G Protein–Coupled Estrogen Receptor Regulates Mammary Tumorigenesis and Metastasis

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Abstract

The role of 17β-estradiol (E2) in breast cancer development and tumor growth has traditionally been attributed exclusively to the activation of estrogen receptor-α (ERα). Although targeted inhibition of ERα is a successful approach for patients with ERα+ breast cancer, many patients fail to respond or become resistant to anti-estrogen therapy. The discovery of the G protein–coupled estrogen receptor (GPER) suggested an additional mechanism through which E2 could exert its effects in breast cancer. Studies have demonstrated clinical correlations between GPER expression in human breast tumor specimens and increased tumor size, distant metastasis, and recurrence, as well as established a proliferative role for GPER in vitro; however, direct in vivo evidence has been lacking. To this end, a GPER-null mutation [GPER knockout (KO)] was introduced, through interbreeding, into a widely used transgenic mouse model of mammary tumorigenesis [MMTV-PyMT (PyMT)]. Early tumor development, assessed by the extent of hyperplasia and proliferation, was not different between GPER wild-type/PyMT (WT/PyMT) and those mice harboring the GPER-null mutation (KO/PyMT). However, by 12 to 13 weeks of age, tumors from KO/PyMT mice were smaller with decreased proliferation compared with those from WT/PyMT mice. Furthermore, tumors from the KO/PyMT mice were of histologically lower grade compared with tumors from their WT counterparts, suggesting less aggressive tumors in the KO/PyMT mice. Finally, KO/PyMT mice displayed dramatically fewer lung metastases compared with WT/PyMT mice. Combined, these data provide the first in vivo evidence that GPER plays a critical role in breast tumor growth and distant metastasis.

Implications: This is the first description of a role for the novel estrogen receptor GPER in breast tumorigenesis and metastasis, demonstrating that it represents a new target in breast cancer diagnosis, prognosis, and therapy. Mol Cancer Res; 12(11); 1644–54. ©2014 AACR.

Introduction

The steroid hormone 17β-estradiol (estrogen, E2) is the primary female sex hormone necessary for the development of secondary sexual characteristics in women. Specifically, E2 mediates the development of breast tissue during puberty and pregnancy by enhancing the proliferation of ductal epithelial cells. Similar to normal development, E2 also promotes breast cancer by augmenting proliferation, migration, and invasion of breast tumor cells, which has been demonstrated both in vitro and in vivo (1). By inhibiting the activity of the classical estrogen receptor (ERα), many cancer-promoting effects of E2 in cultured cells and mice are reduced; therefore, the actions of E2 in breast cancer have been attributed almost exclusively to ERα (2). Clinically, drugs that block the production of estrogen or inhibit ERα, thereby inhibiting E2 signaling, increase long-term survival in patients with breast cancer (3). One of the most commonly prescribed adjuvant treatments for breast cancer is tamoxifen, a selective ER modulator (SERM) that acts as an antagonist for ERα in the breast, inhibiting tumor growth. Treatment with tamoxifen for 5 years following surgery decreases mortality from breast cancer by approximately 30% (3), although the incidence of endometrial cancer increases by 2– to 4-fold (4). However, only patients with ERα+ tumors are eligible for treatment, and many ERα+ tumors either do not respond to tamoxifen, or become resistant during treatment or upon recurrence (5). Although aromatase inhibitors, which inhibit the production of E2, are more efficacious in the prevention and treatment of breast cancer compared with tamoxifen (7), indicating that the inhibition of E2 signaling solely through targeting ERα may be suboptimal and that other E2 receptors may contribute to the initiation and/or progression of breast cancer.
G protein–coupled estrogen receptor (GPER, formerly GPR30) is a novel estrogen receptor (ER) with multiple functions in diverse tissues (8–12), whose role in breast carcinogenesis has yet to be directly determined (13, 14). E2 stimulation of cells expressing GPER, but lacking ERβ, activates the MAPK and PI3K cascades, among other pathways, increasing proliferation of many breast cancer cell lines, suggesting that GPER may play a role in one or more events of breast carcinogenesis (15–17) and particularly triple-negative breast cancer (18, 19). A small number of retrospective studies have examined the relationship between GPER expression in breast tumor samples and clinical outcome or its correlates. A study of 361 patients with breast cancer found GPER expression associated with increased primary tumor size and the prevalence of distant metastasis (20). Another investigation demonstrated an increased recurrence rate in GPER–/– invasive ductal breast cancers (21). More recently, a study of invasive breast cancer samples demonstrated that GPER expression is an independent prognostic factor for decreased disease-free survival in patients treated exclusively with tamoxifen (22), with its cellular localization potentially playing a critical role (18). Although these studies suggest a role for GPER in breast cancer, to this point no direct experimental evidence demonstrates a functional role for GPER in the initiation and/or progression of breast cancer.

In this study, we investigated the contribution of GPER to breast carcinogenesis by introducing a GPER-null mutation [GPER knockout (KO); refs. 23, 24] onto a transgenic mouse model of mammary tumorigenesis through inter-breeding. MMTV-PyMT (PyMT) transgenic mice express the polyoma middle T antigen (PyMT) primarily in mammary tissue, under control of the mammary tumor virus (MMTV) promoter, resulting in the spontaneous development of mammary tumors (25). PyMT mice on the FVB background develop hyperplastic lesions in their mammary glands by 4 weeks of age that progress through the stages of adenoma, early carcinoma, and late carcinoma over the subsequent 8 to 10 weeks (26). Histologically, PyMT tumors at each stage of development closely resemble equivalent-stage human tumors. Furthermore, changes in biomarkers during PyMT mammary tumor development are similar to human breast cancer as tumors progress from hyperplasia to carcinoma. The biomarker changes include loss of ERα, progesterone receptor, and integrin β expression, as well as an increase in the expression of Neu and cyclin D1 (26). Finally, analogous to human breast cancer, early tumor growth in PyMT mice is stimulated by E2, demonstrating the PyMT model of mammary carcinogenesis to be a highly clinically relevant model of human breast cancer (27).

Using the PyMT model, we demonstrate that the presence of GPER promotes growth and metastasis of late-stage mammary tumors in PyMT mice, although we observed no difference in tumor latency between GPER wild-type (WT) PyMT (WT/PyMT) and GPER KO PyMT (KO/PyMT) mice. Furthermore, early development of hyperplasia was not affected by GPER expression; however, upon tumor progression tumors from KO/PyMT mice were smaller with less aggressive histologic features compared to tumors from WT/PyMT and GPER heterozygous (HET/PyMT) mice. Consistent with decreased size, tumors from KO/PyMT mice displayed reduced proliferation compared with tumors from WT/PyMT mice. Perhaps most importantly, KO/PyMT mice exhibited fewer lung metastases compared with WT/ PyMT mice. Taken together, these data demonstrate that silencing GPER decreases tumor size and the number of distant metastases, suggesting that pharmacologic inhibition of GPER may represent a novel approach to reduce morbidity and mortality from breast cancer.

Materials and Methods

Mice

FVB/N-Tg(MMTV-PyVT)634Mul/J (MMTV-PyMT) mice were purchased from The Jackson Laboratory. Mice lacking the GPER gene through targeted deletion (GPER KO) were provided by Jan Rosenbaum (Proctor & Gamble) and have been described previously (24). GPER KO mice were backcrossed 10 generations onto FVB/N mice that were purchased from Harlan Laboratories and subsequently intercrossed with MMTV-PyMT mice to produce MMTV-PyMT mice that were WT, HET, or null (KO) with respect to GPER. Animals were housed at the Animal Research Facility at the University of New Mexico Health Sciences Center (Albuquerque, NM) and maintained under a controlled temperature of 22°C to 23°C with a 12-hour light, 12-hour dark cycle and were fed a soy-free (phytoestrogen-reduced) chow ad libitum. All procedures were approved by the University of New Mexico Institutional Animal Care and Use Committee and carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Tumor and lung resection

Tumors were allowed to grow until mice were 7 weeks or 12 to 13 weeks of age. Tumors and lungs were then resected, fixed in 4% PFA, and paraffin-embedded for subsequent analysis. Hyperplastic mammary glands from 7-week-old mice were analyzed for the extent of hyperplasia and proliferation, while tumors from 12- to 13-week-old mice were assessed for weight (as a measurement of tumor size), proliferation rate, grade, extent of necrosis, and ERα expression. In addition, lungs from 12- to 13-week-old mice were analyzed for the number of metastatic foci.

Ovariectomy

Ovariectomy was performed at 3 weeks of age. PyMT mice were anesthetized with isofluorane. A small incision was made through the skin on the dorsal midline just cranial to the hiphones. Bilateral incisions were made through the underlying muscle lateral to the spine and each ovary was removed. The muscle incisions were closed using polydioxanone synthetic absorbable sutures (PDS® Plus; Ethicon) and the skin incision was closed with 9-mm stainless steel tissue clips (ez CLIPS; Stoelting).

Tamoxifen treatment

When PyMT mice were 4-weeks old, a 60-day release pellet containing tamoxifen (5 mg/pellet; Innovative Research of
America) was subcutaneously implanted on the left dorsal side of the mouse just below the rib cage. Tumors were resected at 12 weeks of age and weighed as a measurement of tumor size.

**Relative quantitation of PyMT gene expression**

Mammary tumors from 13- to 14-week-old intact or ovariec-tomized PyMT mice and 10-week-old WT/PyMT and KO/PyMT were removed and homogenized in TRizol (Sigma-Aldrich) using a Polytron tissue homogenizer. RNA isolation was performed in TRizol (Life Technologies) according to the manufacturer’s instructions. cDNA was synthesized via reverse transcription of 1 μg RNA with the iScript cDNA Synthesis Kit (Bio-Rad) using the GeneAmp PCR system 9700 (Applied Biosystems, Inc.). A relative standard curve for each primer pair was created from a mixture of the sample RNA extracted from the tumors with dilution values of 1.0, 0.25, 0.0625, and 0.0156. The relative concentrations were expressed as arbitrary units and plotted against the logarithm (base 10) of the dilution values. Linear regression was used to create standard curves for each primer that were subsequently used to determine the relative amount of PyMT and cytokeratin 18 in each sample. The relative concentration of PyMT was then normalized to the relative concentration of cytokeratin 18, which served as the endogenous control. The primer sequences were: PyMT forward: 5'-CGG CGG AGC GAG GAA CTG AGG AGA G-3' and reverse: 5'-TCA GAA GAC TCG GAC GTA TTA-3'; and cytokeratin 18 forward: 5'-GAA GTG TGC CGA CAA CAG GTG ATG TTC TGG TTT T-3'.

**Tumor histology**

The largest tumors from 12- to 13-week-old mice were sectioned (5 μm) and stained with hematoxylin (Sigma-Aldrich) and eosin (RICCA Chemical Company). Tumor grade was determined in a blinded manner by a veterinary pathologist (Donna Kusewitt, The University of Texas MD Anderson Cancer Center, Houston, TX). PyMT tumor grade was determined as previously described (26) by tissue architecture, degree of cellular atypia, and invasion into the surrounding stroma. On the basis of these parameters, tumors were assigned grades as follows. (i) Hyperplasia: densely packed acini filled or bridged by epithelial cells that have little to no cellular atypia with no invasion into the surrounding stroma. (ii) Adenoma/mammary intraepithelial neoplasia (MIN): increased proliferation of epithelial cells with acini mostly filled with cells. Minimal cellular atypia and no invasion are present. (iii) Early carcinoma: florid proliferation with loss of acinar definition. Moderate cellular atypia and early stromal invasion is present. (iv) Late carcinoma: solid sheets of cells with very few or no acini present and a high mitotic index consistent with increased proliferation. Marked cellular atypia and pronounced stromal invasion are present. Sections were imaged with a Nikon DS-Fil camera mounted on a Nikon Eclipse E400 microscope running NIS-Elements software.

The extent of tumor necrosis was determined and categorized by the number and size of necrotic areas as follows: 1, few small areas; 2, few larger areas or moderate number of smaller areas; and 3, extensive areas.

**Immunostaining analysis**

For immunostaining, 5-μm sections were deparaffinized, rehydrated, permeabilized in PBS containing 0.1% Triton X-100, and blocked in 3% normal goat serum (NGS) diluted in PBS plus 0.1% Tween-20 (PBS-T). To evaluate cellular proliferation rate, microwave antigen retrieval was performed in 0.1 mol/L sodium citrate (pH 6). Sections were incubated overnight in a 1:100 dilution of the anti–phospho-histone H3 antibody (P-H3, phospho Ser10; EMD Millipore) followed by detection with an anti-rabbit IgG antibody conjugated to Alexa Fluor 488 (Molecular Probes). Coverslips were mounted with Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). One random field from three sections was imaged with a Zeiss 200M Axiovert microscope using MetaMorph software. In a blinded manner, the number of P-H3–positive cells was determined and normalized to the total number of cells per field. Apoptosis was evaluated in the same manner, except that the primary antibody was directed against cleaved caspase-3 (Asp 175; Cell Signaling Technology).

To detect ERα, microwave antigen retrieval was performed in TET buffer (10 mmol/L Tris, 1 mmol/L EDTA, and 0.05% Tween-20; pH 9) and endogenous peroxide activity was quenched in 3% H2O2 before permeabilization. Sections were incubated overnight in a 1:100 dilution of anti-ERα antibody (MC-20; Santa Cruz Biotechnology). The sections were washed in PBS-T and incubated with anti-rabbit IgG antibody conjugated to horseradish peroxidase (HRP; Molecular Probes). 3,3'-Diaminobenzidine (DAB; Sigma-Aldrich) was used as the substrate to detect the presence of the HRP-conjugated antibody. Coverslips were mounted with Permount mounting medium (Fisher Scientific). Three random fields per section were imaged with a Nikon DS-Fil camera mounted on a Nikon Eclipse E400 microscope running NIS-Elements software. The number of ERα" cells was determined and normalized to the total number of cells per field.

**Lung metastasis**

Three 5-μm sections separated by 100 μm were prepared from the lungs of 12- to 13-week-old animals. Sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E). Metastatic tumor foci present in the lung parenchyma are defined as a tightly clustered group of 10 or more hematoxylin-positive cells that excludes eosin-stained stroma. Total metastatic foci were counted in the three lung sections to determine the extent of metastasis. The counting was blinded to the investigator with respect to the genotype.
Whole mount

Number 4 abdominal mammary glands from 7-week-old PyMT mice were fixed in 4% PFA on stretched skin overnight at room temperature. The glands were removed from the skin, incubated overnight in 100% aceton to remove the fat, washed in deionized water, and stained with carmine alum (Sigma-Aldrich) overnight as described (http://mammary.nih.gov). After staining, the glands were washed in deionized water and dehydrated in ethanol before storing in methyl salicylate (Sigma-Aldrich). Glands were imaged with MOTICCam 2300 running MOTIC software on an Olympus SZH dissection microscope.

Statistical analysis

Differences in tumor size in ovariectomized and tamoxifen-treated mice, hyperplasia in 7-week glands, P-histone H3–positive cells, relative concentration of PyMT RNA, ERE expression, and extent of lung metastasis were evaluated using the two-tailed Student t test with a P value threshold for significance of 0.05. Differences in tumor size of WT/PyMT, HET/PyMT, and KO/PyMT mice were assessed using one-way ANOVA with Bonferroni correction for multiple comparisons as a post-hoc test. Tumor grade and extent of necrosis were analyzed using a two-tailed Fisher exact test with a P value threshold for significance of 0.05. Correlation between ERα and tumor grade was assessed using Pearson correlation analysis with a P value threshold for significance of 0.05.

Results

Ovariectomy and tamoxifen treatment reduce mammary tumor size

Although GPER expression has been correlated with human breast tumor growth and metastasis, in vivo studies determining the effects of GPER on breast cancer initiation, growth, and progression have not been reported. To study the role of GPER in breast cancer, we used the MMTV-PyMT (PyMT) model of mammary carcinogenesis, which has been described to be an E2-responsive tumor model (27). To verify the E2-responsiveness of tumor growth, PyMT mice were treated with tamoxifen to remove the majority of circulating E2. Tumors were resected from ovariectomized (OVEX) (n = 5) and intact (n = 3) mice and weighed as a measure of tumor size when mice were 12 weeks old. To further examine the role of E2 signaling, we also examined the effect of E2 withdrawal, either directly or indirectly through the modulation of other hormones, that, because of its E2 responsiveness, this model would be appropriate for examining the role of GPER in mammary tumorigenesis.

GPER deficiency does not affect tumor initiation or early tumor proliferation

To evaluate the effect of GPER expression on tumorigenesis, we generated GPER WT, HET, and KO mice on a PyMT background (WT/PyMT, HET/PyMT, KO/PyMT, respectively). Tumors in both WT/PyMT and KO/PyMT mice were palpable between 7 and 8 weeks of age, suggesting no difference in tumor latency. To assess differences in early tumor development, whole mounts of the number four abdominal mammary glands from 7-week-old WT/
PyMT and KO/PyMT mice were stained with carmine alum to determine the extent of hyperplasia as follows. In ImageJ, a grid was overlaid on the whole mount images and each box was evaluated for the presence of hyperplasia and epithelium (Fig. 2A). The total area of hyperplasia relative to the total area of epithelium was 0.73 ± 0.05 for WT/PyMT and 0.64 ± 0.05 for KO/PyMT, demonstrating no statistical difference ($P > 0.05$; Fig. 2B). To determine whether the lesions in WT/PyMT mice had extended farther into the mammary gland than in KO/PyMT mice, indicating more advanced disease, hyperplasia distal to the midline of the lymph node relative to total epithelium distal to the midline of the lymph node was also evaluated. No difference was evident in the extent of hyperplasia distal to the lymph node between WT/PyMT (0.41 ± 0.08) and KO/PyMT (0.34 ± 0.07) mice ($P > 0.05$; Fig. 2C). Proliferation in early tumors was then examined by staining number 2/3 thoracic mammary glands from 7-week-old mice for P-histone H3 a marker of the M-phase of the cell cycle. No difference in the number of proliferating cells between tumors from WT/PyMT and KO/PyMT mice ($P > 0.05$, Fig. 2D) was evident. Collectively, these data indicate that the absence of GPER does not significantly reduce the development or growth of early mammary tumors in the PyMT model.

**Late-stage mammary tumor growth is reduced in the absence of GPER**

Although early tumor development was not altered in the absence of GPER, tumors from older mice were evaluated to determine whether GPER expression affects the progression to more advanced tumors. When mice were 12- to 13-weeks old, tumors were removed and weighed as a measure of tumor size. Although there was no difference between WT/PyMT and HET/PyMT (0.26 ± 0.02 vs. 0.26 ± 0.01; $P = 0.79$) tumor size, tumors from KO/PyMT mice were 28% smaller than those from WT/PyMT mice ($P < 0.05$; Fig. 3A). To verify that the difference in tumor mass was not indirectly caused by decreased PyMT expression in KO/PyMT mice, real-time PCR was used to determine the level of PyMT mRNA expression relative to cytokeratin 18, a marker of epithelial cells, in tumors from WT/PyMT and KO/PyMT mice. No difference in the relative expression of PyMT RNA between WT/PyMT and KO/PyMT mice was detected (Fig. 3B, $P > 0.05$), indicating differences in tumor mass were not an indirect result of decreased PyMT expression.

Because tumors in KO/PyMT mice were smaller than in WT/PyMT tumors, proliferation and apoptosis of the tumor cells were analyzed to determine the relative contribution of each factor to overall tumor size. To evaluate the...
proliferation rate, the number of P-H3–positive cells was determined and normalized to the total number of cells. Tumors from KO/PyMT mice exhibited 44% fewer P-H3–positive cells than tumors from WT/PyMT mice (P < 0.05; Fig. 3C and D). Apoptosis was determined using an antibody directed against cleaved caspase-3 and similarly analyzed by microscopy. Although a positive control exhibited cleaved caspase-3–expressing cells, the tumors displayed no cleaved caspase-3–positive staining regardless of genotype (not shown). Therefore, we conclude that GPER-expressing tumors are larger, in part, due to increased tumor cell proliferation.

GPER expression is associated with predictors of poor prognosis

To determine the contribution of GPER expression to tumor aggressiveness, sections of the largest tumor from WT/PyMT and KO/PyMT mice were stained with H&E, analyzed to determine grade, and classified as either low-grade (hyperplasia and adenoma) or high-grade (early and late carcinoma; Fig. 4A). The majority of tumors from WT/PyMT mice lost acinar definition appearing as solid sheets of cells, and had invaded through the basement membrane into the surrounding stroma, indicative of carcinoma. In contrast, tumors from KO/PyMT mice frequently maintained acinar structure, although the acini were filled with cells; in addition, fewer tumors invaded through the basal lamina in the KO/PyMT mouse. These histologic parameters revealed that 90% of tumors from WT/PyMT mice were carcinomas versus 50% of tumors from KO/PyMT mice, demonstrating a strong trend for KO/PyMT tumors to exhibit a lower tumor grade than WT/PyMT tumors (P = 0.056; Fig. 4B).

During tumor resection, it appeared that tumors from KO/PyMT mice contained a smaller necrotic center than WT/PyMT mice. Because necrosis is an independent predictor for poor prognosis, a smaller necrotic center suggests less aggressive tumors (31). Necrosis was analyzed by evaluating the number and size of necrotic areas categorized using a scale from 0 to 3 with 0 signifying a lack of necrosis and 3 representing large and/or many areas of necrosis. The majority of tumors (78%) from WT/PyMT mice were scored 2 or greater compared with 21% of tumors from KO/PyMT mice receiving a score of 2 and no tumors from KO/PyMT mice receiving the highest score of 3 (Fig. 4C). Thus, tumors lacking GPER exhibit fewer and smaller areas of necrosis compared with tumors that express GPER (P < 0.05). These data suggest that loss of GPER in KO/PyMT mice leads to less aggressive tumors than mice with normal levels of GPER.

ERα expression is unaffected by GPER deficiency

As ERα drives proliferation in approximately 70% of human breast tumors and tamoxifen successfully inhibited PyMT tumor growth, we considered that eliminating GPER could affect the expression of ERα in the tumors of PyMT mice, thus indirectly affecting tumorigenesis (32). To examine ERα expression, sections of hyperplastic mammary glands from 7-week-old and 12- to 13-week-old mice were stained and analyzed for the number of ERα–positive cells as well as staining intensity. Hyperplastic glands from 7-week-old mice displayed faint ERα staining in the hyperplastic/adenomatous regions, and intense staining in the adjacent normal tissue, suggesting that as tumors form, ERα expression begins to decrease (Fig. 5A), in agreement with published observations (26). The number of ERα–positive cells was not different between mammary glands from 7-week-old WT/PyMT and KO/PyMT mice (Fig. 5B). Tumors from 12- to 13-week-old mice displayed faint ERα immunostaining that was less intense than that of 7-week hyperplastic tissue (Fig. 5A). Consistent with immunostaining in 7-week mammary glands, the number of ERα–expressing cells in tumors from 12- to 13-week-old mice was similar in WT/PyMT and KO/PyMT mice (Fig. 5C). Thus, manipulating the expression of GPER does not affect the expression of ERα. Because patients with ERα–negative tumors have a better prognosis than those with ERα–positive tumors, the number of ERα–expressing cells in tumors from 12- to 13-week-old WT/PyMT and KO/PyMT tumors was analyzed with respect to tumor grade. Consistent with patient data, higher ERα expression correlated with a lower tumor grade (P = 0.05; Fig. 5D).
GPER deficiency yields fewer metastases to the lung

Although tumor size, grade, proliferation rate, and ER status are predictive of aggressiveness in breast tumors, the most reliable predictor of survival in patients is the presence of distant metastases (33, 34). The most common metastatic sites for human breast cancer are the lungs, liver, and bone (35). PyMT tumors predominantly metastasize to the lung, thus representing an appropriate model of human breast cancer for the evaluation of metastasis (1). To assess the extent of metastasis, lungs of 12- to 13-week-old KO/PyMT and WT/PyMT mice were stained with H&E and the number of tumor foci, designated as a group of 10 or more densely packed cells, was determined in three lung sections separated by 100 μm (Fig. 6A). Lung sections from WT/PyMT mice contained 9.0 ± 1.9 metastatic foci, while lungs from KO/PyMT mice contained 3.8 ± 0.6 metastatic foci, representing a 2.4-fold reduction in metastases in KO/PyMT mice (P < 0.05; Fig. 6B). Although the majority of mice had metastasis at this age, 83% of KO/PyMT mice had fewer than five metastases in the analyzed lung tissue compared with 33% of WT/PyMT mice (Fig. 6C). Together, these data demonstrate that PyMT mice lacking GPER have a substantially decreased incidence of metastasis.

Discussion

Several reports have demonstrated that multiple cancer cell lines proliferate in response to the GPER-selective agonist G-1 (36–38) and that E2-dependent proliferation can be reduced upon silencing GPER expression or inhibiting GPER activity (16, 39–41). Although these data suggest that GPER may promote breast tumor growth, its importance in breast cancer initiation, growth, and progression has remained unexplored. This report is therefore the first to describe a role for GPER in both tumor progression and metastasis using an in vivo model of breast carcinogenesis.

In this study, WT/PyMT and KO/PyMT mice were compared to determine the effects of GPER on in vivo mammary tumor growth and metastasis. Assessment of tumor initiation and early proliferation revealed no GPER-dependent effect. However, in more advanced disease, tumors from KO/PyMT mice were smaller than tumors from WT/PyMT mice, due in part to decreased tumor cell proliferation, suggesting a role for GPER in the proliferation and growth of mammary tumors. Although the mechanism of proliferation was not elucidated in vivo, many in vitro models give insight into the mechanisms of GPER-mediated proliferation. GPER has been demonstrated to regulate cellular proliferation in many cancer cell types including, breast, endometrial, and ovarian cancer cells through transactivation of the EGFR, resulting in increased ERK1/2 activation and transcription of genes including cyclins D1, E and A, c-fos, and egr-1 (37, 38, 42). Second, GPER is involved in the E2-dependent activation of fibroblasts in the tumor microenvironment (43–46). Activated fibroblasts act to enhance the growth and metastasis of tumors in vivo. Therefore, GPER expression in both the tumor and microenvironment may contribute to the increased tumor size in WT/PyMT mice.
Although it is unclear why the loss of GPER did not affect early tumor proliferation, we suggest that the delay in effects of GPER deficiency may be due to the classical E2 receptor ERα. ERα dramatically increases the proliferation rate and growth of breast tumors, although its role in the PyMT model has been debated (47). We demonstrated that the growth of PyMT tumors is sensitive to both early estrogen deprivation and tamoxifen administration, strongly suggesting a critical role for ERα in tumor initiation and proliferation. However, the expression of ERα was largely lost as tumors progressed, with early hyperplastic lesions demonstrating higher ERα expression compared with late-stage carcinomas, suggesting ERα expression and function are integral to the early proliferation of neoplastic cells in this model, with diminishing effects as its expression decreases. Therefore, we propose that ERα plays a dominant role in early mammary tumor development through its robust proliferative effects, mitigating the effects of GPER expression or lack thereof at early stages. Therefore, the growth-promoting effects of GPER may become more important in later tumor development and progression as those of ERα decline.

Although tumor size is predictive of more aggressive disease, metastasis is the most important predictor of morbidity and mortality in women with breast cancer (48). We demonstrated that KO/PyMT mice have fewer metastases compared with WT/PyMT mice, indicating that GPER plays a role in mammary tumor cell metastasis consistent with previous in vitro (16, 44, 49, 50) and clinical data (20). Several possible mechanisms have been observed for GPER-dependent tumor cell migration and invasion. In vitro studies have demonstrated GPER-dependent invasion through a recombinant basement membrane due to increased MMP-9 expression and activation (51), one of the first steps of metastasis. Furthermore, in vitro migration is enhanced through GPER activation in tumor cells as well as cells in the microenvironment, specifically fibroblasts, through induction of connective tissue growth factor (CTGF) expression, which is critical for stimulating cell migration (16). Although these mechanisms have been described for GPER mediating tumor cell "escape" from their local environment, the role of GPER at the site of distant metastasis has not been examined. Tumor cell metastasis to distant sites cannot be successful unless the invasive tumor cells can survive and proliferate in the new environment. Therefore, it is important to determine whether GPER augments metastasis through enhancing the ability of cells to escape the tumor microenvironment, supporting the ability of tumor cells to survive and grow in a novel environment, or both. Understanding the mechanisms of GPER-mediated metastasis will enhance our ability to effectively target GPER to inhibit metastasis.

Although these in vitro data have provided insight into the possible role(s) of GPER in breast cancer aggressiveness, it is important to recognize that clinical studies have also demonstrated a correlation between GPER expression and tumor size, metastasis, and recurrence. In one retrospective analysis...
by Filardo and colleagues, high GPER expression in breast tumors correlated with larger tumor size and increased distant metastasis, consistent with results of this study. Furthermore, it has been proposed that GPER plays a role in tamoxifen resistance and recurrence following tamoxifen therapy. Although tamoxifen is a SERM that inhibits ERα in the breast, it ubiquitously activates GPER, possibly contributing to tamoxifen resistance and the development of tamoxifen-associated uterine cancer (52). In a retrospective study, Ignatov and colleagues (22) demonstrated that in patients treated with tamoxifen as a monotherapy, high GPER expression correlated with subsequent tamoxifen resistance and decreased relapse-free survival. Therefore, administering a GPER-selective antagonist following or in combination with tamoxifen could represent an approach to inhibit resistance and improve the efficacy of tamoxifen and other SERMs (40, 52). Furthermore, tamoxifen is only effective in patients with ERα+ tumors, leaving approximately 30% of patients with breast cancer with fewer treatment options (32). However, 60% of patients with ERα- tumors express GPER (20, 22) that may be enhancing tumor growth and metastasis, providing a novel therapeutic target in these patients. Given the possible roles of GPER in tamoxifen-resistant and ERα- tumors, GPER is an appealing target to reduce tumor growth and metastasis, especially in premenopausal women who are not candidates for aromatase inhibitor therapy.

In conclusion, this is the first in vivo study to demonstrate a role for GPER in the progression of breast cancer, identifying a novel target for hormone therapy in breast cancer. As GPER is expressed in 60% of ERα+ tumors, targeting GPER could increase patient survival in women with the more aggressive ERα+ tumors. GPER expression is also associated with increased recurrence after adjuvant treatment with tamoxifen as a monotherapy (20, 22), and, therefore, may be an effective therapeutic target in combination with tamoxifen or other SERMs. Finally, as GPER-selective small-molecule inhibitors (G15 and G36; refs. 53, 54) have been developed and safely administered to mice, GPER represents an even more attractive clinical target. On the basis of an extensive body of evidence, GPER has been demonstrated in vitro, in clinical studies, and now for the first time in an in vivo animal model to be an important regulator of breast cancer growth and metastasis thus representing an important new diagnostic/prognostic (55) and therapeutic target (56) for breast cancer treatment.

**Disclosure of Potential Conflicts of Interest**

E.R. Prossnitz holds a U.S. patent on GPER-selective agonists and antagonists. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: N.A. Marjon, H.J. Hathaway, E.R. Prossnitz

Development of methodology: N.A. Marjon, H.J. Hathaway, E.R. Prossnitz

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N.A. Marjon, C. Hu

Analysis and interpretation of data (e.g., statistical analysis, bios-statistics, computational analysis): N.A. Marjon, C. Hu, H.J. Haynawy, E.R. Prossnitz

Writing, review, and/or revision of the manuscript: N.A. Marjon, H.J. Haynawy, E.R. Prossnitz

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Hu

Study supervision: H.J. Haynawy, E.R. Prossnitz

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G Protein–Coupled Estrogen Receptor Regulates Mammary Tumorigenesis and Metastasis

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