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ARTICLE

G-Protein-Coupled Estrogen Receptor Enhances the Stemness of Triple-Negative Breast Cancer Cells and Promotes Malignant Characteristics

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ABSTRACT

G-protein coupled estrogen receptor (GPER) is a transmembrane receptor that mediates non-genomic effects of estrogen. This study aimed to investigate the role of GPER in the stemness formation and malignancies in triple negative breast cancer (TNBC) cells. Spheroids of MDA-MB-468 cells were induced by mammosphere culture, and the proportion of the $CD44^{+}/CD24^{-/low}$ stem cell subpopulation was detected. Malignant characteristics, expression of GPER and stemness-related markers, and tumorigenesis in a xenograft assay were compared between the mammospheres and adherent cultured cells. The impacts of 17β-estradiol (E2) and the GPER-specific antagonist G15 were studied in *in vitro* assays. The proportion of the $CD44^{+}/CD24^{-/low}$ subpopulation was increased in the mammospheres of MDA-MB-468 cells, which also showed higher expression of GPER and stemness-related markers than adherent cultured cells. The abilities of spherical colonies to proliferate, invade, and form colonies in soft agar were enhanced. Spherical cells exhibited stronger tumorigenesis ability than adherent cells in the xenograft assay. E2 treatment enhanced tumorigenicity of both adherent and spherical cells. Spherical cells treated with E2 had stronger proliferation, invasion, and colony formation abilities than other groups. Pre-treatment with G15 effectively blocked the stimulation by E2. In conclusion, the expression of GPER in TNBC cells is positively related to stemness and malignant features.

KEYWORDS

Triple negative breast cancer; G-protein-coupled estrogen receptor; cancer stem cell; estrogen; mammosphere formation

1 Introduction

Breast cancer is one of the most common malignancies in females worldwide [1]. Triple-negative breast cancer (TNBC) is the most malignant subtype and lacks the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor type 2 (HER2). Due to the lack of targets for endocrine therapy and targeted therapy, TNBC poses a difficult problem in the field of breast cancer treatment. Patients with TNBC have a higher risk of distant metastasis and shorter overall survival [2].

Nuclear ERs (including ERα and ERβ) mediate the estrogen effects on gene expression commonly known as genomic signaling [3]. Estrogen can also induce rapid non-genomic effects, and G-protein-coupled estrogen receptor (GPER, or GRP30) has been reported to mediate these extra-nuclear effects [4]. For TNBC cells lacking ER, GPER is a key receptor for sensing estrogen or estrogen-like substances. Studies in ER^- cell lines have shown that GPER can respond to 17β-estradiol (E2) or tamoxifen and further promote proliferation, motility, and epithelial-mesenchymal transition [5–7]. GPER is a favorable factor for recurrence-free survival in patients without tamoxifen treatment but becomes a risk factor in patients who receive tamoxifen [8]. Different manifestations of GPER in the presence or absence of ER have attracted increasing interest, suggesting possible therapeutic candidates in TNBC. In ER^+ breast cancer, GPER is a favorable factor for prognosis and is negatively related to lymph node metastasis [9]. However, GPER is correlated with a shorter disease-free interval in ER^- breast cancer patients [10]. The recurrence rate of $GPER^+$ TNBC patients is higher than that of $GPER^-$ TNBC patients [11].

The poor prognosis of TNBC is related to a high incidence of distant metastasis [12]. The heterogeneity of tumor cells suggests that not all tumor cells can form distant metastases. Cancer stem cells (CSCs) are characterized by strong self-renewal and differentiation capabilities and can thus promote tumor colonization in a new environment to form new lesions [13]. Breast cancer-related stem cells are enriched in $CD44^+/CD24^{-/low}$ or aldehyde dehydrogenase positive ($ALDH^+$) cells [14]. The cells with these properties are more likely to survive after chemotherapy or neoadjuvant endocrine therapy, suggesting that this subpopulation is important for the emergence of drug resistance [15,16].

Current studies have suggested that the expression and activation of GPER are beneficial to the proliferation of TNBC cells. However, the role of GPER in metastasis or stemness development in TNBC is not completely understood. The present study used mammospheres induced from TNBC cells to investigate the relationships between GPER expression and stemness-related phenotypes. The effect of GPER on the malignant characteristics of both adherent cells and mammospheres in response to estrogen was also studied.

2 Materials and Methods

2.1 Cell Culture

TNBC MDA-MB-468 cells (purchased from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were used in the present study. The cells were maintained in RPMI 1640 (Gibco, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco) and 1% (v/v) penicillin/streptomycin (Beyotime Biotechnology, Shanghai, China) in 5% CO_2 at 37°C.

To investigate the role of GPER under estrogen treatment, the cells were cultured in phenol red-free RPMI medium (without FBS) for 24 h. Adherent cells were cultured in regular 6-well plates and spherical cells were cultured in ultralow attachment 6-well plates (Corning Inc., Corning, NY, USA). On the next day, the cells were treated with 1 nM E2 or pretreated with 1 μM G15 (GPER selective antagonist) for 30 min followed by 1 nM E2. The cells were then cultured for another 24 h before subsequent Western blot, invasion assay, and soft agar assay. For the proliferation assay, cells were seeded in 96-well plates after phenol red-free medium culture, and then treated with E2 or G15/E2 after attachment. The concentrations of the experimental agents were determined according to a previous report [17,18]. Estradiol powder (ST1101, Beyotime) was dissolved in a small amount of ethanol, and normal saline was added to prepare a 20 μM stock solution. G15 (cat. no. 14673, Cayman Chemical, Ann Arbor, MI, USA) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 200 μM.

2.2 Induction of Mammospheres

Mammosphere induction was carried out as previously described [19]. MDA-MB-468 cells were suspended in MammoCult medium (STEMCELL Technologies, Grenoble, Germany) and seeded in

ultralow attachment 6-well plates at a density of 1×10^5 cells/well. After 7 days of culture, mammosphere cells were collected by centrifugation (350 × g for 5 min). MDA-MB-468 cells cannot be maintained in long-term mammospheres after continuous passages [20]; hence, only the first two passages of spherical cells were used for subsequent experiments.

2.3 Flow Cytometry

The expression levels of surface CD44 and CD24 were detected by flow cytometry. Single-cell suspensions of adherent or spherical MDA-MB-468 cells were prepared by detaching the cells with Accutase solution (Yeason Biotech, Shanghai, China) and were adjusted to a density of 10^7 cells/mL with flow cytometry staining buffer (Multi Sciences Biotech, Hangzhou, China). The cells were stained as described [20]. Anti-CD44-APC (product no. A14749, eBioscience, San Diego, CA, USA), anti-CD24-PE (product no. 12-0241-82, eBioscience), or corresponding isotype controls were used as recommended by the manufacturer. After incubation at 4°C for 45 min in the dark, labeled cells were analyzed using a BD LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Signals of 10^6 cells were collected for each sample and analyzed using FlowJo software version 8.8.6 (Tree Star, Ashland, OR, USA).

2.4 Western Blot

Cell lysates were prepared using lysis buffer for Western blotting (P0013, Beyotime) according to the manufacturer's instructions. The protein concentration was determined using a bicinchoninic acid protein assay kit (Beyotime). Total protein lysates (30 μg/lane) were separated by 12% sodium dodecyl sulfate□ polyacrylamide gel electrophoresis and blotted onto a polyvinylidine difluoride membrane (Millipore, Billerica, MA, USA). After blocking with 5% nonfat milk at 37°C for 1 h, the membrane was incubated with the appropriate primary antibodies at 4°C overnight. The primary antibodies included rabbit anti-GPER antibody (1:2000, Solarbio, Beijing, China), rabbit anti-NANOG (1:1000, Solarbio), rabbit anti-sex determining region Y-box 2 (SOX2) (1:2000, Solarbio), rabbit anti-matrix metalloproteinase 2 (MMP2) (1:1000, Solarbio), rabbit anti-CXCL12 (1:1000, Invitrogen, Carlsbad, CA, USA), and rabbit anti-GAPDH (1:3000, Solarbio). GAPDH was used as a loading control. The blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3000, Solarbio), and the bands were visualized using an enhanced chemiluminescence kit (Yeason). Protein bands were quantified using ImageJ software (https://imagej.nih.gov/ij/).

2.5 Cell Proliferation Assay

Spherical or adherent cells (2×10^3 cells/well in 100 μL of medium) were seeded in a 96-well plate. After attachment of the cells, E2 and/or G15 were added according to the experimental requirements. The cells were continuously cultured for 5 days. Cell numbers were determined using a Cell Counting Kit-8 (Yeason) on the day of inoculation and every day after inoculation. The optical density (OD) at 450 nm was measured to calculate the number of cells.

2.6 Invasion Assay

The invasion potency of spherical or adherent cells was tested by a Matrigel invasion assay. Single-cell suspensions of spherical or adherent cells were prepared in serum-free RPMI medium and adjusted to 5×10^5 cells/mL. A total of 5×10^4 cells were seeded in the upper Matrigel-coated chamber (8.0 μm, BD Biosciences). RPMI 1640 containing 10% FBS was added to the lower chamber. After 24 h, noninvasive cells in the upper chamber were gently removed with a cotton swab. Invasive cells remaining on the bottom surface of the chamber were fixed with 100% methanol for 30 min and stained with 0.2% crystal violet for 20 min. For each sample, five random fields under 10× magnification were imaged to count the cells.

2.7 Colony-Forming Assay in Soft Agar

Single-cell suspensions of spherical or adherent cells were prepared in 0.35% agarose in RPMI medium containing 10% FBS and adjusted to 10^3 cells/mL. The suspension was seeded in a 6-well plate (1.5×10^3 cells/well) covered with 0.6% basal agar in RPMI medium containing 10% FBS. After agar solidification, the plates were cultured for 2 weeks and monitored for colonies. The cells were stained with 0.2% crystal violet for 10 min, and the number of colonies >0.5 mm in diameter was counted.

2.8 Xenograft Assay

The animal research protocol was reviewed and approved by the Animal Ethics Committee of Nanjing Medical University (IRB No. 2016-34). Female BALB/c nude mice (6 weeks old) were purchased from Vital River Experimental Animal Technology Co., Ltd. (Beijing, China). Mice were transplanted with various concentrations of adherent or spherical cancer cells (5×10^5, 1×10^6, or 2×10^6 cells in 100 μL of phosphate buffered saline) in the left flank. Tumor growth was monitored for 8 weeks, and mice were euthanized to collect tumor tissues. The tumor tissue volume was calculated using the equation: (length × width^2)/2.

2.9 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). The tumor formation rates in the xenograft assay are expressed as percentages and were compared using Fisher's exact test. Numerical data were tested for normality by Kolmogorov–Smirnov method. When $P > 0.1$, the data were considered to have a normal distribution, and they are presented as the mean ± standard error of the mean (SEM). Cell proliferation rates were compared using two-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test. Other results were compared using Student's *t* test or one-way ANOVA followed by Tukey's post-hoc test. A value of $P < 0.05$ was considered statistically significant. Experiments were performed in triplicate, and representative results are shown.

3 Results

3.1 GPER Expression is Related to Enhanced Malignancies of the Mammospheres of TNBC Cells

Mammospheres usually show strong stem-like characteristics, which are related to acquired anoikis resistance during suspension culture [21]. Initially, mammospheres of MDA-MB-468 cells were induced by suspension culture (Fig. 1A). Comparison with adherent cultured cells indicated that the relative expression of GPER in the mammospheres was significantly increased ($P = 0.0022$, Fig. 1B). The results of flow cytometry analysis indicated that the $CD44^+/CD24^{-/low}$ subpopulation was enriched in spherical cells compared with adherent cultured cells ($P = 0.0193$, Fig. 1C). Hence, the expression of GPER was related to the proportion of the stem cell subgroup. Then, we compared the biological characteristics of spherical cells and adherent cells. The results of the CCK-8 assay indicated that the growth rate of spherical cells was significantly higher than that of adherent cells ($P < 0.0001$, Fig. 2A). The results of the Matrigel invasion assays indicated that spherical cells were more invasive than adherent cells ($P < 0.0001$, Fig. 2B). The results of the colony formation assay in soft agar indicated that the colony-forming ability of spherical cells was significantly higher than that of adherent cells ($P < 0.0001$, Fig. 2C).

To further evaluate the tumorigenesis of MDA-MB-468 mammospheres *in vivo*, we inoculated nude mice with three concentrations of adherent or spherical cells. The results indicated a difference in the minimum tumorigenic concentration between adherent and spherical cells (Table 1). After two months, no visible subcutaneous tumors were detected in mice implanted with 5×10^5 adherent cells, and only one subcutaneous tumor was detected in mice implanted with the same concentration of spherical cells. Subcutaneous tumors were detected in all nude mice implanted with 2×10^6 adherent or spherical cells. At an implantation dose of 1×10^6 cells, visible subcutaneous tumors formed in all nude mice inoculated with spherical cells, while in the adherent cell group, subcutaneous tumors only formed in one mouse.

There was a significant difference in the probability of tumor formation between the two groups at an inoculation dose of 1×10^6 cells ($P = 0.0152$). The volume of subcutaneous tumors in the spherical cell groups was significantly larger than that in the adherent cell groups (Fig. 3).

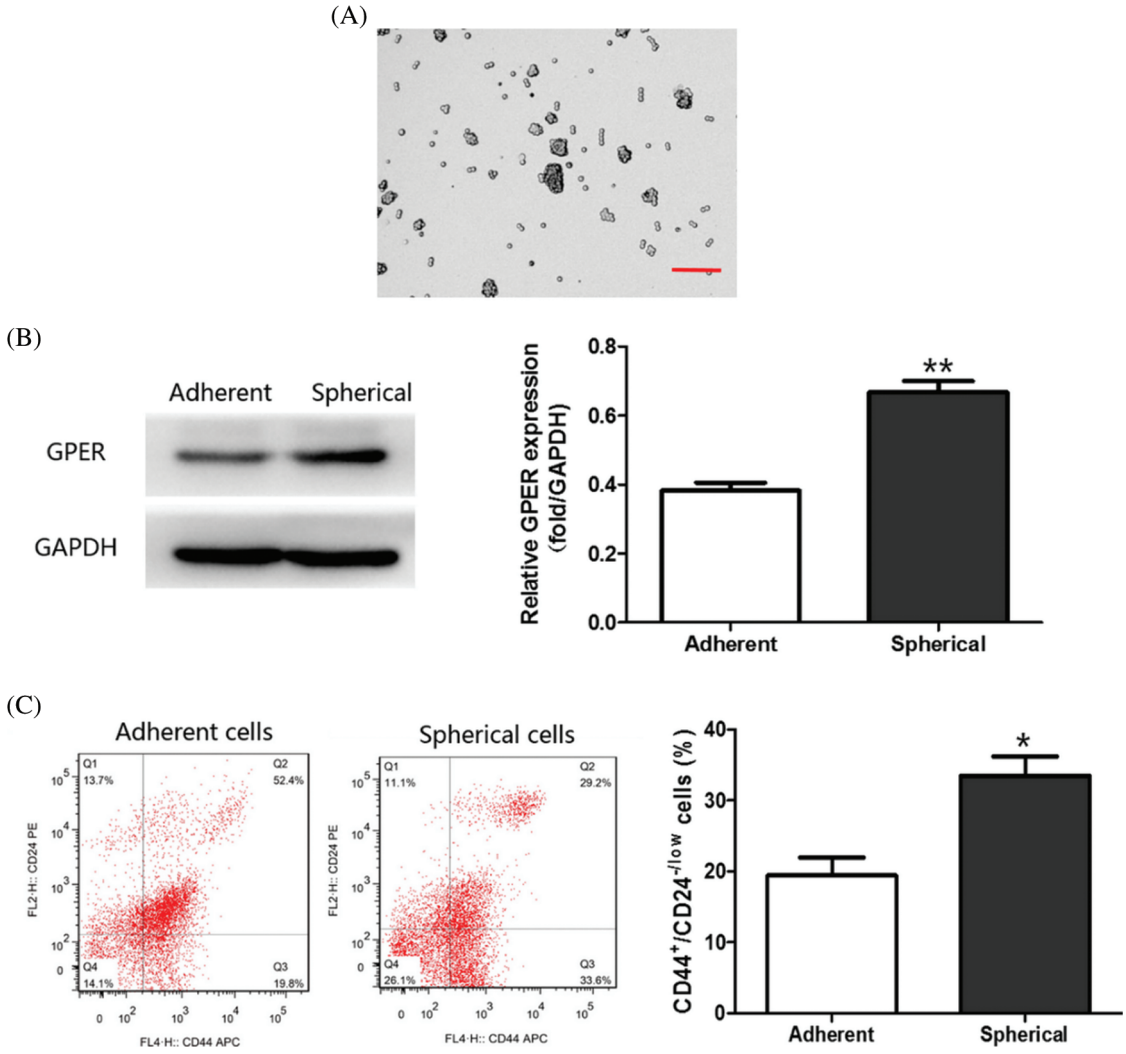

Figure 1: Mammosphere induction in MDA-MB-468 cells. (A) Cell morphology of the mammospheres. Scale bar =100 μm. (B) Relative expression of GPER in adherent and spherical cells. (C) Proportions of the $CD44^+/CD24^{-/low}$ subgroup in adherent and spherical cells. Representative photos of one experiment are shown. *$P < 0.05$ *vs*. adherent cells; **$P < 0.01$ *vs*. adherent cells

Overall, GPER expression and the proportion of the $CD44^+/CD24^{-/low}$ subpopulation were increased in the mammospheres of MDA-MB-468 cells, and spherical cells showed a stronger ability for tumor metastasis. The results suggested that GPER is positively related to stem cell formation and stem cell-related malignancies.

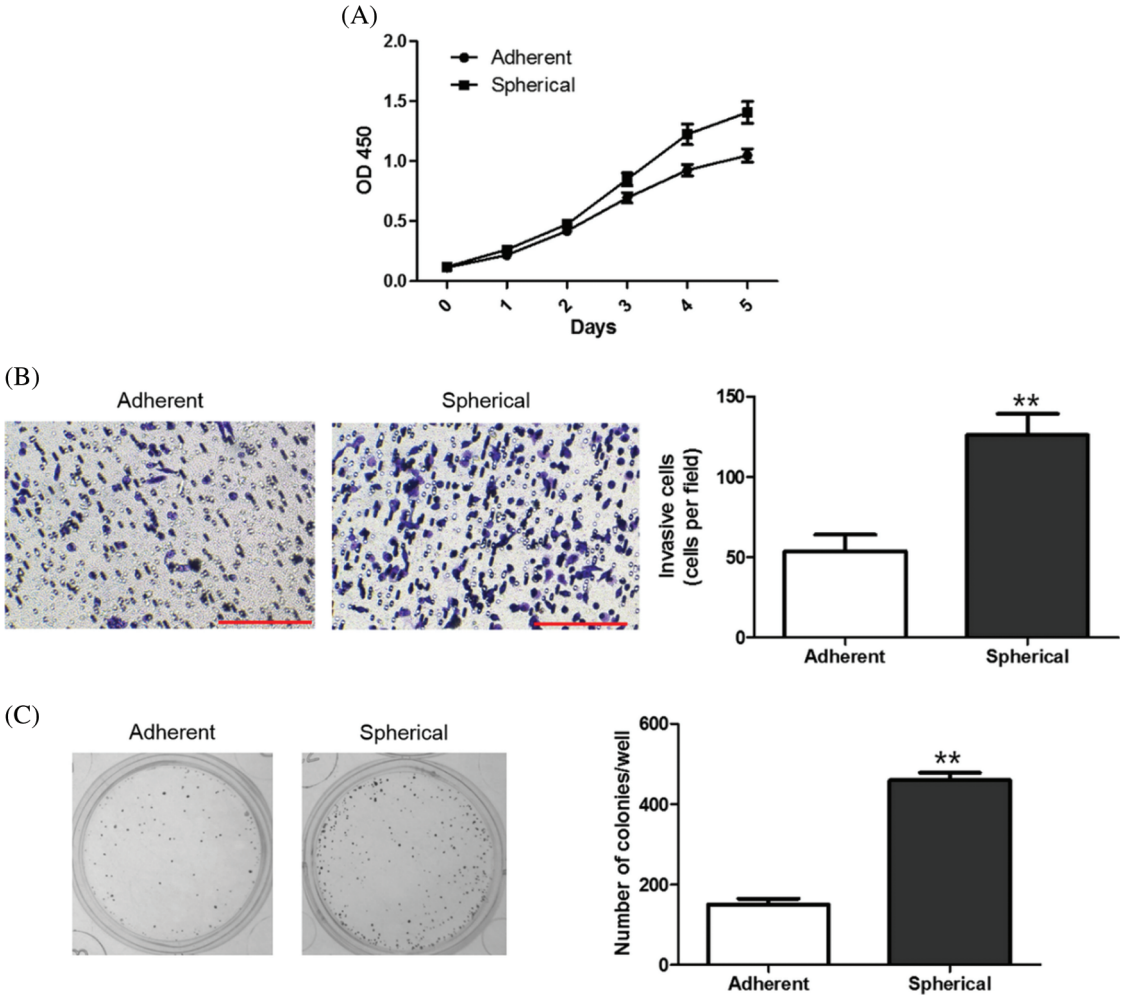

Figure 2: Biological characteristics of adherent and spherical cells *in vitro*. (A) Growth curve of adherent and spherical cells. (B) Invasion ability of adherent and spherical cells. Representative photos of one experiment are shown. Scale bar = 200 μm. (C) Colony-forming ability of adherent and spherical cells. $^{**}P < 0.01$ *vs*. adherent cells

Table 1: Comparison of tumor forming ability between adherent cells and spherical cells

Inoculation number	Tumor forming rate		*P*
	Adherent	Spherical	
5×10^5 cells/site	0/6	1/6	1.0000
1×10^6 cells/site	1/6	6/6	0.0152
2×10^6 cells/site	6/6	6/6	1.0000

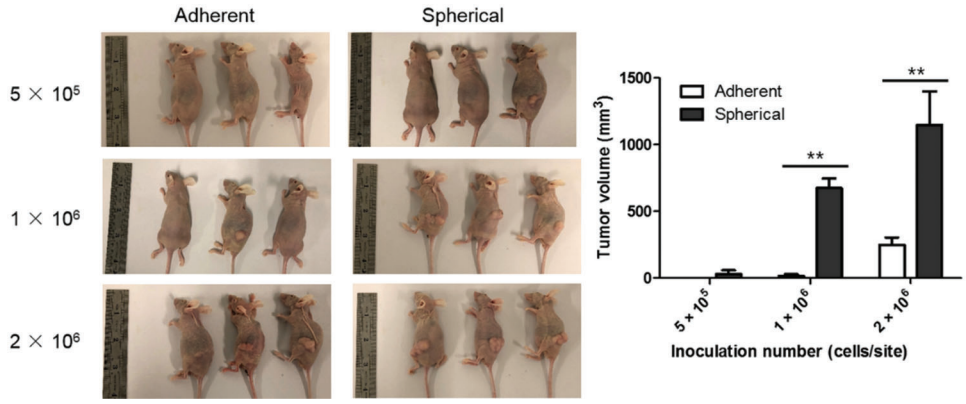

Figure 3: Tumorigenicity of adherent and spherical colonies in a xenograft model. $^{**}P < 0.01$ *vs*. adherent cells

3.2 GPER Mediates the Effect of Estrogen on Malignancies of TNBC Cells

Next, we investigated whether the malignant characteristics of spherical cells and adherent cells were different under estrogen treatment and determined the role of GPER in this process. The results are shown in Fig. 4. The proliferation of adherent cells and spherical cells in the presence of E2 was increased by approximately 50% compared with that in the absence of E2 ($P < 0.01$, Fig. 4A). At the same time, G15 pretreatment decreased cellular proliferation compared with E2 treatment alone ($P < 0.01$, Fig. 4A). E2 treatment doubled the number of migrated cells in the invasion assay ($P < 0.05$, Fig. 4B). Compared with E2 treatment alone, G15 pretreatment significantly reduced the number of migrated cells ($P < 0.05$, Fig. 4B), but there was no significant difference with the respective control groups ($P > 0.05$, Fig. 4B). E2 treatment also significantly promoted the colony formation ability of both adherent and spherical cells ($P < 0.01$, Fig. 4C). Compared with the E2 treatment alone, G15 pretreatment inhibited the colony formation ability ($P < 0.01$, Fig. 4C), and there was no significant difference in the colony formation ability between the E2/G25 groups and the respective control groups ($P > 0.05$, Fig. 4C). Hence, spherical cells treated with E2 showed the strongest proliferation, invasion, and colony-forming abilities. Pretreatment with the GPER antagonist G15 effectively blocked the stimulatory effect of E2. Consistently, the expression level of GPER was enhanced by E2 treatment in both adherent and spherical cells (Fig. 5). GPER expression was increased by 6.3-fold in adherent cells and by 2.8-fold in spherical cells. As expected, GPER expression was blocked by G15. Comparison with the E2 group indicated that GPER expression after G15 blockade was decreased by 58% in adherent cells and by 29% in spherical cells. We also detected the expression of several representative protein markers associated with the metastasis and stemness of breast cancer cells. E2 treatment led to a 1.5–3.5-fold increase in the expression levels of NANOG, SOX2, MMP2, and CXCL12 in both adherent and spherical cells. Similarly, G15 blockade partially reversed the induction effect of E2 and decreased the expression of GPER in adherent and spherical cells. These results indicated that GPER can sense the E2 signal and positively regulate the proliferation, invasion, and colony formation of TNBC cells. Spherical cells with high GPER expression benefit the most from E2 stimulation.

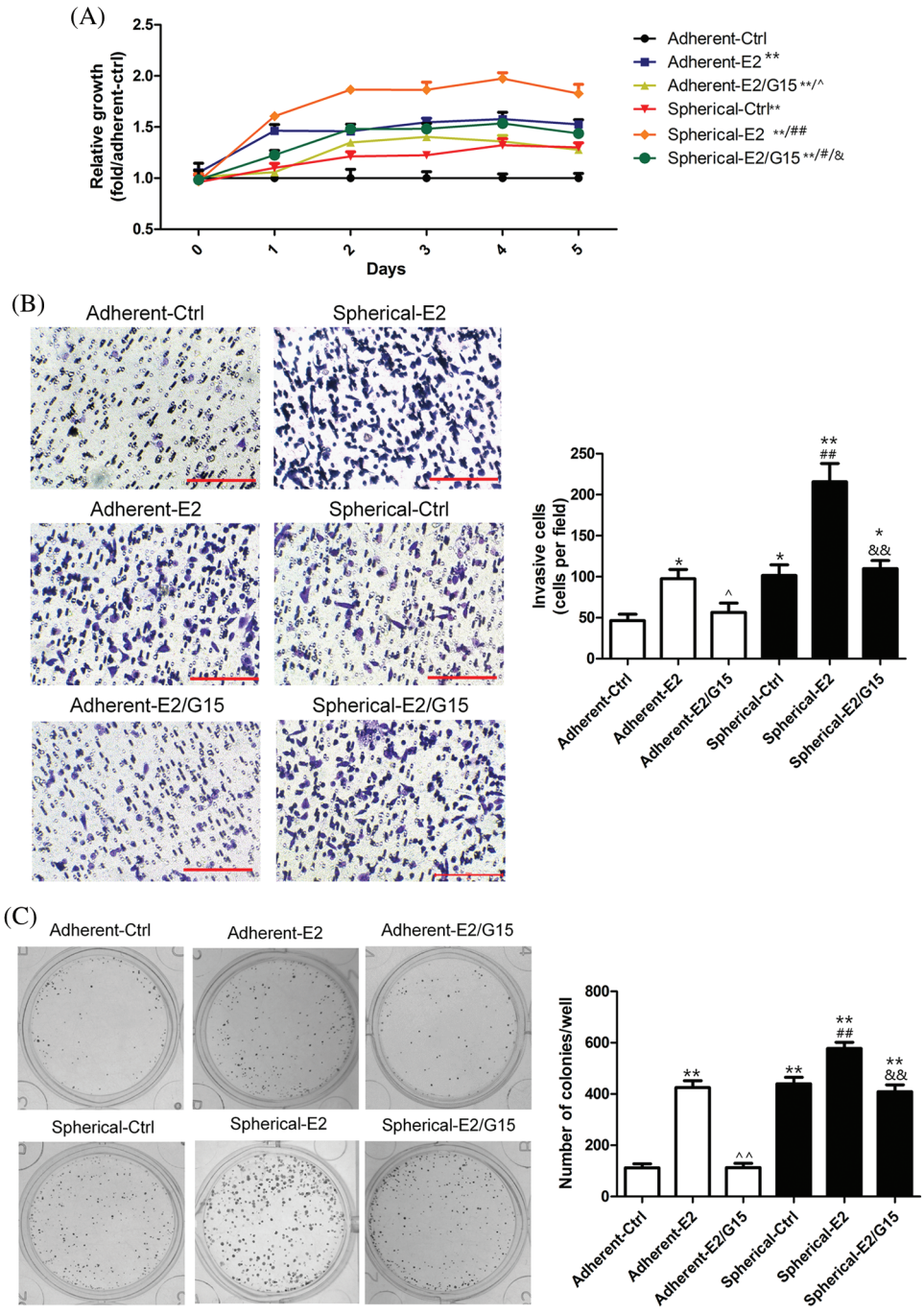

Figure 4: GPER signaling in the regulation of biological characteristics of adherent and spherical cells *in vitro*. Growth curve (A), invasion ability (B), and colony-forming ability (C) of adherent and spherical cells are differentially influenced by estrogen and the GPER antagonist G15. Representative photos of one experiment are shown. Scale bar = 200 μm. $^{*}P < 0.05$ *vs*. adherent-ctrl; $^{**}P < 0.01$ *vs*. adherent-ctrl; $^{\#}P < 0.05$ *vs*. spherical-ctrl; $^{\#\#}P < 0.01$ *vs*. spherical-ctrl; $^{\wedge}P < 0.05$ *vs*. adherent-E2; $^{\wedge\wedge}P < 0.01$ *vs*. adherent-E2; $^{\&}P < 0.05$ *vs*. spherical-E2; $^{\&\&}P < 0.01$ *vs*. spherical-E2

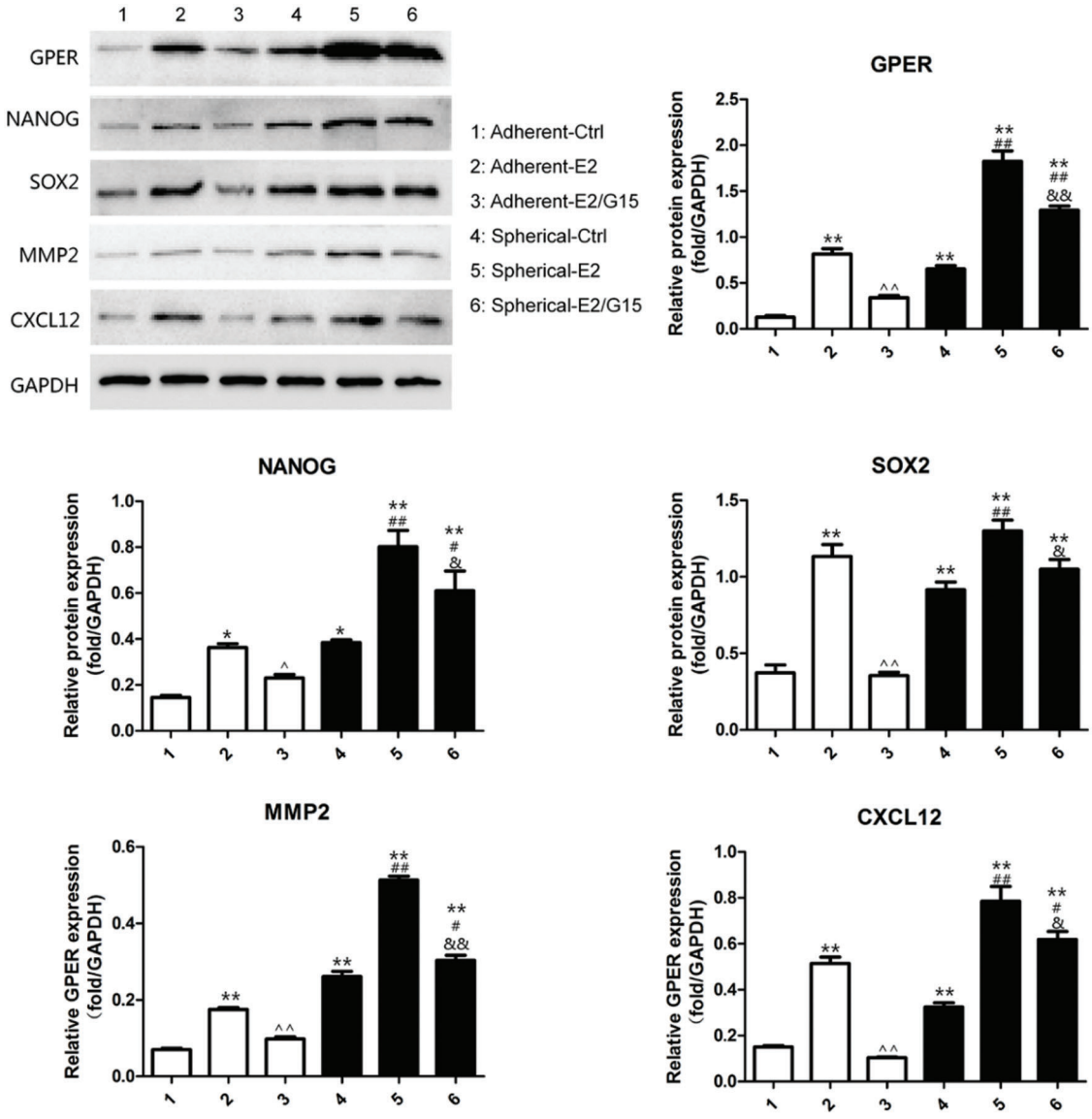

Figure 5: GPER signaling in the regulation of oncogenic biomarkers in adherent and spherical cells. Representative Western blot results of one experiment are shown. GAPDH was used as an internal control. $^{*}P < 0.05$ *vs*. adherent-ctrl; $^{**}P < 0.01$ *vs*. adherent-ctrl; $^{\#}P < 0.05$ *vs*. spherical-ctrl; $^{\#\#}P < 0.01$ *vs*. spherical-ctrl; $^{\wedge}P < 0.05$ *vs*. adherent-E2; $^{\wedge\wedge}P < 0.01$ *vs*. adherent-E2; $^{\&}P < 0.05$ *vs*. spherical-E2; $^{\&\&}P < 0.01$ *vs*. spherical-E2

4 Discussion

GPER is widely expressed in breast cancer, including TNBC [11]. Previous studies have shown that GPER expression is related to poor prognosis of TNBC and participates in the proliferation of TNBC cells [5,11,22,23]. The present study demonstrated that the expression of GPER was positively related to the proportion of stem cell subsets in TNBC cells and to malignant features favorable for metastasis; upregulation of GPER by E2 promoted the proliferation, invasion, and colony formation of TNBC cells.

The results of the present study indicated that high expression of GPER was beneficial to the proliferation of TNBC cells in agreement with the data of previous studies [5,6]. Moreover, the present study demonstrated that high expression of GPER was positively related to the induction of mammospheres of TNBC cells, indicating that GPER expression is involved in the presentation of stemness characteristics. A recent study reported similar results, demonstrating that GPER is upregulated in stem cells using a patient-derived xenograft assay of ER^- breast cancer cells [24]. The main mechanism of this effect involves GPER induction by tamoxifen and subsequent activation of phosphorylation through protein kinase A (PKA) signaling [24]. The results of the present study and studies by other authors suggest that GPER plays an oncogenic role in TNBC cells. However, a study by Chen et al. [25] reported contradictory; they found that GPER is a favorable factor for the prognosis of TNBC patients and that the expression of GPER induced by G1 (GPER agonist) can inhibit the epithelial-mesenchymal transition, migration, and invasion of TNBC cells. Chen et al. [25] speculated that the contradictions may be due to the different agonists used in different studies (i.e., estrogen or tamoxifen *vs*. the specific GPER agonist G-1). Moreover, Chen et al. did not report the age or treatment method of their cases. These are important confounding factors in the study of the relationship between GPER and TNBC prognosis. The TNBC phenotype is a favorable prognostic factor before menopause but a risk factor after menopause [26]. GPER has opposite prognostic roles in tamoxifen and non-tamoxifen treatment [8]. The existence of endogenous estrogen or endocrine therapy regimens has an important impact on the results, which may also explain the contradictions of different studies.

The results of the present study indicated that the oncogenic effect of GPER can be enhanced by E2, and spherical cells with higher GPER expression levels benefitted more than adherent cells with lower GPER expression levels. The expression of GPER in TNBC is related to young age [11], which suggests that the expression of GPER depends on estrogen levels. Ye et al. [23] reported that GPER is induced by estrogen and promotes malignant characteristics of TNBC cells via upregulation of estrogen-related receptor α. The results of the present study further suggested that cells with higher GPER expression levels are more likely to become more malignant under E2 treatment. Thus, E2 applies a selective pressure and may be more conducive to the survival of stem cell subsets of TNBC cells. Therefore, it is reasonable to infer that a high estrogen level is an unfavorable prognostic factor for TNBC patients, especially for patients with a high GPER expression level. A follow-up study of 326 TNBC patients treated with neoadjuvant chemotherapy demonstrated that premenopausal status is a promising factor associated with poor prognosis [27]. Another study demonstrated that a high GPER expression level was associated with poor prognosis only in premenopausal TNBC patients and not in postmenopausal patients [22]. The clinical relevance of GPER expression in TNBC patients remains uncertain. The use of GPER as a prognostic indicator of TNBC requires consideration of the estrogen level of patients. High estrogen levels and high GPER levels may have superposing effects, which further promote the deterioration of TNBC.

There are several limitations of this study. Only one cell line was used in this study due to the affordability of research conditions. The heterogeneity between different TNBC cell lines will lead to an inability to extend the research conclusions. For example, MDA-MD-468 and MDA-MD-231 cells have differences in morphology, proliferative ability, claudin expression, Ki-67 expression, etc. [28,29]. Future studies with multiple cell lines and clinical specimen-derived primary cell culture will expand on this research. In addition, this study is a cell-based basic study. We hope to carry out the collection and follow-up of clinical samples to provide more evidence for the prognostic value of GPER for TNBC and to help clarify the contradictions of different studies.

5 Conclusions

The results of the present study suggest that GPER plays a vital role in the formation of TNBC-related stem cells and the malignant characteristics of TNBC. The promoting effect of GPER on the proliferation,

migration, and invasion of TNBC cells can be further amplified by estrogen. These findings may provide clues for new therapeutic strategies for TNBC.

Authorship: The authors confirm contribution to the paper as follows: study conception and design: Dongliang Zhu; data collection: Dongliang Zhu, Jun Yang, Jiaxin Xu; analysis and interpretation of results: Dongliang Zhu, Jun Yang; draft manuscript preparation: Dongliang Zhu. All authors reviewed the results and approved the final version of the manuscript.

Ethics Approval and Informed Consent Statement: This study was approved by the Animal Ethics Committee of Nanjing Medical University (IRB No. 2016-34).

Availability of Data and Materials: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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