPER-ERα co-expression on patient
385   survival. As shown in Fig 3a, high GPER expression in ERα-positive tumors was significantly
386   associated with prolonged OS. In contrast high GPER expression in ERα-negative tumors evidently
387   had an opposite effect (Fig 3b).

388

389   We studied Gper protein and mRNA expression in the mouse mammary tissues at estrus and
390   diestrus stages. Immunostaning for Gper was detected in both alveolar and ductal epithelium. At the
391   diestrus stage when the glands are smaller, Gper was weak and restricted mainly to the apical region
392   of ductal epithelial cells. At the estrus stage, when the epithelial cells were multi-layered, glands grow
393   in size, abundance of Gper protein per cell was high and strong in the epithelium. The expression of
394   Gper in estrus stage was mainly cytoplasmic and not polarized like that at the diestrus stage (Fig 4a).
395   The staining in the epithelium is specific as no reactivity was noted in these cells when primary
396   antibody was replaced by normal rabbit serum. The staining in the stroma is non-specific as it was
397   detected also in the negative controls. (Fig 4a). RT-qPCR analysis results also corroborate with the
398   immunostaining results and reveal that expression of Gper mRNA was 2 fold higher in estrus stage
399   when compared to diestrus (Fig 4b).

400

401   To test if estrogen induces expression of Gper, we isolated mammary glands from juvenile (5
402   weeks) and adult mice (8 weeks) treated with 17β-estradiol. Gper expression was increased with E2
403   treatment and protein was cytoplasmic (Fig 4c). As compared to untreated controls Gper mRNA

12

levels increased significantly in both juvenile and adult mice (Fig 4d). This increase was statistically significant (p-value <0.001).

The aforementioned *in vivo* results motivated a mechanistic study of the estrogen regulation of GPER in human breast cancer cell lines. Dose-response (Fig 4, e,f) and time-course experiments (Fig 4, g,h) confirmed estrogenic induction of GPER mRNA variants in MCF-7 and T47D cells. PPT, a synthetic non-steroidal ERα agonist, also induced GPER mRNA variants in both the cell lines (Fig 5, a,b). Tamoxifen blocked estrogen- or PPT-mediated induction (Fig 5, a,b); a strong indication of ERα involvement, which was confirmed by ERα knockdown experiments in both the cell lines (Fig 5, c,d). Liganded ERα modulates gene expression by engaging with estrogen response elements in target gene promoters. We hypothesized that GPER is a direct transcriptional target of liganded ERα. To test this hypothesis, we analyzed ChIP-seq data obtained from MCF-7 cells treated by E2 for 24 h. Several ERα enriched regions in the GPER locus were apparent (Fig 5e). Furthermore, both MATINSPECTOR, and JASPAR tools predicted an ERE in the region corresponding to the 5'-most peak revealed in the ChIP-seq data. A ChIP assay employing ERα specific antibody, and primers designed to amplify this region, confirmed the enhanced ERα occupancy in MCF-7 cells exposed to 10 nM E2 for 24 h (Fig 5f).

Discussion

Genes regulated by ERα, or those that constitute ERα co-expression network are key to understanding phenotypic differences between ER-positive, and ER-negative tumors. On this proposition, the differential screening of MCF-7 and MDA-MB-231 cDNA libraries by Carmeci et al. was predicated, which led to the identification of GPER as an ERα co-expressed marker in breast cancer cell lines and tumors [4]. Although the co-expression is confirmed by others [27], the clinical data on the association between ERα and GPER expression are inconsistent. Positive [16, 17, 21], negative [19, 22], and no association [15, 23, 24] were reported in independent cohorts. Here, using an assortment of methods to determine, and analyze GPER mRNA and protein expression in breast tumors from three independent cohorts, we found a positive association.

The TCGA-BRCA breast tumors showed bimodal distribution of ERα. By modeling breast tumors as a mixture of two Gaussian populations, it was possible to classify the tumors into ERα-low, and ERα-high groups. ERα expression varies with patient age or menopausal status, and ERα-positive tumors are more frequent in postmenopausal women [39]. Difference in age or menopausal status, arguably, could explain the existence of ERα-low and ERα-high subgroups. Consistent to this, a significantly lower ERα expression was observed in breast tumors from young (less than median age) versus old (greater than median age), or premenopausal versus postmenopausal women

440 (supplementary data 5). On the contrary GPER mRNA expression did not differ; inconsistent with the

441 positive correlation between ERα and GPER. However, this can be explained by the estrogen-

442 mediated induction of GPER expression, as shown in this study. Despite low levels of ERα, the

443 higher circulating levels of estrogen in young or premenopausal patients may raise the expression of

444 GPER, thereby explaining similar levels of GPER in older and young, or premenopausal and

445 postmenopausal patients.

446

447 The molecular basis of GPER-ERα co-expression has remained elusive. Carmeci et al. have

448 discussed the possible role of AP1 and AP2 transcription factors [4]. In response to EGF and TGFα,

449 the expression of GPER is upregulated in Ishikawa and TAM-R MCF-7 cell lines via the

450 EGFR/MAPK signaling pathway, which involves recruitment of c-fos to the AP1 site of the GPER

451 locus [40]. Demarco et al. reported that the IGF-IR/PKCd/ERK/c-fos/AP1 transduction pathway is

452 involved in the IGF-mediated stimulation of GPER expression [41]. GPER promoter also contains an

453 AP2 binding site [4]. AP1 and AP2 are also involved in the expression of ESR1, the gene encoding

454 ERα [42, 43]. Thus, diverse signalling inputs likely coordinate GPER and ERα expression via

455 activation of common transcription factors that regulate their expression. GPER expression in about

456 50% of ERα-negative breast tumors [24] implies that co-ordinated expression may not be the only

457 basis for co-expression. Induction of GPER expression by ligand-activated ERα, as shown here,

458 presents an alternative basis for GPER-ERα co-expression in ERα-positive breast cancer cells. Even

459 in the absence of the natural ligand, overt growth factor signalling leading to ligand independent

460 activation of ERα [44] may also induce GPER, thereby explaining their co-expression.

461

462 Despite volumes of clinical and cell biological data, little is known about estrogen-mediated

463 transcriptional regulation of GPER, let alone the contribution of ERα in its expression and function.

464 Reporting on the discovery of GPER cDNA (then referred to as GPCR-Br), Carmeci et al. discussed

465 the non-effect of serum stimulation on its expression in MCF-7 breast cancer cells [4]. On the

466 contrary, we found higher expression of GPER mRNA and protein in MCF-7 cells cultured in phenol

467 red-containing medium supplemented with routine serum, compared to those cultured in phenol red-

468 free medium supplemented with charcoal stripped serum (supplementary data 6); strongly favouring

469 estrogen regulation of GPER. Carmeci et al. have discussed decrease in GPER expression post

470 estrogen exposure in MCF-7 cells, which was contradicted by Ignatov et al. with their demonstration

471 of enhanced GPER protein expression following estrogen treatment [13]. In SKBr3 cells, a widely

472 accepted ERα-negative breast cancer cell line, estrogen and progesterone were shown to induce the

473 expression of GPER mRNA and protein by Thomas et al., postulating the involvement of genomic

474 nuclear progesterone receptor and non-genomic estrogen signalling [6]. Here, progesterone had a

475 greater effect than estrogen. Ahola et al. showed that medroxyprogesterone acetate can upregulate

476 GPER in MCF-7 breast cancer cells. However, their experiments were conducted in media containing

14

477   1 nM E2, obscuring the effect of estrogen [45]. Estrogenic regulation is also indicated by higher
478   GPER expression in diestrus hamster endometrium [46]. However, our *in vivo* data shows higher
479   GPER expression in murine mammary tissues, in the estrogen dominant (estrus) phase of the
480   reproductive cycle. Taken together the present study demonstrates the positive influence of estrogen
481   on GPER expression, both *in vivo* and *in vitro*.

482

483   In the context of estrogen-mediated induction, the binding of ERα to the upstream regulatory
484   region of GPER, is a significant insight. The ChIP-seq and chromatin immunoprecipitation data show
485   that GPER is a genomic target of estrogen-ERα signalling. This is not the first instance of
486   transcriptional activation or involvement of ERα in the regulation of GPER expression. Hypoxia
487   transcriptionally induces the expression of GPER via HIF1α binding to the hypoxia responsive
488   element (HRE) in GPER [47]. Interestingly, the induction of GPER via IGF-IR/PKCd/ERK/c-fos/AP1
489   transduction pathway involves recruitment of phospo-ERα, to the AP1 site in the GPER promoter
490   [41]. However, the regulatory regions operative in these instances are distinct from each other. This
491   suggests that different signalling pathways co-opt different regulatory regions to alter GPER
492   expression.

493

494   The knowledge of GPER expression in breast tumors has prognostic or therapeutic value.
495   Being an estrogen receptor, its presence in ERα-negative tumors implies estrogen responsiveness.
496   Given, that GPER activation can lead to cell proliferation, or cell cycle arrest, its potential as an
497   independent prognostic marker or a therapeutic target in ER-negative breast cancer cannot be
498   overemphasized. The significance of GPER-ERα co-expression, however, has not been adequately
499   addressed, although it is appealing on several counts. Analysis of survival data revealed that high
500   GPER expression is associated with significantly longer overall survival of patients with ERα-positive
501   breast tumors. In contrast, patients with ERα-negative tumors with higher GPER expression, have
502   poor overall survival. Thus, GPER-ERα co-expression entails favourable prognosis. 15-20% of ERα-
503   positive breast tumors do not respond to endocrine therapy [48]. Thus, the ability to distinguish
504   between endocrine responsive, and non-responsive ERα-positive breast tumors has therapeutic value.
505   PR, a downstream target of ERα and a gene induced by estrogen-ERα signalling is a classical marker
506   of estrogen responsiveness, and estrogen responsive breast tumors. Given that GPER is a downstream
507   transcriptional target of estrogen-ERα signalling axis, GPER presents itself as a potential alternative
508   marker of endocrine therapy response.

509

510   Estrogen-mediated induction of GPER has cell biological implications. GPER signaling
511   activates the EGFR-MAPK pathway, which in turn activates the unliganded ERα, due to the
512   phosphorylation of serine 118 [11]. This cross-talk between GPER and ERα is a proof of the
513   functional interaction that is made possible by their co-expression. Estrogen- mediated induction of

15

514 GPER via ERα shows that the cross-talk is bidirectional; each impacting the expression or function of
515 the other. It also implies that in the face of estrogenic stimulation cells will be rendered more
516 responsive to GPER activating ligands. Besides, it has greater cell biological implications in the
517 context of breast cancer development and progression. Given the ambivalence in the data on effects of
518 GPER activation, which indicate both promotion and inhibition of cell proliferation, we envisage dual
519 role for enhanced GPER expression. In view of the negative impact of GPER activation on cell
520 proliferation [49], estrogen induction of GPER may serve to produce a balancing effect to counteract
521 the pro-proliferative effects of activated ERα. Such a mechanism would prevent excessive
522 proliferation of the mammary epithelium in the face of estrogenic stimulation, thereby preventing
523 tumorigenesis. On the other hand, in the light of pro-proliferative effects of activated GPER [50, 51],
524 and higher expression of GPER in tamoxifen resistant cells [52], estrogen induction of GPER may
525 render ERα dispensable for growth and proliferation of the mammary epithelium. Such a mechanism
526 would subserve the emergence of endocrine therapy resistant breast tumors.

528 **Legends**

530 **Fig 1: GPER expression in TCGA-BRCA breast tumors stratified according to ERα expression**
531 (a) A scatter-plot of GPER and ERα mRNA levels in primary breast tumors (n = 1097). Note the
532 bimodality of ERα expression, which has been depicted as a histogram in supplementary data 1b. The
533 ERα expression was modelled as a mixture of two Gaussian populations. The green and blue colored
534 clusters represent ERα-low and ERα-high tumors, respectively, which were grouped on the basis of
535 expression level within two standard deviations on either side of the respective means. (b) A boxplot
536 showing the distribution of GPER mRNA expression in ERα-low (n = 286) and ERα-high (n = 790)
537 breast tumors identified in (a). (c) A boxplot showing the distribution of GPER mRNA expression
538 levels in ERα-negative (Negative, n = 179) and ERα-positive (Positive, n = 601) breast tumors on the
539 basis of immunohistochemical assessment. ESR1 symbol for gene encoding human ERα. mRNA
540 expression levels are in terms of $\log_2(RPKM+1)$ values. Data in (b) and (c) were analysed by Mann-
541 Whitney U test (***p < 0.0001)

543 **Fig 2: GPER protein and mRNA expression in ERα-negative and ERα-positive breast tumors**
544 **from the BBCI cohort.** (a) A representative western blot showing GPER protein levels in ERα-
545 positive (n = 4) and ERα-negative (n = 3) breast tumors. The blots were probed with a peptide affinity
546 purified antibody against GPER [26]. (b) Quantitative representation of GPER protein expression in
547 ERα-negative (n = 10) and ERα-positive (n = 12) breast tumors. Chemiluminescence signals were
548 processed and quantified using ImageJ [53]. For each sample, the background-subtracted integrated
549 band intensity for GPER was normalized against that obtained for Histone (H3). Normalized GPER
550 protein expression in tumor samples was expressed relative to that in MCF-7 cells, which was set to 1.

16

551   Data were analyzed by Mann-Whitney U test test (*p < 0.05). The chemiluminescence data for all the

552   samples analysed are compiled as supplementary data 4. (c) GPER mRNA variant expression levels in

553   ERα-negative and ERα-positive breast tumors. Total RNA isolated from ERα-positive (n = 25) and

554   ERα-negative (n = 25) breast tumors were reverse transcribed, and the resultant cDNAs were

555   subjected to RT-qPCR using variant specific primers. RPL35a was used as an internal control. For

556   each sample the average Ct value for a given mRNA variant ($Ct^{variant}$), and RPL35a (Ct^{RPL35a}) obtained

557   from duplicate reactions were determined. The difference $Ct^{variant}$ - Ct^{RPL35a}, was considered as a

558   measure of the normalized variant expression. Samples with no Ct values in duplicate technical

559   replicates were excluded, GPER-v2 (ERα-positive, n = 24; ERα-negative, n = 24), GPER-v3 (ERα-

560   positive, n = 23; ERα-negative, n = 19) and GPER-v3 (ERα-positive, n = 23; ERα-negative, n = 24).

561   Box plots show the normalized GPER variant mRNA expression in the BBCI cohort. Data were

562   analyzed by Mann-Whitney U test. Note that the difference is significantly lower in ERα-positive

563   tumors, indicating higher expression.

564

565   **Fig 3: The influence of GPER expression on overall survival depends on ERα status of breast**

566   **tumors. Relationship between GPER expression and survival of breast cancer patients.** Survival

567   analyses were performed using Kaplan–Meier Plotter online tool [25]. The JetSet best probe

568   "210640_at" was considered for GPER expression. The breast tumors were classified as GPER-high

569   or -low based on "auto select best cut-off" option. OS of patients stratified on the basis of high, or low

570   GPER expression in patients with ERα-positive (a) or ERα-negative (b) breast tumors separately.

571

572   **Fig 4: Estrogen positively affects GPER expression.** (a, b) Immunofluorescence (a) and RT-qPCR

573   (b) study of Gper protein and mRNA expression respectively in mouse mammary tissues during estrus

574   and diestrus phases of the reproductive cycle. Immunostaining for Gper (Green channel), and DAPI

575   stained nuclei (blue channel). Bar is 50 microns. No antibody control is in inset. Mammary tissue was

576   collected from adult cycling mice in estrus (n = 11) & diestrus (n = 8) stages and processed for RT-

577   qPCR. Graph is plotted as mean ± sd. Y-axis shows normalized relative expression. p < 0.05 was

578   taken as statistically significant. **p < 0.01. (c,d) Immunofluorescence (c), and RT-qPCR (d) study of

579   the effect of 17β-estradiol treatment on Gper protein, and mRNA, respectively, in juvenile (5 weeks),

580   or adult (8 weeks) old mice. Juvenile female mice were treated with estrogen for five days and

581   mammary tissue was collected. Immunostaining for Gper (Green channel), and DAPI stained nuclei

582   (blue channel) was performed. Bar is 50 microns. (d) Juvenile (5 weeks) and adult female (8 weeks)

583   mice were treated with estrogen for five days and breast tissue was subjected to RNA extraction and

584   RT-qPCR. Graph is plotted as mean ± sd. Y-axis shows normalized relative expression. p < 0.05 was

585   taken as statistically significant. ***p < 0.001. (e, f) A dose-response study of the effect of E2

586   treatment on GPER mRNA expression in MCF-7 (e), and T47D cells (f). Cells were treated with

587   indicated concentrations of E2 for 72 h. Expression of GPER mRNA variants relative to RPL35a was

588 analyzed using RT-qPCR. Expression levels in vehicle controls (ethanol treated cells) were set to 1

589 and those in E2 treated groups were expressed relative to control. Bars represent mean ± sd (n = 3).

590 Data for each variant were subjected to ANOVA with Tukey's HSD. *p < 0.05, **p < 0.01, ***p <

591 0.001. (g, h) A time-course study of the effect of E2 on MCF-7 (g) or T47D (h) cells. Cells were

592 treated with 10 nM E2 or vehicle for indicated durations. For each sample the expression of GPER

593 mRNA variants relative to RPL35a was analyzed using RT-qPCR. For each time-point the GPER

594 variant expression was expressed relative to vehicle control, which was set to 1. Bars represent mean

595 ± sd (n = 3). For each time-point data were analyzed by Welch two-sample t-test. *p < 0.05, **p <

596 0.01, ***p < 0.001. Colored bars correspond to GPER-v2 (purple), GPER-v3 (light blue), and GPER-

597 v4 (orange) variant mRNAs. The grey bars (g,h) represent control cells, and are included to show that

598 each time point had its ethanol treated control.

599

600

601 **Fig 5: ERα-dependent induction of GPER mRNA by E2 in breast cancer cells.** (a, b) Tamoxifen

602 blocks E2- or PPT- induced expression of GPER mRNA variants. MCF-7 (a) or T47D (b) cells were

603 stimulated with 10 nM E2 or 100 nM PPT, alone or in combination with 10 μM tamoxifen. Relative

604 GPER mRNA variant expression levels were ascertained by RT-qPCR as in dose-response

605 experiments. Cells treated with tamoxifen alone were also included in the experiment design.

606 Expression levels in vehicle controls (ethanol treated cells) were set to 1 and those in E2 treated

607 groups were expressed relative to control. Bars represent mean ± sd (n = 3). Data were subjected to

608 ANOVA with Tukey's HSD. *p < 0.05, **p < 0.01, ***p < 0.001. (c, d) ERα-knockdown blocks E2-

609 mediated induction of GPER mRNA variants. MCF-7 (c), or T47D (d) cells, transfected with

610 scrambled or ERα-specific siRNA for 24 h were stimulated with vehicle or 10 nM E2 for 72 h. GPER

611 mRNA variant expression relative to RPL35a was ascertained by RT-qPCR. For scrambled, or ERα-

612 specific siRNA transfected groups, the relative GPER expression levels in E2 treated cells were

613 expressed relative to vehicle control (ethanol-treated), and analyzed by Welch two-sample t tests. Bars

614 represent mean ± sd (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001. (e) ERα binding sites at the GPER

615 locus in the human genome. (e) ChIP-seq data obtained from MCF-7 cells treated with vehicle, or 10

616 nM E2 (SRA accession no. ERP000380) was analysed using Galaxy. ERα enriched regions were

617 visualized in the UCSC Genome Browser. Note the major peak of ERα binding indicated inside the

618 red rectangle, observed only in estrogen treated sample. An ERE was also predicted within this peak

619 region when analysed using MATINSPECTOR or JASPAR, as indicated in the respective tracks. (f)

620 Validation of ERα binding using ChIP assay. Sonicated chromatin samples from vehicle or 10 nM E2-

621 treated MCF-7 cells were immunoprecipitated with non-specific IgG or ERα-specific antibody.

622 Immunoprecipitated DNA was subjected to PCR using primer pairs designed to specifically amplify

623 the region indicated by black bar (ChIP amplicon track). Colored bars correspond to GPER-v2

624 (purple), GPER-v3 (light blue), and GPER-v4 (orange) variant mRNAs.

625

Acknowledgements

627

637

Statements & Declarations

639

Funding

645

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

648

Author Contributions

Material preparation, data collection and analysis were performed by Uttariya Pal, Mohan Manjegowda, Neha Singh, Snigdha Saikia, Betty S. Philip, Deep Jyoti Kalita, Avdhesh Kumar Rai, Anupam Sarma, The work in respective collaborating institutions were supervised by Vandana Raphael, Deepak Modi, Amal Chandra Kataki and Anil Mukund Limaye. The first draft of the manuscript was written by Mohan Manjegowda and Anil Mukund Limaye and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. Uttariya Pal and Mohan Manjegowda are co-first authors in this manuscript.

657

Data Availability

The TCGA-BRCA dataset analysed during the current study are available in the TCGA repository, https://www.cancer.gov/tcga

661

Ethics approval

The Ethics Committee of Dr Bhubaneshwar Borooah Cancer Institute, Guwahati approved the clinical work Reference no. BBCI-TMC/SC/Appr/15/2019 and BBCI-TMC/Misc-119/3180/2018.The institute ethics committee of North Eastern Indira Gandhi Regional Institute of Health & Medical Sciences, Shillong approved to conduct IHC study on breast cancer tumor samples (NEIGR/IEC/2013/12). The animal study was approved by institution animal ethics committee of National Institute for Research in Reproductive and Child Health, Mumbai (IAEC Project No. 1/16).

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent to publish

Informed consent was obtained from all individual participants included in the study.

References

1.   Giacinti L, Claudio PP, Lopez M, Giordano A (2006) Epigenetic information and estrogen receptor alpha expression in breast cancer. Oncologist 11:1–8. https://doi.org/10.1634/theoncologist.11-1-1

2.   Osborne CK (1998) Steroid hormone receptors in breast cancer management. Breast Cancer Res Treat 51:227–38. https://doi.org/10.1023/a:1006132427948

3.   Osborne CK, Schiff R (2011) Mechanisms of endocrine resistance in breast cancer. Annu Rev Med 62:233–47. https://doi.org/10.1146/annurev-med-070909-182917

4.   Carmeci C, Thompson DA, Ring HZ, et al (1997) Identification of a gene (GPR30) with homology to the G-protein-coupled receptor superfamily associated with estrogen receptor expression in breast cancer. Genomics 45:607–17. https://doi.org/10.1006/geno.1997.4972

5.   Revankar CM, Cimino DF, Sklar LA, et al (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science 307:1625–30. https://doi.org/10.1126/science.1106943

6.   Thomas P, Pang Y, Filardo EJ, Dong J (2005) Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. Endocrinology 146:624–32. https://doi.org/10.1210/en.2004-1064

7.   Filardo EJ, Quinn JA, Bland KI, Frackelton AR (2000) Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. Mol Endocrinol 14:1649–60. https://doi.org/10.1210/mend.14.10.0532

8.   Pandey DP, Lappano R, Albanito L, et al (2009) Estrogenic GPR30 signalling induces

699   proliferation and migration of breast cancer cells through CTGF. EMBO J 28:523–32.

700   https://doi.org/10.1038/emboj.2008.304

701   9.   Albanito L, Madeo A, Lappano R, et al (2007) G protein-coupled receptor 30 (GPR30)

702   mediates gene expression changes and growth response to 17beta-estradiol and selective

703   GPR30 ligand G-1 in ovarian cancer cells. Cancer Res 67:1859–66.

704   https://doi.org/10.1158/0008-5472.CAN-06-2909

705   10.   Filardo EJ, Quinn JA, Sabo E (2008) Association of the membrane estrogen receptor, GPR30,

706   with breast tumor metastasis and transactivation of the epidermal growth factor receptor.

707   Steroids 73:870–3. https://doi.org/10.1016/j.steroids.2007.12.025

708   11.   Hart D, Nilges M, Pollard K, et al (2014) Activation of the G-protein coupled receptor 30

709   (GPR30) has different effects on anxiety in male and female mice. Steroids 81:49–56.

710   https://doi.org/10.1016/j.steroids.2013.11.004

711   12.   Clark S, Pollard K, Rainville J, Vasudevan N (2022) Immunoblot Detection of the

712   Phosphorylation of the Estrogen Receptor α as an Outcome of GPR30 /GPER1 Activation.

713   Methods Mol Biol 2418:25–39. https://doi.org/10.1007/978-1-0716-1920-9_2

714   13.   Ignatov A, Ignatov T, Roessner A, et al (2010) Role of GPR30 in the mechanisms of

715   tamoxifen resistance in breast cancer MCF-7 cells. Breast Cancer Res Treat 123:87–96.

716   https://doi.org/10.1007/s10549-009-0624-6

717   14.   Catalano S, Giordano C, Panza S, et al (2014) Tamoxifen through GPER upregulates

718   aromatase expression: a novel mechanism sustaining tamoxifen-resistant breast cancer cell

719   growth. Breast Cancer Res Treat 146:273–85. https://doi.org/10.1007/s10549-014-3017-4

720   15.   Ignatov A, Ignatov T, Weissenborn C, et al (2011) G-protein-coupled estrogen receptor

721   GPR30 and tamoxifen resistance in breast cancer. Breast Cancer Res Treat 128:457–66.

722   https://doi.org/10.1007/s10549-011-1584-1

723   16.   Filardo EJ, Graeber CT, Quinn JA, et al (2006) Distribution of GPR30, a seven membrane-

724   spanning estrogen receptor, in primary breast cancer and its association with clinicopathologic

725   determinants of tumor progression. Clin Cancer Res 12:6359–66.

726   https://doi.org/10.1158/1078-0432.CCR-06-0860

727   17.   Kuo W-H, Chang L-Y, Liu DL-Y, et al (2007) The interactions between GPR30 and the major

728   biomarkers in infiltrating ductal carcinoma of the breast in an Asian population. Taiwan J

729   Obstet Gynecol 46:135–45. https://doi.org/10.1016/S1028-4559(07)60007-2

730   18.   Samartzis EP, Noske A, Meisel A, et al (2014) The G protein-coupled estrogen receptor

731   (GPER) is expressed in two different subcellular localizations reflecting distinct tumor

732   properties in breast cancer. PLoS One 9:e83296. https://doi.org/10.1371/journal.pone.0083296

733   19.   Arias-Pulido H, Royce M, Gong Y, et al (2010) GPR30 and estrogen receptor expression: new

734   insights into hormone dependence of inflammatory breast cancer. Breast Cancer Res Treat

735   123:51–8. https://doi.org/10.1007/s10549-009-0631-7

736   20.   Ignatov T, Claus M, Nass N, et al (2019) G-protein-coupled estrogen receptor GPER-1
737   expression in hormone receptor-positive breast cancer is associated with poor benefit of
738   tamoxifen. Breast Cancer Res Treat 174:121–127. https://doi.org/10.1007/s10549-018-5064-8

739   21.   Ariazi EA, Brailoiu E, Yerrum S, et al (2010) The G protein-coupled receptor GPR30 inhibits
740   proliferation of estrogen receptor-positive breast cancer cells. Cancer Res 70:1184–94.
741   https://doi.org/10.1158/0008-5472.CAN-09-3068

742   22.   Tu G, Hu D, Yang G, Yu T (2009) The correlation between GPR30 and clinicopathologic
743   variables in breast carcinomas. Technol Cancer Res Treat 8:231–4.
744   https://doi.org/10.1177/153303460900800308

745   23.   Poola I, Abraham J, Liu A, et al (2008) The Cell Surface Estrogen Receptor, G Protein-
746   Coupled Receptor 30 (GPR30), is Markedly Down Regulated During Breast Tumorigenesis.
747   Breast Cancer (Auckl) 1:65–78. https://doi.org/10.4137/bcbcr.s557

748   24.   Luo H-J, Luo P, Yang G-L, et al (2011) G-protein Coupled Estrogen Receptor 1 Expression in
749   Primary Breast Cancers and Its Correlation with Clinicopathological Variables. J Breast
750   Cancer 14:185–90. https://doi.org/10.4048/jbc.2011.14.3.185

751   25.   Györffy B, Lanczky A, Eklund AC, et al (2010) An online survival analysis tool to rapidly
752   assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809
753   patients. Breast Cancer Res Treat 123:725–31. https://doi.org/10.1007/s10549-009-0674-9

754   26.   Manjegowda MC, Gupta PS, Limaye AM (2016) Validation data of a rabbit antiserum and
755   affinity purified polyclonal antibody against the N-terminus of human GPR30. Data Br
756   7:1015–20. https://doi.org/10.1016/j.dib.2016.03.054

757   27.   Manjegowda MC, Gupta PS, Limaye AM (2017) Hyper-methylation of the upstream CpG
758   island shore is a likely mechanism of GPER1 silencing in breast cancer cells. Gene 614:65–73.
759   https://doi.org/10.1016/j.gene.2017.03.006

760   28.   Godbole GB, Modi DN, Puri CP (2007) Regulation of homeobox A10 expression in the
761   primate endometrium by progesterone and embryonic stimuli. Reproduction 134:513–23.
762   https://doi.org/10.1530/REP-07-0234

763   29.   Kumar A, Dhillon A, Manjegowda MC, et al (2021) Estrogen suppresses HOXB2 expression
764   via ERα in breast cancer cells. Gene 794:145746. https://doi.org/10.1016/j.gene.2021.145746

765   30.   Godbole G, Modi D (2010) Regulation of decidualization, interleukin-11 and interleukin-15 by
766   homeobox A 10 in endometrial stromal cells. J Reprod Immunol 85:130–9.
767   https://doi.org/10.1016/j.jri.2010.03.003

768   31.   Afgan E, Baker D, van den Beek M, et al (2016) The Galaxy platform for accessible,
769   reproducible and collaborative biomedical analyses: 2016 update. Nucleic Acids Res 44:W3–
770   W10. https://doi.org/10.1093/nar/gkw343

771   32.   S Andrews (2010) FastQC: A Quality Control Tool for High Throughput Sequence Data.
772   http://www.bioinformatics.babraham.ac.uk/projects/fastqc%0A%0A

773   33.   Blankenberg D, Gordon A, Von Kuster G, et al (2010) Manipulation of FASTQ data with
774   Galaxy. Bioinformatics 26:1783–5. https://doi.org/10.1093/bioinformatics/btq281

775   34.   Langmead B, Trapnell C, Pop M, Salzberg SL (2009) Ultrafast and memory-efficient
776   alignment of short DNA sequences to the human genome. Genome Biol 10:R25.
777   https://doi.org/10.1186/gb-2009-10-3-r25

778   35.   Li H, Handsaker B, Wysoker A, et al (2009) The Sequence Alignment/Map format and
779   SAMtools. Bioinformatics 25:2078–9. https://doi.org/10.1093/bioinformatics/btp352

780   36.   Zhang Y, Liu T, Meyer CA, et al (2008) Model-based analysis of ChIP-Seq (MACS). Genome
781   Biol 9:R137. https://doi.org/10.1186/gb-2008-9-9-r137

782   37.   Kent WJ, Zweig AS, Barber G, et al (2010) BigWig and BigBed: enabling browsing of large
783   distributed datasets. Bioinformatics 26:2204–7. https://doi.org/10.1093/bioinformatics/btq351

784   38.   Pal U, Manjegowda MC, Limaye AM (2021) A novel transcript variant of human G-protein
785   coupled estrogen receptor. Mol Biol Rep 48:2979–2983. https://doi.org/10.1007/s11033-021-
786   06242-y

787   39.   Bajetta E, Martinetti A, Zilembo N, et al (2002) Biological activity of anastrozole in
788   postmenopausal patients with advanced breast cancer: effects on estrogens and bone
789   metabolism. Ann Oncol Off J Eur Soc Med Oncol 13:1059–66.
790   https://doi.org/10.1093/annonc/mdf083

791   40.   Vivacqua A, Lappano R, De Marco P, et al (2009) G protein-coupled receptor 30 expression is
792   up-regulated by EGF and TGF alpha in estrogen receptor alpha-positive cancer cells. Mol
793   Endocrinol 23:1815–26. https://doi.org/10.1210/me.2009-0120

794   41.   De Marco P, Bartella V, Vivacqua A, et al (2013) Insulin-like growth factor-I regulates GPER
795   expression and function in cancer cells. Oncogene 32:678–88.
796   https://doi.org/10.1038/onc.2012.97

797   42.   Tang Z, Treilleux I, Brown M (1997) A transcriptional enhancer required for the differential
798   expression of the human estrogen receptor in breast cancers. Mol Cell Biol 17:1274–80.
799   https://doi.org/10.1128/MCB.17.3.1274

800   43.   McPherson LA, Weigel RJ (1999) AP2alpha and AP2gamma: a comparison of binding site
801   specificity and trans-activation of the estrogen receptor promoter and single site promoter
802   constructs. Nucleic Acids Res 27:4040–9. https://doi.org/10.1093/nar/27.20.4040

803   44.   Bunone G, Briand PA, Miksicek RJ, Picard D (1996) Activation of the unliganded estrogen
804   receptor by EGF involves the MAP kinase pathway and direct phosphorylation. EMBO J
805   15:2174–83

806   45.   Ahola TM, Manninen T, Alkio N, Ylikomi T (2002) G protein-coupled receptor 30 is critical
807   for a progestin-induced growth inhibition in MCF-7 breast cancer cells. Endocrinology
808   143:3376–84. https://doi.org/10.1210/en.2001-211445

809   46.   Wang C, Prossnitz ER, Roy SK (2007) Expression of G protein-coupled receptor 30 in the

810   hamster ovary: differential regulation by gonadotropins and steroid hormones. Endocrinology

811   148:4853–64. https://doi.org/10.1210/en.2007-0727

812   47.   De Francesco EM, Lappano R, Santolla MF, et al (2013) HIF-1α/GPER signaling mediates the

813   expression of VEGF induced by hypoxia in breast cancer associated fibroblasts (CAFs). Breast

814   Cancer Res 15:R64. https://doi.org/10.1186/bcr3458

815   48.   Anurag M, Ellis MJ, Haricharan S (2018) DNA damage repair defects as a new class of

816   endocrine treatment resistance driver. Oncotarget 9:36252–36253.

817   https://doi.org/10.18632/oncotarget.26363

818   49.   Vo D-KH, Hartig R, Weinert S, et al (2019) G-Protein-Coupled Estrogen Receptor (GPER)-

819   Specific Agonist G1 Induces ER Stress Leading to Cell Death in MCF-7 Cells. Biomolecules

820   9:. https://doi.org/10.3390/biom9090503

821   50.   Scaling AL, Prossnitz ER, Hathaway HJ (2014) GPER mediates estrogen-induced signaling

822   and proliferation in human breast epithelial cells and normal and malignant breast. Horm

823   Cancer 5:146–160. https://doi.org/10.1007/s12672-014-0174-1

824   51.   Luo H, Yang G, Yu T, et al (2014) GPER-mediated proliferation and estradiol production in

825   breast cancer-associated fibroblasts. Endocr Relat Cancer 21:355–69.

826   https://doi.org/10.1530/ERC-13-0237

827   52.   Molina L, Bustamante F, Ortloff A, et al (2020) Continuous Exposure of Breast Cancer Cells

828   to Tamoxifen Upregulates GPER-1 and Increases Cell Proliferation. Front Endocrinol

829   (Lausanne) 11:563165. https://doi.org/10.3389/fendo.2020.563165

830   53.   Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image

831   analysis. Nat Methods 9:671–5. https://doi.org/10.1038/nmeth.2089
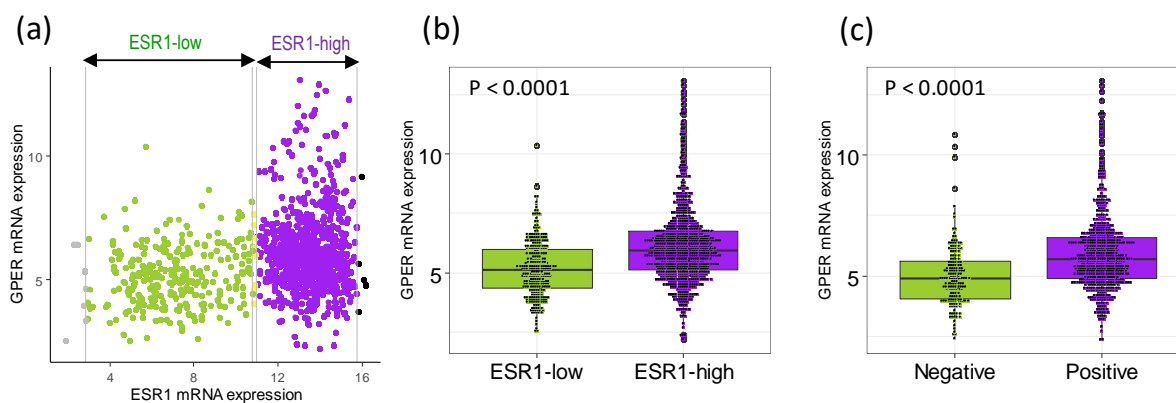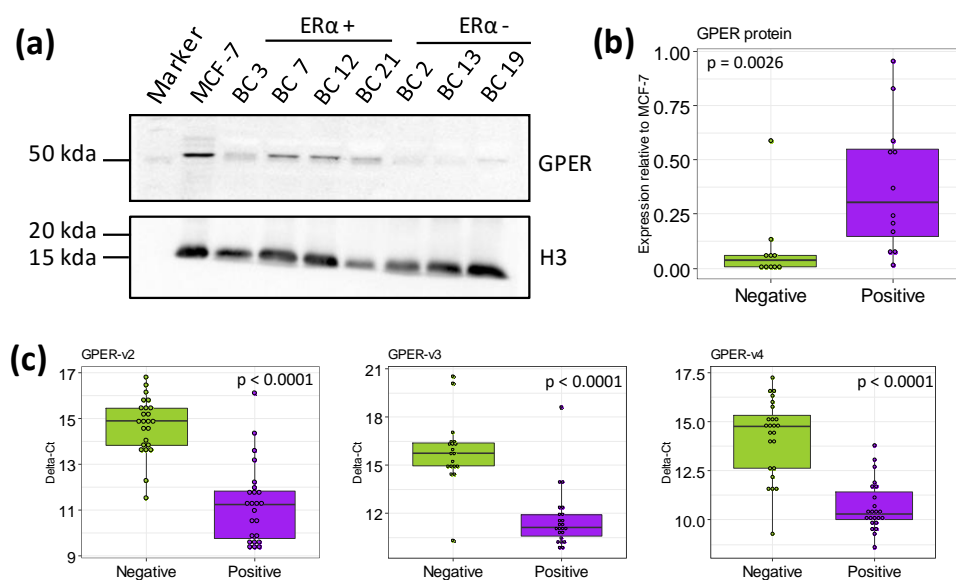
832

833

834

835

836

837

838

839

840

841

842

843

844

845

846

Figure 1

Figure 2

864

865

866

867

(a) ERα-positive

(b) ERα-negative

868

869

870   **Figure 3**

871

872

873

874

875

876
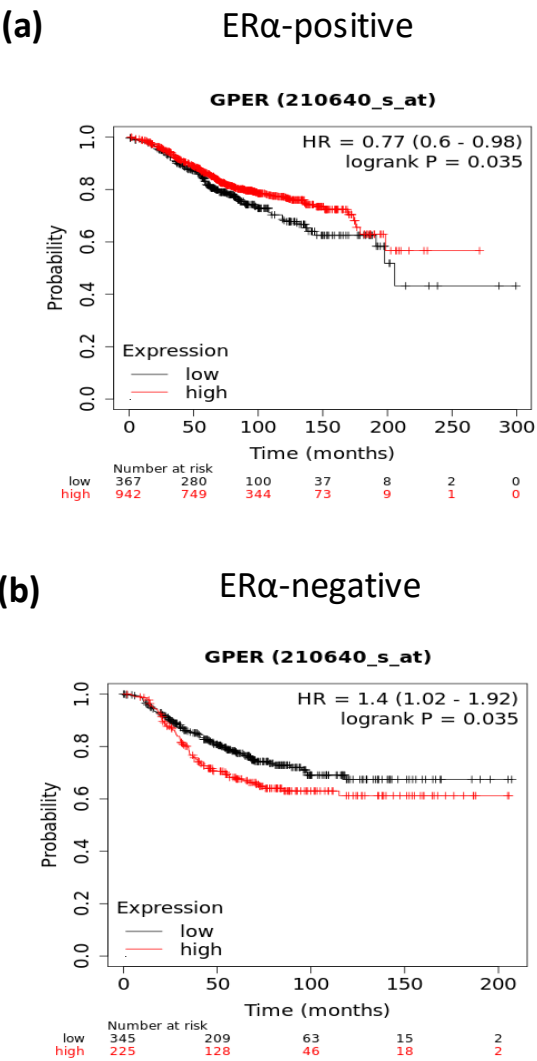
877

878

879

880

881
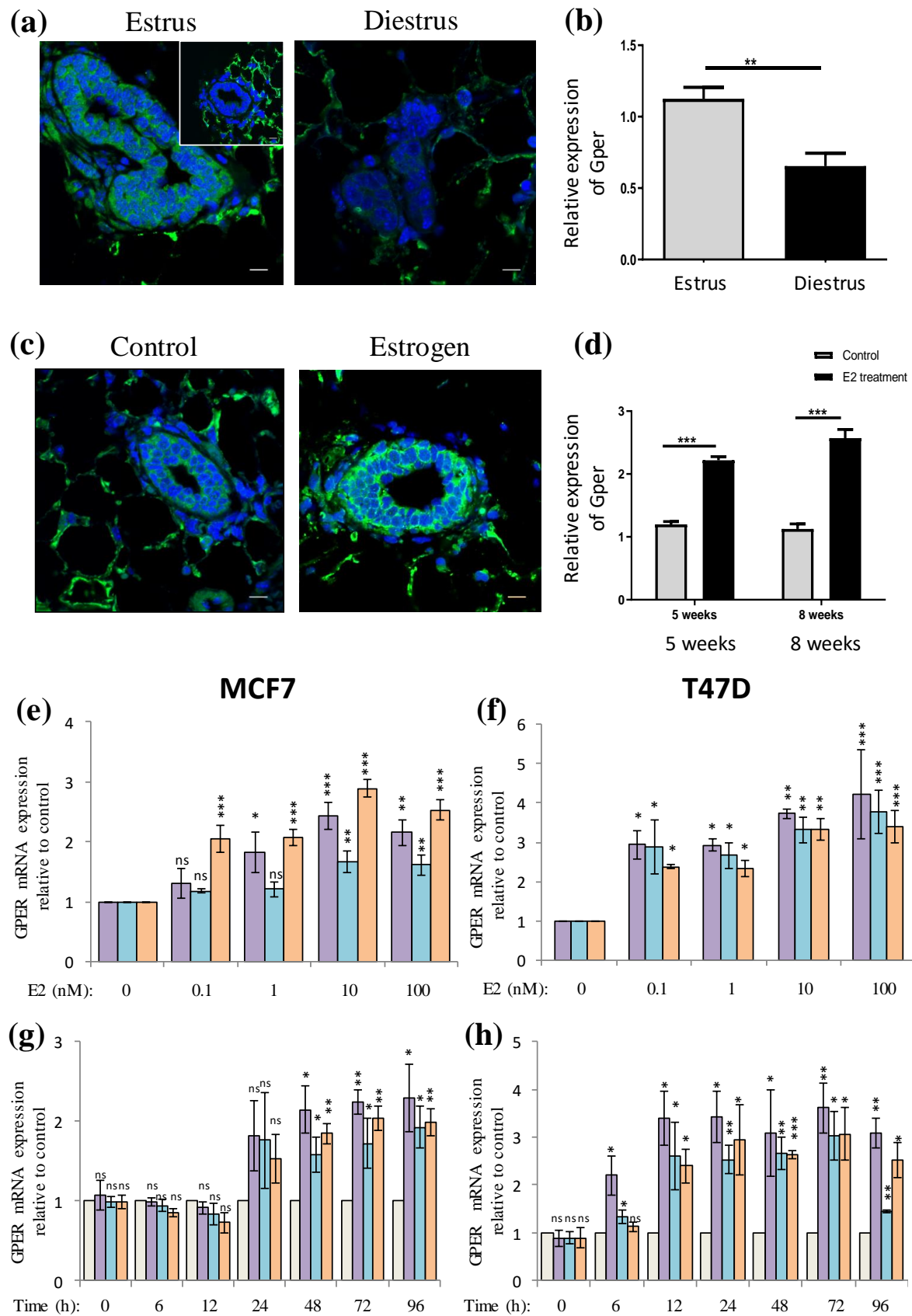
882

Figure 4

27

Figure 5

Table 1. List of primers used for routine RT-PCR, RT-qPCR, and ChIP.

Sl. No	Gene Name	Primer sequence (5'---->3')	Amplicon (base pair)	Annealing temperature (°C)	Remarks
1	RPL35a	Forward- CGGCCTCCAAGCT CT TAAG Reverse- CAGGTCCAGGG CTTGTACT	131	60	Used in qRT-PCR using cDNA synthesized from cell lines and breast tumors
2	GPER-v2	Forward- ATCTGGACGGCAGGT ACC Reverse- GAAGAACAGATGCTCCTCACAC	149	60	Used in qRT-PCR using cDNA synthesized from cell lines and breast tumors
3	GPER-v3	Forward- TGGACGGCAGCCCTGCTC Reverse- GCTGCTCACTCTCTGGGTAC	154	60	Used in qRT-PCR using cDNA synthesized from cell lines and breast tumors
4	GPER-v4	Forward- GCGGGTCTCT TCCTCTCTC Reverse- GCTGCTCACTCTCTGGGTAC	166	60	Used in qRT-PCR using cDNA synthesized from cell lines and breast tumors
5	Gper	Forward- GGGTGCTTCTGTTCCTCTCC Reverse- TTGGGTGTCCTGATCTGTGC	145	66	Used in qRT-PCR using cDNA synthesized from mouse mammary gland
6	18S rRNA	Forward - GGAGAGGGAGCCTGAGAAAC Reverse - CCTCCAATGGATCCTCGTTA	174	62	Used in qRT-PCR using cDNA synthesized from mouse mammary gland
7	Gapdh	Forward- GGCCGGGGCCCACTTGAAG Reverse- TGGATGACCTTGGCCAGGGGG	174	68	Used in qRT-PCR using cDNA synthesized from mouse mammary gland
8	GPER-ChIP	Forward- ATCTGGACAGCCTCACGCAG Reverse- ACGGCCCATGAAGACTGTGC	348	58	Used in ChIP
9	pS2	Forward- CATTGCCTCCTCTCTGCTCC Reverse- ACTGTTGTCACGGCCAAGCC	423	58	Used in ChIP (+ve control)
10	ESR1	Forward- GCCCTACTACCTGGAGAA Reverse- CCCTTGTCATTGGTACTGG	132	60	Used in routine RT- PCR
11	Cyclophilin A	Forward- GGGCCGCGTCTCCTTTGAGC Reverse- GGCGTGTGAAGTCACCACCC	158	60	Used in routine RT-PCR

Table 2. Association of the GPER expression with the histopathological parameters (TCGA-BRCA cohort).

	GPER-high	GPER-low	p
Age			
Mean ± SD	57.6 ± 14.0	58.3 ± 12.7	T: 0.4875
Median	58	58	
Range	26-90	31-90	
ERα			
ERα-positive	319 (57.48)	236 (42.52)	< 0.0001
ERα-negative	39 (24.22)	122 (75.78)	
PR			
PR-positive	283 (58.23)	203 (41.77)	< 0.0001
PR-negative	75 (32.61)	155 (67.39)	
Molecular type			
Normal-like	9 (50.00)	9 (50.00)	
Luminal A	233 (66.38)	118 (33.62)	<0.0001
Luminal B	74 (44.58)	92 (54.42)	
Basal-like	32 (26.02)	91 (73.98)	
HER2-enriched	10 (17.24)	48 (82.76)	
Tumor Stage			
Stage I	68 (56.67)	52 (43.33)	0.2075
Stage II	196 (47.80)	214 (52.20)	
Stage III	85 (52.47)	77 (47.53)	
Stage IV	4 (28.57)	10 (71.43)	
Stage X	5 (50.00)	5 (50.00)	

Number within the braces indicates % of GPER-high or -low in various categories. The p-values (p) are from Chi-squared test, T: student's t-test. In all the tests $p < 0.05$ was considered as significant.

Table 3. Association of GPER expression with immunohistochemical markers in tumor samples of the NEIGRIHMS cohort.
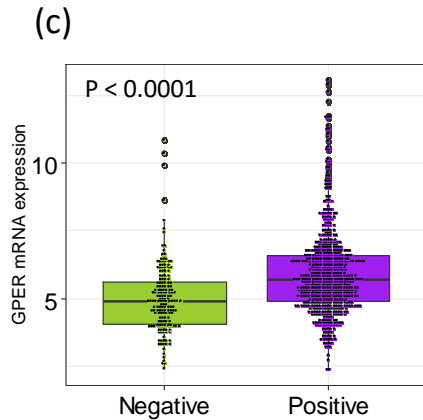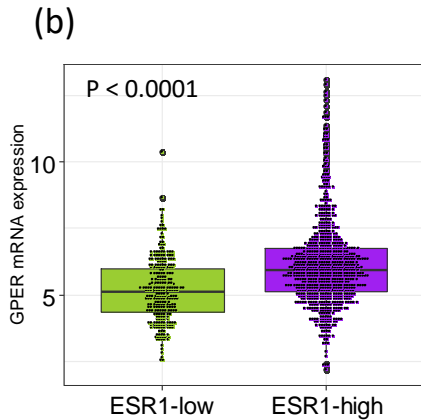
	GPER-positive (44/65)	GPER-negative (21/65)	p
ERα			
Positive	33	10	0.048
Negative	11	11	
PR			
Positive	32	9	0.028
Negative	12	12	

The p values (p) are from Chi-squared test and p < 0.05 was considered as significant.

Table 4. GPER expression in tumor samples of the NEIGRIHMS cohort and its association with clinicopathological parameters

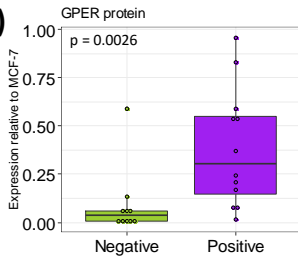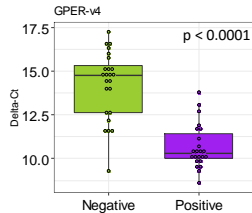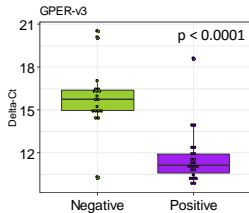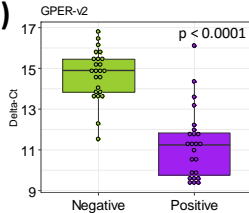|	GPER-positive (44/65)	GPER-negative (21/65)	p
TNM stage			
Early (Stage 1 & 2)	21	9	F: 0.79
Advanced (stage 3 & 4)	23	12	C: 0.92
Bloom Richardson Grade			
1	11	4	F: 0.838
2	18	8	
3	15	9	
Lymphovascular invasion			
Positive	21	4	F: 0.031
Negative	23	17	
Age			
Mean ± SD	47 ± 11.95	48.9 ± 11.17	T: 0.55
Median	46.5	48	M: 0.77
Range	26 to 86 yrs	35 to 86 yrs	
Tumor type			
IDC	39	20	F: 0.65
Others	5	1	
Margin type			
Invasive	39	19	F: 1.00
Pushing	5	2	
Tumor size			
Mean ± SD	4.020 ± 1.896	4.476 ± 1.735	T: 0.355
Median	3.75	4	M: 0.246
Range	1.5 to 11 cm	1.5 to 6 cm	
Lymph node status			
Involved	23	12	F: 0.79
Uninvolved	21	9	
Molecular type			
Luminal A	20	7	F: 0.115
Luminal B	13	3	
Triple negative	2	4	
HER2/neu type	9	7	

The p values are from C: Chi-squared test, T: student's t-test, F: Fisher's exact test, M: Mann-Whitney U test. In all the tests $p < 0.05$ was considered as significant.

(a) ESR1-low / ESR1-high

(b) P < 0.0001

(c) P < 0.0001

(a)

ERα +
ERα -

Marker, MCF-7, BC 3, BC 7, BC 12, BC 21, BC 2, BC 13, BC 19

50 kda — GPER

20 kda
15 kda — H3

(b) GPER protein

p = 0.0026

Expression relative to MCF-7

Negative   Positive

(c)

GPER-v2

p < 0.0001

Delta-Ct

Negative   Positive

GPER-v3

p < 0.0001

Delta-Ct

Negative   Positive

GPER-v4

p < 0.0001

Delta-Ct

Negative   Positive

(a) ERα-positive

GPER (210640_s_at)

HR = 0.77 (0.6 - 0.98)
logrank P = 0.035

Probability

Time (months)

Expression
— low
— high

	Number at risk					
low	367	280	100	37	8	2	0
high	942	749	344	73	9	1	0

(b) ERα-negative

GPER (210640_s_at)

HR = 1.4 (1.02 - 1.92)
logrank P = 0.035

Probability

Time (months)

Expression
— low
— high

	Number at risk				
low	345	209	63	15	2
high	225	128	46	18	2

(a) MCF7

(b) T47D

E2 (10 nM):	-	+	-	-	+	-
PPT (100 nM):	-	-	+	-	-	+
TAM (10 μM):	-	-	-	+	+	+

(c)

| ERα SiRNA: | - | - | + | + |
| E2 (10 nM): | - | + | - | + |

(d)

| ERα SiRNA: | - | - | + | + |
| E2 (10 nM): | - | + | - | + |

(e)

(f)