

NHERF1 inhibits proliferation of triple-negative breast cancer cells by suppressing GPER signaling

YAN WANG¹, ZHIQIANG PENG¹, RAN MENG¹, TAO TAO¹, QIQI WANG¹, CHUNJUAN ZHAO¹,
HUA LIU^{1,2}, RAN SONG^{1,2}, JUNFANG ZHENG^{1,2}, QIONG QIN^{1,2} and JUNQI HE^{1,2}

¹Department of Biochemistry and Molecular Biology, Capital Medical University, Beijing 100069;

²Beijing Key Laboratory for Tumor Invasion and Metastasis, Beijing International Cooperation Base for Science and Technology on China-UK Cancer Research, Beijing 100069, P.R. China

Received November 10, 2016; Accepted April 19, 2017

DOI: 10.3892/or.2017.5649

Abstract. G protein-coupled estrogen receptor (GPER) signaling is activated in triple-negative breast cancer (TNBC); however, the detailed mechanisms of its regulation remain unclear. The present study aimed to elucidate the molecular mechanisms involved in GPER activation in TNBC. In MDA-MB-231 cells, a TNBC cell line, NHERF1 interaction with GPER was verified by co-immunoprecipitation and immunofluorescent staining assays. Overexpression of NHERF1 in MDA-MB-231 cells inhibited GPER-mediated proliferation and phosphorylation of ERK1/2 and Akt. Furthermore, NHERF1 expression levels were negatively correlated with the gene signatures of GPER activation, ERK1/2 and Akt signaling, and cell proliferation in early stage of TNBC tumors from the TCGA data set. Taken together, NHERF1 inhibited the activation of GPER-mediated signaling and suppressed the proliferation of triple-negative breast cancer cells. Loss of NHERF1 expression may play a pivotal role in the early stage of TNBC carcinogenesis.

Introduction

Breast cancer is the second most common cause of death for women around the world. Clinical decisions primarily rely on

the assessment of the expression of the endocrine receptors for estrogen (ER), progesterone (PR) and the aberrant expression of human epidermal growth factor receptor 2 (HER2). Triple-negative breast cancer (TNBC) is a breast tumor that lacks the expression of ER, PR and HER2 (1). Thus, TNBC is not sensitive to endocrine therapy or target therapy such as Herceptin against HER2. Patients with TNBC usually have a poorer outcome compared with other cancer subtypes due to its aggressive clinical behavior and a lack of recognized molecular therapy targets (2,3). Therefore, there is clearly an urgent need to better understand the molecular basis of TNBC.

G protein-coupled estrogen receptor (GPER, also called GPR30), a novel estrogen receptor belonging to GPCR family, is extensively expressed within organs such as breast, ovary, uterus, brain and kidney. Sharing the same ligand as traditional ER, estrogen binding with GPER can activate G protein and lead to production of cAMP, calcium mobilization, ERK1/2 and Akt signaling activation to promote cell growth and proliferation (4). GPER is expressed extensively in TNBC clinical specimens and positively associated with high recurrence of TNBCs (5). The study from Pandey *et al* (6) indicated that GPER activation by estrogen or hydroxytamoxifen, an ER antagonist but GPER agonist, promoted proliferation of SKBR3 ER-negative breast cancer cell line. Several studies reported that GPER activation by estrogen induced proliferation in TNBC cell lines (7-9). Treatment with 17 β -estradiol, G-1 (GPER specific agonist) and tamoxifen led to rapid activation of ERK signaling and significantly promoted the viability and motility of MDA-MB-468 and MDA-MB-436 TNBC cells (8). Therefore, overactivation of GPER has been considered as a key feature during the development and progression of TNBCs (5-7,10). However, the molecular mechanisms of GPER regulation in TNBC are still largely unknown. Better understanding of the mechanisms involved in promoting cell proliferation in TNBC development and progression may aid in the clarity of the pathogenesis of TNBC and advancement of more effective therapy.

GPER contains a PDZ binding motif at its carboxyl terminus. In our previous study, we found that Na⁺/H⁺ exchanger regulatory factor 1 (NHERF1, also identified as EBP50) interacts with GPER. NHERF1 is a PDZ protein with two tandem PDZ domains and an ERM domain (11). The

Correspondence to: Dr Qiong Qin or Professor Junqi He, Department of Biochemistry and Molecular Biology, Capital Medical University, No. 10 Xi Tou Tiao, You An Men, Beijing 100069, P.R. China
E-mail: qqin777@163.com
E-mail: jq_he@ccmu.edu.cn

Abbreviations: NHERF1, Na⁺/H⁺ exchanger regulatory factor 1; EBP50, ERM binding phosphoprotein of 50 kDa; TNBC, triple-negative breast cancer; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor; GPER, G protein-coupled estrogen receptor; GPR30, G protein-coupled receptor 30; TCGA, The Cancer Genome Atlas; GSEA, Gene Set Enrichment Analysis; CCLE, The Cancer Cell Line Encyclopedia

Key words: EBP50, GPR30, triple-negative breast cancer, ERK1/2, Akt, proliferation

interaction of NHERF1 with GPER is mediated by the PDZ2 domain of NHERF1 and the PDZ binding motif of GPER (12). Increasing evidence has shown that NHERF1 is involved in many tumor types such as glioblastoma (13), colorectal (14), and breast cancer (15). In ER-positive breast cancer, NHERF1 was upregulated and positively associated with activation of GPER signaling (12). Conversely, NHERF1 is deficient in two-thirds of the more aggressive ER-negative breast tumors (16,17). Stemmer-Rachamimov *et al* (18) reported that NHERF1 expression was lower or absent in ER-negative breast carcinoma. However, detailed mechanisms of PDZ proteins in regulating GPER activation in TNBC remain elusive.

In the present study, we first analyzed the expression levels of GPER in TCGA breast cancer dataset and found relatively lower levels of GPER mRNA in TNBCs as compared with normal breast tissues. Whereas, gene signatures of GPER activation were enriched in TNBC samples in comparison with normal breast tissue by Gene Set Enrichment Analysis (GSEA), indicating that GPER was over activated in TNBC. Thus, we proposed a hypothesis that GPER underwent some unknown mechanisms to facilitate GPER signaling activation in TNBC. We then used a cell model in further experiments and verified NHERF1/GPER interaction in MDA-MB-231 cells. We also found that NHERF1 overexpression inhibited GPER-mediated proliferation of MDA-MB-231 cells via inhibition of the phosphorylation of ERK1/2 and Akt. Furthermore, downregulation of NHERF1 was detected in TNBC cell lines and stage I of TNBC patients. Gene signatures of GPER activation, ERK1/2 and Akt pathways, and cell proliferation were positively associated with TNBC patients with lower NHERF1 expression. These results provide the first direct evidence that NHERF1 plays a critical role in the regulation of GPER signaling in TNBCs. NHERF1 downregulation in early stage of TNBCs may trigger overactivation of GPER signaling, leading to an enhancement of the proliferation and development of TNBC cancer cells.

Materials and methods

Cell culture and transfection. HEK293, MCF-7 and HTB-126 cells were grown in complete Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. MDA-MB-231, T47D, BT474 and SKBR3 cells were maintained in complete RPMI-1640 medium (RPMI-1640 with 10% FBS and 1% penicillin/streptomycin). MDA-MB-231 and MCF-7 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). HEK293, HTB-126, T47D, BT474 and SKBR3 were purchased from the Cell Resource Center of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. MDA-MB-231 cells that stably expressed HA-NHERF1 or HA-vector were selected with the growth medium containing 400 µg/ml of G418 and maintained in growth medium containing 200 µg/ml of G418 (Calbiochem, San Diego, CA, USA). All cells were cultured in a 37°C/5% CO₂ incubator. For stimulation of ERK and Akt signaling, cells were maintained in phenol red-free medium (Life Technologies, Inc., Carlsbad, CA, USA) for 24 h. The cells were treated with E2 (Sigma-Aldrich, St. Louis, MO, USA) or G-1 (Tocris Bioscience, Minneapolis, MN, USA) at 37°C for

indicated times. After the medium was removed, cells were harvested in sodium dodecyl sulfate (SDS) sample buffer and analyzed via western blotting. The cells were transfected by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The pBK-CMV-HA-vector and pBK-CMV-HA-NHERF1 plasmid were kindly provided by Dr Randy Hall from Emory University.

Antibodies and reagents. Protein A/G PLUS agarose (#sc-2003) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-MAP Kinase1/2 (Erk1/2) (#06-182) and anti-phospho-MAP Kinase1/2 (Erk1/2) (#05-481) antibodies were purchased from Millipore (Bedford, MA, USA). Anti-Akt (#9272) and anti-phospho-Akt (Ser473) (#4060s) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The monoclonal rabbit anti-GPER (#sc-48525-R) antibody was purchased from Santa Cruz Biotechnology. The monoclonal mouse anti-NHERF1 IgG2b (#MA1-19292) was from Thermo Fisher Scientific (Rockford, IL, USA). The polyclonal rabbit anti-HA (#561) antibody was obtained from MBL (Nagoya, Japan). Anti-GAPDH antibody was obtained from ZSGB-BIO (Beijing, China).

Western blotting. Whole cell lysates or immunoprecipitated samples were resolved using 10% SDS-PAGE gels and transferred to PVDF membrane (Millipore). The membranes were blocked with 5% non-fat dried milk for 1 h at room temperature, and the membranes were incubated in primary antibody overnight at 4°C. After washed with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated (ZSGB-BIO) or infrared fluorescent dyes (IRDye)-conjugated (LI-COR Biosciences, Lincoln, NE, USA) secondary antibodies for 1 h. The membranes were washed four times and detected by enhanced chemiluminescence (ECL) detection reagents (Thermo Fisher Scientific) or Odyssey infrared imaging system (LI-COR Biosciences), respectively.

Co-immunoprecipitation assay. Co-immunoprecipitation was performed as previously described (12). The cell lysates were incubated with IgG pre-bound to protein A/G-agarose beads in the presence or absence of anti-GPER antibody at 4°C for 3 h, and then the beads were washed with washing buffer at 4°C, 3,000 rpm for 1 min, 5 times, respectively. Precipitated fractions were resuspended in loading buffer and boiled for 5 min for eluting the proteins from the beads followed by western blotting.

Immunofluorescence. Immunofluorescence was performed as previously described (12). Cells on glass coverslips were rinsed with phosphate-buffered saline (PBS) three times. Cells were fixed in 4% paraformaldehyde for 20 min and perforated with 0.1% Triton X-100 in PBS for 5 min at room temperature. After washing three times with PBS, the cells on glass coverslips were stained with primary antibodies diluted in the blocking buffer (1% BSA in PBS) respectively for 1 h at room temperature. After washing three times, coverslips were incubated with Alexa-488/594-conjugated secondary antibodies (Life Technology; 1:100) for 45 min. After washing three times, nuclei were stained with DAPI. The coverslips were then mounted on glass slide and then were placed at room

temperature for 24 h. The slides were visualized by a confocal microscope (Leica TCS SP8; Leica Microsystems, Heidelberg, Germany) with a 63x oil immersion objective.

Proliferation assay. Cell Counting kit-8 (CCK8) assay was performed to detect the cell proliferation rate. MDA-MB-231 cells were seeded at a density of 3,000 cells/well into 96-well plates with phenol red-free RPMI-1640 medium with 2% charcoal stripped-FBS. Cells were cultured overnight and then treated with 10 nM E2 or control with 2% charcoal stripped-FBS for continuous stimulation and the proliferation rates were detected at 0, 48 and 96 h, respectively (19). The absorbance was detected at 450 nm.

The Cancer Genome Atlas (TCGA) data. Gene expression profiles of breast cancer were downloaded from The Cancer Genome Atlas (TCGA) database (<http://cancergenome.nih.gov/>). The mRNA levels of NHERF1 and GPER were analyzed in the present study. Clinical data were downloaded from cBioPortal database (www.cbioportal.org).

Gene set enrichment analysis. Correlation between the levels of NHERF1 and biological processes was analyzed using Gene Set Enrichment Analysis (GSEA v2.0, <http://www.broad.mit.edu/gsea/>). The gene sets of ERK, Akt and proliferation were downloaded from GSEA website. The gene set associated with GPER activation was obtained from GEO datasets (GSE28645) and analyzed with GEO2R. GSEA calculates a pathway Enrichment Score (ES) that evaluates whether genes from pre-defined gene set were enriched among the highest- (or lowest-) ranked genes or distributed randomly. Default settings were used. Thresholds for significance were determined by permutation analysis (1000 permutations). False discovery rate (FDR) was calculated. A gene set was considered significantly enriched when the FDR score was <0.25 as described on the GSEA website.

Statistical analysis. Statistical analyses were performed using the SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). Results are expressed as mean \pm SD. Two-tailed unpaired Student's t-test and Pearson's Chi-square test was used to determine statistical significance. Statistical significance was accepted at $p < 0.05$.

Results

The levels of GPER are downregulated and GPER signaling is activated in TNBC patients. Based on the reports from other groups which showed that activation of GPER signaling was detected in TNBC (5-7), we firstly analyzed the mRNA levels of GPER in TCGA data containing a group of 112 normal breast tissues and another group of 112 TNBC patients. Data showed that GPER mRNA levels were significantly lower in TNBCs as compared with normal breast tissues (Fig. 1A), and there was no mutation or copy number change of GPER in these specimens. As we analyzed the activation of GPER signaling by GSEA, results showed that gene signatures of GPER signaling were enriched in TNBCs in comparison with normal breast tissues (Fig. 1B). These findings confirmed that the GPER signaling in TNBC is activated in TNBCs.

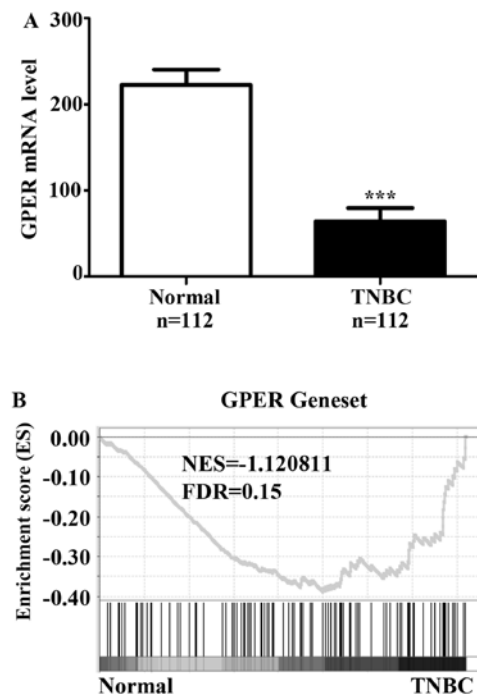


Figure 1. GPER mRNA levels are decreased and GPER signaling is activated in TNBC. (A) The levels of GPER mRNA were significantly downregulated in TNBCs as compared with normal breast tissues. Data of normal breast tissues and TNBC specimens were obtained from TCGA database. Significance between normal tissues (n=112) and TNBCs (n=112) was determined with a two tailed t-test assuming unequal variance. *** $p < 0.001$. (B) GPER signaling was activated in TNBCs (FDR=0.15). The mRNA levels in TNBCs and normal breast tissues were normalized with GPER. Gene signatures of GPER signaling were enriched in TNBCs in comparison with normal breast tissues by GSEA plots.

NHERF1 interacts with GPER in MDA-MB-231 breast cancer cells. We previously found that NHERF1 interacts with GPER in ER-positive breast cancer (12). NHERF1 has been reported to regulate the biological function of multiple receptors via protein-protein interaction (20). It is possible that NHERF1/GPER interaction may be involved in regulating GPER-mediated signaling in TNBCs. In order to confirm NHERF1/GPER interaction in triple-negative breast cancer cells, MDA-MB-231 cells which express low levels of NHERF1, were stably transfected with HA-NHERF1. Ectopic HA-NHERF1 overexpression was confirmed by western blotting (Fig. 2A). Lysates of MDA-MB-231 cells stably expressing HA-NHERF1 were immunoprecipitated (IP) with anti-GPER followed by western blotting. A strong HA-NHERF1 signal was detected in protein-A/G-GPER-IP complex, whereas no detectable immunoreactivity was observed in the protein-A/G control IP complex (Fig. 2B), indicating that GPER interacts with HA-NHERF1 in MDA-MB-231 cells. The co-localization of NHERF1/GPER was investigated by immunofluorescent staining. Cells were immunostained with anti-NHERF1 and anti-GPER antibodies. As shown in Fig. 2C, NHERF1 was found to co-localize with GPER in MDA-MB-231 cells mostly in the cytoplasm. Taken together, these data indicate that NHERF1 interacts with GPER in MDA-MB-231 cells.

NHERF1 overexpression inhibits GPER-mediated proliferation of MDA-MB-231 cells. GPER activation has been

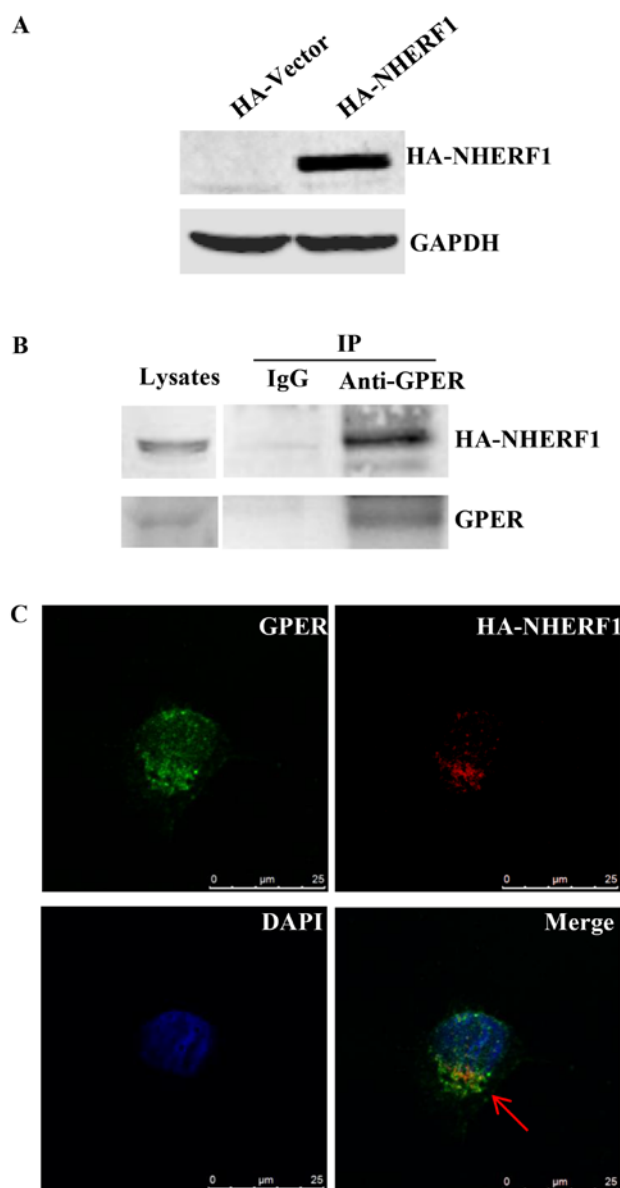


Figure 2. NHERF1 interacts with GPER in MDA-MB-231 cells. (A) NHERF1 was overexpressed in MDA-MB-231 cells. Western blot analysis of NHERF1 expression in MDA-MB-231 cells stably transfected with HA-NHERF1 or HA-vector plasmids. GAPDH was used as a loading control. (B) NHERF1 interacted with GPER in MDA-MB-231 cells. Lysates of MDA-MB-231 cells stably transfected with HA-NHERF1 were incubated with anti-GPER antibody for immunoprecipitation. The precipitated complexes (IP) and cell lysates (Input) were analyzed with anti-GPER or anti-HA antibodies in western blotting. (C) GPER co-localized with HA-NHERF1 in MDA-MB-231 cells. MDA-MB-231 cells stably transfected with HA-NHERF1 were stained with anti-GPER and anti-NHERF1 antibodies followed by Alexa 488- and Alexa 594-conjugated secondary antibodies. Nuclei were stained with DAPI. Co-localization of GPER with NHERF1 is shown in yellow after merging of the two images.

widely found to promote proliferation of triple-negative breast cancer cells (5-7). In order to understand the roles of NHERF1 in GPER-mediated proliferation, cells were serum deprived overnight in phenol red-free medium with 2% charcoal stripped-FBS and then cultured with 17β -estradiol (E2, 10 nM) for 0, 48 or 96 h for CCK-8 proliferation assay. In ER α -negative MDA-MB-231 cells, E2 binds with GPER to activate GPER signaling. As shown in Fig. 3, stimulation of E2

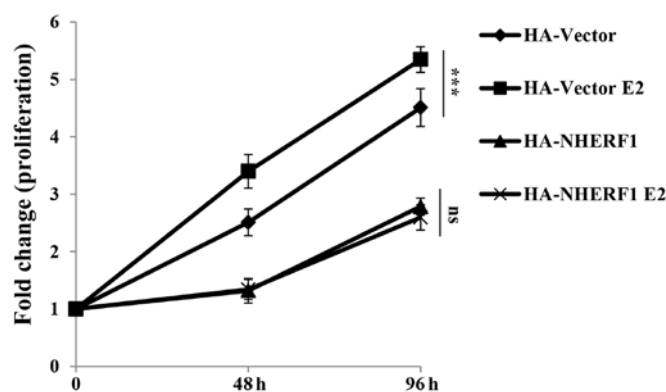


Figure 3. NHERF1 inhibits GPER-mediated proliferation of MDA-MB-231 cells. MDA-MB-231 cells stably overexpressed HA-NHERF1 or HA-vector were treated with or without 10 nM E2 for 0, 48 and 96 h, respectively. Proliferative cells were determined by CCK-8 assay. Values are expressed relative to the absorbance of cells detected at 0 h. Error bars represent SD. *** $p < 0.001$.

significantly promoted the proliferation of MDA-MB-231-HA-vector cells as compared with cells without E2 stimulation. This finding is consistent with reports from other groups which showed GPER stimulation by estrogen enhanced proliferation of TNBC cell lines such as HCC1806 and MDA-MB-468 (7-9). Stable overexpression of NHERF1 inhibited cell proliferation as compared with control cells, which indicated that NHERF1 overexpression inhibited proliferation of MDA-MB-231 cells. However, the proliferation rate of NHERF1 overexpressed cells in groups treated with E2 was similar as compared with the same cells without E2 stimulation. These findings suggested that NHERF1 overexpression inhibited GPER-mediated MDA-MB-231 cell proliferation.

NHERF1 inhibits GPER-mediated ERK1/2 and Akt signaling in MDA-MB-231 cells. Next, we studied the molecular mechanisms underlying NHERF1 inhibition of GPER-mediated proliferation. GPER has been reported to promote cell proliferation via ERK1/2 and Akt signaling (8), thus, we further investigated the effects of NHERF1 overexpression in the activation of GPER-mediated ERK1/2 and Akt signaling. MDA-MB-231 cells stably expressed HA-NHERF1 or control vectors were serum deprived for 24 h in phenol red-free medium and stimulated with E2 (10 nM) or G-1 (1 μ M) for 10 min to detect the levels of phosphorylated ERK1/2 (pERK1/2), or 15 min for the phosphorylated Akt (pAkt). Results showed that the levels of pERK1/2 and pAkt were robustly increased upon E2 or G-1 stimulation in control MDA-MB-231 cells, whereas the total levels of ERK1/2 or Akt had no detectable change (Fig. 4). However, when MDA-MB-231 cells overexpressed NHERF1, the phosphorylation of ERK1/2 and Akt upon E2 or G-1 treatment was significantly attenuated as compared with control cells (Fig. 4). These data suggested that NHERF1 inhibited GPER-mediated ERK1/2 and Akt activation in triple-negative breast cancer cells.

NHERF1 is downregulated in TNBC cell lines and early stage of TNBC specimens. *SLC9A3R1* (NHERF1) gene promoter contains estrogen responsive elements and absence of NHERF1 expression has been found in most of the more aggressive

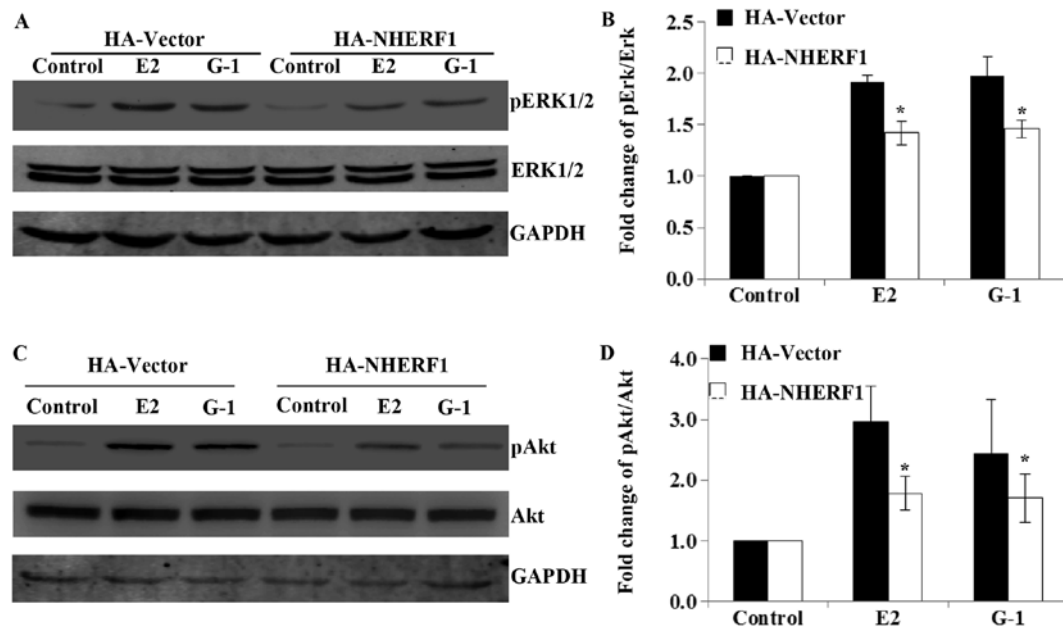


Figure 4. NHERF1 inhibits GPER-mediated ERK1/2 and Akt activation. MDA-MB-231 cells stably transfected with HA-vector or HA-NHERF1 were treated with serum and phenol red-free medium for 24 h followed by stimulation with E2 (10 nM) or G-1 (1 μ M). (A and B) The activation time of ERK1/2 was 10 min. The phosphorylation of ERK was analyzed with anti-phospho-MAP Kinase1/2 antibody and normalized with the levels of ERK in western blotting. (C and D) The activation time of Akt was 15 min. The phosphorylation of Akt was analyzed with anti-phospho-Akt antibody and normalized with the levels of Akt in western blotting. * $p < 0.05$ as compared with the HA-vector group.

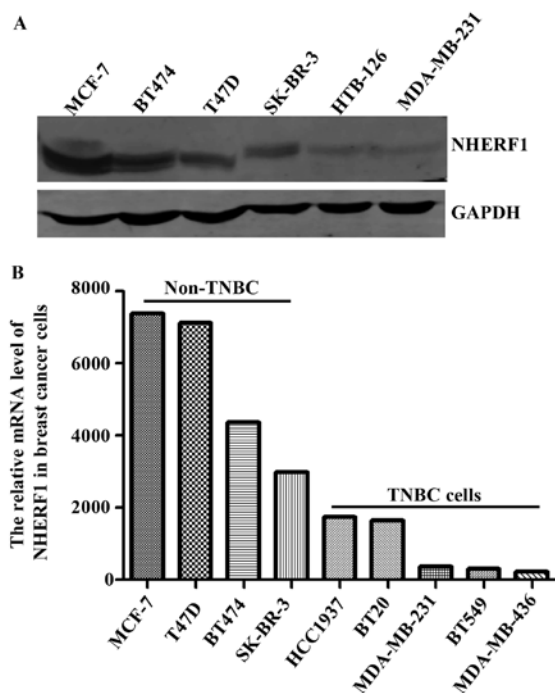


Figure 5. NHERF1 is downregulated in the TNBC cells at protein and mRNA levels. (A) NHERF1 protein levels were significantly decreased in TNBC cells. The protein levels of NHERF1 in breast cancer cells were measured by western blotting. (B) The NHERF1 mRNA levels of TNBC cells were lower than that in ER-positive breast cancer cells. The mRNA expression data of breast cancer cells were downloaded from CCLE (<https://portals.broadinstitute.org/ccle/home>).

ER-negative breast tumors (16,21). However, the expression levels and roles of NHERF1 in subsets of breast tumors are still controversial (18). In order to clarify the expression levels

Table I. Clinical information of TNBC patients in TCGA data set.

Variables	TNBC
Patient, n	112
Age, years, mean (range)	55 (29-90)
Stage, n (%)	
I	20 (18)
II	70 (63)
III/IV	19 (17)
Unknown	3 (2)

TNBC, triple-negative breast cancer.

of NHERF1 protein in TNBCs, we first analyzed the protein levels of NHERF1 in multiple TNBC cell lines. As shown in Fig. 5A, the protein levels of NHERF1 were relatively lower in HTB-126 and MDA-MB-231 TNBC cells as compared with non-TNBC cell lines. We further analyzed the mRNA levels of NHERF1 by using data of TNBC cell lines from The Cancer Cell Line Encyclopedia (CCLE) database. Consistent with data from our cell lines, results from CCLE showed relatively lower levels of NHERF1 mRNA in TNBC cell lines (Fig. 5B).

To verify whether the levels of NHERF1 are also decreased in TNBC clinical specimens, we analyzed the mRNA levels of NHERF1 in TCGA database. Totally 112 TNBC patients were stratified into 3 groups according to stage (Table I). After removing cases with the outlier values of NHERF1 (mean \pm SD) and cases lacking staging information, in total 92 cases of normal breast tissues and 91 cases of TNBC specimens

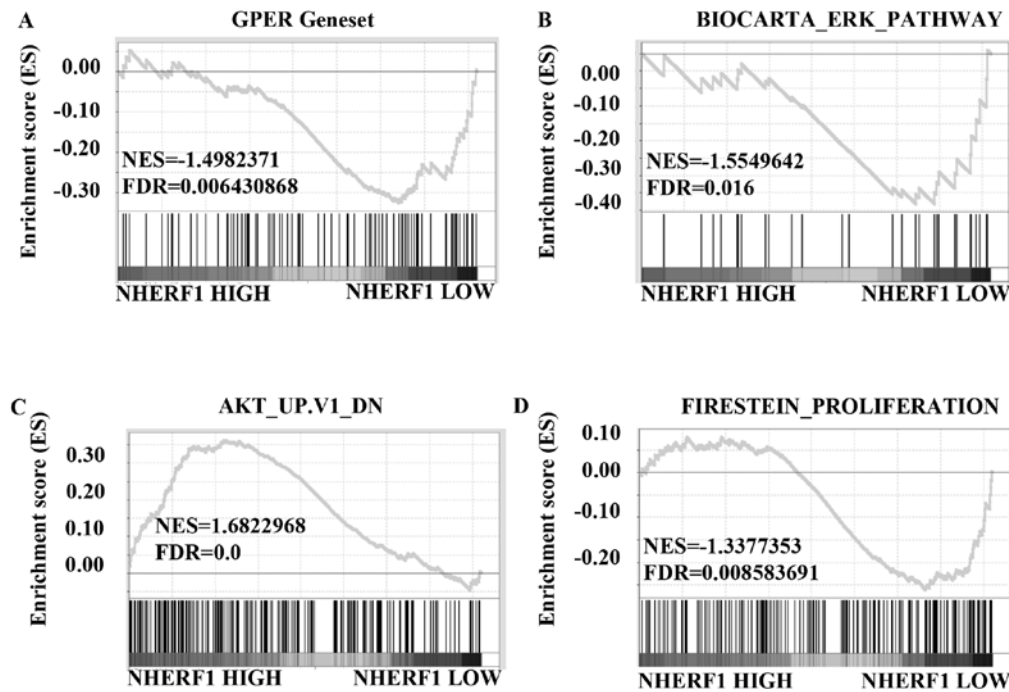


Figure 6. NHERF1 is negatively associated with activation of GPER, ERK and Akt pathways and proliferation genes in stage I TNBC. Enrichment plots of gene expression signatures for GPER, ERK and Akt signaling according to NHERF1 mRNA expression levels by GSEA of TCGA TNBC databases. (A) NHERF1 was negatively correlated with the activation of GPER signaling. Enrichment plots of GSEA showed that gene signatures of GPER signaling were enriched in patients of NHERF1-lower expression group. (B) NHERF1 restrained the activation of ERK1/2 signaling pathway. Enrichment plots of GSEA showed that the gene signatures of ERK1/2 activation (BIOCARTE_ERK_PATHWAY) were enriched with subgroup of NHERF1-Lower expression. (C) NHERF1 suppressed the activation of Akt signaling pathway. Enrichment plots of GSEA showed that the gene signatures of Akt inactivation (Akt_UPV1_DN) were enriched with subgroup of NHERF1-Higher expression. (D) NHERF1 blocked cell proliferation in stage I TNBCs. Enrichment plots of GSEA showed that the gene signatures of cell proliferation (FIRESTEIN_PROLIFERATION) were enriched with subgroup of NHERF1-lower expression. False discovery rate (FDR) gives the estimated probability that a gene set with a given normalized ES (NES) represents a false-positive finding; FDR <0.25 is an accepted cut-off for the identification of biologically significant gene sets.

Table II. Expression levels of NHERF1 in normal and TNBC specimens.

Variables	No. of patients	NHERF1 mRNA expression		P-value
		High n (%)	Low n (%)	
Normal	92	47 (51)	45 (49)	-
Stage I	16	6 (7)	10 (63)	0.046 ^a
Stage II	59	34 (55)	25 (45)	0.571
Stage III/IV	16	7 (44)	9 (56)	0.322

P-values were obtained by Pearson's Chi-square test, ^ap<0.05.

were included for analysis. The tissues from normal breast and different stages (I, II and III) of TNBCs were divided into NHERF1 high/low expression groups, respectively, and the case numbers from three stages of TNBCs with NHERF1 high/low expression were compared with that of normal group by Chi-square test. The mRNA levels of NHERF1 in stage I were significantly lower than that in the normal group (Table II). There was no statistical difference for the later stages of TNBCs. These data indicated that decreased levels

of NHERF1 may contribute to development of early stage TNBCs.

NHERF1 level is negatively correlated with GPER activation and cell proliferation in early stage of TNBCs. To further investigate the correlation between the levels of NHERF1 and GPER-mediated signaling in TNBCs, data from stage I TNBCs were divided into high/low NHERF1 expression groups and further analyzed using GSEA method. The results showed that gene signatures of GPER activation were enriched in stage I TNBC patients with low levels of NHERF1 (Fig. 6A; FDR=0.006). We further analyzed the correlation of NHERF1 levels with the gene signatures of ERK1/2 and Akt signaling activation. Results in Fig. 6B showed that gene signatures of ERK1/2 pathway activation were enriched in stage I TNBC patients with lower NHERF1 levels (FDR=0.016). Similarly, gene signatures of inhibiting Akt pathway were enriched in stage I TNBC patients with higher NHERF1 levels (Fig. 6C; FDR=0.0). Data of GSEA further confirmed that gene signatures of cell proliferation were enriched in stage I TNBC patients with lower NHERF1 levels (Fig. 6D; FDR=0.009), which was in accordance with data in Figs. 6A-C and 3. Taken together, NHERF1 level was negatively correlated with activation of GPER-mediated signaling, including ERK1/2 and Akt activation and proliferation in stage I TNBCs, suggesting that NHERF1 inhibited GPER-mediated activation of ERK and Akt and proliferation in the early stage of TNBCs.

Discussion

GPER has been implicated in the regulation of cancer cell proliferation (22,23) and was considered as an oncogenic receptor for carcinogenesis (10), especially in ER-positive (24) and triple-negative breast cancer (TNBC) (5). The roles of GPER in carcinogenesis are well established and its signaling pathway has become an attractive target for therapy (4,22,25). In a previous study, we found that NHERF1 interacted with GPER and regulated its signaling in ER-positive breast cancer (12). Therefore, it is interesting to know whether NHERF1 also interacts with GPER in ER-negative breast cancers including TNBCs. *SLC9A3R1* (NHERF1) is an estrogen-responsive gene and reports show that NHERF1 was downregulated in ER-negative breast cancer including TNBCs. We further confirmed downregulation of NHERF1 in TNBC cells lines in our present study (Fig. 5 and Table II). Several reports (5-7) and our analysis showed that GPER signaling was activated in TNBCs, thus we further investigated the correlation of NHERF1 downregulation with the activation of GPER signaling in TNBCs. The MDA-MB-231 cells, a TNBC cell line, were used as a cell model to detect the interaction of NHERF1 with GPER. As shown in Fig. 2, Co-IP result showed that NHERF1 interacted with GPER and immunocytochemistry study confirmed that NHERF1 was co-localized with GPER in MDA-MB-231 cells.

Upon binding with estrogen, GPER activation induces rapid cAMP signaling and calcium mobilization, as well as activation of ERK and Akt signaling to promote cell growth and proliferation. We observed that E2 treatment induced GPER-mediated proliferation of TNBC cells, whereas overexpression of NHERF1 inhibited E2-induced proliferation of MDA-MB-231 cells (Fig. 3). This finding was further confirmed by data in Fig. 4, in which NHERF1 overexpression inhibited E2- or G-1-induced activation of ERK1/2 and Akt in MDA-MB-231 cells. These data suggest that NHERF1 functions as a tumor suppressor in TNBCs by inhibition of GPER-mediated proliferation. Our findings are consistent with findings from other groups which demonstrated an oncogenic role of GPER in TNBC cells (5-9). In these reports, the activation of GPER was induced mostly by E2 or Tamoxifen. However, it seems that there are contradictory reports from several groups which showed that after stimulation by G-1, GPER had tumor suppressive activity in ER-negative breast cancer cells, including TNBC cells such as MDA-MB-231 and MDA-MB-468 cells (26,27). In the present study, we found that stimulation by E2 or G-1 respectively led to activation of ERK1/2 and Akt (Fig. 4). Difference between E2 and G-1 property, cell lines and experimental conditions may be accountable for the result discrepancy from different research groups.

As a downstream target effector of ER signaling, NHERF1 was downregulated both in mRNA and protein levels in TNBC cells in our laboratory and CCLE dataset (Fig. 5), which is consistent with other reports using TNBC cells (21). Data from TCGA further showed that NHERF1 was significantly downregulated in early stage of TNBC (Table II). NHERF1 has been reported to inhibit growth of ER-positive breast cancer cells such as MCF-7 and T47D in culture and a xenograft model (28). In the present study, we also found that NHERF1

elicited inhibitory effects of growth in MDA-MB-231 TNBC cells (Fig. 3). Therefore, it is possible that decreased levels of NHERF1 may also contribute to the growth of TNBCs. GSEA of TCGA data showed that NHERF1 expression levels were negatively correlated with the gene signatures of GPER activation, ERK1/2 and Akt signaling, and cell proliferation in early stage of TNBC tumors (Fig. 6). These findings provided clinical evidence for a potential suppressive role of NHERF1 in TNBC. Why there was no correlation of NHERF1 with GPER signaling in later stages was elusive. It is possible that mechanisms other than NHERF1 may be involved in promoting TNBCs development into later stage. It is necessary to point out the limitation in Fig. 6A regarding to GPER gene set. To date, there is no established GPER gene set available to verify the activation of GPER downstream genes in TNBC. The GPER gene signatures obtained via public GEO data in current study need further verification.

NHERF1 has been reported to interact with several GPCRs such as PTHR and β 2AR by regulating G protein or cAMP concentration to regulate downstream signaling (29), and NHERF1 is also found to interact with GPER (12). In the present study, NHERF1 was found to inhibit GPER-mediated Akt and ERK1/2 signaling in TNBC cells. How NHERF1 interacts with GPER to regulate the receptor signaling needs further investigation. NHERF1 has been reported to interact with epidermal growth factor receptor (EGFR) and stabilize EGFR at the cell surface to regulate the receptor downstream signaling (30). Since activation of GPER can lead to trans-activation of EGFR (31), it is possible that in early stage of TNBC development, multiple regulatory pathways of NHERF1 may coexist.

In summary, the present study showed that GPER levels were downregulated and GPER signaling was activated in the early stage of TNBCs. By using TNBC cell model, it was demonstrated that NHERF1 inhibited GPER-mediated proliferation via inhibition of ERK1/2 and Akt signaling. Activation of GPER-mediated signaling was correlated with lower NHERF1 expression in early stage of TNBCs. These findings indicate that GPER-mediated proliferation in TNBCs may be attributed to downregulation of NHERF1 levels.

Acknowledgements

The present study was supported by the National Natural Science Foundation of the People's Republic of China (nos. 81272887 and 81302373), the Beijing Municipal Natural Science Foundation (no. 7152014) and the Scientific Research Common Program of Beijing Municipal Commission of Education (KM201410025001).

References

1. Bianchini G, Balko JM, Mayer IA, Sanders ME and Gianni L: Triple-negative breast cancer: Challenges and opportunities of a heterogeneous disease. *Nat Rev Clin Oncol* 13: 674-690, 2016.
2. Collignon J, Lousberg L, Schroeder H and Jerusalem G: Triple-negative breast cancer: Treatment challenges and solutions. *Breast Cancer (Dove Med Press)* 8: 93-107, 2016.
3. Kalimutho M, Parsons K, Mittal D, López JA, Srihari S and Khanna KK: Targeted therapies for triple-negative breast cancer: Combating a stubborn disease. *Trends Pharmacol Sci* 36: 822-846, 2015.

4. Prossnitz ER and Barton M: Estrogen biology: New insights into GPER function and clinical opportunities. *Mol Cell Endocrinol* 389: 71-83, 2014.
5. Steiman J, Peralta EA, Louis S and Kamel O: Biology of the estrogen receptor, GPR30, in triple negative breast cancer. *Am J Surg* 206: 698-703, 2013.
6. Pandey DP, Lappano R, Albanito L, Madeo A, Maggiolini M and Picard D: Estrogenic GPR30 signalling induces proliferation and migration of breast cancer cells through CTGF. *EMBO J* 28: 523-532, 2009.
7. Girgert R, Emons G and Gründker C: Inactivation of GPR30 reduces growth of triple-negative breast cancer cells: Possible application in targeted therapy. *Breast Cancer Res Treat* 134: 199-205, 2012.
8. Yu T, Liu M, Luo H, Wu C, Tang X, Tang S, Hu P, Yan Y, Wang Z and Tu G: GPER mediates enhanced cell viability and motility via non-genomic signaling induced by 17 β -estradiol in triple-negative breast cancer cells. *J Steroid Biochem Mol Biol* 143: 392-403, 2014.
9. Girgert R, Emons G and Gründker C: Inhibition of GPR30 by estradiol prevents growth stimulation of triple-negative breast cancer cells by 17 β -estradiol. *BMC Cancer* 14: 935, 2014.
10. Lappano R, Pisano A and Maggiolini M: GPER function in breast cancer: An Overview. *Front Endocrinol (Lausanne)* 5: 66, 2014.
11. Hung AY and Sheng M: PDZ domains: Structural modules for protein complex assembly. *J Biol Chem* 277: 5699-5702, 2002.
12. Meng R, Qin Q, Xiong Y, Wang Y, Zheng J, Zhao Y, Tao T, Wang Q, Liu H, Wang S, *et al*: NHERF1, a novel GPER associated protein, increases stability and activation of GPER in ER-positive breast cancer. *Oncotarget* 7: 54983-54997, 2016.
13. Molina JR, Agarwal NK, Morales FC, Hayashi Y, Aldape KD, Cote G and Georgescu MM: PTEN, NHERF1 and PHLPP form a tumor suppressor network that is disabled in glioblastoma. *Oncogene* 31: 1264-1274, 2012.
14. Hayashi Y, Molina JR, Hamilton SR and Georgescu MM: NHERF1/EBP50 is a new marker in colorectal cancer. *Neoplasia* 12: 1013-1022, 2010.
15. Bellizzi A, Malfettone A, Cardone RA and Mangia A: NHERF1/EBP50 in breast cancer: Clinical perspectives. *Breast Care (Basel)* 5: 86-90, 2010.
16. Song J, Bai J, Yang W, Gabrielson EW, Chan DW and Zhang Z: Expression and clinicopathological significance of oestrogen-responsive ezrin-radixin-moesin-binding phosphoprotein 50 in breast cancer. *Histopathology* 51: 40-53, 2007.
17. Ediger TR, Kraus WL, Weinman EJ and Katzenellenbogen BS: Estrogen receptor regulation of the Na⁺/H⁺ exchange regulatory factor. *Endocrinology* 140: 2976-2982, 1999.
18. Stemmer-Rachamimov AO, Wiederhold T, Nielsen GP, James M, Pinney-Michalowski D, Roy JE, Cohen WA, Ramesh V and Louis DN: NHE-RF, a merlin-interacting protein, is primarily expressed in luminal epithelia, proliferative endometrium, and estrogen receptor-positive breast carcinomas. *Am J Pathol* 158: 57-62, 2001.
19. Yao W, Feng D, Bian W, Yang L, Li Y, Yang Z, Xiong Y, Zheng J, Zhai R and He J: EBP50 inhibits EGF-induced breast cancer cell proliferation by blocking EGFR phosphorylation. *Amino Acids* 43: 2027-2035, 2012.
20. Saponaro C, Malfettone A, Dell'Endice TS, Brunetti AE, Achimas-Cadariu P, Paradiso A and Mangia A: The prognostic value of the Na⁺/H⁺ exchanger regulatory factor 1 (NHERF1) protein in cancer. *Cancer Biomark* 14: 177-184, 2014.
21. Dai JL, Wang L, Sahin AA, Broemeling LD, Schutte M and Pan Y: NHERF (Na⁺/H⁺ exchanger regulatory factor) gene mutations in human breast cancer. *Oncogene* 23: 8681-8687, 2004.
22. Barton M: Not lost in translation: Emerging clinical importance of the G protein-coupled estrogen receptor GPER. *Steroids* 111: 37-45, 2016.
23. Jacenik D, Cygankiewicz AI and Krajewska WM: The G protein-coupled estrogen receptor as a modulator of neoplastic transformation. *Mol Cell Endocrinol* 429: 10-18, 2016.
24. Filardo EJ, Graeber CT, Quinn JA, Resnick MB, Giri D, DeLellis RA, Steinhoff MM and Sabo E: Distribution of GPR30, a seven membrane-spanning estrogen receptor, in primary breast cancer and its association with clinicopathologic determinants of tumor progression. *Clin Cancer Res* 12: 6359-6366, 2006.
25. Srivastava DP and Evans PD: G-protein oestrogen receptor 1: Trials and tribulations of a membrane oestrogen receptor. *J Neuroendocrinol* 25: 1219-1230, 2013.
26. Weißenborn C, Ignatov T, Ochel HJ, Costa SD, Zenclussen AC, Ignatova Z and Ignatov A: GPER functions as a tumor suppressor in triple-negative breast cancer cells. *J Cancer Res Clin Oncol* 140: 713-723, 2014.
27. Wei W, Chen ZJ, Zhang KS, Yang XL, Wu YM, Chen XH, Huang HB, Liu HL, Cai SH, Du J, *et al*: The activation of G protein-coupled receptor 30 (GPR30) inhibits proliferation of estrogen receptor-negative breast cancer cells in vitro and in vivo. *Cell Death Dis* 5: e1428, 2014.
28. Pan Y, Wang L and Dai JL: Suppression of breast cancer cell growth by Na⁺/H⁺ exchanger regulatory factor 1 (NHERF1). *Breast Cancer Res* 8: R63, 2006.
29. Ardura JA and Friedman PA: Regulation of G protein-coupled receptor function by Na⁺/H⁺ exchange regulatory factors. *Pharmacol Rev* 63: 882-900, 2011.
30. Lazar CS, Cresson CM, Lauffenburger DA and Gill GN: The Na⁺/H⁺ exchanger regulatory factor stabilizes epidermal growth factor receptors at the cell surface. *Mol Biol Cell* 15: 5470-5480, 2004.
31. Filardo EJ: Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: A novel signaling pathway with potential significance for breast cancer. *J Steroid Biochem Mol Biol* 80: 231-238, 2002.