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ORIGINAL PAPER

GPER Mediates Estrogen-Induced Signaling and Proliferation in Human Breast Epithelial Cells and Normal and Malignant Breast

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Abstract 17β-Estradiol (estrogen), through receptor binding and activation, is required for mammary gland development. Estrogen stimulates epithelial proliferation in the mammary gland, promoting ductal elongation and morphogenesis. In addition to a developmental role, estrogen promotes proliferation in tumorigenic settings, particularly breast cancer. The proliferative effects of estrogen in the normal breast and breast tumors are attributed to estrogen receptor α. Although in vitro studies have demonstrated that the G protein-coupled estrogen receptor (GPER, previously called GPR30) can modulate proliferation in breast cancer cells both positively and negatively depending on cellular context, its role in proliferation in the intact normal or malignant breast remains unclear. Estrogen-induced GPER-dependent proliferation was assessed in the immortalized nontumorigenic human breast epithelial cell line, MCF10A, and an ex vivo organ culture model employing human breast tissue from reduction mammoplasty or tumor resections. Stimulation by estrogen and the GPER-selective agonist G-1 increased the mitotic index in MCF10A cells and proportion of cells in the cell cycle in human breast and breast cancer explants, suggesting increased proliferation. Inhibition of candidate signaling pathways that may link GPER activation to proliferation revealed a dependence on Src, epidermal growth factor receptor transactivation by heparin-bound EGF and subsequent ERK phosphorylation. Proliferation was not dependent on matrix metalloproteinase cleavage of membrane-bound pro-HB-EGF. The contribution of GPER to estrogen-induced proliferation in MCF10A cells and breast tissue was confirmed by the ability of GPER-selective antagonist G36 to abrogate estrogen- and G-1-induced proliferation, and the ability of siRNA knockdown of GPER to reduce estrogen- and G-1-induced proliferation in MCF10A cells. This is the first study to demonstrate GPER-dependent proliferation in primary normal and malignant human tissue, revealing a role for GPER in estrogen-induced breast physiology and pathology.

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Introduction

Normal growth and differentiation of the breast are under tight endocrine control. This is highlighted by the fact that further development of the mammary gland rudiment is not initiated until the gland is exposed to circulating 17β-estradiol (E2) at puberty [16, 38]. The actions of E2 in the breast involve genomic signaling via activation of ligand-dependent transcription factors, including estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) [12, 54]. E2 acts through ERα to promote proliferation of the epithelium in the developing gland at puberty, consequently promoting ductal elongation and outgrowth [8]. ERβ appears dispensable for pubertal mammary gland growth and development in murine models [38], but is instead responsible for terminal

differentiation of the mammary gland in late pregnancy, in preparation for lactation [28]. The proliferative effect of E2 can be reproduced in normal human breast tissue cultured in a physiologically relevant model ex vivo [22].

Although E2 is required for normal breast development, it also has a well-established role in breast carcinogenesis [32] with lifetime E2 exposure (i.e., early menarche, late first full-term pregnancy, and late menopause) linked to the risk of breast and other hormone-responsive tissue cancers [6, 15, 32, 60]. E2 signaling through ERα can directly induce proliferation of breast epithelial cells, increasing the chance of mutations in rapidly dividing breast epithelium [27, 69], while indirectly, E2 metabolism into oxidative byproducts can lead to DNA damage and breast carcinogenesis [79]. Whereas E2-induced proliferation in a nontumorigenic setting is highly regulated by paracrine mechanisms, in which the ER-negative cells represent the proliferative population, in a tumorigenic setting paracrine regulation is lost, and markers for proliferation and estrogen receptors overlap [49, 71, 78].

More recently, it has become accepted that, in addition to genomic signaling, E2 can modulate rapid cellular signaling, in part through the classical estrogen receptors [59, 62] associated with the plasma membrane [42]. These signaling pathways include the second messengers calcium and nitric oxide, receptor tyrosine kinases including the epidermal growth factor receptor (EGFR) and IGF, various G protein-coupled receptors (GPCRs), as well as nonreceptor kinases including phosphoinositide-3 kinase (PI3K), MAPK, Src, and protein kinases A and C [43].

It is now well documented that rapid E2-dependent signaling also occurs through the novel estrogen receptor GPER, a G protein-coupled receptor (originally designated GPR30) [63, 72]. E2 activation of GPER leads to transactivation of the EGFR and downstream activation of MAPK and PI3K signaling cascades [26]. Previous studies have shown that activation of GPER can promote proliferation in cancer cells, including ER-negative breast cancer cells [57, 74] and in vivo in the murine endometrium [19]; however, there is also evidence that GPER activation has an inhibitory role on proliferation in ER-positive MCF7 cells [4]. GPER expression has been observed in both normal breast tissue and breast tumors [3, 25, 34, 40, 47]. In a large retrospective study, high GPER protein expression was correlated with increased tumor size, the presence of distant metastasis, and HER-2/*neu* expression [25], suggesting GPER expression may be a predictor of more aggressive forms of breast cancer. Studies examining GPER expression and function in breast cancer highlight the importance of determining the contribution of GPER to E2-dependent functions in normal breast tissue and cells.

Given the established link between estrogen exposure and the risk of developing breast cancer, in the present study, we determined whether GPER contributes to E2-induced epithelial proliferation in immortalized nontumorigenic human breast cells (MCF10A) and in explants from normal human breast and human breast tumors. As E2 nonspecifically activates all three estrogen receptors, ERα, ERβ, and GPER, in order to selectively study the contributions of GPER, we have recently identified ligands with high selectivity towards GPER, including an agonist, G-1 [7], and an antagonist, G36 [20]. In the present study, we demonstrate that GPER is expressed in MCF10A cells, which express neither ERα nor ERβ [1, 18, 46, 61], and that both E2 and the GPER agonist G-1 stimulate an increase in mitotic in these cells, suggesting increased proliferation. E2-induced proliferation in MCF10A cells is dependent on EGFR transactivation via heparin-binding EGF (HB-EGF) and subsequent activation of ERK; however, ERK activation and proliferation are not dependent on the activation of matrix metalloproteinases (MMPs), a mechanism previously described for GPER-dependent ERK activation in breast cancer cell lines [26]. Proliferation is also induced in both normal and tumorigenic human breast tissue explants in response to E2 and G-1, and we demonstrate that proliferation is in part mediated by GPER, as the GPER-selective antagonist G36 partially abrogates this effect. Our results indicate that alongside ERα, GPER contributes to E2-induced proliferation in the breast, the first demonstration of GPER-mediated proliferation in primary normal human tissue.

Research Design and Methods

Reagents

DMEM, E2, fetal bovine serum (FBS), normal goat serum (NGS), insulin, cholera toxin, transferrin, hydrocortisone, and prolactin were from Sigma (St. Louis, MO, USA). Recombinant epidermal growth factor (EGF) and penicillin/streptomycin (P/S) were from Invitrogen (Grand Island, NY, USA). Bovine serum albumin (BSA) was from AMRESCO (Solon, OH, USA). Growth factor-reduced phenol red-free Matrigel™ was from BD Biosciences (San Jose, CA, USA). G-1 was synthesized as described [7] and provided by Jeffrey Arterburn (New Mexico State University, Las Cruces, NM, USA). Lipofectamine 2000 was from Invitrogen. Small-interfering RNA (siRNA) was from Dharmacon RNAi Technologies (Lafayette, CO, USA): ON-TARGET plus SMARTpool siRNA for GPER (L-005563-00) and ON-TARGETplus siControl Non-Targeting siRNA (D-001810-02).

Inhibitors and Antibodies

EGFR inhibitor tyrphostin AG1478, PI3K inhibitor LY294002, Src inhibitor PP2, MEK inhibitor U0126, and MMP inhibitor GM6001 were from Calbiochem (Billerica, MA, USA). Diphtheria toxin mutant CRM-197 (Berna Products, Coral Gables, FL, USA) and HB-EGF neutralizing antibody (R&D Systems,

Minneapolis, MN, USA) were a gift from Edward Filardo (Rhode Island Hospital, Providence, RI, USA). G36 was synthesized as described [20] and provided by Jeffrey Arterburn (New Mexico State University). Polyclonal antibody against a C-terminal peptide in the human GPER protein was used for GPER localization assays as previously described [63]. Rabbit anti-histone H3 antibody (phospho-Ser10) (anti-pH3) and mouse anti-β-actin antibody were from Millipore (Billerica, MA, USA). Rabbit anti-phospho-44/42 MAPK (ERK1/2) (Thr202/Tyr204) antibody was from Cell Signaling (Danvers, MA, USA). Rabbit anti-Ki67 and rabbit anti-ERα antibodies were from Neomarkers/Lab Vision (Thermo Fisher, Waltham, MA, USA). Mouse anti-α-tubulin antibody was from Sigma. Goat anti-rabbit IgG-Alexa 488-conjugated secondary antibody and goat anti-mouse IgG-Alexa 533-conjugated secondary antibody were from Invitrogen. Goat anti-rabbit IgG-HRP-conjugated antibody was from GE Healthcare (Waukesha, WI, USA) and goat anti-mouse IgG-HRP-conjugated antibody was from Cell Signaling.

Cell Culture

MCF10A human breast epithelial cells (ATCC, Manassas, VA, USA; catalog number CRL-10317) were maintained in MCF10A complete medium (DMEM/F-12 supplemented with 5 % horse serum, 10 μg/mL insulin, 100 ng/mL cholera toxin, 0.5 μg/mL hydrocortisone, 20 ng/mL recombinant epidermal growth factor, and 1 % penicillin/streptomycin (P/S)) [18]. Cells were cultured in a humidified atmosphere containing 5 % CO_2 at 37 °C. For proliferation assays, cells were passaged onto 12-mm glass coverslips (Electron Microscopy Sciences, Hatfield, PA, USA) and cultured for 24 h in phenol red-free MCF10A medium with all supplements listed above, except that 5 % charcoal stripped dextran-treated FBS was substituted for 5 % horse serum. Overnight, cell synchronization for proliferation and immunoblot analysis was performed as previously described [1] in phenol red-free growth medium, charcoal-stripped FBS reduced to 1 %, omitting EGF. Under these conditions, MCF10A cells growth arrest and remain viable [13]. After overnight synchronization, cells were stimulated for 24 h with vehicle control (dimethylsulfoxide, DMSO), 17β-estradiol (E2, 1 to 100 nM), G-1 (GPER-selective agonist, 1 to 100 nM), and G36 (GPER-selective antagonist, 5 to 500 nM) fixed in 4 % paraformaldehyde (PFA) in PBS for 15 min at room temperature. For some experiments, MCF10A cells were grown in 60-mm cell culture dishes and transfected with siRNA using Lipofectamine 2000 per manufacturer's instructions. For immunoblot analysis, cells were grown on 60-mm plates in phenol red-free MCF10A medium and stimulated following overnight synchronization.

For 3D assays, MCF10A cells were grown in growth factor reduced phenol red-free Matrigel™ on eight-well chamber slides (BD Falcon, San Jose, CA, USA). Approximately 5,000 MCF10A cells were seeded on 40 μL of Matrigel™ per chamber. Growth medium (described above) was supplemented with 2 % Matrigel™. The medium was changed every 2 days, and after 4 days in culture, the treatments were added to growth medium. Matrigel™ cultures were continued until day 10, and then they were fixed with 4 % PFA in PBS for 15 min at room temperature. Immunofluorescence assays were conducted on 2D and 3D MCF10A cells as previously described [18]. Images were captured on either a Zeiss 200M Axiovert inverted microscope (Carl Zeiss Inc. Thornwood, NY, USA), at 400× total magnification (2D cultures) or a Zeiss LSM 510 confocal microscope (3D cultures) at 400× total magnification and an optical thickness of 0.7 μM (3D cultures).

Tissue Samples

Human breast tissue was acquired from female patients undergoing reduction mammoplasty surgery between November 2007 and January 2011. Malignant and normal breast tissue remaining after pathological testing was collected for this study. Specimens were obtained from the University of New Mexico Hospital (UNMH) or from the Cooperative Human Tissue Network (CHTN Western division, Vanderbilt University, Nashville, TN, USA), a division of the National Cancer Institute. The University of New Mexico Health Sciences Center Institutional Review Board (IRB) approved this study protocol; all samples were de-identified. Tissue collected at UNMH was transported to the laboratory on ice in DMEM/F-12 medium containing 1 % P/S within 1–2 h of surgery. Tissue obtained from CHTN was shipped overnight on ice in RPMI medium (Sigma) supplemented with 1 % P/S. All tissue was dissected into 3 mm^3 pieces in phenol-red free DMEM/F-12 medium. For normal breast samples, the collagenous connective tissue containing epithelial elements were retained for explant culture and adipose tissue was excluded.

Explant Culture

Normal breast tissue was cultured as previously described [22], with a few modifications. Briefly, 1–2 mm pieces of mechanically minced breast tissue were placed on sterile lens paper supported by grids (500 μM Nitex nylon mesh, Tetko Inc., Briarcliff Manor, NY, USA) atop 35-mm tissue culture dishes (no lid), placed inside a 10-cm dish. The 35-mm dish was filled with complete medium (see below) so that the Nitex grid and lens paper were saturated with, but not submerged in, medium (i.e., at the liquid–air interface). The larger dish also contained 10 mL complete medium, to maintain high local humidity. Tumor tissue was fully submerged in medium in 24-well tissue culture dishes. Tissue was incubated overnight in a humidified atmosphere with a mixture of 5 % CO_2 and 95 % air at 37 °C in phenol-red free DMEM/F-12 medium supplemented with 1 % P/S, 10 μg/mL insulin, 3 μg/mL prolactin,

4 mg/mL transferrin, and 1 μg/mL hydrocortisone [22]. Following overnight incubation to allow the tissue to equilibrate, additions were made to the medium as described above for MCF10A cultures. Growth medium was changed every 2 days, and fresh treatments were added. Tissue was collected after 7 days of treatment and fixed in 4 % PFA in PBS overnight at room temperature.

Indirect Immunofluorescence (Tissue)

For immunofluorescence staining, paraffin sections (5 μm) were mounted on Super-Frost Plus slides (Menzel-Gläser, Thermo Fisher, Waltham, MA, USA). After rehydrating sections through a graded alcohol series to PBS, the slides were treated for antigen retrieval by boiling in a microwave oven in 0.01 M citrate buffer (pH 6.0) for 20 min. After three washes in PBS, the sections were incubated with PBS containing 0.1 % Triton X-100 and 3 % NGS (PBS-TN) for 30 min at room temperature to permeabilize cells and block nonspecific antibody binding. Tissue sections were then incubated with primary antibodies diluted in PBS-TN overnight at 4 °C in a humid chamber. Tissue sections were then washed and incubated with species-matched Alexa Fluor®-conjugated secondary antibodies (Invitrogen) diluted in PBS-TN for 1 h at room temperature in a dark chamber. Sections were mounted with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Labs, Burlingame, CA, USA) and sealed with nail polish. Images were captured on a Zeiss 200M Axiovert inverted microscope at 400× total magnification. For immunohistochemical analysis of ERα and GPER, tissue sections were incubated as described above with primary antibodies diluted in PBS-TN overnight at 4 °C in a humid chamber. Tissue sections were then washed and incubated with species-matched horseradish peroxidase (HRP)-conjugated secondary antibodies (Invitrogen) diluted in PBS-TN for 1 h at room temperature. After a series of wash steps, sections were incubated in 3,3′-diaminobenzidine (DAB) until reaction product was visible. Sections were then counterstained with hematoxylin, dehydrated through a graded alcohol series, and mounted with Permount® mounting medium (Fisher). Images were captured on a Nikon Eclipse E400 microscope with a Nikon DS-Fi1 camera (Nikon Corp., Melville, NY, USA) at 400× total magnification.

Western Immunoblotting

Cells were lysed in radioimmunoprecipitation (RIPA) buffer supplemented with sodium fluoride (50 mM), sodium orthovanadate (1 mM), phenylmethylsulfonylfluoride (1 mM), and protease inhibitor cocktail (1×). Protein concentration in cell lysates was determined by Bradford assay (BioRad, Hercules, CA, USA). Equal protein concentration was loaded on a 4–20 % gradient SDS-PAGE gel (Thermo-Scientific, Rockford, IL, USA) and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After blocking in Tris-buffered saline with 0.01 % Tween (TBS-T) containing 5 % nonfat dry milk for 1 h at room temperatures, the membranes were incubated with primary antibodies in TBS-T with 3 % BSA overnight at 4 °C with gentle rocking. After a series of washes in TBS-T, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG at 1:10,000 in TBS-T with 3 % BSA for 1 h at room temperature with gentle rocking. The blots were developed using Supersignal West Pico Chemilumiscent Substrate (Thermo Fisher). Films were then scanned and quantified using ImageJ software (National Institutes of Health).

Mitotic Index and Proliferation Quantitation and Statistical Analysis

For Ki67 and pH3 detection, immunostained cells were quantitated and expressed as a percentage of the total number of cells in each treatment sample (as determined by counting total DAPI-counterstained nuclei). For reduction mammoplasty tissue sections, quantitation was confined to immunostained luminal epithelia relative to total luminal epithelial cells. Quantitation was performed blind, and fields of view were chosen at random while viewing DAPI-stained nuclei to identify ductal and alveolar structures.

Data was graphed and analyzed using GraphPad Prism version 4.03 (GraphPad Software, San Diego, CA, USA). Statistical analysis performed with a one-way analysis of variance (ANOVA) within Prism estimates the correlation of variables (e.g., protein expression and proliferation) between treatment groups (e.g., control, E2, G-1, and G36). Pairwise comparisons of results between different treatment groups were determined using a one-way ANOVA followed by a Dunnett's test. Data represent the mean±SEM of three or more separate experiments. p Values less than or equal to 0.05 were considered to be significant.

Results

Estrogen Increases the Mitotic Index in MCF10A Cells

MCF10A cells have been used extensively as a model to study the behavior of normal breast epithelia in vitro because although they are immortalized, they are nontransformed and therefore nontumorigenic, and can recapitulate normal breast epithelial morphogenesis when cultured in three-dimensional (3D) recombinant basal lamina (i.e., Matrigel™) culture [18]. Because these cells are ERα and ERβ negative, they are not typically used in studies of E2 responsiveness. However, since GPER has been shown to mediate E2 signaling in ERα/β-negative breast cancer cell lines [26, 48], we sought to

determine whether GPER might mediate effects of E2 in ER-negative, human breast epithelial cells. To determine if MCF10A cells proliferate upon E2 stimulation, cells were cultured on tissue culture plastic in the presence of either vehicle control or E2 for 24 h, then fixed and immunostained with an antibody that recognizes a mitosis-specific phosphorylated form of histone H3 (phospho-ser10; pH3) [76]. We observed a statistically significant dose-dependent increase in the mitotic index of cells with E2 treatment, from 1 up to 100 nM, with a near-maximal difference (threefold) in the presence of 10 nM E2 yielding an EC_{50} of ~5 nM (Fig. 1a).

MCF10A Cells Express GPER

Since we observed that MCF10A cells are indeed E2 responsive, we first determined if they are ERα and ERβ-negative by RT-PCR, as previously reported [1, 18, 46, 61]; Supplemental Fig. 1). We also measured mRNA levels of the ERα splice variant, ERα36, which has been demonstrated both to mediate and inhibit E2-dependent signaling [45, 75, 82], although it has also been reported to be absent in MCF10A cells [75]. RT-PCR of ERα, ERβ, and ERα36 in MCF10A cells showed negligible expression compared to positive control cells [MCF7 for ERα [5], the melanoma cell line SK-MEL-29 for ERβ (Torres and Berwick, personal communication), and the neuroblastoma cell line U87 for ERα36 (our unpublished observations)] (Supplemental Fig. 1A–C). Moreover, ERα, ERβ, and ERα36 expression was not induced following 24 h of E2 treatment (Supplemental Fig. 1D). We next asked if GPER expression in MCF10A cells could explain the observed E2-induced increase in mitotic index, suggesting increased proliferation. GPER protein expression in MCF10A cells was demonstrated by both immunofluorescence detection and Western immunoblotting using a polyclonal antibody generated against a C-terminal peptide from the human GPER protein sequence [63] (Fig. 1b, c). GPER immunostaining revealed an intracellular pattern for GPER, consistent with previously described [63] endoplasmic reticulum/Golgi localization (Fig. 1b). GPER immunostaining decreased considerably in intensity following transfection with a GPER-specific siRNA (GPER siRNA), but not with transfection of nonspecific, control siRNA (Supplemental Fig. 2). Western immunoblotting using the anti-GPER antibody detected a specific polypeptide of MW ~55 kDa (Fig. 1c), consistent with published reports [75, 65], and which was diminished in cells transfected with GPER-specific siRNA (Fig. 1c, d). An additional polypeptide of lower molecular weight (~45 kDa) was also reduced by GPER siRNA

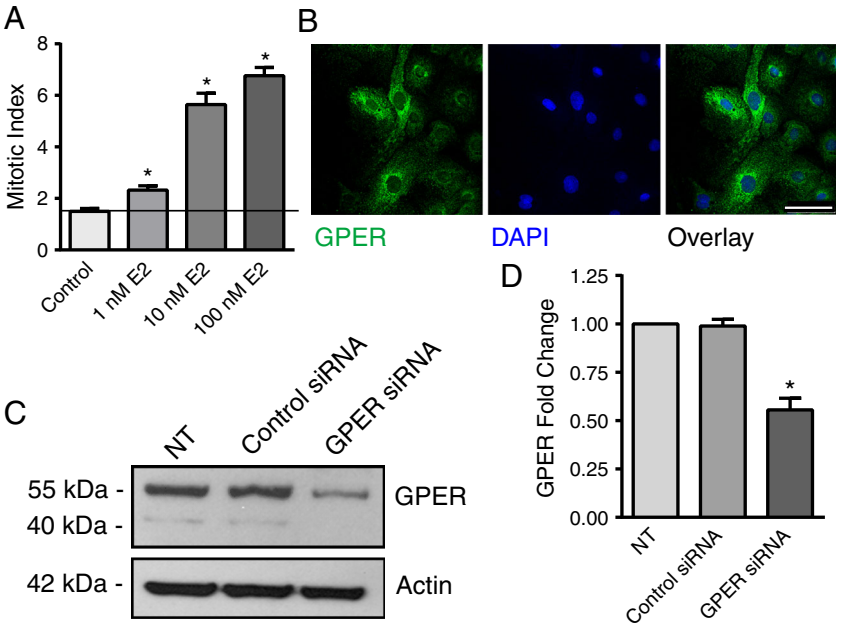

Fig. 1 17β-Estradiol stimulates proliferation in MCF10A cells. Mitotic index was assessed as a surrogate for proliferation by immunofluorescence using an anti-histone H3 (phospho-ser10) (pH3) antibody in MCF10A cells cultured in the presence of vehicle control or the indicated concentrations of E2 for 24 h (**a**). Data represents the average of three independent experiments. Results are expressed as mean ± SEM and statistical significance ($p \leq 0.05$) was assessed by one-way ANOVA followed by a Dunnett's test (*significantly different relative to control). GPER expression was assessed in MCF10A cells by immunofluorescence (**b**; *scale bar* 75 μm) and western immunoblotting (**c**), probing with an anti-human GPER C-terminal peptide antibody. Reduced GPER protein and RNA expression following siRNA knockdown was also confirmed (representative experiment shown in **c**). Cells transfected with nonspecific (scrambled) control siRNA express normal levels of protein (**c**). **d** Densitometric quantitation of three independent GPER immunoblots following no transfection (NT), or 72 h following transfection with control siRNA or GPER-specific siRNA. Quantitation is normalized to β-actin immunodetection. Results are expressed as mean ± SEM, and statistical significance ($p=0.0176$) was assessed by one-way ANOVA followed by a Dunnett's test (*statistically significant relative to nontransfected cells)

(Fig. 1c), suggesting the presence of hypo-glycosylated isoforms [65]. In some instances, we detect a higher molecular weight (~85 kDa) polypeptide (Supplemental Fig. 3A), likely reflecting a detergent-resistant complex as has been reported for GPER [65] and other GPCRs [51, 64]. We also demonstrated specificity of the C-terminal GPER peptide-specific antibody by peptide competition in both Western immunoblotting (Supplemental Fig. 3A) and immunohistochemistry of human breast reduction mammoplasty samples (Supplemental Fig. 3B).

Estrogen-Induced Proliferation is Mediated by GPER in MCF10A Cells

Given that GPER is expressed in MCF10A cells and E2 stimulation promoted proliferation, we evaluated the effect of the GPER-selective agonist G-1 on MCF10A proliferation. Cells stimulated with G-1 for 24 h exhibited a dose-dependent increase in mitotic index, with a near maximal (cf. E2) difference (threefold) at 100 nM compared to control (Fig. 2a). When MCF10A cells were stimulated with either E2 or G-1 combined with GPER-selective antagonist G36, proliferation was blocked. In contrast, G36 had no effect on EGF-induced proliferation (Fig. 2b). To further demonstrate that both E2- and G-1-induced proliferation are GPER-dependent, proliferation was assessed in MCF10A cells after GPER-targeted siRNA treatment. GPER siRNA transfection significantly reduced E2- and G-1-induced proliferation compared with control siRNA-transfected cells (Fig. 2c), but had no effect on EGF-induced proliferation (Fig. 2c). Reduced GPER protein expression following siRNA knockdown was confirmed by Western immunoblotting (Fig. 2d).

E2 and G-1 Induce ERK Activation in MCF10A Cells

As GPER has been reported to promote ERK phosphorylation in multiple tumor cell lines [26, 66] and ERK activation is frequently associated with cellular proliferation [81], we tested whether GPER activation in MCF10A cells results in ERK phosphorylation. In preliminary experiments, we determined that E2 and G-1 stimulation resulted in a time-dependent increase in pERK as assessed by densitometric quantitation of Western blots, standardized to actin loading controls, with peak activation occurring at 15 min (data not shown). All subsequent experiments were therefore conducted at 15 min. E2-and G-1-induced ERK phosphorylation compared to control-treated cells (Fig. 3a) and G36 significantly inhibited both E2- and G-1-induced ERK phosphorylation; G36 alone had no effect. Furthermore,

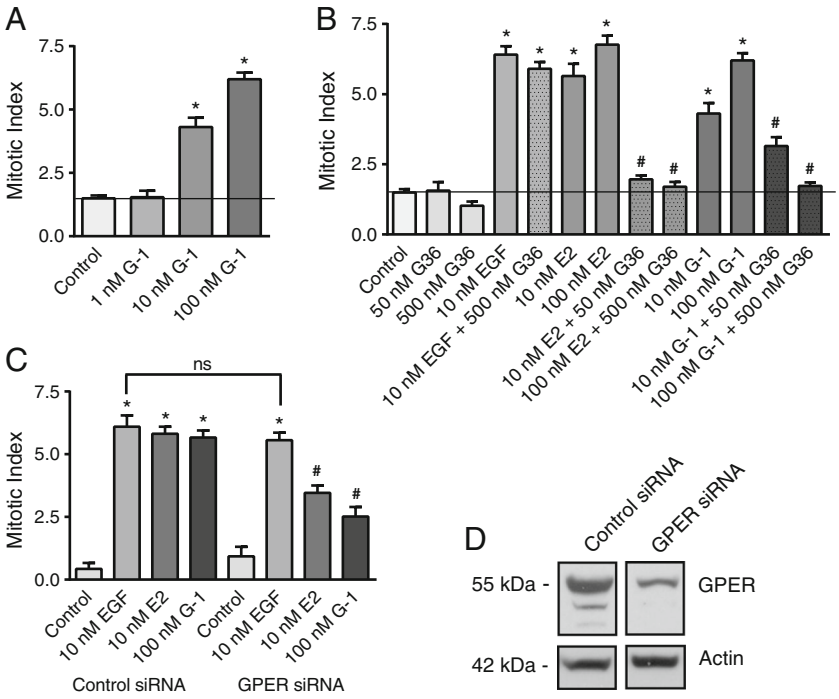

Fig. 2 E2 and G-1-induced proliferation is dependent on GPER in MCF10A cells. Mitotic index as a surrogate for proliferation was assessed in MCF10A cells grown on glass cover slips in the presence of indicated concentrations of GPER agonists (E2, G-1), antagonist (G36), or combinations, for 24 h (**a**, **b**). Proliferation was also assessed after GPER siRNA or control siRNA transfection followed by 24-h stimulation with E2 or G-1 (**c**). Mitotic index was quantified by immunofluorescence using an anti-pH3 antibody. GPER knockdown was confirmed by Western immunoblotting (**d**). Data is representative of a minimum of three independent experiments. Results are expressed as mean ± SEM and statistical significance ($p \leq 0.05$) was assessed by one-way ANOVA followed by a Dunnett's test (*significantly different relative to control; #significantly different relative to E2 or G-1; *ns* not significant)

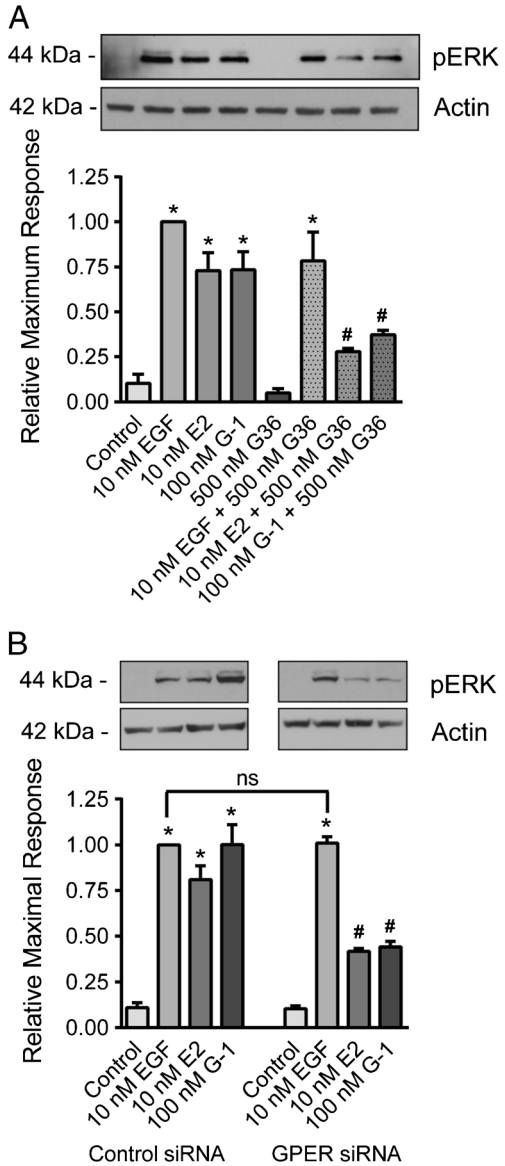

Fig. 3 GPER activation induces activation of the MAPK signaling cascade. MCF10A cells were stimulated with indicated concentrations of E2 or G-1 alone or in combination with GPER antagonist G36, for 15 min (**a**). Lysates were prepared and immunoblotted with antibodies specific to phospho-ERK (pERK). Equal protein loading was confirmed by β-actin immunoblotting. Histograms represent fold change (pERK relative to actin) in pERK protein expression, relative to control-treated cells. pERK was also assayed in cells transfected with control or GPER siRNA-treated cells 72 h after transfection and then stimulated with E2 or G-1 for 15 min (**b**). Data are representative of three independent experiments. Results are expressed as mean ± SEM and statistical significance ($p \leq 0.05$) was assessed by one-way ANOVA followed by a Dunnett's test (*significantly different relative to control; #significantly different relative to E2 or G-1)

GPER-targeted siRNA knockdown in MCF10A cells significantly reduced both E2- and G-1-induced ERK phosphorylation compared to control siRNA (Fig. 3b), while GPER knockdown had no effect on the level of EGF-induced ERK phosphorylation.

GPER-Dependent ERK Activation Requires EGFR Transactivation

Since GPER has been shown to transactivate the EGFR in breast cancer cell lines [26], we tested the ability of the EGFR-specific tyrosine kinase inhibitor, AG1478, to block E2- and G-1-induced ERK phosphorylation in MCF10A cells (Fig. 4a). In addition, we tested the ERK inhibitor, U0126

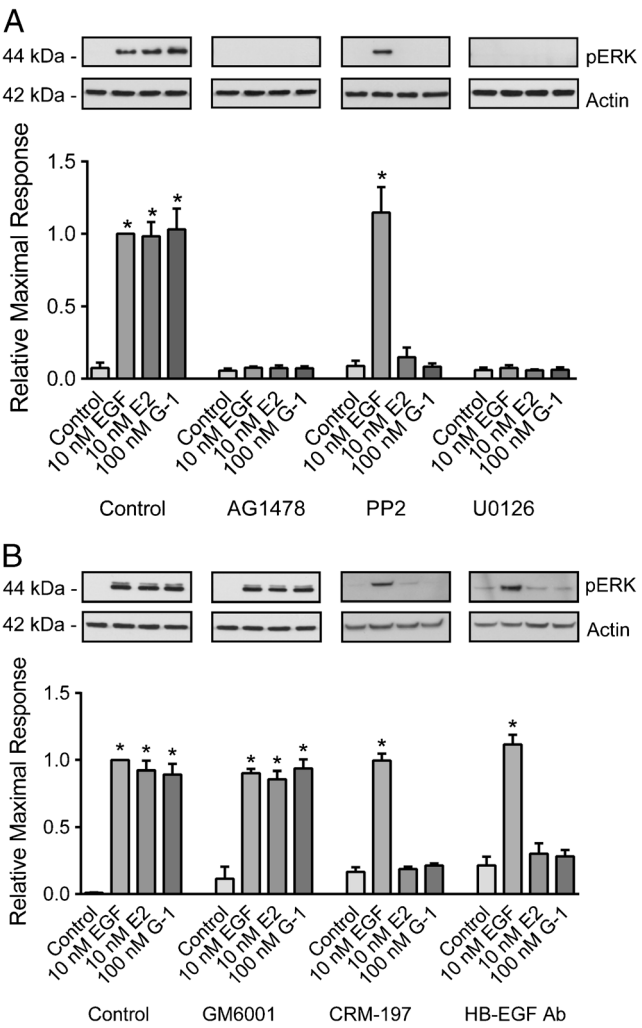

Fig. 4 GPER-dependent activation of MAPK (ERK1 and ERK2) is dependent on Src activation but not MMP activation in MCF10A cells. Signal transduction inhibitors were tested for their ability to block GPER-dependent ERK activation in MCF10A cells. Cells were pre-incubated for 30 min with either control, AG1478 (**a** 250 nM, inhibitor of EGFR), U0126 (**a** 10 μM, inhibitor of MEK), PP2 (**a** 10 nM, inhibitor of Src), GM6001 (**b** 25 μM, inhibitor of MMPs), CRM-197 (**b** 0.2 mg/mL, inhibitor of HB-EGF or HB-EGF neutralizing antibody (**b** 6 ng/mL), then stimulated with 10 nM EGF, 10 nM E2 or 100 nM G-1 for 15 min. Lysates were immunoblotted with anti-phospho-ERK antibody. Histograms represent fold change in pERK protein expression relative to β-actin loading control. Data are representative of three independent experiments. Results are expressed as mean ± SEM and statistical significance ($p \leq 0.05$) was assessed by one-way ANOVA followed by a Dunnett's test (*significantly different relative to control)

(as a positive control) and the nonreceptor tyrosine kinase Src inhibitor, PP2 (Fig. 4a), for their ability to block E2- and G-1-induced ERK phosphorylation. Previous reports demonstrate Src is frequently activated downstream of GPCR activation in cancer cell lines [30], and evidence suggests that Src can directly activate the intracellular domain of the EGFR [50] as well as play a role in MMP activation [39]. AG1478 or U0126 pretreatment blocked E2- and G-1-induced ERK phosphorylation (Fig. 4a), demonstrating that EGFR transactivation is a consequence of E2- and G-1-dependent GPER activation. PP2 pretreatment blocked E2- and G-1-induced ERK phosphorylation (Fig. 4a); however, PP2 did not affect EGF-induced ERK phosphorylation (Fig. 4a).

These results suggest that Src activation is required for GPER-dependent EGFR transactivation in MCF10A cells. A mechanism for transactivation has been described in MDA-MB-231 breast cancer cells, in which GPER-dependent Src activation leads to the release of extracellular MMP, which in turn cleaves membrane-bound pro-HB-EGF, allowing soluble HB-EGF to bind EGFR [26]. To determine whether this mechanism also occurs in the immortalized, nontransformed MCF10A cells, we tested the ability of a broad-spectrum MMP inhibitor, GM6001, to inhibit E2- and G-1-induced, GPER-dependent ERK phosphorylation. Unexpectedly, we found that GM6001 had no effect on ERK activation (Fig. 4b). We confirmed that GM6001 was active as it inhibited MMP activity in conditioned medium of HT-1080 cells (known to overexpress MMPs) [68] in a gel zymography assay (Supplemental Fig. 4). Taken together, our observations indicate that Src is activated in a GPER-dependent manner in MCF10A cells, and that Src activation is required for EGFR transactivation and subsequent ERK activation. However, classical MMPs do not appear to be required for E2- and G-1-induced, GPER-dependent ERK phosphorylation.

This unexpected result led us to ask if production of HB-EGF is required for GPER-dependent EGFR transactivation in these cells, perhaps in an MMP-independent manner or via other proteases. To address this, we performed ERK activation assays using two reagents that interfere with the production or availability of soluble HB-EGF. First, we tested a diphtheria toxin mutant, CRM-197, that sequesters and downmodulates surface-expressed pro-HB-EGF, inhibiting its mitogenic activity [53], and, second, we tested an HB-EGF-specific antibody that blocks the ability of the ligand to bind and transactivate EGFR. Both CRM-197- and HB-EGF-neutralizing antibody blocked E2- and G-1-induced, GPER-dependent ERK phosphorylation, but, as expected, neither CRM-197 nor neutralizing antibody had any effect on the ability of exogenous EGF to phosphorylate ERK (Fig. 4b). These results suggest that GPER-dependent EGFR transactivation requires HB-EGF, but that MMPs (inhibited by GM6001) are not required for HB-EGF activity as they are in multiple cancer cell lines.

E2- and G-1-Induced Proliferation in MCF10A Cells Require GPER-Dependent EGFR Activation

Removal of exogenous EGF is sufficient to arrest MCF10A cells in the G_1 phase of the cell cycle, but does not result in apoptosis [13]. Since we have shown that E2 and G-1 promote proliferation as measured by an increase in mitotic index in the absence of exogenous EGF (Fig. 2b), we tested the ability of a variety of kinase, protease, and HB-EGF inhibitors to block E2- and G-1-induced, GPER-mediated proliferation. Both AG1478 (EGFR inhibitor) and U0126 (MEK inhibitor) completely blocked E2- and G-1-induced proliferation (Fig. 5a); AG1478 also blocked EGF-induced proliferation as expected (Fig. 5a), and U0126 was able to partially block EGF-induced proliferation. We also tested the ability of the PI3Kinase (PI3K) inhibitor LY294002 to block E2- and G-1-induced proliferation since PI3K is a downstream mediator of EGFR action [24, 83] and PI3K is activated in a GPER-

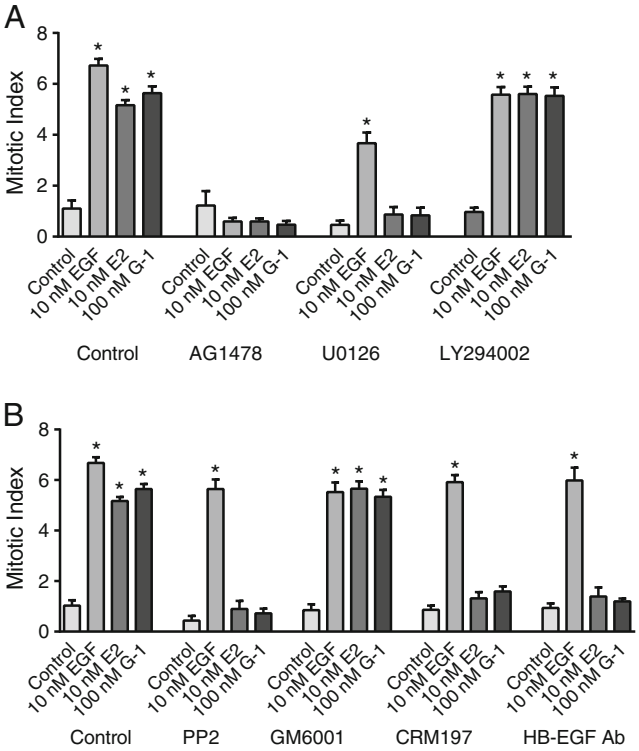

Fig. 5 GPER-dependent proliferation requires transactivation of EGFR. Signal transduction inhibitors were tested for their ability to block GPER-dependent proliferation in MCF10A cells. Cells were pre-incubated for 30 min with either vehicle (control), AG1478 (**b** 250 nM, EGFR inhibitor), U0126 (**a** 10 μM, MEK inhibitor), LY294002 (**a** 10 μM, PI3K inhibitor), PP2 (**b** 10 nM, Src inhibitor), GM6001 (**b** 25 μM, MMP inhibitor), CRM197 (**b** 0.2 mg/mL HB-EGF release inhibitor) or HB-EGF-neutralizing antibody (**b** 6 ng/mL) and then stimulated with 10 nM EGF, 10 nM E2, or 100 nM G-1 for 24 h. Mitotic index as a surrogate for proliferation was quantified by immunofluorescence using an anti-pH3 antibody. Data are representative of a minimum of three independent experiments. Results are expressed as mean ± SEM and statistical significance ($p \leq 0.05$) was assessed by one-way ANOVA followed by a Dunnett's test (*significantly different relative to control)

dependent manner [63]. Pretreatment of MCF10A cells with LY294002 had no effect on E2- and G-1-induced proliferation (Fig. 5a), suggesting that GPER-dependent proliferation occurs independently of PI3K activation. Pretreatment with PP2 (Src inhibitor), CRM-197 (HB-EGF inhibitor), or HB-EGF neutralizing antibody all blocked E2- and G-1-induced, GPER-mediated proliferation (Fig. 5b); however, like U0126, they did not block exogenous EGF-dependent proliferation (Fig. 5b). The MMP inhibitor GM6001, which did not block E2- and G-1-induced ERK phosphorylation (Fig. 5b) also had no effect on E2- and G-1-induced proliferation (Fig. 5b), suggesting that although Src is activated in a GPER-dependent manner, subsequent activation of MMP is not required for E2- and G-1-induced proliferation in MCF10A cells.

E2 and G-1 Induce Proliferation in a 3D Model of Breast Morphogenesis

Collectively, our observations demonstrate that activation of GPER via either E2 or G-1 promotes proliferation in MCF10A cells in monolayer culture (Fig. 2b), and, moreover, that GPER-stimulated proliferation is dependent on EGFR transactivation and subsequent ERK phosphorylation (Fig. 3). To test whether this mechanism is also active in a more physiologically relevant environment, we assessed whether GPER activation promoted mitotic index increases, suggesting proliferation of MCF10A cells cultured in a 3D basal lamina-rich environment. MCF10A cells cultured in 3D mimic several important features of breast epithelial morphogenesis [18]. Seeded as single cells, MCF10A cells proliferate over a period of 14 days to form multicellular spheroids. Apoptosis of cells in the center of the spheroid leads to a hollow structure, similar to alveolar structures found in the human breast. Single cells were seeded on Matrigel™ with 2 % Matrigel™ added to the medium, cultured for 3 days. On day 4, treatments were added and were continued for 6 days. Cells were fixed on day 10 of culture and mitotic index was measured by immunodetection of pH3 (Fig. 6a). Cells were co-stained with an antibody directed against α-tubulin to label microtubules (to visualize cell shape and boundaries); nuclei were counterstained with TO-PRO®-3 (Fig. 6a). pH3 staining revealed E2 and G-1 increased proliferation relative to control (Fig. 6b). Additionally, E2 and G-1 treatment led to an increase in average cell number per spheroid (Fig. 6c) indicating that E2 and G-1 promote completion of the MCF10A cell cycle.

GPER Contributes to E2-Induced Proliferation in Human Breast Tissue

Since GPER activation led to proliferation of MCF10A breast cells (monolayers and spheroids), we next investigated whether E2-dependent proliferation in normal human breast tissue can also be mediated in part by GPER. Normal, nontumorigenic breast tissue is reported to express both GPER and ERα [10, 25], confirmed in our reduction mammoplasty samples by immunohistochemistry (Fig. 7a, b; specificity of anti-GPER antibody demonstrated in Supplemental Fig. 3B). To determine if GPER activation increased proliferation in the human breast, tissue from reduction mammoplasty surgeries was cultured as described [22]. Immunodetection of proliferation marker Ki67 was used to determine the effect of GPER activation on proliferation in mammary explants after 7 days in culture. Ki67 was used instead of pH3 in this assay because Ki67 labels a greater numbers of cells as it detects cells at any stage of the cell cycle (excluding G_0), whereas pH3 only labels mitotic cells [76]. The proliferation rates in breast alveolar epithelia are lower than in MCF10A cells in vitro; therefore, immunodetection of

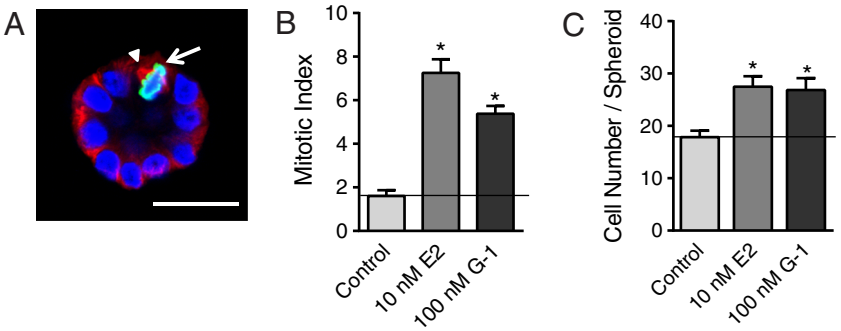

Fig. 6 Estrogen-induced GPER activation stimulates proliferation in a 3D model of breast morphogenesis. MCF10A cells were grown in 3D on Matrigel™ basal lamina in the presence of 10 nM E2 or 100 nM G-1 for 6 days. Mitotic index as a surrogate for proliferation (**b**) was quantified by immunofluorescence using an anti-pH3 antibody. A representative spheroid immunolabeled with anti-pH3 (*green*) and anti-gamma tubulin (*red*) is shown (**a** *arrow* indicates anti-pH3 immunolabeled chromatin; *arrowhead* indicates mitotic spindle). Total cell number per spheroid was quantified for each treatment group (**c**). Data are representative of three independent experiments (*scale bar* 25 μm). Results are expressed as mean ± SEM and statistical significance ($p \leq 0.05$) was assessed by one-way ANOVA followed by a Dunnett's test (*significantly different relative to control)

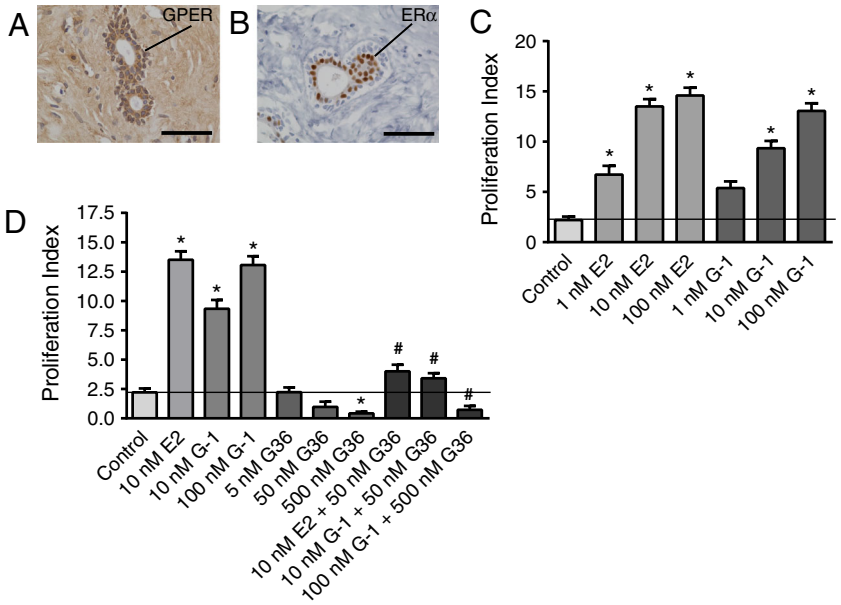

Fig. 7 E2 and G-1 promote proliferation in normal human breast tissue. **a** GPER protein expression was detected in epithelia and stroma by immunohistochemistry on tissue sections. **b** ERα protein expression was detected in nuclei of breast epithelia. Breast epithelial proliferation was quantified by immunofluorescence using anti-Ki67 antibody in the presence of GPER agonists E2 and G-1 (**c**, **d**) and antagonist G36 (**d**) in alveolar structures within normal human breast tissue explants. Each treatment group consisted of tissue samples from a minimum of five different patients (*scale bars* 50 μm). Results are expressed as mean ± SEM, and statistical significance ($p \leq 0.05$) was assessed by one-way ANOVA followed by a Dunnett's *t* test (*significantly different relative to control, #significantly different relative to E2 or G-1)

Ki67 allowed us to detect sufficient numbers of proliferating cells to achieve statistical significance. Our results demonstrate that like MCF10A cells, E2 and G-1 increased luminal epithelial cell proliferation in breast tissue explants (Fig. 7c). G36 treatment significantly reduced both E2- and G-1-dependent proliferation, although G36 alone (at 5 or 10 nM) had no effect on proliferation (Fig. 7d). At 500 nM, G36 alone significantly reduced proliferation relative to control. This may reflect the fact that breast adipose tissue synthesizes low levels of E2 locally, and, therefore, very high G36 concentrations may abrogate the GPER-dependent proliferative activity resulting from E2 derived from adipose tissue present in the explants [31]. These results suggest that in addition to ERα, GPER contributes to E2-induced proliferation in primary human breast tissue.

We also investigated whether GPER contributed to E2-induced proliferation in human breast tumor tissue, since GPER expression in breast tumors correlates with poor prognosis [25]. We confirmed the expression of GPER on breast tumors used in these assays (a representative sample is shown in Fig. 8a). Treatment of breast tumor tissue explants with E2 or G-1 for 7 days significantly increased epithelial cell proliferation, compared to control (Fig. 8b). While treatment of tumor explants with G36 alone did not affect proliferation, G36 co-treatment significantly reduced E2- and G-1-dependent proliferation (Fig. 8b), suggesting that GPER activation contributes to E2-induced proliferation in primary breast tumor explants.

Discussion

The proliferative effects of E2 in the breast are well established and have long been attributed to the classical estrogen receptor ERα [8, 33]. Alternatively, ERβ is thought to be antiproliferative in the presence of E2 [29], downregulating transcription of genes involved in DNA replication, cell

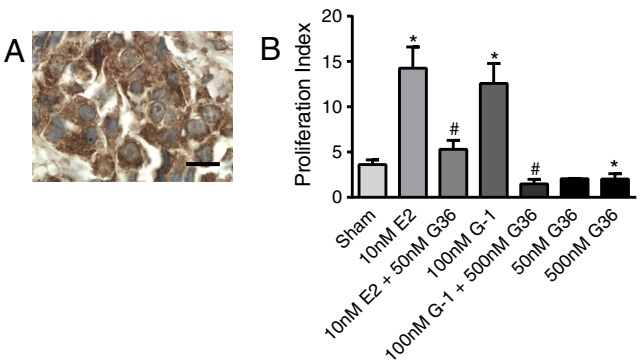

Fig. 8 E2 and G-1 promote proliferation in human breast tumors. **a** GPER protein expression was detected in breast tumor cells by immunohistochemistry on tissue sections. **b** Tumor cell proliferation was quantified by immunofluorescence using anti-Ki67 antibody in the presence of GPER agonists E2 and G-1 and antagonist G36. Each treatment group consisted of tissue samples from a minimum of five different patients (*scale bars* 50 μm). Results are expressed as mean ± SEM, and statistical significance ($p \leq 0.05$) was assessed by one-way ANOVA followed by a Dunnett's *t* test (*significantly different relative to control, #significantly different relative to E2 or G-1)

cycle regulation and proliferation including c-myc and cyclin D1 [11, 44, 77], and increasing expression of antiproliferative genes p21 and p27 [11], thus inducing G_2 cell cycle arrest in breast epithelial cells [58]. To date, it is unknown if the third estrogen receptor GPER can mediate E2-induced proliferation in the normal human breast. Unlike mice in which ERα is deleted through homologous recombination, mice lacking GPER display no overt mammary or reproductive phenotypes, suggesting that E2-dependent GPER activation does not recapitulate ERα activation in normal female murine reproductive function. Moreover, in human breast cancers, GPER has been linked to markers of poor prognosis and aggressive cancer progression [25], underscoring the importance of understanding how GPER activity impacts cellular physiology. Previous studies have shown that GPER binds E2 [72] and promotes E2-dependent proliferation in SKBr3 breast cancer cells that express GPER but not ERα or ERβ [57], endometrial cancer cells [74], and ovarian cancer cells [2] as well as in vivo in the murine endometrium [19]; however, there is also evidence that GPER inhibits proliferation of ER-positive MCF7 breast cancer cells [4], and one report employing GPER knockout mice concluded that GPER did not promote proliferation in the murine mammary gland [55, 56]. Because these studies report that GPER can promote, inhibit, or have no effect on proliferation depending on context (e.g., cell type, in vitro vs. in vivo, or mouse vs. human, perhaps reflecting variation in estrogen receptor status and widely differing treatment regimens), we reasoned that directly testing GPER function in regulating proliferation in nontumorigenic breast epithelial cells and tissue could resolve some of the discrepancies. As normal human breast expresses all three estrogen receptors, E2 actions are likely influenced by multiple receptors [10, 25]. We first measured GPER-dependent proliferation as measured by increases in mitotic index [using anti-histone H3 (phospho-Ser10) antibody] in the immortalized, nontransformed human breast epithelial cell line, MCF10A, and subsequently in explants from normal human breast tissue (using anti-Ki67 antibody) derived from reduction mammoplasty surgery and human breast tumors. Others have detected a slight, statistically insignificant increase in MCF10A cell number [1, 9] or a decrease in doubling time [61] in response to E2; however, to our knowledge, this is the first report measuring E2-dependent mitosis specifically in these cells. We showed that E2 and the GPER-selective agonist G-1 induce an increase in mitotic index, suggestive of proliferation, in MCF10A cells both in standard monolayer culture, and in a 3D model of breast epithelial morphogenesis, where growth control cues similar to those found in the normal breast are present. In 3D culture, E2 and G-1 treatment also increased cell number, providing additional confirmation of proliferation. These cells express GPER but not ERα, ERβ, or ERα36 [1, 18, 46, 61, 75], suggesting that E2-induced proliferation is dependent on GPER alone in MCF10A cells. To confirm that the E2-induced proliferation was GPER dependent, we showed that a GPER-selective antagonist, G36, as well as GPER-targeted siRNA, inhibited proliferation induced by E2- and G-1. Inhibition of basal proliferation by high (500 nM) G36 concentrations may reflect its effects at antagonizing the actions of adipose-derived E2 [31] or may be due to off-target effects.

Our results also demonstrate that E2 promotes proliferation in normal human breast tissue explants, consistent with previous findings [22]. The GPER-selective agonist G-1 also stimulated proliferation in explant cultures, albeit at a slightly reduced level compared to E2. This may reflect the fact that G-1 has a higher Ki for GPER (11 nM) [7] compared to E2 (6.6 nM) [63] in estrogen receptor-negative cells transfected with GPER alone, in addition to the fact that G-1 does not activate ERα/β. Whereas G36 completely blocked G-1-induced proliferation, it also partially blocked E2-induced proliferation in normal human breast tissue explants, suggesting that maximal E2-dependent proliferation in the human breast likely involves both ERα and GPER. We also interrogated GPER function in modulating proliferation in a small set of breast tumor explants and found E2- and G-1-dependent proliferation to be enhanced, while G36 abrogated these effects (partially for E2, completely for G-1), similar to that found in normal breast explants. The tumor explants represented a mixed group with respect to ER status (though predominantly ERα positive); therefore, these results suggest that the GPER agonist G-1 promotes proliferation in these breast tumors. In this regard, there is evidence that ER status does not always predict E2-dependent proliferative responses [14, 17, 80], and although ERα-negative patients are not generally given anti-estrogen therapy, in a clinical trial, the response to letrozole was nearly equal across patients with ERα Allred scores from 3 to 6, suggesting in patients with lower ERα expression that other factors could contribute to letrozole response [23]. While the role of GPER in breast cancer progression remains unclear, and in the letrozole clinical trial GPER expression was not measured, it is possible that GPER could modulate therapy response, and studies are ongoing to directly address this question. Collectively, these results demonstrate for the first time GPER-mediated proliferation in a human tissue. Moreover, physiologic concentrations of E2 in breast tissue have been reported in the nanomolar range [31], which is higher than that typically reported in serum, and equivalent to the dose range used in this study, where we observed significant responses at 1 nM E2. These results suggest that our findings are relevant with respect to physiological E2 concentrations in the breast. We had hypothesized that proliferation induced by E2 would be significantly higher compared to G-1 because E2 activates both ERα and GPER, whereas G-1 activates only GPER. The E2-dependent antiproliferative role of ERβ [11, 33, 41, 58, 67, 74] may explain this result. It is likely that E2 produces

both proliferative (through activation of ERα and GPER) and antiproliferative (through activation of ERβ) signals in breast tissue, which would limit the overall extent of E2-induced proliferation. Finally, since both ERα and GPER are likely expressed in a heterogeneous pattern in any given breast cancer, it remains to be determined whether estrogen receptor expression coincides with, or is distinct from, those cells that are proliferating [37, 35, 36, 45]. Because the importance of GPER in breast cancer progression remains unclear, our results argue that further investigation of GPER expression and activity in human breast tumors is warranted.

Filardo and colleagues previously demonstrated that E2-mediated GPER activation leads to EGFR transactivation, with subsequent ERK-1 and ERK-2 activation in breast cancer cells [26]. Consistent with this, we previously demonstrated that E2-dependent GPER activation stimulates the PI3K pathway in an EGFR activation-dependent manner [63]. Therefore, in order to dissect the molecular pathway through which GPER promotes proliferation in a normal, nontumorigenic setting, we targeted components of the EGFR/MAPK signaling pathway. Our results reveal that E2- and G-1-induced GPER activation lead to EGFR transactivation and subsequent ERK activation, and that these events are required for E2- and G-1-induced proliferation in MCF10A cells. Interestingly, PI3K inhibition had no effect on E2- and G-1-induced proliferation, suggesting that GPER-dependent PI3K activation is not required for proliferation. We also determined that in MCF10A cells, although activation of the nonreceptor tyrosine kinase Src is required for GPER-dependent activation of ERK and proliferation, MMP activity is not required for EGFR transactivation (measured by ERK activation) or proliferation, as was previously reported for breast cancer cell lines [26]. In that report, HB-EGF was identified as the ligand required for EGFR activation, and it was demonstrated that MMP activity was necessary for pro-HB-EGF cleavage and production of soluble HB-EGF ligand. Despite the fact that our data suggest that MMPs are not required, we confirmed a requirement for HB-EGF to promote E2- and G-1-induced, GPER-mediated phosphorylation of ERK and proliferation both by sequestering and downmodulating pro-HB-EGF with CRM-197 and by blocking its ability to bind EGFR with neutralizing antibodies. Based on these observations, it is possible that an alternate protease, activated in a GPER-dependent manner, is responsible for cleaving pro-HB-EGF. However, in our experiments, the concentration of GM6001 used (25 μM) is known to be sufficient to inhibit other extracellular proteases such as ADAMs, as well as MMPs [52]. An alternative hypothesis is that pro-HB-EGF may be transactivating EGFR without cleavage, e.g., in a juxtacrine manner, independent of cleavage by proteases, following GPER activation [21, 70]. Juxtacrine pro-HB-EGF signaling has been previously reported in MCF10A cells [78] in which formalin-fixed MCF10A cells were able to activate the EGFR on MCF10A cells in vitro. In this study, we show for the first time that GPER mediates E2-induced proliferation in immortalized, nontransformed breast epithelial cells and importantly, in normal human breast tissue. We have also demonstrated a novel mechanism for transactivation of the EGFR in MCF10A cells in response to GPER activation. Given the ability of GPER to promote proliferation in normal breast tissue as well as breast cancer cells, and the correlation between GPER expression and predictors of poor outcome in a breast tumor setting, understanding the mechanism of E2-induced, GPER-dependent signaling and proliferation is critical. In this regard, the ability of the GPER-selective antagonist G36 to block E2-induced proliferation in vitro in cell lines as well as in human tissue suggests that this agent could have preventative or therapeutic potential against carcinogenesis in breast and other E2-responsive tissues.

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Authors' Contributions ALS, ERP, and HJH participated in experiment design. ALS performed research. ALS, ERP, and HJH participated in data analysis. ALS drafted the manuscript. ERP and HJH contributed equally to the manuscript. All authors read and approved the final manuscript.

Conflict of Interest ERP holds a United States patent on G-1 and G36 (US patent number 7,875,721). HJH and ALS declare that they have no conflict of interest.

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